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Acute Toxicity of Electronic Cigarette Refill Liquids in Aquatic Vertebrates: Comparative Responses of Fish Embryos, Fish Juveniles and Amphibian Larvae

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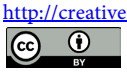

Abstract

The increasing use of electronic cigarettes has raised concerns regarding the potential environmental impact of e-cigarette refill liquids and their release into aquatic ecosystems. Despite their growing consumption worldwide, information concerning their ecotoxicological effects on aquatic vertebrates remains limited. In the present study, the acute toxicity of an electronic cigarette refill liquid was evaluated using different aquatic vertebrate models, including fish eggs (*Danio rerio*), juvenile fish (*Jenynsia multidentata*), and amphibian larvae (*Rhinella arenarum*). Acute toxicity assays were performed using a stock solution of e-cigarette liquid (1 mL·L^{-1}), from which different dilutions were prepared. Juvenile fish and amphibian larvae were exposed to concentrations ranging from 5% to 50% of the stock solution for 96 h, while fish eggs were exposed to concentrations between 3% and 50% for 72 h. Mortality was recorded at regular intervals throughout the exposure period. No mortality was observed in *J. multidentata* juveniles or *R. arenarum* larvae at any of the concentrations tested, indicating low acute toxicity under the experimental conditions employed. In contrast, *D. rerio* eggs exhibited concentration-dependent mortality, with the highest effects observed at 50% of the stock solution, where mortality ranged between 66% and 100% in duplicate assays. Lower concentrations produced variable responses, although embryonic stages consistently showed greater sensitivity than juvenile organisms. The results demonstrate that the toxicological response to e-cigarette refill liquids is strongly influenced by developmental stage. While juvenile fish and amphibian larvae appeared relatively resistant

to exposure, fish embryos showed increased susceptibility, suggesting that early developmental stages may represent sensitive indicators for assessing the environmental risk of emerging contaminants associated with electronic cigarettes. These findings contribute to the understanding of the ecotoxicological effects of vaping-related products and highlight the importance of including multiple biological models and life stages in environmental risk assessments.

Subject Areas

Environmental Chemistry

Keywords

Electronic Cigarettes, Vaping Liquids, Ecotoxicology, *Danio rerio*, *Jenynsia multidentata*, *Rhinella arenarum*, Aquatic Pollution

1. Introduction

Electronic cigarettes (e-cigarettes) and vaping devices have experienced a remarkable increase in popularity over the last decade, particularly among adolescents and young adults. These products are frequently promoted as safer alternatives to conventional tobacco cigarettes; however, growing evidence suggests that they may also represent a source of environmental contamination. The widespread use and disposal of e-cigarette liquids, cartridges, and associated residues introduce a variety of chemical substances into aquatic and terrestrial ecosystems, including nicotine, flavoring agents, humectants, and degradation products whose environmental effects remain poorly understood [1]-[3].

In contrast to conventional cigarette waste, which has been extensively recognized as an emerging environmental pollutant, the ecotoxicological implications of e-cigarette residues have received comparatively less attention. Previous studies have demonstrated that cigarette butts constitute one of the most abundant forms of anthropogenic litter worldwide and are capable of releasing numerous toxic substances into aquatic environments, including nicotine, polycyclic aromatic hydrocarbons, heavy metals, aromatic amines, volatile organic compounds, and microplastic fibers [4]-[6]. These contaminants have been associated with adverse effects on aquatic organisms, including mortality, behavioral alterations, oxidative stress, developmental abnormalities, and impairment of ecosystem functions [7]-[9].

Among the compounds present in tobacco-derived products, nicotine is considered one of the most relevant due to its high biological activity and environmental occurrence. Recent work by our research group developed a sensitive fluorescence-based methodology employing a luminescent europium-terbium metal-organic framework for the determination of trace concentrations of nicotine in environmental waters contaminated by cigarette butt residues [10]. Furthermore, acute toxicity assays performed using *Poecilia reticulata* demonstrated that ciga-

rette butt leachates can produce significant mortality and behavioral alterations even when nicotine concentrations are substantially lower than the experimentally determined LC50 value for pure nicotine, suggesting the participation of additional toxic compounds released from tobacco-derived residues [11].

Although the environmental impact of conventional cigarette waste has increasingly been investigated, considerably less information is available regarding the potential ecotoxicological effects of e-cigarette refill liquids. Most studies have focused primarily on human health concerns, while the effects of these products on aquatic organisms remain largely unexplored. This lack of information is particularly relevant considering the increasing consumption of vaping products and the potential release of their chemical constituents into aquatic ecosystems through improper disposal, accidental spills, wastewater discharges, or environmental weathering processes [2] [3].

Fish and amphibians constitute valuable biological models for ecotoxicological studies because of their sensitivity to environmental contaminants and their ecological relevance in freshwater ecosystems. In particular, early developmental stages are frequently more susceptible to chemical exposure than juvenile or adult organisms due to incomplete physiological development, high metabolic activity, and reduced detoxification capacity [12]-[14]. Consequently, the use of multiple biological models and developmental stages may provide a more comprehensive understanding of the environmental risks associated with emerging contaminants.

Therefore, the aim of the present study was to evaluate the acute toxicity of an electronic cigarette refill liquid using different aquatic vertebrate models, including fish eggs (*Danio rerio*), juvenile fish (*Jenynsia multidentata*), and amphibian larvae (*Rhinella arenarum*). The comparative responses obtained from these biological systems were analyzed in order to assess potential differences in sensitivity among developmental stages and taxonomic groups and to contribute to the growing body of knowledge regarding the environmental impact of contaminants associated with electronic cigarettes.

2. Materials and Methods

2.1. Biological Models

Larvae of *Rhinella arenarum*, juveniles of *Jenynsia multidentata*, and embryos of *Danio rerio* were used as biological models. Juvenile fish and embryos were obtained from laboratory cultures and maintained under standard aquarium conditions at approximately 25˚C. Amphibian larvae were obtained by collection in a natural environment (33˚17'44"S, 66˚20'16"W). Prior to experimentation, organisms were acclimated under laboratory conditions, and only healthy individuals showing normal behavior were selected for toxicity assays.

Embryos of *D. rerio* were obtained from breeding adults maintained under controlled laboratory conditions. Fertilized and viable embryos were identified under stereoscopic observation based on normal cleavage patterns, transparent chorion, and the absence of coagulation or developmental abnormalities. Unfertilized eggs

and embryos showing signs of non-viability prior to exposure were discarded and were not included in the mortality analysis.

2.2. Acute Toxicity Assays with E-Cigarette Liquid

Acute toxicity assays were carried out using a commercially available electronic cigarette refill liquid. According to the product label, the formulation contained nicotine (50 $mg \cdot mL^{-1}$), propylene glycol, vegetable glycerin, and natural and artificial flavoring agents. The tested product corresponded to a sour strawberry-dragonfruit flavored formulation (batch number EC081653). No quantitative information regarding the propylene glycol/vegetable glycerin ratio was provided by the manufacturer. The product was used as received without further modification. A stock solution was prepared at a nominal concentration of 1 $mL \cdot L^{-1}$. From this stock solution, different dilutions were prepared using standardized aquarium water to obtain final exposure concentrations expressed as percentages of the stock solution.

All assays were performed under static exposure conditions at 25˚C ± 1˚C under a natural photoperiod. No aeration or feeding was provided during the exposure period. Test solutions were not renewed throughout the experiment. Organisms were maintained in standardized aquarium water prepared under laboratory conditions. The protocol was adapted from the guidelines proposed by Johnson and Finley [15] and further modified by our research group.

Juveniles of *Jenynsia multidentata* and larvae of *Rhinella arenarum* were exposed to 5%, 10%, 20% and 50% dilutions of the stock solution. Each experimental system contained, for fish, 1000 mL of test solution and 10 organisms, and, for amphibians, 400 mL of test solution and 10 organisms. Assays were performed in duplicate. For *Jenynsia multidentata*, each treatment consisted of two independent vessels, each containing 10 organisms (n = 20 organisms per concentration). For *Rhinella arenarum*, each treatment consisted of two independent vessels, each containing 10 larvae (n = 20 larvae per concentration).

For *Danio rerio* embryos, each concentration was tested in duplicate in two independent wells, each containing three embryos (n = 6 embryos per concentration). Mortality was recorded after 24, 48, 72, and 96 h of exposure.

For embryo assays, the methodology described in OECD Guideline 236 [16] was followed.

In parallel, acute toxicity assays were carried out using eggs of *Danio rerio* as early developmental-stage models. Eggs were exposed to 3%, 6%, 12.5%, 25%, and 50% dilutions of the same stock solution in wells of 5 mL. Each well contained three eggs, and all treatments were performed in duplicate. Mortality was recorded after 24, 48, and 72 h of exposure.

Control systems were maintained under the same experimental conditions without the addition of e-cigarette liquid. Organisms were considered dead when a complete absence of movement and response to external stimulation was observed.

2.3. Statistical Analysis

Mortality data were expressed as percentages. Due to the exploratory nature of the study and the limited number of organisms used in some treatments, results were evaluated descriptively. Average mortality values obtained from duplicate assays were used for graphical representation and comparison among biological models.

3. Results and Discussion

3.1. Acute Toxicity in *Jenynsia multidentata* and *Rhinella arenarum*

The stock solution was prepared from a commercial electronic cigarette essence containing a declared nicotine concentration of 50 mg·mL^{-1}. An initial dilution of 1 mL·L^{-1} yielded a stock solution equivalent to 50 mg·L^{-1} nicotine. The different exposure concentrations were subsequently prepared from this stock solution.

No mortality was observed in either *Jenynsia multidentata* juveniles or *Rhinella arenarum* larvae during the 96 h exposure period. All organisms survived throughout the experiment, regardless of the concentration tested (5% - 50% of the stock solution, corresponding to 2.5 - 25 mg ·L^{-1} nicotine). No differences were detected among treatments or between duplicate assays. These results indicate that the electronic cigarette refill liquid did not produce acute lethal effects in juvenile fish or amphibian larvae under the experimental conditions employed.

The absence of mortality at concentrations up to 25 mg·L^{-1} nicotine suggests a relatively low acute toxicity of the tested formulation toward juvenile fish and amphibians under the experimental conditions employed.

3.2. Acute Toxicity in *Danio rerio* Embryos

Unlike the results obtained for fish and amphibians, *Danio rerio* embryos exhibited sensitivity to the electronic cigarette essence. Mortality was observed as early as 24 h post-exposure and reached the highest values in the treatment corresponding to 50% of the stock solution (25 mg·L^{-1} nicotine).

In general, mortality increased with exposure concentration, although a strictly linear dose-response relationship was not observed. This variability may be associated with the limited number of embryos tested per treatment and the inherent biological variability commonly observed in exploratory embryo toxicity assays. A limitation of the present study is the relatively small number of embryos used per treatment. Therefore, the observed mortality patterns should be interpreted as preliminary evidence of embryotoxicity and should be confirmed in future studies employing larger sample sizes (See Table 1).

The highest mortality (83%) was recorded at 25 mg·L^{-1} nicotine, indicating that embryonic stages are considerably more sensitive to exposure than juvenile fish and amphibians. These findings are consistent with previous reports describing the vulnerability of early developmental stages to nicotine and other constituents

of electronic cigarette liquids.

Table 1. Average mortality (%) of *Danio rerio* embryos exposed to different concentrations of electronic cigarette essence.

Treatment (% v/v)	Equivalent Nicotine Concentration (Mg·L^{-1})	24 h (%)	48 h (%)	72 h (%)
Control	0	0	0	0
3	1.5	16.7	50.0	50.0
6	3.0	0	33.3	33.3
12.5	6.25	16.7	50.0	50.0
25	12.5	33.3	33.3	33.3
50	25.0	83.3	83.3	83.3

The observed embryotoxicity may result not only from nicotine itself but also from the combined action of other formulation components, including propylene glycol, vegetable glycerin, and flavoring agents. Therefore, the toxicity detected in this study likely reflects the overall effect of the commercial formulation rather than nicotine alone (See Figure 1).

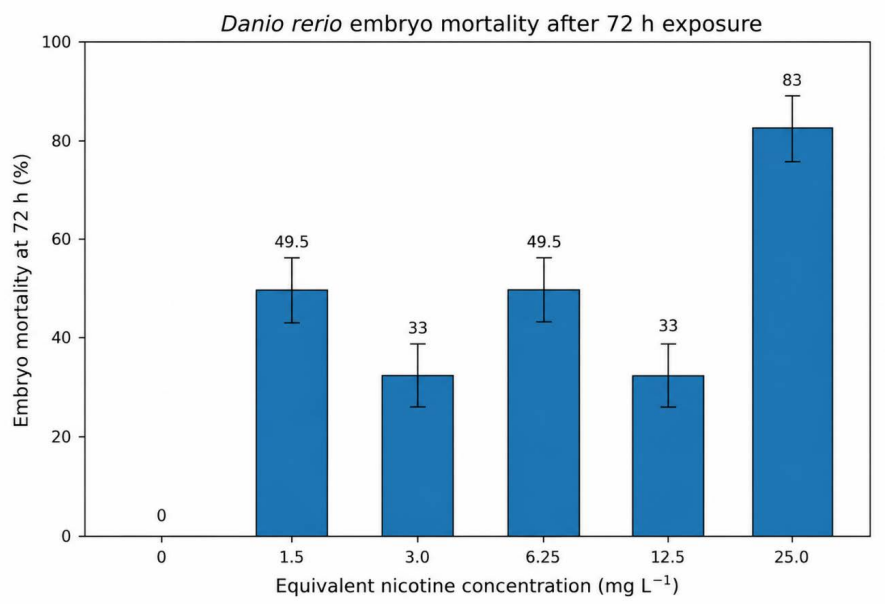

Figure 1. Mortality of *Danio rerio* embryos after 72 h of exposure to increasing concentrations of electronic cigarette essence. Concentrations are expressed as equivalent nicotine concentrations calculated from the manufacturer's declared nicotine content (50 mg·mL^{-1}). The highest mortality (83%) was observed at 25 mg·L^{-1} nicotine, indicating increased sensitivity of embryonic stages compared with juvenile fish and amphibians.

A comparison of the maximum mortality values recorded for each biological model is presented in Figure 2. The marked contrast between fish embryos and juvenile organisms highlights the importance of developmental stage as a deter-

minant of toxicological sensitivity.

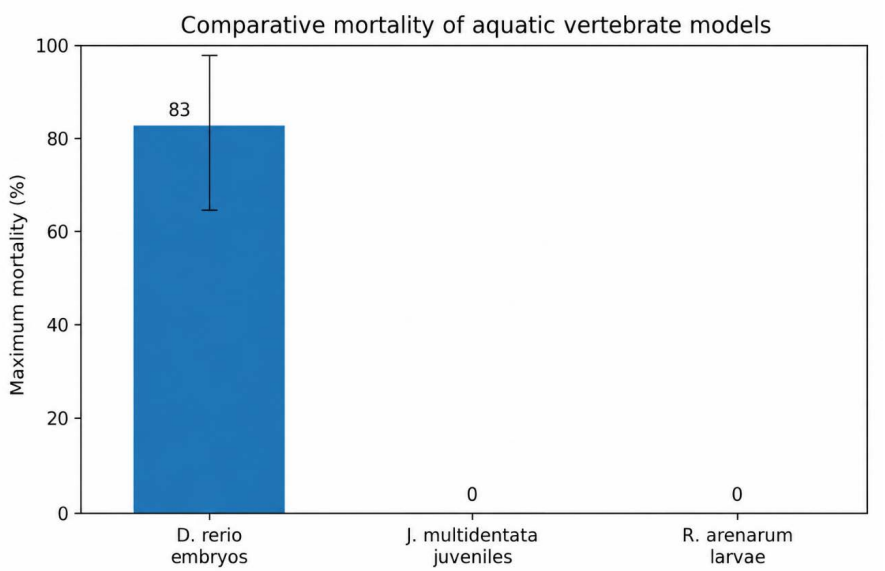

Figure 2. Comparative mortality observed in aquatic vertebrate models exposed to electronic cigarette liquid. Values correspond to the maximum mortality recorded during the exposure period.

3.3. Ecotoxicological Implications

The results obtained in this study demonstrate marked differences in sensitivity among the biological models evaluated. While no mortality was observed in juvenile fish (*J. multidentata*) or amphibian larvae (*R. arenarum*), embryos of *D. rerio* exhibited evident susceptibility to the tested e-cigarette liquid.

The higher sensitivity observed in embryos is consistent with previous reports indicating that early developmental stages are generally more vulnerable to environmental contaminants because of incomplete physiological development and limited detoxification capacity. Embryonic stages are particularly sensitive to compounds capable of interfering with cellular differentiation, organogenesis, and metabolic regulation.

The absence of mortality in juvenile fish and amphibian larvae suggests that the tested formulation presents relatively low acute toxicity toward these organisms under the exposure conditions employed. However, the mortality observed in embryos indicates that early life stages may represent more sensitive bioindicators for evaluating the environmental risks associated with electronic cigarette products.

Previous studies performed by our research group using pure nicotine reported acute toxicity in fish at concentrations lower than those evaluated here [11]. These differences suggest that species, developmental stage, exposure conditions, and the composition of the tested matrix may strongly influence toxicological responses. Therefore, direct comparisons between studies should be made with caution.

Although nicotine is considered one of the principal biologically active compounds present in vaping liquids, the observed effects cannot be attributed exclusively to nicotine. Commercial formulations also contain propylene glycol, vegetable glycerin, flavoring compounds, and other additives that may contribute to the overall toxicological response. Consequently, the embryotoxic effects observed in this work likely result from the combined action of several formulation components.

These findings reinforce the importance of incorporating multiple biological models and developmental stages into ecotoxicological evaluations of emerging contaminants associated with electronic cigarettes.

It should be noted that direct comparisons among biological models should be interpreted with caution because exposure duration and concentration ranges differed among embryo, juvenile fish, and amphibian assays. Therefore, the observed differences in sensitivity provide qualitative evidence of developmental-stage susceptibility rather than a quantitative ranking of species sensitivity.

Although the concentrations evaluated in this study are higher than those typically expected in natural waters, similar exposure scenarios may occur locally following accidental spills, improper disposal of refill liquids, leakage from discarded vaping devices, or inputs from wastewater systems. Consequently, the concentrations tested represent realistic worst-case scenarios that may be relevant for assessing the environmental risks associated with increasing consumption of electronic cigarette products.

4. Conclusions

The present study evaluated the acute toxicity of a commercial electronic cigarette refill liquid using different aquatic vertebrate models, including fish embryos (*Danio rerio*), juvenile fish (*Jenynsia multidentata*), and amphibian larvae (*Rhinella arenarum*).

No mortality was observed in juvenile fish or amphibian larvae exposed to concentrations up to 50% of the stock solution during the experimental period, indicating low acute toxicity under the tested conditions. In contrast, *D. rerio* embryos exhibited clear susceptibility to the electronic cigarette liquid, with mortality increasing at the highest exposure concentrations.

The results demonstrate that the developmental stage plays a critical role in determining sensitivity to contaminants associated with electronic cigarette products. Embryonic stages were considerably more vulnerable than juvenile organisms, highlighting their value as sensitive biological indicators in ecotoxicological studies. However, direct comparisons among biological models should be interpreted with caution because exposure durations and concentration ranges differed among the assays performed with embryos, juvenile fish, and amphibian larvae.

Furthermore, the observed effects are likely associated with the combined action of nicotine and other constituents present in the commercial formulation, including humectants, flavoring agents, and additional additives. Consequently,

the environmental risks associated with electronic cigarette liquids should not be evaluated solely on the basis of nicotine content.

Overall, the findings contribute to the growing body of knowledge regarding the environmental impact of vaping-related products and emphasize the importance of incorporating multiple biological models and developmental stages into ecological risk assessments. Additional studies involving chronic exposure, developmental endpoints, behavioral biomarkers, and environmentally relevant concentrations are recommended to improve understanding of the ecological consequences associated with electronic cigarette residues.

Together with previous studies on nicotine and cigarette butt leachates, the present results support the need for a broader ecotoxicological assessment of tobacco-derived and vaping-related contaminants in aquatic ecosystems.

Ethics Statement

All experimental procedures involving animals were carried out according to institutional guidelines and were approved by the CICUAL of the Universidad Nacional de San Luis (Resolution No. 291/2025).

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Conflicts of Interest

The authors declare no conflicts of interest.

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