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Short-term exposure to environmental levels of nicotine and cotinine impairs visual motor response in zebrafish larvae through a similar mode of action: Exploring the potential role of zebrafish α7 nAChR

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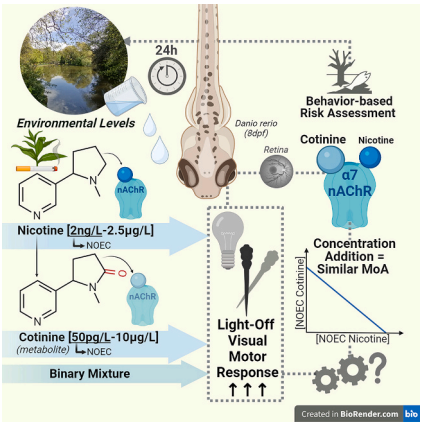
HIGHLIGHTS

- Both nicotine and cotinine are nAChR agonists.
- Environmental levels of nicotine and cotinine impair zebrafish larvae behavior.
- Nicotine and cotinine impair zebrafish behavior through a similar mode of action.
- Retinal α7nAChR could play an essential role in the observed effect.

GRAPHICAL ABSTRACT



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ABSTRACT

The current view is that environmental levels of nicotine and cotinine, commonly in the ng/L range, are safe for aquatic organisms. In this study, 7 days post-fertilization zebrafish embryos have been exposed for 24 h to a range of environmental concentrations of nicotine (2.0 ng/L-2.5 μg/L) and cotinine (50 pg/L–10 μg/L), as well as to a binary mixture of these emerging pollutants. Nicotine exposure led to hyperactivity, decreased vibrational startle response and increased non-associative learning. However, the more consistent effect found for both nicotine and cotinine was a significant increase in light-off visual motor response (VMR). The effect of both pollutants on this behavior occurred through a similar mode of action, as the joint effects of the binary mixture of both chemicals were consistent with the concentration addition concept predictions. The results from docking studies suggest that the effect of nicotine and cotinine on light-off VMR could be mediated by zebrafish α7 nAChR

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expressed in retina. The results presented in this study emphasize the need to revisit the environmental risk assessment of chemicals including additional ecologically relevant sublethal endpoints.

1. Introduction

More than six trillion conventional cigarettes are produced and consumed worldwide each year (Araújo and Costa, 2019). Nicotine, a highly lipophilic alkaloid found at high concentrations in tobacco leaves, is one of the main chemicals found in tobacco products, including cigarettes, and the most addictive (Hukkanen et al., 2005). In addition to this source of nicotine, the use of electronic cigarettes (e-cigarettes), devices that heat liquid nicotine, has increased dramatically in the last years (Beutel et al., 2021). Once in the blood stream, nicotine crosses the blood-brain-barrier (BBB) and is accumulated at the central nervous system (CNS) (Hukkanen et al., 2005). There, nicotine binds with high affinity to a heterogeneous family of ligand gated cation channels, the neuronal nicotinic acetylcholine receptors (AChRs), leading to the release of excitatory or inhibitory neurotransmitters depending on the neuronal-type expressing these receptors (Zoli et al., 2015). At CNS level, nAChRs are involved in cognitive function and their impairment has been associated to different neuropsychiatric disorders such as schizophrenia, epilepsy, anxiety, depression or nicotine addiction (Higley and Picciotto, 2014; Picciotto, 2003). Cotinine, the main metabolite of nicotine, is also able to cross BBB and it has been demonstrated to exhibit neuroactive effects (Tan et al., 2021). Cotinine is considered a weak agonist of nAChRs, although its potency depends on the subunit composition of nAChR. The potential role of cotinine as a positive allosteric agonist of nAChR has also been suggested (Moran, 2012). The suitability of cotinine in the treatment of depression, schizophrenia, Alzheimer's disease, and Parkinson's disease is currently under study (Moran, 2012; Tan et al., 2021). Despite the fact that both chemicals can be found in the CNS of smokers, and that their interaction could be really relevant from a pharmacological and/or toxicological point of view, information on these potential interactions is still scarce (Hatsukami et al., 1998; Riah et al., 1999).

Nicotine and cotinine are also found together in many aquatic ecosystems (Buerge et al., 2008). While the primary source of nicotine and cotinine in wastewater treatment plants is excretion from smokers, nicotine can leach into aquatic ecosystems throughout the whole tobacco life cycle, and specially by the littering of cigarette butts (CB) and e-liquid containers from e-cigarettes (Araújo and Costa, 2019; Beutel et al., 2021). In addition to the filter, CBs can include tobacco residues, ash and chemicals and tar from tobacco smoke. With about 4.5 trillion CBs littered into the environment each year, they are the most prevalent form of solid waste from tobacco products worldwide (Beutel et al., 2021; Novotny and Slaughter, 2014). Many littered CBs find their way into urban waterways and aquatic ecosystems (Araújo and Costa, 2019; Beutel et al., 2021). It has been found that nicotine rapidly leaches from test CBs, and that one CB can contaminate 1000 L of water with nicotine to levels above the predicted no effect concentration (PNEC) (Roder Green et al., 2014). Environmental relevant concentrations of these emerging pollutants are usually at the ng/L level. For instance, levels of 24.4 and 44.8 ng/L have been reported for nicotine and cotinine, respectively, in the Guadalquivir river (Spain) (Robles-Molina et al., 2014). Similar levels, 44–59 ng/L for nicotine and 33–69 ng/L for cotinine, were reported in Tagus river (Spain), decreasing to 15 and 8 ng/L, respectively, in tap water (Valcárcel et al., 2013). The median levels of these pollutants found in different rivers of Madrid (Spain), downstream the main wastewater treatment plants (WWTPs), were about ten-fold higher, 527.5 ng/L for nicotine and 496.5 ng/L for cotinine (Valcárcel et al., 2011). Moreover, in a recent global-scale study in 258 rivers from 104 countries from all continents, the reported median levels and the detection frequency were 322 ng/L and 42.6 % for nicotine and 245 ng/L and 36.9 % for cotinine (Wilkinson et al., 2022). Maximum and minimum levels determined in that study were 11.7 μg/L and 5.06 ng/L for nicotine and 9.57 μg/L and 11.2 ng/L for cotinine (Wilkinson et al., 2022).

In influent and effluent samples from different WWTPs from the Llobregat river (Spain), the levels of nicotine and cotinine were even higher, with median values of 32.5 and 3.5 μg/L for nicotine, and 5.1 and 2.4 μg/L for cotinine, respectively (Huerta-Fontela et al., 2008). The predicted no effect concentrations (PNEC) of nicotine and cotinine reported in the bibliography for fish, in the range of 4.0–58 μg/L (Bouzas-Monroy et al., 2022; Valcárcel et al., 2011) for the former and 970 ng/L (Bouzas-Monroy et al., 2022) for the latter, suggest only low hazard or no adverse effects to fish communities in most of the non-directly exposed aquatic ecosystems.

Zebrafish (*Danio rerio*) is a valuable and versatile vertebrate model extensively used in ecotoxicology, drug discovery, and safety pharmacology studies (Raldúa and Piña, 2014). With a general organization of the nervous and neurotransmitter systems very similar to that of humans, and a wide behavioral repertoire, zebrafish is also widely used in translational neuroscience (Faria et al., 2021a). Recently, video-tracking methodologies have been developed to assess ecologically relevant behaviors in zebrafish larvae (Faria et al., 2021b, 2020; Faria et al., 2019b). Moreover, the analysis of neurotransmitters in the CNS of this organism has been refined (Bellot et al., 2021). Therefore, the adverse effects of environmental levels of different neuroactive pollutants have been determined on zebrafish larvae. Zebrafish embryos, larvae and adults have been also used as experimental model for assessing pharmacological and toxicological effects of nicotine (Babin et al., 2014; Mora-Zamorano et al., 2016; Parker and Connaughton, 2007; Thomas et al., 2009). However, the concentrations of nicotine commonly tested are several orders of magnitude higher than those commonly found in freshwater aquatic ecosystems. Moreover, the effect of cotinine or the potential interactions between nicotine and cotinine have not yet been addressed.

Thus, the main objective of this study has been to better characterize the potential hazard of current levels of nicotine, cotinine, as well as the interaction of both compounds, on fish larvae by analyzing changes in highly ecologically relevant behaviors. We hypothesized that both compounds may act similarly and hence their joint effect in a mixture should be additive and accurately predicted by the concentration addition concept (Altenburger et al., 2003). In this study, 7 days post-fertilization zebrafish larvae have been exposed for 24 h to a range of environmental relevant concentrations of nicotine (2 ng/L–2.5 μg/L), cotinine (50 pg/L–10 μg/L), as well as a binary mixture of these pollutants at their NOEC levels. Then, the basal locomotor activity (BLA), light-off visual motor response (light-off VMR), startle response (SR) evoked by a vibrational stimulus, as well as the habituation to series of vibrational stimuli were determined in both control and exposed larvae. The content of the main neurotransmitters, including the monoaminergic (serotonin, dopamine, norepinephrine, epinephrine, histamine) and cholinergic (acetylcholine) systems, as well as excitatory (glutamate) and inhibitory (GABA) amino acids has been determined in the head of the control and exposed larvae. Differences between nicotine and cotinine binding to zebrafish α7 nAChR have been explored using in silico docking analysis. Finally, the environmental risk of nicotine and cotinine in freshwater systems has been revisited.

2. Material and methods

2.1. Chemicals and reagents

The highly pure analytical standard of nicotine and cotinine were

provided by Sigma-Aldrich (St. Louis, Missouri, USA). LC-MS grade organic solvents, methanol (MeOH) and acetonitrile (ACN) were purchased from VWR Chemicals Prolabo (Leuven, Belgium). Ammonium formate and ammonium acetate were supplied by Sigma-Aldrich (St. Louis, MO, USA), while formic acid (FA) was supplied by Fisher Scientific (Loughborough, UK). Ultra-pure water was obtained daily through the Millipore Milli-Q purification system (Millipore, Bedford, MA, USA).

For neurotransmitter analysis, pure reference standards of acetylcholine (ACh), dopamine (DA), epinephrine (Epi), γ-aminobutyric acid (GABA), glutamic acid (Glu), histamine (His), serotonin (5-HT) and were supplied by Sigma-Aldrich (St. Louis, USA), while norepinephrine (NE) was obtained from Tocris Bioscience (Ellisville, USA). A standard solution mixture containing 5 ng/μL of each metabolite was prepared in the extractant solvent mixture (ES, ACN: H_2O (90:10) + 1 % FA), and used to spike quality controls (QCs) as well as to prepare the calibration curve. Moreover, isotopically labelled standards used as internal standards, including NE-d6, DA-1,1,2,2-d4, and 5-HT-d4, and were purchased from Toronto Research Chemicals (TRC, Toronto, Canada). A mixture of labelled standards (ISM) was prepared at different concentrations (between 0.01 and 0.5 ng/μL, depending on the metabolite) and added to every sample, QC, or calibration standard.

2.2. Fish husbandry

Adult wild-type zebrafish were acquired from Pisciber (Terrassa, Spain) and housed in the Research and Development Centre of the Spanish Research Council (CID-CSIC) facilities, where they were maintained in fish water at a temperature of 28 ± 1 °C. The zebrafish larvae used in the study were obtained through natural mating and subsequently kept in fish water in a thermostatic chamber (POL-EKO APARATURA Climatic chamber KK350, Poland) at a temperature of 28.5 °C, with a 12-hour light/12-hour dark photoperiod.

All experimental procedures involving zebrafish were conducted in accordance with the guidelines set forth by the Institutional Animal Care and Use Committees at the CID-CSIC. The protocols were approved, and the study was carried out under a license obtained from the local government (agreement number 11336). Stringent adherence to ethical standards ensured the welfare and appropriate treatment of the zebrafish used in the study.

2.3. Exposure protocol

Stock solutions of nicotine and cotinine were prepared using dimethyl sulfoxide (DMSO) and subsequently diluted in fish water to achieve a final concentration of 0.1 % DMSO in all treatment groups. The nominal concentrations of the working solutions were 2, 4, 20, 100, 500, 2500 ng/L for nicotine and 0.05, 0.10, 1, 10, 100, 1000, 10,000 ng/L for cotinine. While the initial selected concentrations were in the range of 20–2000 for nicotine and 10–10,000 for cotinine, during the study lower concentrations of both compounds has to be added in order to identify the non-observed effect concentrations (NOEC).

At 7 days post-fertilization (dpf), zebrafish larvae were carefully transferred to 48-well plates, each well containing 1 larva in 1 mL of treatment solution, for 24 h. All exposures were performed at a temperature of 28.5 °C using the same climatic chamber (POL-EKO APARATURA Climatic chamber KK350, Poland) where larvae grew, with a 12-hour light/12-hour dark photoperiod.

2.4. Nicotine and cotinine stability in water

Nicotine and cotinine experimental concentration, as well as their stability in fish water, were assessed using solid phase extraction procedure (to preconcentrate samples) followed by LC-MS/MS analysis. Water samples for the stability study were prepared exactly as in the exposure experiment, and solutions were kept for 24 h under the same conditions of temperature and light. Due to the low concentration levels (ng/L), a preconcentration step was required. Solid phase extraction procedure was adapted from studies that measured nicotine and its main metabolites in human urine (Xu et al., 2004). To do so, OASIS HLB cartridges were first conditioned with 5 mL of MeOH, followed by 5 mL of MilliQ water. Afterwards, the samples were loaded into the cartridges, under vacuum and pumped for 30 min after finishing the sample volume to ensure complete water removal. Subsequently, the analytes (nicotine and cotinine) were eluted from the cartridge using 5 mL of 10 mM ammonium acetate in MeOH. Finally, samples were dried under N_2 stream, and reconstituted in 0.5 mL of MilliQ water. Samples were kept at −20 °C until LC-MS/MS analysis.

Regarding the instrumental conditions, samples were analyzed through LC-MS/MS in an UPLC H-Class Acquity coupled to tandem mass spectrometry detector Xevo TQ-s micro from Waters (Milford, MA, USA). Both alkaloids were analyzed using a BEH C18 column (100 × 2.1 mm, 1.7 μm). The mobile phase consisted of a binary mixture of 0.1 % FA in MilliQ water (A) and 0.1 % FA in ACN (B). The elution was performed using an isocratic mode (10 % B for 3 min). The flow rate was set at 0.25 mL/min, and 20 μL were injected. Detection, identification, and quantification were performed using a multiple reaction monitoring mode (MRM). Flow injection analyses (FIA) were used to obtain the precursor ion, and fragments for each analyte, as well as the optimum cone voltage and collision energy. All MS parameters are summarized in the Supplementary Table ST1. MassLynx v4.1 software package was used for data processing.

2.5. Behavioral analysis

The experiments were carried out using 48-well plates, with one zebrafish larva per well as described in previous studies (Faria et al., 2022). Each plate was placed into a DanioVision observation equipped with a temperature control unit (Noldus Information Technology, Leesburg, VA), and kept for 30 min to acclimate to the dark before the initiation of video recording with a digital video camera (Basler acA1300-60 g m, Basler Inc., Exton, PA). Videos were analyzed with the EthoVision XT 13 video tracking system (Noldus, Wageningen, Netherlands).

To assess the vibrational startle response (VSR) for each larva, the experiments were conducted under near-infrared light. After the 10 min of conditioning, a series of 50 tapping stimuli were administered at a rate of one stimulus per second, for 1 min. VSR corresponds to the distance moved (cm) following the first tapping stimuli. Moreover, the habituation to the vibrational stimuli was evaluated by calculating the area under the curve of the plots of distance moved relative to the response of the 50 vibrational stimuli. Basal locomotor activity (BLA) was determined by measuring the total distance (cm) travelled by each larva over a 10-minute dark period, while light-off visual motor response (light-off VMR) was assessed by measuring the difference in the total distance (cm) travelled by the larvae during the last 2-minute dark period and the first 2-minute light period.

2.6. Neurotransmitters extraction and LC-MS/MS analysis

2.6.1. Extraction

The procedure for extracting the target metabolites from larval heads was adapted from a previous study that elucidated the different regulation of some metabolites between whole larvae and head metabolomic profiling analysis (Bellot et al., 2021).

Initially, 300 μL of a cold ES were added to pools of 15 zebrafish larvae heads. Each sample was also spiked with labelled neurotransmitter mix (ISM). Three stainless steel beads (3 mm diameter) were placed in each pool, and the samples were homogenized and ground using a bead mill homogenizer (TissueLyser LT, Quiagen, Hilden, Germany), for 90 s at 50 oscillations per second. Samples were then centrifuged for 20 min at 13,000 rpm (4 °C). Finally, the supernatant was filtered through 0.20 μm PTFE filters and stored at −20 °C until LC-

MS/MS analysis.

2.6.2. UPLC-MS/MS

To determine the metabolomic profile, the analysis of target metabolites was performed using a UPLC H-Class Acquity coupled to tandem mass spectrometry detector Xevo TQ-s micro from Waters (Milford, MA, USA). Analytes retention and separation was achieved using an Acquity UPLC BEH Amide column (150 × 2.1 mm, 1.7 mm) provided with an Acquity UPLC BEH Amide pre-column (5 × 2.1 mm, 1.7 mm) (Waters, Milford, MA, USA). The mobile phase consisted of a binary mixture of aqueous and organic solution (A and B). Solvent A was composed of Milli-Q water and ACN (95:5) containing 100 mM ammonium formate, while solvent B was Milli-Q water and ACN (15:85) containing 30 mM ammonium formate. The pH of both solvents was acidified to pH 3 with formic acid (FA). The elution of the analytes followed a gradient mode, lightly increasing polarity along the gradient. It began 100 % B, decreased to 80 % B within 4 min, and maintained for 1 min. From 5 to 7 min, solvent B was linearly increased to 100 %. Finally, the initial conditions were re-equilibrated in 3 min, resulting in a total run time of 10 min. All the gradient was performed at 30 °C, while samples were kept at 8 °C during the analysis period. The flow rate was set at 250 μL/min, and10 μL of each sample were injected. The concentration levels of target neurotransmitters in the pools were normalized based on the number of heads in each pool (15 heads).

The main conditions for MS are summarized in the Supplementary Table ST1. The acquisition was performed in MRM mode. The precursor ion, its fragmentations, and the optimized cone voltage (CV) and collision energies (CE) are also depicted in Supplementary Table ST1. The system and data management were processed using MassLynx v4.1 software package.

2.6.3. Quality assurance

The linearity of the method was studied in the range between 0.005 and 2.5 ng/μL, using at least 6 calibration points for each metabolite. Blanks of extractant solvent were analyzed to control carryover or cross-contamination during the process. To correct the extraction procedure and the chromatographic analysis, ISM was employed as internal standard for each analyte. To determine the extraction recoveries, three quality controls (QCs) were used, spiking 250 ng of each target metabolite.

Furthermore, the instrumental detection limits (IDLs) were assessed by determining the concentration that produced a signal-to-noise ratio (S/N) of 3, using the standard with the lowest concentration as a reference. Similarly, the method detection limits (MDLs) were calculated using QCs samples as a reference. Moreover, intra-day precision was evaluated by injecting four consecutive standard solutions, while inter-day precision was determined by measuring the same standard solution on three different days. Finally, the matrix effect (ME) for each neurotransmitter was obtained by comparing the peak area from the QCs with the peak area from the most similar standard solution. The quality parameters of this method are summarized in Supplementary Table ST2, and additional details are provided at the Supplementary material.

2.7. Docking studies using Alphafold's modeled zebrafish α7 nAChR structure

Modeling studies were performed using MOE2022.02 software (Inc, 2016) and Amber10 forcefield. The RCSB Protein Data Bank (PDB) 7EKT (Berman et al., 2000; Liu et al., n.d.) (human α7 nAChR) structure cocrystallized with the EVP-6124 ligand was used as a reference to locate the allosteric binding site. The zebrafish receptor was modeled and superposed to the original human receptor using AlphaFold (Jumper et al., 2021; Varadi et al., 2022) B3DH13 predicted structure of the cholinergic receptor in *Danio rerio*. 7EKT complex was prepared using QuickPrep module and B3DH13 was pentaplicated and overlapped with the analogous human model matching the pocket residues (Fig. 3A). Docking studies were performed on the active site of both receptors using nicotine, cotinine and EVP ligands and discarding all the water molecules of the system. The ligand placement was performed via triangle matcher using London dG scoring function and two case studies were executed using induced fit (flexible) and rigid receptor conditions as refinement and GBVI/WSA dG as final scoring methodology. Finally, the best conformation, in terms of score value, for each ligand was selected for discussion (Fig. 3B–C). The docking results are summarized in Table 2.

2.8. Behavioral-based risk assessment

The environmental risk of nicotine, cotinine and their mixture, have been determined by using the hazard quotient (HQ), the ratio between the measured environmental concentration (MEC) and the predicted non-effect concentration (PNEC). MEC values used were based in the median and maximum levels reported in the studies cited in the Introduction, while the NOEC for nicotine and cotinine in the light-off VMR were used to determine the PNEC. Whereas compounds with HQ >1 are potentially hazardous for aquatic ecosystems, those with HQ < 1 values are considered as slightly or not hazardous.

2.9. Data analysis

Data analysis was performed with IBM SPSS v25 (Statistical Package 2010, Chicago, IL). First of all, normality and homogeneity of variance of the data was determined using Shapiro-Wilk test and Levene's test, respectively. When the assumptions of normality and homoscedasticity were tenable, Student's *t*-test or one-way ANOVA followed by Dunnett's multiple comparison test was used. In those cases where assumptions of normality and homoscedasticity were not met, Kruskal–Wallis test followed by Dunn's multiple comparison test was used. Significance was set at $p < 0.05$.

3. Results

3.1. Actual concentrations and stability of nicotine and cotinine

The actual concentration and stability of the experimental solutions at the end of the exposure period (24 h at 28 °C, 12/12 light/dark) were determined at two nominal concentrations of nicotine (20 and 200 ng/L) and cotinine (1 and 10 ng/L), selected under the criterion that they were in the range of the effective concentrations. As shown in Table 1, no differences were found between the nominal and actual concentrations determined in the experimental solutions of nicotine and cotinine. Moreover, when the stability of the experimental solutions was determined using the same temperature (28 °C) and photoperiod (12/12, L/D) conditions as used during the exposure, no differences were found between the initial and final concentrations.

3.2. Environmental concentrations of nicotine and cotinine treatment impair light-off VMR

Zebrafish larvae exposed for only 24 h to a wide range of environmental concentrations of nicotine (2 ng/L to 2.5 μg/L) and cotinine (50 pg/L to 10 μg/L) did not exhibit any sign of systemic toxicity (impaired gross morphology or lethality). Therefore, potential behavioral effects of the exposure to these environmental concentrations of both neuroactive pollutants were analyzed using a battery of assays including BLA, VSR, habituation to a series of vibrational stimuli, and light-off VMR (Fig. 1).

As shown in Fig. 1A–B, exposure to nicotine, but not to cotinine, resulted in a significant effect on BLA, with a mild to moderate hyperactivity at 2.5 μg/L ($H(6) = 26.28$, $p = 1.97 \times 10^{-4}$). Nicotine had also a significant effect on the VSR ($H(6) = 33.29$, $p = 9{,}23 \times 10^{-6}$), with larvae exposed to 4 ng/L nicotine exhibiting a decreased response to the

Table 1
Actual concentrations and stability during the first 24 h in the experimental conditions for nicotine and cotinine (mean ± SE, n = 3).

	Actual concentration (T0), ng/L	Nominal vs actual p (Student's t-test)	Concentration at 24 h (T24), ng/L	T0 vs T24 p (Student's t-test)
20 ng/L nicotine	21.58 ± 1.20	0.26	19.35 ± 0.27	0.15
200 ng/L nicotine	204.74 ± 1.81	0.06	193.96 ± 4.64	0.10
1 ng/L cotinine	1.04 ± 0.16	0.81	0.94 ± 0.03	0.57
10 ng/L cotinine	10.11 ± 0.10	0.36	9.46 ± 0.35	0.15

vibrational stimulus (Fig. 1C). In addition, a significant effect between nicotine and non-associative learning was also found ($H(6) = 34.89$, $p = 4.52 \times 10^{-6}$), with larvae exposed to 4 ng/L nicotine exhibiting a faster habituation to a series of vibrational stimuli than the corresponding controls Cotinine exposure, however, produced no effect on VSR or habituation (Fig. 1D and F).

Interestingly, exposure to nicotine and cotinine resulted in a significant effect on light-off VMR ($H(6) = 32.60$, $p = 1.25 \times 10^{-5}$ for nicotine and $H(7) = 43.80$, $p = 2.34 \times 10^{-7}$ for cotinine). The observed effect was similar for both chemicals, an increased motor response in response to a sudden transition from light to dark. The effect of these chemicals on light-off VMR was non-monotonic, with the strongest effects at the lowest concentrations. As shown in Fig. 1G–H, the potency of cotinine for this effect was higher than that for nicotine, and the range of effective concentrations for cotinine, 0.1–1000 ng/L, was wider than that for nicotine, 4–500 ng/L. The low-observed effect concentration (LOEC) for light-off VMR was 4 ng/L for nicotine and 100 pg/L for cotinine, while the non-observed effect concentration (NOEC) was 2 ng/L for nicotine and 50 pg/L for cotinine. These results show that cotinine is about 40-fold more potent than nicotine in altering light-off VMR.

Since nicotine and cotinine share a common MoA, as agonists of nAChR, the hypothesis that a binary mixture of both chemicals in the water should have an additive effect on light-off VMR was tested. Consequently, 7 dpf zebrafish were exposed for 24 h to a mixture of both chemicals at half of their LOECs (2 ng/L nicotine and 50 ng/L cotinine). A significant increase on the distance moved in response to the visual stimulus ($t(88) = -2.011$, $p = 0.047$, Student's *t*-test) was found in the exposed larvae compared to the respective controls (Fig. 1I).

3.3. Effects of nicotine and cotinine on the neurotransmitter levels in the head of zebrafish larvae

In order to understand if the observed behavioral changes found after the exposure to effective concentrations of nicotine and cotinine were associated to changes in the pool of neurotransmitter levels, concentrations of acetylcholine, dopamine, norepinephrine, epinephrine, serotonin, histamine, glutamate and GABA were determined in the head of larvae control and exposed to 20–100 ng/L nicotine and 1–10 ng/L cotinine (Fig. 2). No changes in the levels of any of the selected neurotransmitters were found after 24 h exposure to nicotine. Glutamate was the only neurotransmitter modulated by cotinine [$H(4) = 15.31$, $p = 4.10 \times 10^{-3}$], decreasing the levels of this excitatory amino acid after 24 h exposure to 10 ng/L cotinine (Fig. 2).

3.4. Docking studies using Alphafold's modeled zebrafish α7 nAChR structure predict the binding mode of nicotine and cotinine

In order to provide a deeper understanding of the observed differences between nicotine and cotinine at the molecular level we decided to model the α7 nAChR of *Danio rerio* and perform a docking study to predict their binding modes. Given the absence of any crystal structure of the α7 nAChR of *Danio rerio* deposited in the PDB (Berman et al., 2000), we used the Alphafold (Jumper et al., 2021; Varadi et al., 2022) structure B3DH13 generated by artificial intelligence (AI), pentaplicated, and superposed it to the human structure (PDB ID: 7EKT) (Berman et al., 2000; Liu et al., n.d.). As it can be observed in Fig. 3A, both structures are highly similar (85 % similarity), share the same binding site residues, and largely superpose (RMSD = 1.221 Å). Next, we used MOE software (Inc, 2016) to dock nicotine and cotinine to this structure and compared it to the cocrystallized ligand in 7EKT (EVP). As it can be observed in Table 2, the docking score is significantly larger (more negative) for cotinine than for nicotine in both the rigid and induced-fit docking, suggesting that cotinine could bind more potently to *Danio rerio*'s α7 nAChR protein.

3.5. Behavioral-based risk assessment for nicotine and cotinine

The finding that environmental concentrations of nicotine and cotinine are able to alter an ecologically relevant behavior such as the light-off VMR in zebrafish larvae strongly suggests that the environmental risk of this pesticide should be revisited. The predicted non-effect concentration (PNEC) of nicotine and cotinine in zebrafish larvae can be determined by using the non-observed effect concentration (NOEC) for VMR, 2 ng/L for nicotine and 50 pg/L for cotinine. Therefore, the environmental risk of nicotine and cotinine will be high at those aquatic ecosystems with concentrations of these neuroactive chemicals above these PNECs. Considering the worldwide levels of nicotine and cotinine reported by Wilkinson et al. (2022) in aquatic ecosystems, the range of hazard quotients (HQ) for nicotine were from 2.53 to 5850, with a value of 161 for the worldwide median concentration. For cotinine, 40-fold more potent than nicotine in altering light-off VMR, the range of HQ were from 224 to 191,400, with a value of 4900 for the worldwide median concentration. Therefore, the levels of nicotine and cotinine commonly reported in most of the freshwater ecosystems are able to alter fish larvae behavior.

4. Discussion

Nicotine and cotinine are two neuroactive chemicals commonly found, at ng/L concentrations, in freshwater ecosystems all over the world. Since concentrations of these chemicals in freshwater are, in most cases, lower than the reported PNEC for fish, it is generally accepted that the current levels of both chemicals are safe for fish communities. In this manuscript we have analyzed the effect of the exposure of zebrafish larvae, for only 24 h, to a wide range of environmental concentrations of nicotine and cotinine. We have found that some environmental concentrations of nicotine, but not cotinine, were able to induce hyperactivity (2.5 μM) and decrease in both the startle response evoked by a vibrational stimulus and the habituation time (4 ng/L). Although an increase in locomotor activity has also been reported in rodents (Ksir, 1994) and zebrafish (Bencan and Levin, 2008; Gómez-Canela et al., 2017) exposed to high doses of nicotine, the effect of this alkaloid on locomotion appears to be time- and dose-dependent (Wronikowska et al., 2020). The effects of 4 ng/L nicotine on the startle response and its habituation seem to be non-monotonic, as these endpoints return to the control values when nicotine concentrations increase. In fact, when in previous studies 7 dpf larvae were exposed for 24 h to 25 mM nicotine, a concentration about 1600-fold higher than the highest one tested in this study, no effects on the startle response were found and there was an increase in the habituation time (Faria et al., 2019b; Faria et al., 2019a). The non-monotonic concentration response (NMCR) relationship between neuroactive compounds and zebrafish larvae behaviors has been

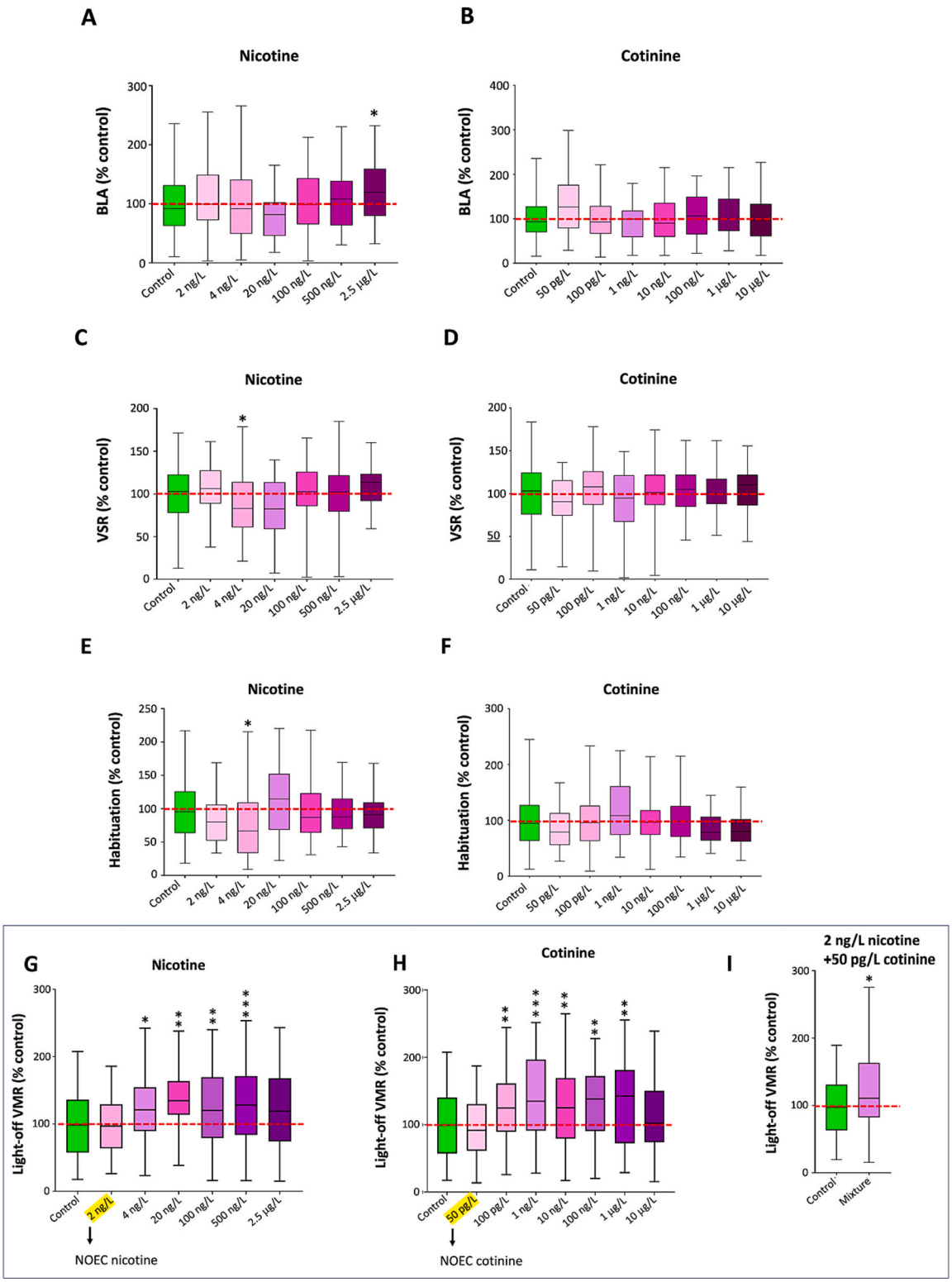

Fig. 1. Behavioral effects on 7 days post-fertilization zebrafish exposed for 24 h to low environmental levels of nicotine and cotinine. (A–B) Effects on basal locomotor activity (BLA; n = 36–248 for nicotine and n = 44–227 for cotinine); (C–D) effects on vibrational startle response (VSR; n = 35–201 for nicotine and n = 42–197 for cotinine); (E–F) effects on habituation to a series of 50 vibrational stimuli (inter-stimuli distance: 1 s) (n = 39–257 for nicotine and n = 41–209 for cotinine); (G–H) effects on light-off Visual Motor Response (VMR). Non-observed effect concentrations (NOEC) of these chemicals for this behavioral endpoint are marked (n = 39–177 for nicotine and n = 47–140 for cotinine); (I) effect of a binary mixture of nicotine and cotinine, with a concentration of each chemical being half of the low-observed effect concentration (LOEC) for the light-off VMR (n = 45–85). 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, **p < 0.01, ***p < 0.001; Kruskal Wallis test with Bonferroni correction; Data from 2 to 5 independent experiments.

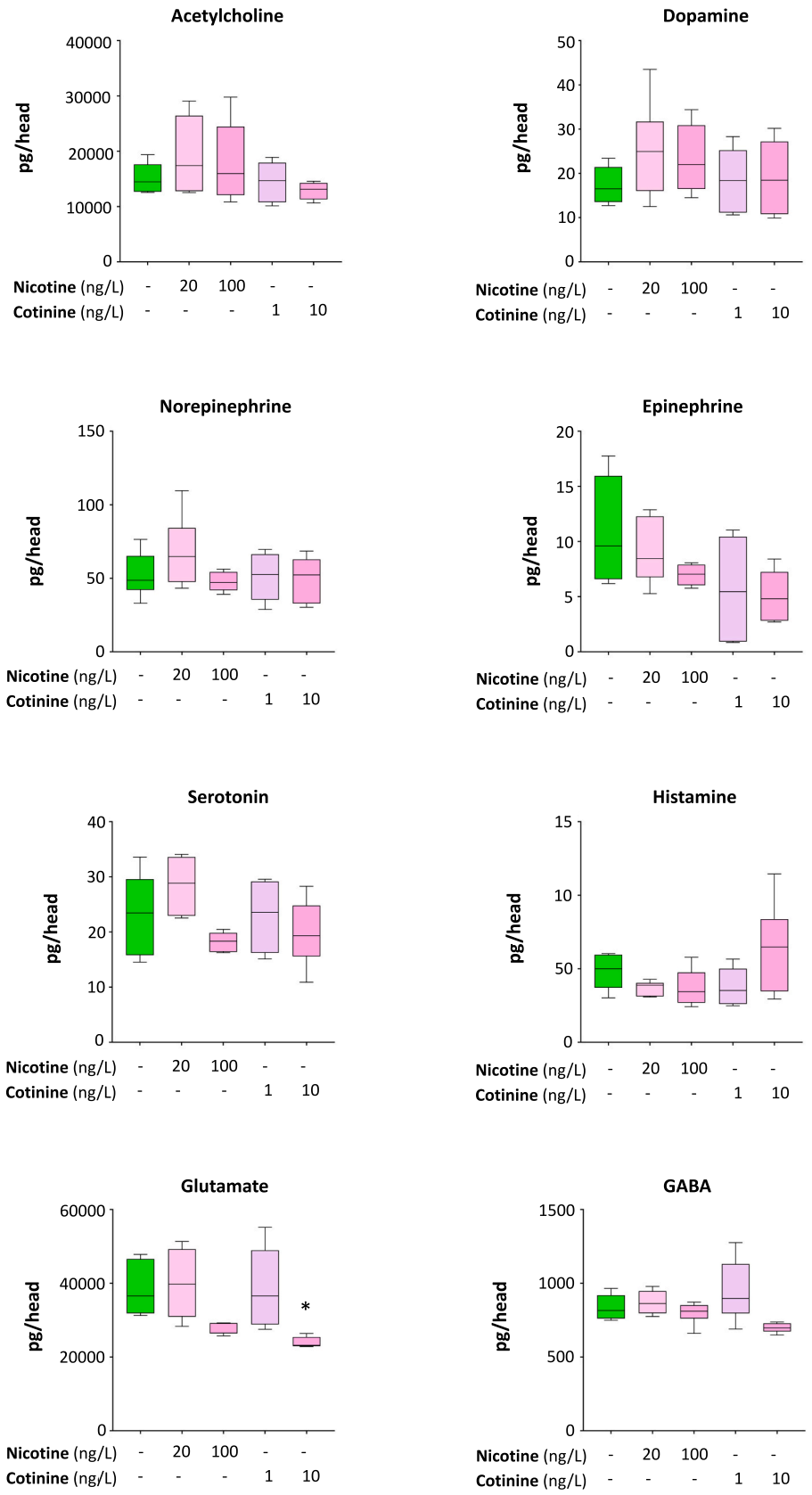

Fig. 2. Effects on the main neurotransmitters in the head of 7 days post-fertilization zebrafish exposed for 24 h to low environmental levels of nicotine (20–100 ng/L) and cotinine (1–10 ng/L). Boxplot representation of pg of neurochemical per head with the box indicating the 25th and 75th percentiles and the whiskers the maximum and minimum values. The thin line within the box marks the median (n = 6 pools). *$p < 0.05$ Kruskal Wallis test with Bonferroni correction; Data from 2 independent experiments.

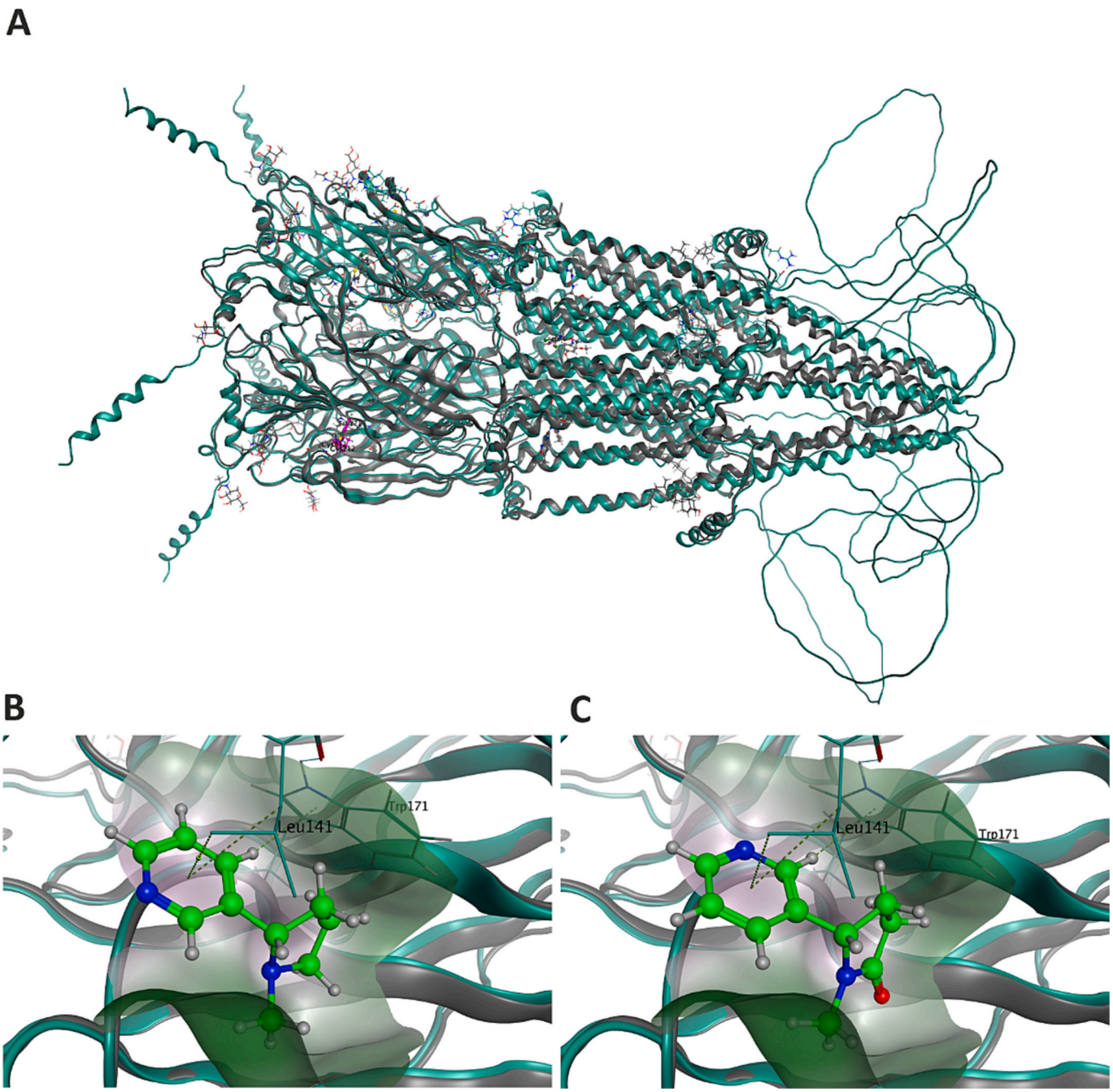

Fig. 3. Docking studies using Alphafold's modeled zebrafish α7 nAChR structure predict the binding mode of nicotine and cotinine. (A) Superposition of the human α7 nAChR crystal structure (PDB ID: 7EKT; in grey) and the pentaplicated AlphaFold structure of the α7 nAChR zebrafish protein (AF B3DH13; in turquoise). The original EVP ligand is shown in pink to locate the active site; (B) first induced-fit docking conformation of R-nicotine in AF B3DH13 (in turquoise) shown with the residues involved in its key interactions; (C) first induced-fit docking conformation of R-cotinine in AF B3DH13 (in turquoise) shown with the residues involved in its key interactions.

previously reported by us (Bedrossiantz et al., 2023; Faria et al., 2022, 2021b) and others (Agathokleous, 2022; Agathokleous et al., 2021).

While the exposure to some environmental concentrations of nicotine resulted in changes in BLA, VSRA or habituation, the most consistent biological effect found in this study led for environmental concentrations of nicotine and cotinine has been a significant increase in the light-off VMR. Sudden decrements in light intensity trigger acute locomotor response in zebrafish larvae (Fero et al., 2011). Apparently, the main component involved in a sudden decrease in illumination is the retinal OFF channels, a neural circuit relaying visual signals from photoreceptors to specific OFF bipolar and ganglion cells and ultimately to higher visual centers (Tian and Copenhagen, 2003). Activation of this circuit evocates a large turn-angle, the O-bend, a few milliseconds after this stimulus. These O-bends are initially followed by routine high frequency turns and then, during the dark adaptation period, by increased swimming bouts (Fernandes et al., 2012). Therefore, the effect of nicotine and cotinine on light-off VMR could due to modulation of the retinal OFF-channels.

The major excitatory neurotransmitter in the retina is glutamate (Slaughter and Miller, 1983). Light decrements cause photoreceptor depolarization resulting in the release of glutamate, activating the AMPA/Kainate receptors expressed in the dendrites of the OFF bipolar

Table 2
Docking scores and energy interactions with specific residues measured for the first pose observed for EVP, R-nicotine and R-cotinine using a rigid and induced fit (flexible) receptor system for the Alphafold, AI-predicted α7 protein structure of zebrafish (AF B3DH13).

Docking: AF B3DH13	Rigid receptor		Induced fit	
	Score	Interactions (kcal/mol)	Score	Interactions (kcal/mol)
EVP	−5.9697	Met60 (−2.1) Leu141 (−0.7) Thr172 (−0.5)	−6.7462	Met60 (−3.4) Asp186 (−1.5) Leu141 (−1.30, −1.3)
Nicotine	−5.8524	Thr172 (−0.7) Leu141 (−0.7)	−5.6543	Leu141 (−1.4, −1.4) Trp171 (−1.2)
Cotinine	−6.1631	Thr172 (−0.9) Leu141 (−0.5)	−6.0583	Leu141 (−1.0, −0.5) Trp171 (−1.4)

cells (Slaughter and Miller, 1983). Activation of these ionotropic receptors results in depolarization of OFF bipolar cells and increased firing rates of OFF ganglion cells (Smith et al., 2014). Another excitatory neurotransmitter expressed in the retina, specifically in subtypes of amacrine cells, is ACh, and interestingly, α7 nAChRs are expressed in subpopulations of bipolar, amacrine and ganglion cells (Smith et al., 2014). It has been suggested that activation of α7 nAChRs may modulate the response of bipolar and ganglion cells. In this manuscript, levels of glutamate in the head (including eyes) of larvae exposed to the highest concentrations of nicotine and cotinine, 100 and 10 ng/L, were lower than those of the corresponding control, although this effect was only statistically significant for cotinine. Heads were collected at the end of the behavioral assays, before the effect on light-off VMR was known. However, considering that glutamate in the retina represent only a small percentage of the total glutamate in the head, further experiments specifically assessing retinal glutamate will be necessary to fully understand the significance of this finding for VMR. No differences in ACh were observed in treated larvae. However, as nicotine is a prototypic nAChR agonist, it should be able to activate the cholinergic modulation of retinal activity without any change in the ACh levels (Ariel and Daw, 1982). Therefore, cholinergic activation of subpopulations of amacrine, bipolar and ganglion cells of the retina could be involved in the observed effect on light-off VMR.

One of the hypotheses tested in this study is that both nicotine and cotinine share the same MoA, activation of nAChR. In aquatic toxicology the concept of concentration addition (CA) describes the relationship between chemicals sharing a similar MoA (Barata et al., 2006). Therefore, in order to assess if the increased light-off VMR produced by exposure to both nicotine and cotinine was through a similar MoA, a binary mixture of both chemicals was prepared by adding 2 and 0.05 ng/L of nicotine and cotinine, respectively, concentrations representing the half of the LOEC for each compound and their NOEC. The results presented in this manuscript support that the effect of both chemicals on light-off VMR is consistent with the CA concept and, therefore, that both chemicals share a similar MoA to produce this effect.

It has been reported that the potency of cotinine as nAChR is much lower than that of nicotine (Tan et al., 2021). However, cotinine was found to be much more potent than nicotine in inducing the effect on light-off VMR. The affinity of any agonist for the receptors varies according to the different animal species and therefore, in zebrafish cotinine might be a more potent agonist of α7 than nicotine. Modeling of the zebrafish α7 nAChRs structure and docking of nicotine and cotinine predicted a similar binding mode of both compounds (Fig. 3) but with stronger interactions in the case of cotinine (Table 2). These results suggest that cotinine may indeed bind more potently to zebrafish α7 nAChRs, providing a potential molecular explanation for the higher light-off visual motor response that we observe in vivo. The differences between cotinine and nicotine in the docking scores are interesting, since the interactions predicted by the docking are similar for both chemicals, with any additional interaction involving the carbonyl in cotinine, its only structural difference with nicotine (Fig. 3B–C). Therefore, we speculate that the carbonyl could slightly modify the electrostatics allowing for a better interaction and stacking of cotinine with Trp171 and/or Thr172 and Leu141 (as it can be observed in Table 2 the predicted interaction energy with these residues is always more potent for cotinine). Small differences in decoration of heterocycles affecting stacking energy have been previously described (Bootsma et al., 2019). However, it is important to stress that scoring functions are not devoid of limitations in predicting binding affinity. Furthermore, docking with Alphafold structures has been reported to be less accurate than docking with crystal structures, even when using induced fit (Scardino et al., 2023). Therefore, our docking study provides an initial hypothesis for the observed effects of cotinine at the molecular level that should be further validated experimentally with structural and biochemical studies such as resolving a cocrystal structure.

There is still controversy about the ecological significance of light-off VMR. One hypothesis is that abrupt reductions in illumination represent the shadow of a potential predator, and that the O-bend evoked by this stimulus should be considered as a visual startle response (Yoo et al., 2018). However, more recent studies have demonstrated that light-off VMR is not a true startle response suggesting that this response is primarily navigational, one of the mechanisms used by the larvae to reach illuminated environments, increasing the probability of a successful feeding (Burgess and Granato, 2007).

The results presented in this manuscript are relevant to the environmental risk assessment for different reasons. First, both predatory avoidance and bottom-feeding are ecologically relevant behavior and therefore, altered light-off VMR is also ecologically relevant. Second, since the relationship between nicotine and cotinine and light-off VMR is non-monotonic, it is more difficult to fix freshwater quality criteria for these chemicals. Moreover, as cotinine is the main metabolite of the nicotine, both chemicals are found simultaneously in the same ecosystems, increasing the risk of additive effects. The currently opinion is that the levels of nicotine and cotinine found in most of the freshwater aquatic ecosystems worldwide are safe for the protection of fish communities, as they are below the NOECs of these compounds for lethality. However, the results presented in this study show that in fact these levels are very often above the NOEC of these compounds for light-off VMR and that, therefore, the environmental risk for nicotine and cotinine should be revisited.

Since nicotine leached from e-cigarette components can reach aquatic ecosystems, often without passing through wastewater treatment plants, this waste poses a major threat to the environment. To reduce this source of nicotine in our aquatic ecosystems, different measures have recently been proposed including the implementation of specific strategies for the management of CBs and e-cigarette litter in cities and probably more important, a public education approach, working on an effective change in littering behavior through awareness (Roder Green et al., 2014).

CRediT authorship contribution statement

Marina Bellot: Conceptualization, Data curation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **Leticia Manen-Freixa:** Data curation, Investigation, Writing – original draft, Writing – review & editing. **Eva Prats:** Data curation, Formal analysis, Investigation, Writing – review & editing. **Juliette Bedrossiantz:** Investigation, Methodology, Visualization, Writing – review & editing. **Carlos Barata:** Conceptualization, Formal analysis, Writing – original draft, Writing – review & editing. **Cristian Gómez-Canela:** Data curation, Formal analysis, Project administration, Supervision, Writing – original draft, Writing – review & editing. **Albert A. Antolin:** Conceptualization, Data curation, Formal analysis, Software, Writing –

original draft, Writing – review & editing. **Demetrio Raldúa:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Project administration, Supervision, Writing – original draft, Writing – review & editing.

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2023.169301.

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