Comparative Biochemistry and Physiology, Part C 180 (2016) 49–55

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part C

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

Acute exposure to the biopesticide azadirachtin affects parameters in the gills of common carp (*Cyprinus carpio*)

Camila R. Murussi [a], Maiara D. Costa [a], Jossiele W. Leitemperger [a], Fábio Flores-Lopes [b], Charlene C. Menezes [c], Luisa Loebens [c], Luis Antonio de Avila [d], Tiele M. Rizzetti [e], Martha B. Adaime [e], Renato Zanella [e], Vania L. Loro [a,c,*]

[a] Programa de Pós-graduação em Bioquímica Toxicológica, Departamento de Bioquímica e Biologia Molecular, Centro de Ciências Naturais e Exatas, Universidade Federal de Santa Maria, Santa Maria, RS, Brazil
[b] Departamento de Ciências Biológicas, Universidade Estadual de Santa Cruz, Ilhéus, BA, Brazil
[c] Programa de Pós-graduação em Biodiversidade Animal, Departamento de Bioquímica e Biologia Molecular, Centro de Ciências Naturais e Exatas, Universidade Federal de Santa Maria, Santa Maria, RS, Brazil
[d] Programa de Pós-Graduação em Fitossanidade, Faculdade de Agronomia Eliseu Maciel, Universidade Federal de Pelotas, Pelotas, RS, Brazil
[e] Laboratório de Análises de Resíduos de Pesticidas, Departamento de Química, Centro de Ciências Naturais e Exatas, Universidade Federal de Santa Maria, Santa Maria, RS, Brazil

ARTICLE INFO

Article history:
Received 3 September 2015
Received in revised form 25 November 2015
Accepted 7 December 2015
Available online 9 December 2015

Keywords:
Fish
Histology analyses, Na^+K^+-ATPase
Mucus layer
Oxidative profile
Toxicity

ABSTRACT

The biopesticide, azadirachtin (Aza) is less hazardous to the environment, but may cause several toxic effects in aquatic organisms. The *Cyprinus carpio* (n = 12, for all concentrations) after 10 days of acclimation under controlled conditions, were exposed at 20, 40, and 60 μL/L of Aza during 96 h. After this period, fish were anesthetized and euthanized then mucus layer and gills collected. In this study, the effects of exposure to different Aza concentrations were analysed through a set of biomarkers: Na^+/K^+-ATPase, lipid peroxidation (TBARS), protein carbonyl (PC), superoxide dismutase (SOD), glutathione-S-transferase (GST), catalase (CAT), glutathione peroxidase (GPx), non-protein thiols (NPSH), ascorbic acid (AsA) and histological parameters and, yet, protein and glucose concentration in the surface area of mucous layer. Na^+K^+-ATPase was inhibited at 40 and 60 μL/L compared to control. TBARS decreased at 40 μL/L compared to control. PC, SOD and GST increased at 60 μL/L in comparison to control. CAT increased at 20 and 60 μL/L, and GPx increased in all Aza concentrations compared to control. NPSH decreased and AsA increased in all concentrations in comparison to control. Histological analyses demonstrated an increase in the intensity of the damage with increasing Aza concentration. Alterations in histological examination were elevation and hypertrophy of the epithelial cells of the secondary filament, hypertrophy and hyperplasia of the mucous and chlorate cells and lamellar aneurism. Glucose and protein concentrations in mucus layer increased at 60 μL/L compared to control. In general, we suggest that 60 μL/L Aza concentration affected several parameters causing disruptions carp metabolism.

1. Introduction

Aquaculture, is widely used in the world due to its high potential to preserve biodiversity by decreasing the pressure on wild stocks and producing animal protein for consumption by the growing global population (Bostock et al., 2010; Nomura, 2010). According to the FAO (2013), per capita consumption of fish was estimated at approximately 20 kg/year. In Brazil the consumption is 11.17 kg per capita/year, still considered low, with the potential for increased production. Nevertheless, *Cyprinus carpio* is the third most important farmed freshwater species in the world, together with tilapia representing about 80% of the tropical inland aquaculture production (Arthur et al., 2010; Ljubojevic et al., 2015). The common carp is rustic, omnivorous, shows fast growth, has commercial value and is widely distributed geographically.

When this species and others are intensively rearing in aquaculture systems the stock density may lead to increases in the incidence of diseases as bacterial infection and stress situation may affect the growth (Drennan et al., 2005; Biswas et al., 2006; Menezes et al., 2015a, 2015b). Natural substances such as the neem-based biopesticide azadirachtin (Aza) are most promising compounds for the control of some these problems. Several studies have showed that Aza is used to the control of bacterial parasites such as *Aeromonas hydrophila*, *Aeromonas salmonicida*, *Citrobacter freundii* (Harikrishnan et al., 2003; Thomas et al., 2013; Thanigaivel et al., 2015).

Aza ($C_{35}H_{44}O_{16}$) is a tetranortriterpinoid derived from the tree *Azadirachta indica* A. Juss. The compounds Aza A and B are found in several parts of the plant, which is cultivated in warm regions, and considered tolerant to harsh climates (Bajwa and Ahmad, 2012; Debashri and Tamal, 2012). However, information on the dosage and frequency of use

* Corresponding author at: Department of Biochemistry and Molecular Biology, Federal University of Santa Maria, 97105.900, Santa Maria, RS, Brazil.
E-mail address: vania.loro@gmail.com (V.L. Loro).

http://dx.doi.org/10.1016/j.cbpc.2015.12.003
1532-0456/

of chemical and biological products for the control of diseases and pathogenic bacteria in aquatic organisms is very limited. Previous studies by our research group demonstrated toxic effects of Aza, such as changes in haematological and behavioural parameters of *C. carpio* (Murussi et al., 2015). Other studies recorded changes in several fish species as osmolarity and histology of *Prochilodus lineatus* (Winkaler et al., 2007) blood electrolytes in *Heteropneustes fossilis* (Kumar et al., 2011), neurotoxicity in *Danio rerio* (Bernardi et al., 2013), abnormalities such as erratic and rapid movement, body imbalance and surface floating to exposure *Lepidocephalichthys guntea* (Mondal et al., 2007). Alterations in reserves of glycogen and protein levels of *Labeo rohita* also were observed (Saravanan et al., 2010).

Tissue damage in fish gills is easily observed when they are in contact with toxic substances. The gill surface represents more than half of the entire body surface area. This important organ is multifunctional and responsible for osmoregulation, nitrogenous waste excretion, acid–basic balance and respiration (Kumar et al., 2010; Flores-Lopes and Thomaz, 2011). ATPases are membrane-bound enzymes and transport ions through biological membranes and thus regulate their movement. Na^+K^+-ATPase is found abundantly in the tubular system of chloride cells and has a major role in maintenance of ion balance across the gills (Sancho et al., 2003; Parvez et al., 2006; Suvetha et al., 2010).

Several other biomarkers, such as lipid peroxidation, protein carbonylation and enzymatic and non-enzymatic antioxidants are very useful for evaluating oxidative status and gill membrane fragility. Oxidative stress is a result of in adequate removal of reactive oxygen species (ROS) formed when pro-oxidant forces overcome antioxidant defences (van der Oost et al., 2003; Kumar et al., 2012). The integration of a set of analyses including Na^+K^+-ATPase, biochemical and histological is a sensitive tool for detecting possible changes in the gills of *C. carpio* exposed the Aza concentrations. Histological analyses are important as sensitive and reliable indicators of health status in fish species (Raskovic et al., 2013). In addition, the investigation of the possible of Aza on the mucous layer is important to verify changes in the protective barrier. The mucous layer has functions such as disease resistance, respiration, ionic and osmotic regulation, locomotion, reproduction, communication and feeding (Subramanian et al., 2008).

The main aim of this study was investigate Na^+K^+-ATPase as well as biochemical and histological parameters in gills of *C. carpio* after exposure to Aza for 96 h. Also, evaluate possible responses of toxicity and describe effective biomarkers in the response to Aza exposure for this fish species widely reared in aquaculture system.

2. Materials and methods

2.1. Aza formulation

Neenmax™ (manufactured by Insetimax, Brazil) used in present study is a biopesticide that contains Aza with a maximum of 1200 mL/L (0.12%) of Aza A and B as active ingredient. Neenmax™ was dissolved in ethanol (99.9%-high purity, manufactured by Tedia, USA) (1:1) and then added to dechlorinated tap water to obtain the desired concentration. In the ethanol group the same concentrations of ethanol used were add at 60 μL/L.

2.2. Specimen and acclimation period

Male and female carp (*C. carpio*) (weight, 10.0 ± 2.5 g; length, 6.5 ± 1.0 cm) were obtained from the fish farm at the Federal University of Santa Maria (UFSM). The fish were acclimated in dechlorinated tap water in 250 L tanks for 10 days. They were maintained in continuously aerated water with a natural photoperiod (12 h light/12 h dark). Water quality parameters were measured every day and were recorded as: temperature 22.5 ± 2.0 °C, pH 6.5 ± 1.0 units, dissolved oxygen 7.8 ± 0.2 mg/L, nonionized ammonia 0.52 ± 0.04 μg/L, nitrite 0.08 ± 0.01 mg/L and alkalinity 20.0 ± 1.5 mg/L $CaCO_3$. All water parameters were determined according to Boyd and Tucker (1992). During acclimation, the fish were feeding once a day with commercial fish pellets (42% crude protein, Supra, Brazil). Faeces and pellet residues were removed by suction. All protocols used in this study were approved by the Committee on Ethics and Animal Welfare of the Federal University of Santa Maria, protocol number 029-2014.

2.3. Experimental design

After the acclimation period the fish were distributed randomly in 45 L boxes with dechlorinated tap water. Water quality parameters during the treatment period were similar to those in the acclimation period and did not change during the experiment. The experiment was constituted of a control group (0.0 μL/L Aza), an ethanol group (60 μL/L ethanol), and three concentrations of Aza (20, 40 and 60 μL/L) that corresponded the 25, 50 and 75% the values of LC_{50} Aza for *C. carpio*, 80 μL/L (Murussi et al., 2015). Groups of six fish per box (duplicate, n = 12) were exposed for 96 h. After this period the fish were anesthetized with benzocaine hydrochloride (0.1 g/L) according Antunes et al. (2008) and were euthanized by section of the spinal cord. The concentration of Aza in the water was monitored during the experiment (1st and 4th day) and analysed by LC–MS/MS using the method described by Menezes et al. (2004).

2.4. Biochemical analysis

2.4.1. Na^+/K^+-ATPase activity

Gill Na^+/K^+-ATPase activity was assayed using a modification of the method described by Bianchini and Castilho (1999). Na^+/K^+-ATPase activity was determined as the difference between phosphate liberated from ATP in the presence of K^+ (medium A) and in the absence of K^+ with 1 mM of ouabain (medium B). For each assay, 20 μL of the homogenate fraction was added and mixed to 2.0 mL of assay media containing the following final concentrations. The medium A was: 77 mM NaCl, 19 mM KCl, 6 mM $MgCl_2$, 3 mM ATP, and buffer Tris–HCl 0.1 M at pH 7.6. The medium B was: 96 mM NaCl, 6 mM $MgCl_2$, 3 mM ATP, 1 mM ouabain, and buffer Tris–HCl 0.1 M at pH 7.6. The reaction started with the addition of the homogenate and was incubated at 30 °C for 30 min. The reaction was stopped by adding 0.2 mL of trichloroacetic acid (20%) to the reaction medium. Phosphate concentration in the reaction medium was determined using a modification of the method of Fiske and Subbarow (1925). Enzyme specific activity was expressed as μmol Pi/mg protein/h.

2.4.2. Determination of oxidative stress indicators

All the analyses described in this topic were performed using gills of carp. Lipid peroxidation estimation was determined according to method of Buege and Aust (1978). Protein carbonyl (PC) assay was determined according with Yan et al. (1995). The antioxidant enzyme superoxide dismutase (SOD) follows method described by Misra and Fridovich (1972). Glutathione-S-transferase (GST) activity was measured according with Habig et al. (1974). Catalase (CAT) and glutathione peroxidase (GPx) were determined according Nelson and Kiesow (1972) and Paglia and Valentine (1987), respectively. Non-protein thiols (NPSH) and ascorbic acid (AsA) levels were determined by the method of Ellman (1959) and Roe (1954) respectively.

2.4.3. Mucus layer analyses

The mucus layer was carefully scraped from dorsal body surface (total area of 4 cm^2) using a cotton swab. After scraping, the cotton was immersed in 2 mL of distilled water, and the sample was used to determine soluble sugar represented by glucose (Duboie et al., 1956) and protein concentrations (Bradford, 1976).