Aquatic Toxicology 170 (2016) 390–399

Contents lists available at ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aquatox

# Carbendazim exposure induces developmental, biochemical and behavioural disturbance in zebrafish embryos

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ARTICLE INFO

*Article history:*
Received 29 June 2015
Received in revised form
11 November 2015
Accepted 18 November 2015
Available online 22 November 2015

*Keywords:*
Zebrafish
Locomotor response
Biomarkers
Sublethal
Swimming behaviour

ABSTRACT

Carbendazim is a widely used broad spectrum benzimidazole fungicide; however, its effects to non-target aquatic organisms are poorly studied. The aim of this study was to investigate the toxic effects of carbendazim to zebrafish early life stages at several levels of biological organization, including developmental, biochemical and behavioural levels. The embryo assay was done following the OECD guideline 236 and using a concentration range between 1.1 and 1.8 mg/L. Lethal and developmental endpoints such as hatching, edemas, malformations, heart beat rate, body growth and delays were assessed in a 96 h exposure. A sub-teratogenic range (from 0.16 to 500 μg/L) was then used to assess effects at biochemical and behavioural levels. Biochemical markers included cholinesterase (ChE), glutathione-S-transferase (GST), lactate dehydrogenase (LDH) and catalase (CAT) and were assessed at 96 h. The locomotor behaviour was assessed using an automated video tracking system at 120 h.

Carbendazim (96 h-$LC_{50}$ of 1.75 mg/L) elicited several developmental anomalies in zebrafish embryos with $EC_{50}$ values ranging from 0.85 to 1.6 mg/L. ChE, GST and LDH activities were increased at concentrations equal or above 4 μg/L. The locomotor assay showed to be extremely sensitive, detecting effects in time that larvae spent swimming at concentrations of 0.16 μg/L and thus, being several orders of magnitude more sensitive that developmental parameters or lethality. These are ecological relevant concentrations and highlight the potential of behavioural endpoints as early warning signs for environmental stress. Further studies should focus on understanding how the behavioural disturbances measured in these types of studies translate into fitness impairment at the adult stage.



## 1. Introduction

Carbendazim (methyl-1-H-benzimidazol-2-yl-carbamate) is one of the most widely used benzimidazole fungicides. It is highly toxic to target organisms, inhibiting the development of a wide variety of fungi even at low doses. It is used in agriculture, horticulture, forest and home gardening and as a preservative in paint, papermaking, textile, leather industry and fruits (Selmanoğlu et al., 2001). Carbendazim is a metabolite of benomyl and it is known to target the tubulin in cells, causing disruption of microtubule assembly and cell division (Davidse, 1986).

Low concentrations of carbendazim ranging from 0.2 to 200 μg/L have already been detected in surface waters near agriculture and forestry areas (Palma et al., 2004; Readman et al., 1997). Moreover, carbendazim has shown to be very persistent in the water with a half-life of 6–25 weeks (Cuppen et al., 2000a). Many studies have reported the adverse effects of carbendazim on mammals, mainly on reproductive organs (Farag et al., 2011; Ireland et al., 1979; Lim and Miller, 1997; Nakai et al., 2002; Urani et al., 1995), but unlike mammals, effects on aquatic organisms are poorly studied. The majority of studies available focus on zooplankton and macroinvertebrate communities in which chronic exposures to carbendazim decreased survival, reproduction and feeding rates (Cuppen et al., 2000b; Daam et al., 2010; Ferreira et al., 2008; Ribeiro et al., 2011; Van den Brink et al., 2000). To our knowledge, only two studies report carbendazim effects on fish early life stages. Ludwikowska et al. (2013) showed that carbendazim affects the survival and hatching success of *Prussian carp* embryos at concentrations above 0.036 mg/L and Jiang et al. (2014) demonstrated that embryonic exposure to carbendazim led to significant

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http://dx.doi.org/10.1016/j.aquatox.2015.11.017
0166-445X/

changes in the expression of genes related to apoptosis, immunotoxicity and endocrine disruption in zebrafish (*Danio rerio*). In this later study concentrations between 4 and 500 μg/L of carbendazim were tested.

Risk characterization is better achieved by studying chemical effects at several levels of biological organization. Recently, behavioural parameters such as locomotion (whose evaluation have been considered time consuming and lacking objectivity) have been increasingly used due to the development of technology for automated analysis. In the case of zebrafish, locomotion has been used as an endpoint to assess the neurotoxic effects of chemicals in early life stages (Irons et al., 2010; Padilla et al., 2011; Selderslaghs et al., 2010) and the sublethal toxicity of pollutants (Ulhaq et al., 2013). In fact, many contaminants disrupt fish behaviour at concentrations much lower than those causing mortality, e.g.: Klüver et al. (2015) recorded alteration of behaviour of fish embryos at concentrations 375-fold lower than the $LC_{10}$. Thus, behaviour has proven to provide very sensitive measures of stress exposure; furthermore it has high ecological relevance as effects can be translated long term health and survival of populations (Scott and Sloman, 2004; Tierney, 2011).

Recently, the approval of the OECD test guideline 236 (fish embryo toxicity test) has consolidated the zebrafish embryo test as a true alternative for the acute fish toxicity test with adults (Braunbeck et al., 2014) in the European Union. This test has been increasingly used to assess the toxicity of chemicals and waste waters as reviewed by Scholz et al. (2008, 2013). The low volume of test solutions needed (tests are deployed in 24 or 96-wells microplates) and the rapid development and transparency of embryos that allow the monitoring of the entire organogenesis are among the advantages of this test. Zebrafish embryos also comprise an excellent model for determining the effects of chemicals at biochemical level (Oliveira et al., 2009).

Thus, based on the hypothesis that carbendazim could have serious adverse effects in fish early life stages, the aim of this work was to assess the toxic effects of carbendazim in zebrafish embryos at several organizational levels, namely:

i) lethality,
ii) developmental (including embryo development delays and malformations),
iii) biochemical (including the measurement of the enzymes ChE-cholinesterase, GST-glutathione S-transferase, LDH-lactate dehydrogenase and CAT- catalase) and
iv) behavioural (by measuring locomotion of zebrafish eleutheroembryos expressed by distance moved and time spent moving)

The parameters selected to be evaluated at biochemical levels include not only parameters directly related to neurobehavioral action as it is the case of ChE but also enzymes representative of different metabolic pathways such as GST (involved in the phase II of the detoxification process), LDH (involved in the anaerobic way of energy production) and CAT (involved in the antioxidant defence).

Is this way an integrated analysis of carbendazim can be done contributing to understand the mechanisms of toxicity of this compound.

## 2. Materials and methods

### 2.1. Zebrafish maintenance and embryo collection

All the embryos used in the present study were provided by the zebrafish facility established at the Department of Biology, University of Aveiro (Portugal). Adults were maintained in carbon-filtered water, complemented with 0.34 mg/L salt ("Instant Ocean Synthetic Sea Salt", Spectrum Brands, USA) and automatically adjusted for pH and conductivity. Water temperature was kept at 26.0 ± 1 °C, conductivity at 750 ± 50 μS, pH at 7.5 ± 0.5 and dissolved oxygen equal or above 95% saturation. A 16:8 h (light:dark) photoperiod cycle was maintained. This reconstituted water was used in the preparation of test solutions of all assays performed. The above mentioned temperature and photoperiod conditions were constant in all assays. Zebrafish eggs were obtained by crossbreeding of individuals in aquaria; after 30 min of natural mating, eggs were rinsed in water and checked under a stereomicroscope (Stereoscopic Zoom Microscope—SMZ 1500, Nikon Corporation); those unfertilized, with cleavage irregularities, injuries or other kind of malformations were discarded.

### 2.2. Test chemicals and preparation of test solutions

Carbendazim (Methyl 2-benzimidazolecarbamate, 97% purity) was purchased from Sigma–Aldrich. Carbendazim solutions were carefully prepared by dissolving carbendazim on the zebrafish water system. Ten millilitres of each tested concentration was sampled at the beginning and at the end of the assay and preserved at −20 °C for further chemical analysis. The chemical analysis aimed to assess the degradation of carbendazim in the test solutions and was performed at Laboratory of Environmental Chemistry and Biochemistry, University of South Bohemia in České Budějovice, Czech Republic.

### 2.3. Embryo assay

The assay was based on the OECD testing guideline 236 on Fish Embryo Acute Toxicity (FET) Test (OECD, 2013). A range finding test was conducted in which no mortality was observed at concentrations equal or below 1 mg/L of carbendazim and 100% of mortality was recorded at concentrations equal or higher than 2 mg/L(data not shown). Thus, based on these results the following carbendazim concentrations were used: 1.1, 1.19, 1.3, 1.41, 1.53, 1.66 and 1.8 mg/L. Ten embryos (approximately 3 hpf) were used per replicate (3 replicates were used per treatment) and distributed individually in 24-wells microplates (2 ml of test solution per well). As carbendazim showed to be stable throughout the period of the test, the medium was not renewed. Test run for 96 h. Embryos were daily observed under a stereomicroscope (Stereoscopic Zoom Microscope—SMZ 1500, Nikon Corporation, Japan) and the following parameters were evaluated: survival, somite formation, incidence of pericardial edema, heart beat, malformations (general, spinal, tail and head), hatching, body length (total length: snout to tail tip), yolk sac length and developmental delay. The heartbeat (beats/15 s) was measured by counting heart beats under a stereomicroscope in 3 randomly selected embryos of each replicate ($n = 9$ per concentration) at 48 h. The body and yolk sac length was measured using digital images of the embryos with the software NIS Elements D (Nikon Corporation, Tokyo, Japan). Development delay was obtained by matching the developmental stage of a given embryo with the developmental stages defined by Kimmel et al. (1995).

A sublethal range of carbendazim concentrations (0.16, 0.8, 4, 20, 100 and 500 μg/L) was used to set up the test for biochemical determinations and locomotory analysis. This test was deployed in the same conditions as the above described test. At 96 h of exposure, 10 clusters of eight larvae per treatment were snap-frozen in microtubes containing 0.8 ml of K-phosphate buffer (0.1 M, pH 7.4) and stored at −80 °C for further enzymatic analysis (see Section 2.4). For locomotory assay, larvae were exposed until 120 h