Moxidectin toxicity to zebrafish embryos: bioaccumulation and biomarker responses

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List of abbreviations:

MLs – Macrocyclic lactones; Hpf – Hours post-fertilization; Dpf – Days post-fertilization; DMSO – Dimethyl sulfoxide; OECD – Organisation for Economic Co-operation and Development; FET – Fish Embryo Acute Toxicity; AChE – Acetylcholinesterase; GST – Glutathione S-transferase; CAT – Catalase; LDH – Lactate dehydrogenase; MALDI-MSi – Matrix-assisted laser desorption/ionization - Mass Spectrometry imaging; HCCA – α-cyano-4-hydroxycinnamic acid; UPLC-QToF-MS -

Ultraperformance liquid chromatography quadrupole-time-of-flight mass spectrometry; BCF – Bioconcentration factor; GluCls - Glutamate-gated chloride channels; $GABA_A$ receptors. - γ-aminobutyric acid type A–gated chloride channels; BBB – Blood-brain barrier.

Abstract

Moxidectin is an antiparasitic drug belonging to the class of the macrocyclic lactones, subgroup mylbemicins. It is used worldwide in veterinary practice, but little is known about its potential environmental risks. Thus, we used the zebrafish embryo as a model system to study the potential effects of moxidectin on aquatic non-target organisms. The analyses were performed in two experimental sets: (1) acute toxicity and apical endpoints were characterized, with biomarker assays providing information on the activity levels of catalase (CAT), glutathione S-transferase (GST), lactate dehydrogenase (LDH), and acetylcholinesterase (AChE); and (2) internal concentration and spatial distribution of moxidectin were determined using ultraperformance liquid chromatography quadrupole-time-of-flight mass spectrometry (UPLC-QToF-MS) and matrix-assisted laser desorption/ionization-MS imaging (MALDI-MSi). The acute toxicity to zebrafish embryos (96 hpf) appeared mainly as a decrease in hatching rates (EC_{50} = 20.75 µg/L). It also altered the enzymatic activity of biomarker enzymes related to xenobiotic processing, anaerobic metabolism, and oxidative stress (GST, LDH, and CAT, respectively) and strongly accumulated in the embryos, as internal concentrations were 4 orders of magnitude higher than those detected in exposure solutions. MALDI-MSi revealed accumulations of the drug mainly in the head and eyes of the embryos (72 and 96 hpf). Thus, our results show that exposure to moxidectin decreases hatching success by 96 h and alters biochemical parameters in the early life stages of zebrafish while accumulating in the head and eye regions of the animals, demonstrating the need to prioritize this compound for environmental studies.

Keywords: aquatic toxicology, veterinary antiparasitics, emerging pollutants, MALDI-MS imaging, macrocyclic lactones.

Capsule:

54 Exposure to moxidectin prevents hatching at 96 h, alters enzymatic activity, and
55 accumulates in the heads and eyes of embryos.

1. Introduction

Veterinary antiparasitics are emerging pollutants. They are routinely used in farms around the globe to treat and prevent disease in several species, especially those in grazing systems (Kools et al. 2008; Kim et al. 2008; Di Nica et al. 2015). These chemicals obviously play an important role in animal welfare and food security but are likely to pose a higher risk to aquatic life than antibiotics (Kools et al. 2008; Carlsson et al. 2013; Kołodziejska et al. 2013; Di Nica et al. 2015; Bundschuh et al. 2016). The preventive nature of its use may drive a continuous presence of these compounds in the ecosystems surrounding agricultural land, warranting a detailed understanding of their environmental risks. In cattle farming, they can reach adjacent aquatic ecosystems through two routes: (1) being deposited in the soil during the application of drugs via topical sprinkler or bath; (2) being excreted, along with its metabolites, in feces and urine, which are then used as fertilizer and gain leaching potential (Boxall et al. 2003). The latter route is especially important when animals are raised extensively along the banks of rivers or in flooded areas (Mesa et al. 2017, 2018, 2020; Yopasá-Arenas and Fostier 2018).

Moxidectin is a veterinary antiparasitic belonging to the chemical group of macrocyclic lactones (MLs) and represents the most important compound in the subgroup of the milbemycins (Prichard et al. 2012). MLs are believed to be the most widely used antiparasitic agents worldwide in veterinary practice, being authorized in more than 60 countries and having their popularity attributed to the fact that they offer broad-spectrum protection and low toxicity to the host (Lumaret et al. 2012). The most representative compound in this group is ivermectin, whose environmental effects are relatively well characterized. Along with other MLs, such as doramectin and abamectin, ivermectin is considered to be of high environmental risk due to its high toxicity to non-target organisms and high frequency of use (Kools et al. 2008; Lumaret et al. 2012).

Milbemycins possess structural variations that may impact their mode of action, suggesting that their effects may differ from those caused by other MLs and that it may not be possible to extrapolate results from other MLs to milbemycins (Prichard et al. 2012). In the United States, moxidectin was approved with mitigation requirements, as it showed varying degrees of toxicity to non-target organisms, being more toxic to fish than to invertebrates (Fort Dodge Animal Health 1997). In addition to presenting a high risk of occurrence in aquatic environments, the drug was classified by the European Union as persistent in the environment and bioaccumulative in fish (Kools et al. 2008; European Medicines Agency 2017; Health Products Regulatory Authority 2019). Despite mounting evidence pointing to the need for prioritization of moxidectin, research on the environmental effects of this frequently used milbemycin is very limited (Prichard et al. 2012; Hentz et al. 2019).

Considering the gaps in hazard characterization of moxidectin, particularly when it comes to its effect in aquatic organisms, we aimed to contribute to the understanding of the environmental impact of this drug by investigating its toxic effects on the early life stages of zebrafish. To this end, we investigated the acute toxicity of moxidectin through apical endpoints and enzymatic biomarkers, as well as its internal concentration and spatial distribution within the embryos. To the best of our knowledge, this is the first report on the effects of moxidectin on zebrafish embryos.

2. Materials and Methods

2.1. Chemicals

Moxidectin (CAS No. 113507-06-5) was supplied by AK Scientific (Union City, California, USA) and Sigma-Aldrich (Germany). The matrix α-cyano-4-hydroxycinnamic acid (HCCA) was purchased from Sigma-Aldrich (Germany). Moxidectin was dissolved in dimethylsulfoxide (DMSO, Sigma-Aldrich, Germany) to

prepare stock solutions that were stored at -18 ºC. All chemicals and solvents were of analytical grade.

2.2. Animals

The experiments for morphological and biochemical analyses were approved by the Ethics Committee on the Use of Animals at UFPB, with authorization documented by protocol No. 8881290419. Adult wild-type animals were kept in mixed groups (with males and females) in tanks with a water recirculation system, at a temperature of 26 ± 1ºC and a photoperiod of 14:10 h light/dark. After spawning, the embryos (approximately 1 hpf) were washed, cultured in adapted embryonic medium E3 (5.0 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl, and 0.33 mM MgSO4) and analyzed under an inverted light microscope (Televal 31, Zeiss, Germany) for selection based on developmental stage and viability. Selected embryos were distributed in 96-well microtiter plates, where they were incubated with moxidectin, DMSO, or E3 medium for exposure assays.

For exposure experiments for internal concentration and spatial distribution, embryos were obtained from adults of the OBI-UFZ lineage (*Danio rerio*, generation F16). The adults were originally obtained through a local breeder, then kept for several generations at the UFZ facilities (Fetter et al. 2015). Animals were kept and used according to the animal protection standards of Germany and the European Union, with procedures approved by the Government of Saxony, Landesdirektion Leipzig, Germany (Aktenzeichen 75-9185.64).

2.3. Acute toxicity and biomarker analysis

2.3.1. Range-finding and EC_{50} exposure assays

Prior to exposures, solutions of moxidectin in E3 medium (DMSO = 0.01%) were prepared to a maximum concentration of 20 mg/L, which was the highest concentration at which the compound was efficiently diluted. To determine the range of toxicity, successive exposures were conducted with dilutions spaced by a factor of 2, with nominal concentrations starting at 20 mg/L and ending at 10 µg/L. Once the range of toxicity was determined (when concentrations with an effect below 100% were found), new exposures were performed to determine the EC_{50}, in the same conditions as described above and in three independent biological repetitions. These exposures were performed with the concentrations of 80, 40, 20, and 10 µg/L, plus controls in E3 medium only and solvent controls in DMSO 0.01%.

Exposure assays were performed according to OECD 236 (OECD 2013), with modifications. The embryos (≈3 hpf, n = 20/group) were distributed individually in 96-well plates where they were exposed to successive dilutions of moxidectin stocks in E3 medium with DMSO (0.01%), plus control groups only in E3 medium (n = 20) and solvent control groups in 0.01% DMSO (n = 20). Every 24 h, embryos were observed under an inverted light microscope (Televal 31, Zeiss, Germany) to verify the appearance of lethal and sub-lethal endpoints for a total period of 96 h. Additionally, the exposure media was renewed every 24 h in order to maintain moxidectin concentrations with a variation of less than 20% of the nominal concentrations. Previous experiments showed that moxidectin showed degradation of 17% after 24 h of incubation in our experimental conditions (Supplementary Information).

2.3.2. Spontaneous movements and heart rate

Spontaneous movements or coils are unprovoked motor responses observable in embryos between 17 and 27 hpf (Saint-Amant and Drapeau 1998), and changes in the frequency of these movements are thought to be early behavioral manifestations of

158 neurotoxicity (Weichert et al. 2017; Ogungbemi et al. 2019, 2020; Tao et al. 2020), while
159 heart rates may provide insight into cardiovascular alterations at concentrations below
160 those that cause morphological changes (Fraysse et al. 2006; Sun and Liu 2017; Cheng et
161 al. 2020). To measure these variables, three concentrations below the EC_{50} at which no
162 effect was observed in the exposed groups (1.25, 2.5, and 5 µg/L) were chosen to indicate
163 whether these could be more sensitive toxicity indicators than the observed endpoint.
164 Groups of 20 embryos were exposed to these concentrations in 96-well microtiter plates
165 in the same conditions as described in item 2.3.1. Under a light microscope, the
166 spontaneous movements of 10 random embryos were observed individually for 1 min at
167 24 hpf (± 1 h) (Ogungbemi et al. 2020; Tao et al. 2020). At 48 hpf (± 1 h), the heartbeats
168 were observed for another 1 min (Fraysse et al. 2006; Sun and Liu 2017). Both variables
169 were counted manually in less than 60 minutes to avoid age differences between embryos
170 by the end of the observations. These analyses were performed in four independent
171 biological replicates.
172
173 *2.3.4. Enzymatic activity assays*
174 As a general depiction of the toxicity induced by moxidectin, we measured the
175 enzymatic activity of classic biomarkers related to: (i) antioxidant response – catalase
176 (CAT) and glutathione S-transferase (GST); (ii) neurotransmission – acetylcholinesterase
177 (AChE); and (iii) metabolism – lactate dehydrogenase (LDH). Groups of 20 embryos
178 were exposed to three concentrations below the $EC_{50,}$ at which no effect was observed
179 (1.25, 2.5, and 5 µg/L). After 96 h, pools of 20 organisms were transferred to microtubes
180 containing 1 mL of sodium phosphate buffer solution (0.1 M, pH 7.4) and quickly frozen,
181 then stored at -4ºC. These analyses were performed in four independent biological
182 replicates.

The homogenization of samples for all enzymatic activity assays was carried out following the procedures described by Rivero-Wendt et al. (2016). The stored samples were thawed in an ice bath, homogenized in phosphate buffer (0.1 M, pH 7.4), and centrifuged for 20 min at 11,500 *g* to obtain the post-mitochondrial supernatant (PMS).

To measure AChE, acetylcholine was used as a substrate. 50 µL of PMS were incubated with 250 µL of reaction solution (0.075 M acetylcholine and 0.01 M 5.5'-acid dithiobis- [2-nitrobenzoic] [DTNB]) for 5 min, at 25 ºC. The formation of thiocholine, a degradation product of acetylcholine, was determined with a spectrophotometer (Multiskan G0, Thermo Fischer, USA) at 414 nm, according to the method of Ellman et al. (1961).

The quantification of GST activity was used as an indicator of detoxification capacity since GST catalyzes the conjugation of the reduced thiol group of glutathione to electrophilic xenobiotics. The test was carried out according to the methodology previously described by Habig and Jakoby (1981), using 100 µL of PMS from the larvae homogenate and 200 µL of the reaction solution (10 mM reduced glutathione and 60 mM 1-chloro-2,4-dinitrobenzene, CDNB). The absorbance was measured at 340 nm every 20 s for 5 min.

To assess CAT activity, 15 µL of PMS were added to 135 µL of a reaction solution containing hydrogen peroxide (30 mM) and 150 µL of phosphate buffer (0.05 mM). The absorbance of the mixture at 240 nm was monitored every 10 s, for 2 min, according to the methodology of (Claiborne 1986).

Finally, LDH was measured by adding 40 µL of PMS to a reaction solution containing 250 µl of NADH (0.24 mM) and 40 µl of pyruvate (10 mM) in Tris–NaCl buffer (0.1 M, pH 7.2). The oxidation of NADH results in a continuous decrease in

absorbance, which was measured for 5 min at 340 nm (Diamantino et al. 2001). All enzymatic assays were performed with four technical replicates.

2.4. Chemical analysis and spatial distribution

2.4.1. Exposure assays

Moxidectin was first dissolved in DMSO and then diluted to 640 µg/L (1 µM with <0.01% DMSO) in ISO water (ISO 2007) in order to reach internal concentrations within the embryos that were sufficient for the ultraperformance liquid chromatography quadrupole-time-of-flight mass spectrometry (UPLC-QToF-MS) analysis. This concentration was necessary to ensure that the internal concentrations would surpass the limit of detection, according to previous experiments (data not published). Groups of nine embryos (≥ 2 hpf) were exposed in 18 mL in glass vials for 24, 48, 72, and 96 h. Incubation occurred in static conditions at 26 °C, 14:10 light/dark cycle with 75 rpm horizontal agitation (Edmund Buehler, Germany). Four replicates were set up for each time point, as well as negative control groups (n = 9/group), which were exposed only to ISO water and maintained under the same conditions as the treatment groups. These were used as negative controls (for moxidectin quantification and MALDI-MS imaging), as well as quality controls for recovery and matrix effect measurements. At this concentration, the delayed hatching was also observed in all embryos of the OBI-UFZ lineage.

After exposure, embryos were dechorionated, washed twice with Milli-Q water (Milli-Q® Advantage, Merck Chemicals GmbH, Germany), and transferred to 2 mL

FastPrep tubes (MP Biomedicals, Solon, USA) containing 0.75 mm glass beads. Excess water was removed. The tubes were snap-frozen in liquid nitrogen and stored at -20 °C until extraction and analysis. Samples of the exposure solution (300 µL) were also taken at the beginning and the end of exposure times, then stored at -20 °C.

For matrix-assisted laser desorption/ionization-MS imaging (MALDI-MSi) analysis, embryos exposed for 72 h and 96 h were washed twice and deposited in cryomolds containing Neg-50™ embedding medium (Thermo Scientific, Germany), frozen on dry ice, wrapped in aluminum foil, and stored at -80 °C.

2.4.2. Sample preparation and chemical measurements by UPLC-QToF-MS measurements

For the measurements of internal concentrations, the embryos in FastPrep tubes were extracted with 500 µL acetonitrile, homogenized in a FastPrep homogenizer (MP Biomedicals, 30 s, 6.5 m/s), placed in an ultrasonic bath for 15 min, and centrifuged for 15 min at 13000 rpm. 175 µL of the supernatant was then transferred to 1.5 mL glass vials filled with 175 µL Milli-Q water and stored at -20°C until analysis.

Both the aliquots taken from the exposure solutions and the embryo extract were analyzed by UPLC-QToF-MS with detailed instrumentation and procedures as described by Halbach et al. (2020). The sampe preparation was also applied to identify the potential occurrence of transformation products with UPLC-QToF-MS (Brox et al. 2016) by comparing exposed and negative control embryo extracts. Moxidectin was serially diluted in a solution of water and acetonitrile (50:50) for calibration ranging from 0.78 to 100

ng/mL. Moxidectin was detected as sodium adduct (m/z 662.367) at a retention time of 13.02 min. The limit of detection was 1 ng/mL and the limit of quantification 3 ng/mL. However, the dilution factor chosen for T0 aliquotes (DF = 10) did not allow for the detection of moxidectin in those samples; therefore, it is assumed that the concentration is similar to the one measured after 24, 48, 72, and 96 h of exposure which were measured undiluted.

For the calculation of matrix effects, nine pooled embryos (≈ 100 hpf) from controls were extracted with 500 µL acetonitrile and spiked with 10 µL of 2.5 µg/L moxidectin solution just before the measurements. For recovery experiments, nine pooled embryos (≈ 100 hpf) from controls were extracted with 500 µL acetonitrile spiked with 50 ng/mL of moxidectin, in triplicates. Samples were diluted 1:1 (v/v) with Milli-Q water and measured with UPLC-QToF-MS using the same procedures and parameters described above. The matrix effects were 49.3 ± 2.4%. The recovery was 40.8 ± 0.8%, and the internal concentrations in embryos were corrected for the results of the recovery experiments.

2.4.3. Slide Preparation for MALDI-MSi

Embryos in embedding medium were transferred into a -17 °C cryotome chamber (Cryo-Star HM 560, Microm International, Walldorf, Germany) 24 h prior to sectioning. Sections (12 µm thick) were cut and subsequently deposited onto ITO-coated glass slides (Bruker, Bremen, Germany). The slides were then dried in a desiccator (300 mbar) for 30 min before teaching marks were drawn around the sections in the form of white marker crosses. Positive controls were produced by depositing one drop (0.5 µL) of a 10 ppm

moxidectin solution in ethanol on negative control sections. After drying, slides were weighted to account for the amount of matrix to be deposited on them, as well as scanned for imaging (OpticLab H850, Plustek, Ahrensfelde, Germany). Slides were then stored in the desiccator until spraying, which was carried out with an automatic sprayer (Image Prep, Bruker Daltonik, Bremen, Germany). Standard settings were used for α-cyano-4-hydroxycinnamic acid (HCCA) application. The slides with embryo sections were sprayed with 7 µg/L HCCA in methanol/water/trifluoroacetic acid 49.9/49.9/0.2. MALDI-MSi measurements were carried out within 24 h after spraying.

2.4.5. MALDI-MSi

The slides were loaded into a Fourier-transform ion cyclotron resonance mass spectrometer (FT-ICR-MS; Solarix XR 12T, Bruker Daltonics, Germany) coupled to a MALDI ion source equipped with a 1 kHz Laser of 355 nm (Smartbeam II, Bruker Daltonics, Germany). The software ftmsControl (Bruker Daltonics, Germany) was used for setting optimal parameters and for measurements, while measurement areas were set with the FlexImaging software (Bruker Daltonics, Germany). The following MALDI settings were applied: 14% laser power, 100 laser shots, 2000 Hz frequency, small laser focus, 50 µM raster width. Moxidectin was measured as potassium adduct (calculated *m/z* 678.340) in positive mode, and an isolation window of *m/z* 662 (± 30) was applied. MALDI-MSi data and scanned images were imported into SCiLS Lab 2020a (SCiLS GmbH, Bremen, Germany) for data analysis. Total ion count normalization, weak denoising, and hotspot removal were applied.

296

297 *2.5. Data and statistical analysis*

298 The effect concentration (EC_{50}) was obtained with Probit analysis (Finney 1952).
299 The results for biomarkers, heartbeat, and spontaneous movements were analyzed using
300 the software GraphPad Prism 8 and are represented as mean ± standard deviation. The
301 data were tested for normality and homogeneity of variances using the Shapiro-Wilk and
302 Brown-Forsythe tests. Data fulfilling these requirements were analyzed by one-way
303 ANOVA and a *post-hoc* Dunnet's test ($p < 0.05$), whereas non-compliant datasets were
304 evaluated with a Kruskal–Wallis analysis and a *post-hoc* Dunn's test.

305 The data retrieved from the UPLC-QToF-MS were analyzed using OriginPro
306 2020. Internal concentrations were calculated per volume (µM) and per dry weight (pg/µg
307 DW) of individual embryos at different developmental stages as previously described by
308 Halbach et al. (2020). The bioconcentration factor (BCF, unitless) was calculated with
309 the internal concentrations per volume divided by the concentration measured in the
310 exposure solution (Massei et al. 2015; Kristofco et al. 2018; Vogs et al. 2019). The
311 variations in internal concentrations, as well as concentrations in exposure solutions, are
312 expressed as mean ± standard deviation.

313

314 **3. Results**

315 *3.1. Acute toxicity*

316 At the highest nominal concentration tested (20 mg/L), 100% of the embryos
317 failed to hatch, but no mortality was observed (Table 1). Successive exposure assays
318 revealed a delay in hatching that appeared in a concentration-dependent manner (Figure
319 1), yielding an EC_{50} of 20.75 µg/L.

320

321 *3.2. Enzymatic activity, spontaneous movements, and heartbeats per minute*

322 At concentrations under the EC_{50} (1.25, 2.5, and 5 µg/L) that did not induce lethal
323 or sub-lethal endpoints, changes in the activity levels of LDH, GST, and CAT were
324 observed while AChE remained unchanged (Figure 2, A-D). Both CAT and LDH
325 increased at 1.25 and 2.5 µg/L, but GST increased only at 2.5 µg/L. No changes in enzyme
326 activities were observed at 5 µg/L. Moreover, DMSO did not affect enzyme activity, as
327 no solvent controls differed from the negative controls in E3 medium only.

328 At these concentrations, the number of spontaneous movements at 24 hpf and the
329 heart rate at 48 hpf were measured, but no differences between treated groups and the
330 control were observed (Figure 2, E-F).

331

332 *3.3. Chemical analysis in embryos and exposure solutions*

333 The measured external concentration was strongly below the nominal
334 concentration (1 µM) . This may be explained with the known rapid photolysis of
335 moxidectin and possible sorption to equipment due to the high lipophilicity (log P = 6;
336 Prichard et al. 2012).

337 On the other hand, concentrations in whole embryos (Figure 3) were stable in the
338 samples measured (24 – 96 h), averaging 94.91 µM (± 74.33). At this exposure
339 concentration, no embryos hatched by the end of the assay, and the concentration in the
340 embryos was much higher than the measured concentration in the exposure solution (four

orders of magnitude). These values yielded a very high BCF at the end of the exposure time based on the measured external concentration ($1.11x10^4$, 96 h). Considering the nominal concentration, a lower BCF (151.95, 96 h) is obtained, which still indicates a strong enrichment of the embryos. No transformation products were found in the external solution or the embryo extracts.

The strong deviation between the concentration in the exposure medium and the embryo points towards a stronger stability of moxidectin in the embryos. Nevertheless, measurements of several time points within the first 24h might be necessary to better elucidate the dynamics of moxidectin in the water, and how it compares to the uptake by the embryo.

3.4. Spatial distribution

MALDI-MSi measurements detected moxidectin as a sodium adduct (m/z 678.340) in two 72 hpf embryos and two 96 hpf embryos. The distribution within these embryos was similar, with a signal concentrated in the head and eye regions compared to the rest of the embryo (Figure 4). No peaks were detected in negative control sections, which were sampled from embryos exposed only to ISO water. Meanwhile, the peak found in exposed embryos was also found in positive control sections, which were sampled from negative control embryos, but the sections were spiked with a fresh solution of moxidectin (10 ppm).

4. Discussion

Despite the absence of lethal endpoints as defined by the OECD (OECD 2013), moxidectin caused a marked decrease in hatching rates at 96 h. The decline in hatching rates is an outcome of particular environmental relevance, as it can translate into decreased hatching viability and altered population dynamics (Parra et al. 2005; Navis et al. 2013). The EC_{50} value found in this tudy (20.75 µg/L; 0.03 µM) indicates that moxidectin in highly capable of decreasing the hatching rates of zebrafish embryos as late as 96 h, compared to other hatching-inhibiting macrocyclic lactones. In studies of the effects of ivermectin on different zebrafish life stages, Oliveira et al. (2016) found an EC_{50} value of 482.51 µg/L for inhibition of hatching by 96 h, while Sanches et al. (2018) documented unhatched embryos at 96 h, but found an EC_{50} value <500 µg/L. On the other hand, we did not record mortality up to 20 mg/L, while a previous regulatory study on moxidectin found much lower LC_{50} values for bluegill and rainbow trout exposed to moxidectin: 0.62 µg/L and < 0.15 µg/L, respectively (Fort Dodge Animal Health, 1997).

However, it can be difficult to determine how close our EC_{50} value is to environmentally relevant concentrations because studies quantifying moxidectin in environmental samples are scarce. Zrnčić et al. (2014) detected moxidectin in a sample from a Spanish river at a concentration of 1.84 ng/L, with samples collected mostly near pig farms. However, the level of moxidectin contamination in a given location depends on the predominance and composition of herds, as well as the volume of use relative to other antiparasitics (Kools et al. 2008; Di Nica et al. 2015). Additionally, moxidectin strongly binds to organic matter, indicating that this chemical is more likely to enter the food web and be taken up by aquatic organisms (Kools et al. 2008; Mesa et al. 2018), highlighting the relevance of measuring moxidectin in samples from sediment and from aquatic organisms to clarify its environmental effects.

Another aspect of the environmental relevance of moxidectin is its high potential to enrich in zebrafish embryos. Our data resulted in a high BCF value ($1.11x10^4$, 96 h), which may still be underestimated, as a more dynamic exposure regimen avoids this drastic decrease in moxidectin concentration (Supplmentary Materials). Similar to ivermectin, moxidectin is a highly hydrophobic compound (log P = 6; Prichard et al. 2012) that binds to organic matter, allowing for high intake by living organisms through direct contact with the chemical or by consumption of contaminated organic matter (Mesa et al. 2018, 2020). In fact, moxidectin is persistent in dung and soil while being degraded in water by photolysis (with a half-life of 6.8 h) (Fort Dodge Animal Health 1997; Hentz et al. 2019). Our results showed that, despite its comparatively low concentration in the medium, moxidectin is quickly taken up by the embryo in high amounts. As the concentration in the embryo did not drastically change over the 96 hours of exposure, organic constituents in the embryo might also increase the stability of moxidectin in the embryo compared to the exposure solution. This also points out the need to consider the toxicity of compounds rapidly degraded in water bodies to aquatic organisms. This result may be particularly relevant in chronic or tandem exposure situations, as this rapid uptake facilitates the persistence of the compound within the organism.

When embryos were exposed to concentrations of moxidectin that were too low to prevent hatching (1.25, 2.5, and 5 µg/L), increased activity levels of LDH, GST, and CAT were observed, but not AChE. However, this trend was not reflected by the spontaneous movements at 24 hpf or the heart rate at 48 hpf, indicating that these parameters were not sensitive measures of moxidectin toxicity in concentrations below those that lead to observable endpoints. This may happen because heart rate and spontaneous movements are complex tertiary-level stress responses, which are influenced by many biochemical and tissue-level components (Schreck and Tort 2016).

412 Although the changes in GST activity observed in this study were discreet, alterations
413 related to this enzyme are expected because it participates directly in xenobiotic
414 metabolism. GST facilitates the excretion of toxicants by conjugating them with reduced
415 glutathione (Domingues et al. 2016), and it has been implicated in ML detoxification in
416 adult fish. After orally feeding Atlantic salmon (*Salmo salar* L.) for seven days with
417 medicated feed containing 50 µg emamectin benzoate per kg of fish, Olsvik et al. (2008)
418 registered decreased GST gene expression shortly after the treatment (day 7) and
419 increased expression on day 35. Additionally, Oliveira et al. (2016) described a decrease
420 in GST activity in the gills and livers of adults exposed to ivermectin for 96 h, but the
421 enzyme remained unchanged in embryos. In a longer-term study with zebrafish exposed
422 to ivermectin, (Domingues et al. 2016) found that GST activity decreases after 21 days
423 of exposure.

424 In regards to LDH, it is widely distributed in the organism and it is involved in
425 anaerobic metabolism, so its activity can be differentially impacted in several body parts
426 of fish, indicating losses in tissue integrity or low oxygen availability (Arya et al. 2010;
427 da Silva Santos et al. 2018; Dar et al. 2020), as well as stress responses in fish that include
428 thermal and chemical stress (Dalvi et al. 2017; Khare et al. 2019; Dar et al. 2020). At the
429 lowest concentrations tested (1.25, 2.5, and 5 µg/L), moxidectin did not produce a
430 significant difference in heartbeats per minute or spontaneous movements, but LDH
431 activity was higher in groups exposed to 1.25 and 2.5 µg/L, indicating a cytotoxic fallout
432 via upregulation of anaerobic metabolism (Tonomura et al. 2009; Arya et al. 2010). To
433 further elucidate these questions, histopathological examinations may provide a
434 qualitative and spatial understanding of moxidectin damage on tissues, and larval motility
435 studies could be more sensitive to moxidectin toxicity at lower concentrations.

436 Meanwhile, exposure to low concentrations of moxidectin (1.25, 2.5, and 5 µg/L) did
437 not affect AChE activity. This may happen because the mode of action of MLs is
438 associated with GABAergic signaling though interatcions with γ-aminobutyric acid type
439 A–gated chloride channels ($GABA_A$ receptors), in addition to glutamate-gated chloride
440 channels (GluCls) in invertebrates (Sieghart, 2010; Lumaret, 2012; Prichard, 2012). In
441 mammals, GABA receptors are predominantly located in the brain, and their interaction
442 with both ivermectin and moxidectin are responsible for toxicity to the host (Ménez et al.
443 2012). Despite that, MLs have also been shown to affect AChE activity: while
444 eprinomectin inhibits brain AChE in jundiá fish (*Rhamdia quelen*), ivermectin inhibits it
445 in zebrafish embryos but not in adults (Oliveira et al. 2016; Serafini et al. 2019). However,
446 our results show that AChE is not involved in the toxicity of moxidectin to early life
447 stages of zebrafish.

448 The MALDI-MS analysis detected moxidectin in the heads of 74 and 96 hpf embryos,
449 which may be due to an underdeveloped blood-brain barrier at this stage (BBB) (Fleming
450 et al. 2013). As with vertebrates in general, GABA receptors also concentrate in the brains
451 of zebrafish embryos, which express GABA patterns in the brain within the first 32 h
452 (Martin et al. 1998; Higashijima et al. 2004; Mueller et al. 2006). However, MLs do not
453 easily reach these receptors in mammals because they do not readily cross the BBB,
454 mainly due to P-glycoprotein activity, which is an efflux transporter encoded by multi-
455 drug resistance genes (*mdr*) that prevents the accumulation of MLs in the brain (Kircik et
456 al. 2016; Geyer et al. 2009). Likewise, this enzyme is a key factor for limiting moxidectin
457 in the brain (Kiki-Mvouaka et al. 2010), and sensitivity to intoxication by MLs in
458 mammals is attributed to deficient P-glycoprotein activity in the BBB (Dowling 2006;
459 Geyer et al. 2009). The function and structure of the BBB in zebrafish are conserved,
460 developing between 3 and 10 days post fertilization (dpf) (Fleming et al. 2013; Kim et al.

2017; O'Brown et al. 2018). However, in an investigation of BBB development in zebrafish embryos, Fleming et al. (2013) identified P-glycoprotein orthologs that show efflux transporter activity from 8 days post fertilization. Furthermore, the authors investigated the spatial distribution of five drugs known to either cross or not cross the BBB in mammals, all of which were equally distributed in the heads and trunks of embryos at 5 dpf (120 hpf), suggesting that a functional BBB was not fully present at this stage.

Meanwhile, the deposition of moxidectin in the eyes is likely related to the neuronal components of sight, since zebrafish eyes are structurally conserved but concentrate half of the neurons in early larvae (7-8 dpf), accounting for nearly a quarter of their total body volume (Zimmermann et al. 2018). Accodingly, Monesson-Olson et al. (2018) demonstrated through in-situ hybridization that GABA orthologs are present and show distinct patterns of expression in the retinas of 96 hpf embryos.

5. Conclusions

Our results show that the effects of moxidectin on zebrafish embryos manifest as a decline in hatching rates as late as 96 h, as well as alterations in enzymatic activity of GST, LDH, and CAT. These changes in enzyme activity appeared at concentrations below 5 µg/L (when no more macroscopic effects were observed), but neither the spontaneous tail movements nor the heart rate were sensitive enough to reflect them, indicating that behavioral assays may be necessary to provide a reliable picture of moxidectin toxicity in lower concentrations.

Moreover, we analyzed internal concentration and distribution of moxidectin in zebrafish embryos. We demonstrated that the distribution of chemicals within the embryo

485 is important to consider when investigating internal concentration, and that the spatial
486 distribution can support the understanding of toxicity to non-target organisms.
487 Moxidectin is highly enriched in the embryos and was detected in the head and eye
488 regions, which are rich in nervous tissue and contain GABA receptors. The results thus
489 corroborate the need to prioritize moxidectin as pointed out by previous studies to further
490 elucidate the risks posed by this drug to non-target organisms and to subsidize mitigation
491 strategies accordingly. To this end, a better understanding of the ecotoxicological profile
492 and environmental fate of moxidectin is urgently needed.

493

494 **6. References**

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692

693 **Figure captions**

694

695 **Figure 1.** Zebrafish embryos at 96 hpf. Negative controls (A) hatched between 48 and 72
696 hpf, while moxidectin-exposed embryos failed to hatch by 96 hpf (B, T 96 h, 10 µg/L).
697 The effect was documented as percentage (%) of unhatched embryos at 96 h (C). Results
698 are expressed as mean ± standard deviation, with asterisks (*) indicating significant
699 difference ($p < 0.05$) between treatment groups and the control.

700

701 **Figure 2.** Enzymatic activity of acetylcholinesterase (AChE, A), lactate dehydrogenase
702 (LDH, B), glutathione S-transferase (GST, C) and catalase (CAT, D), of zebrafish
703 embryos exposed to moxidectin for 96 h. Additionally, spontaneous movements (24 hpf
704 ± 1 h) and heart rate (24 hpf ± 1 h, F) were measured. Results are expressed as mean ±
705 standard deviation, with asterisks (*) indicating significant difference ($p < 0.05$) between
706 treatment groups and the control.

707

708 **Figure 3.** Moxidectin concentrations in embryonic bodies expressed per volume (a; µM)
709 and dry weight (b; pg/µg DW) (n = 3, detected with UPLC-QToF-MS as a Na-adduct,
710 *m/z* 662.367). Values are expressed as mean ± standard deviation.

711

712 **Figure 4.** Spatial distribution of moxidectin in zebrafish embryo determined with
713 MALDI-MSi. Color-scale pixels represent the peak intensity of the K-adduct of
714 moxidectin (m/z 678.340). MALDI-MSi results are depicted (left) and original scanned

715 images before the measurement (right). Arrows indicate the eyes. NC = negative control,
716 embryos exposed to ISO water only. PC = positive control, sections of embryos exposed
717 to ISO water spiked with 0.05 µL of moxidectin solution (10 ppm).