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Androgenic activation, impairment of the monoaminergic system and altered behavior in zebrafish larvae exposed to environmental concentrations of fenitrothion

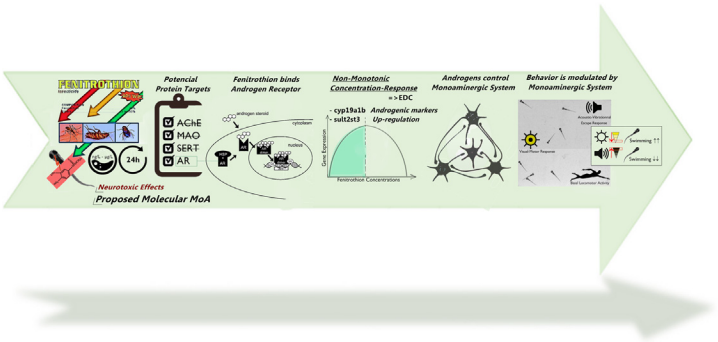
Melissa Faria [a], Eva Prats [b], Jonathan Ricardo Rosas Ramírez [c], Marina Bellot [d], Juliette Bedrossiantz [a], Maria Pagano [e], Arnau Valls [f], Cristian Gomez-Canela [d], Josep M. Porta [f], Jordi Mestres [g], Natalia Garcia-Reyero [h], Caterina Faggio [e], Leobardo Manuel Gómez Oliván [c], Demetrio Raldua [a,*]

[a] Institute for Environmental Assessment and Water Research (IDAEA-CSIC), Jordi Girona, 18, 08034 Barcelona, Spain
[b] Research and Development Center (CID-CSIC), Jordi Girona 18, 08034 Barcelona, Spain
[c] Laboratorio de Toxicología Ambiental, Facultad de Química, Universidad Autónoma del Estado de México, Paseo Colón intersección Paseo Tollocan s/n. Col. Residencial Colón, 50120 Toluca, Estado de México, Mexico
[d] Department of Analytical Chemistry and Applied (Chromatography section), School of Engineering, Institut Químic de Sarrià-Universitat Ramon Llull, Via Augusta 390, 08017 Barcelona, Spain
[e] Department of Chemical, Biological, Pharmaceutical and Environmental Sciences, University of Messina, Viale Ferdinando Stagno d'Alcontres, 31, 98166 Agata-Messina, Italy
[f] Institut de Robòtica i Informàtica Industrial, CSIC-UPC, Barcelona, Spain
[g] Systems Pharmacology, Research Group on Biomedical Informatics (GRIB), IMIM Hospital del Mar Medical Research Institute and Universitat Pompeu Fabra, Parc de Recerca Biomèdica, Chemotargets SL, Parc Científic de Barcelona, Barcelona, Spain
[h] Environmental Laboratory, US Army Engineer Research and Development Center, Vicksburg, MS, USA

HIGHLIGHTS

- Environmental concentrations of fenitrothion induce behavioral changes in zebrafish.
- Fenitrothion alter the expression of neurotoxicity markers in a non-monotonic fashion.
- At low environmental concentrations, fenitrothion effects are AChE-independents.
- Binding to androgen receptor is the most suitable candidate to MIE.

GRAPHICAL ABSTRACT



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ABSTRACT

Fenitrothion is an organophosphorus insecticide usually found in aquatic ecosystems at concentrations in the range of low ng/L. In this manuscript we show that 24 h exposure to environmental concentrations of fenitrothion, from ng/L to low μg/L, altered basal locomotor activity, visual-motor response and acoustic/vibrational escape response of zebrafish larvae. Furthermore, fenitrothion and expression of *gap43a*, *gfap*, *atp2b1a*, and *mbp* exhibited a significant non-monotonic concentration-response relationship. Once determined that environmental concentrations of fenitrothion were neurotoxic for zebrafish larvae, a computational analysis identified potential protein targets of this compound. Some of the predictions, including interactions with acetylcholinesterase, monoamine-oxidases and androgen receptor (AR), were experimentally validated. Binding to AR was the most suitable candidate for molecular initiating event, as indicated by both the up-regulation of *cyp19a1b* and *sult2st3* and the non-monotonic relationship found between fenitrothion and the observed responses. Finally, when the integrity of the monoaminergic system was evaluated, altered levels of L-DOPA, DOPAC, HVA and 5-HIAA were

* Corresponding author.
E-mail address: drpqam@cid.csic.es (D. Raldua).

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Risk assessment

found, as well as a significant up-regulation of *slc18a2* expression at the lowest concentrations of fenitrothion. These data strongly suggest that concentrations of fenitrothion commonly found in aquatic ecosystems present a significant environmental risk for fish communities.

1. Introduction

Fenitrothion (*O,O*-dimethyl *O*-[3-methyl-4-nitrophenol] phosphorothiate) is an inexpensive organophosphorus insecticide widely used in agriculture, forestry and public health to control insects, especially those that suck or bite. Whereas the most relevant toxicological mode of action of fenitrothion is widely accepted to be the direct inhibition of the activity of the enzyme acetylcholinesterase (AChE) in the central and peripheral nervous system (Sancho et al., 1997), other molecular targets have been described for this compound, including the androgen receptor and some members of the CYP450 family (Sebire et al., 2009; Spaggiari et al., 2016; Tamura et al., 2001). Its application is usually delivered through an aerial spray, which makes it easily transported to the surface water *via* spray drift. Fenitrothion concentrations between 15 - 75 μg/L have been reported in the immediate vicinity of the sprayed area (Hossain et al., 2015; Mallet and Volpe, 1982). However, fenitrothion concentrations usually drop by dilution and physicochemical and microbial degradation to less than 1 μg/L within a few days after spraying (Hatakeyama et al., 1990). Fenitrothion concentrations in non-directly exposed areas are usually low, in the range of 6–600 ng/L (Carrasco et al., 1987; Derbalah et al., 2019; Köck et al., 2010).

Whereas fenitrothion exhibits high systemic toxicity in aquatic invertebrates, with LC50 values ranging μg/L for most species, fish are less sensitive, exhibiting 96-LC50 varying between 1.7 and 10 mg/L (ICPS, 1992). However, as neurotoxic compound, specifically neurotoxic endpoints are more sensitive than those analyzed for systemic toxicity, and relevant behavioral changes have been reported in fish exposed to concentrations of this compound in the low μg/L range (Morgan and Kiceniuk, 1990). Therefore, additional efforts should be made to determine whether environmental concentrations of fenitrothion, in the range of ng/L, are able to impair ecologically relevant behaviors of fish.

Zebrafish (*Danio rerio*) is a small cyprinid commonly used as a model species in ecotoxicological studies (Blahova et al., 2020; Vossen et al., 2020; Wang et al., 2019). Furthermore, zebrafish is currently a recognized vertebrate model for neurotoxicity studies. Whereas the acute toxicity of fenitrothion for zebrafish embryos and larvae has been found to be moderate, with 24-LC50 in the range of 0.23–0.62 mg/L (Shadiqur et al., 2020), behavioral effects have been recently reported in larvae exposed to environmental concentration of this chemical (Faria et al., 2020). Ecologically relevant fish larvae behaviors commonly tested in zebrafish include visual motor response, a complex behavior integrating visual and motor functions (Neuhauss, 2010), the basal locomotor activity and the vibrational-startle response (Faria et al., 2020; Prats et al., 2017). Anti-predatory or prey capture behaviors, essential for survival of fish larvae, lay on the integrity of their sensory and motor circuits (Bhattacharyya et al., 2019; Faria et al., 2019; Scott and Sloman, 2004). Therefore, any impairment in these behaviors due to exposure to pollutants may be detrimental for larvae survival under natural conditions.

In this study, the effect of five realistic environmental concentrations of fenitrothion (1.7 ng/L-17 μg/L) on the basal locomotor activity, visual motor response and acoustic/vibrational-evoked escape response, has been determined in zebrafish larvae 24 h after exposure. Then, the expression of *mbp*, *gfap*, *syt1a*, and *gap43*, transcriptional markers of neurotoxicity, has also been determined. Moreover, the molecular pathways behind the observed behavior have been explored by combining *in silico* target profiling, *in vitro* test, gene expression and biochemical analyses and neurotransmitter profiling. Finally, the environmental risk of fenitrothion in aquatic ecosystems has been estimated.

2. Material and methods

2.1. Fish husbandry and larvae production

Adult wild-type zebrafish were purchased from Piscicultura Superior SL (Parets del Vallès, Barcelona) and maintained in fish water [reverse-osmosis purified water containing 90 μg/mL of Instant Ocean (Aquarium Systems, Sarrebourg, France) and 0.58 mM $CaSO_4 \cdot 2H_2O$] at 28 ± 1 °C in the Research and Development Center of the Spanish Research Council (CID-CSIC) facilities under standard conditions. Embryos were obtained by in-tank group breeding with a 5:3, female: male ratio per tank. Breeding tanks are homemade and include a solid external tank and an internal plastic net. Embryos deposited in the bottom of the tank were collected and maintained in 500 mL glass containers at 1 individual/mL density in fish water at 28.5 °C on a 12 light:12 dark photoperiod. Larvae were not fed before or during the experimental period (from 7 to 8 days post fertilization (dpf)). All procedures were approved by the Institutional Animal Care and Use Committees at the CID-CSIC and conducted in accordance with the institutional guidelines under a license from the local government (agreement number 9027).

2.2. Experimental concentrations and stability of fenitrothion in fish water and

Fenitrothion (Pestanal®, analytical standard, purity 95.4%) was obtained from Sigma-Aldrich (Steinheim, Germany). Experimental concentrations in fish water of the five nominal concentrations used in this study (1.7 ng/L, 17 ng/L, 170 ng/L, 1.7 μg /L and 17 μg /L) were determined using ultra-high performance liquid chromatography with triple quadrupole detector (UPLC-MS/MS) analysis (see Supplementary Methods for additional details). Three samples of each concentration were analyzed. Furthermore, the stability of fenitrothion in fish water was assessed by preparing in triplicate solutions with a nominal concentration of 0.17 and 17 μg/L (representing the higher and lower levels of the concentrations studied), which were maintained under the same conditions used for zebrafish larvae (28 °C and 12 L:12D photoperiod). Aliquots of these solutions were analyzed at time 0 and 24 h and analyzed as described above.

2.3. Experimental protocol

Five fenitrothion concentrations have been tested in this study, 1.7 ng/L, 17 ng/L, 170 ng/L, 1.7 μg/L, and 17 μg/L. The two highest concentrations are representative of the situation in the vicinity of sprayed areas and the three lowest concentrations represent the situation in non-directly exposed areas. Fenitrothion stocks were prepared in DMSO, with a final carrier concentration of 0.1% in both vehicle control and treatment groups. The use of vehicle controls with 0.1% DMSO has been reported to be safe and is widely used to screen libraries of small chemicals in zebrafish (Maes et al., 2012; Vliet et al., 2017). The highest concentration of fenitrothion tested in this study, 17 μg/L, is already an environmentally relevant concentration that in a previous study was reported to reduce the magnitude of the vibrational-evoked startle response in zebrafish larvae after 24 h exposure (Faria et al., 2020).

Exposures were conducted in 48-well microplates with 1 larva per well and 1 mL of working solution (total 48 larvae per plate). After

24 h of exposure (larvae from 7 to 8 days dpf, behavior of the larvae was directly tested without further manipulation, or larvae were collected for the different analyses. All the exposures were performed at 28.5 °C (POL-EKO APARATURA Climatic chamber KK350, Poland) with 12 L:12D photoperiod. For each variable investigated, samples were collected from 2 to 3 trials of the same experiment setup, which were conducted in different days and with different larvae batches.

2.4. Behavioral analysis

Vibrational startle response assay was performed as described in Faria et al. (2020). This assay is based in the escape response evoked in zebrafish larvae by a tapping stimulus. Video tracking acquisition was controlled and the escape response was analyzed using the EthoVision XT 9 software (Noldus, Wageningen, The Netherlands). Trials were performed at 28 °C with near-infrared light. Tapping stimulus was selected at the highest intensity (intensity level: 8) and one stimulus was delivered after a 15 min acclimation period to the chamber Videos were recorded at 30 frames per second and the vibrational startle response (VSR) was analyzed for each individual larva by measuring the distance traveled (cm) over the 1 s period after the stimulus.

Basal locomotor activity (BLA) and visual-motor response (VMR) analyses of 8 dpf zebrafish larvae were performed using a DanioVision system running an Ethovision XT 11 software (Noldus, Wageningen, the Netherlands), essentially as described by Faria et al. (2015) (see Supplementary Methods for additional details). Whereas BLA is defined as the distance traveled by the larvae during the first period of 20 min in the dark, VMR is based in the hyperactivity period evoked by a sudden reduction in light intensity (Fernandes et al., 2012).

2.5. Kinematic analysis

The kinematics of the acoustic/vibrational-evoked escape response is a highly stereotyped complex behavior constituted by three sequential modules: a very fast and large C-bend followed by a high amplitude counterbend and, finally, a bout of fast swimming oriented away from the stimulus. The experiments have been carried out on the setup shown in Supplementary Fig. S1, that mimics the one described in Burgess and Granato (2007a). The setup is composed by a 50 mm Petri dish with nine arenas arranged in a 3 × 3 squared grid. During the experiments, each arena holds a larva swimming in 400 μL of fish water. The dish is attached to a shaker (Mini Shaker 4810, Brüel&Kajær) with a custom, 3D-printed holder. The shaker produces the vibrational stimuli required in the experiments. Such vibrations are controlled by a microprocessor (STM32 F4 Discovery) and modulated by an amplifier (Power Amplifier Type 2718, Brüel&Kajær) set to a gain of 10 dB, a current limit of 1.8 A, and a variable gain of 50%.

Larvae reactions in the nine arenas are recorded with a high-speed camera (Photron Fastcam Mini UX100) equipped with a 50 mm lens (Sigma 50 mm F1.4 DG). When active, the camera takes 512 × 512 pixel images at 1000 frames per second. The trigger of the camera is activated by the same microprocessor taking care of controlling the vibrations. In this way, a precise synchronization between the captured videos and the stimuli is obtained. To improve image quality, the Petri dish is retro-illuminated with a led light of variable intensity (Backlight Led LF-100SWZ-IU, CCS Inc.) also controlled by the mentioned microprocessor.

To facilitate the use of the experimental setup, we developed *LarvaCam*, a software suitable both for experiments aiming the analysis of the startle response and for experiments analyzing the habituation process after multiple vibrational-evoked startle responses. *LarvaCam*, which runs on a Windows-based PC, allows designing experiments composed by a variable number of phases that are executed in sequence. Each phase can include several steps where, at each step, a vibration is transmitted to the larvae (Supplementary Fig. S2). Two different vibration intensities can be selected for all the steps of each phase: soft vibration (1 kHz, 0.6 V), and strong vibration (1 kHz, 6 V). In this study, a design with only one phase and only one step has been used. Moreover, the strong vibration was selected as the vibrational stimulus, as we found that using this intensity there were about 70% short-latency C-bends in control larvae. The camera was activated 30 ms before the vibration, and the total length of the captured video was 120 ms. *LarvaCam* software translates the configured experiment into low-level commands that are sent to the microcontroller *via* USB connection. Moreover, the software also takes care of downloading of the videos captured by the high-speed camera, which is connected to the PC *via* a Gigabit ethernet cable.

To process the videos, we developed *LarvaTrack*, a software that automatically identifies the patches of each frame corresponding to the nine arenas using line detection and morphological image processing techniques (see Supplementary Fig. S3). Then, each patch is processed, correcting the intensity and also using background subtraction and morphological image operators to detect the region corresponding to the larva. The skeleton of this region is computed next and, finally, the head of the larva is identified. The skeleton is approximated by three straight segments, and the angles shown in Supplementary Fig. S4 are computed. Namely, the computed angles are the one between the first and second segment (α), the one between the second and third segment (β) and the sum of both (γ), which is used to characterize the amplitude of the bend. The temporal evolution of such angles gives an accurate account of the reaction of the larvae to the stimuli.

Finally, some experiments were performed to determine the distance traveled by control and fenitrothion-exposed larvae during the short-latency C-bend (SLC). In this case, Petri dishes without grid, containing 20 larvae, were used in the setup. The analyses of kinematic parameters of these larvae, including SLC, long-latency C-bend (LLC), and the distance traveled, were obtained by using Flote software package (Burgess and Granato, 2007a, 2007b).

2.6. RNA preparation and qRT-PCR analysis

Gene expression analysis was performed as previously reported by Prats et al. (2017). Total RNA was extracted from a total of 6–8 pools of 4 larvae (8 dpf) using the Trizol Reagent (Invitrogen Life Technologies, Carlsbad, CA). RNA concentration was measured by spectrophotometric absorption at 260 nm in a NanoDrop™ ND-8000 spectrophotometer (Fisher Scientific) and the quality checked in an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). RIN (RNA Integrity Number) values ranged between 9 and 10. After DNaseI treatment (Ambion, Austin, TX), 1 μg of total RNA was used to synthesize the first strand of cDNA with First Strand cDNA synthesis Kit (Roche Diagnostics, Mannheim, Germany) using oligo(dT), following the manufacturer's instructions.

Real Time PCR was performed in a LightCycler ® 480 Real-Time PCR System using SYBR Green PCR Master Mix (Roche Diagnostics, Mannheim, Germany). Cycling parameters were 95 °C for 15 min followed by 45 cycles of 95 °C, 10 s and 60 °C, 30 s. For each experimental condition, qPCR analyses were performed from two independent experiments, with 4 biological replicates on each experiment and three technical replicates for each sample. The sequences of primers for the sixteen selected genes (*gap43a*, *mbp*, *gfap*, *syt1a*, *atp2b1a*, *cyp19a1b*, *sult2st3*, *mao*, *comtb*, *slc6a4a, th1, th2, slc6a3, slc18a2, tph1a, tph1b*) are reported in Supplementary Table ST1. The housekeeping gene *ppia2* was used as reference gene for normalization purposes (Prats et al., 2017). Primers were synthesized by Sigma-Aldrich (Steinheim, Germany). Efficiency and specificity of all primers were checked before the analyses. Results were normalized to *ppia2* and the relative abundance of mRNA was calculated following the ΔΔCt method (Livak and Schmittgen, 2001) deriving fold-change ratios from these values.

2.7. Computational analyses

Based on the recently defined PHASE initiative from the US Food and Drug Administration's Center for Drug Evaluation and Research (Ellis et al., 2019, 2020), targets for fenitrothion were predicted using two platforms, namely, CLARITY (Chemotargets CLARITY v4, 2019) and SEA (Keiser et al., 2007). Both platforms use two-dimensional chemical structures to predict potential binding targets (Keiser et al., 2009; Vidal and Mestres, 2010). SEA uses descriptor-based similarity to compare the structure of a molecule to the chemical structures with known *in vitro* binding affinity in ChEMBL (Keiser et al., 2007, 2009; Vidal and Mestres, 2010). Approximately 2300 protein targets are covered. For each predicted target, a *p*-value and the similarity of the closest molecule are provided. CLARITY uses six ligand-based approaches that rely on descriptor-based molecular similarity, an implementation of the similarity ensemble approach, fuzzy fragment-based mapping, quantitative structure-activity relationships, a set of machine learning methods and target cross-pharmacology indices (Gregori-Puigjané and Mestres, 2006; Vidal et al., 2011). The training set for the 4799 protein target models is generated from *in vitro* affinity data contained in both public and patent sources (Sharma et al., 2016). For each target prediction, the projected affinity and mode of action are provided alongside with a confidence score based on the number and type of methods that independently contribute to the prediction.

2.8. Mammalian in vitro functional assays

In vitro functional assays to validate some of the predicted interactions of fenitrothion with proteins were performed using human proteins, since these assays are not currently commercially available for zebrafish proteins. Binding assays [AR Human Androgen NHR Binding (Agonist Radioligand) and Human Serotonin Transporter Binding (Antagonist Radioligand)] and Enzymatic assays [Acetylcholinesterase Human, Monoamine Oxidase A (MAO-A) Human and Monoamine Oxidase B (MAO-B) Human] assays were performed by Eurofins Cerep (Celle l'Evescault, France). The aim of these mammalian *in vitro* functional assays was to determine the validity of some predicted interactions between fenitrothion and the human proteins and not to assess if these interactions occur at environmentally relevant concentrations. Therefore, 100 nM and 10 μM fenitrothion, two concentrations commonly used in these *in vitro* assays, were selected instead of using the same range of concentrations selected for our *in vivo* assays in zebrafish larvae. Compound binding was calculated as a % inhibition of the binding of a radioactively labeled ligand specific for each target, whereas compound enzyme inhibition effect was calculated as a % inhibition of control enzyme activity. Results showing an inhibition or stimulation higher than 50% are considered to represent significant effects of the test compounds. Additional details can be found in Supplementary Methods.

2.9. Zebrafish acetylcholinesterase activity

Acetylcholinesterase (AChE) activity was determined in individual larvae ($n = 9$ larvae per experimental group), essentially as described by Faria et al. (2015). Thus, larvae were homogenized by TissueLyser®, by adding two 3-mm stainless steel beads to each sample, and homogenizing at 50 oscillations/s during 30 s, in 200 μl of ice-cold 0.1 M phosphate buffer (pH 7.4) with 150 mM KCl and 0.1 mM EDTA and further centrifuged at 10,000 ×*g* for 10 min at 4 °C, collecting the resulting supernatant for AChE analysis. Then, 2 mM acetylthiocholine and 0.33 mM 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) were added to the S9 fraction and the formation of the product resulting from the reaction between thiocholine and DTNB ion was monitored at 405 nm for 15 min. The final results were expressed in μmol min-^{1} mg protein using the extinction coefficient of 13.6×10^3 M^{-1} cm^{-1}.

2.10. Zebrafish monoamine-oxidase activity

Zebrafish larvae (8 dpf) collected in pools of 20 individuals were homogenized with ice-cold 10 mM Phosphate Buffer pH 7.6 containing 1 mM of EDTA, to a final tissue volume concentration of 100 larvae/mL of buffer. Homogenization was conducted using a TissueLyser®. Homogenates were then centrifuged at 5000 rpm for 5 min at 4 °C and MAO activity was immediately determined in the carefully collected supernatant using the peroxidase-linked spectrophotometric assay described by Holt et al. (1997) and adapted to zebrafish tissue by Anichtchik et al. (2006), which is based on the determination of the amount of H_2O_2 released during the oxidation of amines. In this assay, 4-aminoantipyrine is oxidized and condensed with vanillic acid to produce a red quinoneimine dye. The assay was performed in a 96-well plate where 50 μL extracts were incubated in the presence of 100 μL of amine substrate tyramine, 10 mM final concentration and of 50 μL of a chromogenic solution containing final concentrations of 500 μM 4-aminoantipyrine, 1 mM vanillic acid and 4 U/mL horseradish peroxidase type II in 10 mM Phosphate Buffer pH 7.6. The reaction was left to stabilize for 15 min at room temperature and then incubated for a further 60 min at 28 °C in the microplate reader (Synergy 2, Bio Tek) where the formation of the red quinoneimine dye was recorded at 490 nm. MAO activity results were presented as nmol/min/mg obtained using the molar absorption coefficient for quinoneimine dye at pH 7.6 (4656 M^{-1} cm^{-1}) and normalized with total protein in assay.

2.11. Extraction and analysis of neurotransmitters

Monoaminergic neurotransmitters were extracted from 8 pools of 5 larvae (8 dpf) following an extraction procedure adapted from Mayol-Cabré et al. (2020). The extraction process was based on the use of a solvent of polarity similar enough to the neurotransmitters to be able to extract them from the sample.

The monoaminergic neurotransmitters profile was assessed analyzing the extractions using the LC-MS/MS technique. The analysis was performed by ultra-high-performance liquid chromatography (Acquity UPLCH-Class Waters, Milford, MA, USA) coupled to a triple quadrupole mass spectrometer equipped with an electrospray (ESI) source (Xevo, TQS micro, Waters, USA). The quality parameters of this method are summarized in Table 1. Additional details on the extraction, analysis and quality assurance of the methodology used are provided in the Supplementary Methods.

2.12. Risk assessment

The environmental risk of pesticides in aquatic ecosystems is commonly estimated using the hazard quotient (HQ), the ratio between the measured environmental concentration (MEC) and the predicted non-effect concentration (PNEC):

$$HQ = MEC/PNEC$$

MEC values used are based in the median and maximum detected concentrations, whereas PNEC values commonly use non-observed effect concentration (NOEC) for the most sensitive endpoints and species. Whereas compounds with HQ >1 are potentially hazardous for aquatic ecosystems, those with HQ < 1 values are considered as slightly or not hazardous.

2.13. Statistical analysis

Data were analyzed with IBM SPSS v25 (Statistical Package 2010, Chicago, IL), and were plotted with GraphPad Prism 8.31 for Windows (GraphPad software Inc., La Jolla, CA) and Microsoft Excel for Mac 2011 (v14.5.7; Microsoft Corp., Redmond, WA, USA). Normality was assessed using Kolmogorov-Smirnov and Shapiro-Wilk tests. One-way

Table 1
Quality parameters obtained by LC-MS/MS for the 10 monoaminergic neurochemicals. F: slope, r^2: regression coefficient; IDL: instrumental detection limit; %R: recovery; RSD: relative standard deviation; %ME: matrix effect; MDL: method detection limit.

Monoamine NTs	Linearity (ng µL^{-1})	Calibration type	F	r^2	IDL (pg)	MDL (ng larvae^{-1})	Intra-day precision (RSD, %)	Inter-day precision (RSD, %)	%R ± RSD	%ME ± RSD
5-HT	0.005–2.5	External	0.41	0.99997	1.7	1.7	4.1	4.3	77 ± 4	126 ± 7
3-MT	0.005–2.5	Internal	5.18	0.99741	0.51	14.7	8.9	13.2	123 ± 12	63 ± 8
HVA	0.005–2.5	Internal	0.10	0.99776	24.6	53.0	2.9	8.0	64 ± 17	109 ± 16
DOPAC	0.005–2.5	Internal	2.51	0.99717	0.46	11.4	2.2	4.4	115 ± 16	59 ± 8
Tryp	0.005–0.5	Internal	0.41	0.99566	2.6	4.3	0.2	2.6	126 ± 8	134 ± 17
L-DOPA	0.005–2.5	Internal	3.93	0.99973	51.4	57.8	4.3	5.4	79 ± 8	61 ± 6
DA	0.005–2.5	External	0.20	0.99860	14.2	3.8	5.8	6.9	113 ± 5	91 ± 5
NE	0.005–2.5	External	1.08	0.99831	19.8	19.6	5.2	8.2	90 ± 2	79 ± 4
HIAA	0.005–2.5	Internal	4.05	0.99593	6.4	8.8	2.2	4.9	76 ± 3	82 ± 3
Tyr	0.005–2.5	Internal	0.90	0.99974	18.2	11.3	3.7	9.2	99 ± 10	117 ± 17

ANOVA followed by Dunnett's multiple comparison test was used to test for differences between normally distributed groups, whereas the Kruskal-Wallis test followed by Dunn's multiple comparison test against the control value was used to test for differences between groups that did not meet parametric assumptions. Data are presented as the mean ± SEM or median and 25–75 percentile of 2–3 independent experiments, unless otherwise stated. Significance was set at $P < 0.05$.

3. Results

3.1. Analytical chemistry

Table 2 shows the experimental concentration of fenitrothion for the five nominal concentrations used in this study. The experimental concentrations were calculated as the mean values of three replicates, showing values between 94 and 102% of the nominal concentrations (Table 2). Regarding the degradation assay, 0.17 and 17 µg/L were further analyzed 24 h after sample preparation. No degradation was found for the 0.17 µg/L solution whereas the fenitrothion content in the 17 µg/L solution decreased only by 7% (Table 2).

3.2. Environmental concentrations of fenitrothion impair locomotor behaviors

None of the five selected concentrations resulted in signs of systemic toxicity (impaired gross morphology or lethality). Locomotor behavior of the control and fenitrothion-treated larvae was analyzed using a battery of tests including basal locomotor activity (BLA), visual-motor response (VMR) and acoustic/vibrational escape response.

A significant effect on BLA was found in zebrafish larvae across the selected fenitrothion concentrations ($F_{5,587} = 13.831$, $P = 8.6 \times 10^{-13}$). This behavior decreased in larvae exposed to 1.7–170 ng/L and 17 µg/L fenitrothion compared to controls (Fig. 1A).

The analysis of VMR reported also significant effects on this behavior across fenitrothion concentrations ($F_{5,538} = 18.440$, $P = 6.4 \times 10^{-17}$). VMR increased in larvae exposed to 170 ng/L to 17 µg/L (Fig. 1B).

We then analyzed the effect of the five environmental concentrations of fenitrothion on the acoustic/vibrational escape response in zebrafish larvae,. Fig. 1C shows that fenitrothion exhibited a non-monotonic dose-response effect on this behavior ($F_{5,528} = 18.778$, $P = 3.4 \times 10^{-17}$), with an increase in the startle response at 17 ng/L and then decreasing the startle magnitude from 170 ng/L to 17 µg/L.

Finally, we analyzed the effect of fenitrothion on the kinematic of the acoustic/vibrational-evoked escape response, a highly stereotyped complex behavior constituted by three sequential modules: a very fast and large C-bend followed by a high amplitude counterbend and, finally, a bout of fast swimming oriented away from the stimulus. Two different types of escape responses can be evoked by an acoustic/vibrational stimulus, a short latency C-bend (SLC) and a long latency C-bend (LLC), although only SLC can be considered a startle response (Burgess and Granato, 2007a). In order to determine if the decrease in the magnitude of the vibrational-evoked escape response found by tracking the larvae on the DanioVision was in fact the result of a change in the SLC/LLC ratio, an automatized setup was built including a vibration exciter to provide the stimuli and a high-speed camera to record the larvae movement at 1000 fps. The intensity of the vibrational stimulus was selected to evoke SLC in about 70% of the control larvae (Wolman et al., 2011). Fig. 1D shows that after 24 h of exposure to 17 µg/L fenitrothion there was a significant increase in the SLC/LLC ratio. As the distance traveled during SLC has been reported to be higher than during LLC (Marquart et al., 2019), the increased percentage of SLC found in fenitrothion-exposed larvae should lead to an increase in the total distance traveled after the stimulus rather than a decrease in the distance traveled by the larvae consistently found in the DanioVision response to the vibrational stimulus. One potential explanation would be that fenitrothion specifically targeted the last step of SLC in zebrafish larvae, the fast swimming bouts, reducing the total distance traveled. In order to assess this hypothesis, a different design was used, recording the movement of the larvae for 300 ms (30 ms before the stimulus delivery and 270 ms after) in a Petri dish without any grid. In this case, kinematic analysis was performed using *Flote* software. Surprisingly, kinematic analysis showed that the main parameters characterizing SLC, including latency (9.06 ± 0.32 ms *vs* 8.28 ± 0.34 ms for control and fenitrothion-treated larvae, respectively; $n = 50–53$; $P = 0.100$, Student's *t*-test), amplitude of the C-bend (118.34 ± 3.53 degrees *vs* 119.64 ± 2.05 degrees for control and fenitrothion-treated larvae, respectively; n = 50–53; $P = 0.749$, Student's t-test), duration of the C-bend (10.16 ± 0.11 ms *vs* 9.85 ± 0.13 ms for control and fenitrothion-treated larvae, respectively; n = 50–53; $P = 0.075$, Student's t-test) and distance traveled in 270 ms after the stimulus (11.82 ± 0.34 mm *vs* 12.42 ± 0.45 mm

Table 2
Experimental concentrations of fenitrothion, recoveries obtained in the SPE procedure and stability of two of the concentrations 24 h after preparation.

Nominal concentration (µg/L)	Experimental concentration (µg/L)	% Recovery	Experimental concentration (µg/L) after 24 h
0.00170	0.00174 ± 1.95 × 10^{-5}	102.1 ± 1.15	–
0.0170	0.0166 ± 1.97 × 10^{-5}	97.5 ± 0.12	–
0.170	0.169 ± 4.46 × 10^{-4}	99.4 ± 0.37	0.171 ± 0.624 × 10^{-4}
1.70	1.643 ± 0.0135	96.7 ± 0.79	–
17.0	16.063 ± 0.02	94.5 ± 0.1	14.947 ± 0.377

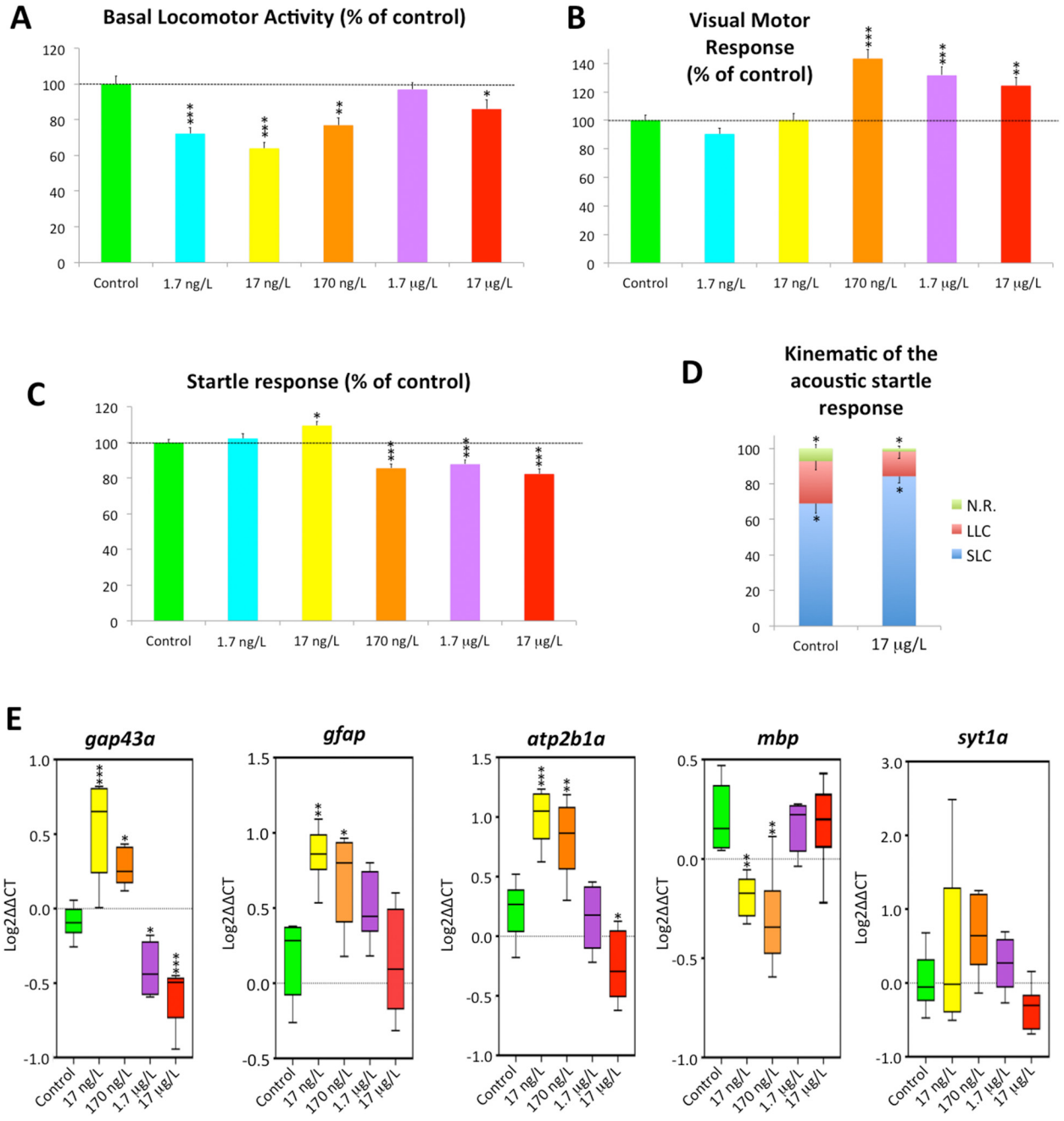

Fig. 1. Short-term (24 h) exposure to environmental concentrations of fenitrothion induces neurotoxicity in 8 days-post fertilization zebrafish larvae. (A) Analysis of the basal locomotor activity (BLA), showing hypoactivity of larvae exposed to 1.7–170 ng/L and 17 µg/L. (B) Analysis of the visual-motor response (VMR), showing a significant increase in the mobility of larvae exposed to 0.17–17 µg/L during the first 2 min of the light/dark challenge. (C) Analysis of the acoustic/vibrational escape response shows a significant decrease of the escape response evoked by one tapping stimulus in larvae exposed to 0.17–17 µg/L fenitrothion, and an increase in the response in larvae exposed to 17 ng/L. (D) Kinematics of the vibrational escape response show an increase in the SLC/LLC ratio in larvae exposed to 17 µg/L (N.R.: non-responder; SLC: short-latency C-bend; LLC: long-latency C-bend). Data reported as mean ± SEM (n = 81–167 for BLA, n = 76–154 for VMR, n = 73–148 for acoustic/vibrational escape response and n = 12–15 for kinematic analysis SLC/LLC ratio) (E) Gene expression of five selected transcriptional markers of neurotoxicity in zebrafish larvae control or exposed to four Environmental concentrations of fenitrothion. Boxplot representation of ΔΔCt values, with the box indicating the 25th and 75th percentiles and the whiskers the maximum and minimum values. The thin line within the box marks the median (n = 6–8 pools). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; one-way ANOVA with Dunnett's multiple comparison test. Data from 2 to 4 independent experiments.

for control and fenitrothion-treated larvae, respectively; n = 50–53; P = 0.298, Student's t-test) remained unchanged in fenitrothion-treated larvae.

3.3. Non-monotonic concentration-response relationship between fenitrothion and some transcriptional neurotoxicity markers

In order to better characterize the neurotoxic effects of fenitrothion at molecular level, the expression of five genes involved in different functions in the central and peripheral nervous system was analyzed (Fig. 1E). An non-monotonic concentration-response curve (NMCRC) with an inverted U-shape was found to describe the relationship between fenitrothion and *gap43a* expression (Fig. 1B), with a significant up-regulation in the expression of this gene at the two lowest concentrations and a down-regulation at the two highest concentrations ($F_{4,25} = 32.983$, $P = 1.2 \times 10^{-9}$).

An inverted U-shaped NMCRC also described the relationship between fenitrothion concentrations and *gfap* expression, with a

significant increase in the expression of this gene at the two lowest concentrations and no significant effects at the higher concentrations ($F_{4,25} = 8.003$, $P = 0.00026$). Glial fibrillary acid protein (GFAP), the protein encoded by *gfap*, is a classical marker of astrogliosis.

Expression of *atp2b1a*, a marker of the integrity of the hair cells in inner ear and neuromasts (Go et al., 2010), exhibited also a U-shaped non-monotonic relationship with fenitrothion concentrations ($F_{4,25} = 21.548$, $P = 8.5 \times 10^{-8}$). Whereas larvae exposed to 17–170 ng/L fenitrothion exhibited a significant up-regulation of *atp2b1a*, the expression of this gene in larvae exposed to 17 μg/L fenitrothion was down-regulated.

In contrast to *gap43*, *gfap* and *atp2b1a*, the relationship between myelin basic protein (*mbp*) expression and fenitrothion was better described with an inverted U-shaped NMCRC, since the expression of this gene was significantly reduced at 17–170 ng/L fenitrothion, but not at higher concentrations.

Finally, expression of *syt1a*, a transcriptional marker of synaptic vesicle cycling, was not significantly altered by any of the tested concentrations of fenitrothion.

3.4. Predicted target profile for fenitrothion

Different toxicological modes of action have been reported for fenitrothion, including the direct inhibition of the activity of the enzyme acetylcholinesterase (AChE) (Sancho et al., 1997; Zinkl et al., 1991), an antagonistic effect on the androgen receptor (AR) (Tamura et al., 2001; Sebire et al., 2009) and interaction with some members of the CYP450 family (Abass and Pelkonen, 2013; Spaggiari et al., 2016). In order to explore the toxicological targets of fenitrothion potentially behind the observed neurotoxic effect, the CLARITY (Chemotargets CLARITY v4, 2019) and SEA (Keiser et al., 2007) platforms recommended in the PHASE initiative (Ellis et al., 2019; Ellis et al., 2020) were used to predict its off-target pharmacology.

The list of predicted human targets for fenitrothion is collected in Table 3. Taking the confidence/significance thresholds recommended by each platform, a total of six and four targets were identified by CLARITY and SEA, respectively. Among them, the only common target of fenitrothion predicted by both platforms is carboxylic ester hydrolase, a protein with acetylcholinesterase activity.

Focus was then put in selecting for further consideration any predicted targets known to be linked to neurotoxic effects. Within the list of predictions from CLARITY above the recommended confidence score, two neurotoxic-associated targets were detected, namely, the androgen receptor (AR) and the sodium-dependent serotonin transporter (SLC6A4, also referred as SERT). In addition, five additional neurotoxic-associated targets could be recovered from predictions below the recommended confidence scores. These are the amine oxidase [flavin-containing] A and B (MAOA and MAOB), the tyrosine 3-monooxygenase (TH), the dopamine beta-hydroxylase (DBH) and the catechol *O*-methyltransferase (COMT).

Further experimental studies were then designed to explore the possibility that any of these predicted targets could be responsible for the observed neurotoxic effect of fenitrothion in zebrafish larvae.

3.5. Validating in silico predictions with human in vitro functional assays

In vitro binding and enzymatic assays were performed to validate some of the fenitrothion interactions suggested by the *in silico* predicted target profile (Table 2). Whereas no inhibition of human AChE, MAO-A and MAO-B activities was found in these assays, the binding of fenitrothion to the human AR was confirmed, resulting in inhibitions of 44.3% and 96.4% of the binding of the synthetic radiolabeled androgen [^{3}H] methyltrienolone to the human AR when cells were obtained after exposure to 100 nM and 10 μM fenitrothion, respectively. These results demonstrate that the primary toxicological target of fenitrothion in humans is AR and not AChE.

Table 3
Predicted targets of fenitrothion by CLARITY and SEA. Confidence scores from CLARITY and *p*-values from SEA are provided. Predictions below the respective recommended cutoffs are colored in grey.

Protein(s)	Gene name	CLARITY	SEA
Carboxylic ester hydrolase	ACE2	1.00	6.2E-31
Krueppel-like factor 10	KLF10	-	8.2E-33
Salicylate synthetase	LRP9	-	9.2E-30
Free fatty acid receptor 4	FFAR4	-	9.9E-20
Tubulin polymerization-promoting protein	TPPP	-	2.5E-13
Monolgyceride lipase	MGLL	-	5.2E-06
Androgen receptor	AR	0.51	-
G protein-coupled receptor 35	GPR35	0.47	-
Hydroxycarboxylic acid receptor 2/3	HCAR2/3	0.42	-
Hepatocyte nuclear factor 4-alpha	HNF4A	0.38	-
Sodium-dependent serotonin transporter	SLC6A4	0.36	-
Amine oxidase [flavin-containing] A	MAOA	<0.35	-
Amine oxidase [flavin-containing] B	MAOB	<0.35	-
Tyrosine 3-monooxygenase	TH	<0.35	-
Dopamine beta-hydroxylase	DBH	<0.35	-
Catechol O-methyltransferase	COMT	<0.35	-

Both human and rat serotonin transporter (SERT) have been reported to be inhibited by fenitrothion (https://comptox.epa.gov/dashboard) (Williams et al., 2017). Therefore, in addition to validating the interactions predicted in our *in silico* target profile, an *in vitro* binding assay was performed to determine potential interactions of fenitrothion with human SERT. While exposure to 10 μM fenitrothion resulted in a 66.5% inhibition of the binding of [^{3}H] imipramine to human SERT, no effect was found when using 100 nM fenitrothion.

3.6. Exploring fenitrothion interactions with zebrafish proteins

In vitro functional assays to validate some of the predicted interactions of fenitrothion with proteins were performed using human proteins, since these assays are not currently commercially available for zebrafish proteins. Therefore, we decided to directly explore the potential interactions of fenitrothion with zebrafish proteins *in vivo* (Fig. 2).

It is generally assumed that the molecular initiating event of fenitrothion neurotoxicity is AChE inhibition. Thus, in order to understand the molecular mechanisms involved in the behavioral changes observed in zebrafish larvae exposed to environmental concentrations of fenitrothion, the AChE activity of control and fenitrothion-exposed larvae was determined. As Fig. 2A shows, concentrations of fenitrothion in the range 17 ng/L to 17 μg/L were not able to significantly inhibit AChE activity ($F_{4,39} = 0.531$, $P = 0.713$). In fact, in the exposed larvae fenitrothion was unable to inhibit AChE activity even at concentrations as high as 1.7 mg/L (Supplementary Table ST3). These results indicate that the effect of fenitrothion on larvae behavior was mediated through AChE-independent mechanisms.

A potential androgen receptor agonistic role of fenitrothion at environmentally concentrations was then explored by analyzing the expression of two androgen-responsive genes, *cyp19a1b* and *sult2st3* (Fig. 2B). An inverted U-shaped NMCRC described the relationship between the expression of *cyp19a1b* ($F_{4,26} = 4.490$, $P = 0.007$) and *sult2st3* ($F_{4,26} = 5.185$, $P = 0.003$) and the fenitrothion levels, with a significant up-regulation of both genes after exposure to the lowest concentrations and no effect at higher concentrations.

In order to validate the interactions of fenitrothion with zebrafish SERT, the expression of *slc6a4a*, the gene encoding this transporter, was determined (Fig. 2C). However, as Fig. 2C shows, fenitrothion had

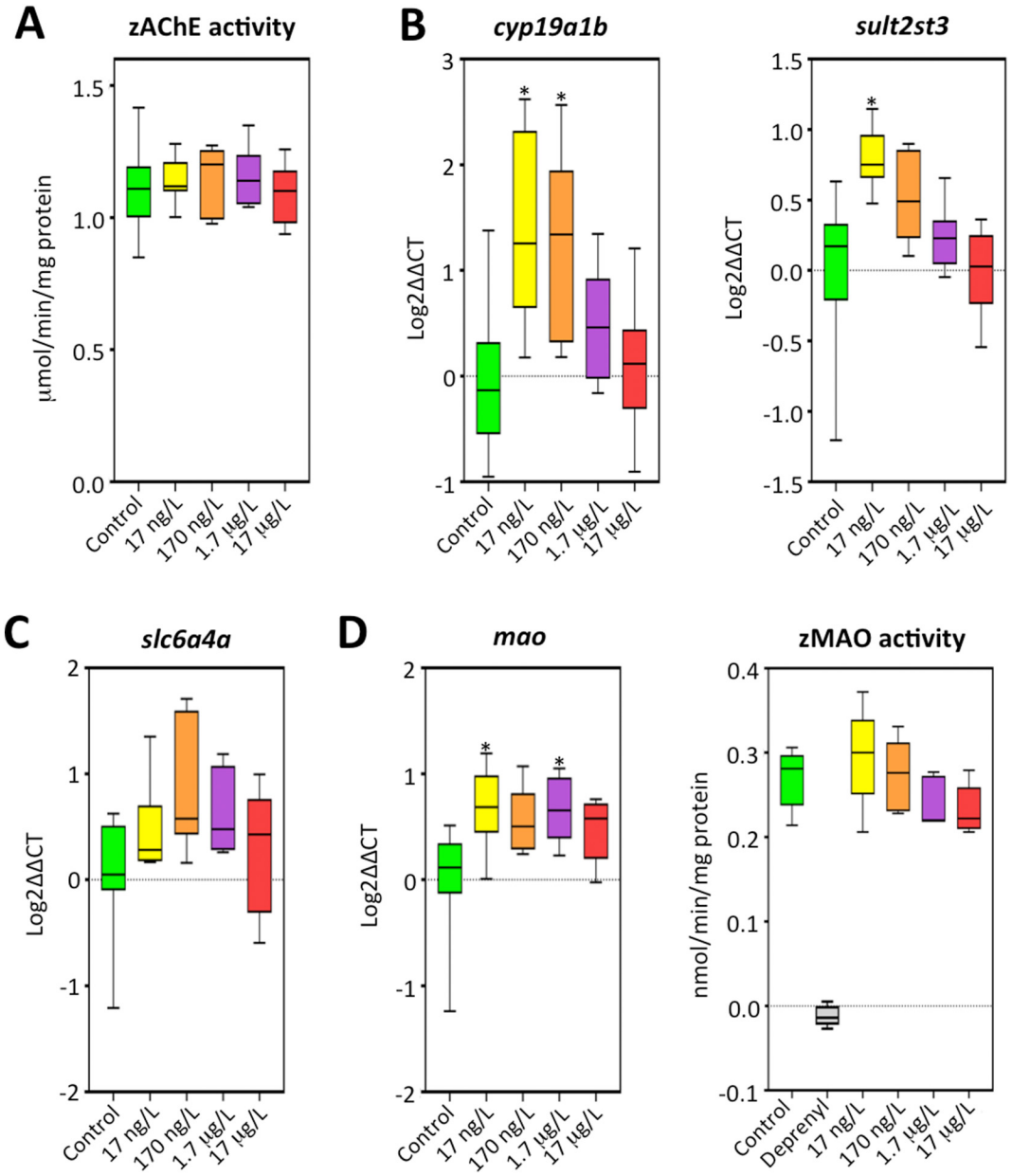

Fig. 2. Validation in zebrafish larvae of *in silico* predicted molecular targets of fenitrothion. (A) Zebrafish acetylcholinesterase (zAChE) activity in control and fenitrothion exposed 8 days-post fertilization zebrafish larvae, showing that the selected concentrations of fenitrothion are not able to induce a significant inhibition of AChE activity ($P > 0.05$; one-way ANOVA with Dunnett's multiple comparison test). Data from 2 independent experiments (n = 8–9). (B) The potential androgenic or anti-androgenic effect of fenitrothion on zebrafish arvae was assessed by analyzing the expression of *cyp19a1b* and *sult2st3*, two well established markers of androgenicity in zebrafish. (C) Expression of *slc6a4a* (zebrafish SERT) in control and fenitrothion exposed zebrafish larvae. (D) Expression of *mao* and MAO activity in control and fenitrothion exposed zebrafish larvae. Boxplot representation with the box indicating the 25th and 75th percentiles and the whiskers the maximum and minimum values. The thin line within the box marks the median. *$P < 0.05$; One way ANOVA followed by Dunnett's multiple comparison test was used for zAChE activity and *cyp19a1b*, *sult2st3* and *mao* expression, whereas Kruskal Wallis test followed by Dunn's multiple comparison test against the control values was used for *slc6a4* expression and MAO activity.

no a significant effect on the expression of this gene ($H(4) = 7.368$, $P = 0.118$).

Finally, in order to validate potential interactions of fenitrothion with zebrafish MAO, both gene expression and enzymatic activity were determined in control and treated larvae (Fig. 2D). Fenitrothion was able to alter *mao* expression ($F_{4,26} = 3.122$, $P = 0.032$), and a mild but significant up-regulation in the expression of this gene was found in larvae exposed to 17 ng/L and 1.7 µg/L fenitrothion. Larvae exposed to 170 ng/L fenitrothion also exhibited a similar trend to up-regulate *mao* expression ($P = 0.069$), while no effect was found for larvae exposed to 17 µg/L ($P = 0.114$).). Then, in order to better understand the potential role of MAO in the observed behavioral changes, zebrafish MAO activity was determined in larvae exposed to 17 ng/L - 17 µg/L fenitrothion for 24 h, using 1.1 mg/L deprenyl, a specific MAO inhibitor, as positive control (Fig. 2D). Despite the significant up-regulation of *mao* expression found in larvae exposed to 17 ng/L and 1.7 µg/L fenitrothion, this insecticide was no able to inhibit MAO activity at any tested concentration ($H(4) = 6.771$, $P = 0.149$).

3.7. Effect of fenitrothion on the monoaminergic system

The *in silico* predictions suggested that fenitrothion may also directly interact with key proteins involved in the synthesis (TH) or metabolism (DBH, COMT) of monoaminergic neurotransmitters. Therefore, the monoaminergic profile of the control and exposed larvae, as well as

the expression of main monoaminergic-related genes have been determined (Fig. 3 and Supplementary Figs. 5–6).

As Fig. 3A shows, whereas dopamine (DA) levels remained unchanged, fenitrothion significantly altered the levels of its precursors tyrosine ($F_{4,35} = 21.392$, $P = 5.3 \times 10^{-9}$) and L-DOPA ($F_{4,35} = 43.261$, $P = 4.4 \times 10^{-13}$), with the former increasing at concentrations in the range 17–170 ng/L and the latter at all tested concentrations. Fenitrothion also exhibited a significant effect on the levels of the DA metabolites DOPAC ($H(4) = 23.354$, $P = 0.0001$) and HVA ($F_{4,35} = 14.306$, $P = 5.1 \times 10^{-7}$), with a significant decrease of the former in larvae exposed to all tested concentrations and a significant increase of the latter only in those larvae exposed to 17–170 ng/L. Norepinephrine levels were also significantly altered by fenitrothion ($F_{4,35} = 2.799$, $P = 0.041$), with decrease in the levels of this catecholaminergic neurotransmitter after exposure to 1.7 μg/L fenitrothion. Similarly to DA, serotonin levels remained unchanged after fenitrothion exposures (Supplementary Fig. S5). Finally, although fenitrothion did not show a significant effect ($F_{4,35} = 2.031$ $P = 0.111$) on 5-HIAA, the main serotonin metabolite, its levels were found significantly reduced after exposure to 17 ng/L fenitrothion (Supplementary Fig. S5).

Whereas the expression of the monaminergic-related genes *slc6a4a* and *mao* was already analyzed (Fig. 2C, D) as a part of the validation process of the *in silico* predictions, expression of genes involved in the synthesis of dopamine (*th1* and *th2*), serotonin (*tph1a* and *tph1b*) and norepinephrine (*dbh*), the vesicular monoamine transporter 2 (*slc18a2*, encoding VMAT2), dopamine active transporter (*slc6a3*, encoding DAT), as well as the gene involved in dopamine metabolism catechol-*O*-methyltransferase (*comtb*) was determined to evaluate the effect of fenitrothion exposure on this essential modulatory neurotransmitter system (Fig. 3 and Supplementary Fig. S6). Whereas no effect of fenitrothion on *th1, th2, tph1a, tph1b, slc6a3* and *comtb* expression levels was found when compared with the control values ($P > 0.05$; One way ANOVA followed by Dunnett's multiple comparison test; Supplementary Fig. S6), an inverted U-shaped NMCRC described the relationship between the *slc18a2* expression and the fenitrothion levels ($F_{4,33} = 15.189$, $P = 3.8 \times 10^{-7}$), with a significant up-regulation of this gene after exposure to the lowest concentrations and no effect at higher concentrations (Fig. 3B). Finally, fenitrothion exhibited also a significant effect on *dbh* expression ($F_{4,33} = 6.397$, $P = 0.0006$), although only larvae exposed to 170 ng/L of this chemicals presented *dbh* levels significantly higher than the corresponding controls (Fig. 3B).

3.8. Revisiting the risk assessment for fenitrothion

The finding that environmental concentrations of fenitrothion are able to alter zebrafish larvae behavior strongly suggests that the environmental risk of this pesticide should be revisited. The predicted non-effect concentration (PNEC) of fenitrothion in zebrafish larvae can

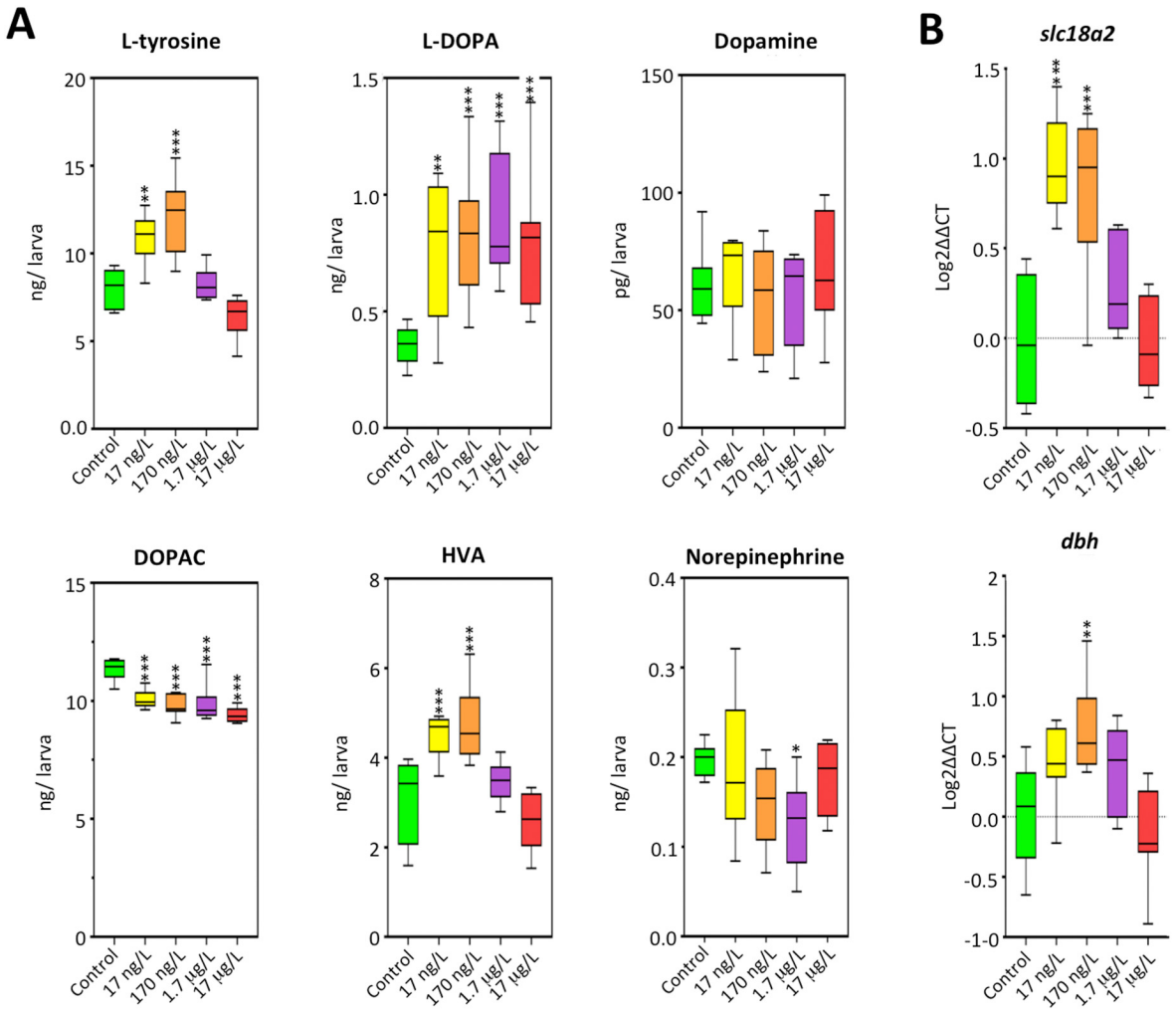

Fig. 3. Effect of environmental concentrations of fenitrothion on the monaminergic system in zebrafish larvae. (A) Effects of fenitrothion on the levels of some neurochemicals from the monaminergic system. (B) Effects of fenitrothion on the expression levels of *slc18a2* (gene encoding zebrafish VMAT2) and *dbh*. Boxplot representation of ng or pg of neurochemical per larva (A) or ΔΔCt values (B), with the box indicating the 25th and 75th percentiles and the whiskers the maximum and minimum values. The thin line within the box marks the median [n = 8 pools (A) or 6–8 pools (B)].*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; one-way ANOVA with Dunnett's multiple comparison test. Data from 2 to 4 independent experiments.

be determined by using the non-observed effect concentration (NOEC) for the different behaviors analyzed. Whereas the basal locomotor activity of the larvae was altered even at the lower concentration tested, NOEC for the vibrational startle response was 1.7 ng/L fenitrothion. Therefore, the environmental risk of fenitrothion will be high for fish larvae at those aquatic ecosystems with fenitrothion levels above 1.7 ng/L, with hazard quotient (HQ) values 8000–44,000 in the sprayed areas (Hossain et al., 2015; Lockhart et al., 1977; Mallet and Volpe, 1982; Symons, 1977) and HQ values 3–350 in most of the non-directly exposed areas (Carrasco et al., 1987; Derbalah et al., 2019; Eidt and Sundaram, 1975).

4. Discussion

Fenitrothion is an organophosphorus insecticide usually found in aquatic ecosystems at concentrations in the range of ng/L (Carrasco et al., 1987; Derbalah et al., 2019), whereas the acute systemic toxicity of this compound in fish is in the range of mg/L (IPCS, 1992). As a result, it is generally assumed that the environmental risk of fenitrothion for the fish communities is very low. Therefore, the first objective of this study was to determine if short-term exposures to environmental concentrations of fenitrothion were able to induce neurotoxicity in fish. In order to answer this biological question, an apical and ecologically relevant endpoint, behavior, and a molecular endpoint, changes in the expression of different transcriptional markers of neurotoxicity, have been analyzed. After only 24 h exposure, fenitrothion concentrations ranging from low ng/L to low μg/L altered three essential behaviors, BLA, VMR, and acoustic/vibrational escape response, in zebrafish larvae. Since VMR and the acoustic/vibrational escape response integrates sensory and motor responses, the effect of fenitrothion could be mediated by the impairment of the mechanisms involved in the detection of the stimulus or by an effect on the specific motor circuits involved in the evoked motor response (Emran et al., 2007; Fero et al., 2011). The fact that fenitrothion increases the motor activity in response to a sudden decrease in light intensity but decreases this activity in response to vibrational stimuli strongly suggests that sensory and/or integrative circuits at the CNS, and not the neuromuscular system, are the main targets of fenitrothion. Interestingly, different effects on the visual system have also been reported after exposure to fenitrothion in humans and experimental animal models (Mecklenburg and Schraermeyer, 2007). Although the effect of 17 μg/L fenitrothion on the vibrational escape response was previously reported (Faria et al., 2020), our data show that fenitrothion is able to impair this response even at low ng/L concentrations. One interesting finding during the behavioral analysis is the apparently contradictory results obtained with the vibrational startle analysis performed with the DanioVision and the kinematic analysis. Whereas larvae exposed to 17 μg/L fenitrothion exhibited a reduced response to the vibrational stimulus in the DanioVision setup, the percentage of larvae responding with an SLC to a similar stimulus increased when the kinematic of the vibrational evoked escape response was analyzed. The important methodological differences between the DanioVision and the setup used for the kinematic analysis, including the type of vibration (solenoid *vs* mini shaker) and the time of the analysis (1000 ms *vs* 270 ms), could explain the apparent differences observed between the two approaches analyzing the vibrational-evoked escape response.

After determining the effects on behavior, the neurotoxic potential of fenitrothion was also determined through the analysis of the expression of *gap43a*, *gfap*, *atp2b1a, mbp, and syt1a*. Growth-associated protein 43, the protein encoded by *gap43a*, is expressed in both neurons and astrocytes, and neurotoxic damage induces up-regulation of *gap43a* in both neurons, where this protein promotes axonal regeneration, and in astrocytes, where attenuates the astrogliosis-induced neurotoxicity (Hung et al., 2016). Increased levels of GFAP have been also found in mammalian and zebrafish brain after exposure to different neurotoxicants and measurement of this protein has been included in the neurotoxicity screening panel recommended by the US Environmental Protection Agency (McGrath and Li, 2008). Interestingly, the expression of *gap43* and *gfap* is regulated by STAT3, a transcription factor involved in astrogliosis activation (Tsai et al., 2007; Yeo et al., 2013). Down-regulation of *atp2b1a* has been reported in zebrafish larvae exposed to ototoxic compounds, probably reflecting the death of the hair cells of neuromasts (Han et al., 2019; Monroe et al., 2015; Sonnack et al., 2018). Interestingly, a non-monotonic concentration-response curve (NMCRC) with inverted U-shape was found to describe the relationship between fenitrothion concentrations and the levels of transcripts of *gap43a*, *gfap,* and *atp2b1a*, with a significant up-regulation in the expression of these genes at the lowest fenitrothion concentrations and a down-regulation at the highest concentrations. The concept of non-monotonic dose-response relationship is well-established in ecological risk assessment, and many endocrine disrupting compounds have been reported to produce non-monotonic effects at environmental concentrations (Agathokleous et al., 2019; Vandenberg et al., 2012). Moreover, the up-regulation of *gap43a* and *gfap* in zebrafish larvae exposed to a similar range of fenitrothion concentrations strongly suggest that this chemical is able to trigger astrogliosis even at ng/L concentrations. On the other hand, the *atp2b1a* up-regulation found in the exposed larvae strongly suggests that the observed increase on the vibrational escape response is not the result of a specific toxic effect on the neuromasts and/or inner ear.

Once demonstrated that short-term exposures to environmental concentrations of fenitrothion resulted in relevant neurotoxic effects in zebrafish larvae, the molecular modes of action behind these effects were further explored. Different toxicological modes of action (MoA) have been proposed for fenitrothion, including the direct inhibition of the activity of the enzyme acetylcholinesterase (AChE) (Sancho et al., 1997; Zinkl et al., 1991) and binding to the androgen receptor (AR) (Sebire et al., 2009; Sohoni et al., 2001; Tamura et al., 2001, 2003). Our computational analysis predicted several potential safety-associated human targets, including AChE, AR, MAO-A, MAO-B, tyrosine hydroxylase (TH), dopamine beta hydroxylase (DBH), and catechol *O*-methyltransferase (COMT). During the validation process, however, AChE was discarded as a toxicological target, since no inhibition of this activity was observed in either the *in vitro* (human AChE) or the *in vivo* (zebrafish AChE) assays. As fenitrothion needs to be oxidized into fenitrooxon by the cytochrome P450 monooxygenase system to be able to irreversible inhibit AChE (Escartín and Porte, 1996), results from the *in vitro* test, without any metabolic activation, were somehow expected. Moreover, considering that 24 h exposure to 20 μg/L fenitrothion has been reported to significantly inhibit AChE activity in eel brain (Sancho et al., 1997), the absence of inhibition in 8 dpf zebrafish larvae exposed 24 h to 17 ng/L-1.7 mg/L fenitrothion strongly suggests that at this developmental stage larvae lack fully functional biotransformation enzymes specifically involved in oxidizing fenitrothion into its oxon form (Yang et al., 2011).

MAOA from rat brain has been found to be inhibited by fenitrothion in *in vitro* assays (https://comptox.epa.gov/dashboard) (Williams et al., 2017), and both MAOA and MAOB were identified as potential targets of fenitrothion in our *in silico* predictions. Furthermore, inhibition of MAO with deprenyl in zebrafish larvae has been reported to induce a decrease in the BLA (Sallinen et al., 2009), a similar effect to that observed in fenitrothion-treated larvae. Despite of the mild but significant up-regulation of *mao* expression found in larvae exposed to 17 ng/L and 1.7 μg/L fenitrothion, the absence of MAO inhibition in larvae exposed to all fenitrothion concentrations indicates that this enzymatic activity is not directly involved in the observed neurotoxic effects.

Our *in vitro* functional assays show that human SERT was significantly inhibited by fenitrothion, a result consistent with a previous report with human and rat SERT (https://comptox.epa.gov/dashboard) (Williams et al., 2017). Moreover, in a recent study (Faria et al., 2019) using also zebrafish larvae, inhibition of the serotonin transporter (SERT; *slc6a4a*) with fluoxetine resulted in a decreased startle response,

a behavioral phenotype similar to that observed in larvae exposed to 170 ng/L-17 μg/L fenitrothion in our study. However, the fact that *slc6a4* expression remained unchanged in larvae exposed to all fenitrothion concentrations suggests that zebrafish SERT is not a highly relevant target of fenitrothion at the selected concentrations.

TH, COMT and DBH are three essential proteins of the monoaminergic system that were also predicted to interact with fenitrothion in our *in silico* analysis. However, expression of *th1*, *th2* and *comtb* in fenitrothion exposed larvae were similar to the control values. Moreover, only larvae exposed to 170 ng/L fenitrothion exhibited altered *dbh* expression, whereas the only effect on NE levels were found at 1.7 μg/L. These results suggest that fenitrothion interaction with TH, COMT or DBH is not playing an essential role in the observed neurotoxic effects of this insecticide.

Androgen receptor (AR) binding properties of fenitrothion were first reported about 20 years ago (Freyberger and Ahr, 2004), and these properties have been confirmed in the present study by an *in vitro* binding assay with human AR. Although fenitrothion is generally considered as a weak anti-androgenic compound (Freyberger and Ahr, 2004; Tamura et al., 2003; Sebire et al., 2009), other reports indicate that fenitrothion could also behave as an androgen agonist in biological context with very low concentrations of androgens (Tamura et al., 2003), as probably happens in 7 dpf zebrafish larvae (Gorelick et al., 2008). In order to better understand if environmental concentrations of fenitrothion were able to bind zebrafish AR inducing an androgenic/anti-androgenic effect on zebrafish larvae, the expression *cyp19a1b* and *sult2st3*, transcriptional markers commonly used to identify androgenic compounds in zebrafish (Fent et al., 2018; Fetter et al., 2015) was analyzed, and a significant up-regulation of both genes was found. Interestingly, the relationship between fenitrothion concentration and *cyp19a1b* and *sult2st3* expression was also described by an inverted U-shaped NMCRC, the typical relationship found between endocrine disruptors concentrations and biological effects (Agathokleous et al., 2019; Campos et al., 2013; Vandenberg et al., 2012). Moreover, the relationship found in this study between fenitrothion and *mbp* expression, a U-shaped NMCRC, is similar to the relationship previously reported for *mbp* expression and 17β-trenbolone, a potent androgen receptor agonist, in the medial prefrontal cortex of mice (Zhang et al., 2020). Interestingly, in addition to the altered *mbp* expression, mice exposed to 17β-trenbolone also exhibited behavioral changes. All these results, in addition with the higher potency exhibited by the binding fenitrothion-AR respect other predicted interactions and the absence of evidences supporting the involvement of other predicted targets, strongly support the hypothesis that fenitrothion binding to AR is the molecular initiating event (MIE) of the neurotoxic effect induced by this chemical in zebrafish larvae.

AR are widely expressed in the brain and endogenous and exogenous androgens may regulate neuronal activity, synaptic plasticity, cognition and different behaviors (Sarkey et al., 2008). For instance, administration of exogenous testosterone to animals and humans has an anxiolytic effect, reducing vigilance, startle response and threat detection, increasing risk-taking (Domonkos et al., 2018; Hodosy et al., 2012; Oliveira and Oliveira, 2014). Treatment with the anti-androgen flutamide has been associated with depression in humans (Dinh et al., 2016). Although the monoaminergic system, and in particular the dopaminergic system, are controlled by androgens (Barclay and Harding, 1990; Fabre-Nys, 1998; Jardí et al., 2018; Sotomayor-Zarate et al., 2014), the precise mechanisms of this control remain to be established. Moreover, our *in silico* predictions suggested that fenitrothion may not only impair the dopaminergic system through its androgenic effect, but might also directly interact with some key components of the dopaminergic system involved in the synthesis (TH) and metabolism (COMT, DBH) of dopamine. The significant decrease in DOPAC levels with a concomitant increase in HVA observed in zebrafish larvae exposed to low fenitrothion concentrations (17–170 ng/L) was previously reported in the brain of rats treated with amphetamines (Westerink and Korf, 1976) and methamphetamines (Yang et al., 1997), drugs targeting the vesicular monoamine transporter. Even knowing that results from neurochemical analyses performed on whole larvae should be always taken with caution, as many of the analyzed chemicals are also expressed also in non-neural tissue, the fact that expression of *slc18a2*, the gene encoding zebrafish VMAT2, was also significantly altered at the same two fenitrothion concentrations suggests that VMAT2 could play an important role in the observed changes in behavior. Furthermore, *slc18a2* expression in fenitrothion-exposed larvae follows an inverted U-shaped NMCRC typical of endocrine disrupters and it has been recently demonstrated in rats an increase in *slc18a* expression after treatment with an androgenic drug, propionate of testosterone has been recently demonstrated in rats (Wang et al., 2016).

The results presented in this manuscript are not only relevant for environmental risk assessment, but also for the human risk assessment methodology. The reference dose (RfD) for fenitrothion, 0.0013 mg/kg/day, was established by US EPA based upon a NOEL for systemic effects (histopathological changes in lymph nodes) and plasma cholinesterase inhibition observed in a long-term feeding study in dogs (EPA, 1995). Our results show that relevant adverse effects on behavior are observed at fenitrothion concentrations several orders of magnitude lower than those inhibiting AChE activity or producing changes in morphology. Therefore, the potential behavioral effects of low levels of fenitrothion should be tested in neonatal rodents and, if necessary, the current RfD for fenitrothion should be re-adjusted.

CRediT authorship contribution statement

Melissa Faria: Investigation, Methodology, Formal analysis, Writing - original draft; **Eva Prats**: Investigation, Writing - review & editing; **Jonathan Ricardo Rosas Ramírez**: Investigation; **Marina Bellot**: Investigation; **Juliette Bedrossiantz**: Investigation; **Maria Pagano**: Investigation; **Arnau Valls**: Software development; **Cristian Gomez-Canela**: Resources, Writing - review & editing; **Josep M. Porta**: Hardware and software development, Writing - review & editing; **Jordi Mestres**: Molecular predictions, Writing - review & editing; **Natalia Garcia-Reyero**: Resources, Writing - review & editing; **Caterina Faggio**: Resources, Writing - review & editing; **Leobardo Manuel Lopez Olivan**: Resources, Writing - review & editing; **Demetrio Raldúa**: Conceptualization, Resources, Supervision, Funding acquisition, Writing - review & editing, Supervision.

Declaration of competing interest

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

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Data availability

The source code of the LarvaCam and LarvaTrack software is released under GNU v3.0 license, and can be accessed at https://github.com/jmporta/.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2021.145671.

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