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Exposure to low-level metalaxyl impacts the cardiac development and function of zebrafish embryos

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ABSTRACT

Metalaxyl is an anilide pesticide that is widely used to control plant diseases caused by Peronosporales species. In order to study the toxic effects, zebrafish embryos were exposed to metalaxyl at nominal concentrations of 5, 50 and 500 ng/L for 72 hr, and the cardiac development and functioning of larvae were observed. The results showed that metalaxyl exposure resulted in increased rates of pericardial edema, heart hemorrhage and cardiac malformation. The distance between the sinus venosus and bulbus arteriosus, stroke volume, cardiac output and heart rate were significantly increased in larvae exposed to 50 and 500 ng/L metalaxyl compared to solvent control larvae. Significant upregulation in the transcription of *tbx5*, *gata4* and *myh6* was observed in the 50 and 500 ng/L treatments, and that of *nkx2.5* and *myl7* was observed in the 5, 50 and 500 ng/L groups. These disturbances may be related to cardiac developmental and functional defects in the larvae. The activity of Na^+/K^+-ATPase and Ca^{2+}-ATPase was significantly increased in zebrafish embryos exposed to 500 ng/L metalaxyl, and the mRNA levels of genes related to ATPase (*atp2a11*, *atp1b2b*, and *atp1a3b*) (in the 50 and 500 ng/L groups) and calcium channels (*cacna1ab*) (in the 500 ng/L group) were significantly downregulated; these changes might be associated with heart arrhythmia and functional failure.

Introduction

Metalaxyl is an acylanilide fungicide used worldwide to control plant diseases caused by Peronosporales species in crops, vegetables and fruits (Tomlin, 2006; Malhat, 2017). Due to its low absorption in soil and high solubility in water (7100 mg/L), metalaxyl easily flows into bodies of water via leaching caused by rainfall (Meite et al., 2018). It was reported that metalaxyl levels ranged from 0.007 to 0.67 μg/L in water samples from 29 streams in the United States (Battaglin et al., 2011). In Rwanda, metalaxyl was measured in the surface water collected from Muhazi Lake, Mugesera Lake and Sebeya River at concentrations of 0.06–4.8 μg/L (Houbraken et al., 2017). In surface waters from southern Ontario during base flow conditions, the overall mean concentration of metalaxyl was 18 ng/L over the course of one study (2007–2010), and the highest concentration reached 1330 ng/L (Struger et al., 2016). Metalaxyl was detected at 0.001–0.191 μg/L in water samples from 24 urban wetlands in

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Melbourne, Australia (Allinson et al., 2015), at 383–807 ng/L in ditch water in Sinaloa (Mexico) (Moeder et al., 2017), and at 0.25–0.29 μg/L in farm ditches flowing to the Lower Fraser River tributary fish streams of British Columbia, Canada (Wan et al., 2006). The concentrations of metalaxyl ranged from ND to 128 ng/L in water samples from the Jiulong River and estuary in southern China (Zheng et al., 2016).

Metalaxyl is often detected in fruits. This chemical was found in 97% of mangosteen samples from Thailand at concentrations ranging from 0.003 to 0.239 μg/L, which exceeds its maximum residue limit (0.05 μg/L); 50% remained in the fruit after washing (Phopin et al., 2017). After application at the recommended dosage (262.5 g a.i./ha), the mean initial concentration of metalaxyl detected in/on tomato was 2.39 mg/kg fresh weight (Malhat, 2017). Metalaxyl residues in herbs with dual medicinal and food purposes collected from China were 37–267 μg/kg dry weight (Du et al., 2012).

Widespread contamination by metalaxyl probably poses a threat to both wildlife and human health. A few studies have been conducted to evaluate the toxicity of metalaxyl to animals. The 96-hr LC_{50} of metalaxyl is >100 mg/L for *Cyprinus carpio* (USEPA, 1995). The 24-hr LC_{50} of its enantiomers, rac-metalaxyl and R-metalaxyl, are 258.47 and 237.67 mg/L for zebrafish (*Danio rerio*) embryos, respectively (Yao et al., 2009). In amphibians, larval *Rana pipiens* treated with metalaxyl exhibited 35% mortality before metamorphosis (Hayes et al., 2006). Treatment with metalaxyl caused nephrotoxicity in albino mice (Sakr et al., 2011). Exposure of human lymphocytes to metalaxyl in vitro resulted in micronucleus formation and sister-chromatid exchange (Demsia et al., 2007). However, there have not yet been any studies on the toxic effects of metalaxyl on cardiac development and function.

Zebrafish have been well accepted as excellent model vertebrates for ecotoxicological investigation (He et al., 2014), since they have many advantages for developmental studies. Their advantages, such as high fecundity, small size and ease of culture, make it probable to synchronously obtain a large number of embryos and larvae for experiments. The transparency of the embryos and their rapid development provide a convenient way to comprehensively observe and assess their developmental status. In particular, their transparency is favorable for assessing heart morphology, rhythm and physical action (Bakkers, 2011; Liu and Stainier, 2012). Additionally, the zebrafish genome, including the genetic basis of its development, is well characterized, which is beneficial to the rapid assessment of the mechanisms of action leading to abnormal phenotypes (Fernández et al., 2018).

The expression of some specific genes affects cardiac morphogenesis and conduction system development. As master cardiac transcription factors, Nkx2.5, Tbx5 and Gata4 play a crucial role in cardiac development (Välimäki et al., 2017). In zebrafish, the expression of *nkx2.5* requires activation of the bone morphogenetic proteins such as Bmp2b (Reiter et al., 2001); both cardiac troponin T2 (Tnnt2) and myosin light polypeptide 7 (Myl7) play an essential role in heart muscle differentiation and functioning (Maves et al., 2009); cadherin2 (coded by *cdh2*), a cell-adhesion molecule, is necessary for cardiovascular development (Bagatto et al., 2006). Heart rhythm and contractility depend on the processes of cardiomyocyte excitation and excitation–contraction (EC) coupling. At the cellular level, the action potential and EC coupling are controlled by the electrochemical gradients of Na^+, K^+ and Ca^{2+} ions (Incardona, 2017). In cardiac myocytes, Na^+, K^+ and Ca^{2+} homeostasis across the plasma membrane is stringently controlled by their corresponding carriers, channels or pumps, such as Na^+/K^+-ATPase (encoded by *atp1*) (Richards et al., 2003), sarcoplasmic reticulum Ca^{2+}-ATPase (encoded by *atp2a*) and the Na^+, Ca^{2+}-exchanger (Ebert et al., 2005; Barth and Tomaselli, 2009). Therefore, the transcript of these genes and ATPase activity were investigated in this study.

The objective of this study was to investigate the toxic effects of metalaxyl on cardiac development in zebrafish embryos at environmentally relevant concentrations, and to examine the mechanisms involved.

1. Materials and methods

1.1. Zebrafish husbandry and embryo collection

Adult zebrafish (wild-type, strain TU) were housed in an aquaculture system with a stable photoperiod of 14 hr:10 hr light:dark, a water temperature of (28 ± 1)°C, a pH of 7.2–7.3, and a dissolved oxygen concentration of 7–8 mg/L. The fish were fed live brine shrimp twice daily. All fish experiments were conducted in accordance with the ethical guidelines of Xiamen University. Sexually mature fish without any signs of disease were selected as breeders.

Adult fish were mated at a ratio of 1:2 (male/female). The spawned eggs were collected within 1 hr. Fertilized eggs were washed with zebrafish culture liquid and placed in petri dishes for the exposure experiments.

1.2. Embryonic exposure and sampling

Metalaxyl (>98% purity) was purchased from the Agro-Environmental Protection Institute, Ministry of Agriculture, China. It was dissolved in analytical-grade hexane to obtain stock concentrations of 1, 10 and 100 μg/mL. The metalaxyl exposure solutions were obtained by adding the appropriate volume of the stock concentration to zebrafish culture medium (3.5 g/L NaCl, 0.05 g/L $NaHCO_3$, 0.05 g/L KCl, 0.05 g/L $CaCl_2$) (AR, Taicang Hushi Reagent Co., LTD, Shanghai, China). Embryos within 1 hr post fertilization (hpf) were collected, randomly distributed into multiple petri dishes, and exposed to metalaxyl at concentrations of 0, 5, 50 and 500 ng/L. One hundred embryos were cultured in 30 mL of exposure solution, and there were five replicates for each treatment. Similar criteria were applied to the solvent control group, which received an equal volume of hexane (5 μL/L) (AR, Taicang Hushi Reagent Co., LTD, Shanghai, China). The exposure solutions were changed twice daily. The development of the embryos was observed every 12 hr using an Olympus SZ51 stereomicroscope (Nikon, Tokyo, Japan). The larvae exposed to metalaxyl for 72 hr were collected for analysis.

1.3. Cardiac malformation and function assessment

The larvae exposed to metalaxyl for 72 hr were randomly selected and immobilized in 3% methylcellulose (CP, Shantou

Xilong Chemical Co., LTD, Guangdong, China) to allow the capture of lateral-view images. The morphology and configuration of the larval heart were observed and photographed using a Nikon TE300 microscope (Nikon, Tokyo, Japan). Pericardial edema is identified as swelling due to an increased volume of fluid in the pericardium, which is a portion of the coelomic cavity separating the heart from the body wall (Westerfield, 2000). The SV-BA distance was defined as the length of a straight line connecting the sinus venosus (SV) and bulbus arteriosus (BA) (Appendix A Fig. S1); it was measured in pixels from digital images of the lateral view of whole-mount embryos as previously described (Antkiewicz et al., 2005). In addition, heart hemorrhage was observed, and the rates of occurrence were recorded.

The heart rate (HR) and quantitative assessment of cardiac arrhythmia were obtained from 20-sec video segments collected from the exposed larvae based on the method of Incardona et al. (2009). The end-diastolic volume (EDV), end-systolic volume (ESV), stroke volume (SV) and cardiac output (CO) of the larvae were measured and assessed following the method of Chen et al. (2008). SV was calculated using the equations SV = EDV − ESV, and CO was calculated as CO = SV × HR.

Measurements of cardiac arrhythmia were obtained by determining the interbeat variability (Incardona et al., 2009). The number of frames between cardiac contraction initiations was calculated using NIS-Elements Imaging Software (Nikon, Tokyo, Japan). The means and standard deviations (SDs) were analyzed for each larva. The SD is a measure of heart rate irregularity, since a regular rhythm would have essentially the same number of frames between beats and therefore a low SD.

The aforementioned indices were assessed in three larvae from each replicate to obtain a mean for this replicate. The values from five replicates in each treatment were used for statistical analysis.

1.4. Real-time quantitative PCR (qPCR)

Fifty larvae from each replicate were pooled into a subsample. RNA extraction and reverse transcription were performed following the methods described by Huang et al. (2012). Total RNA was extracted from the whole embryos using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. First-strand cDNA was synthesized from 1 μg of total RNA using a Revert Aid Mu-MLV cDNA synthesis kit (TransGen Biotech, Beijing, China) based on the manufacturer's protocol.

QPCR analysis was performed on an Mx3000P Real-Time PCR system (Stratagene, La Jolla, CA, USA) using a Brilliant SYBR Green QPCR reagent kit (TransGen Biotech, Beijing, China) following the manufacturer's protocol. Standard curves and primer efficiencies were determined for all the genes analyzed by qPCR.

The cycling parameters were 94°C for 10 min followed by 45 cycles of 94°C for 20 sec, 55°C for 20 sec and 72°C for 20 sec. The threshold cycles and dissociation curves were determined with Rotor-Gene 6000 software to confirm that only one PCR product was amplified and detected, and the gene expression levels were normalized to those of zebrafish *gapdh*.

The real-time quantitative PCR primers (Appendix A Table S1) were designed using Primer Premier 5.0 (Primer company, Canada). The Relative Expression Software Tool (REST-MCS-version 2) was employed to calculate the relative expression of the target gene mRNA (Pfaffl et al., 2002).

1.5. ATPase activity analysis

Approximately 40 larvae from each replicate were pooled and homogenized to obtain a supernatant, which was used as the source of enzyme. The ATPase activity was measured using an ultramicro Ca^{2+}-ATPase kit and Na^{+}/K^{+}-ATPase kit from Nanjing Jiancheng Bioengineering Institute (Nanjing, China) according to the manufacturer's instructions. Protein content was measured with a Coomassie blue protein assay kit (Nanjing Jiancheng Bioengineering Institute, China); ATPase activity was expressed as mol Pi liberated per mg protein per hour (mol Pi/(mg prot·hr)).

1.6. Determination of metalaxyl in exposure solutions

The exposure solutions, freshly made up with the stock solutions, were collected three times at random for metalaxyl determination. The metalaxyl concentrations were measured based on the method of Zhang et al. (2017), with slight modification. Briefly, 1 L of exposure solution was mixed with simeconazole (purity > 97%) (Witega Laboratories Berlin-Adlershof GmbH, Berlin, Germany) as a surrogate and extracted using a liquid–liquid extraction method with 50 mL of CH_2Cl_2 (purity ≥ 99.99%, Tedia, USA) in a separatory funnel. The organic phase was collected and dried with anhydrous sodium sulfate (AR, WuSi Chemical Reagent Co. LTD, Shanghai, China), and the extracts were concentrated to dryness by a rotary evaporator (1N-1001, Alon Instruments Co. LTD, Shanghai, China) and then diluted with an acetone/*n*-hexane (1:1, *V*/*V*) solution. The metalaxyl concentration was detected using a GC/MS/MS system (Agilent Technology, USA) following the description of Zhang et al. (2017).

The recovery of metalaxyl was 94% ± 2.5% (n = 3), and the limit of detection was 1.0 ng/L. The detected concentrations of metalaxyl in the exposure medium were 0, 4.62 ± 0.13, 47.41 ± 0.59 and 457.03 ± 15.77 ng/L in the nominal 0, 5, 50 and 500 ng/L groups, respectively.

1.7. Data processing

The results are reported as the means ± SE (standard error). The data were first checked for normality and homogeneity, and significant differences between the treatments were subsequently statistically analyzed with one-way analysis of variance (ANOVA) followed by the Duncan test via SPSS 16.0 software (SPSS Inc., Chicago, IL, USA). A value of $p < 0.05$ was taken to indicate a significant difference.

2. Results

2.1. Solvent effects

No significant changes were observed for any of the tested indices between the solvent treatment group and the blank control group; thus, the changes in the indices were compared to those of the solvent group.

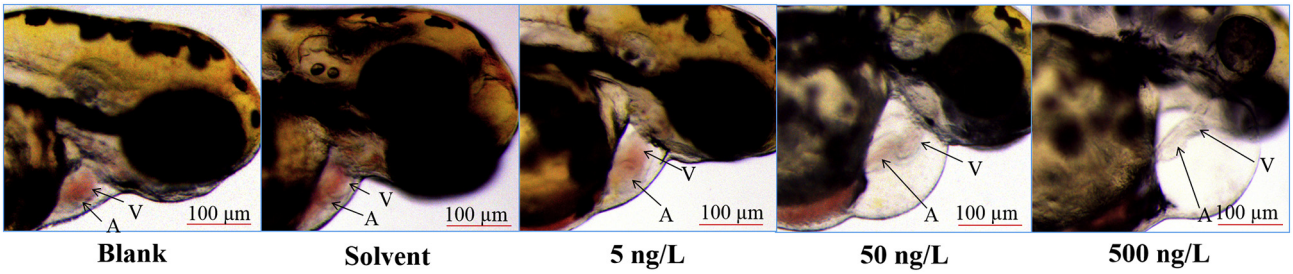

Fig. 1 – Effects of metalaxyl on larval heart morphology. Fertilized embryos were exposed to metalaxyl for 72 hr. Heart malformation was enhanced with increasing concentrations of metalaxyl. A: Atrium; V: Ventricle.

2.2. Effects of metalaxyl on cardiac development and function

Exposure to metalaxyl resulted in abnormal development of the heart in zebrafish embryos, including pericardial edema, heart hemorrhage and morphological malformation (Fig. 1). The pericardial edema rates were significantly increased (by 1.33- and 2.67-fold, respectively) in the 50 and 500 ng/L groups, and the heart hemorrhage rates were significantly increased (by 1.58-, 3.12-, 3.00-fold, respectively) in all the metalaxyl-treated groups (Table 1). In the control larvae, the ventricle and atrium overlapped each other in the lateral view, showing a normal looping shape. In the metalaxyl-treated larvae, the hearts were stretched, and the atria and ventricles were elongated and separated without overlapping (Fig. 1). The SV-BA distance increased with increasing metalaxyl doses and reached a significant difference (1.31- and 1.63-fold higher, respectively) in the 50 and 500 ng/L groups (Table 1).

Compared to the solvent control larvae, metalaxyl-treated larvae showed no significant changes in EDV and ESV (Fig. 2A). A significant increase in SV (by 1.29- and 1.28-fold, respectively) and CO (by 1.41- and 1.38-fold, respectively) was observed in the 50 and 500 ng/L groups (Fig. 2B and C). The heart rate was significantly increased (both by 1.10-fold) in the 50 and 500 ng/L groups (Fig. 2D), and the 500 ng/L metalaxyl-treated larvae displayed significantly increased rhythm irregularity with interbeat variability (±10.9 msec) (Fig. 2E).

2.3. Quantitative analysis of the transcript levels of selected genes

QPCR analysis showed that the mRNA expression levels of 11 genes were altered in the metalaxyl-treated larvae (Fig. 3). No significant changes in *tnnt2* or *cdh2* transcription were observed in the larvae treated with metalaxyl, but the mRNA levels of *tbx5* (by 1.45- and 1.80-fold), *gata4* (by 1.59- and 2.30-fold) and *myh6* (by 1.71- and 1.81-fold) were significantly upregulated in the 50 and 500 ng/L groups, respectively, compared to the control group. The mRNA levels of *nkx2.5* (by 2.20-, 2.90- and 2.70-fold) and *myl7* (by 1.48-, 1.44- and 1.70-fold) were significantly upregulated in the 5, 50 and 500 ng/L groups, respectively, while the mRNA levels of *atp2a11* (by 1.15- and 1.21-fold), *atp1b2b* (by 1.16- and 1.28-fold), and *atp1a3b* (by 1.41- and 1.49-fold) were significantly downregulated in the 50 and 500 ng/L groups, respectively. The mRNA levels of *cacna1da* were significantly decreased (by 1.54-fold) in the 500 ng/L group compared to the control group.

2.4. ATPase activity

The activity of Na^+/K^+-ATPase and Ca^{2+}-ATPase was significantly increased (by 1.65-fold and 1.87-fold, respectively) in the 500 ng/L group compared to the control group (Fig. 4).

3. Discussion

Because of the prohibition of organochlorine and organophosphorus pesticide application, the use of replacement "low-toxic" pesticides has rapidly increased. However, the safety of these current pesticides has not been adequately evaluated. Previous studies have shown that some replacement pesticides result in abnormal development, especially regarding cardiac morphology and function, in zebrafish. For example, exposure to 1.66 mg/L pyriproxyfen caused heart elongation, yolk sac edema and hyperemia, and increased heart beat rate in zebrafish embryos (Maharajan et al., 2018); and zebrafish embryos exposed to 25 and 50 mg/L 2,4-dichlorophenoxyacetic acid displayed pericardial edema, increased heart rate, and upregulation of the transcripts of marker

Table 1 – Cardiac developmental effects of zebrafish embryos exposed to metalaxyl.

Index	Group				
	blank	Solvent	5 ng/L	50 ng/L	500 ng/L
Pericardial edema rate (%)	2.0 ± 0.4^{a}	2.4 ± 0.4^{a}	3.5 ± 0.4^{ab}	4.2. ± 0.8^{b}	8.4 ± 0.6^{c}
Heart hemorrhage rate (%)	6.0 ± 1.8^{a}	5.2 ± 1.8^{a}	8.2 ± 2.1^{b}	16.2 ± 3.8^{c}	15.6 ± 2.3^{c}
SV-BA distance (μm)	71.80 ± 4.48^{a}	70.38 ± 5.98^{a}	81.88 ± 4.44^{a}	92.00 ± 8.97^{b}	114.44 ± 10.54^{b}

Data are presented as mean ± SE (n = 5). Means of exposures not sharing a common letter are significantly different at $p < .05$ as assessed by one-way ANOVA followed by the Duncan test.
SV-BA distance: distance between the sinus venosus (SV) and bulbus arteriosus (BA).

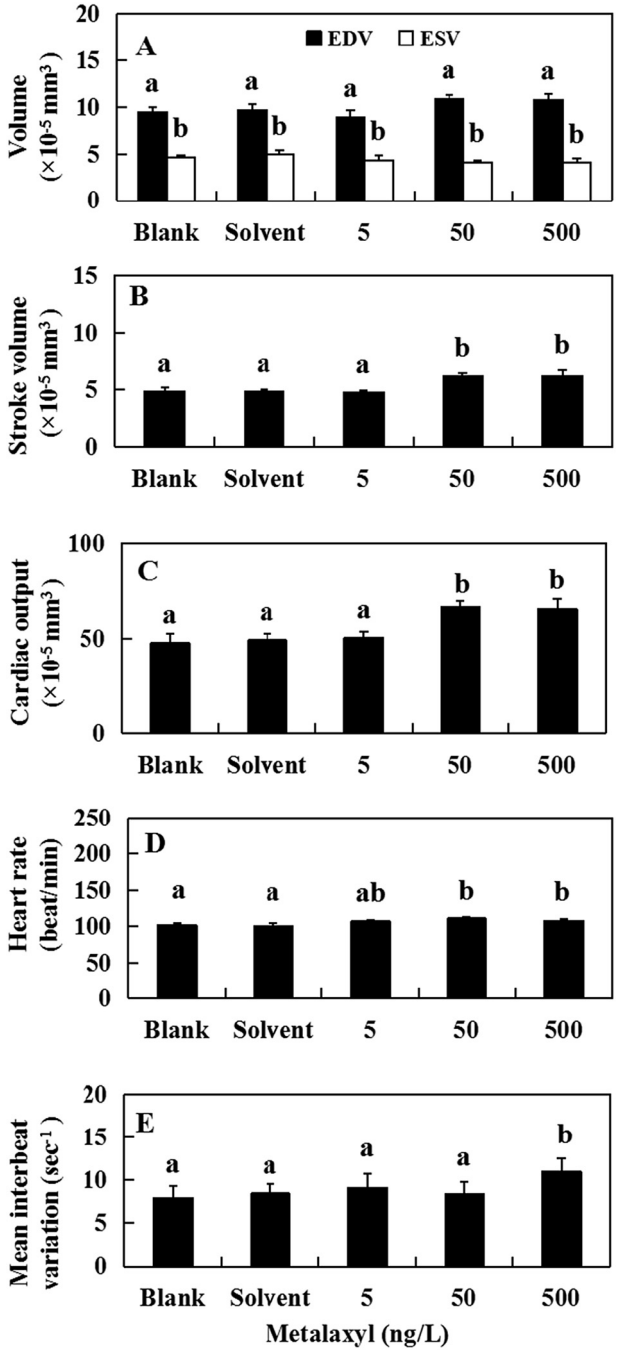

Fig. 2 – Cardiac function in zebrafish larvae after exposure to metalaxyl for 72 hr. (A) Volume of the ventricle at end-diastole (EDV) and end-systole (ESV); (B) Stroke volume; (C) Cardiac output; (D) Heart rate; (E) Cardiac arrhythmia. Each bar indicates the mean ± SE (n = 5). The means for exposures not sharing a common letter are significantly different at $p < 0.05$ as assessed by one-way ANOVA followed by the Duncan test.

genes of cardiac development (*vmhc, amhc, hand2, vegf* and *gata1*) (Li et al., 2017). The pesticides fenitrothion (2.5 and 3.5 mg/L), cymoxanil (5–20 mg/L) and tebuconazole (7–21 mg/L) significantly reduced heart rate, but pyriproxyfen (0.16–0.66 mg/L) did not (Horie et al., 2017). Exposure to 20 and 40 mg/L carbaryl caused defects in heart formation and decreased heart rate (Schock et al., 2012), and 10 mg/L exposure resulted in pericardial edema, red blood cell accumulation and bradycardia (Lin et al., 2007). Heart rates were significantly decreased in zebrafish embryos treated with 30, 100 and 300 μg/L chlorpyrifos for 48 hr (Jin et al., 2015) or with 25–200 mg/L propoxur (Pandey and Guo, 2014), and exposure to procymidone at environmental levels resulted in elongated atria and elevated heart rate (Wu et al., 2018a). The 96-hr LC_{50} of rac-metalaxyl and R-metalaxyl are 416.41 and 320.65 mg/L for zebrafish (*Danio rerio*) embryos respectively, and both enantiomers at concentrations of 200–500 mg/L significantly increased the pericardial edema rate in zebrafish embryos (Zhang et al., 2016). The results of this study indicated that metalaxyl at environmentally relevant concentrations impacted cardiac development and function in zebrafish embryos, suggesting that the developing heart could be a sensitive target of metalaxyl.

In zebrafish, cardiac developmental processes, including conduction system development and cardiac morphogenesis, are controlled by several key genes, including *tbx5*, *nkx2.5*, *tnnt2*, *gata4*, *bmp2b*, *myh6*, *myl7* and *cdh2* (Välimäki et al., 2017). Nkx2.5 is a key factor triggering initial cardiomyocyte differentiation (Balci and Akdemir, 2011), which can determine qualitative and quantitative ventricular characteristics (Targoff et al., 2013). Tbx5 is an essential regulator of heart development (Hiroi et al., 2001; Kathiriya et al., 2003) and plays a crucial role in the differentiation of contracting cardiomyocytes (Takeuchi and Bruneau, 2009) as well as in cardiac conduction system function (Moskowitz et al., 2004). Gata4 plays an important role in cardiac morphogenesis (Holtzinger and Evans, 2005) and cooperates with *nkx2.5* to play a synergistic role in programming cells toward a cardiomyocyte fate (Välimäki et al., 2017). In zebrafish, both myosin light polypeptide 7 (encoded by *myl7*) and atrial myosin heavy chain polypeptide 6 (encoded by *myh6*) are essential for heart muscle differentiation and functioning (Maves et al., 2009). Previous studies have reported that some currently used pesticides impact the cardiac development of zebrafish embryos and cause disturbances in the expression of genes related to cardiac morphogenesis (Wu et al., 2018a, 2018b). In the present study, the transcription of the aforementioned genes (including *tbx5, nkx2.5, gata4, myh6* and *myl7*) was significantly increased in treated zebrafish embryos, indicating that metalaxyl exposure obstructed the developmental expression of these genes and led to disturbed cardiac morphogenesis.

As in humans, the balance between ions such as potassium and calcium plays a critical role in maintaining normal heart rhythm and function in zebrafish (Pott et al., 2014). The transport and coordination of K^+ and Ca^{2+} is essential for the stable action potential of cardiac myocytes (Xu et al., 2005). ATP enzymes play an important role in the maintenance of ion balance (Yadwad et al., 1990; Richards et al., 2003). In cardiac myocytes, Ca^{2+}-ATPase is responsible for the regulation of Ca^{2+} uptake into the sarcoplasmic reticulum (Xu et al., 2009). Changes in Ca^{2+}-ATPase activity will affect cardiac function (Kodde et al., 2007). Na^+/K^+-ATPase (encoded by *atp1*) is also involved in ionic balance in fish (Richards et al., 2003). Genes such as *cacna1ab* and *cacna1da* are controllers of voltage-dependent calcium channels. A previous study showed that triadimefon (18.7–47.2 μg/mL) exposure significantly decreased the transcription of genes related to ATPase (*atp2a11, atp1b2b, atp1a3b*) and

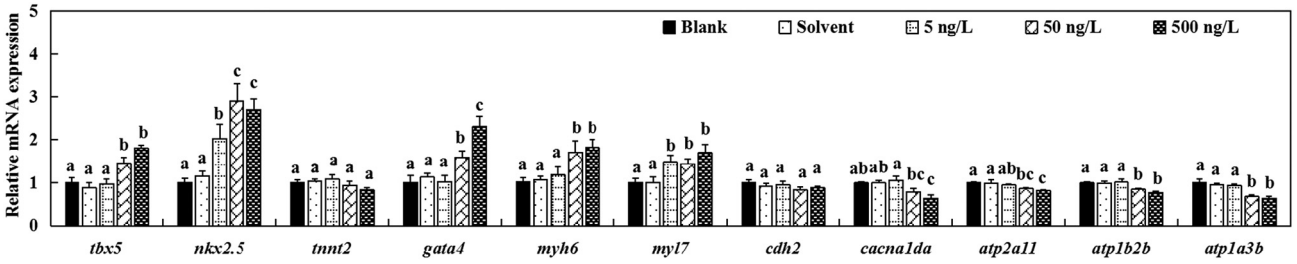

Fig. 3 – Transcription levels of genes in zebrafish larvae after embryonic exposure to metalaxyl for 72 hr. The values were normalized against those of *gapdh*. The results are reported as the mean ± SE (n = 5). The means for exposures not sharing a common letter are significantly different at $p < 0.05$ as assessed by one-way ANOVA followed by the Duncan test.

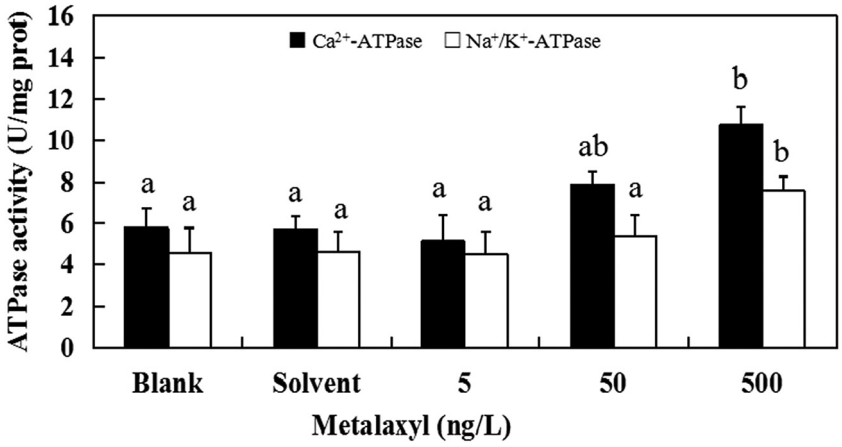

Fig. 4 – Na^+/K^+-ATPase and Ca^{2+}-ATPase activity in zebrafish larvae after embryonic exposure to metalaxyl for 72 hr. The data are presented as the mean ± SE (n = 5). The means for exposures not sharing a common letter are significantly different at $p < 0.05$ as assessed by one-way ANOVA followed by the Duncan test.

calcium channels (*cacna1ab, cacna1da*); these changes could be associated with the impairment of cardiac function (Liu et al., 2017). In adult zebrafish, on exposure to 10 mg/L R-metalaxyl for 48–96 hr, Na^+/K^+-ATPase activity was increased, while 70 mg/L R-metalaxyl treatment elevated enzyme activity after 24 hr and reduced the activity after 96 hr. On the contrary, the enzyme activity was not increased until 96 hr in the fish exposed to 10 and 70 mg/L rac-metalaxyl (Yao et al., 2009). In the present study, Ca^{2+}-ATPase and Na^+/K^+-ATPase activity was increased in zebrafish embryos exposed to metalaxyl, which would be associated with heart arrhythmia and functional failure.

In summary, our results showed that exposure to metalaxyl at environmental concentrations can cause adverse effects on cardiac development in zebrafish embryos, including pericardial edema, heart hemorrhage and cardiac malformation and dysfunction. QPCR analysis showed that the transcription of genes related to cardiac development and function were disturbed by metalaxyl exposure. These results can provide a reference concentration for metalaxyl risk assessment.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jes.2019.03.019.

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