Basagran® induces developmental malformations and changes the bacterial community of zebrafish embryos

Jacinta M.M. Oliveira a,*, 1, Victor Galhano a,** 1, Isabel Henriques b, Amadeu M.V.M. Soares a, Susana Loureiro a

a Department of Biology & CESAM, University of Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal
b Department of Biology, CESAM & iBIMED, University of Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal

ARTICLE INFO

Article history:
Received 3 May 2016
Received in revised form 7 October 2016
Accepted 8 October 2016
Available online xxx

Keywords:
Basagran®
Zebrafish
Embryotoxicity
Bacterial communities
DGGE

ABSTRACT

This study aimed to assess the effects of Basagran® on zebrafish (Danio rerio) embryos. The embryos were exposed to Basagran® at concentrations ranging from 120.0 to 480.6 mg/L, and the effects on embryo development (up to 96 h) and bacterial communities of 96 h-larvae were assessed. The embryo development response was time-dependent and concentration-dependent (106.35 < EC50 < 421.58 mg/L). The sensitivity of embryo-related endpoints decreased as follows: blood clotting in the head and/or around the yolk sac > delay or anomaly in yolk sac absorption > change in swimming equilibrium > development of pericardial and/or yolk sac oedema > scoliosis. A PCR-DGGE analysis was used to evaluate changes in the structure, richness, evenness and diversity of bacterial communities after herbicide exposure. A herbicide-induced structural adjustment of bacterial community was observed.

In this study, it was successfully demonstrated that Basagran® affected zebrafish embryos and associated bacterial communities, showing time-dependent and concentration-dependent endpoints’ developmental response and structural changes in bacterial community. Thus, this work provides for the first time a complementary approach, which is useful to derive robust toxicity thresholds considering the embryo-microbiota system as a whole. The aquatic hazard assessment will be strengthened by combining current ecotoxicological tests with molecular microbiology tools.

© 2016 Elsevier Ltd. All rights reserved.

1. Introduction

Herbicides are widely used and often applied indiscriminately without an appropriate awareness of their environmental consequences. The herbicide Basagran® [containing 480 g/L of bentazon sodium salt as active ingredient (a.i.)] is one of the commercial formulations available worldwide to effectively control broadleaf weeds and sedges in several crops, particularly in the most economically relevant ones such as rice and maize (FAOSTAT, 2015). In integrated weed management programmes, the recommended doses for field application of Basagran® vary between the two crops (2.5–3 and 3–4 L/ha for maize and rice, respectively) (Tomlin, 2011). Basagran® shows a strong potential to contaminate surface waters through spraying, leaching and/or run-off, depending, among other factors, on the application procedures (prior flooding in rice and at any developmental stage in maize, also a frequently irrigated crop). Even when applied at recommended doses, groundwater contamination can be at risk (EFSA, 2015). Consequently, Basagran® may pose a serious threat to aquatic organisms, including fish, invertebrates and non-target plants.

The effect of the commercial formulation is not foreseen for the majority of non-target aquatic biota and microbiota. With regard to fish, to the best of our knowledge, only two studies have reported Basagran® toxicity: in rainbow trout juveniles ( Oncorhynchus mykiss), the lethal concentration for half of tested organisms after 96 h (96 h-LC50) was higher than 100 mg/L (EFSA, 2015), and the 96 h-LC50 for western mosquitofish juveniles ( Gambusia affinis) was 3874 mg/L (Leung et al., 1983). For the a.i. of Basagran®, that is bentazon, the only known study was carried out with goldfish juveniles ( Carassius auratus; Cyprinidae; 4–7 g), reporting significant
effects on behaviour (e.g. orientation, intensity of swimming, comfort activities, social relations) at 10 mg/L comparatively to controls. Surprisingly, no information is currently available on the effects of Basagran® on zebrafish (Danio rerio; Cyprinidae), juveniles or embryos, though this is one of the most suitable and well-known model organisms used in aquatic toxicity studies (Dai et al., 2014; Hill et al., 2005; McGrath, 2011).

Fish might be exposed to chemicals during their life cycle. Therefore, understanding the effects of xenobiotics throughout all fish life stages is considered crucial to establish an accurate hazard assessment. Particularly at early life stages, fish eggs can be exposed to xenobiotics, and consequently, it can be hypothesized that the associated microbiota of eggs might be affected as well. The zebrafish-associated microbiota changes during the organism development, presenting stage-specific features as well as extensive inter-individual variations within the same developmental stage (Stephens et al., 2016; Giatís et al., 2014; Gómez and Balcázar, 2008; Hansen and Olafsen, 1999; Olafsen, 2001). Each microbial assembly is fundamental to the overall fish health, particularly at early stages, when microbiota can act as a first protective barrier (Giatís et al., 2014; Kanther and Rawls, 2010). Any shift that occurs in such early stages (before the gut opening) might jeopardize the normal development of microbial assembly during the fish development and influences the organism strategies to cope with stress. Therefore, it is particularly important to identify potential stressors that might affect microbial colonization of fish at early stages to improve the hazard assessment (Giatís et al., 2014; Gómez and Balcázar, 2008; Olafsen, 2001). The early developmental stages of fish are highly sensitive to stress caused by biotic and abiotic factors, including anthropogenic stressors (Giatís et al., 2014). In particular, chemical stress may create an additional pressure, thereby determining the shifts that occur in embryo-microbiota assembly, with possible repercussions to the overall system’s stability and to the development and/or survival of the organism (Olafsen, 2001).

The microbiota of zebrafish juveniles and adults has been extensively investigated (Cantas et al., 2012; Kanther and Rawls, 2010; Rawls et al., 2004; Semova et al., 2012). However, the majority of the studies on teleost microbiota have focused mainly on fish gut, particularly at the juvenile and adult developmental stages, and information is still lacking regarding the microbiota associated with early developmental stages. Only one study is available on the effects of chemical stress, namely triclosan, potassium dichromate and prochloraz on bacterial communities of zebrafish embryos (Oliveira et al., 2016). On the contrary, several studies have addressed the microbiota of early life stages in other fish by both culture-dependent (Cahill, 1990) and culture-independent procedures (Brunvold et al., 2007; Griffiths et al., 2001; Jensen et al., 2004; Romero and Navarrete, 2006). The present work is focused on a very early developmental stage, that is 96 h [100-h post fertilization (hpf)], a crucial life stage of fish development. The effects of chemical stress on microbiota of zebrafish juveniles and adults were previously addressed (Gaulke et al., 2016; Narrowe et al., 2015), but not with Basagran®. Thus, to the best of our knowledge, studies with herbicides (including Basagran®) on zebrafish early developmental stages are non-existent.

According to the aforementioned evidence, it is therefore reasonable to hypothesize that Basagran® might function as an important stressor to fish embryos up to 96 h, thereby inducing important changes in development and affecting their microbiota. Considering this, this study aims to evaluate, for the first time, the effects of Basagran® on zebrafish embryos, focusing on ecotoxicological and microbiological perspectives, through the: (1) assessment of the main embryotoxicological endpoints; and (2) analysis of shifts on the associated bacterial community using the Polymerase Chain Reaction-Denaturing Gradient Gel Electrophoresis (PCR-DGGE) approach.

2. Materials and methods

2.1. Herbicide and stock solutions

The commercial herbicide formulation Basagran® (480 g/L bentazon a.i. as sodium salt; BASF Crop Protection, Prior Velho, Portugal) was used in this study. Stock solutions were prepared in autoclaved rearing water, protected from light and stirred overnight to obtain a pale rose transparent solution.

2.2. Zebrafish maintenance and egg production

Zebrafish (Danio rerio) wild-type AB eggs were obtained from the facility at the University of Aveiro, Department of Biology bioterium, following conditions reported elsewhere (Domíngues et al., 2013), except for water temperature which was 26 ± 1 °C. All described procedures were in accordance with both the European (EC, 2010) and national (MAMAO, 2013) laws and regulations for animal welfare, being approved by the local ethical commission (CREBEA from the Department of Biology of the University of Aveiro, Portugal).

Within 30 min after natural mating, eggs were collected, rinsed briefly with fish system water and screened by stereomicroscopy (Stereoscopic Zoom Microscope-SMZ 1500, Nikon Corporation, Tokyo, Japan). Unfertilized eggs with irregularities during cleavage or injuries were discarded.

2.3. Zebrafish embryo toxicity test (FET)

The experimental design of the FET was based on the Organization for Economic Co-operation and Development Guideline No. 236 (OECD, 2013), although adapted to include only sublethal endpoints (Lammer et al., 2009). As recommended by Braunbeck et al. (2014) and Wigh et al. (2015), the extension of the FET to cover sublethal endpoints enables toxicity detection with higher sensitivity. This enables detecting sublethal effects earlier than embryo’s death, thus making the extended FET a powerful tool in aquatic ecotoxicology.

A semi-static exposure method was adopted. Briefly, 20 eggs were randomly distributed in 24-well culture plates and exposed at 4 hpf to different Basagran® nominal concentrations for up to 96 h (i.e. until 100 hpf) (Beekhuizen et al., 2015; OECD, 2013). A range finding test was previously performed with Basagran®, and the 96 h-LC50 derived was 575.64 mg/L (Fig. S1). The nominal concentrations of Basagran® (prepared with autoclaved rearing water) were 120.0, 125.9, 132.1, 145.4, 152.5, 160.0, 180.2, 360.4 and 480.6 mg/L, hereafter referred as C1—C10, respectively. These concentrations were calculated by considering the respective a.i. and based on previous studies [data not shown and Galhano et al. (2010)]. In addition, the highest concentration used (480.6 mg/L) was ~100 × the predicted environmental concentration (PEC) for surface waters [474.66 µg/L (EPSA, 2015)]. Culture plates were covered with plastic lids to avoid changes in volume due to evaporation (Beekhuizen et al., 2015) and placed on an orbital shaker (SM 30A Control, Edmund Bühler GmbH, Hechingen) at 100 rpm (Galhano et al., 2010, 2011) to ensure a uniform suspension throughout the experiment duration. Incubation was performed at the same growth conditions as described above in Section 2.2. All solutions were renewed twice during the experiment: (1) at 0 h, before egg placement, to “wash” the original culture plates and ensure a potential adsorption of formulation molecules to the plate.