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Effects of Tebuconazole on the Earthworm *Dendrobaena veneta*: Full Life Cycle Approach

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Abstract: Tebuconazole (TEB), a widely used triazole fungicide, is effective against soil-borne and foliar fungal pathogens. Toxicants can exhibit varying effects depending on the life stage of organisms, although standard toxicity tests typically focus on adult individuals. This study aimed to assess TEB's potential adverse effects on the earthworm *Dendrobaena veneta* throughout its life cycle. Effects were evaluated by exposing cocoons to varying TEB concentrations, monitoring hatching, newly hatched juvenile mass, and growth to adulthood. A reproduction test assessed impacts on adults, offering insights into how these results compared with cocoon exposure findings. Results revealed that TEB delayed hatching at concentrations of 25, 50, and 100 mg/kg by 6, 8, and 15.5 days, respectively. Newly hatched juveniles exhibited a 15.96% (50 mg/kg) and 27.37% (100 mg/kg) reduction in body mass compared to controls, with no subsequent compensation during growth. Results from the reproduction tests showed no adverse effects on adult survival, but the effects are observed on juveniles, indicating a higher sensitivity of this developmental stage. While several adverse effects were observed, it is important to note that these occurred at concentrations exceeding recommended application rates. TEB appears safe for earthworms when used correctly, but the presence of multiple contaminants and stressors warrants consideration.

Keywords: fungicide; reproduction; hatching dynamics; growth model; non-target organisms

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1. Introduction

Today's intensive agriculture, characterized by increased pesticide use, poses various environmental risks, making pesticides significant pollutants in both agricultural and natural habitats. Many adverse effects of pesticides have been uncovered over time; yet, despite concerted efforts, global pesticide consumption continues to rise, reaching an estimated 4.2 million tons in 2019 [1]. This corresponds to a market size of nearly USD 84.5 billion, and projections show that by the end of 2023 the total value of all pesticides used is expected to grow to nearly USD 130.7 billion [2]. Multiple scenarios indicate that total food consumption will need to increase by 35%, to 56%, between 2010 and 2050 [3], imposing an additional environmental burden through increased pesticide usage.

Tebuconazole (TEB) is a triazole fungicide, which is commonly used in agricultural practices to control soil-borne and foliar fungal pathogens in many crop plants [4]. Its antifungal activity is a result of the inhibition of C14-demethylase in sterol biosynthesis and disruption of the formation of the cell membrane in pathogens [5]. TEB stands out as one of the most commonly detected pesticides in agricultural soil samples, often found at the highest concentrations [6]. Over 70% of investigated Central European soils have at least one conazole fungicide present in them, with the second most frequent being TEB (36% soil), and it often exceeds the threshold of 0.01 mg/kg [7]. Silva et al. ([6]) reported a median TEB concentration of 0.02 mg/kg, with maximum values ranging from 0.16 to 0.31 mg/kg in European agricultural soils. TEB is considered as a pesticide that has some high-risk properties, such as very high bioaccumulation potential, strong adsorption in soil, low-to-moderate water solubility, and long persistency [8]. Considering that reported

half-lifes (DT_{50}) range from 120 days to >1 year [9], it has a strong potential to affect various soil non-target organisms.

In aquatic ecosystems, it has been mostly studied in zebrafish (*Danio rerio*) and water fleas (*Daphnia magna*). In zebrafish (*Danio rerio*), it is known to increase malformation rates, decrease swimming speed, and induce thyroid disruption in offspring through maternal transfer of TEB [10]. Furthermore, it has been linked to developmental toxicity, immunotoxicity, and the induction of oxidative stress and apoptosis in developing zebrafish [11]. In *D. magna*, exposure to TEB decreases survival, body size, reproduction, and growth rates [12], and induces disruptions in energy metabolism, which subsequently impairs overall metabolic functions in these organisms [13]. In terrestrial ecosystems, it has been mostly studied in earthworms (*Eisenia fetida*) and enchytraeids (*Enchytraeus crypticus*). In *Eisenia fetida*, exposure to 142 mg/kg of TEB for 14 days resulted in noticeable morphological alterations, weight loss, the development of hemo-limphatic edemas, and occasional necrosis in the circular and longitudinal muscular layers [14]. TEB also induces nerve and immunological toxicity, while also posing potential carcinogenic risks and triggering oxidative stress in these organisms [15]. Furthermore, it has been well-documented that TEB tends to bioaccumulate and biomagnify in earthworms, giving rise to reproductive toxicity [16], an effect largely attributed to its disruption of earthworm metabolism, particularly in the AMP pathway, which significantly impacts their reproductive processes [17]. In *E. crypticus*, reproductive toxicity was observed as evident in the reduction in the number of juveniles after the reproduction test [18].

Soil invertebrates are essential contributors to the enhancement of soil structure and fertility. Among these invertebrates, earthworms are of particular significance due to their roles in decomposing organic matter, facilitating nutrient cycling, and contributing to the formation of soil [19,20]. Due to their ecological significance, substantial biomass, and susceptibility to pollutants, they are considered as well-suited organisms for evaluating the ecological risks of pesticides in terrestrial ecosystems [21].

While it is known that toxicants can have varying effects on organisms depending on their developmental stage [22,23], most standard toxicological tests employ adult individuals to assess endpoints such as survival and reproduction. Examining the effects of toxicants on different developmental stages can provide valuable information for modeling the population-level effects of chemicals, significantly enhancing ecological relevance. The main aim of this research was to evaluate the potential adverse effects of TEB on the earthworm *Dendrobaena veneta* throughout its life cycle. Those effects are evaluated by exposing cocoons to different concentrations of TEB and monitoring hatching dynamics in a mass of newly hatched juveniles, and their growth to adulthood. In parallel, a reproduction test was performed to evaluate the negative effects on adults, and also to see how the results of the reproduction test compare with the results obtained from the cocoon exposure.

D. veneta is an earthworm species used mainly for vermicomposting; however, it is also naturally occurring in various habitats across the Northern Hemisphere [24], which increases the ecological relevance of our study. While epigeic species such as *E. fetida* and *D. veneta* are less prevalent in agricultural habitats, they are frequently chosen as model species, and findings related to them can often be extrapolated to earthworm species from different ecological categories. Moreover, given that epigeic species tend to be less sensitive to xenobiotics, any substantial impacts observed should prompt a more cautious approach or additional research concerning endogeic or anecic earthworm species [25].

2. Materials and Methods

2.1. Test Organisms

Adult earthworms (*Dendrobaena veneta*: Rosa, 1886) were obtained from a culture maintained at the Department of Biology (Osijek, Croatia). *D. veneta* is commonly used in vermicomposting and is characterized by a relatively short life cycle, relatively fast growth rate, and efficient reproduction [24]. All earthworms were adults with a well-developed

clitellum. Before each experiment, earthworms were removed from the culture, and stored on a damp filter paper for 24 h to void the gut contents.

2.2. Test Materials and Soil Spiking

All reagents used in the experiments were of analytical grade. For the preparation of exposure concentrations, a commercial product of TEB was used: Folicur EW 250® (Bayer Ltd., Zagreb, Croatia, 250 g/L TEB). Concentrations were chosen based on the maximum application rate of this fungicide (2 L/ha). A control and five concentrations were used: C0 = 0 mg/kg, C1 = 1 mg/kg, C2 = 5 mg/kg, C3 = 25 mg/kg, C4 = 50 mg/kg, and C5 = 100 mg/kg, with concentrations C1 and C2 being environmentally relevant. Concentrations are expressed as mg of active ingredient per kg of dry weight of soil.

Experimental conditions are adjusted according to the OECD protocol [26]. In all of the experiments, an artificial soil mixture was used. The mixture consisted of 70% fine quartz sand, 20% kaolinite clay, and 10% sphagnum peat, with its pH carefully adjusted to 6.0 ± 0.5 using $CaCO_3$. Folicur fungicide was diluted with distilled water to achieve the desired concentrations and then added separately to each test vessel. The control group contained only distilled water. Soil moisture was adjusted to 50% of its water-holding capacity (WHC). After the prepared fungicide solutions were introduced, the soil was thoroughly mixed to ensure uniform distribution and was allowed to stabilize for 24 h before introducing the earthworms. The temperature in the experiments is adjusted to 20 ± 1 °C, according to the standard protocol. Also, the natural habitat of *D. veneta* is characterized by a mean temperature of ~15 °C [27], but it is also used as a species in vermicomposting and is successfully grown at higher temperatures. So, although OECD guidelines are designed for *Eisenia fetia*/*Eisenia andrei*, we assumed that 20 °C is optimal for our experiments.

2.3. Experimental Design

2.3.1. Cocoon Hatching Success and Juvenile Growth Dynamics (Cocoon Exposure)

Cocoons were collected from the earthworm culture by careful hand sorting. In order to obtain a homogenous cocoon cohort and minimize the age difference of the cocoons, they are first separated by their color. Newly hatched cocoons are slightly greener in color, so all yellow/amber and brown cocoons are discarded, and only green ones are used for the experiments. After sorting them by color, we also removed all cocoons that were too small or too big. For the cocoon exposure, 6-well microplates were used and each well was filled with 10 g of artificial soil. Five cocoons were randomly selected and carefully buried in the soil. For each concentration, six replicates were used. The plates were incubated at 20 ± 1 °C and the WHC was adjusted weekly.

Cocoon hatching dynamics were evaluated every two days and monitored for 58 days. Cocoons that did not hatch until that time were considered to never hatch. Newly hatched earthworms were weighed and transferred to a jar with clean artificial soil. As they are very fragile, we tried to disturb them as little as possible. They were carefully collated from the plates, gently washed with water to remove dirt, and put briefly on filter paper prior to weighing to remove excess water. It assumed that the content of their digestive tract will have minimal effect on their weight, and will not introduce significant bias in our results. The development of juveniles was further monitored by carefully extracting the juveniles from the soil and weighing them until attaining adulthood (development of clitellum). Juveniles were initially weighed every 14 days (3 times), but this was then moved to a 21-day period to avoid disturbing the organisms too often. In these experiments, the endpoints taken were hatching dynamics, hatching success, number of juveniles per cocoon, juvenile hatching mass, juvenile growth dynamics, and mass at adulthood.

2.3.2. Reproduction Test

The effects of TEB were also assessed using an earthworm reproduction test with some modifications. The soil was placed in a test vessel and 400 g of artificial soil was added in each vessel. The prepared TEB concentrations were mixed in soil while the control contained only distilled water. For each concentration, 7 replicates were used and in each jar 5 adult earthworms (not exposed to TEB previously during the life cycle) with a well-developed clitellum were added. Earthworms were fed once a week with horse manure. Horse manure was collected from the local farm; it was air-dried, heat-treated at 70 °C (pasteurized), and finely ground before feeding the earthworms. Test vessels were kept at 20 ± 1 °C and 16:8 light/dark photoperiod. After 28 days, all adult worms were removed and weighed, and the cocoons were counted and returned to the soil for the next 28 days. After the additional 28 days, the test vessels were immersed in a warm water bath (60 ± 5 °C), and extracted juveniles were weighted and counted.

2.4. Data Presentation and Statistical Analysis

For the entire data analysis and data presentation, R software [28] and RStudio [29] were used. Prior to the analysis, data were tested for normality using the Shapiro–Wilks test and variance homogeneity was tested using Bartlett's test.

Hatching dynamics were analyzed using the Kaplan–Meier survival method, using a survival package [30], and differences among hatching curves were compared with the Log-rank and tests using a survminer package [31].

To estimate differences in the growth dynamics of juveniles, their mass was fitted to a logistic growth model (1):

$$N_t = \frac{N_0 K}{N_0 + (K - N_0)e^{-rt}} \quad (1)$$

where N_t corresponds to the juvenile mass at time t, N_0 is the initial juvenile mass, K corresponds to final mass when reaching adult stage (clitellum development), and r is the growth rate. For fitting growth curves, a growthcurver package was used [32]. After model-fitting, parameters of the individual curves are compared to the parameters estimated for the control treatment using one-way ANOVA, followed by a Dunnett post hoc test.

To determine any differences in juvenile mass at hatching, as well as to test the results from the reproduction test, one-way ANOVA was used. After this, significant ANOVA differences compared to the control treatment were tested using a Dunnett post hoc test.

3. Results

3.1. Cocoon Hatching Dynamics, Juvenile Mass Hatching, Juvenile Growth Dynamics

3.1.1. Cocoon Hatching Dynamics

The hatching dynamics of cocoons exposed to varying concentrations of TEB differed significantly (Log-rank; $p < 0.001$) (Figure 1). In the control treatment group, the median hatching time was estimated to be 21 days, and there were no statistically significant differences in median hatching times between C1 and C2, both of which correspond to environmentally relevant concentrations (Table 1). However, when examining the hatching time at concentrations C3, C4, and C5 (25, 50, and 100 mg/kg), a substantial lengthening of the median hatching time became apparent when compared with the control. Notably, the median hatching times at these concentrations were prolonged by 6, 8, and a remarkable 15.5 days, respectively.

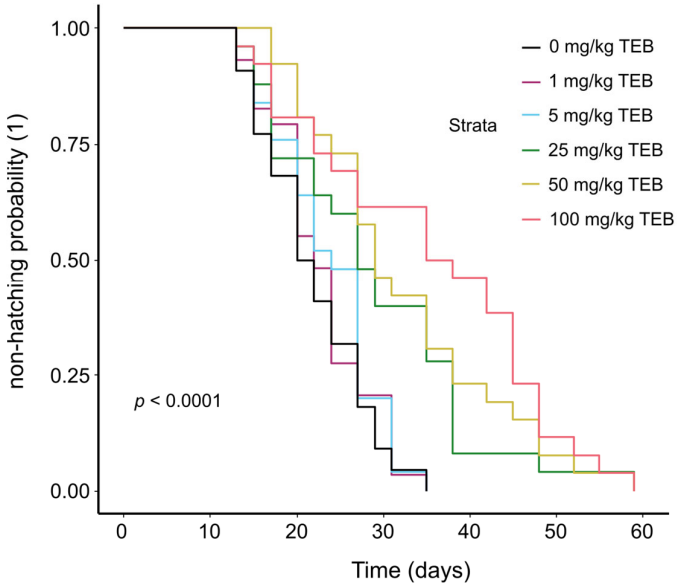

Figure 1. Cocoon hatching dynamics at different TEB concentrations are presented as Kaplan–Meier curves. *p*-value marks statistically significant differences among hatching curves.

Table 1. Median hatching time of cocoons exposed to different TEB concentrations. A 95% confidence interval for the median is presented in square brackets. Statistically significant differences in median hatching time compared to the control treatment are marked with an asterisk (Log-rank tests; $p < 0.05$). Average hatching success, average number of juveniles per hatched cocoons, and average survival probability of juveniles are presented as mean ± standard deviation. A dot represents a statistically significant difference compared to the control (ANOVA, Dunnett post hoc test, $p < 0.05$).

Treatment (mg/kg TEB)	Median Hatching Time	Average Hatching Success (%)	Average Number of Juveniles per Cocoon	Juvenile Survival Probability (%)
0	21 [20, 27]	73.3 ± 27.3	1.03 ± 0.08	0.95 ± 0.04
1	22 [20, 24]	86.6 ± 16.32	1.03 ± 0.08	0.70 ± 0.16
5	24 [20, 27]	83.3 ± 23.4	1.13 ± 0.33	0.77 ± 0.06
25	27 [22, 38] *	76.7 ± 26.6	1.07 ± 0.16	0.63 ± 0.15
50	29 [27, 38] *	70.0 ± 20.9	1.03 ± 0.08	0.40 ± 0.10 •
100	36.5 [27, 45] *	83.3 ± 23.4	1.10 ± 0.11	0.33 ± 0.10 •

Although there were no significant differences in the average hatching success between treatments, 10–13% higher values were observed at environmentally relevant concentrations (C1 and C2). The average number of juveniles per cocoon also did not differ significantly between treatments (Table 1), with an average of 1.03 to 1.1 juveniles hatched per cocoon. Juvenile survival probability was significantly lower compared to the control treatment at C4 and C5 ($F = 4.518$; $p = 0.0034$), where a 57.8% and 65.2% decrease was observed (Table 1).

3.1.2. Juvenile Mass at Hatching and Growth Dynamics

The mass of newly hatched juveniles differed significantly between treatments (ANOVA; $F = 6.457$; $p < 0.001$) (Figure 2). Significant differences were observed at concentrations C4 and C5, in which the body mass of newly hatched juveniles was on average 15.96% and 27.37% smaller compared to the control treatment.

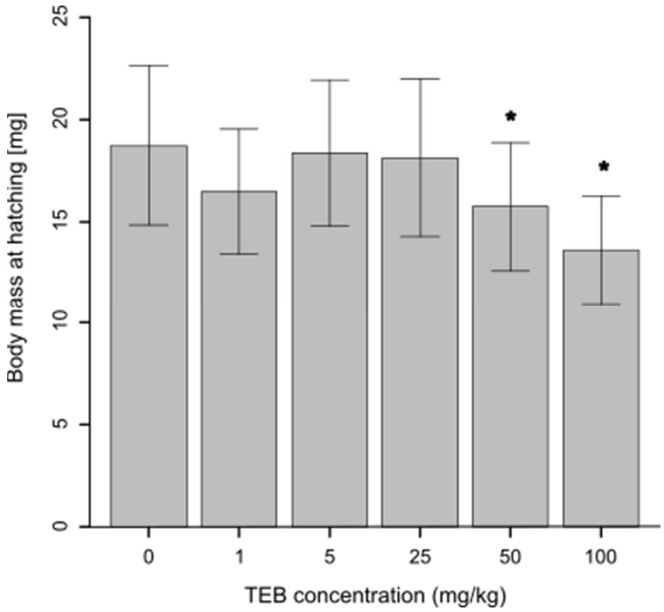

Figure 2. Body mass of *D. veneta* juveniles hatched from the cocoons exposed to TEB. Results are presented as mean ± standard deviation. An asterisk (*) represents statistically significant differences compared to the control treatment (ANOVA, Dunnett post hoc test; $p < 0.05$).

The growth dynamics of juveniles followed a classical logistic model (Figure 3), and parameters estimated from the growth model show that the growth rate (r) was significantly lower at the highest tested concentration, in which r was reduced by 56.7% compared to the control (Table 2). Although there were no statistically significant differences in the growth rate parameter (r) at concentrations C3 and C4, adult earthworms at those same concentrations attained a significantly lower body mass (estimated as K), with reductions of 59.2% and 38.4%, respectively, compared to the control. As expected, adult body mass at C5 also displayed a significant reduction, of 47.3%, compared to the control.

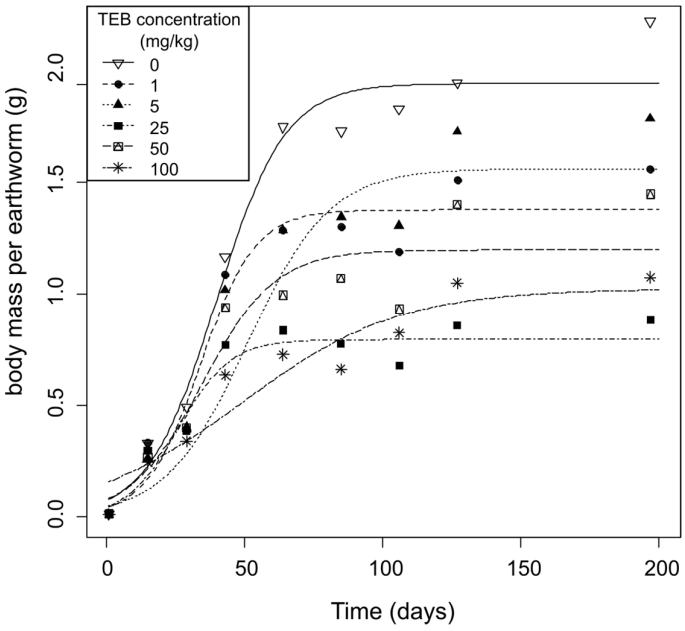

Figure 3. Growth dynamics (body mass/g) of juveniles exposed to TEB as cocoons. The results are from a logistic growth model.

Table 2. Logistic growth model parameters estimated from the body mass of juveniles exposed to TEB as cocoons. *r*—growth rate, *K*—final body mass of juveniles (adult mass), σ—residual standard error on 6 degrees of freedom. An asterisk (*) signifies a statistically significant difference in parameters compared to the control.

Model Parameters	0 mg/kg TEB	1 mg/kg TEB	5 mg/kg TEB	25 mg/kg TEB	50 mg/kg TEB	100 mg/kg TEB
r	0.081	0.101	0.068	0.106	0.077	0.035 *
K	1.943	1.377	1.561	0.793 *	1.197 *	1.024 *
σ	0.154	0.154	0.183	0.092	0.195	0.126

3.2. Reproduction Test

The results from the reproduction test revealed that adult survival remained unaffected by TEB across all tested concentrations (Table 3). However, a significantly lower number of juveniles was observed at C3, C4, and C5 (ANOVA, F = 7.675, $p < 0.001$), with reductions of 28.9%, 30.3%, and 47.5%, respectively, when compared to the control treatment. Moreover, the average mass per juvenile exhibited significant declines at concentrations C4 and C5, with notable decreases of 29.4% and 51.1% in body mass compared to the control treatment. At these same concentrations, the average number of juveniles per earthworm was also significantly lower, with reductions of 28.8% and 44.2% (ANOVA, F = 5.094, $p = 0.0012$). Interestingly, the number of unhatched cocoons after 56 days was significantly lower at the lowest tested concentration (ANOVA, F = 6.143, $p = 0.0003$).

Table 3. Effects of TEB on the survival and reproduction of the earthworm *D. veneta* after 56 days of exposure. Values are presented as mean ± standard deviation. Significant differences compared to the control are labelled with an asterisk (*) (ANOVA, Dunnett post hoc test; $p < 0.05$).

Treatment (mg/kg TEB)	Adult Survival	Number of Juveniles	Mass per Juvenile (mg)	Number of Juveniles per Earthworm	Number of Unhatched Cocoons
0	0.94 ± 0.09	22.1 ± 3.3	30.9 ± 2.8	5.2 ± 1.6	4.3 ± 0.95
1	0.97 ± 0.08	20.2 ± 2.9	25.1 ± 9.0	4.2 ± 0.6	2.1 ± 1.3 *
5	0.91 ± 0.11	20.3 ± 5.0	27.5 ± 5.1	4.4 ± 0.8	2.7 ± 1.1
25	0.83 ± 0.14	15.7 ± 4.9 *	27.0 ± 2.0	4.9 ± 0.9	4.9 ± 1.2
50	0.91 ± 0.11	15.4 ± 4.2 *	21.8 ± 5.8 *	3.7 ± 0.6 *	4.6 ± 1.1
100	0.83 ± 0.18	11.6 ± 1.3 *	15.1 ± 2.2 *	2.9 ± 0.77 *	4.1 ± 1.2

4. Discussion

Limited data are available on the effects of TEB, a fungicide, on the earthworm species *Dendrobaena veneta*, as most studies investigating TEB used the model species *Eisenia fetida*. To our knowledge, this is the first study investigating the effects of TEB on this earthworm species by examining the effects on the entire life cycle. Although fungicides like TEB are designed to control fungal diseases in plants and are not specifically targeted at earthworms, the results of this study demonstrated its numerous negative effects on the survival and development of the earthworm *Dendrobaena veneta*. Observed effects differ depending on the developmental stage. Previous research has confirmed variations in sensitivity among the different life stages of soil organisms when exposed to various classes of chemicals [18,33–35]. In cocoons, a significant delay in hatching time was observed at concentrations higher than 25 mg/kg (C3, C4, and C5). Similar effects in hatching delay were observed by Bart et al. ([35]) in the earthworm *Aporectodea caliginosa* exposed to fungicide copper oxychloride (Cuprafor micro®), where a concentration of 232.5 mg Cu/kg caused a 5-day delay in hatching. But, contrary to our results, they observed a simultaneous reduction in hatching success, while in our study the hatching success of exposed cocoons was not affected by TEB.

It is demonstrated that TEB induces oxidative stress in earthworms [36], and research by Li et al. [15] showed that in earthworms exposed to TEB, increased P450s gene expression is observed to alleviate the harmful consequences of oxidative stress. Earthworms exposed to xenobiotics can modify their energy management and decrease or increase the energy used for detoxification processes [37]; however, although this strategy increases the chances of survival, the energy deficiency due to detoxification must manifest itself elsewhere in the life cycle. This is probably evident in the reduced body mass of newly hatched earthworms (exposed as cocoons), as a significantly lower body mass was observed after exposure to TEB at concentrations C4 and C5. Also, in the reproduction test, a significantly lower body mass of juveniles was observed at those same concentrations. The research of Bart et al. [35] showed that cocoons exposed to fungicides were less affected in comparison with the cocoons produced by exposed adults; however, in our study the effects on both types of cocoons were similar. Nevertheless, this difference could be due to the fact that the authors used a fungicide that is a combination of epoxiconazole and dimoxystrobin and other earthworm species (*Aporrectodea caliginosa*), or a result of species-specific differences in sensitivity to pesticides [38].

This effect of a lower body mass of newly hatched juveniles was not compensated during the growth period, resulting in all earthworms, exposed as cocoons to C3, C4, and C5, having a lower body mass in adulthood. Although no significant decrease in the body mass of newly hatched juveniles was observed in C3, this indicates that TEB probably affected cocoons at the subcellular level and the effects were not observable in body mass, although a significantly prolonged hatching time was observed. What is interesting is that only the growth rate (r) at the highest concentration was significantly lower compared to the control treatment. Other researchers have also noted that the growth rate is a sensitive parameter that can be used to assess the impact of pollutants on earthworms, and these effects have been observed in various earthworm species. For instance, in the case of *Eisenia fetida*, significant reductions in growth rates were observed following exposure to higher acetochlor concentrations (20–80 mg/kg) [39]. Similarly, *Aporrectodea caliginosa* exhibited reduced growth rates when exposed to aldicarb, cypermethrin, profenofos, chlorfluazuron, atrazine, and metalaxyl [40]. Additionally, the growth rate of the anecic earthworm *Lumbricus terrestris* was found to decrease when exposed to endosulfan and aldicarb [41]. However, those negative effects are usually observed at higher exposure concentrations that are usually not expected in natural environments, as observed in our study as well.

While no significant effect was observed in adult survival, significant effects were evident among juveniles. A reduced survival probability was observed at C4 and C5 for juveniles exposed as cocoons, and a significantly lower number of juveniles was observed at C3, C4, and C5 after the reproduction test. These findings indicate that juveniles are more sensitive to TEB than adults, as they exhibited adverse effects at lower concentrations than adults. Similar sensitivity patterns have been observed in the research of other authors. For instance, cypermethrin had a more pronounced impact on *E. fetida* juveniles compared to adults, affecting the growth of juvenile earthworms at lower concentrations than those required to affect adults, which in turn leads to delayed development and reproduction, especially in cases of chronic exposure [42]. Booth and O'Halloran's [43] research also indicated that earthworms exposed as juveniles exhibited a greater sensitivity to organophosphorus insecticides compared to those exposed as adults. The authors thus suggested that the responses of juvenile earthworms may provide a more predictive insight into the long-term impacts of organophosphorus insecticide applications.

The differences in sensitivities among developmental stages can be attributed to various factors. Zhou et al. [42] proposed that juvenile earthworms may not have the ability to escape from pesticide-contaminated soil into clean soil in the field, potentially leading to continuous exposure to pesticides. Furthermore, reaching sexual maturity in earthworms is often associated with a notable increase in tegument thickness [44], which could potentially make it easier for pesticides to penetrate juvenile tegument and

impact their internal systems. Also, it is possible that juvenile earthworms may have less developed detoxification mechanisms, such as enzymes responsible for breaking down and detoxifying pesticides, which can result in a reduced ability to metabolize and eliminate pesticides from their bodies.

An interesting effect was observed in the reproduction test, as a significantly lower number of unhatched cocoons was observed at the lowest tested concentration, which could indicate a stimulatory or hormetic effect. The stimulatory effect of low pesticide concentrations on earthworms has been observed in several other studies [25,45] and is typically considered a reaction to stress, resulting in an increase in reproductive rate [46]. However, as mechanisms explaining the hormetic effect are still unknown, further experiments are needed.

In general, our experiments revealed several adverse effects of TEB on earthworm survival, development, and growth. However, it is important to note that these negative effects were observed at concentrations higher than the recommended application rate and are not expected to occur in a natural environment. Similar findings and conclusions were reported by Costa et al. [47], who investigated the effects of TEB at field-relevant concentrations in *E. fetida* and did not observe any negative effects. While this research and our study confirm the safety of TEB when used correctly, caution should be taken due to the possibility of cumulative effects with other pollutants commonly found in agricultural soils, as well as potential interactions with repeated and multiple stressors in the environment, especially in light of ongoing climate change [48,49].

5. Conclusions

Standard toxicity tests evaluate the effects of toxicants usually only during the adult stage, with endpoints taken for adult survival and reproduction. Our primary aim was to investigate the potential impact of TEB on the earthworm *D. veneta* across their entire life cycle, from early developmental stages to adulthood. The effects observed differed depending on the developmental stage. The exposure of cocoons caused delayed hatching at concentrations higher than 25 mg/kg, and juveniles were more sensitive to TEB than adults, as they exhibited adverse effects at lower concentrations than adults.

In general, our experiments revealed several adverse effects of TEB on earthworm survival, development, and growth, but those effects are observed at concentrations higher than the recommended application rate. This suggests that TEB is relatively safe for earthworms if used according to the manufacturer's instructions. However, other stressors commonly present in the environment must be considered as it is known that they can induce additive or synergistic effects. This is especially important as not all developmental stages display the same level of sensitivity to toxicants. Thus, a stronger impact on one developmental stage can lead to shifts in population structure, potentially resulting in adverse population-level effects, even when standard tests indicate the product's safety.

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Conflicts of Interest: The authors declare that they have no known competing financial interest or personal relationships that could have appeared to influence the work reported in this paper.

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