Marine Pollution Bulletin xxx (2017) xxx–xxx

Contents lists available at ScienceDirect

Marine Pollution Bulletin

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

Oxidative stress and genotoxicity biomarker responses in tilapia (*Oreochromis niloticus*) exposed to environmental concentration of 1-nitropyrene

Eugene T. Bacolod [a], Seiichi Uno [b,*], Shiela S. Villamor [b], Jiro Koyama [b]

[a] Department of Chemistry, School of Arts and Sciences, University of San Carlos, 6000 Cebu City, Philippines
[b] Education and Research Center for Marine Resources and Environment, Faculty of Fisheries, Kagoshima University, 4-50-20 Shimoarata, Kagoshima 890-0056, Japan

ARTICLE INFO

Article history:
Received 31 August 2016
Received in revised form 26 January 2017
Accepted 30 January 2017
Available online xxxx

Keywords:
NPAHs
Genotoxicity
Oxidative stress
Environmental pollution
1-nitropyrene

ABSTRACT

The present study aimed to assess whether environmental 1-nitropyrene (1-NP) concentration will induce genotoxicity and oxidative damages in tilapia, lives in estuarine and brackish water. Tilapia were exposed to waterborne 1-NP. Cellular antioxidant enzyme activity of glutathione peroxidase and oxidative damage, i.e., lipid peroxidation, protein and DNA oxidation were used as biomarkers of oxidative stress, while the micronucleus test was used for evaluation of chromosomal damage and was used as an indication of genotoxicity. Results showed that all biomarkers for oxidative stress positively responded, and micronucleus and other nuclear abnormalities frequencies significantly increased ($p < 0.001$). This study showed that environmentally relevant 1-NP concentration in test water (0.15 ng/L) and in fish (3 ng/kg) induced genotoxicity and oxidative stress. Micronuclei and other nuclear abnormalities were probably formed as a result of oxidative stress. In conclusion, exposure to lower waterborne 1-NP concentration can pose a risk to freshwater and estuarine organisms through accumulation.

1. Introduction

Oxidative stress occurs when the balance between oxidants and antioxidants is disrupted due to the depletion of antioxidants, the excessive accumulation of reactive oxygen species (ROS), or both of them (Scandalios, 2005). This imbalance can result in oxidative damage to lipids, proteins, and DNA. Thus, changes in antioxidant defenses and oxidative damage are often used as biomarkers of oxidative stress (Livingstone, 2001). Biomarkers can be characterized as a functional measure of exposure to a stressor, and are typically expressed at the sub-organism level of biological organization (Adams et al., 2001). Sub-organism responses to environmental stressors occur before other disturbances, such as disease, mortality, or population changes, and thus may offer early warnings of pollution (Depledge and Fossi, 1994).

Genotoxic pollutants induce changes in the genetic material of aquatic organisms, including DNA damage and gene or chromosomal alteration. Many types of environmental contaminants, including metals, polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), and pesticides exhibit genotoxic properties (De Flora et al., 1991). Biomarkers of DNA and chromosomal damage are used to assess genotoxic effects in aquatic organisms (Ohe et al., 2004).

Fish are an ideal model for the evaluation of pollution in aquatic ecosystems. Biomarkers of oxidative stress and genotoxicity have been used in several fish species to assess pollution in laboratory and field experiments (Cheikyula et al., 2009; Tsangaris et al., 2011; Bacolod et al., 2013a; Bacolod et al., 2013b). In the laboratory, exposure to a mixture of nitrated polycyclic aromatic hydrocarbons or nitroarenes (NPAHs) resulted in genotoxic effects by induction of micronuclei in marbled flounder, *Pleuronectes yokohamae* (Bacolod et al., 2013a). Although the IPCS (2003) has categorized some NPAHs as potential carcinogens that may cause genotoxicity, there has been little empirical evidence to support this, except for our prior study (Bacolod et al., 2013a).

In the current study, we evaluated the effects of 1-nitropyrene (1-NP) in tilapia *Oreochromis niloticus*. Since *O. niloticus* is an estuary fish and can easily adapt to different water environment such as sea, brackish, or fresh waters, the influences of pollutants from upper of a river to estuarine and coastal areas can be successively evaluated by only this fish. 1-NP is a member of the 2A group of NPAHs, meaning it is probably carcinogenic to humans (IPCS, 2003). 1-NP has been detected in river water (100 to 1000 pg/L) (Ohe and Nukaya, 1996), in sea-water collected from the Sea of Japan (0.2 to 0.5 pg/L) (Murahashi et al., 2001), in river and sea sediments (Ozaki et al., 2010), and in aquatic organisms (Uno et al., 2011). Despite this, little is known about the effect of NPAHs exposure, including 1-NP, on oxidative stress and genotoxicity in aquatic organisms.

* Corresponding author.
E-mail address: uno@fish.kagoshima-u.ac.jp (S. Uno).

http://dx.doi.org/10.1016/j.marpolbul.2017.01.077
0025-326X/

2. Materials and methods

2.1. Reagents

We purchased 1-nitropyrene (1-NP, 99.9% purity) from Sigma-Aldrich (St. Louis, MO, USA) and 1-nitropyrene-d_9 from Central Chemicals Inc. (Canada). The remaining reagents were laboratory grade or analytical grade and were purchased from Wako Pure Chemical Industries (Japan), Merck (Germany), or Sigma-Aldrich (Japan).

2.2. Exposure and sampling

Tilapia, *O. niloticus*, juveniles were purchased from a private aquaculture facility in Ibusuki city, Kagoshima prefecture, Japan, and reared in a 200 L tank in the laboratory until they reached a size of ~50 g. The tank was supplied with aerated, dechlorinated tap water. The fish were regularly fed daily a commercial fish diet (6P pellet, Nippon Formula Feed Manufacturing, Tokyo) at a rate of 1% before exposure to the treatment media and during the experimental period.

A total of 60 tilapia (body weight: 55 ± 7 g, total body length: 15 ± 0.8 cm) were used in the experiment. The exposure set-up consisted of four 40 L stock aquaria that contained either 250 (low), 2500 (medium), or 25,000 pg/L (high) 1-NP stock solution (dissolved in acetone), or dechlorinated tap water (control). The outflow from each stock aquaria was setup to flow continuously to a single exposure aquaria at a rate of 1.7 L/h. The exposure aquaria consisted of four 40 L (60 cm) glass aquaria (low, medium, high exposure groups, and a control group) each containing 15 tilapia. The stock solution was replaced every 24 h until the end of the experiment (14 d).

Five fish were removed from each exposure aquaria 3, 7, and 14 d after starting the test. These fish were anaesthetized by immersion in a 0.5 mL/L solution of 2-phenoxyethanol. Blood was collected from the caudal vein by syringe for micronuclei (MN) and nuclear abnormality (NA) analysis (see Section 2.5). The remaining blood was centrifuged at 16,000 ×*g* for 5 min at 4 °C and the supernatant was collected for DNA oxidation analyses. Additionally, the liver was collected and separated for biochemical analyses (see Section 2.3). The procedure for the biochemical analyses is described in Section 2.3. The remaining tissue from each individual was used for 1-NP analysis. Water samples were collected after 1, 5, and 12 d for the measurement of 1-NP concentrations. The analysis of 1-NP is described in Sections 2.6 and 2.7. During the experimental period, the mean (± standard deviation) water temperature, dissolved oxygen, and pH in all test groups were 25.3 ± 0.2 °C, 6.8 ± 0.1 mg/L, and 7.60 ± 0.10, respectively.

2.3. Biochemical analyses

We quantified the levels of oxidative biomarkers, except 8-hydroxydeoxyguanosine (8-OHdOG), in the liver tissue. Liver samples were homogenized in 0.1 M phosphate buffer, pH 7.4 using 0.1 g liver per 1 mL buffer in a micro tube placed on ice using a Power Masher II (Nippi Ltd. Japan). The homogenates were centrifuged at 16,000 ×*g* for 10 min at 4 °C and the supernatant was kept at −80 °C until analysis. All preparation procedures were performed at 4 °C. The biochemical analyses were completed within 3–5 d from the end of the exposure period. Oxidative biomarker activity was assayed using the supernatant.

Glutathione peroxidase (GPx) activity was assayed using the procedure of Gallo and Martino (2009). Briefly, 0.5 mL supernatant was added to 1 mL mixed reagent containing 0.5 M K_2HPO_4 (pH 7.0), 2.5 mM EDTA, 0.18 units/mL glutathione reductase (GR), 100 mM glutathione, and 10 mM reduced nicotinamide adenine dinucleotide phosphate (NADPH). The reaction was initiated by the addition of 0.1 mL cumene hydroperoxide. Enzyme activity was then measured at 340 nm every 30 s for 5 min using a UV spectrophotometer (UV-1600, Shimadzu, Japan). GPx activity was calculated as follows: GPx Activity = (Absorbance $_{T1\&T2}$ − Absorbance $_{blank}$) / (T2-T1) × V $_{sample}$, where T1 and T2 represents the change in absorbance at any time point; T2-T1 represents the difference between initial and final time; and V $_{sample}$ represents sample volume. GPx activity was expressed as units (U)/mg protein/min.

Lipid peroxidation was measured using the procedure of Chauhan et al. (2004). Approximately 1 mL of supernatant was added to 2 mL 0.37% thiobarbituric acid (w/v):15% trichloroacetic acid (w/v) in 0.25 M HCl. The mixture was heated in a boiling water bath for 15 min and allowed to cool. The resulting solution was then centrifuged at 16,000 ×*g* for 10 min. The thiobarbituric reactive substances (TBARS) were measured in the supernatant at 532 nm using a UV spectrophotometer. The results were expressed as nmoL TBARS/mg protein based on the extinction coefficient for MDA $2.56 \times 10^5\ M^{-1}\ cm^{-1}$.

Carbonyl protein was assayed using the procedure of Levine et al. (1990). Approximately 0.5 mL of supernatant was reacted with 4 mL of 10 mM 2,4-dinitrophenylhydrazine (DNPH) in 2 M hydrochloric acid for 1 h at room temperature with shaking at 15-min intervals. The mixture was then precipitated with 20% trichloroacetic acid (TCA) for 1 h and centrifuged at 16,000 ×*g* for 10 min at 4 °C. The precipitated protein was washed thrice by resuspension in 4 mL ethanol/ethyl acetate (1:1). The proteins were then solubilized in 2 mL 6 M guanidine in 20 mM K_3PO_4 and centrifuged at 16,000 ×*g* for 5 min. The carbonyl protein content was measured spectrophotometrically with a UV spectrophotometer at 366 nm. The results were expressed as nanomoles of DNPH incorporated/mg protein based on a molar extinction coefficient of $22{,}000\ M^{-1}\ cm^{-1}$.

The amount of oxidized DNA was measured using 8-OHdG levels in the serum sample. Serum was prepared by centrifugation of the blood sample at 16,000 ×*g* for 10 min and 8-OHdOG levels were quantified using a competitive ELISA kit (New 8-OHdOG Check, Japan Institute for the Control of Aging, Japan). The ELISA assay was performed according to the manufacturer's instructions. Briefly, 50 μL of blood serum or standard was used during the analysis. After a series of sample preparation and color development steps, the absorbance was measured at 450 nm using a microplate reader (MTP-32, Corona Electric, Japan). The levels of 8-OHdOG was expressed as ng/mL serum calculated from the standard curve.

2.4. Sample protein analysis

The protein content of each sample was analyzed in the supernatant using a DC protein kit (Bio-Rad, Hercules, USA) based on the Lowry method (Lowry et al., 1951). Optical absorbance was measured at 660 nm on a microplate reader with bovine serum albumin protein standards.

2.5. Genotoxicity analyses

Genotoxicity analyses were based on the methods described in our previous paper (Bacolod et al., 2013a). Briefly, blood was collected from the caudal blood vessel using a heparinized syringe with a 23-gauge needle (Terumo, Japan). Sampling days coincided with the days fish were sampled. Additionally, a blood smear was air-dried for 24 h, fixed in absolute methanol for 30 s, washed with distilled water, and air-dried for another 24 h before staining. The dried smear was supplemented with May-Grunwald solution and stained with 10% Giemsa stain in phosphate buffer. Duplicate smears were performed for each fish. The frequency of micronucleus (MN) and nuclear abnormalities (NA) were counted using a microscope (Eclipse E600, Nikon, Japan) at 1000 magnification. MN and types of NA (binucleated cell, blebbed cell, lobbed cell, and notched cell) were identified based on descriptions in Al-Sabti and Metcalfe (1995) and Fenech et al. (2003). A total of 1000 erythrocytes were observed per slide. The final frequency was estimated by calculating the average MN and NA of two slides from one fish, then calculating the average MN and NA from five fish.

Please cite this article as: Bacolod, E.T., et al., Oxidative stress and genotoxicity biomarker responses in tilapia (*Oreochromis niloticus*) exposed to environmental concentration of 1-nitropyrene..., Marine Pollution Bulletin (2017), http://dx.doi.org/10.1016/j.marpolbul.2017.01.077