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Quinoxyfen 1995-2019 is the story's end? Evaluation of its adverse effects on head size and nervous system genes involved in synaptic maturation

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ABSTRACT

In 2013 quinoxyfen (QXY) was included in the list of priority hazard pollutants of the European Water Framework Directive due to its toxicity to aquatic organisms. The use of products formulated containing QXY was banned from all commercials starting from 27th June of 2019. QXY is an organic pollutant with potential persistence, bioaccumulation and this must not lower attention to this pollutant. To date the effects of this compound on development are not completely clear, thus this research tries to elucidate the adverse effects of QXY on zebrafish development. The study aims to understand the toxicological effects of QXY using the zebrafish as in vivo model and performing toxicological and molecular investigations. Considering the FET test results two sublethal concentrations, 0.4 mg/L and 0.8 mg/L were chosen for subsequent analysis. The expression of the *gad1b, cyp19a1b, shank3a, nrxn1a* and *c-fos* genes, involved in the development of the nervous system and the regulation of synaptic transmission, were evaluated. To confirm the potential neurotoxic effects of the treatment on the development of the central nervous system, both a transgenic *Tg(neuroD:gfp ia50)* line was used for confocal microscopy and Orange Acridine was used on wild type larvae to assess the presence of neuronal apoptosis. The results showed sub-lethal alterations, particularly affecting craniofacial and brain development highlighting as QXY may represent a possible endocrine disruptor able to induce severe cartilage defects, small head and tremor phenotype in zebrafish larvae and a strong modulation of the selected genes.

1. Introduction

Quinoxyfen (QXY) belongs to the azanapthalene group, and it is a preventive fungicide used to control the powdery mildew diseases of wheat, barley and grapes: it is a potent inhibitor of appressorium formation of these fungi (Bernhard et al., 2002). The mechanism of action of QXY consists in interfering with the pre-infectious developmental stages, suppressing the germination and formation of the fungus appressories through the interruption of the first cell signaling events (Bernhard et al., 2002). Moreover, QXY seems to interfere also at the level of the cutinoses enzymes present in some phytopathogenic fungi. These enzymes degrade the surface of the host damaging plant tissues and releasing monomers that facilitate the fungi entrance (Lee et al., 2008). It is an extremely lipophilic product which moves and enters in all environmental compartments with preference for hydrophobic solid matrices (Ferri et al., 2017). Although agricultural soil is the main recipient of pesticides, water bodies adjacent to agricultural areas are usually the final recipients of pesticide residues (Pereira et al., 2009; Ramya et al., 2023; Kannan et al., 2023). In fact, QXY has been identified as a substance dangerous for aquatic environments, given the indication of high acute toxicity for aquatic invertebrates such as *Daphnia magna* and moderate acute toxicity for some aquatic plants such as *Lemna gibba*, or sediment-dwelling organisms such as *Chironomus riparius*. Moreover, in several fish species such as *Leuciscus cephalus*, *Gobio gobio*, *Cobitis taenia* and *Alburnus alburnus alborella*the presence of QXY has been detected, highlighting its bioaccumulation potential (Barbieri et al., 2019).

In 2013 (Directive 201/39 EU) QXY was included in the European Union list of priority pollutants and in 2018 (Directive 2018/1914/EU) its use was banned, however, it is still present in the environment due to its persistence (EFSA, 2018). Considering the lack of toxicological and environmental accumulation data of QXY probably due to the only few

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published analytical methods and high analysis costs (Duncan et al., 2018) the aim of the present study was to investigate the effects of this compound in the zebrafish early life stage (*Danio rerio*), considering in particular the central nervous systems damages. The study was conducted in two phases. In the first phase, the main toxicological end points (CL_{10}, CL_{20} and CL_{50}), sublethal alterations and any abnormal behavior were evaluated by using the Fish Embryo Acute Toxcity (FET) test, while in the second phase, considering the results obtained by the FETs, two sublethal concentrations were selected for deeper investigations. Considering the sublethal alterations observed in the first phase, such as tremors and craniofacial deformities, the second phase focused on evaluating potential impairments in neural and craniofacial development in specimens exposed to QXY.

This evaluation involved assessing the expression of genes associated with nervous system development, including *cytochrome P450, family 19* (*cyp19a1b*) (Mouriec et al., 2009; Vosges et al., 2010) and *glutamate decarboxylase 1b* (*gad1b*) (Wirbisky et al., 2014) as well as genes implicated in synaptic formation and maturation, such as *neurexin1a* (*nrxn1a*) (Kabashi et al., 2011) and *SH3 and multiple ankyrin repeat domains 3a* (*shank3a*) (Costales and Kolevzon, 2015; Zhu et al., 2018), were examined. Finally, the study also investigated *cellular Finkel–Biskis–Jinkins Fos* (*c-fos*), a gene involved in neuronal activity (Rodríguez-Berdini et al., 2020).

Morphometric analyses of the head were performed to detect alterations in craniofacial cartilage and apoptosis, visualizing neuronal cell death in vivo. Finally, brain neurogenesis was investigated using a zebrafish transgenic line expressing GFP in the promoter region of the *neuroD* (*nrd*) gene, a basic Helix-Loop-Helix transcription factor that promotes neuronal differentiation (Blader et al., 1997; Schwarzer et al., 2020). Exactly, *neuroD* is a neuronal differentiation factor significant for the terminal differentiation of neurons (Ross et al., 2003; Chae et al., 2004).

The enzyme glutamate decarboxylase (GAD) is responsible for gamma-aminobutyric acid (GABA) synthesis. There are two isoforms of this enzyme: GAD65 and GAD67. In zebrafish, these two GAD isoforms are produced by the genes *gad2* and *gad1b*, respectively (Wirbisky et al., 2014). GABA (gamma-aminobutyricacid) plays a crucial role as the primary inhibitory neurotransmitter in the central nervous system (CNS). GABAergic neurons are distributed widely in the brain and have a key function in regulating neural activity. Their main role is to modulate neural systems and the activity of cells after a synaptic connection. Perturbations in the GABAergic system have been linked to conditions such as epilepsy, depression, schizophrenia, and sleep disorders (Horzmann and Freeman, 2016). GABA and GABAergic signaling have been shown to have important roles outside the CNS, particularly GABAergic signaling has also been implicated in craniofacial development in mammals (Asada et al., 1997; Condie et al., 1997; Culiat et al., 1995; Homanics et al., 1997; Kanno et al., 2004; Scapoli et al., 2002) and in zebrafish particularly GAD67 isoforms encoded by *gad1b* (O'Connor et al., 2018). Two *cyp19a1* genes, *cyp19a1a* and *cyp19a1b*, encoding the aromatase enzymes, exist in zebrafish. These two genes have different expression patterns: the *cyp19a1a* gene, which encodes aromatase A, is primarily expressed in the gonads, while the *cyp19a1b* gene, which encodes aromatase B, is expressed in the brain. Both aromatases synthesize estrogens from androgens. In teleosts, *cyp19a1b* expression is limited to radial glial cells, mainly found in regions of the forebrain, such as the olfactory bulbs, telencephalon, preoptic area, and hypothalamus. *Cyp19a1b* is expressed in the radial glial cells that are neuronal and glial progenitor cells, suggesting a crucial role of estradiol on brain development and brain repair (Mouriec et al., 2009). *Shank3* gene encodes for a protein found in the postsynaptic density, acting as a scaffold by binding to other proteins that contribute to ensuring proper synaptic formation and function (Costales and Kolevzon, 2015). The orthologous gene of human shank3 is duplicated in zebrafish as shank3a (on chromosome 18) and shank3b (on chromosome 4). Both share high amino acid identity and are predicted to have a similar function in zebrafish. Neurexins are primarily presynaptic cell adhesion molecules. There are three neurexin genes (NRXN1, 2, and 3), each of which encodes two major variants (alpha and beta). In zebrafish, two orthologs of NRXN1 (*Nrxn1a and 1b*) have been identified with a protein identity to the human counterpart of over 70 % (Rissone et al., 2006). Expression analysis showed that all three Nrxn genes are expressed during zebrafish embryonic development, and specific isoforms of Nrxn1a are expressed in different stages of development (Kabashi et al., 2011). *nrxn1a* is known to be involved in the maturation and organization of synapses in vertebrates. In the mature nervous system, synapses form connections necessary for neural processing and function, and the assembly of individual synapses is mediated by bidirectional signaling between pre- and postsynaptic neurons. Synaptic cell adhesion molecules, including neurexin (Nrxn) and neuroligin (Nlgn), control the recognition events between pre- and postsynaptic neurons to orchestrate the structural organization of synaptic junctions. The interaction between Nrxn and Nlgn is involved in neural plasticity mechanisms and is expected to influence the balance between excitatory and inhibitory synapses in the brain. Additionally, *Nrxns* overexpressed in neurons, selectively suppressed GABAergic synaptic communication without reducing synapse numbers (Zhang et al., 2010). Finally we investigate the expression levels of c-fos an excellent biomarker neuronal activity (Torres-Hernández et al., 2016); *c-fos* belong to immediate early gene family, induced in different cell types by diverse stimuli such as growth factors and neurotransmitters (Rodríguez-Berdini et al., 2020), *c-fos* promoter region is binding by C/EBPs and NeuroD that trigger its expression, once in the nucleus *c-fos* trigger the genomic program of differentiation (Calella et al., 2007).

2. Materials and methods

2.1. Chemicals

QXY (CAS number 124495-18-7, PESTANAL®, analytical standard), dimethyl sulfoxide [DMSO (CAS number 67-68-5, >99.9 % purity;)] Alcian Blue (CAS number 33864-99-2), Acridine Orange (CAS number 10127-02-3) 3,4-dichloroaniline (CAS number 95-76-1, >98 % purity), 10 % neutral- buffered formalin (CAS number 30525-89-4) were purchased from Merck Life Science (Milan Italy). Dilution water (DW) was prepared according to OECD TG 203, Annex 2 (OECD, 1992).

2.2. Experimental design

Both, the Tg(*neuroD:gfp ia50*) line and the wild-type AB zebrafish strain were raised in the facility of the University of Teramo in a "ranks and tanks" housing system with recirculating water (protocol number n. 4236). The light/dark cycle was set at 14 and 10 h, respectively. Physico-chemical parameters of the water were maintained constant with the following values: water temperature at 28 ± 1 °C, pH of 7, nitrites <0.05 mg/L, and ammonia <0.1 mg/L. The day before collecting the embryos, adult zebrafish (in a 1:1 male-to-female ratio) were placed in "breeding tanks". The following morning, after mating, the eggs were collected and cleaned with DW. Initially, a macroscopic selection was performed to remove coagulated eggs, and then microscopic selection was carried out to choose eggs at the appropriate stage of development without irregularities, morphological asymmetries, or corion lesions for the execution of the FET test. The work was carried out following the Italian law for the protection of research animals D.L. n. 26, 4 March 2014 and the European regulation directive 2010/63/U for animal experiments. Treatments were performed in non-feeding embryos and larvae used for Alcian Blue staining, Real-Time PCR and confocal microscopy were euthanized before the analyses using an overdose of tricaine pharmaq (PHARMAQ AS, Norway).

2.3. Fish embryo acute toxicity test (FET test)

The FET tests were conducted in accordance with OECD guideline 236 (OECD, 2013). The following concentrations of QXY were tested: 0.25 mg/L, 0.4 mg/L, 0.8 mg/L, 1 mg/L, and 2 mg/L. For each concentration 20 embryos were exposed individually in a 24-well plate and three independent replicates were analyzed ($n = 60$). The negative control was DW, the solvent control was prepared with a 0.02 % DMSO solution, and the positive control was prepared with 3,4-dichloroaniline at a concentration of 4 mg/L. The tested solutions were renewed every 24 h up to 96 hpf, when the test ended. Furthermore, at 96 hpf, considering the observed sublethal alterations and results of FET tests, two concentrations (0.4 and 0.8 mg/L) were chosen to conduct the alcian blue staining, the molecular investigation and confocal imaging. The first neurological effects appeared at the concentration of 0.4 mg/L while the concentration of 0.8 mg/L was chosen because under the LC_{10} value.

2.4. Alcian blue staining

The larvae at 96 h post-fertilization (hpf) were collected and euthanized to perform morphometric analysis by Alcian Blue staining. 4 specimens of zebrafish larvae exposed to 0.4 mg/L, 0.8 mg/L and DMSO (0.02 %) as negative control, were used for these investigations. The larvae were euthanized and fixed in 10 % neutral- buffered formalin for 2 h, followed by washes in PBS-T and then were incubated in 0.1 % Alcian Blue with a 80: 20 mixture of ethanol-glacial acetic acid. Later they were rehydrated in ethanol at 80 %, 20 %, and 10 %, and then subjected to a PBS wash. They were subsequently placed in trypsin for 50 min and fixed in 4 % PFA for 20 min. After several washes in PBS, the larvae were bleached in a solution composed of 3 % H2O2 and 1 % KOH for 20 min. Finally, each colored larva was positioned both laterally and ventrally to allow observation through the Olympus CKX 41microscope and image acquisition using an EC3 camera. The following measurements were taken head length, distance between the left and right Meckel's cartilages (M-M distance), angle between the Meckel and palatoquadrate cartilages (M-PQ angle), and distance between the Meckel and ceratohyal cartilages (M-C distance).

2.5. Wholemount fluorescent imaging

Tg(neuroD:gfp ia50) zebrafish were collected at 96 hpf and fixed in 4 % formaldehyde and mounted on glass microscope slide to be observed using a Leica TCS SP5II confocal microscope. 4 specimens of zebrafish larvae exposed to 0.4 mg/L, 0.8 mg/L and DMSO (0.02 %) as negative control, were used for this investigation.

2.6. Neuronal apoptosis assay in live larvae

At 96 hpf, neuronal apoptosis was evaluated in 10 specimens for each experimental condition: DMSO 0.02 %, QXY 0.4 mg/L, and QXY 0.8 mg/L. The specimens were stained with Acridine Orange following this protocol: larvae were anesthetized with 0.02 % tricaine. After several washes in 1× PBS, they were incubated for 30 min at 28 °C in a 2 mg/L Acridine Orange solution. The dorsal side of the larvae was oriented towards the microscope for the image acquisition. Quantitative analysis was conducted using a Time-lapse microscope (Nikon Eclipse Ti), evaluating the mean fluorescence intensity from apoptotic foci with ImageJ. Qualitative analysis was performed with a confocal microscope (Leica TCS SP5II).

2.7. Gene expression analysis

Three biological replicates were collected. In each replicate, twenty larvae for each condition (0.4 mg/L, 0.8 mg/L and DMSO 0.02 %) were homogenized with 200 μL of Trizol™ Reagent (Invitrogen, CA, USA). Nucleic acid purity and RNA concentration were determined by NanoDrop™ 2000 Spectrophotometer (Thermo Fisher Scientific, DE, USA) and Qubit 2.0 fluorometer (Invitrogen, CA, USA), respectively. cDNA was synthesized using SuperScript™ IV VILO™ ezDNase™ Enzyme (Invitrogen, Life Technologies Corporation, CA, USA) according to the manufacturer's instructions. Real-Time PCRs were performed with CFX Opus 96 Real-Time PCR system (Bio-rad, CA, USA) using Sso Advanced™ Universal Probes Supermix (Bio-rad, CA, USA) and gene-specific probes (Bio-rad, CA, USA) for *cyp19a1b* (ID: qDreCEP0043698), *shank3a* (ID: qDreCIP0038934), *gad1b* (ID: qDreCIP0044792), neurexin 1a (*nrxn1a*) (ID: qDreCEP0050779), *c-fos* (IDqDreCEP00401109).

The amplification procedure was the following: 95 °C × 3 min, then 40 cycles of 15 s denaturation at 95 °C and 1 min annealing/extension at 60 °C. The results were normalized using β-actin (qDreCEP0045468) as the reference gene. We used the $2^{-\Delta\Delta Ct}$ method to calculate the expression levels (Livak and Schmittgen, 2001).

2.8. Statistical analysis

The results obtained from the FET tests were analyzed using ToxRat software version 3.3 (ToxRat Solutions GmbH, Germany). For the statistical analysis of cartilage angles and length, GraphPad Prism 8 software (GraphPad Software) was employed, and the measurements were expressed as the mean ± SD. For RT-PCR the results were expressed as the mean ± SEM. The statistical difference between the exposed and control groups was assessed using one-way ANOVA. For statistical analysis of apoptosis, Rstudio software (v2024.09.1) was used. For the quantitative analyses data between exposed and control groups, were analyzed with Shapiro-Wilk normality test. Data resulting with non-parametric distribution were then evaluated using Dunn's Many-to-One Rank Comparison Test.

3. Results

3.1. Fish embryo acute toxicity test (FET test)

In accordance with OECD guideline no. 236, toxicological endpoints of QXY were calculated using ToxRat software. Lethal effects including embryo coagulation, failure of tail detachment from the yolk sac, lack of somites, absence of heartbeat and sub-lethal effects were assessed. Among the toxicological endpoints, LC_{10} was the only parameter reached, with a recorded value of 0.9 mg/L. The main lethal effect was embryo coagulation, which was 13.3 % at the highest concentration of 2 mg/L at 96 hpf. In addition to lethality parameters, during the FET tests, sub-lethal alterations were observed (Table 1). Specifically, at 48 hpf from the lowest concentration of 0.25 mg/L, sub-lethal effects were observed in the cardiovascular system, including yolk sac edema, pericardial edema, reduced blood circulation, and blood stasis. At 96 hpf, starting from 0.4 mg/L, in addition to the previously mentioned cardiovascular alterations, a likely involvement at the neurological level was observed.

At the lowest concentration (0.25 mg/L), 30 % of larvae exhibited tremors in the oral apparatus, with this percentage increasing to 90 % in specimens exposed to the highest concentration (2 mg/L) (Videos 1 and 2). Additionally, the same zebrafish larvae displayed smaller head sizes and altered mouth morphology.

3.2. Alcian blue staining

These morphometric alterations of the craniofacial structure seemed due to the modification of cartilage parameters (Fig. 1). In particular, zebrafish larvae treated with QXY at 0.4 and 0.8 mg/L, showed a decreased head length, an increase of M-PQ angle and an increase of M-C distance ($p < 0.005$).

Table 1
Total number of survived larvae and larvae with sub-lethal effects at 96 hpf.

	Survived larvae	Larvae with deformed head	Larvae with generalized tremors	Larvae with oral apparatus tremors	Larvae with blood stasis	Larvae with pericardial edema	Larvae with yolk sac edema
NK	60	0	0	0	0	0	0
DMSO 0.02 %	59	0	0	0	0	0	0
0.25 mg/L	56	7	3	18	4	2	0
0.4 mg/L	51	49	40	50	1	0	2
0.8 mg/L	59	51	51	51	4	0	5
1 mg/L	55	55	55	55	7	5	1
2 mg/L	54	54	54	54	6	3	5

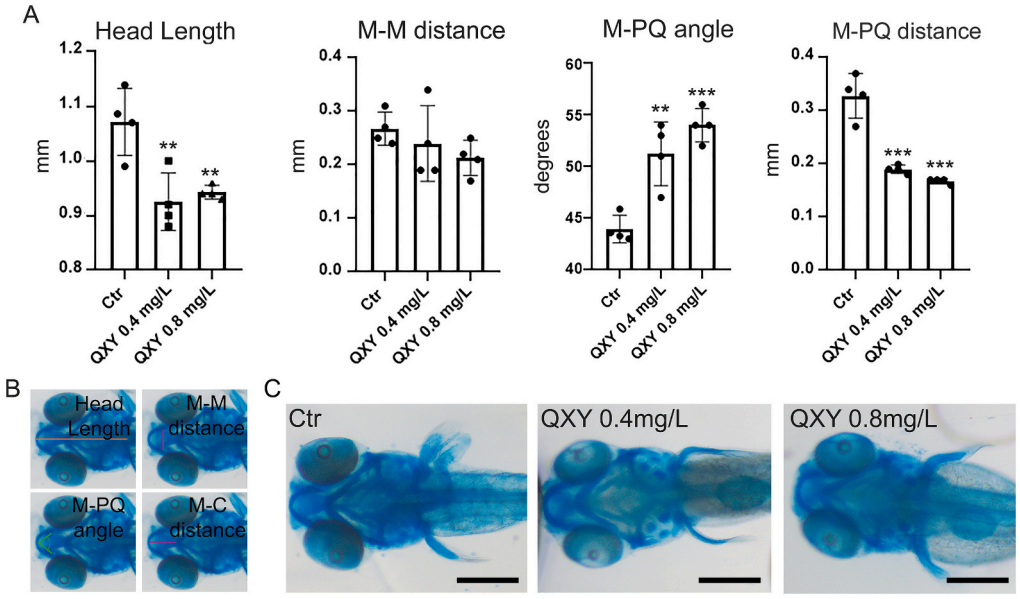

Fig. 1. Alcian blue staining of 96 hpf zebrafish larvae: A) Morphometric analysis. The data is expressed as the mean +/− SD of 4 larvae for each experimental condition. Statistical significance is shown as $p < 0.05$*; $p < 0.005$**; $p < 0.0005$***. B) Graphical representation of distance between left and right Meckel's cartilage (M-M distance), angle between Meckel and palatoquadrate cartilage (M-PQ angle) Meckel and ceratohyals cartilage distance (M-C distance) in zebrafish larvae control. C) Ventral projection Ctr (0.02 % DMSO), 0.04 mg/L QXY; 0.08 mg/L QXY. Scale bar = 0.5 mm.

3.3. Wholemount fluorescent imaging

In *tg(neuroD:gfp ia50)* zebrafish line GFP is expressed in immature neurons, treatment induces a decrease of neuron expressing *nrd* particularly in optical tectum, midbrain and cerebellum (Fig. 2).

3.4. Neuronal apoptosis assay in live larvae

In addition, Acridine Orange staining allowed the identification of neuronal apoptosis. Specifically for the concentration of 0.4 mg/L the apoptosis was present at the level of the optical tectum and telencephalon and for the concentration of 0.8 mg/L at the level of optical tectum, telencephalon and cerebellum with a statistically significant difference ($p < 0.001$) for both concentrations compared to the control (Fig. 3).

3.5. Gene expression analysis

Finally, the molecular investigation results demonstrated as QXY, at concentrations of 0.4 and 0.8 mg/L, upregulates the expression of *shank3a, gad1b,* and *nrxn1a* genes, which are involved in synaptogenesis. Simultaneously, it leaded to a decrease in the expression of *cyp19a1b*, a gene encoding for aromatase and specific to radial glia (Fig. 2) and *c-fos* which is implicated in neuronal activity.

4. Discussion

The FET tests provided only one toxicological endpoint, the LC_{10} of 0.9 mg/L, while LC_{20} and LC_{50} could not be determined because the tested concentrations were too low to reach these thresholds.

The existing QXY data indicates that it possesses low acute toxicity via the oral, dermal and inhalation routes in rat and mouse (Environmental Protection Agency, 2009). Our results show a dose-dependent effect on sub-lethal alterations, particularly affecting craniofacial and brain development on zebrafish QXY exposure. At 96 hpf, exposed larvae exhibited reduced head size, alterations in cartilage morphology, leading to changes in mouth morphology, and general tremors, especially in the oral region. These morphological alterations, along with the observed neurotoxic phenotype, could be attributed to the downregulation of the cyp19a1b gene and the upregulation of gad1b at both concentrations. Cyp19a1b is expressed during development and is essential for proper estradiol-dependent chondrogenesis (Cohen et al., 2014; Lassiter and Linney, 2007) and is involved in brain development (Diotel et al., 2010). In line with Cohen et al., 2014, a decrease in estrogen production, controlled by aromatases, including cyp19a1b, leads to alterations in craniofacial cartilage formation, as observed in this study. Moreover, the M-PQ angle has been proposed as a reliable high-throughput standard parameter for evaluating the effects of chemical exposure (Staal et al., 2018; Zoupa et al., 2020), and its increase can be interpreted as a measure of distortions in the head and jaw, leading to

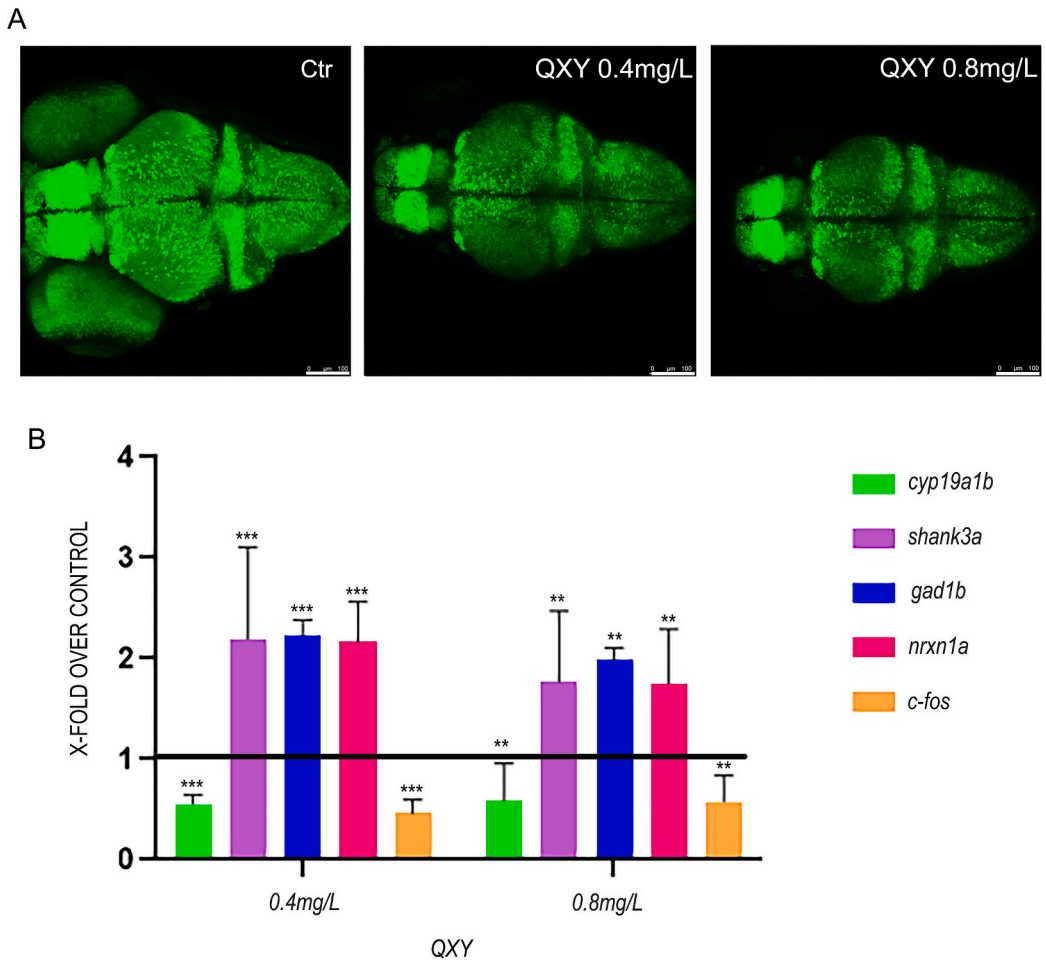

Fig. 2. A) confocal microscope image of 96 hpf brain of tg(*neuroD:gfp ia50*) line. 4 specimens of zebrafish larvae exposed to 0.4 mg/L, 0.8 mg/L and DMSO (0.02 %) as negative control, were used for this investigation. B) gene expression levels of *cyp19a1b, shank3a, gad1b*, *nrxn1a* and *c-fos* after exposure to QXY concentrations of zebrafish larvae at 96 hpf. Data are shown as the mean ± SEM of 150 larvae for each condition (50 larvae for each condition in three biological replicates). Statistical significance is shown as **p < 0.005; ***p < 0.0005 versus control. Scale bar = 100 μm.

microcephaly and micrognathia (Raterman et al., 2020), supporting the action induced by QXY, where the M-PQ angle was altered, resulting in compromised functionality, manifested by repetitive movements, particularly in the mouth area. To reinforce the data obtained on the downregulation of cyp19a1b, the upregulation of gad1b induced by QXY could confirm the hypothesis that this compound acts as an endocrine disruptor, interfering with steroid hormone regulation. Consistent with our results, the expression and activity of enzymes involved in the biosynthesis of 17-β estradiol (E2) in fish are primarily controlled by gonadotropins (GtHs), including FSH and LH, derived from the pituitary. These hormones regulate steroidogenesis by binding to their receptors in the gonads and activating specific signaling pathways. GtHs are under the control of gonadotropin-releasing hormones (GnRHs) produced in the hypothalamus, which are further regulated by neurotransmitters and neuropeptides, including γ-aminobutyric acid (GABA), which stimulates GnRH and GtH levels in fish. GABAergic signaling is implicated in craniofacial development in mammals (Condie et al., 1997; Kanno et al., 2004), and in zebrafish, the isoform GAD67, encoded by *gad1b*, is a key factor for craniofacial morphology (O'Connor et al., 2018). Therefore, the observed increase in *gad1b* could represent a compensatory mechanism to enhance GABA production and the release of E2 through the hypothalamic-pituitary-gonadal axis. However, considering that GABA is one of the main inhibitory neurotransmitters, the increase in gad1b, along with the reduction of cyp19a1b, leads to an imbalance between excitatory and inhibitory neurotransmission and alters craniofacial development, as evidenced by the morphological and neurological phenotypes observed in this study, with smaller head sizes and tremors, especially in the oral apparatus. Supporting the potential neurotoxic effect of QXY, the concurrent decrease in cyp19a1b, NeuroD-positive neurons, and *c-fos* expression could be responsible for the neurological phenotype observed. NeuroD is a crucial factor during cortical and central nervous system development, including the cerebellum, brainstem, and spinal cord (Tutukova et al., 2021), and is expressed in both late mitotic and early post-mitotic neurons. NeuroD is a transcription factor necessary for *c-fos* expression (Calella et al., 2007), a gene essential for initiating the neuronal differentiation program and activating phospholipid synthesis, a key process that allows neurons to continue differentiating (Rodríguez-Berdini et al., 2020). Furthermore, cyp19a1b is involved in the development of serotonergic neurons in zebrafish embryos and larvae (Ulhaq and Kishida, 2018) and is implicated in dyskinesia observed in Parkinson's disease (Huot and Fox, 2013). A reduction in cyp19a1b, along with decreased NeuroD activity, results in a failure of proper neurogenesis induced by QXY, leading to neuronal apoptosis, which manifests as the neurological phenotype observed in this study. The alteration in neurogenesis is reflected in the results concerning synaptogenesis-related genes such as *shank3a* and *nrxn1a*, which were both upregulated at concentrations of 0.4 and 0.8 mg/L of QXY. Nrxn1a is a synaptic adhesion molecule essential for promoting communication between neurons and modulating synaptic activity. The upregulation of nrxn1a could represent a compensatory mechanism to restore synaptic communication, which is disrupted due to failed neurogenesis leading to neuronal apoptosis, as observed in our

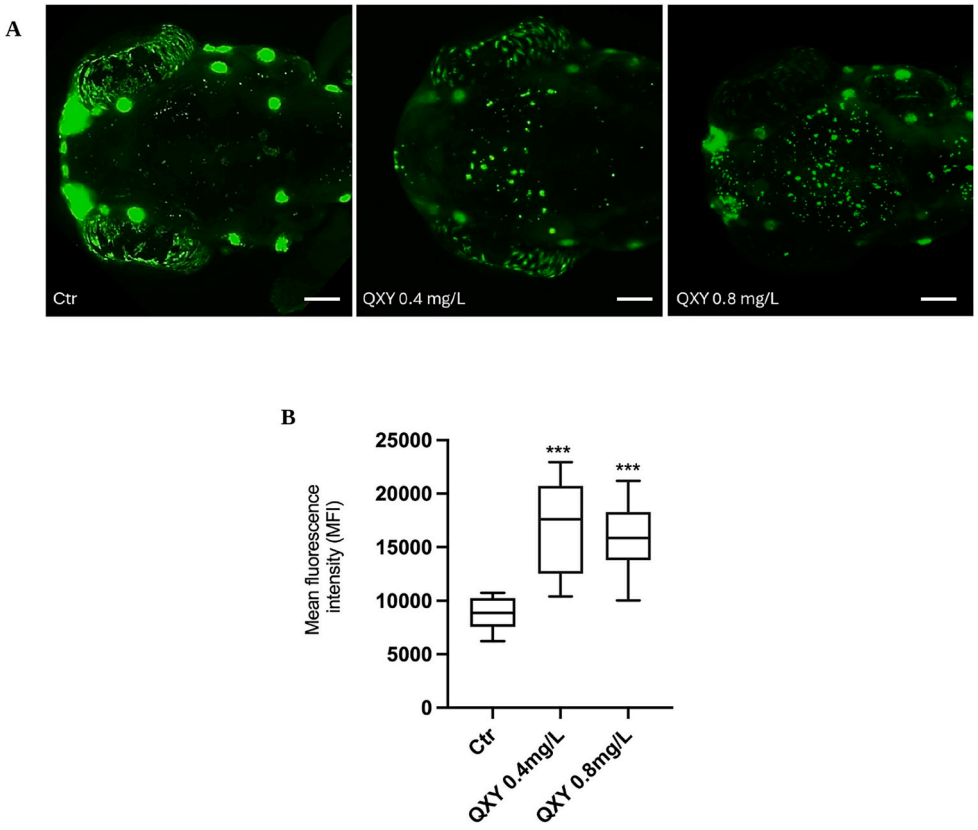

Fig. 3. Neuronal Apoptosis Assay in zebrafish larvae subjected to acridine orange staining. A) Confocal microscope image at 96 hpf. 3 specimens of zebrafish larvae exposed to 0.4 mg/L, 0.8 mg/L and DMSO (0.02 %) were used for these investigations. B) Quantification of neuronal apoptosis. Statistical significance is shown as ***$p < 0.0005$ versus control. 10 specimens of zebrafish larvae for each concentration were used. Scale bar = 100 μm.

results. Shank3a is localized in the cortex, thalamus, striatum, hippocampus, dentate gyrus, and granular cells of the cerebellum (Monteiro and Feng, 2017), suggesting important functions in synaptic plasticity underlying cortical organization, sensory processing, behavioral control, and cognition (Liu et al., 2021). Considering that homozygous knockout mice for shank3 exhibited abnormalities in corticostriatal circuits and that monkey models showed impaired social interactions, repetitive behaviors, delayed vocalization, and reduced brain network activity (Liu et al., 2021), it is plausible that the neurotoxic action induced by QXY, leading to neuronal apoptosis in the brain and reduced *shank3a* expression in the central nervous system, triggers upregulation of this gene in surviving neurons. Together with altered glutamatergic signals from the upregulation of gad1b, this contributes to the neurological manifestation with generalized and localized tremors, particularly affecting the oral apparatus. QXY acts as an endocrine disruptor, interfering with both steroid hormone regulation and the expression of the *cyp19b* and *gad1b* genes, which are important for proper craniofacial development and neurogenesis. This finding is further supported by the analysis of apoptosis, where at a concentration of 0.4 mg/L, the presence of apoptotic individuals is higher compared to 0.8 mg/L. However, at the latter concentration, the apoptotic foci are more extensive and localized in multiple brain structures. QXY induces a decrease in NeuroD-positive neurons and a reduction in *c-fos* expression, causing defects in brain development, resulting in an imbalance between excitatory and inhibitory neurotransmission and the manifestation of tremors, demonstrating a strong effect of this azanafthalene on brain and cartilage development.

5. Conclusions

This work highlighted the toxic effects of QXY on zebrafish early life stage and the alteration of *cyp19a1b* gene expression, suggesting an altered production of estradiol in the brain, accentuating its potential role as endocrine disruptor. These results could represent a starting point to study the potential role of QXY on neurodevelopment and possible link with birth defect induced by organism exposure by bioaccumulation effects.

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CRediT authorship contribution statement

Annamaria Iannetta: Writing – original draft, Investigation, Data curation. **Silvana Zugaro:** Investigation, Data curation. **Giovanni Angelozzi:** Investigation. **Francesca Mazza:** Writing – review & editing. **Marco Minacori:** Supervision, Investigation. **Tommaso Silvestrini:** Investigation. **Elisabetta Benedetti:** Writing – original draft, Supervision, Resources, Data curation. **Monia Perugini:** Writing – review & editing, Resources, Project administration, Conceptualization.

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

Data will be made available on request.

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