Ecotoxicology and Environmental Safety 99 (2014) 21–27

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

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

Effects of *in vivo* chronic exposure to pendimethalin on EROD activity and antioxidant defenses in rainbow trout (*Oncorhynchus mykiss*)

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ARTICLE INFO

Article history:
Received 20 February 2013
Received in revised form
13 September 2013
Accepted 14 September 2013
Available online 29 October 2013

Keywords:
Pendimethalin
Oncorhynchus mykiss
EROD
GSH
SOD
GPx

ABSTRACT

Pendimethalin, an herbicide active substance frequently used in terrestrial systems, has detected in European aquatic ecosystems. Reliable indicators still need to be found in order to properly assess the impact of pesticides in fish. After an *in vivo* chronic exposure to pendimethalin, the detoxification process and the antioxidant defense system were assessed in 120 adult rainbow trout, *Oncorhynchus mykiss*. Four nominal exposure conditions were tested: control (C), 500 ng L^{-1} (P500), 800 ng L^{-1} (P800) and the commercial formulation Prowl® at 500 ng L^{-1} (Pw500). Fish samples were made after a 28 day exposure period (D28) and after a fifteen day recovery period in clean fresh water (D43). At D28, ethoxyresorufin-O-deethylase (EROD) activity was not activated in liver in spite of the pendimethalin uptake in fish. At D43, EROD activity in fish exposed to the commercial product was lower than in control fish, which may be explained by the high presence of herbicide in fish (613 ± 163 ng g bile^{-1}). Furthermore, antioxidant defense responses were set up by trout in gills and liver following chronic exposure to 800 ng L^{-1} of pendimethalin concentration. While the glutathione content (GSH) decreased in gills, it increased in liver associated with higher activities of glutathione peroxidase (GPx) and superoxide dismutase (SOD). These disturbances could lead to reactive oxygen species production and oxidative stress in the vital organs in fish. After fifteen days in clean water, while the SOD activity was restored, the GSH content and GPx activity were still significantly disturbed in fish exposed to pendimethalin in comparison with control. These significant differences between treatments in antioxidant defenses parameters measured, attesting to the irreversibility of the effects.

1. Introduction

Pendimethalin (*N*-(1-ethylpropyl)-2,6-dinitro-3,4-xylidine) is a dinitroaniline herbicide active substance, frequently used in terrestrial systems. Due to the common usage of various formulations composed of pendimethalin, this chemical compound has been detected at high concentrations in European aquatic ecosystem *i.e.* 352 ng L^{-1} in Denk (Asman et al., 2005), 370 ng L^{-1} in Spain (Barba-Brioso et al., 2010) and 840 ng L^{-1} in France (CORPEP, 2010). Taken up by fish due to its high bioconcentration factor (5100, Agritox database), pendimethalin is accumulated in specific organs and could disturb physiological parameters such as immune system components (Danion et al., 2012a,b). Despite these facts, reliable indicators still need to be found in order to measure and properly assess the impact of low and prolonged exposure to pesticides in fish. Danion et al. (2012a) have already demonstrated that chronic exposure to 200 ng L^{-1} of pendimethalin involves a high bioconcentration in flesh, attesting to the uptake of the pesticide. Indeed, following exposure to pollutants, organisms usually attempt to metabolize and depurate them, minimizing some of the cellular damage they cause and hence acquire fast elimination rates of compounds through bile and urine (Oliveira-Ribeiro et al., 2005). Phase I and phase II biotransformation parameters such as ethoxyresorufin-O-deethylase (EROD) and glutathione-S-transferase activities take part to a set of biochemical parameters which were applied to monitor biological effects in fish in the context of the Joint Assessment and Monitoring Program (JAMP) developed in the European framework (Sanchez and Porcher, 2009). The first step is usually catalyzed by cytochrome P450-dependent monooxygenases (phase I) and their products are subsequently coupled to endogenous metabolites (phase II) (Buhler and Williams, 1988; James and Whitlock, 1999).

Moreover, depends on their chemical structure and their biotransformation, pesticides are known to cause the generation of reactive oxygen species (ROS), leading to oxidative stress.

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http://dx.doi.org/10.1016/j.ecoenv.2013.09.024

Pesticide-induced oxidative stress has been a focus of toxicological research for the last decade as a possible mechanism of toxicity (Banerjee et al., 2001; Abdollahi et al., 2004; Dorval et al., 2003; Moraes et al., 2009; Jin et al., 2011). In response to these potential harmful impacts, every living organism has a variety of antioxidant system defenses to protect themselves against the production of oxygen radicals uncoupling at various electron transfer sites or *via* autoxidation reactions. In this context, thiols play an important role against the pernicious effects of pro-oxidant challenges, and glutathione in particular provides a first line of defense against ROS (Pastore et al., 2003). This non-enzymatic antioxidant participates in many cellular reactions taking away ROS directly (Fang et al., 2002). Antioxidant enzymes are also commonly used to understand the associated toxic mechanisms of xenobiotics (Jensen et al., 1991; Sanchez et al., 2005). Superoxide dismutase (SOD) catalyzes the transformation of superoxide radicals to H_2O_2 and O_2 and is the first enzyme to deal with oxyradicals. Then, H_2O_2 can be reduced to water and oxygen by glutathione peroxidase (GPx) and catalase (CAT) (Kappus, 1985; Oliveira et al., 2008). So, variations in antioxidant defenses can be very sensitive in revealing a pro-oxidant condition and have been used as oxidative stress markers in fish exposed to pesticides (Anguiano et al., 2001; Dorval and Hontela, 2003; Kavitha and Rao, 2008). Previous studies focusing on xenobiotic effects in fish have found that antioxidant enzyme activities may differ considerably between organs (Ahmad et al., 2003; Jee and Kang, 2005), highlighting the need to assess antioxidant responses in the main target organs when a comprehensive evaluation of the oxidative stress risk at individual level is intended. So, antioxidant defense parameters should be assessed in gills and liver, selected on the basis of functional criteria which made them preferential targets, *i.e.* xenobiotic uptake (gills) and xenobiotic metabolism (liver).

The aim of the present study was to evaluate the effects of chronic exposure to 200 ng L^{-1} pendimethalin, active substance alone or the commercial product (Prowl®) on the detoxification process and the antioxidant defense system were tested *in vivo* in rainbow trout (*Oncorhynchus mykiss*). To this end, the pendimethalin concentration in bile, the EROD activity and antioxidant defense parameters were monitored in gills and liver.

2. Materials and methods

This experiment was conducted in accordance with the European Commission recommendation 2007/526/EC on revised guidelines for the accommodation and care of animals used for experimental and other scientific purposes. The center of documentation, research and experimentation on accidental water pollution (CEDRE) is authorized to conduct experimentation on animals in its facilities as a certified establishment according to the administrative order no. 2006-0429. Furthermore, the experimentation carried out as part of this study was conducted under the responsibility and supervision of Dr. Claire Quentel, who holds a certificate to direct scientific experimentation on animals (certificate no. 29-008).

2.1. Fish

The 120 rainbow trout, 79.8 ± 1.6 g, used for this experiment were sourced from virus-free fish rearing facilities at ANSES Laboratory (French Agency for Food, Environmental and Occupational Health and Safety, Plouzané, France). At CEDRE (Brest, France), the fishes were acclimated for one week in eight tanks (300 L) with a flow of 8 L h^{-1} at 15 ± 1 °C. The fishes were fed with commercial dry pellets at 1.5 percent body weight (Neo Prima 4, Le Gouessant Aquaculture) once a day.

2.2. Tested substances

The chemical and physical characteristics of pendimethalin have previously been described by Strandberg and Scott-Fordsmand (2004). The active substance and the commercial herbicide formulation were obtained from Sigma–Aldrich (98.8 percent purity; Germany) and BASF (400 g L^{-1}; France), respectively. The stock solutions were prepared and stored as previously described by Danion et al. (2012a).

2.3. Experimental design

2.3.1. Experimental system

The experimental system has already been described by Danion et al. (2012a). Briefly, the experimental system consisted of four similar independent units, each composed of three tanks. One stock tank containing a concentrated solution of the pollutant supplied two exposure tanks to expose fish in duplicate using a peristaltic pump with a two-channel pump head. All the exposure tanks were also provided with clean fresh water to dilute the concentrated solution in order to obtain the required pendimethalin concentration. The four units were placed in a thermo-regulated greenhouse, in which the air was totally renewed every 6 h and with a natural light/dark cycle (12/12 h approximately).

2.3.2. Exposure conditions and recovery period

Four nominal exposure conditions were tested, one per unit: (i) fresh water as control (C), (ii) 500 ng L^{-1} of active substance (P500), (iii) 800 ng L^{-1} of active substance (P800) and (iv) 500 ngCL^{-1} of pendimethalin with the commercial herbicide formulation of Prowl® (Pw500). P500 represents the nominal concentration just below the predicted no-effect concentration (PNEC) estimated at 550 ng L^{-1} by Council Directive 2003/31/EC, while P800 represents the maximal concentration measured in rivers in Brittany in 2007. For the exposure period, the concentrated solution was flowed at 1 L h^{-1} from the stock tanks to the exposure tanks. In addition, a clean fresh water flow at a rate of 5 L h^{-1} was supplied to the exposure tanks to dilute the exposure concentration. Then, 120 trout were randomly distributed among the four units (or fifteen fishes/exposure tank). During the 28 days of the exposure period, the trout were exposed to a diluted pendimethalin concentration. At the end of the exposure period, the stock tanks were disconnected and clean fresh water was flowed into the exposure tanks at a rate of 8 L h^{-1} during the two week recovery period. Throughout the experiment, the trout were maintained in the same tanks and were fed once a day.

2.3.3. Samples and sampling date

The oxygen level and temperature were monitored daily in each tank (Oxymeter WTW-OXI315I), while nitrate and nitrite levels were measured weekly (Colorimetric test JBL®). Fresh water parameters were stable during the acclimation period and throughout the experiment: dissolved oxygen 93 ± 3 percent, pH 8 ± 0.1, temperature 15 ± 1 °C, free of nitrate and nitrite. Ten fishes were sampled from each unit (or five fishes/exposure tank) on the last day of exposure and recovery periods. The fishes were killed with an overdose of anesthetic phenoxy-2-ethanol and weighed. From each fish, the bile, liver and two branchial arcs were carefully removed and immediately frozen at −80 °C to measure the EROD activity and antioxidant defense parameters.

2.4. Analytical methods

2.4.1. Pendimethalin concentration in bile

The extraction technique used was stir bar sorptive extraction (SBSE) and the quantification was performed using gas chromatography equipment coupled with a mass spectrometer (GC–MS). From each fish, 300 μL of bile was diluted in 100 mL of distilled water with 100 μL of a solution of quintozene Pestanal® (internal standard; Sigma–Aldrich). The SBSE and GC–MS analysis conditions and the quantification of pendimethalin were as described by Danion et al. (2012a).

2.4.2. Liver EROD activity

The livers were homogenized in an ice-cold HEPES buffer using the tissue homogenizer Precellys 24 (Bertin Technologies, France). EROD activity in the liver S9 fraction was measured as described by Couillard et al. (2004) by a microplate spectrofluorometric assay using a Spectrofluorometer® plate reader (excitation 530 nm, emission 585 nm). Reaction mixture contained S9 in HEPES buffer (0.1 M, pH 7.8), 7-ethoxyresorufin (0.024 mg mL^{-1} in DMSO) and NADPH (20 mg mL^{-1}). The activity was quantified by measuring the fluorescence of resorufin at 60 s intervals over a 13 min total scan time. Liver EROD activity was calculated as nmol min^{-1} mg^{-1} protein with resorufin used as standard.All samples were assayed in duplicate.

2.4.3. Antioxidant defenses

The gills and livers were homogenized in an ice-cold phosphate buffer (0.1 M, pH 7.8) with 20 percent glycerol and 0.2 mM phenylmethylsulfonyl fluoride as a serine protease inhibitor with the tissue homogenizer Precellys 24 (Bertin Technologies, France). The homogenates were centrifuged at 9000 × *g* at 4 °C, for 15 min and the supernatant was splitted into five aliquots and stored at −80 °C for biochemical assays. The total protein concentrations in gill and liver samples were spectrophotometrically estimated using the method of Bradford (1976) with bovine serum albumin (Sigma–Aldrich Chemicals, France) used as a standard. Biomarker assays including GSH content and activities of SOD, GPx and CAT in the gills and liver of rainbow trout were adapted for use in microplates after preliminary tests using several dilutions. All samples were assayed in duplicate.