Toxicology International, *32(3) : 623-634; 2025. DOI: 10.18311/ti/2025/v32i3/45263*

Online ISSN : 0976-5131; Print ISSN : 0971-6580

Toxicology International

Journal Homepage: https://informaticsjournals.co.in/index.php/toxi



Ecotoxicity Profiling of Indoxacarb: A Multi-Model Approach Using ECOSAR, T.E.S.T., SeqAPASS and Bioassays

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Abstract

The intensification of agriculture has led to widespread use of synthetic pesticides, raising growing concerns about their unintended impacts on non-target organisms and the ecosystem. Indoxacarb, a widely used oxadiazine insecticide, has been increasingly reported in aquatic environments, prompting concerns about its ecotoxicological effects on non-target species. This study evaluated the acute toxicity and ecological risk of indoxacarb using an integrated approach that combined *in vivo* bioassays and *in silico* predictive models. Acute toxicity testing on *Daphnia pulex* was conducted in accordance with the Organisation for Economic Cooperation and Development (OECD) Guideline 202, and the 1-hour LC_{50} was estimated to be 36.95 µg/mL. The rapid mortality observed in the temporal kill kinetics and survival analysis suggests that indoxacarb exhibits an acute onset of action. Quantitative Structure–Activity Relationship (QSAR)-based predictions using Ecological Structure-Activity Relationship Model (ECOSAR) (v2.2) aligned moderately well with observed toxicity in *D. pulex* but showed inconsistent estimates for various fish and algal species. In contrast, the Toxicity Estimation Software Tool (T.E.S.T) model significantly overestimated LC_{50} values, underpredicting *D. pulex* sensitivity. SeqAPASS analysis based on sodium channel homology identified high cross-species susceptibility among non-target taxa, especially Hymenoptera and Lepidoptera, including ecologically critical pollinators and endangered butterflies.

Major Findings: The findings of the study indicate that indoxacarb demonstrates acute toxicity to *D. pulex* in both *in vivo* and *in silico* assessments. Furthermore, *in silico* analysis indicate a high ecological risk to non-target taxa, including pollinators and endangered Lepidoptera, underscoring its potential adverse impact on ecosystem. The study underscores the need for integrating predictive models with empirical data through a multifaceted ecotoxicological approach to achieve more accurate and sustainable pesticide risk assessments.

Keywords: *Daphnia pulex*, ECOSAR, Ecotoxicity, Indoxacarb, SeqAPASS, Temporal Kill Kinetics, T.E.S.T

1. Introduction

Agriculture plays a pivotal role in the genesis and evolution of human civilisation. The contemporary agricultural practices have integrated innovative modern methodologies aimed at enhancing crop yield, notably the utilisation of chemical fertilisers and pesticides. While such chemicals have significantly contributed to the upsurge in global food production and consumption, they have also been associated with potential health and ecological adversities. Furthermore, the indiscriminate use of pesticides has altered the biome of non-target species, contaminated the ground

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Article Received on: 01.08.2024 Revised on: 14.06.2025 Accepted on: 17.06.2025

and surface water resources, and degraded soil quality, along with other environmental concerns[1,2]. The various application methods of agrochemicals, including spray drift, volatilisation, run-off and leaching, inadvertently channel these xenobiotics into soils and adjacent water bodies.

The conventional ecotoxicological assessments often rely on laboratory-based *in vivo* bioassays. While these methods are considered reliable and provide integrative data on biological effects such as growth, reproduction, and mortality, they are often limited by several significant drawbacks: high costs, time-intensive methods and ethical issues associated with animal experimentation[3]. To address these limitations, QSAR models, including tools like ECOSAR and T.E.S.T., have become increasingly utilised for preliminary toxicity screening. However, these models, which predict toxicity based on molecular structure, often fail to account for species-specific physiological differences and environmental variables[4]. Consequently, there is a growing need to validate these models against experimental data to assess their reliability. In parallel, cross-species extrapolation tools, such as Sequence Alignment to Predict Across Species Susceptibility (SeqAPASS), are emerging as powerful platforms to identify potentially vulnerable species by comparing protein target homology across taxa[5,6]. These tools are particularly valuable in predicting risks to non-target and endangered species, which are seldom included in standard toxicity assays.

Indoxacarb, an oxadiazine insecticide, has gained prominence for its selective action against lepidopteran pests through sodium channel blockade[7]. Despite its targeted mechanism, several recent studies have raised concerns over its broader environmental impact. Indoxacarb and its toxic metabolite, Decarbomethoxyllated JW062 (DCJW), have been detected in surface waters and sediments, raising questions about their potential toxicity to aquatic organisms and beneficial insects[8,9]. Chronic exposure to indoxacarb has been shown to cause significant adverse effects on non-target organisms across both aquatic and terrestrial ecosystems. In *Chironomus riparius*, it impairs growth and emergence while triggering oxidative stress and detoxification responses, indicating sublethal risks to aquatic insects[10]. Likewise, in *Theba pisana* (white garden snail), a 28-day dietary exposure to environmentally relevant concentrations of indoxacarb induced physiological, biochemical, and histopathological alterations, highlighting its terrestrial ecotoxicity[11]. Laboratory studies from our research demonstrated a median Lethal Dose (LD_{50}) of 1098 mg/kg in rats and noted elevated serum biochemical markers in layer birds following sub-acute exposure[12,13]. Additionally, indoxacarb showed high toxicity to *Artemia* species, with an LC_{50} of 5.13 µg/mL, pointing to ecological hazards for non-target aquatic crustaceans[14]. Therefore, while evaluating the potential adverse impacts of pesticides, it is imperative to consider non-target organisms present in terrestrial as well as aquatic environments[2,15].

Although several previous studies have examined the acute and chronic toxicity of indoxacarb in fish and aquatic invertebrates, there is a lack of comprehensive evaluations that integrate computational predictions with experimental data and cross-species sensitivity assessments. This investigation addresses the research gap through a multidimensional approach that integrates *in vivo* toxicity testing in *D. pulex*, *in silico* toxicity predictions using QSAR-based models (ECOSAR and T.E.S.T.) and cross-species susceptibility analysis through SeqAPASS. This integrated strategy offers a novel and holistic framework for evaluating the ecological risks of indoxacarb, enhancing the accuracy and relevance of environmental risk assessments for non-target species.

2. Materials and Methods

2.1. *In silico* Ecotoxicity Assessment

2.1.1. Toxic Hazard Assessment of Indoxacarb on Aquatic Organisms

The software ECOSAR (version 2.2)[16] was used to predict the hazard of indoxacarb on aquatic organisms. The program employed the predictive Structure-Activity Relationship (SAR) to assess both acute and chronic toxicity of a chemical to marine and freshwater organisms. ECOSAR is developed from extensive data of ecotoxicity studies from the ECOTOX database, encompassing 130 structural classes, which complies with the regulations of the U.S. EPA Office of Chemical Safety and Pollution Prevention[17]. The Chemical Abstracts Service (CAS) number of the chemical indoxacarb (144171-61-9) was entered into the software ECOSAR for the prediction of toxicity. The model predicted the median lethal concentration (LC_{50}), Effective Concentration (EC_{50}) and Chronic Value (ChV) by log K_{ow} (octanol-water partition coefficient) dependent linear regression models per species and per chemical class. ChV is calculated as the geometric mean of the No Observed Effect Concentration (NOEC) and the Lowest Observed Effect Concentration (LOEC)[18]. If the measured

log K_{ow} is not available, the U.S. EPA's Estimation Programs Interface Suite (EPISuit) model was used to generate the log K_{ow} using the KOWWIN application[17]. Three model organisms (green algae, daphnids and fishes) were used for the assessment of acute LC_{50}s and chronic toxicity values.

2.1.2. Prediction of Cross-Species Susceptibility of Indoxacarb

The program developed by the U.S. Environmental Protection Agency, SeqAPASS version 4 software, was used to predict the toxicological susceptibility of indoxacarb across different species. The tool is designed as a quick and cost-effective method for extrapolating the toxic hazard assessment of chemicals from model species to species of concern by evaluating protein structural similarities and differences. SeqAPASS precisely compares the amino acid sequence of the protein target for a chemical in a known sensitive species with the sequences of over 95 million proteins in the National Centre for Biotechnology Information (NCBI) protein database. This comparison yields sequence similarity metrics, which are utilised to infer the potential cross-species susceptibility to chemicals[5]. Since indoxacarb acts on sodium channels, the query protein used for evaluating primary amino acid sequence conservation was given as sodium channel protein Nach-like (NCBI Reference Sequence accession XP_046452024) of *D. pulex*[19].

2.1.3. In silico LC_{50} of Indoxacarb in Daphnia pulex Using Toxicity Estimation Software Tool

The Toxicity Estimation Software Tool (T.E.S.T.) is a computerised predictive system available online from the Environmental Protection Agency, which incorporates QSAR mathematical models[20]. T.E.S.T. employs a range of toxicity endpoints to predict acute toxicity values based on the physical properties of the molecular structure[19]. T.E.S.T. offers various prediction methods, including hierarchical clustering, nearest neighbour approaches, consensus-based methods, the Food and Drug Administration (FDA) method, and a single model approach using a multiple linear regression model. Among the various clusters, the model from the appropriate cluster is utilised to make the predictions for test compounds. In the current study, we employed the consensus method, where the predicted toxicity is determined by averaging the predictions from the various QSAR methods.

2.2 *In vivo* Ecotoxicity Assessment on *Daphnia pulex*

2.2.1 Test Organism

Daphnia pulex, popularly known as the "water flea," is a highly sensitive organism that can be easily cultivated in laboratory conditions, making it an excellent model organism for assessing ecotoxicity. It is a freshwater crustacean and a pivotal species in the pelagic zone of most freshwater habitats, serving as a crucial link between primary production and higher trophic levels in the aquatic ecosystem. The organism, *D. pulex*, for the study was obtained from laboratory cultures maintained at the Department of Veterinary Pharmacology and Toxicology (CVAS, Thrissur). The model was cultivated as clonal cultures for several generations before the experiments, using dechlorinated distilled water as the culture medium with a pH of 6–9, hardness of 140–250 mg/L, and dissolved oxygen concentration of 4.0 mg/L[12]. *D. pulex* were maintained under a 16-hour light: 8-hour dark photoperiod, at a temperature of 20 ± 1 °C, and were fed yeast powder and papaya leaves[21]. Adult *D. pulex* (gravid females) were sorted 24 hours before the test, and the neonates produced by these adults were utilised in the experiment.

2.2.2 Test Chemical

Technical grade indoxacarb was procured from M/s Urban Crop Science, India. The ten different concentrations of indoxacarb solution, ranging from 15 µg/ml to 1000 µg/ml, were prepared by dissolving the required amount of indoxacarb into known volumes of distilled water.

2.2.3 In vivo Acute Toxicity of Indoxacarb

A modified test method of OECD guideline 202 ("Biological Test Method: Acute Lethality Test Using Daphnia spp") was used to conduct the acute (1 h) *D. pulex* bioassay[22]. The concentration range linked with indoxacarb toxicity was unknown before the experiment. Therefore, preliminary range-finding tests were carried out at concentrations of 10, 1 and 0.1 mg/mL, using groups of five test organisms. The preliminary range-finding tests helped to obtain the concentration range to be employed in the definitive tests[23]. Based on the results of the range-finding test, ten different concentrations—1000, 500, 375, 250, 125, 100, 62.5, 50, 31.25, and 15.625 µg/mL—were selected for the main study. The experiments were conducted under static non-renewal

conditions. The test was carried out by placing 5 neonates in 10ml of indoxacarb solutions at specified concentrations. The experiment was performed in quadruplicate, and a control test was also performed in distilled water. *D. pulex* that did not exhibit motility or swimming movement for 15 seconds following gentle stirring were taken as immobile or dead. The number of living and dead neonates was noted at 1 hour after the initiation of each trial.

2.2.4 *In vivo* Temporal Kill Kinetics of Indoxacarb

Besides the initial *in silico* toxicological studies and acute *in vivo* toxicity tests, the temporal kill kinetics of indoxacarb were also later analysed in *D. pulex*. The study for temporal kill kinetics was conducted according to the previous method, OECD guideline 202 (Biological Test Method: Acute Lethality Test Using Daphnia spp), whereas only a single concentration of indoxacarb was selected. Based on the findings from the acute toxicity assay conducted on *D. pulex*, a concentration of 250 µg/ml of indoxacarb solution was taken for the current study, since it is the lowest dose which can induce complete mortality of organisms in 1 hour. The test was carried out by placing 5 neonates in 10ml of indoxacarb solution at a concentration of 250 µg/ml for 1 hour. The experiment was carried out in four replicates, and a control group was included using distilled water. The time of death of each organism was recorded up to 1 hour after the initiation of each trial[16].

2.3 Statistical Analysis

The data from the experiment were analysed using GraphPad Prism 5 software for estimating the mortality pattern of organisms. The median LC_{50} of indoxacarb for *D. pulex* for one hour was calculated using log agonist versus per cent mortality in non-linear regression analysis.

3. Results

3.1 *In silico* Ecotoxicity Assessment

3.1.1 Toxic Hazard Assessment of Indoxacarb on Aquatic Organisms

Table 1 summarises the acute toxicity predictions by ECOSAR for the test compound indoxacarb on aquatic organisms. The software assigned the test substance, indoxacarb, into four different chemical classes, namely hydrazines, neutral organics, carbamate esters and carbonyl urea, based on structural characteristics and estimated the acute toxic hazard of the compound to the aquatic biome. The selected organisms represent a simplified aquatic food chain, comprising algae, an aquatic insect, and their predator, fish. The 96-hour LC_{50} (acute toxicity) and chronic toxicity values corresponding to the above substructures of indoxacarb were also presented in Table 1. According to Golbamaki *et al.*,[24] if ECOSAR assigns the substance to multiple classes, resulting in more than one toxicity value, the lowest value should be considered, as it represents the highest toxicity. Hence, in the current study, indoxacarb contains a carbonyl urea linkage, which shows the lowest toxicity values (48-h LC_{50} and ChV values of 0.095 mg/L and 0.012 mg/L, respectively, for *D. pulex*). More important is the Chronic toxicity Value (ChV) for fish, where the same urea linkage present in may contribute to increased toxicity in aquatic ecosystems. Algae were also sensitive, whereas fish exhibited greater tolerance.

3.1.2 Prediction of Cross-Species Susceptibility of Indoxacarb Using Daphnia pulex Sodium Channel Protein

The primary amino acid sequence comparison of the *D. pulex* sodium channel protein in SeqAPASS software

Table 1. ECOSAR-predicted acute toxicity of Indoxacarb to aquatic organisms representing various trophic levels, based on different chemical substructures.

Organism	ECOSAR Prediction Parameters	Hydrazines	Neutral Organics	Carbamate Esters	Carbonyl Urea
Fish	96 h LC50 (mg/L)	0.078	0.567	0.335	0.968
	ChV (mg/L)	0.036	0.079	0.035	0.0074
Daphnid	48 h LC50 (mg/L)	0.643	0.433	0.293	0.095
	ChV (mg/L)	0.027	0.096	0.059	0.012
Green Algae	96 h EC50 (mg/L)	0.297	1.10	0.316	0.053
	ChV (mg/L)	0.048	0.557	0.194	0.011

*ChV: Chronic toxicity value: the geometric mean of the No Observed Effect Concentration (NOEC) and the Lowest Observed Effect Concentration (LOEC)[16].

resulted in sequence alignment across 42 taxonomic classes. Among them, 276 species from different classes, such as *Insecta*, *Arachnida*, *Aves*, *Mammalia*, and *Amphibia,* were predicted to be susceptible to chemicals known to act on the *D. pulex* sodium channel protein (Figure 1). The percentage similarity of the primary amino acid sequences across different species, when compared with the sodium channel protein of *D. pulex*, ranged from 99.14% to 4.45%. A cut-off value of 13.72% was used to predict species susceptibility. The various species aligned with different types of proteins, such as the acid-sensing ion channel, amiloride-sensitive sodium channel, degenerin (epithelial sodium channel), and pickpocket protein (epithelial sodium channel in Drosophila) for cross-species predictability. The proportion of different taxonomical classes that showed cross-species susceptibility (except insects) is shown in Figure 1b. The maximum number of susceptible species was found for chromadorea (nematode), followed by aves (birds), arachnids and mammals (Figure1b).

In the present study, the SeqAPASS software of the US EPA was utilised to predict the cross-species susceptibility of Indoxacarb. The initial *in silico* evaluation of indoxacarb toxicity on the ECOSAR program predicted the toxicity of the test compound to aquatic organisms such as fish, daphnid, and green algae. Since daphnids are a model organism for ecotoxicological studies, their sodium channel protein has been adopted as the query protein in the prediction of cross-species susceptibility in SeqAPASS[25]. As illustrated in Figure 1a, according to the prediction of SeqAPASS, the majority of susceptible species to the toxicity of indoxacarb belong to the class Insecta. Most of the current evidence supports the notion because indoxacarb is a candidate from the latest generation of insecticides, and as per previous literature, it is effective against a broad range of insect pests from several orders, including Hemiptera, Homoptera, Coleoptera, and Lepidoptera[7,26]. However, the SeqAPASS-based prediction of insect toxicity offers a different perspective since it is based on the ecotoxicological aspect. Most of the species analysed in the software belong to a beneficial class of insects rather than agricultural pests. As per SeqAPASS, the order Hymenoptera has the highest number of susceptible species to indoxacarb

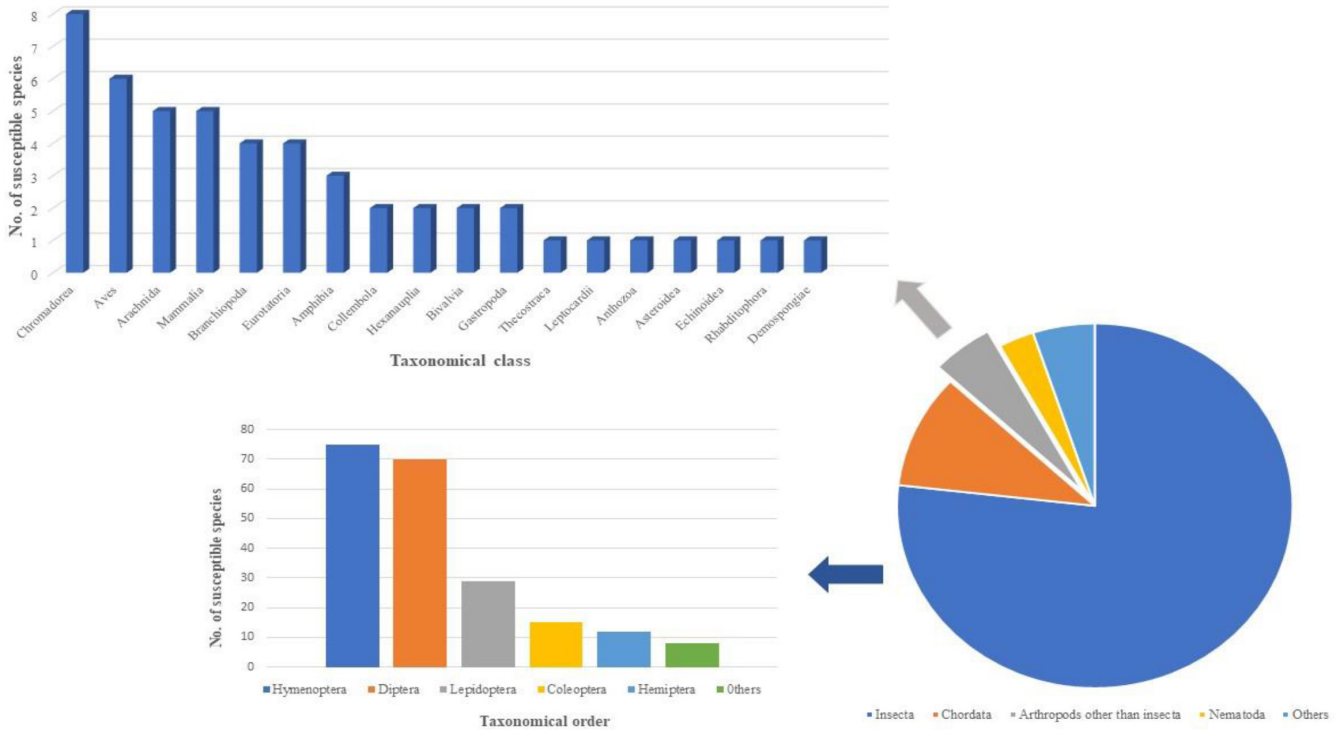


Figure 1. **(A)**: Pie chart showing the distribution of different taxonomical classes with cross-species susceptibility to the chemicals known to act on the *D. pulex* sodium channel protein. **(B):** The bar graph on the top shows the no. of susceptible species in the different taxonomical classes of arthropods (excluding insects). **(C):** The bar graph on the lower part shows the no. of susceptible species in the taxonomical class of Insecta.

toxicity, which is depicted in Figure 1c. Hymenoptera is among the largest insect orders, comprising a wide range of species including bees, wasps, hornets, sawflies, and ants [27]. The toxicity of indoxacarb to these species has also been reported earlier by different researchers[28,29].

3.1.3 *In silico Prediction of 48h LC_{50} of Indoxacarb in Daphnia pulex Using T.E.S.T.*

The consensus LC_{50} value of indoxacarb predicted by the T.E.S.T. software was 44.3 µg/mL. The predicted value for the hierarchical clustering model was 54.9 µg/mL, the single model was 37.5 µg/mL, and the nearest neighbour was 42.2 µg/mL. In the current investigation, the consensus model was adopted to predict toxicity because it compensates for the shortcomings of the individual models by averaging estimates from the individual models.

3.1.4 *In vivo Acute Toxicity of Indoxacarb*

To evaluate the acute bioassay concentration v/s response of indoxacarb, the average immobility of *D. pulex* across treatment groups was analysed using a non-linear regression curve. The model estimated the 1 h LC_{50} of indoxacarb as 36.95 mg/L with its 95% confidence limit range of 27.4 to 49.79 mg/L. The logarithmic concentrations of indoxacarb showed a strong correlation with the mortality of organisms (R^2 = 0.8010). At the lower concentration of 15 µg/ml, no mortality was observed after 1 hour, while 100% mortality was observed in concentrations of 250 µg/ml and above. Even though sub-lethal behavioural changes were not an evaluation endpoint, some alterations in swimming behaviour were noticed in exposed organisms, like a reduction in hopping, sinking of organisms, as well as minimal movement of organisms.

3.1.5 *In vivo Temporal Kill Kinetics of Indoxacarb*

The death of each neonate of *D. pulex* during a period of 1 hour was obtained from the bioassay and used to calculate the cumulative mortality of neonates of *D. pulex* corresponding to a specific duration. The graph plotted with time on the X-axis and cumulative mortality percentage on the Y-axis revealed a polynomial regression, which is shown in Figure 2. The above data was analysed using non-linear regression analysis using online resources (www.mycurvefit.com), and the following polynomial equation was obtained. Per cent motility (Y) = $0.0001x^5$-$0.0104x^4$+$0.3541x^3$-$5.8112x^2$+48.615x-88.183, where x = time (minutes) and the time of death of the various mortality percentage of neonates (1,25,50, 75, 90, 95, and 99%) was calculated, which is given in Table 2. Using the above equation, the mortality percentage of indoxacarb and its corresponding time was calculated when used as the dose rate of 250 µg/mL, and the results are expressed in Table 2. From this result, it is evident that 50% deaths occurred by 5.19 minutes and 99% deaths occurred by 20.30 minutes, indicating that Indoxacarb is a quick-acting ecotoxin, especially for aquatic arthropods.

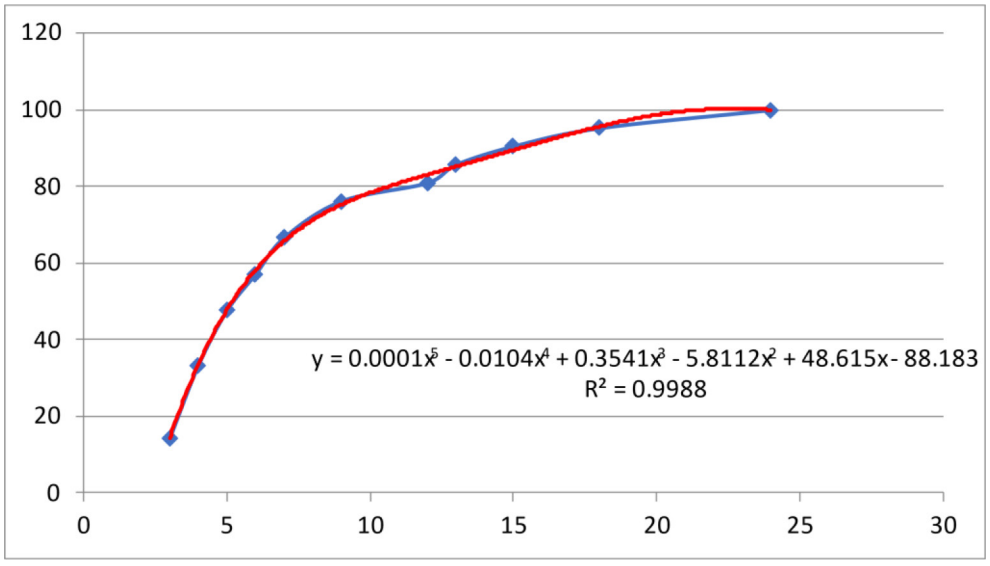


Figure 2. Graph of cumulative mortality percentage versus time. The X axis indicates time (in minutes), and the Y axis is per cent mortality

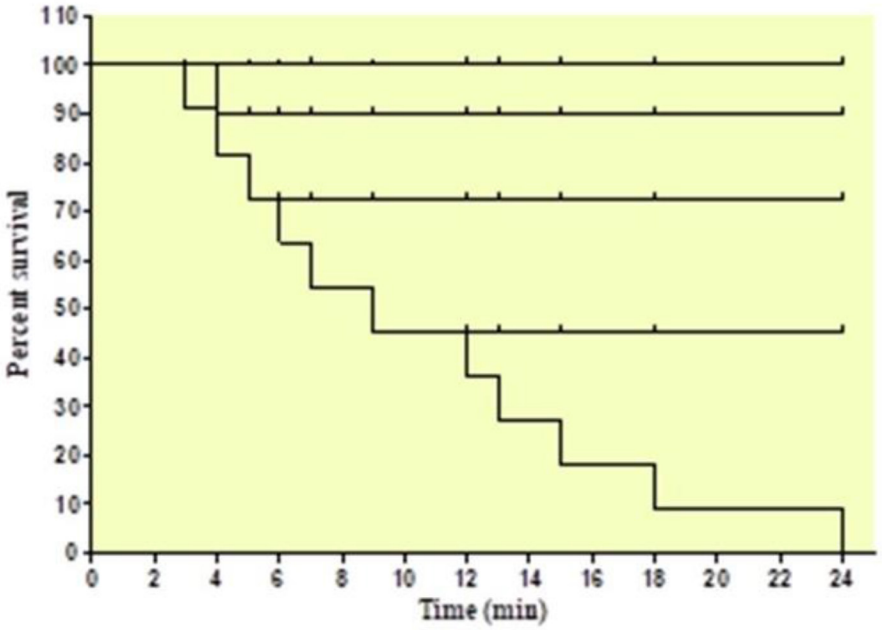


Figure 3. Aggregate survival percentage of *D. pulex*.

Table 2. Temporally extrapolated mo80rtality rate of *D. pulex* in the presence of Indoxacarb @ 250 µg/mL

Per cent Mortality of *D. pulex* (%)	Predicted Time of Death (min)
1	2.45
25	3.53
50	5.19
75	8.93
90	15.26
95	17.68
99	20.30

From the predicted time of death of organisms, the survival percentage of organisms was calculated using GraphPad Prism 5 software, which is depicted in Figure 3. The survival analysis shows that the rate of death is maximum between 9-12 minutes.

4. Discussion

The present study aims to evaluate the ecotoxicity of indoxacarb on non-target organisms by integrating *in vivo* toxicity data with QSAR-based *in silico* predictions and cross-species susceptibility analysis. The software tools ECOSAR, T.E.S.T., and SeqAPASS were employed to generate *in silico* toxicity data, while *in vivo* analysis was conducted using *D. pulex* as a model organism. Furthermore, the results from the *in vivo* study were compared with the *in silico* predictions to assess the reliability and predictive accuracy of the computational models.

The US EPA software, ECOSAR, predicted that the major contributing factor to the aquatic ecotoxicity of indoxacarb is the presence of carbonyl urea in its structure[24] and evaluated the acute as well as chronic toxicity parameters of indoxacarb in aquatic organisms. The earlier reported acute toxicity studies of indoxacarb on fish have shown that the 96-hour LC_{50} values vary depending on the taxonomic class of the species. For instance, the acute toxicity tests conducted on the Indian major carp (*Labeo rohita*) revealed that the 96-h LC_{50} falls in the range of 0.0521 - 0.0531 mg/L, whereas in Rainbow trout it showed a lower value of 0.024 mg/L[30-32]. In contrast, the 96-h LC_{50} value predicted by ECOSAR was significantly higher, at 0.968 mg/L, suggesting an underestimation of toxicity in these sensitive species. Meanwhile, the studies in *Cyprinus carpio* found out the 96-h LC_{50} of indoxacarb was 16.85 mg/L[33,] and the ECOSAR value appears to overestimate toxicity, predicting the compound to be more harmful than observed. These discrepancies highlight the limitations of QSAR-based models like ECOSAR, which rely on structural similarity and do not account for species-specific physiological and biochemical differences[34,35].

The acute toxicity assessment of Indoxacarb on *D. pulex* reveals variation between empirical findings and

predictions generated by different QSAR-based models. A 2018 study conducted in China reported a 48-hour LC_{50} value of 0.0237 mg/L for *D. pulex*, which closely aligns with the ECOSAR-predicted value of 0.095 mg/L, indicating a reasonable predictive performance for this model[19]. However, the T.E.S.T. software predicted a markedly higher 48-hour LC_{50} of 44.3 mg/L, indicating a substantial overestimation of the tolerance level in *D. pulex*, exceeding the 1-hour LC_{50} value of 36.95 mg/L determined in the present study. This inconsistency suggests that T.E.S.T. significantly underestimates the sensitivity of *D. pulex* to Indoxacarb, even during short-term exposure. On the other hand, ECOSAR provided a toxicity estimate that aligns more closely with empirical long-duration data, demonstrating better accuracy and reliability for environmental risk assessment of indoxacarb. This discrepancy highlights the variability in prediction accuracy among QSAR-based models and emphasises the need for careful model selection and validation[36,37]. Golbamaki *et al*., assessed and compared eight *in silico* modelling packages for predicting acute toxicity to *D. pulex*. They reported that variability in experimental data for *D. pulex* is likely the primary reason for the suboptimal performance of the predictive models. High-quality data in the training set is crucial for developing reliable QSARs[24].

ECOSAR predicted a 96-hour EC_{50} value of 0.053 mg/L for green algae, indicating high toxicity. However, experimental data, such as the 72-hour EC_{50} reported for *Pseudokirchneriella subcapitata*, indicate considerably higher values, with estimates exceeding 0.6 mg/L[38]. This variation may be attributed to differences in exposure duration, as longer test periods generally yield lower EC_{50} values due to extended chemical interaction[39,40]. Additionally, predictive models like ECOSAR rely on quantitative structure–activity relationships and may not fully capture environmental complexity, potentially leading to under- or overestimation. The stability and bioavailability of indoxacarb in test conditions can also influence toxicity outcomes[41,42]. These findings highlight the need to integrate both predictive and experimental data when evaluating environmental risk, as reliance on a single approach may not provide an accurate toxicity profile.

The ECOSAR model predicted the 96-hour LC_{50} and chronic toxicity (ChV) values for fish and green algae, and 48-hour LC_{50} values for *D. pulex*. The 96-hour LC_{50} values were extrapolated to 48 hours using Haber's rule, which assumes a constant concentration-time–time ($C \times t$) relationship[43,44]. Based on this, the extrapolated 48-hour LC_{50} values were approximately 1.936 mg/L for fish and 0.106 mg/L for green algae. In comparison, *D. pulex* exhibited a 48-hour LC_{50} of 0.095 mg/L, indicating higher sensitivity than fish and algae. Chronic toxicity (ChV) values further reinforce this pattern, with *D. pulex* (ChV: 0.012 mg/L) and fish (ChV: 0.0074 mg/L) showing greater vulnerability compared to green algae (ChV: 0.011 mg/L). Overall, the data suggest that *D. pulex* is among the most sensitive species to indoxacarb exposure.

In the present acute bioassay, *D. pulex* exhibited concentration-dependent mortality upon exposure to Indoxacarb, with a 1-hour LC50 value of 36.95 mg/L. Notably, Herrero-Hernández *et al*. (2020) reported Indoxacarb concentrations in water bodies of Spanish vineyard regions exceeding the legally permissible limit of 0.1 µg/L, raising concerns about chronic environmental exposure[45]. Furthermore, recent findings by Ren *et al*. highlight the bioaccumulation potential of indoxacarb, suggesting that even low environmental concentrations can lead to toxic internal doses over time[46]. These observations imply that the experimentally determined LC50 value may have environmental relevance under prolonged exposure, posing a serious threat to aquatic ecosystems.

The temporal mortality kinetics of *D. pulex* in the present study revealed a rapid initial increase in mortality, likely due to the bioactivation of indoxacarb to its toxic metabolite DCJW. At a test concentration of 250 µg/mL, the predicted time for 99% mortality was 20.3 minutes, indicating the acute action of indoxacarb. The wider time gap between 75% and 90% mortality suggests some variability in individual tolerance but still reflects a steep dose-response curve. Decarbomethoxyllated JW062 (DCJW), a potent sodium channel blocker, exerts its lethal effects by stabilising the slow-inactivated state of voltage-gated sodium channels, thereby inhibiting their recovery and reducing the availability of excitable channels. This mechanism disrupts normal neural signalling and results in rapid, acute toxicity, as supported by survival analysis[46-49]. The rate of bioactivation of indoxacarb is a crucial factor in determining the toxicity of this compound in various species[7].

The SeqAPASS analysis highlighted that species within the class Insecta are the most susceptible to indoxacarb, aligning with its known mode of action as

a sodium channel blocker targeting insects. Although susceptibility was expected in pest orders like Hemiptera, Homoptera, Coleoptera, and Lepidoptera, the analysis notably identified a high number of vulnerable species within Hymenoptera—a group that includes numerous key pollinators and ecologically vital species like bees and wasps. This raises concerns about potential non-target impacts on beneficial insect populations. Other insect orders, including Diptera and Coleoptera, were also identified as sensitive to varying degrees, emphasising the need for careful ecotoxicological evaluation of indoxacarb.

SeqAPASS extended its predictions of indoxacarb toxicity beyond Insecta, indicating potential sensitivity in taxa such as Chordata, nematodes, and other arthropods, suggesting broader ecotoxicological risks. A notable finding in the present study was the predicted susceptibility of 11 butterfly species, including the Mountain Apollo (*Parnassius apollo*), which is listed under Appendix II of the Convention on International Trade in Endangered Species (CITES)[50,51]. But the limited reports on *in vivo* toxicity on non-pest Lepidoptera highlight a critical research gap. Overall, SeqAPASS serves as a valuable tool for identifying non-target species at risk, guiding species selection for testing, supporting cross-species extrapolations, and enhancing the ecological relevance of pesticide risk assessments.

5. Conclusion

This study demonstrates that integrating *in vivo* experiments with *in silico* modelling provides a more comprehensive understanding of the ecotoxicity profile of indoxacarb, while also demonstrating the constraints of relying solely on computational predictions. Although ECOSAR provided reasonably accurate predictions for certain species, such as *D. pulex*, it failed to capture the full extent of toxicity in various fish species, and T.E.S.T. significantly underestimated *D. pulex* sensitivity. These discrepancies highlight the importance of validating QSAR-based predictions with empirical data, as model-based estimates may overlook species-specific responses and environmental variables. The SeqAPASS analysis further reveals the broad ecotoxicological risk of indoxacarb, particularly to beneficial non-target insects like pollinators and endangered Lepidoptera species, suggesting a need for expanded testing and risk assessment beyond pest organisms. Collectively, the findings emphasise the necessity of a multifaceted ecotoxicity evaluation framework that combines computational modelling with targeted bioassays and cross-species susceptibility analysis to ensure more comprehensive and ecologically relevant pesticide risk assessments.

6. Acknowledgements

The authors are thankful for the funding provided by Kerala State Council for Science, Technology and Environment vide KSCSTE/919/2022-SRSLS. The Authors are thankful to Kerala Veterinary and Animal Sciences University, Pookode, for providing facilities and Mr Sudheesh Kalthil for providing the cultures of *D. pulex*

7. Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request

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