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Endocrine and physiological effects of linuron and S-metolachlor in zebrafish developing embryos

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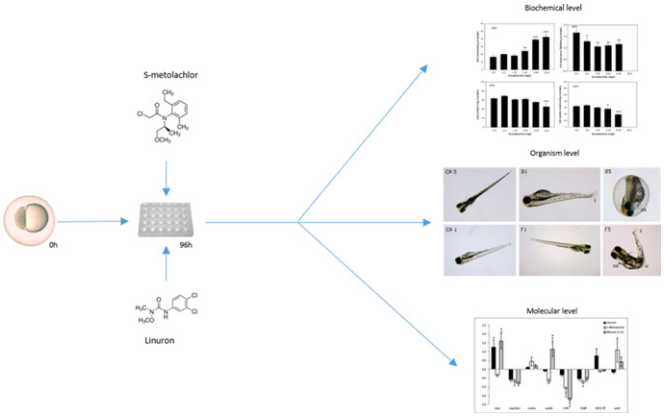
HIGHLIGHTS

- Effects of two herbicides in zebrafish early life stages.
- S-metolachlor induces detoxification, impair neurotransmission and anaerobic metabolism.
- Linuron seems to have an estrogenic mode of action.
- S-metolachlor seems affect steroidogenesis and Hypothalamus-pituitary (HP)-thyroid and HP-adrenal-axis.

GRAPHICAL ABSTRACT



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ABSTRACT

Evaluation of the effects of linuron and S-metolachlor on apical, biochemical and transcriptional endpoints in zebrafish (*Danio rerio*) early life stages was the main purpose of this work. Embryos were exposed for 96 h to a range of concentrations of each herbicide to determine lethal and sub-lethal effects on apical (e.g. malformations, hatching) and biochemical parameters (cholinesterase, ChE; catalase, CAT; glutathione S-transferase, GST; lipid peroxidation, LPO and lactate dehydrogenase, LDH). To evaluate endocrine disruption effects, embryos were exposed during 96 h to 0.88 mg/L linuron and 9.66 mg/L S-metolachlor, isolated or in binary mixture. Expression of a suite of genes involved in HPT, HPG and HPA-axis was then assessed. Highest concentration of linuron (5.0 mg/L) decreased hatching rate to 5% and 70.0 mg/L S-metolachlor completely inhibited hatching, about 100%. Both herbicides impaired development by inducing several malformations (100% in 5.0 mg/L linuron and 70.0 mg/L S-metolachlor). Linuron only affected GST and CAT at concentrations of 0.25 and 0.0025 mg/L, respectively. S-metolachlor induced GST (to 256%), inhibited ChE (to 61%) and LDH (to 60%) and reduced LPO levels (to 63%). Linuron isolated treatment seems to have an estrogenic mode of action due to the observed induction of *vtg1*. Exposure to S-metolachlor seems to interfere with steroidogenesis and with HPT and HPA-axis, since it has inhibited *cyp19a2*, *TSHβ* and *CRH* gene expression. In addition to *vtg1* induction and *CRH* inhibition, herbicide combination also induced *sox9b* that has a role in regulation of sexual development in zebrafish. This study pointed out adverse effects of linuron and S-metolachlor, namely impairment of neurotransmission and energy production, induction of steroidogenesis, and interference with HPT and HPA-axis. These results contributed to

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elucidate modes of action of linuron and S-metolachlor in zebrafish embryo model. Furthermore, gene expression patterns obtained are indicative of endocrine disruption action of these herbicides.

1. Introduction

Herbicides or their products can be bioavailable for aquatic organisms and, depending of their lipid affinity, they can be accumulated in fatty tissues (Kime, 1998). Nevertheless, biota is usually not only exposed to a single pesticide, but to an amalgam of chemicals resulting either from single or mixed applications in agricultural fields. Therefore, their concomitant presence, even at low concentrations, needs to be evaluated as may lead to toxicity increases resulting on higher effects than expected.

In last decades, industry related to agriculture has developed chemicals with capability to mimic hormones or with endocrine disruption activity (Dias et al., 2014; Romano et al., 2009). Hormones play a fundamental role at cell differentiation and thus, exposures to endocrine disruption compounds (EDCs) at early life stages can change normal development of organisms (Hachfi et al., 2012). A major scientific challenge in experimental toxicology is the identification of EDCs and their modes of action (MOA) due to different organs, tissues and signal networks involved (Lorenzetti et al., 2011). Commonly MOA of EDCs of concern include estrogen and androgen antagonists and agonists, aromatase inhibitors, and also thyroid disruptors. Studies with complex EDCs mixtures have shown substantial combined effects, even though each individual chemical is present at low doses that individually do not induce observable effects (Christiansen et al., 2012; Kortenkamp, 2007).

Linuron and S-metolachlor are two pesticides suspected of endocrine disruption. Linuron is a systemic and selective herbicide from urea family used for pre- and post-emergence control of annual grass and broad-leaved weeds in cultures of several cereals, fruit and vegetables (Caux et al., 1998). It acts through inhibition of photosynthesis by disrupting photosystem II and blocking electron transport, leading to a range of oxidants' production and fast destruction of plant cells (USEPA, 1984; Webster et al., 2015). Concentrations detected in surface waters receiving agricultural runoff include 1.05 μg/L in a Canadian river (Woudneh et al., 2009) and 4.42 μg/L in a Florida stream (Schuler and Rand, 2008). Linuron has been described to have anti-androgenic activity, being antagonistic to androgen receptor, which can lead to decreases in testosterone production (Wilson et al., 2009). Different studies also suggest that linuron may also exert anti-estrogenic effects directly by estrogen-mediated processes (e.g. Marlatt et al., 2013).

Some commercial formulations contain linuron in combination with another active ingredient, the metolachlor (Caux et al., 1998), which can be also applied alone. The S conformation of metolachlor, S-metolachlor, is a selective herbicide from chloroacetamides group that reduces seed germination through inhibition of mitosis and cell division (Vallotton et al., 2008). This pre-emergence herbicide is used to control certain broad-leaf and annual grassy weeds (O'Connell et al., 1998). Metolachlor has been found in concentrations as high as 138 μg/L (Howard, 1991) and 293 μg/L (Buttle, 1990) in surface waters associated with runoff from agricultural areas. Also, Cerejeira et al. (2003) found concentrations up to 56 μg/L in groundwater of Portuguese (Europe) agricultural areas. Laville et al. (2006) have shown metolachlor to increase activity of aromatase enzyme in a human cell line, which is responsible to convert testosterone into estradiol. Alterations on this enzyme might result on impairment of male reproduction. Mai et al. (2013) found significantly reduced fertilization success and increased development defects of oyster embryos with low concentrations of S-metolachlor. Moreover, Zeilinger et al. (2009) reported that acetochlor, another acetamide herbicide, have potential to disrupt the thyroid system in a fish species at environmental relevant concentrations. In addition to the described endocrine disruption effects, in which several studies were focused, less is known about the toxicity of these herbicides at biochemical level. Analysis of alterations on activity of, antioxidant, biotransformation and neurotransmission related enzymes, as well as increases of lipid peroxidation levels, offer rapid means to assess toxic effect and MOA of these herbicides on fish species. These biochemical biomarkers have been used to assess potential impacts of chemicals on aquatic ecosystems and can represent a complement to whole organism responses helping to unravel adverse outcome pathways (AOPs) of toxicants.

One of the most promising alternative methods to replace animal testing concerning aquatic toxicology is the zebrafish embryo toxicity test (FET) (OECD, 2013). A good correlation between fish acute and fish embryo toxicities favours FET as a viable alternative to fish acute toxicity test (e.g. Scholz et al., 2013). Furthermore, with recent developments, zebrafish early life stage testing has been suggested as a tool to bridge the gap between in vitro cell-based models and in vivo mammalian models (van Woudenberg et al., 2013).

From the above, this study aims to evaluate lethal and sub-lethal effects of linuron and S-metolachlor at different levels of biological organization in early life stages of zebrafish. To achieve this, embryos were exposed to the herbicides, isolated or/and in binary mixture, performing an integrative approach, including a suite of apical, biochemical and transcriptional endpoints, in order to unravel possible adverse outcomes of EDCs. Effects on embryo development and on key enzymes of different processes (neurotransmission, oxidative stress and energy metabolism) were evaluated. A suite of genes was chosen for gene expression evaluation during early development of zebrafish due to their important role in hypothalamus-pituitary-thyroid (HPT), gonadal (HPG) and adrenal (HPA)-axis, which are potential targets for EDCs. Molecular and biochemical responses have the potential to give an insight into physiological status of an individual under stress conditions and to be translated to higher levels of biological organization, which enables a better understanding of chemicals' MOA.

2. Material and methods

2.1. Chemicals

All the chemicals used, including the herbicides linuron (CAS: 330–55-2; 99.9%) and S-metolachlor (CAS: 87,392–12-9; 98.4%) and 3,4-dichlorianiline (3,4-DCA; CAS: 95–76-1; 98%) were purchased from Sigma-Aldrich (The Netherlands), with the exception of the Bradford reagent, which was purchased from Bio-Rad (Germany).

2.2. Adult zebrafish maintenance and eggs collection

Adults of zebrafish (*Danio rerio* Hamilton-Buchanan, 1822) were maintained at the facility at Department of Biology of University of Aveiro. Fish were maintained in a flow-through system with carbon-filtered water at 27.0 ± 1 °C with a 16:8 h photoperiod (light:dark) and fed twice a day with commercial available artificial diet and brine shrimp. Conductivity is kept at 550 ± 50 μS/cm^{1}, pH at 7.5 ± 0.5 and saturation of dissolved oxygen at 95%. Water from this system was used to prepare solutions in all toxicity assays conducted.

In the day before spawning, male and female fish were separated with a board in aquariums with marbles in the bottom. In following day, the isolation board was removed at beginning of light cycle for the natural mating. Eggs were collected 30 min after spawning, rinsed in system water and checked under a stereomicroscope (Stereoscopic Zoom Microscope - SMZ 1500, Nikon Corporation) for unfertilized or