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Is the Synthetic Fungicide Fosetyl-Al Safe for the Ecotoxicological Models *Danio rerio* and *Enchytraeus crypticus*?

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Abstract: Worldwide, pesticides have contaminated the environment, affecting non-target species. The aim of this work was to evaluate the effects of fosetyl-Al (FOS) on model organisms. Based on the 3 Rs for animal research and described guidelines, the OECD 236 and 220 were applied with some modifications. The FOS test concentrations were 0.02–0.2–2–20–200 mg/L for *Danio rerio* and 250–500–750–1000–1250 mg/kg for *Enchytraeus crypticus*. Besides the standard endpoints, additional endpoints were evaluated (*D. rerio*: behavior and biochemical responses; *E. crypticus*: extension of exposure duration (28 d (days) + 28 d) and organisms' sizes). For *D. rerio*, after 96 h (h), hatching was inhibited (200 mg/L), proteins' content increased (2 and 20 mg/L), lipids' content decreased (2 mg/L), glutathione *S*-transferase activity increased (2 mg/L), and, after 120 h, larvae distance swam increased (20 mg/L). For *E. crypticus*, after 28 d, almost all the tested concentrations enlarged the organisms' sizes and, after 56 d, 1250 mg/kg decreased the reproduction. In general, alterations in the organisms' biochemical responses, behavior, and growth occurred at lower concentrations than the effects observed at the standard endpoints. This ecotoxicological assessment showed that FOS may not be considered safe for the tested species, only at higher concentrations than the predicted environmental concentrations (PECs). This research highlighted the importance of a multi-endpoint approach to assess the (eco)toxic effects of the contaminants.

Keywords: pesticides; soil and freshwater organisms; reproduction; survival; embryo development; biochemical and behavior biomarkers

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1. Introduction

Despite the worldwide aim being driven toward more sustainable agriculture, pest management clearly depends on the usage of diverse kinds of pesticides. In Europe, around 380,000 tons of synthetic and inorganic pesticides are sold per year (average between 2011 and 2017 considering 28 European countries) [1,2]. Fungicides are extensively used to control or eradicate fungal phytopathogens. Nevertheless, fungicides employed on soils not only affect the target phytopathogens, but also the non-target autochthonous soil organisms, which have crucial functions in the terrestrial ecosystems [3]. Indeed, pesticides and their transformation products or metabolites may continue in the environment [2,4], contaminating soil and water [2,5] and affecting non-target terrestrial and aquatic organisms [2,6–8], representing a risk for human health [2,9].

Based on the European Food Safety Authority (EFSA), fosetyl-Al (FOS) is one of the active substances listed in Regulation (EU) No 686/2012 [10]. FOS that is sold under the trade name Aliette is an inorganic phosphorous systemic fungicide applied to control various plant pathogenic phycomycetes and ascomycetes, damping off and rotting of plant roots, stems, and fruit. This active substance is utilized for the prevention of crops and for the inhibition of fungal spore propagation and infiltration of pathogens into plants. It is applied as a plant dip treatment and a drench for transplants by incorporating it into the soil prior to planting and by applying it to foliage [11]. A dose of 2.5 g/L FOS is

recommended to apply on foliage against fungi that affect several crops, such as citrus, lettuce, and hops [11]. According to EFSA (2018) [10], FOS is a phosphonate compound; its structure and mode of action vary from most of the organophosphorus compounds applied as pesticides. EFSA also reported a data gap for the FOS risk to aquatic organisms and to non-target terrestrial plants.

There is no information regarding the lethal and sub-lethal effects of FOS on the ecotoxicological model aquatic and terrestrial species, *Danio rerio* and *Enchytraeus crypticus*, respectively. Considering the effects of other pesticides to *D. rerio*, a median lethal concentration (LC_{50}) of 0.235 mg/L, a decreased hatching success, and several malformations were found on embryos after exposure to the insecticide sumithion [12]. Additionally, a strong inhibition of hatching by triazole fungicides [13] and diazinon insecticide [14] exposure was already reported in zebrafish embryos. For *E. crypticus*, organisms were not affected by the insecticide toxaphene [15] and fungicide prosaro [16]. However, the insecticide dimethoate increased the survival and limited the growth of enchytraeids, and fungicides mancozeb and amistar decreased the survival and reproduction [16,17].

Zebrafish (*D. rerio*) is a widely used model organism for aquatic (eco)toxicology being employed for the assessment of effects at different levels of biological organization [18]. Particularly, the fish embryo toxicity (FET) test with zebrafish embryos has been shown to be a valuable alternative to the common fish toxicity test [19]. The FET test [20] may be seen as a refinement or even a potential replacement of animal experiments according to the 3 Rs principle (refinement, reduction, and replacement) [21], as the embryos are assumed to experience less or no pain compared to adult fish. In specific, zebrafish was already employed to assess the toxicity of several pesticides, such as imidacloprid, atrazine, chlorpyrifos, butachlor, λ-cyhalothrin, sumithion, linuron, and propamocarb [12,18,22,23]. Enchytraeids are ecologically important, soil-dwelling annelids, playing an essential role in organic matter decomposition and soil bioturbation [24]. Enchytraeid reproduction test (ERT) guidelines [25] have been established for the genus *Enchytraeus*. *E. crypticus* gained growing attention in soil ecotoxicological studies due to practical advantages: (1) Is cultured in agar medium, (2) has a high reproductive rate, (3) relatively short generation time and test period, and (4) wider tolerance range to different soil properties (pH, texture, and organic matter content) [24]. Several studies already showed that *E. crypticus* is a valuable bioindicator of contaminated environments from a large range of anthropogenic sources, including pesticides [15–17,26].

Therefore, the current research was developed to know and describe the potential toxicological effects of FOS on these two models for freshwater and soil ecosystems, *D. rerio* embryos and *E. crypticus*, respectively. A multi-endpoint approach, including standard (described on the guidelines) and additional endpoints, was performed to reach a more complete evaluation of the potential toxicity of FOS. For *E. crypticus*, survival, reproduction, and organisms' sizes were evaluated after 28 days (d) of exposure. Moreover, the exposure duration was extended (plus 28 d) to evaluate the FOS effects on the organisms' reproduction after 56 d. For *D. rerio*, after 96 h (h) of exposure, survival, hatching, development malformations, heartbeat rate, and biochemical responses involved in energy budgets, antioxidants, and neurotoxicity were analyzed. After 120 h, the larvae locomotor behavior was also assessed.

2. Material and Methods

2.1. Test Chemical: Fosetyl-Al (FOS)

FOS used on the present study was fabricated by LGC Labor GmbH, Germany (CAS number: 39148-24) with a purity of 88.90% (g/g) and acquired as a wettable powder. It has the molecular formula of $3C_2H_6O_3P.Al$.

2.2. Test Species: Danio rerio and Enchytraeus crypticus

Two biological model organisms were used for the bioassays.

Danio rerio (zebrafish—Cyprinidae, Teleostei) eggs were obtained from a culture maintained at the Department of Biology, University of Aveiro (Aveiro, Portugal). The Zebrafish Facility complies with the Portuguese law (Portaria 1005/02 and Portaria 1131/97), which follows the European Guideline 86/609/EC and conforms with the Council Regulation (EC) No. 2005/01 EC regarding animal transit. It also follows the FELASA (Federation of European Laboratory Animal Science Associations) guidelines and recommendations concerning animal welfare in the care and use of laboratory animals, and in the design and conduct of research projects in which animals are used (including the proper education and training of the people involved in all animal work). Zebrafish adults were kept in a recirculating system with reverse osmosis and activated carbon-filtered tap water, supplemented with instant ocean synthetic salt automatically adjusted for pH and conductivity. The fish were maintained at 27 ± 1 °C, under a 12:12 h light:dark photoperiod, with conductivity at 750 ± 50 µS/cm, pH at 7.5 ± 0.5, salinity of 0.35, and dissolved oxygen at 95% saturation. Adult fish were fed daily with a commercial, artificial diet Gemma Micro 500 (Skretting®, Burgos, Spain). Reproduction groups were placed in a sloping breeding tank on the day before the test (at 4 pm). In the next morning, two hours after the opening of the illumination, the eggs were collected, counted and cleaned of residues. Zebrafish eggs with normal development (3 h post-fertilization (hpf)) were chosen for the bioassay, using a Stereoscopic Zoom Microscope—SMZ 1500 (Nikon, Amsterdam, The Netherlands). Unfertilized, irregular, or damaged eggs were rejected.

Enchytraeus crypticus (potworms—Enchytraeidae, Oligochaeta) was also used for the bioassays. *E. crypticus* adults were obtained from laboratorial cultures maintained at CESAM, University of Aveiro (Aveiro, Portugal). The organisms were kept in agar, consisting of Bacti-Agar medium (Agar No. 1, Lab M Limited, Lancashire, UK) and a mixture of four salt solutions (2 mM $CaCl_2 \cdot 2H_2O$, 1 mM $MgSO_4$, 0.08 mM KCl, and 0.75 mM $NaHCO_3$) at 20 ± 1 °C, under a 16:8 h light:dark photoperiod. Organisms were fed ground, autoclaved oats twice per week. For the bioassay, synchronized-age cultures of *E. crypticus* were set by transferring, from the laboratorial cultures, adults with well-developed clitellum into fresh agar plates to lay cocoons. The number of adults to transfer should be 2 $\frac{1}{2}$ times the number of cocoons necessary. After 2 d, cocoons were relocated to fresh agar plates. Juveniles of 17–19 d were used. According to Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010, invertebrates (like *E. crypticus*) are allowed biological models for scientific experimentation and are free of an Ethical Statement.

2.3. Bioassays

2.3.1. *Danio rerio*

Fish Embryo Acute Toxicity (FET) Test

The FET test was based on the Organization for Economic Co-operation and Development (OECD) guideline number 236 [20]. The selected embryos were distributed in 24-well plates, each well containing one embryo per 2 mL of the test solution. Twenty replicates (n = 20) were used per each experimental condition. Zebrafish embryos were exposed to 0, 0.02, 0.2, 2, 20, and 200 mg/L of FOS and kept at 27 ± 1 °C and a 12:12 h light:dark photoperiod. The FOS concentration range was based on 10-fold increases, from the predicted environmental concentrations (PECs) in surface water described by EFSA [10]. FOS, at the tested concentrations, easily dissolved in the zebrafish water; therefore, it was not necessarily a solvent. The test ran at a static system for 96 h and embryos were observed daily with Stereoscopic Zoom Microscope (SMZ 1500, Nikon) to evaluate the survival, hatching, and the appearance of malformations (in specific, pericardial edema, yolk-sac edema, notochord curvature, loss of equilibrium, and abnormal pigmentation). The lack of heartbeat was checked daily from 48 and 96 h and the heartbeat rate was evaluated at 48 h. The number of heartbeats per embryo was counted for 30 s using a mechanical counter. The heartbeat rate (number of heartbeats/minute) was evaluated at 48 h because at this

time embryos are in a favorable stage of development and a good position (lateral position) to allow an accurate heartbeat rate evaluation.

Locomotor Behavior Assay

The FET test exposure was extended until 120 h and larvae locomotor behavior was analyzed using the Zebrabox tracking system (Viewpoint, Lyon, France) over a period of 12 min. Dead larvae or larvae exhibiting malformations were not included in this assay. Ten replicates (n = 10) were used per each experimental condition. The temperature was maintained at 27 ± 1 °C and larvae movement was stimulated by alternating light and dark periods. The test consisted of a cycle with two periods: 6 min of light and 6 min of darkness. The larvae total time and distance swimming were recorded for each period. Larvae path angle was also calculated through the vector of fish swimming direction and the turn path performed by the organisms. The angles of movements were organized in four classes [27]: Class 1 included big amplitude angles (90–180°), classes 2 and 3 included medium amplitude angles (30–90° and 10–30°, respectively), and class 4 included small amplitude angles (0–10°). Three types of movements were considered: no movements (NM, 0 mm/s), medium-velocity movements (MVM, between 0 and 10 mm/s), and high-velocity movements (HVM, above 10 mm/s).

Biochemical Markers' Assessment

Based on the results from the FET test, embryos were exposed to 2 and 20 mg/L of FOS. Seven replicates (n = 7) of 15 embryos each were used per experimental condition and kept in Petri dishes at 27 ± 1 °C. After 96 h of exposure, the embryos were frozen in liquid nitrogen and stored at −80 °C until further analyses. Samples were homogenized in ultrapure water, on ice, using an ultrasonic homogenizer (Sonifier 250, Branson sonicator). The homogenates were divided for the biochemical analyses: total glutathione (TG) content, catalase (CAT) activity, glutathione *S*-transferase (GST) activity, cholinesterase (ChE) activity, protein quantification, and energy budgets. Phosphate buffer (0.2 M; pH 7.4) was added to the homogenate aliquots reserved to TG, CAT, GST, and CHE, which were then centrifuged (10,000× g; 20 min; 4 °C) to obtain the post-mitochondrial supernatant (PMS). A Labsystem Multiskan EX microplate reader was utilized for all the biochemical determinations. TG content was assessed based on the method of Tietze (1969) [28]. CAT activity was measured according to Claiborne (1985) [29]. The measurement of GST activity was performed following the method of Habig et al. (1974) [30]. The measurement of ChE activity was achieved following the protocol defined by Ellman et al. (1961) [31], adapted to a 96-well microplate by Guilhermino et al. (1996) [32]. The quantification of the protein was done following the Bradford method [33], adapted to a 96-well microplate, using bovine γ-globulin as a standard. The energy budgets (lipids', carbohydrates', and proteins' contents) were measured following De Coen and Janssen (1997) [34], with minor modifications for 96-well microplate reading [35].

2.3.2. *Enchytraeus crypticus*

Enchytraeid Reproduction Test (ERT)

For the ERT, the natural standard LUFA 2.2 soil (Speyer, Germany) was used, with the following main characteristics: pH (0.01 M $CaCl_2$) = 5.8; organic carbon = 1.71%; cation exchange capacity = 9.2 meq/100 g; maximum water holding capacity (WHC) = 44.8%; grain size distribution of 7.2% clay, 8% silt, and 77.5% sand.

The soil was dried (48 h; 60 °C) prior to use. The control soil (0 mg FOS/kg soil) was prepared by adding deionized water to correct the moisture content (50% of the WHC maximum). Concerning FOS treatments, the aqueous solutions of the fungicide were added to the pre-moistened soil considering 50% of the WHC maximum and mixed manually [25]. Tests started 1 d after soil spiking. The following FOS nominal concentrations: 250, 500, 750, 1000, and 1250 mg/kg were considered for soil spiking. These concentrations were selected based on the recommended dose for FOS application (2.5 g/L) [11].

The ERT procedures followed the OECD 220 guideline [25] with some adaptations [36]. Briefly, 10 synchronized-age *E. crypticus* were introduced in each test container (diameter of 4 cm) with 20 g of moist soil and food supply (24 ± 1 mg autoclaved oats). The test ran for 28 d at 20 ± 1 °C and a 16:8-h light:dark photoperiod. During the test, food (24 ± 1 mg autoclaved oats) and water content (based on weight loss) were replenished weekly. Per experimental conditions, four replicates were used (n = 4). An extra replicate per experimental condition (without organisms) was included to measure the pH values. At the end of the test period, the *E. crypticus* were fixed with ethanol and stained with Bengal rose (1% in ethanol). After 24 h, soil samples were sieved through meshes with decreasing pore size (1.6, 0.5, and 0.3 mm) to separate the organisms from most of the soil and facilitate counting. For survival and reproduction evaluation, adult and juvenile enchytraeids were counted, respectively, using a stereomicroscope. For the exposure extension of 28 d (i.e., 56 d), four extra replicates (n = 4) were done and, hence, larger test containers (diameter of 5.5 cm) were utilized, with 40 g of soil per replicate because of the expected higher density of organisms. For these replicates, at day 28, adults (the original synchronized-age juveniles) were carefully removed from the soil, after which the soil was left, replenishing water and food (24 ± 1 mg autoclaved oats) weekly. At 56 d, the number of juveniles was assessed, as performed at 28 d. The size (length) of the adults (i.e., the 10 synchronized-age organisms) collected at day 28 was also evaluated.

2.4. Data Analysis

Graphics and statistical analyses were performed using the Sigma Plot 12.5 software package. Shapiro–Wilk and Levene's tests were performed to evaluate the normality and homoscedasticity of data, respectively. One-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison post hoc test was applied to assess differences between control and FOS treatments. When data failed the normality and/or homoscedasticity tests, a non-parametric Kruskal–Wallis test was applied. Significant differences were considered for $p < 0.05$.

3. Results and Discussion

3.1. Danio rerio

3.1.1. Fish Embryo Acute Toxicity (FET) Test

FOS caused no significant effects on the organisms' survival during 96 h of exposure ($p > 0.05$, Figure 1A). In terms of hatching, organisms did not hatch at 200 mg/L FOS ($p < 0.05$, Figure 1B). FOS did not cause significant malformations' induction and did not affect the heartbeat rate ($p > 0.05$, Figure 1C,D).

Based on the mentioned results, FOS showed low toxicity to zebrafish embryos. No study was found about the effects of FOS to zebrafish. A LC_{50} of 428 mg/L was reported for the fish rainbow trout after 96 h exposure to FOS [37]. FOS has a period for 50% dispersion (DT_{50}) of 3 d in surface waters [10]. Thus, the obtained effects on hatching may be mainly due to the parental compound FOS and not its metabolites: phosphonic acid and ethanol. A 20% effect concentration (EC_{20}) to hatching of *Pimephales promelas* was determined as 0.44 mg of active substance (FOS WG80 of Bayer CropScience)/L, for a chronic exposure [10]. Comparing both freshwater fish (*P. promelas* versus *D. rerio*), the fungicide FOS WG80 was more toxic than the FOS used in the current study. Based on the literature, EFSA (2018) considered FOS as a compound with lower acute/chronic risk to aquatic organisms [10]. Regarding FOS metabolites' effects, in a study using disodium phosphonate (related to phosphonic acid), a $LC_{50} > 61.26$ mg/L for *Daphnia magna* (48 h, static exposure) was calculated [38], which showed a greater (severe) effect in comparison to the parental compound (FOS).

Considering the effects of other pesticides to zebrafish embryos, a LC_{50} of 0.235 mg/L was found after 24 h exposure to sumithion [12]. Increasing sumithion concentrations also decreased hatching success and induced several malformations on the organisms [12]. Triazole fungicides [13] and the insecticide diazinon [14] induced a strong inhibition of

hatching in zebrafish embryos. From five tested pesticides, chlorpyrifos, λ-cyhalothrin, and butachlor had the highest toxicities to *D. rerio* with a LC_{50} value ranging from 0.28 to 0.45 mg/L [22]. Comparing the results from studies including other pesticides [12,22] with the ones found in our study (malformations, heartbeat, hatching, and survival), FOS induced less toxicity for zebrafish embryos than the other referred pesticides.

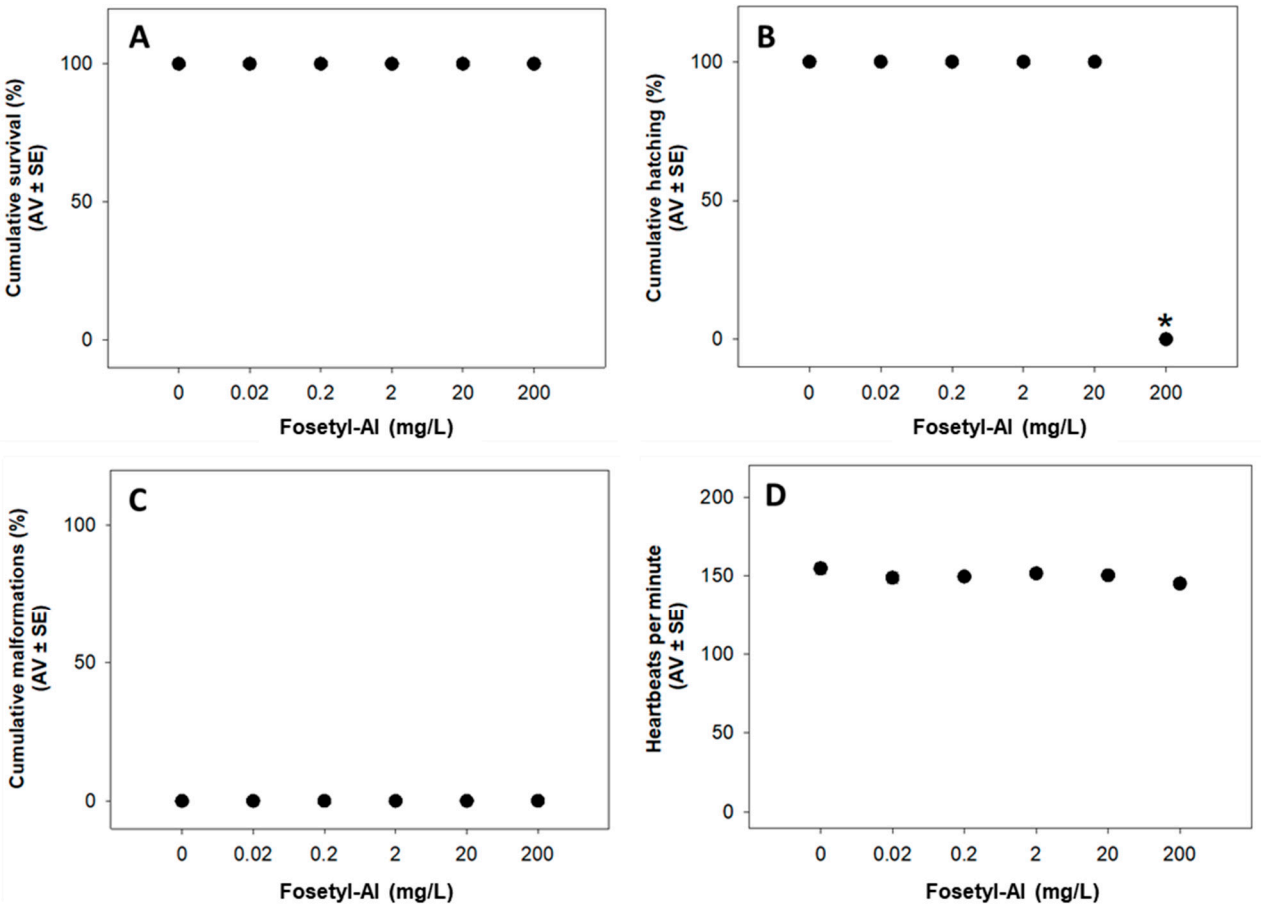

Figure 1. Effects of 96 h of exposure to fosetyl-Al on zebrafish embryos for survival (**A**); hatching (**B**); appearance of malformations (**C**); and heartbeat at 48 h (**D**). Data are expressed as average value (AV) ± standard error (SE). * Significant differences to control ($p < 0.05$).

The organisms' hatching occurs from the activity of the hatching enzyme chorinase, muscle contraction, perivitelline pressure, and water uptake by the embryo [14]. FOS may disturb directly these processes, e.g., through the inhibition of the chorinase or less energy could be available for hatching since exposed organisms assign part of their energy to metabolic pathways for FOS elimination. Inhibition of hatching is generally classified as a sub-lethal effect; however, it can result in death or delay in the organism development/maturation if it is not reversed in a short term. Thus, hatching inhibition may cause a negative impact at the population level. There is a data gap concerning the involvement of hormones in the hypothesized mode of action of FOS as an endocrine disruptor. A study using a mixture of fungicides (without FOS) showed impairments in the reproductive performance of *Hyalella azteca* (amphipod) [39]. It will be important to explore this issue, applying a multigenerational approach where multi-level hierarchical responses can be elucidated (e.g., at molecular, biochemical, and population level).

3.1.2. Locomotor Behavior Assay

Zebrafish larvae typically present low levels of activity during light and increase their locomotor activity upon a sudden switch to darkness. Thus, only the data obtained

during the 6-min dark period were analyzed and will be shown. The organisms exposed to 200 mg/L of FOS did not hatch; therefore, it was not possible to assess the locomotor behavior. After 120 h, FOS induced no effect in terms of total swimming time ($p > 0.05$, Figure 2A); however, 20 mg/L FOS increased the distance swam by zebrafish larvae ($p < 0.05$, Figure 2B). Larvae path angle and types of movement were not altered by FOS exposure comparing with the control group ($p > 0.05$, Figure 2C,D). Considering other pesticides, pendimethalin, diazinon, hexazinone, methomyl, molinate, and dimethoate decreased the distance moved by zebrafish larvae [40]. However, other pesticides (paraquat and amitrole) increased the distance moved by larvae [40], as found in our study. In fact, even at low concentrations, neurobehavioral toxicity of different pesticides, including organophosphates, to non-target organisms are very well documented [41,42]. These effects can be, in most cases, severe, for long periods, or irreversible. Moreover, it can be more adverse during early development of the organisms [42]. The detected increased total distance that was moved by larvae at 20 mg/L of FOS suggests hyperactivity. FOS may be metabolized to phosphorous acid, interfering with ATP synthesis [43,44]. The effects on fish larvae locomotion behavior may be relevant in some ecological functions of the organisms, such as feeding, reproduction, and escaping from predators [45]. However, the effects detected in the current study for larvae locomotor behavior (at 20 mg/L) are not at PECs.

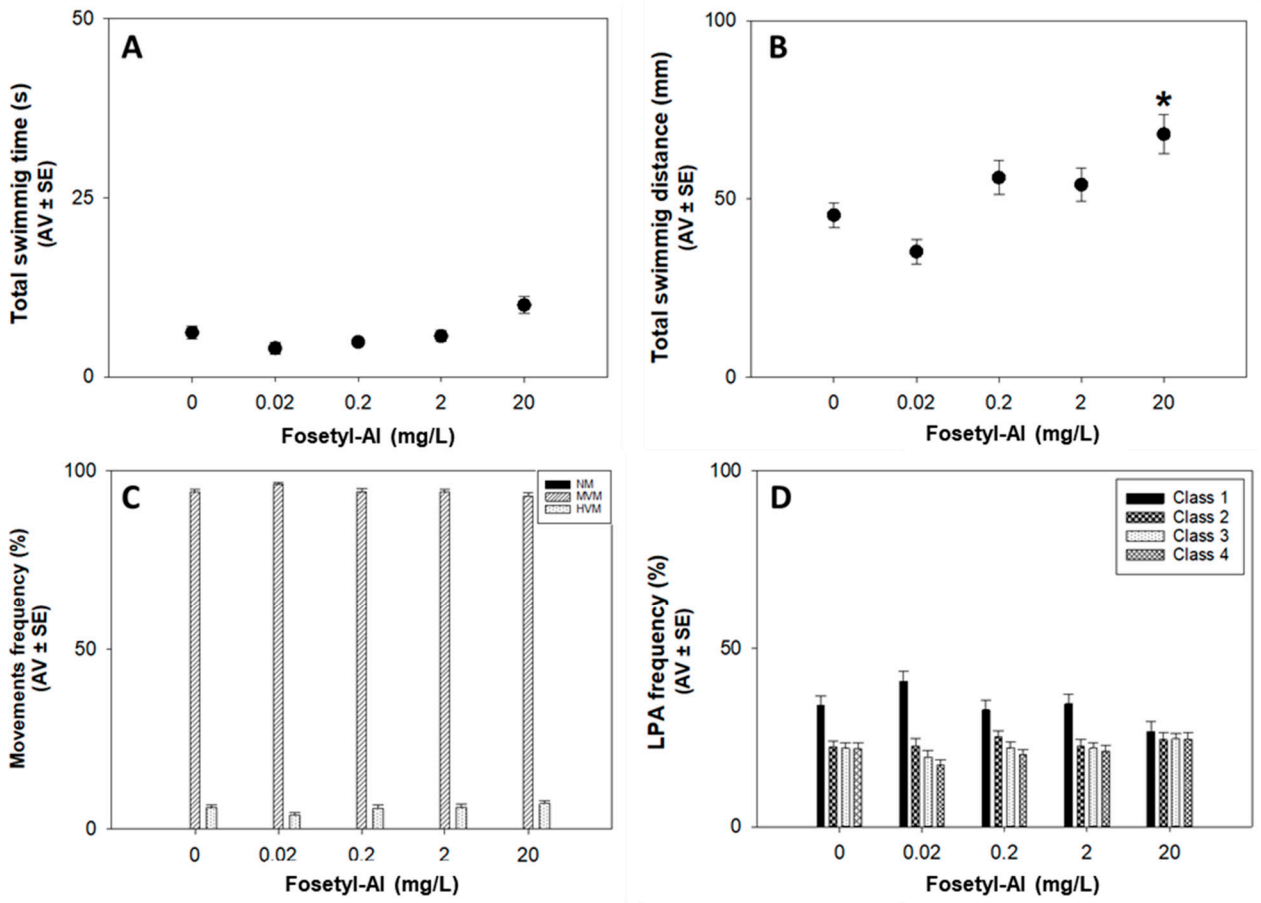

Figure 2. Effects of 120 h of exposure to fosetyl-Al on zebrafish larvae for total swimming time (**A**) and distance (**B**); types of movements (**C**); and larvae path angle (LPA) (**D**). NM, no movements; MVM, medium-velocity movements; HVM, high-velocity movements. Class 1: big amplitude angles (90–180°); Class 2: medium amplitude angles (30–90°); Class 3: medium amplitude angles (10–30°); Class 4: small amplitude angles (0–10°). Data are expressed as average value (AV) ± standard error (SE). * Significant differences to control ($p < 0.05$).

3.1.3. Biochemical Markers

After 96 h, FOS did not cause any significant variation in the glycogen content of zebrafish larvae ($p > 0.05$, Figure 3A); however, the proteins content increased at 2 and 20 mg/L FOS ($p < 0.05$, Figure 3B) and a decrease in lipids content was found at 2 mg/L FOS ($p < 0.05$, Figure 3C). Contaminants may change the total energy available on the organisms, which originates compensatory corrections in the energy metabolism to keep the homeostasis [46]. Lipids are considered the primary energy source mobilized when organisms are exposed to contaminants and, with the carbohydrates, they are rapidly mobilized to supply a sudden energy demand while proteins are the last choice of energy source, being usually mobilized only under severe conditions [46]. Decreased lipid contents may be a result of physiological stress response caused by FOS [47]. The detected effects on energy metabolism of zebrafish embryos after FOS exposure (increased protein content and decreased lipids' content) may compromise other biological functions, such as growth, reproduction, development, and locomotor activity. In our study, no effects were found on zebrafish development (e.g., in terms of hatching, heartbeat rate, and malformations' appearance) by 2 and 20 mg/L FOS. However, an increase in the distance swam by zebrafish larvae was found to 20 mg/L FOS, which may be related with the detected alterations on the energy metabolism.

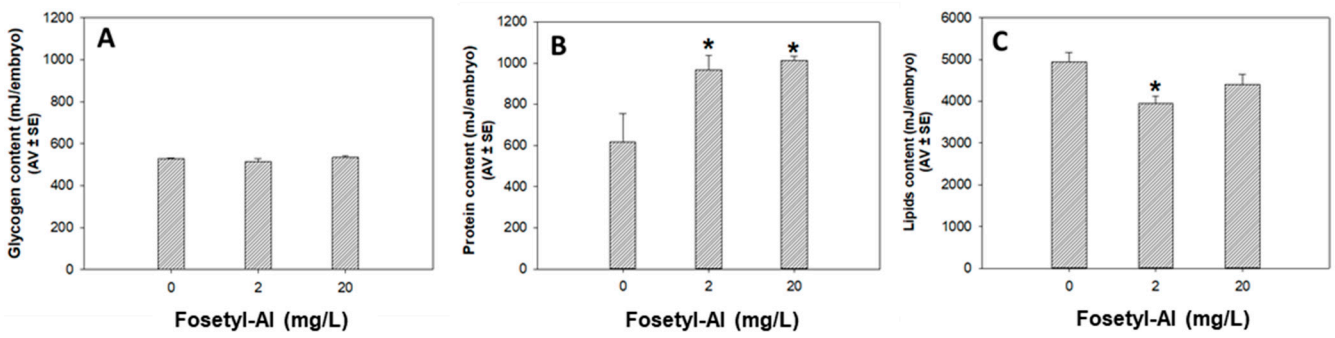

Figure 3. Effects of 96 h of exposure to fosetyl-Al on zebrafish larvae for carbohydrates' (**A**); proteins' (**B**); and lipids' (**C**) contents. Data are expressed as average value (AV) ± standard error (SE). * Significant differences to control ($p < 0.05$).

After 96 h, FOS induced no significant effects on ChE, CAT activities, and TG content of zebrafish larvae ($p > 0.05$, Figure 4A,C,D). However, FOS (2 mg/L) increased GST activity ($p < 0.05$, Figure 4B). Oxidative damage results from an imbalance between oxidants and antioxidant levels in the organism, which increases reactive oxygen species (ROS) generation. ROS may react with biomolecules, such as lipids and proteins, affecting cell viability [48,49]. However, fish have effective antioxidant defenses comprised by various antioxidant enzymes (e.g., CAT and GST) and low-molecular-weight scavengers (e.g., glutathione (GSH)) [50–52]. GST is a phase II detoxification family of enzymes and an oxidative stress biomarker. The increased GST activity detected in zebrafish larvae after exposure to FOS may be associated to the elimination of lipid peroxides by GST-conjugated products [49]. GST may not be considered a highly sensitive biomarker regarding organophosphate exposures [17,50,51], but may be useful as a biomarker for long-term exposure scenarios, as it seems that it requires a longer exposure period to inhibit GST by organophosphates [18,53,54]. FOS did not also significantly alter the activity of CAT (another oxidative stress-related biomarker). Therefore, the lack of an effect on the tested enzymatic biomarkers could be due to the short time of exposure (96 h) [49]. However, a previous study reported a relevant increase of GSH after 1 h of exposure of zebrafish larvae to the pesticides diazinon and diuron [18]. Diazinon also caused an inhibition of ChE activity in zebrafish larvae [18]. Indeed, the primary target of organophosphates pesticides is the enzyme acetylcholinesterase (AChE), which hydrolyzes the neurotransmitter acetylcholine in the peripheral and central nervous system [42]. Organophosphates are known to inhibit the activity of ChE [18,55], whereas, in the current study, no effect was

detected in the ChE activity after the exposure to FOS, suggesting that FOS may have a distinct mode of action than the organophosphates pesticides. Moreover, the behavioral effects detected in the current study cannot be related to the alterations on ChE activity. A further neurotoxicity study with FOS should be provided to comprehend its role on the nervous system of non-target organisms.

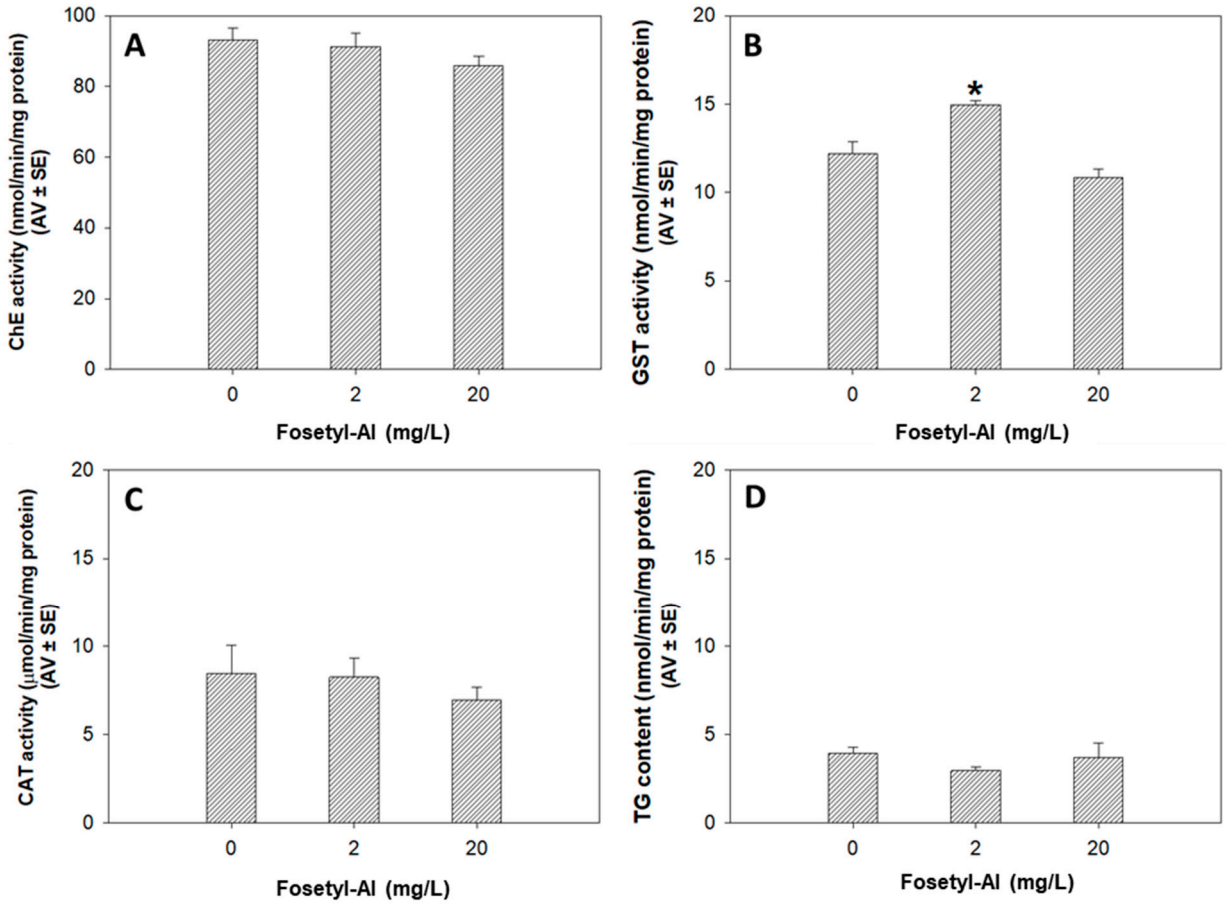

Figure 4. Effects of 96 h pf exposure to fosetyl-Al on zebrafish larvae for cholinesterase (ChE) (**A**); glutathione *S*-transferase (GST) (**B**); catalase (CAT) (**C**) activities; and total glutathione (TG) content (**D**). Data are expressed as average value (AV) ± standard error (SE). * Significant differences to control ($p < 0.05$).

3.2. Enchytraeus crypticus

Enchytraeid Reproduction Test (ERT)

FOS induced no effects in terms of survival and reproduction of *E. crypticus* after 28 d of exposure ($p > 0.05$, Figure 5A). However, a decrease in the population number after 56 d was found at 1250 mg/kg FOS ($p < 0.05$, Figure 5B). The toxicity of FOS to *E. crypticus* seems to have increased with the increase of the exposure period. This corroborates the idea that, for some endpoints (in this case reproduction), FOS seems to have a toxicological action analogous to the organophosphates, since it only caused toxic effects after a longer-term exposure [18,49,53,54]. In addition, all the tested concentrations of FOS (except for 500 mg/kg) enhanced the size (length) of adults after 28 d ($p < 0.05$, Figure 3C). No study was found about the effects of FOS to *E. crypticus*. However, for a *Folsomia candida* chronic exposure with FOS water-dispersible granule containing 800 g/kg (WG80) (Bayer CropScience) an EC_{20} of 958.8 mg/kg for reproduction was calculated [10]. In soil, FOS can be rapidly transformed to their metabolites, phosphonic acid and ethanol (DT_{50} = 0.1 d). Phosphonic acid is an important degradation product of FOS due to its persistence with a DT_{50} of 28 to 130 d in soil [10]. Hence, we cannot discard the hypothesis that the

observed effects in the present study may be due to FOS metabolite, phosphonic acid. A value of LC_{50} > 615 mg/kg was estimated for earthworms after acute exposure (14 d) to phosphonic acid [38]. Based on the current data gap, a phosphonic acid risk assessment for soil invertebrates is needed (this is also in line with the Terrestrial Guidance Document from the European Commission) [38].

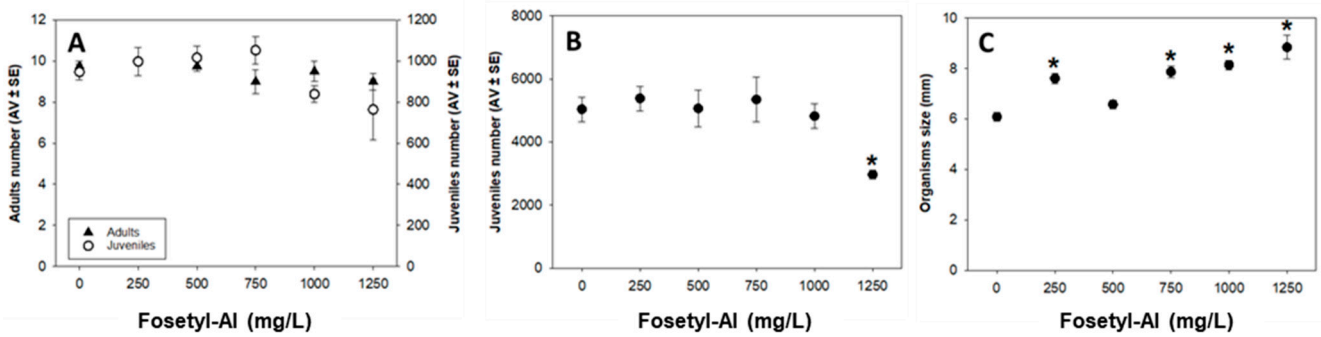

Figure 5. Effects of fosetyl-Al on *Enchytraeus crypticus* for survival (number of adults) and reproduction (number of juveniles) after 28 d of exposure (**A**); reproduction (number of juveniles) after 56 d of exposure (**B**); and adults' sizes after 28 d of exposure (**C**). Data are expressed as average value (AV) ± standard error (SE). * Significant differences to control ($p < 0.05$).

For *E. crypticus*, a recent work with various fungicides showed that 21 d exposure of prosaro did not also affect reproduction and survival of the organisms [16]. On contrary, another fungicide, amistar, had a toxic effect to *E. crypticus*, with the reproduction being the most sensitive endpoint [16]. Another study reported that *E. crypticus* were not affected by the pesticide toxaphene even at the highest tested concentration (1000 mg/kg) [15]. Dimethoate increased the survival and mancozeb decreased the survival and reproduction of enchytraeids [17]. The size of the adults was significantly affected by dimethoate, increasing the dimethoate concentration limited growth [17]. This is an opposite effect considering the one detected in our study: FOS increased the organisms' sizes. Growth is a very relevant ecological endpoint [56]. Effects of the contaminants on the organisms' sizes may induce harmful consequences in terms of organisms' performances, e.g., change in age structure and, hence, population dynamics [57]. The detected increase of size may be a trade-off, i.e., an investment of the organisms in terms of optimal size for their survival, after the exposure to FOS. Indeed, no significant alterations were found on survival of *E. crypticus* after 28 d of exposure. It is of interest to note that the increase of adults' sizes in the first generation (as mentioned, probably an investment for survival) did not prevent the impact on reproduction at 56 d, which was significantly reduced when parents were largest (Figure 5B,C).

3.3. Integration of Knowledge for Risk Assessment

The risk assessment of pesticides to non-target organisms usually is based on standard tests. The impact of the contaminants is measured through standard endpoints, such as reproduction and survival. However, these biological responses only give information about the potential ecological impact of the selected contaminant, being the assessment of other endpoints highly recommended [58]. Indeed, data covering a larger spectrum are welcomed, but when it is not possible to obtain detailed answers about the mechanistic issues involved, there is a need to look behind the apical effects. As detected in our study, alterations in terms of biochemical responses, behavior, and growth of the organisms occurred at lower concentrations than the effects observed at the standard endpoints (e.g., hatching and reproduction). Comparing the data obtained with the previously published ones, the mechanisms of toxicity of the pesticides seem dependent on the type of the pesticide and the test organism. Although, for FOS, PECs did not cause toxic effects to the tested organisms, for other pesticides, the effects may be detected at environmentally relevant concentrations. Another important aspect is that the toxicity tests should consider longer

exposure periods than the ones recommended by the guidelines [59]. In the environment, pesticides (and their metabolites and transformation products) may persist for long periods of time and the organisms may be continuously exposed [57]. Indeed, the toxic effect of FOS (decrease of organisms' reproduction for the highest concentration) was only seen after a long-term exposure (56 d). This has been the case for other contaminants, e.g., multi-walled carbon nanotubes (MWCNT) [60] or tungsten carbide cobalt (WCCo) [36] materials, where the effects were minute after 28 d and significantly higher at 56 d.

4. Conclusions

Some adverse effects were found after the exposure to FOS (*D. rerio*: hatching, behavior, and biochemical responses; *E. crypticus*: reproduction and growth). Answering to the main question of the current study, FOS may not be considered safe for *D. rerio* and *E. crypticus* for concentrations higher than the PECs described for soil (e.g., 1.067 mg/kg) and for surface water (e.g., 0.06496 mg/L). For a better evaluation of the risk, the obtained results showed the importance of assessing the short- and long(er)-term effects of different pesticides and their metabolites (as is the case of phosphonic acid) to various non-target organisms. Additionally, a multi-endpoint approach, with standard (described by the guidelines) and extra endpoints, is highly recommended for further studies. Multigenerational studies might also be required to address the potential endocrine-disrupting properties of FOS and phosphonic acid in non-target organisms (this is a data gap).

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Institutional Review Board Statement: The experiments were in accordance with the current laws of the country in which they were performed. Zebrafish Facility complies with the Portuguese law (Portaria 1005/02 and Portaria 1131/97), which follows the European Guideline 86/609/EC, and with the Council Regulation No. 2005/01 EC. It also follows the FELASA (Federation of European Laboratory Animal Science Associations) guidelines and recommendations concerning animal welfare and the design and conduct of research projects in which animals are used. Animal housing, welfare, and experimentation were under constant monitoring from the veterinary service.

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Data Availability Statement: The data are available on request from the corresponding author.

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