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Cadmium bioaccumulation and oxidative-stress-induced DNA alterations in the freshwater cladoceran *Moina macrocopa* (Straus 1820) following consecutive short-term exposure assessments

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Abstract

Moina macrocopa is a toxicologically less explored, yet widely distributed freshwater cladoceran found throughout the world. The present study is aimed at determining the cadmium (Cd) bioaccumulation and Cd-induced molecular and biochemical biomarkers, including DNA damage and antioxidant enzyme responses (i.e., catalase (CAT) and glutathione S-transferase (GST) activities), in *M. macrocopa* neonates. Cd bioaccumulation was determined using a Cd-sensitive fluorescence probe after acute exposure to Cd. The results indicated a concentration-dependent Cd bioaccumulation, especially in the gut region, where it was statistically significant in neonates exposed to ≥ 5 Cd $\mu\text{g/L}$. DNA damage was determined by the randomly amplified polymorphic DNA (RAPD) technique after a short-term exposure to sublethal Cd concentrations. Significant DNA alterations were observed in individuals exposed to ≥ 10 Cd $\mu\text{g/L}$. Short-term exposure to sublethal Cd concentrations did not change CAT levels. However, low Cd concentrations (10 $\mu\text{g/L}$) significantly induced GST levels, and higher concentrations (50 $\mu\text{g/L}$) significantly inhibited the GST levels. Taken together, the results of the present study indicated that bioaccumulation of Cd in *M. macrocopa* causes oxidative stress even at sublethal concentrations, which may be one of the underlying causes of the observed DNA damage. Furthermore, the findings of the present study highlight the importance of utilizing molecular and biochemical biomarkers for toxicity assessment in cladocerans owing to their high sensitivity and rapid responses even under short-term exposure conditions with low concentrations.

Introduction

Heavy metal contamination in aquatic environments is one of the most critical global environmental concerns (Shaw et al. 2006). Among the various heavy metals, cadmium (Cd) can be commonly found in water bodies around the world (Gama-Flores et al. 2017). Natural processes such as weathering and volcanic emissions, as well as domestic, industrial, and other anthropogenic activities can contaminate aquatic environments with Cd (Gama-Flores et al. 2017; Garcia et al. 2004).

Cd does not have any biological function and it is highly toxic to aquatic organisms even at a concentration of a few micrograms per liter (Shaw et al. 2006; Wang et al. 2009). However, several studies have revealed the occurrence of Cd in freshwater environments at concentrations above background levels (0.1–2 µg/L) (Barata et al. 2002; Campbell 2006). Freshwater invertebrates and vertebrates are highly susceptible to Cd, which causes a series of changes in cellular homeostasis in organisms at molecular and biochemical levels, such as changes in gene expression, DNA damage, oxidative stress, and interaction with bioelements (Đukić-Ćosić et al. 2020; Gama-Flores et al. 2017; Sadeq and Beckerman 2019; Wang et al. 2018). These sub-organism-level changes can ultimately lead to lethal or sublethal effects such as changes in feeding rate, somatic growth rate, and reproduction at organism levels. This can also lead to changes in population and community structures in aquatic habitats (Barata et al. 2002; Freitas and Rocha 2014). Furthermore, owing to its ability to induce metallothioneins, Cd can be bioaccumulated inside organisms (Fan et al. 2009). Cd accumulated at lower trophic levels can transfer to higher trophic levels through food chains (Ruangsomboon and Wongrat 2006).

Cladocerans, an essential component of freshwater zooplankton, are a useful group of organisms in aquatic toxicological studies for several reasons. Their role as a food link between phytoplankton and fish makes them useful in understanding the trophic dynamics of freshwater bodies (Mangas-Ramírez et al. 2004). Moreover, characteristics such as appropriate body size, sensitivity to toxicants, short generation time, high fecundity, and availability of basic data on culture conditions make them attractive candidates for ecotoxicological studies (Mangas-Ramírez et al. 2004). Furthermore, their parthenogenetic lifecycle results in genetically identical clonal study populations; hence, undesirable genetic differences can be controlled (Barata et al. 2002). Therefore, they are universally employed in routine bioassays of water (Gama-Flores et al. 2007). However, most toxicological studies have been carried out with species belonging to the genus *Daphnia*, especially *Daphnia magna*, irrespective of their limited occurrence in tropical water bodies (Gama-Flores et al. 2007; Garcia et al. 2004). Moreover, Sadeq and Beckerman (2019) reported that there is a marked variation in the susceptibility to metals across cladoceran species, where *D. magna* was found to be the least sensitive species to sublethal concentrations. On the other hand, genera such as *Moina* and *Diaphanosoma* show a wide distribution in both tropical and temperate water bodies and they have been less explored in scientific studies (Gama-Flores et al. 2007; Sarma and Nandini 2006). Therefore, the generation of toxicity data on members of these genera will be beneficial (Borase et al. 2019). *M. macrocopa* is one such widely distributed nontarget freshwater cladoceran, small in size (~1.0 mm) with a relatively thin carapace having a high sensitivity to pollutants. It is generally short-lived (2–3 weeks) and shows a high growth rate making it suitable for ecotoxicological studies (Borase et al. 2021; Gama-Flores et al. 2007; Garcia et al. 2004).

The limited number of previous reports on Cd toxicity in *M. macrocopa* in the literature is largely confined to identifying lethal effects (Garcia et al. 2004; Hatakeyama and Yasuno 1981; Pokethitiyook et al. 1987) and the effects of sublethal concentrations of Cd on life span (Gama-Flores et al. 2007), reproductive rate (Gama-Flores et al. 2007; Hatakeyama and Yasuno 1981; Wong and Wong 1990) generation time (Gama-Flores et al. 2007; Wong and Wong 1990), and population growth (Gama-Flores et al. 2006; Gama-Flores et al. 2007; Garcia et al. 2004; Wong and Wong 1990). In addition, Gama-Flores et al. (2017) have reported the effects of Cd on transgenerational demographic characteristics (i.e., life expectancy at birth, reproductive rates, generation times, and population increase rate) for multiple generations of Cd-exposed *M. macrocopa*, and Mangas-Ramírez et al. (2004) reported the recovery patterns of previously Cd-treated *M. macrocopa* in terms of life history variables (i.e., life span, life expectancy, reproductive rate, generation time, and population increase rate). However, in addition to the individual- and population-level responses, knowledge on subindividual (i.e., molecular and biochemical)-level responses is important as it enables a more realistic assessment of the impacts and provides insight into the causal mechanisms responsible for the effects observed at higher levels of organization (Jemec et al. 2010; Jemec et al. 2007). Furthermore, molecular and biochemical responses can be observed even at low concentrations, and such responses are considered to be rapidly responding endpoints (Jemec et al. 2008). Phromchaloem et al. (2018) have studied nuclear damage in *M. macrocopa* exposed to a single concentration of Cd (160 µg/L) by single-cell gel electrophoresis (Comet assay). They observed a significantly higher level of nuclear damage in the treated group than the control group. Other than this work, molecular-level responses of Cd exposure have not been studied in *M. macrocopa*. In fact, to our best knowledge, biochemical level responses (e.g., antioxidant enzyme activities) in *M. macrocopa* upon Cd exposure were not evaluated previously.

Studies have shown that Cd stimulates the production of reactive oxygen species (ROS) (e.g., superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{\bullet}$)), which subsequently cause oxidative damage of lipids, proteins, and DNA (Đukić-Ćosić et al. 2020). To minimize the oxidative damage to cellular components, organisms have developed antioxidant defense systems that include several antioxidant enzymes such as catalase (CAT, EC 1.11.1.6) and glutathione S-transferases (GST, EC 2.5.1.18) (Barata et al. 2005). CAT reduces H_2O_2 into water and GST catalyzes the conjugation of glutathione (GSH) with various xenobiotics and cytotoxic aldehydes produced during lipid peroxidation (Barata et al. 2005; Jemec et al. 2010). In several freshwater invertebrates, including cladocerans, alterations in the levels of antioxidant enzymes have been observed after exposure to Cd, which is an indication of oxidative stress responses (Barata et al. 2005; Kim et al. 2018; Parolini et al. 2018; Wu et al. 2013; Xie and Buchwalter 2011). In fact, these enzymatic responses are sensitive to very small concentrations of heavy metals including Cd (Kim et al. 2018).

The aim of this study was to determine Cd bioaccumulation in one of the least explored freshwater cladocerans, *M. macrocopa*, after a short-term exposure to different concentrations of Cd, and to establish relationships between and among Cd bioaccumulation and more sensitive suborganism-level molecular and biochemical endpoints including DNA alterations and antioxidant enzyme (i.e., CAT, GST) responses, respectively. To visualize and quantify Cd bioaccumulation, we utilized a novel Cd-sensitive synthetic fluorescence probe that has been successfully used to determine Cd internalization in an *in vitro* experiment (Hagimori et al. 2021). DNA alterations were determined using the randomly amplified polymorphic DNA (RAPD) technique. The RAPD

technique has the potential to detect a variety of DNA damage at the whole genome level and is more sensitive than the Comet assay (Plaire et al. 2013). Furthermore, the variations in whole-body CAT and GST enzyme activities were determined spectrophotometrically to gain an understanding of the oxidative stress responses and the toxicity mechanism of Cd in *M. macrocopa*. The results of this study provide insight into the dose–response relationships of bioaccumulation, DNA damage, and oxidative stress biomarkers to Cd exposure in *M. macrocopa*. Furthermore, this study highlights the applicability of molecular and biochemical biomarkers in toxicological assessments conducted with cladocerans.

Materials and Methods

Laboratory cultures of test organisms and holding conditions

Female *M. macrocopa* were initially obtained from a commercial aquarium and successfully cultured in 12 L glass aquaria at the Applied Ecological Engineering Laboratory, Saitama University, for several generations. Before their use in the experiments, clonal populations of *M. macrocopa* were established using single healthy parthenogenetic females (Fig. 1a). Clonal cultures were maintained in 1 L glass beakers with the stocking density controlled at ~100 individuals per beaker. Aerated (≥ 24 h) tap water was used as the culture medium and as the dilution water for all exposure experiments. The cultures were fed daily with dry baker's yeast suspension (25 mg/mL) until the water became cloudy in appearance (0.5–5 mL).

Regular checkups were carried out in all cultures for any signs of stress conditions (e.g., presence of non-parthenogenetic females (Fig. 1b) and ephippia (Fig. 1c)). The culture medium was renewed twice a week. Cultures were maintained at 22 ± 2 °C, 7.5 ± 0.8 pH, 7.3 ± 1.2 mg/L dissolved oxygen, 10.12 ± 0.66 mS/m conductivity and ~75 mg/L hardness as CaCO₃ with a photoperiod of a 16:8 light/dark cycle and under gentle aeration. All exposure experiments were conducted with female *M. macrocopa* neonates (<24 h old) (Fig. 1d) obtained from clonal cultures excluding the first brood progenies.

Acute toxicity tests

Acute toxicity tests were conducted in accordance with the Organization for Economic Cooperation and Development (OECD) guideline for testing of chemicals No. 202 (OECD 2004). Anhydrous CdCl₂ was obtained from FUJIFILM Wako Pure Chemical Corporation, USA (Wako 1st grade). On the basis of the results of a preliminary range finding test, nine test concentrations (1, 5, 10, 50, 100, 250, 500, 750, 1000 µg/L) were prepared using a 100 mg/L Cd stock solution. *M. macrocopa* neonates <24 h old were exposed to each test concentration and dilution water (control) in three replicates per test concentration and control, with 10 individuals per test vessel (glass petri dishes) placed with 25 mL of test solution. The toxicity tests were conducted at 22 ± 2 °C with a 16:8 light/dark photoperiod without aeration for 48 h under a semistatic renewal condition where the test medium was renewed after 24 h using freshly prepared stock solution and dilutions. The organisms were not fed during the tests. Survival and immobilization, the latter being defined as incapability of swimming within 15 s after gentle agitation, were recorded in each test vessel after 24 and 48 h of exposure. Immobilization results were used to

calculate the 24- and 48-h EC₅₀ values by probit analysis. Upon the termination of exposure, all surviving individuals (including the immobilized) were used to determine Cd bioaccumulation.

Cadmium bioaccumulation in *Moina macrocopa*

A novel fluorescence probe (Fig. 2) with high selectivity towards Cd ions (Hagimori et al. 2021) was used to visualize and quantify Cd bioaccumulation in *M. macrocopa*. This low-molecular-weight fluorescence probe (MW 218.06) possess a pyridine–pyrimidine core structure that functions as both a Cd²⁺ coordination site and a fluorophore and produces a fluorescence turn–on response when it binds with Cd²⁺ (Hagimori et al. 2021).

The surviving *M. macrocopa* neonates exposed to different Cd concentrations and the control were washed with 0.01 mol/L phosphate-buffered saline three times to remove Cd ions attached to the body surface. The washed neonates were incubated with 100 µmol/L Cd fluorescence probe prepared in 1% dimethyl sulfoxide (v/v %) for 1 h at 25°C in 6-well plates in the dark. After the incubation period, the *M. macrocopa* neonates were imaged using a fluorescence microscope (BZ-X810, Keyence, Osaka, Japan) with a 4x objective lens, BZ-X filter DAPI (Model OP-87762), and excitation and emission wavelengths at 360/40 and 460/50 nm, respectively. Fluorescence intensities of the images were determined using ImageJ software (National Institutes of Health, USA) with background corrections (Fitzpatrick 2014). For image merging, BZ-X analyzer software (Keyence, Osaka, Japan) was used.

Toxicity tests for the analysis of DNA damage and antioxidant enzymes

On the basis of the estimated 48-h EC₅₀ values, four sublethal Cd concentrations (1, 5, 10, and 50 µg/L), as well as a control, were selected to determine Cd-induced DNA damage and the levels of antioxidant enzymes. Because of the large sample size required for the molecular and biochemical analyses, two consecutive experimentally identical trials were carried out in 200 mL transparent glass jars with 150 mL of test solution with 60 neonates of *M. macrocopa* per replicate and 2–3 replicates for DNA analysis and enzyme assay, respectively. All the other experimental conditions were maintained the same as in the acute toxicity test described above.

Analysis of DNA damage

Genomic DNA extraction

At the end of the exposure, total genomic DNA was extracted from ~25 (pooled) surviving *M. macrocopa* neonates from each test concentration and control (2 replicates). Genomic DNA extraction was carried out under sterile conditions using a DNeasy® blood & tissue kit (QIAGEN, Hilden, Germany) in accordance with the manufacturer's instructions with slight modifications. Briefly, the neonates were placed in 1.5 mL microcentrifuge tubes, homogenized in 180 µL of buffer ATL and 20 µL of proteinase K using a micropestle and mixed by vortexing for 15 s. The samples were then incubated at 56°C (Eyela SLI-220, Tokyo, Japan) for 3 h for tissue lysis with occasional vortexing. At the end of the incubation period, the lysates were vortexed for 15 s, 200 µL of buffer

AL was immediately added, mixed, and again incubated at 56°C for 10 minutes. After incubation, 200 µL of 99.5% ethanol was added and mixed by vortexing. The mixtures were pipetted into DNeasy Mini spin columns placed in 2 mL collection tubes and centrifuged at 6,000 g (KINTARO-18, TOMY, Japan) for 1 min at room temperature. The flow-through and collection tubes were discarded, and the spin columns were sequentially washed with buffer AW1 (500 µL) and buffer AW2 (500 µL) by centrifuging at 6,000 g (1 min) and 17,000 g (3 min) at room temperature, respectively, using new 2 mL collection tubes each time. The flow-through and collection tubes were discarded each time, and the DNA was eluted into new 1.5 mL microcentrifuge tubes with 200 µL of buffer AE by centrifuging for 1 min at 6,000 g after an incubation period of 1 min at room temperature after adding buffer AE. The DNA purity and concentration were determined using a WPA Biowave DNA Life Science UV/Vis spectrophotometer (Biochrom Cambridge, UK). The software provides the DNA concentration in µg/mL and the absorption ratios of A260/280 and A260/230 to estimate the DNA purity. Furthermore, the integrity of the extracted DNA was confirmed by capillary electrophoresis. The extracted DNA was subjected to RAPD PCR on the same day.

RAPD-PCR analysis

Cd-induced genotoxicity assessment of *M. macrocopa* was carried out by the RAPD-DNA technique as described for *D. magna*, by Atienzar et al. (1999), Atienzar et al. (2001), and Parisot et al. (2015). Ten RAPD primers (10-mer oligonucleotides) that were previously used in the genotoxicity assessment of *D. magna* (Atienzar et al. 2001; Atienzar and Jha 2004) were selected after a preliminary analysis. The primers (Table 1) were obtained from Eurofins Genomics, Tokyo, Japan. The RAPD polymerase chain reaction (PCR) was carried out using the QIAGEN® multiplex PCR plus kit (QIAGEN, Hilden, Germany) in accordance with the manufacturer's instructions with slight modifications. Each reaction mixture contained 25 µL of the multiplex PCR master mix, 10 µL of the primer mix (25 pmol from each primer), and variable volumes of RNase-free water and template DNA (100 ng) to make a final volume of 50 µL. DNA amplification was carried out on 2720 Thermal Cycler (Applied Biosystems®, Thermo Fisher Scientific, USA) with an initial activation at 95°C (5 min) followed by 45 cycles of denaturation at 95°C (30 s), annealing at 36°C (90 s) and extension at 72°C (90 s). A final extension and a cooling step (until capillary electrophoresis) were carried out at 68°C (10 min) and 15°C, respectively.

Capillary electrophoresis and analysis of DNA profiles

The PCR products were electrophoresed with the QIAxcel Advanced instrument (QIAGEN, Hilden, Germany) using the QIAxcel DNA screening kit in accordance with the protocol for the determination of DNA fragment sizes using the QIAxcel ScreenGel Software (QIAGEN, Hilden, Germany). Method AM320 with a sample injection time of 10 s was employed for the migration parameters. QS DNA Size Marker 100 bp – 2.5 kb (Cat. 929559) and QS Alignment Marker 15 bp/ 3 kb (Cat. 929522) (QIAGEN, Hilden, Germany) were used to determine DNA fragment sizes. RAPD profiles were analyzed using QIAxcel ScreenGel Software (QIAGEN, Hilden, Germany).

Estimation of the percentage genomic template stability

Genomic template stability (GTS, %) was estimated in accordance with Atienzar et al. (2001) and Baurand et al. (2015). Briefly, the RAPD profiles of the Cd-treated groups were scored on the basis of different changes observed compared with the control. The changes considered were (1) the appearance of a new band, (2) the disappearance of a band, (3) increased band intensity, and (4) decreased band intensity (Fig. 3). Each change was given an arbitrary value of +1. GTS (%) was calculated for each Cd-treated group using,

$$GTS (\%) = \left(1 - \frac{a}{n}\right) \times 100,$$

where a is the average number of changes observed in a DNA profile, and n is the total number of bands in the control profile.

Analysis of antioxidant enzymes

Sample preparation

At the end of the exposure, the surviving *M. macrocopa* neonates in each test vessel were washed twice with 100 mM potassium phosphate buffer of pH 7.4 and 40–50 individuals were pooled from each replicate in 1.5 mL centrifuge tubes. The pooled individuals were homogenized in 1 mL of ice-cold potassium phosphate buffer (100 mM, pH 7.4) using an ultrasonic tissue homogenizer (Astrason XL2020, New York, USA). Homogenates were centrifuged at 10,000 g for 10 min at 4°C, and the supernatants were immediately used as the source for enzymes and for protein assays (Barata et al. 2005). Each measurement was performed in triplicate.

Catalase

CAT activity was determined in accordance with Claiborne (1985). The enzyme supernatant (50 µL) was mixed with 19 mM hydrogen peroxide (950 µL) prepared in 50 mM potassium phosphate buffer of pH 7.0 and the decrease in absorbance at 240 nm was measured for 2 min using a spectrophotometer (UV-1280, Shimadzu, Kyoto, Japan). CAT activity was expressed as micromoles of hydrogen peroxide consumed per minute per milligram of protein sample (µmol/min/mg protein), taking 0.0436 mM⁻¹cm⁻¹ as the molar extinction coefficient of hydrogen peroxide.

Glutathione S Transferase

GST activity was determined in accordance with Habig et al. (1974), as described by Im et al. (2020) for *D. magna*. 1-Chloro-2,4-dinitrobenzene (CDNB) was dissolved in 15 mL of ethanol (99.5%) and diluted to 250 mL with 100 mM potassium phosphate buffer (pH 6.5) to make a 3 mM stock solution. A solution of reduced glutathione was prepared in 100 mM potassium phosphate buffer (pH 6.5) to a final concentration of 0.01 M. One milliliter of CDNB (3 mM) and 300 µL of reduced glutathione (0.01 M) were mixed with 1.65 mL of 100 mM potassium

phosphate buffer (pH 6.5). After adding 50 μ L of the enzyme supernatant, the increase in absorbance at 340 nm was measured for 5 min. GST activity was expressed as micromoles of S-2,4dinitrophenyl glutathione conjugate formed per minute per milligram of protein sample (μ mol/min/mg protein), taking 9.6 mM⁻¹cm⁻¹ as the molar extinction coefficient of the conjugate.

Protein

Protein content of the supernatant was measured using the BCA Protein Assay Kit (Pierce™, ThermoFisher Scientific) in accordance with the manufacturer's instructions using the standard test tube protocol.

Statistical analysis

All statistical analyses were performed using Minitab® 19 statistical software. The data were tested for normality using the Anderson–Darling test at $p < 0.05$. One-way analysis of variance (ANOVA) followed by Tukey's post hoc test was conducted to evaluate the statistically significant differences ($p < 0.05$) among Cd bioaccumulation, GTS (%), and oxidative stress responses in the control and treated samples.

Results

Survival and immobilization

Survival percentages of *M. macrocopa* were decreased with increasing Cd concentration and exposure duration (Fig. 4a), whereas immobilization percentages were increased with increasing Cd concentration and exposure duration (Fig. 4b). All individuals exposed up to 50 μ g/L showed 100% survival until 48 h but showed clear signs of immobilization even in the lowest concentration tested (1 μ g/L). Control groups did not show any sign of immobilization until the end of the exposure assessment. Neonates exposed to Cd ≥ 750 μ g/L did not survive to 48 h.

On the basis of immobilization data, the 24- and 48-h median effective concentrations (EC₅₀) were calculated as 260.84 and 107.64 Cd μ g/L, respectively, using nominal Cd concentrations (Table 2).

Cadmium bioaccumulation

Bioaccumulation of Cd in *M. macrocopa* was estimated using a novel synthetic fluorescence probe that has a high selectivity towards Cd. The probe was previously used to examine Cd internalization in mouse macrophage cells *in vitro* (Hagimori et al. 2021).

Analysis of the fluorescence images of live *M. macrocopa* incubated with the fluorescence probe after treating with different concentrations of Cd showed a concentration-dependent Cd bioaccumulation (Figs. 5 & 6) where a statistically significant fluorescence enhancement was detected in neonates treated with ≥ 5 μ g/L compared with

the control group ($p<0.05$). However, appreciably higher fluorescence signals were detected in *M. macrocopa* treated with ≥ 100 $\mu\text{g/L}$. As shown by the fluorescence images, clear fluorescence enhancements can be seen in the intestinal tracts of the neonates, indicating that Cd is localized mostly in the gut region (Fig. 6).

DNA damage

Referring to the results of the acute toxicity study, four sublethal Cd concentrations ($\sim 50\%$, $\sim 10\%$, $\sim 5\%$, and $\sim 1\%$ of 48 h- EC_{50}) were selected to study the genotoxic effects of Cd in *M. macrocopa* by the RAPD technique, which is considered as one of the sensitive methods that can detect variations in genome profiles of different organisms (Baurand et al. 2015; Kokina et al. 2020; Maselli et al. 2017).

As Cd concentrations increased, there was an observable corresponding increase in the changes in the RAPD profiles of *M. macrocopa*. The changes seen in RAPD profiles included the appearance of a new band, disappearance of a normal band, increased band intensity, and decreased band intensity in compared with the RAPD profile of the control group. Bands with such changes were considered as polymorphic bands. The number of polymorphic bands observed in each group treated with different Cd concentrations is given in Table 3. The largest average number of polymorphic bands (7.5) was observed in neonates treated with 50 $\mu\text{g/L}$ of Cd, where the lowest (2.5) was observed in the neonates treated with 1 $\mu\text{g/L}$ of Cd.

The GTS (%) of each test group was calculated using the number of polymorphic bands observed in each experimental group. The GTS of the control group was fixed as 100% and the rest of the GTS values were calculated relative to the control group. *M. macrocopa* treated with ≥ 10 Cd $\mu\text{g/L}$ showed significant GTS reductions ($> 20\%$ from the control) ($p<0.05$) where the highest GTS reduction was observed in the group treated with 100 Cd $\mu\text{g/L}$ ($>30\%$ from the control) (Fig. 7).

Oxidative stress

The same set of sublethal Cd concentrations were selected to study oxidative stress enzyme activity responses in *M. macrocopa*. Activities of two important antioxidant enzyme systems, CAT and GST, which play a major role in ROS-associated stress, were evaluated in each sublethal Cd concentration.

Numerically, a concentration-dependent enhancement of CAT activity was observed; however, statistically, the values were less significantly different from each other ($F_{4,10}=3.11$, $p=0.066$) (Fig. 8a). In contrast, GST activities showed a concentration-dependent enhancement up to 10 $\mu\text{g/L}$ where it was significantly higher than that of the control group ($p<0.05$) (Fig. 8b). Interestingly, GST activities of the neonates exposed to 50 $\mu\text{g/L}$ showed a significantly lower level ($p<0.05$), indicating possible GST inhibition at higher concentrations.

Discussion

In the present study, the bioaccumulation of Cd in one of the less explored, yet important freshwater cladocerans, *M. macrocopa*, was assessed, and associated molecular and biochemical changes (i.e., DNA damage and antioxidant enzyme responses, respectively) were determined in an attempt to understand the mechanism of Cd toxicity in *M. macrocopa*.

Since there is little published work on Cd toxicity in *M. macrocopa* and variation in the limited number of previous findings, the EC₅₀ value on immobilization in *M. macrocopa* was determined using neonates <24 h old, where a basic understanding of Cd toxicity in the organism was obtained. In the present study, the 24- and 48-h EC₅₀ values were estimated as 260.84 and 107.64 Cd µg/L, respectively. In previous studies, Garcia et al. (2004) reported a 24-h LC₅₀ value of 680 Cd µg/L for neonates 24 ± 2 h old, Hatakeyama and Yasuno (1981) reported 48- and 72-h LC₅₀ values of 71 and 28 Cd µg/L, respectively, for neonates 12 ± 12 h old, and Pokethitiyook et al. (1987) reported 24- and 48-h LC₅₀ values of 218 and 13 µg/L, respectively, for neonates 12 ± 2 h old. The differences among these toxicity values may have been attributed to the type of endpoint considered (lethality or immobilization), different test media, age of the test organisms, purity of tested heavy metals, different salts of heavy metals, and exposure conditions.

Cd can be bioaccumulated in freshwater organisms including cladocerans (Ruangsomboon and Wongrat 2006). Cd accumulated by organisms is mostly bound to metallothionein, a low-molecular-weight, cysteine-rich protein. This sequestration of Cd reduces the amount of free metal ions responsible for toxic effects, and hence is regarded as a method of detoxification (Fan et al. 2009; Hamilton and Mehrle 1986). Nevertheless, the determination of Cd bioaccumulation is important as it is the link between exposure and toxicity, and it also yields information on the trophic transfer of Cd. In some previous studies, Cd bioaccumulation in *M. macrocopa* has been determined by measuring Cd-binding protein levels (Yamamura et al. 1983), or by measuring total Cd levels in the body by analytical techniques such as atomic absorption spectrometry (Ruangsomboon and Wongrat 2006; Tan and Wang 2011). However, toxicologically, the free Cd ions in the body are responsible for toxicological effects. In this regard, fluorescence sensors for metal ions are attractive alternatives (Carter et al. 2014; Hagimori et al. 2015). In the literature, various fluorescence sensors have been developed to target different metal ion, but only a few have been used for the *in vivo* detection of metal ions in different aquatic organisms. For example, Yang et al. (2007) used a rhodamine-based fluorescence probe to detect mercury ions in zebrafish embryo, He et al. (2019) used an aggregation-induced emission fluorogen to detect the bioaccumulation of mercury ions in the freshwater zooplankton *Daphnia carinata*, Liu et al. (2016) used a rhodamine-based fluorescence probe to detect palladium ions in zebra fish and *D. magna*, Liu et al. (2016) used a two-photon ratiometric fluorescence probe to detect zinc ions in zebrafish, and Lv et al. (2020) used a benzopyran-based fluorescence probe to detect the bioaccumulation of mercury ions in zebrafish. In the present study, we used a low-molecular-weight fluorescence probe with a high sensitivity towards Cd ions, which was previously used to detect cellular Cd ions (Hagimori et al. 2021). The results of the present study indicated a dose-dependent Cd bioaccumulation in *M. macrocopa*, especially in the gut region. Using radioactively labeled Cd in the exposure media, Carney et al. (1986) found that Cd taken up from solution is concentrated in the exoskeleton of *D. magna*. In another study, the distribution of radioactively

labeled Cd was studied in the freshwater cladoceran *Ceriodaphnia dubia*, and Cd was found to be mainly accumulated in the diverticula of the gut (Munger et al. 1998). In different cladoceran species, the uptake and bioaccumulation patterns of Cd are different, and it is documented that this is because Cd is taken up by aquatic organisms as a mimic of Ca through Ca uptake pathways, the mechanisms of which differ from species to species (Smirnov 2017; Tan and Wang 2011).

The genotoxicity of Cd is well documented for several aquatic organisms (Zhang and Reynolds 2019); however, little is known about the genotoxic effects of Cd in *M. macrocopa*. In the present study, we utilized the RAPD technique to determine the genotoxic effects of Cd in *M. macrocopa*. In genotoxicity assessment, the most widely used technique is single-cell gel electrophoresis or the Comet assay. Phromchaloem et al. (2018) used the latter technique to determine nuclear damage in *M. macrocopa* (<24 h old) by exposing them to 160 µg/L for 48 h and observed significant DNA damage. However, they reported that the nuclear damage was at a minimal level when compared with the results of another study by Pellegrini et al. (2014) who observed significant nuclear damage in *D. magna* exposed to 10 µg/L for 24 h using the same technique. In the present study, we observed significant DNA damage in *M. macrocopa* exposed to ≥ 10 µg/L Cd by the RAPD technique. The RAPD technique has been used in several previous studies to determine the genotoxic effects in *D. magna* exposed to different stressors including heavy metals (Atienzar et al. 2001; Plaire et al. 2013), polycyclic aromatic hydrocarbons (Atienzar et al. 2002; Atienzar and Jha 2004), nano-particles (Maselli et al. 2017), and ionizing radiation (Parisot et al. 2015). In general, the most important advantages of the RAPD technique in genotoxicity assessment are that this technique does not require any prior knowledge of the genome and it has the potential to detect a wide range of DNA damage (e.g., DNA adducts, DNA breakage) and mutations (e.g., insertion, rearrangement, deletion) (Baurand et al. 2015). However, there are some limitations of this method, such as its qualitative or semi-quantitative nature, and the possibility of exhibiting same modification of the DNA profile for different types of DNA alterations (Baurand et al. 2015).

Most of the studied mechanisms of Cd-induced DNA alterations are those that are linked with oxidative stress and the ability of Cd to influence different cellular proteins and their functions in the cell (e.g., DNA damage repair proteins) (Đukić-Ćosić et al. 2020; Zhang and Reynolds 2019). In the present study, the levels of two important antioxidant enzymes (i.e., CAT, GST) were measured to gain insight on the mechanism of the observed DNA damage. To our best knowledge, this is the first report providing information about antioxidant enzyme activities in *M. macrocopa* upon Cd exposure. CAT activity was found to be increased numerically with increasing exposure concentrations, but these values were less statistically significant from each other. In contrast, GST activities increased significantly up to 10 µg/L, but a significant reduction was observed in the neonates exposed to 50 µg/L. These results indicate that, although Cd is a redox inactive metal (Đukić-Ćosić et al. 2020), increasing Cd concentration increases the intracellular ROS level. It was observed in previous studies that although Cd does not appear to generate free radicals, lipid peroxidation is elevated in tissues soon after exposure (Barata et al. 2005; Stohs and Bagchi 1995). To minimize the oxidative damage that may be caused by these ROS, antioxidant levels may increase as a detoxification process. Similar patterns of antioxidant responses in other types of cladocerans upon exposure to Cd have been observed elsewhere. Barata et al. (2005) observed a significant increase in GST activities in *D. magna* exposed to 5 µg/L Cd (highest concentration tested) compared with the

control group. However, in the same study, CAT activities were significantly reduced in *D. magna* exposed to 5 µg/L Cd. Kim et al. (2018) reported a significant increase in GST activity in *D. magna* exposed to 10 µg/L Cd (the highest concentration tested). In support of this finding, the same group reported a significant reduction of GSH content when the exposure Cd concentration was increased. Jemec et al. (2008) reported that there were no significant changes in CAT or GST activities in *D. magna* exposed to Cd up to 1.31 µg/L. However, in the present study, we found that the GST activity in neonates exposed to 50 µg/L is significantly lower than the GST activity in neonates exposed to 10 µg/L. This may be an inhibition of GST activity by the high Cd concentration. In fact, Cd has the ability to bind to thiol (-SH) groups of proteins such as GSH, which is the substrate for GST. This inactivation of GSH will reduce the cellular antioxidant potential and increase the oxidative stress (Kim et al., 2018).

In conclusion, findings from the present study indicated that the bioaccumulation of Cd in *M. macrocopa* causes oxidative stress even at sublethal concentrations (≥ 10 Cd µg/L) via the production of ROS, as well as a potential inhibition of cellular antioxidants (i.e., GST) at higher concentrations (50 Cd µg/L). These oxidative stress responses could be one of the underlying mechanisms of the DNA damage observed in neonates exposed to ≥ 10 Cd µg/L. Taken all together, the results of our study improve the understanding of molecular and biochemical responses in one of the least explored freshwater cladocerans, *M. macrocopa*, upon exposure to Cd. Furthermore, we suggest that the molecular and biochemical endpoints we used in the present study provide more sensitive toxicity details than the lethal and life history variables commonly employed in previous studies, in a short-exposure duration. Therefore, these molecular and biochemical biomarkers could be used for routine toxicity assessments in *M. macrocopa*. To facilitate further comparisons to expand knowledge about the toxicity of Cd in *M. macrocopa*, as well as to compare sensitivity differences with other commonly used species, more research on molecular and biochemical endpoints is needed in future studies.

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Statements and Declarations

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Competing Interests

The authors declare that they have no financial or non-financial interests to disclose.

Author Contributions

Takeshi Fujino and Thilomi Samarakoon conceptualized and designed the experiment. Experiments and data analyses were performed by Thilomi Samarakoon under the supervision of Takeshi Fujino. The novel Cd selective fluorescence probe was provided by Masayori Hagimori. Rie Saito assisted in DNA analysis. The first draft of the manuscript was written by Thilomi Samarakoon, and all authors have commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Data Availability

The data generated and analyzed during this study will be available upon proper request to the corresponding author.

Figures

Fig. 1 *M. macrocopa* from laboratory cultures: (a) parthenogenetic (asexual) female with the brood pouch, (b) non-parthenogenetic (sexual) female with ephippia, (c) a free ephippium, and (d) neonate <24 h old

Figure 1

Please See image above for figure legend.

Fig. 2 4-(Methylsulfany)-6-(pyridin-2-yl)pyrimidin-2-amine (λ ex = 334 nm, λ em = 412 nm)

Figure 2

Please See image above for figure legend.

Fig. 3 Example of RAPD profile of *M. macrocopa* treated with different concentrations of Cd (0–50 µg/L). Different numbers indicate different changes compared with the control: (1) appearance of a new band, (2) disappearance of a new band, (3) increased band intensity, and (4) decreased band intensity.

Figure 3

Please See image above for figure legend.

a

b

Fig. 4 (a) Survival percentages and (b) immobilization percentages of *M. macrocopa* neonates exposed to different concentrations of Cd for 48 h. Data are presented as mean $\pm$ SEM from three replicates ($n=10$ per replicate).

Figure 4

Please See image above for figure legend.

Fig. 5 Fluorescence intensities of the fluorescence images of *M. macrocopa* neonates exposed to Cd (0–500 µg/L) for 48 h followed by incubation with the Cd-sensitive fluorescence probe for 1 h. Data are presented as mean $\pm$ SEM from three replicates ($n=5$ per replicate). In each concentration, mean values with different letters are significantly different from each other ($p<0.05$).

Figure 5

Please See image above for figure legend.

Fig. 6 (a) Brightfield, (b) fluorescence, and (c) merged images of *M. macrocopa* treated with different concentrations of Cd (0–500 µg/L) for 48 h followed by 1 h incubation with 100 µmol Cd fluorescence probe.

Figure 6

Please See image above for figure legend.

Fig. 7 GTS (%) of *M. macrocopa* neonates after 48 h exposure to different sublethal concentrations of Cd (0–50 µg/L). Data are presented as mean $\pm$ SEM from two replicates ($n=25$ pooled per replicate). Mean values with different letters are significantly different from each other ($p<0.05$).

Figure 7

Please See image above for figure legend.

a

b

Fig. 8 (a) CAT and (b) GST activities in *M. macrocopa* neonates exposed to different sublethal Cd concentrations (0–50 μg/L) for 48 h. Data are presented as mean ± SEM from three replicates ($n=40$ –50 pooled per replicate). Mean values with different letters are significantly different from each other ($p<0.05$).

Figure 8

Please See image above for figure legend.

Supplementary Files

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