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High-speed optical mapping of heart and brain voltage activities in zebrafish larvae exposed to environmental contaminants ☆

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ABSTRACT

Environmental contaminants represent a poorly understood ecotoxicological and health risk. Here, we advanced a high-speed optical mapping (OM) technique to non-invasively track voltage dynamics in living zebrafish larvae's heart and brain and investigate the effects of selected pesticides.

OM allowed high resolution (~17x) and fast acquisition (100 to 200 frames/s) of the voltage signal generated in the heart and brain after immersion of the zebrafish larvae in a voltage-sensitive dye. First, we used varying temperatures (20 °C to 25 °C) to test the adequacy of OM in capturing cardiac and brain voltage changes. Then, we tested the effects of glyphosate or a selected pesticide cocktail (2 to 120 h post-fertilization), accounting for their environmental thresholds and mimicking high-level exposure. Glyphosate (0.1 and 1000 μg/L) and the pesticide cocktail (0.1 and 10 μg/L) did not alter cardiac activity, except for a trend increase in heart rate variability at high glyphosate dose. Fourier transform (FT) analyses indicated that glyphosate reduced the abundance of low-amplitude voltage activities in the brain at the target low-frequency range of 0.2–15 Hz. The anatomical fragmentation of the brain into four regions, right and left diencephalon (RD and LD) and right and left optic tectum (ROT and LOT), confirmed the impact of glyphosate on the larvae brain and revealed a specific adaptation to the pesticide cocktail in the RD and ROT regions.

In summary, OM captured heart and brain voltage changes in zebrafish larvae, with discrete patterns of brain depolarization in the presence of specific water contaminants. Here we discuss the relevance of these findings to ecotoxicology and exposome research.

Abbreviations: Optical mapping, OM; Right and left diencephalon, RD and LD; Right and left optic tectum, ROT and LOT; Days post-fertilization, dpf; Hours post-fertilization, hpf; *N*-phenylthiourea, PTU; Fourier transform, FT; Control group, Contr

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1. Introduction

The widespread use of pesticides raises essential questions about their impact on ecotoxicology and human health. Environmental matrices contain complex mixtures of pesticides (Thrupp et al., 2018) with multiple interactions between compounds (Martin et al., 2021). Accumulating epidemiological studies suggest that exposure to different pesticides during critical developmental phases could represent a risk factor for neurological and cardiac pathological adaptations (Roberts et al., 2019) associated with neuroinflammation (Cunningham et al., 2013; Forner-Piquer et al., 2021a; Martínez et al., 2020), neurodegenerative modifications, and frail physiology (Cattani et al., 2014; Cunningham et al., 2013; Martínez et al., 2020; Peng et al., 2010). Therefore, there is an urgent need to develop non-invasive modalities to examine the impact of environmental contaminants on organ functionality *in vivo* (Breitholtz et al., 2006; Legradi et al., 2018).

Recently, zebrafish has emerged as a valuable tool in environmental toxicology, with a prospective application to experimental cardiology and neuroscience (de Abreu et al., 2020; Turrini et al., 2017). The zebrafish heart presents electromechanical features equivalent to those of mammals, beating at a similar rate to that of humans (Arel et al., 2022). Furthermore, the zebrafish nervous system consists of precise networks, with neuronal populations intertwined with glial cells and the microvasculature, a mimicry of the human brain (Burrows et al., 2020). Here, we took advantage of the zebrafish larvae's transparency to apply high-speed optical mapping (OM) in combination with the voltage sensitive-dye Di-4-ANEPPS. This approach allowed us to record the spatiotemporal dynamics of excitable events across the whole heart and brain surface *in vivo*. OM with voltage-sensitive dyes is a non-invasive substitute for field-potential electrophysiology (O'Shea et al., 2020). Previously, OM enabled analysis of the heart and brain activities, although limited to *ex vivo* settings (Baudot et al., 2020; Liu and Baraban, 2019; Torrente et al., 2015). Here, we examined the voltage activities in physiological conditions and after developmental exposure to the herbicide glyphosate and an environmentally relevant cocktail of six pesticides (boscalid, chlorpyrifos, ziram, captan, thiophanate, thiacloprid). The molecules of choice, dosages, and water exposure protocols are based on our previous work and existing guidelines (Cunningham et al., 2013; Forner-Piquer et al., 2021a,b; Martínez et al., 2020). We report and discuss OM-based ecotoxicological assessment to screen heart or brain voltage depolarization patterns in the environmental exposome context.

2. Materials and methods

2.1. Zebrafish husbandry

Experiments were conducted following the European Directive on using laboratory animals (2010/63/EU) and the local approval (B34-172-41). Wild-type adult Zebrafish (*Danio rerio*) were reared under standardized conditions (28 ± 0.5 °C, pH 7, 14/10 h light/dark photoperiod) in a recirculation system (Techniplast) and fed twice a day with dry pellets (Gemma Micro 500ZF, Skretting). Zebrafish larvae were obtained by crossing adult zebrafish (AB wild-type strain). Once a week, adult males and females (3 to 6 months) were separated the afternoon before the experiments in different crossing tanks (with enriched environment), where 4–8 breeding couples were separated by plastic dividers and left undisturbed until the following day. The next morning, the plastic partition was removed, letting the breeding couples naturally spawn. After one hour, eggs were collected and washed, and dead or malformed eggs were removed. We exclusively select the fertilized eggs at the same developmental stage and randomly allocate them in groups of 30 to 50 in 50 mL E3 medium (5 mM NaCl, 0.17 mM KCL, 0.33 mM CaCl2, and 0.33 mM MgSO4) and placed them inside an incubator at 28 °C (±0.5 °C). To ensure transparency until 5 days post-fertilization (dpf), eggs were exposed to 0.2 mM *N*-phenylthiourea (PTU) from the epiboly stage (8.5–9 h post-fertilization, hpf). Although this treatment could introduce a co-exposure effect, it improves the acquisition of the fluorescent signals, and it has been applied in all the experimental groups. Moreover, we previously showed that this molecule at 0.2 mM does not induce locomotor, morphology, and muscle structure modifications when used alone or in combination with glyphosate (Forner-Piquer et al., 2021a).

We randomly allocated 30 to 50 embryos per petri dish and per day for our experiments. Five to 10 fish per group were analyzed at each experiment session, having always control and treated larvae for each session. For each experiment, two fish were positioned in parallel in the agarose drop for the experiments. All the larvae were exposed to the environmental contaminants (glyphosate and pesticide cocktail) from 2 to 120 hpf and then prepared for OM imaging. The water (control and treatments) with PTU was renewed on the third day. All the larvae were euthanized at the end of the OM with an overdose of tricaine methane sulfonate (300 mg/L; MS222 Sigma-Aldrich).

2.2. Glyphosate and pesticide exposure protocols

Zebrafish larvae were exposed to 2 concentrations of glyphosate (Sigma-Aldrich, purity 98.5%, CAS number 1071-83-6): 0.1 μg/L (5.91×10^{-4} μM/L) and 1000 μg/L (5.91 μM/L) in E3 medium from 2 to 120 hpf. Solutions were diluted on the first and third days. The concentrations selected for the present study were based on previous experiments from our group (Forner-Piquer et al., 2021a) and followed the guidelines established for regional environmental concentrations (Székács and Darvas, 2018). Notably, the lowest concentration tested in our experiments (0.1 μg/L) represents the maximum European concentration allowed for an individual pesticide in drinking and groundwater (Revised Drinking

Water Directive 2020/2184 and 2006/118/EC), while the higher concentration (1000 μg/L) was selected based on our previous results showing motor-behavior changes without anatomical malformations (Forner-Piquer et al., 2021a).

The pesticide mixtures contained equal parts of six selected pesticides purchased from Sigma-Aldrich: boscalid (purity ≥ 98.0%, CAS 188425-85-6), chlorpyrifos (purity ≥ 98.0%, CAS 2921-88-2), captan (purity ≥ 98.0%, CAS 133-06-2), thiacloprid (purity ≥ 98.0%, CAS 111988-49-9), thiophanate-methyl (analytical standard, CAS 23564-05-8) and ziram (analytical standard, CAS 137-30-4), relevant to agriculture, and already used in our previous publications (Forner-Piquer et al., 2021b; Klement et al., 2020; Lukowicz et al., 2018; Smith et al., 2020). The pesticide mixture concentrations tested were 0.1 and 10 μg/L, based on the established threshold in water and our previous results indicating motor defects (Forner-Piquer et al., 2021b). All pesticides were dissolved with dimethyl sulfoxide (DMSO, Sigma-Aldrich) to obtain a 100 mg/L stock solution. Aliquots were kept at −20 °C. For every single pesticide, 100 μL of the respective stock solution was mixed and diluted with E3 to obtain the final working mixture concentrations. With this procedure, the DMSO present in the stock solution was diluted, reaching the final concentrations of 0.001% for the 10 μg/L and 0.00001% for the 0.1 μg/L pesticide cocktail. Two DMSO control groups with the mentioned concentrations were included, although DMSO does not induce embryotoxicity at the concentrations tested (Forner-Piquer et al., 2021b; Hoyberghs et al., 2021; Kais et al., 2013). Nominal concentrations of glyphosate and pesticides in the water were verified in our previous publications (Forner-Piquer et al., 2021a,b), where we used identical experimental paradigms and equipment. The molar concentration of the pesticides and their half-life in water matrices (DT50) are noted below. Pesticides were renewed on the third day according to their long half-life in the water, except for captan (short half-life).

	Boscalid	Captan	Ziram	Chlorpyrifos	Thio.-meth.	Thiacloprid
0.1 μg/L	$2.91 \cdot 10^{-4}$ μM	$3.33 \cdot 10^{-4}$ μM	$3.27 \cdot 10^{-4}$ μM	$2.85 \cdot 10^{-4}$ μM	$2.92 \cdot 10^{-4}$ μM	$3.96 \cdot 10^{-4}$ μM
10 μg/L	0.0291 μM	0.0333 μM	0.0327 μM	0.0285 μM	0.0292 μM	0.0396 μM

Half-life Glyphosate: 4.2 days to 10 weeks (Gill et al., 2017; Mercurio et al., 2014; Vera et al., 2010) (https://pubchem.ncbi.nlm.nih.gov/compound/3496). Boscalid: 3–9 days (Review Report 2008: https://ec.europa.eu/food/plant/pesticides/eu-pesticides-database/start/screen/active-substances/details/472). Ziram: 2–18 days (https://pubchem.ncbi.nlm.nih.gov/compound/8722) Captan: 0.2 days (https://pubchem.ncbi.nlm.nih.gov/compound/8606). Thiophanate-methyl: 2.9–3.7 days (Review Report 2005: https://ec.europa.eu/food/plant/pesticides/eu-pesticides-database/start/screen/active-substances/details/807). Chlorpyrifos: 3–51 days (Review Report 2005: https://ec.europa.eu/food/plant/pesticides/eu-pesticides-database/start/screen/active-substances/details/548). Thiacloprid: 6–27 days (Review Report 2004: https://ec.europa.eu/food/plant/pesticides/eu-pesticides-database/start/screen/active-substances/details/841)

2.3. Optical mapping

To avoid motion artifacts, we tested pancuronium dibromide (300 or 600 mM, Abcam; Forner-Piquer et al., 2021b) and blebbistatin (5 μM, 8.5 μM, and 10 μM, Tocris Bioscience). We chose the myosin inhibitor blebbistatin, supported by previous tests indicating no effects on zebrafish cardiac activity (Marchant et al., 2022). To record voltage changes in the brain and heart of zebrafish larvae, we tested different concentrations of the voltage-sensitive probe Di-4-ANEPPS (3, 6 or 9 μM; AAT Bioquest), selecting 9 μM as the more appropriate concentration for our experiments. Zebrafish larvae were incubated in the dark for 40 min with Di-4-ANEPPS (9 μM). Blebbistatin (8.5 μM) was also diluted in the E3-staining medium to avoid muscular contraction and motion artifacts during imaging acquisition. The larvae were washed from the voltage-sensitive probe and transferred into an E3 medium containing only blebbistatin (8.5 μM) while always maintained in the dark. Next, the larvae were immobilized in dorsal or lateral position (Fig. 1 B and F) using a drop of low-melting point agarose (LMA, Sigma-Aldrich) on a small glass bottom petri dish. In addition, 2 mL of E3-blebbistatin solution (8.5 μM) were added to the petri dish to cover the zebrafish larvae embedded in the agarose drop. The cardiac or cerebral signals were recorded for 30 s to 2 min with an acquisition frequency of 100 to 200 frames/s using a 150 W halogen lighting system (SciMedia) as an excitation source, coupled with a 531/50 nm excitation filter, a 580 nm dichroic mirror, and a 580 long-pass emission filter. The SDF PLAN FLUOR 0.3X stereo microscope objective mounted as an eyepiece lens (Olympus), the Plan Apo 5.0 X/0.50 LWD mounted as magnification lens (Leica) and a MiCAM03 optical camera with a sensor of 17.6 × 17.6 mm (SciMedia), corresponding to 256 × 256 pixels, completed the optical mapping system. This setting allowed us to obtain a field of view of 1.06 × 1.06 mm, thus an optical magnification of 16.6 X and a pixel size of 4.1 × 4.1 μm. The MiCAM03 camera was connected to the BV Workbench software (ver 2.7.2, SciMedia) to allow the recording at 100 or 200 frames/s and the subsequent analysis of fluorescent signals (Fig. 1). Fluorescence changes indicative of cardiac depolarization and heart activity were recorded with the larvae positioned in lateral view. For those recordings, we analyzed the fluorescence detected by one pixel of the camera sensor, located in a position corresponding to the larvae heart's ventricular region. These recordings were consistent with cyclical changes of fluorescence representative of the larvae heart rate traces. For brain depolarization, the dorsal position provided better acquisition (Fig. 1). The fluorescent signal was recorded as the average of the whole brain area or the average of each of the four regions in which we subdivided the brain. The temperature inside the petri dish was recorded by a sensor in the warming chamber (QE-1, Warner Instruments), connected with a dual automatic temperature controller (TC-344B, Warner Instruments).

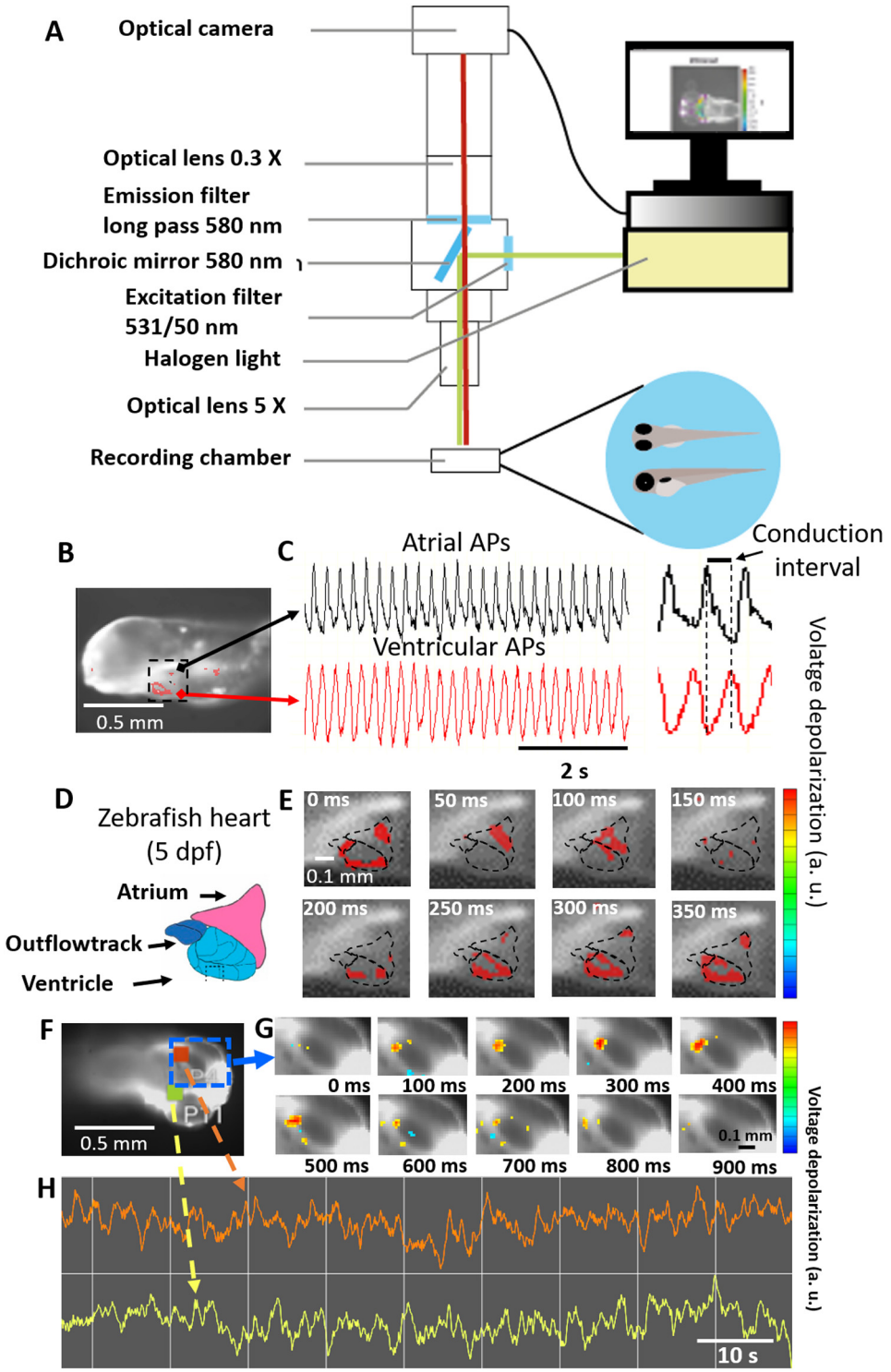

Fig. 1. Optical mapping (OM) set-up to record heart and brain voltage activities in living zebrafish larvae. A: Optical set-up for zebrafish larvae *in vivo*. B: Zebrafish larvae in lateral position exposing the heart charged with a voltage-sensitive dye (red indicates depolarization). C: Action potentials (APs) recorded in the atrial and ventricular regions of the zebrafish heart. D: Cartoon representing the zebrafish heart at 5 dpf (Brown et al., 2016). E: Example of time series of the voltage depolarization signal (red) across heart regions. The image at time 0 corresponds to the beginning of atrial depolarization and the end of ventricular depolarization. F: Zebrafish larvae in the dorsal position exposing the brain. G: Time series in the brain regions. H: Example of voltage signal recorded in two zebrafish brain regions at 120 hpf. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

2.4. Data analysis

After video recording of fluorescence changes indicative of heart and brain depolarization, raw data were processed with a BV Workbench mean filter and drift removal of the baseline to reduce background noise. On these spatiotemporal maps, cardiac and brain areas of interest were selected. The fluorescent signals were extracted for analyses (ClampFit ver. 10.0.7, Molecular Devices, LLC). Effects of temperature changes or contaminants were studied at the cardiac level by analyzing heart rate and the coefficient of variability of the interval between two heartbeats, recorded as optical action potential in the cardiac region. Under similar conditions, we ran a Fourier transform (FT, Clampfit 8.2) of the signal recorded in the brain (whole or regional) to examine the pattern of low-amplitude voltage activities. To avoid possible artifacts associated with the cardiac signal, we removed the frequencies between 2–3 Hz and 4–5 Hz from the FT analysis of brain signals. We specifically assessed the FT frequency range between 0.2–2 Hz, 3–4 Hz, and 5–15 Hz (Newson and Thiagarajan, 2019), therefore not exceeding 15 Hz to allow robust calculations (Nyquist sampling law, Colarusso et al., 1999) within the available sampling rate (100 frames/s for the brain). The recordings were visually inspected for high-amplitude spike activities and to exclude artifacts due to uncontrolled muscle contractions and an inadequate positioning of the zebrafish in the agarose gel. Y-axes unit in brain activity plots derives from the mathematical FT elaboration, which indicates the relative abundance of low-amplitude waves in the targeted frequency ranges and for the executed recording time. This solution is widely used to analyze the frequency range of activities emerging from excitable cells (Vogel, 2010).

2.5. Statistics

Data were analyzed using GraphPad Prism 8.0. T-test or One-way ANOVA followed by Bonferroni *post hoc* test were used according to the required comparisons between data groups. If data did not fulfill the parametric test criteria, we used a Kruskal–Wallis test followed by Dunn's *post hoc* test. Asterisks indicate statistical differences compared to the control group (Contr): * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$), **** ($p < 0.0001$). Data are expressed as means ± standard error of the mean (SEM). Data considered outliers by GraphPad Prism 8.0 were removed from the graphics and the statistical analysis.

3. Results

3.1. Optical mapping settings and effect of temperature

To perform OM of zebrafish larvae (set-up in Fig. 1), we used a voltage-sensitive probe (Di-4 ANEPPS) that intercalates in the cell membranes and emits a red fluorescence in response to variations of membrane potential. Initially, we tested which larval orientation provided the most robust recording of the voltage changes in cardiac or brain tissues. Our data indicate that the optimal orientation for cardiac recording is when the larvae are positioned on their left side (Fig. 1), which allowed us to track the signal from its generation in the atrial region to its conduction in the ventricular region, according to the profile drawn by the fluorescent signal and the anatomy of the zebrafish heart (Fig. 1 and Suppl. movie. 1). Dorsal orientation of the larvae allowed for the adequate recording of voltage changes at the cerebral surface (Fig. 1 and Suppl. movie 2), in coherence with a previous report analyzing intracellular calcium signals in the same region (Baraban, 2021).

Next, we tested the ability of OM to record voltage signals under varying temperature conditions (20 °C and 25 °C). These settings are consistent with the natural zebrafish habitats (Lin et al., 2014). Changing the temperature adjusted heart and brain voltage activity patterns, showing that OM is appropriately sensitive. We found a temperature-dependent increase in heart rate (Fig. 2A–B) and an increase in low-frequency voltage activity in the whole brain (0.2–2, 3–4, and 5–15 Hz; Fig. 2C–D), as calculated by FT. These results agree with previous protocols (Arel et al., 2022; Lin et al., 2014). From these tests, we selected 20 °C as the experimental temperature, allowing a rapid agarose solidification to position the zebrafish larvae reproducibly across experiments.

3.2. Effect of glyphosate and pesticide cocktail exposure on heart and whole brain voltage activity

We applied OM to examine the impact of selected contaminants on cardiac and brain voltage activities in zebrafish larvae. First, we exposed the larvae (2 to 120 hpf) to two broad-range concentrations of the herbicide glyphosate (0.1 μg/L and 1 mg/L) based on existing environmental guidelines (see Materials and Methods) and our previous results (Forner-Piquer et al., 2021b,a). As mentioned above, 0.1 μg/L represents the maximum levels accepted for an individual pesticide in environmental waters, while 1 mg/L is a high concentration that we associated with locomotor modifications without anatomical malformations (Forner-Piquer et al., 2021a). We found that glyphosate did not affect heart rate, except for a trend increase ($p = 0.2$) in the variability of the inter-beat interval (Fig. 3A–B). In the whole brain surface, glyphosate exposure modified low-amplitude voltage activities, decreasing the percentage of events abudance in the 0.2–2, 3–4, and 5–15 Hz ranges (Fig. 3C–D).

Next, we exposed the larvae (2 to 120 hpf) to a specific pesticide cocktail (boscalid, chlorpyrifos, captan, thiacloprid, thiophanate, and ziram) with relevance to the environmental exposome (Reilly et al., 2012; Sánchez-Bayo and Hyne,

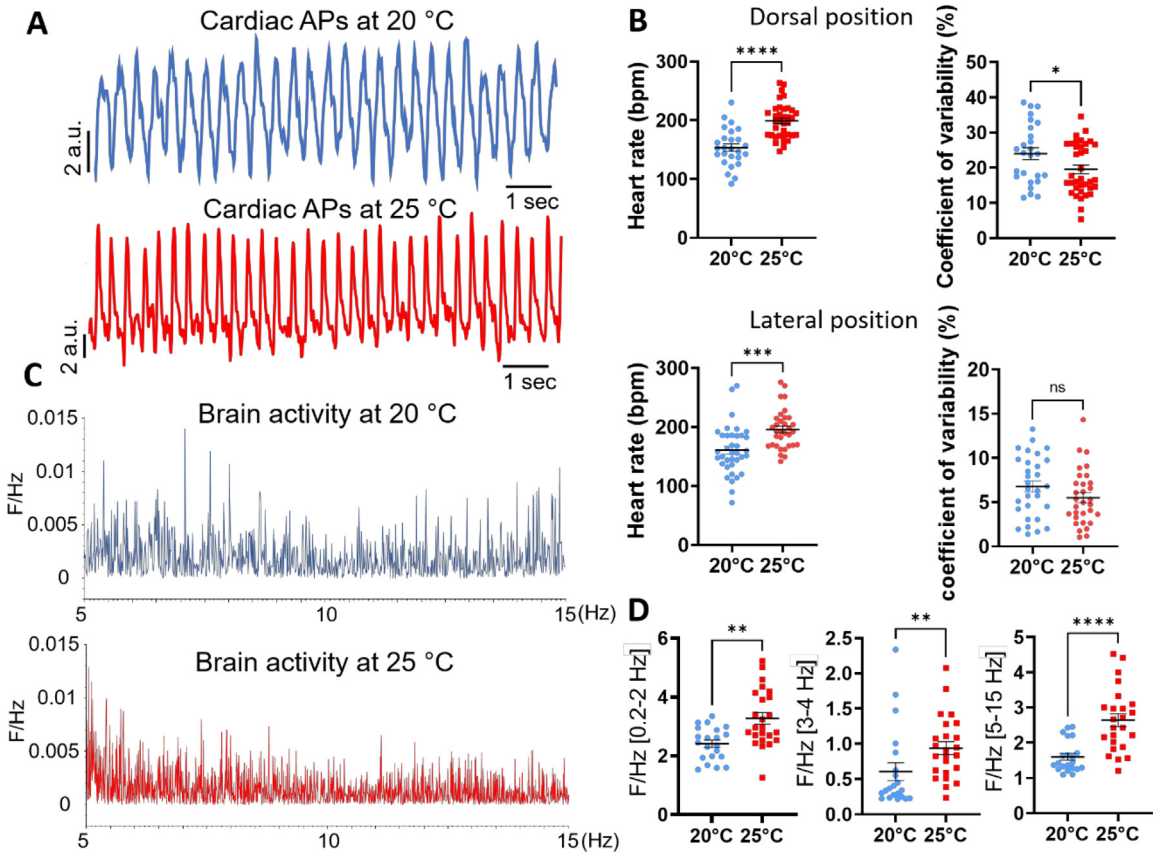

Fig. 2. Capturing the influence of the temperature on heart and brain voltage patterns. A: Examples of AP traces recorded in the heart of zebrafish larvae (120 hpf) on the lateral position at 20 (blue) and 25 (red) °C. B: Quantification of cardiac APs rate and coefficient of variability (interval between two APs) in zebrafish larvae on the dorsal (n = 26 at 20 °C and n = 36 at 25 °C) and lateral (n = 30 at 20 °C, n = 27 at 25 °C) positions at the same temperature and with the same color code reported in A. C: Example of Fourier Transform (FT) of brain voltage activity recorded in zebrafish larvae on the dorsal position (conditions and colors as in A) and for the 5–15 Hz interval. D: Quantifications of brain voltage activities for the intervals 0.2–2, 3–4, and 5–15 Hz (n = 21 at 20 °C and n = 24 at 25 °C, respectively), using the same color code in A. T-test for heart activity and Mann–Whitney test for brain activity; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

2014). The concentrations examined, reported in the methods, are based on current environmental limits in water and are associated with motor modifications in zebrafish larvae, as we previously showed (Forner-Piquer et al., 2021b). This pesticide cocktail did not modify heart rate, inter-beat variability, or whole brain voltage activities compared to control vehicle conditions (Fig. 4C–D).

3.3. Region-specific impact of glyphosate and pesticide cocktail in the brain

Lastly, we examined whether glyphosate or the pesticide cocktail could preferentially impact specific brain regions in zebrafish larvae. From previous studies (Baraban, 2021), we segmented the brain into four regions of interest (ROI): the right diencephalon (RD), left diencephalon (LD), right optic tectum (ROT), and left optic tectum (LOT). We then analyzed the respective voltage activities separately (Fig. 5A–B). In control, the four ROI display a comparable abundance of low-frequency voltage signals analyzed by FT (0.2–15 Hz; Fig. 5C). We then performed individual ROI analyses after glyphosate or pesticide cocktail exposure. As for the whole brain, glyphosate decreased the activity of the four regions analyzed (Tables 1 and 2). Notably, the pesticide cocktail (0.1 μg/L) specifically reduced RD and ROT activity in the 5 to 15 Hz range. This regional analysis unveils voltage activity adaptations that could not be captured when assessing the zebrafish brain as a whole.

4. Discussion

Our research has potential ecotoxicology and human health values within the exposome framework. Contaminants, such as pesticides, present in water bodies can impact wildlife and reach the human body through varying exposure

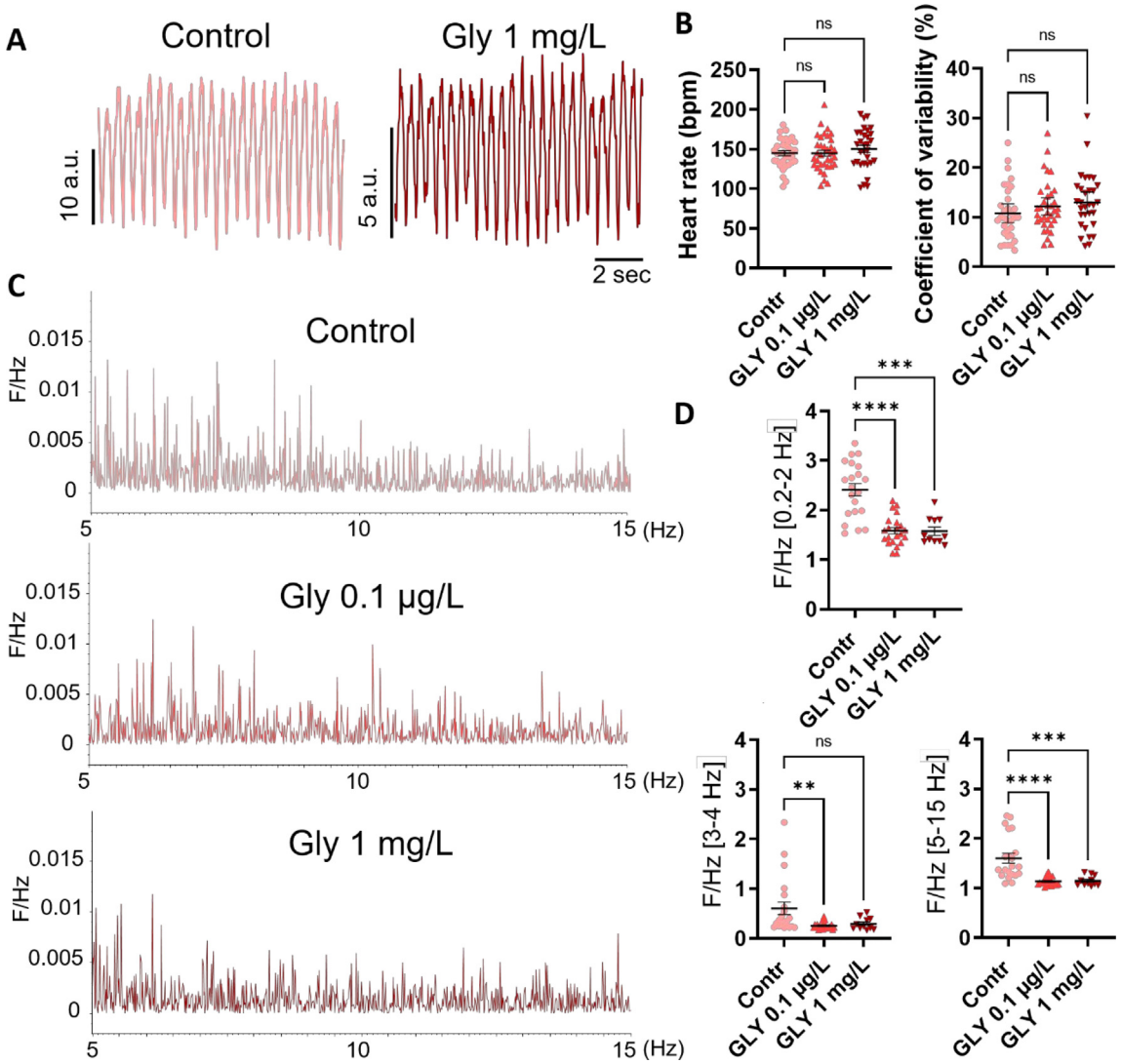

Fig. 3. Effects of glyphosate on heart and brain voltage patterns. A: Examples of AP traces recorded in the heart of zebrafish larvae in control (clear red) or after exposure to 1 mg/L of glyphosate (Gly; dark red). B: Quantification of cardiac APs rate and coefficient of variability (control n = 33 in clear red, Gly 0.1 µg/L n = 35 in red, Gly 1 mg/L n = 31 in dark red). C: Example of FT of the brain voltage activity for the interval 5–15 Hz, in control (clear red) and Gly 0.1 µg/L (red), and 1 mg/L (dark red). D: Quantifications of brain voltage activities for the intervals 0.2–2, 3–4, and 5–15 Hz (control n = 21, Gly 0.1 µg/L n = 22, Gly 1 mg/L n = 11), using the same color code in C. One-way ANOVA for heart activity, Kruskal and Wallis for brain activity; ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

modalities, such as ingestion or contact. In humans, continuous exposure to low-level environmental contaminants such as glyphosate or specific pesticides, like those studied herein, represents an emerging factor for long-term risks and adverse neurological and cardiac trajectories (Cresto et al., 2023). Therefore, it is essential to capture the effects of contaminants at the cellular level in relevant models.

We have applied high-speed OM and a voltage-sensitive dye to monitor voltage changes consistent with heart and brain depolarization in living zebrafish larvae with high temporal and spatial precision. During optimization, we found that augmenting temperature increased the heartbeat frequency and the percentage of low-frequency, low-amplitude voltage events in the whole brain. When testing environmentally relevant contaminants, we captured specific adaptations in cardiac and brain voltage activities elicited by glyphosate and a particular pesticide cocktail. Collectively, our research supports the continuous optimization of OM to screen the impact of contaminants on spatiotemporal voltage dynamics in excitable tissues or whole organs. Importantly, taking advantage of the transparency of zebrafish larvae, OM allows non-invasive monitoring of activity across the whole brain or heart regions and provides a modality to create time series of voltage changes and depolarization maps in these organs.

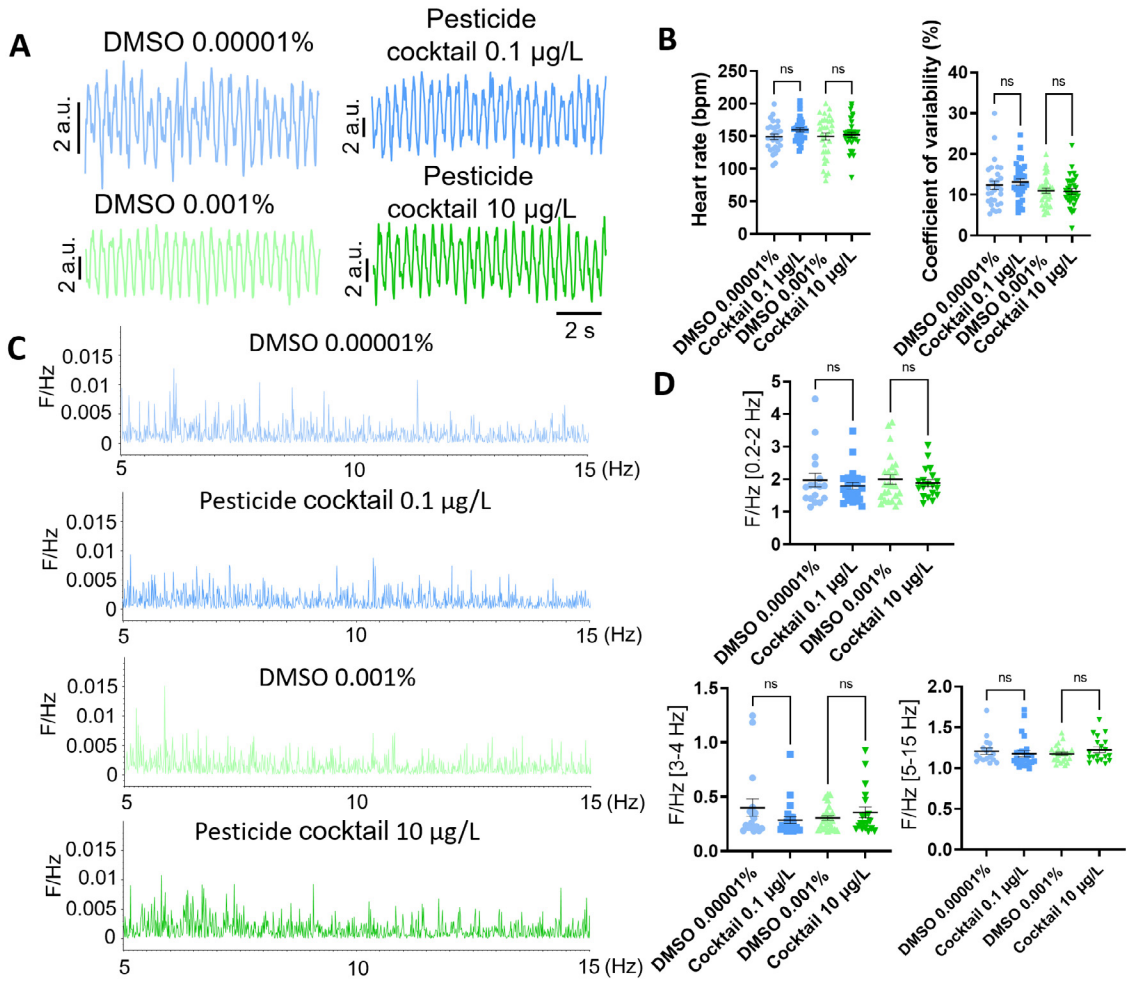

Fig. 4. Effects of pesticide cocktail on heart and brain voltage patterns. A: Examples of AP traces recorded in the heart of zebrafish larvae in DMSO 0.00001% or 0.001% (clear blue and clear green, respectively) or after exposure to the pesticides cocktail at a concentration of 0.1 µg/L or 10 µg/L (dark blue and dark green, respectively). B: Heart rate and coefficient of variability of the interbeat interval under the conditions and with the same colors in A. (DMSO 0.00001% n = 29, pesticide cocktail 0.1 µg/L n = 32, DMSO 0.001% n = 34, pesticides cocktail 10 µg/L n = 32). C: Example of FT of the brain voltage activity for the interval 5–15 Hz with the same color in A. D: Quantifications of the brain voltage activities for the intervals 0.2–2, 3–4, and 5–15 Hz (DMSO 0.00001% n = 17, pesticide cocktail 0.1 µg/L n = 25, DMSO 0.001% n = 25, pesticides cocktail 10 µg/L n = 19), with the same color in A. One-way ANOVA for heart activity, Kruskal and Wallis for brain activity; ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

4.1. From glyphosate to pesticides cocktail: heart–brain voltage activity adaptations

The chemical characteristics of glyphosate allow it to permeate the chorion and enter into the zebrafish embryo during a critical period when the nervous system is under development. Using OM and voltage-sensitive dyes, we report no changes in the heart rate of zebrafish embryos exposed to 1 mg/L of glyphosate. However, a trend increase in heart rate variability generates a possible frail condition. A recent systematic review analyzed the varying cardiac effects of a broad-range exposure to glyphosate (0.001 to 100 mg/L) in zebrafish embryos (48 and 72 hpf) (Ames et al., 2022). Among the studies examined, Zhang et al. (2021) showed an increase in heart rate under 10 ng/mL of glyphosate. By contrast, Liu et al. (2022), Lanzarin et al. (2019), and Gaur and Bhargava (2019) found decreased heart rate in concentrations ranging from 5 to 100 mg/L, while Pompermaier et al. (2022) reported no difference with 4.8 µg/L of glyphosate. These studies were performed by placing the larvae under a microscope and manually counting the heartbeats, which could induce errors. To avoid those errors, Gaur et al. used a ZebraPace (Zebrafish Precise Algorithm for Cardiac-rhythm Estimation) based on measuring pixel intensity from a video (Gaur et al., 2018). Here, OM provides a reliable method to measure heart rate and its variability, making it possible also to discern between action potentials generated in the atrial and ventricular regions.

Our previous studies reported no gross cardiac malformations in glyphosate groups at 120 hpf, although heart development was not assessed in detail (Forner-Piquer et al., 2021a). Furthermore, embryos exposed for 96 h to glyphosate

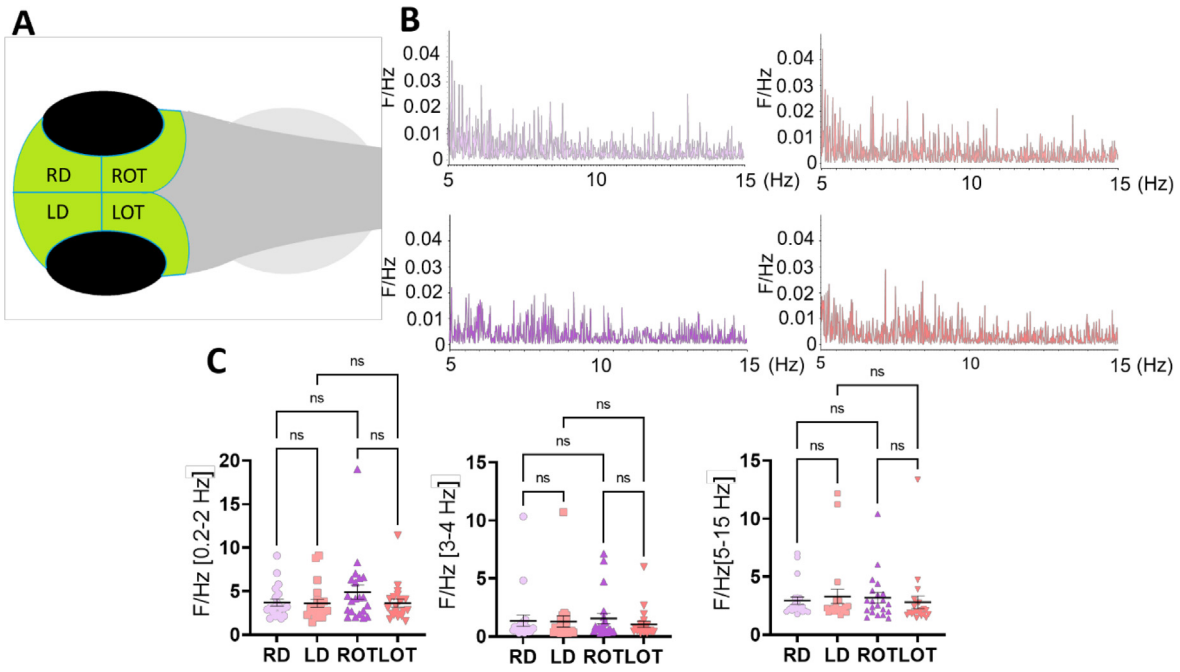

Fig. 5. Brain voltage patterns in four brain regions of interest. A: Cartoon showing the four regions of interest (ROIs) that we selected in the brain, the right diencephalon (RD), left diencephalon (LD), right optic tectum (ROT), and left optic tectum (LOT). B: Example of FT of brain voltage activity for 5–15 Hz interval: RD (clear purple), LD (clear red), ROT (dark purple), LOT (dark red). C: Quantifications of the brain voltage activities for the intervals 0.2–2, 3–4, and 5–15 Hz in control zebrafish larvae not treated with any contaminant (n = 21). Kruskal–Wallis test; *** $p < 0.001$, **** $p < 0.0001$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

Table 1
Effect of glyphosate on brain regions of interest. Analysis of the activity of four brain ROI, under control (n = 21) and after exposure to Glyphosate (Gly 0.1 µg/L, n = 22 and 1 mg/L, n = 11). Kruskal–Wallis test; Asterisks indicate statistical difference *versus* the control group; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

		0.2 – 2 Hz	*p*	3 – 4 Hz	*p*	5 – 15 Hz	*p*
RD	Control	3.688 ± 0.398		1.359 ± 0.495		2.941 ± 0.328	
	GLY 0.1 µg/L	1.946 ± 0.120	****	0.375 ± 0.029	****	1.898 ± 0.074	****
	GLY 1 mg/L	1.995 ± 0.215	***	0.839 ± 0.404	*	2.039 ± 0.233	**
LD	Control	3.605 ± 0.452		1.296 ± 0.487		3.304 ± 0.625	
	GLY 0.1 µg/L	1.947 ± 0.108	***	0.496 ± 0.070	*	1.908 ± 0.073	***
	GLY 1 mg/L	1.887 ± 0.185	***	0.680 ± 0.273	ns	1.909 ± 0.158	***
ROT	Control	4.891 ± 0.821		1.553 ± 0.439		3.199 ± 0.442	
	GLY 0.1 µg/L	1.934 ± 0.160	***	0.398 ± 0.051	****	1.695 ± 0.095	****
	GLY 1 mg/L	2.389 ± 0.488	**	0.591 ± 0.152	ns	1.610 ± 0.065	**
LOT	Control	3.631 ± 0.461		1.054 ± 0.279		2.795 ± 0.557	
	GLY 0.1 µg/L	2.041 ± 0.156	***	0.380 ± 0.035	**	1.642 ± 0.071	**
	GLY 1 mg/L	1.743 ± 0.264	***	0.626 ± 0.300	**	1.757 ± 0.255	**

Table 2
Effects of the pesticide cocktail on brain regions of interest. Data refer to control (DMSO 0.001 and 0.00001%; n = 25 and n = 17) and pesticide exposure (0.1 and 1 μg/L, n = 25 and n = 19). Kruskal–Wallis test; Asterisks indicate statistical difference versus the control groups; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

		0.2 – 2 Hz	*p*	3 – 4 Hz	*p*	5 – 15 Hz	*p*
RD	DMSO 0.00001 %	2.071 ± 0.133		0.720 ± 0.273		1.994 ± 0.091	
	Cocktail 0.1 μg/L	1.990 ± 0.122	ns	0.330 ± 0.015	ns	1.770 ± 0.034	*
	DMSO 0.001 %	2.622 ±0.198		0.426 ± 0.055		2.061 ± 0.067	
	Cocktail 10 μg/L	2.464 ± 0.208	ns	0.536 ± 0.085	ns	2.117 ± 0.079	ns
LD	DMS 0.00001 %	1.970 ± 0.117		0.657 ± 0.204		1.979 ± 0.076	
	Cocktail 0.1 μg/L	1.998 ± 0.120	ns	0.346 ± 0.023	ns	1.792 ± 0.045	ns
	DMSO 0.001 %	2.693 ± 0.215		0.493 ± 0.078		1.979 ± 0.066	
	Cocktail 10 μg/L	2.722 ± 0.445	ns	0.902 ± 0.287	ns	2.329 ± 0.193	ns
ROT	DMS 0.00001 %	4.032 ± 1.346		1.042 ± 0.274		2.027 ± 0.266	
	Cocktail 0.1 μg/L	2.322 ± 0.240	ns	0.508 ± 0.139	ns	1.756 ± 0.203	*
	DMSO 0.001 %	2.731 ± 0.497		0.554 ± 0.088		1.742 ± 0.096	
	Cocktail 10 μg/L	3.220 ± 0.674	ns	0.599 ± 0.119	ns	1.902 ± 0.185	ns
LOT	DMS 0.00001 %	3.720 ± 1.730		0.502 ± 0.098		1.642 ± 0.047	
	Cocktail 0.1 μg/L	2.566 ± 0.448	ns	0.496 ± 0.085	ns	1.645 ± 0.077	ns
	DMSO 0.001 %	2.570 ± 0.241		0.598 ± 0.095		1.903 ± 0.123	
	Cocktail 10 μg/L	3.996 ± 0.861	ns	0.650 ± 0.151	ns	2.006 ± 0.214	ns

(10, 100, 1000 μg/L) showed a temporary decrease in heart rate, indicating that the effect on heart rate could be transient and not result in persistent cardiovascular toxicity (Terrazas-Salgado et al., 2022). Together, these results illustrate glyphosate's time and concentration-dependent effects on cardiac read-outs in zebrafish larvae.

In the brain, OM captured voltage dynamics in the low-amplitude signals simultaneously and coordinated in all brain regions, which was previously challenging when using single-electrode and invasive field potential recording limited to the zebrafish larvae forebrain. The tissue damage induced by electrode-based recording could represent a confounding factor when applied to zebrafish. The OM spatial and temporal improvement enables the study of low-amplitude voltage signals corresponding to specific spectral frequencies and without tissue damage. Furthermore, the fragmentation of the whole brain into four areas unveiled regional voltage changes with a potential application to neurological trajectories in this model. In our experimental condition, the lowest concentration of glyphosate (0.1 μg/L) was the saturating level for developmental brain effects, while 1 mg/L did not elicit additional adaptations. Concerning the impact of the pesticide cocktail, 0.1 μg/L specifically reduced the voltage activity of the RD and ROT regions. The precise developmental and translational values of these regional changes remain to be elucidated. However, this result suggests complex interactions of these pesticides within specific brain areas. It highlights how low levels of pesticides could sufficiently promote brain adaptations in this ecotoxicology-relevant model. Moreover, the different sensitivity of distinct brain areas to pesticides highlights the interest in developing voltage dyes encoded by a specific population of neurons. Such an approach will allow more precise tracking of the voltage activity, helping to characterize the traits of varying neuron populations in the brain of zebrafish larvae, perhaps associated with specific transcriptomic adaptions.

5. Limitations and conclusions

We advanced the use of a non-invasive imaging tool to study the effect of selected environmental pollutants on cardiac and brain voltage activities. Nevertheless, we are aware that the 100 to 200 frames/s sampling rate likely impedes the detection of fast high-amplitude neuronal spike activity occurring in the single-digit ms range. Therefore, we cannot rule

out the presence of these pathological signatures, especially in pesticide-exposed settings. Using traditional electrode-based field potential recordings with a 1 KHz sampling rate, we previously identified high-amplitude spikes in zebrafish exposed to 1 mg/L glyphosate, although with high variability among zebrafish (Forner-Piquer et al., 2021a). Increasing the sampling rate capability can be achieved contingent on the progression of this research, allowing sufficient data to capture these events in the future.

We are also aware that, although we worked in the temperature range consistent with the natural zebrafish habitats (Lin et al., 2014), the heartbeat frequency slowing recorded at 20 °C could mask eventual alterations of cardiac activity related to contaminant exposure. Future development should focus on genetically encoded fluorophores to improve and stabilize signal intensity and to capture the activity of a specific population of excitable cells in the brain and heart of zebrafish larvae (Turrini et al., 2017).

In summary, we used the zebrafish larvae and OM to pursue a practical and non-invasive analysis of the adaptations related to the diffusion of environmental compounds. We identified distinct heart–brain changes in response to specific chemical exposome protocols. We endorsed investigating the impact of contaminants commonly present in ecological matrices, extending to ecotoxicology and one-health frameworks.

CRediT authorship contribution statement

Solène Micou: Experimental excution, Data analysis. **Isabel Forner-Piquer:** Conceptualization, Writing – original draft, Experimental execution, Data analysis. **Noémie Cresto:** Support to experiment execution. **Tess Zassot:** Experimental excution, Data analysis. **Aurélien Drouard:** Support to experiment execution. **Marianna Larbi:** Experimental excution, Data analysis. **Matteo E. Mangoni:** Writing – original draft, Read, Commented, Approved the manuscript. **Etienne Audinat:** Writing – original draft, Read, Commented, Approved the manuscript. **Chris Jopling:** Writing – original draft, Read, Commented, Approved the manuscript. **Adèle Faucherre:** Conceptualization, Funding acquisition, Supervision, Writing – original draft, Read, Commented, Approved the manuscript. **Nicola Marchi:** Conceptualization, Funding acquisition, Supervision, Writing – original draft, Read, Commented, Approved the manuscript. **Angelo G. Torrente:** Conceptualization, Funding acquisition, Supervision, Writing – original draft, Participate in the execution of the experiments and in the data analysis, Read, Commented, Approved the manuscript.

Declaration of competing interest

Authors disclose any financial or non-financial interests that are directly or indirectly related to this work.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary material related to this article can be found online at https://doi.org/10.1016/j.eti.2023.103196.

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