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Assessment of Copper Total Bioaccumulation and Genotoxicity in Boac River, Marinduque Island, Philippines Two Decades Post-Mining Disaster: *Pseudodon* sp. as Aquatic Fauna Indicator

Glenn L. Sia Su[1]✉ • Ma. Liezel L. Del Mundo[1] • Eliza Katrina D. Barredo[1] • Maria Lilibeth L. Sia Su[1] • Gliceria B. Ramos[2]

[1] University of the Philippines Manila, PHILIPPINES
[2] De La Salle University Manila, PHILIPPINES

Abstract

In 1996, a mining drainage tunnel burst and released copper wastes into the Boac River—rendering it dead. Two decades after the disaster, the river is now used for aquaculture farming. This study assessed the total bioaccumulation of copper and its genotoxicity in the mussel *Pseudodon* sp. farmed in the river. A total of 30 *Pseudodon* sp. were collected randomly from the downstream tributaries of the river in September 2015. Total bioaccumulation in terms of total copper concentrations was determined by atomic absorption spectrometry and the genotoxicity by micronucleus cytome assay. The total copper concentrations were present in the soft tissues (Mean + Standard Error of the Mean [SEM] of 0.0550 + 0.0003 ppm). Genotoxic indicators of nuclear alterations showed the formation of micronuclei yielding the highest frequency (Mean + SEM of 10.257 + 0.793 per 1000 cells). Binucleated, nuclear buds, agranular, apoptotic, and necrotic nuclear anomalies were likewise evident. Total copper concentrations and the frequency of the hemocyte micronuclei occurrences showed a significant relationship ($r=0.366$; $p = 0.047$) while the other nuclear abnormalities were not significantly related to the total copper concentrations. Continuous biomonitoring of mining-affected areas is necessary to safeguard people's health from the effects of mining wastes.

Keywords: bioaccumulation · copper · energy use · genotoxicity · heavy metal · mining · nuclear abnormalities

Correspondence: GL Sia Su. Department of Biology, College of Arts and Sciences, University of the Philippines Manila, Padre Faura St., Ermita, Manila 1000, Philippines. Contact number: +639478198532. Email: glsiasu@up.edu.ph

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Assessment of Copper Total Bioaccumulation and Genotoxicity in Boac River, Marinduque Island, Philippines Two Decades Post-Mining Disaster: *Pseudodon* sp. as Aquatic Fauna Indicator

Glenn L. Sia Su[1]✉ • Ma. Liezel L. Del Mundo[1] • Eliza Katrina D. Barredo[1] • Maria Lilibeth L. Sia Su[1] • Gliceria B. Ramos[2]

[1] University of the Philippines Manila, PHILIPPINES
[2] De La Salle University Manila, PHILIPPINES

Introduction

Marinduque is an island province situated in the southern part of Luzon, Philippines. It lies between Tayabas Bay in the north and Sibuyan Sea in the south. The island is located south and west of Quezon Province, east of Mindoro Island, and north of Romblon Province. The municipality of Boac serves as its capital and the 26-km long Boac River divides the island into the northern and southern areas as well as serves as the town's major water system.

The province relies on its rich agricultural land and vast fishing grounds. Mining used to be its principal industry where copper ores were extracted. The mining operation started in 1969 and initially used an open-pit mine in Mount Taipan where the copper concentrate was disposed of (Hamilton-Paterson 1997). From 1975 up to 1992, a mining company operated on the island and used the nearby Calancan Bay as their main dumping site for tailings. The mining corporation also built a tunnel where the water from the mining pit drained to the Boac River. This tunnel was later sealed after the reserve in Mount Taipan was depleted and the corporation made use of the Mount Taipan pit as their tailing dam for the new operation in the neighboring municipality of San Antonio. On 24 March 1996, the plug that sealed the tunnel from Mount Taipan pit to the Boac River ruptured. This disaster resulted in the release of mine copper wastes directly into the river at a rate of 5-10 cubic meters per second (Coumans 2006).

The disaster resulted in the heavy metal contamination of the Boac River, which killed the aquatic organisms and affected the livelihood of the locals. The Boac River was declared dead and unusable by the government. The presence of copper-containing compounds, such as copper silicates and copper sulfates, were recognized as the primary pollutant of the river system (David 2003). In addition, the oxidation of copper or iron sulfide-containing minerals in the sediment also posed a problem as it made the river water acidic (Soriano 2015; David and Plumlee 2006).

The Boac River showed gradual recovery over the years. Small fish and thin vegetation have been spotted even though the river may still be contaminated with heavy metals. Hence, there remains a need to undertake environmental monitoring in the mine-affected sites, especially because continuous exposure to the mine wastes has detrimental risks to the community. Despite this, the locals still rely their livelihood on the river. Fisher folks practice mussel farming in the river system, oblivious of the accumulation of copper on the soft tissues of mussels and the possible genotoxic effects.

Freshwater mussels are the aquatic organisms commonly used to determine biological responses to heavy metals. Mytilid and Unionid mussels (Vosloo et al. 2011; Kinn 2013) are used in biomonitoring programs as they are efficient accumulators of contaminants (Nicholson 1999; Fedato et al. 2010). Genotoxicity biomarkers in aquatic biomonitoring programs help in establishing associations between the genotoxicity and the chronic health effects of the species as responses to the current environmental stress over changes that occurred during their exposure (Bolognesi et al. 1995).

With this, the study aims to assess the total bioaccumulation of copper and its genotoxic impact on the mussels being farmed in the Boac

River and provide updated information on the condition of the water ecosystem in the area using aquatic fauna as an indicator.

Methodology

Sampling collections of mussel samples *Pseudodon* sp. (Figure 1) were done along the creek side of Boac River, Barangay Buliasnin (Figure 2). The sample size for estimating a single proportion was used with a Z of 1.645 for an alpha of 90% and desired error of 10%. The species richness estimate was at 88% (Miller et al. 2017). A total of 30 mussels were picked individually by hand within a 30-meter transect line along the length of the river at a depth of 2.0 ft. The collection was done in September 2015. The specimens were identified and certified by the Philippine National Museum's Zoology Division (Control # Concho-2015-17).

FIGURE 1 Profile of the mussel sample, *Pseudodon* sp. identified and certified by the Zoology, Division, Philippine National Museum (Control # Concho-2015-17)

The samples were placed in a styropor box (40-liter capacity), spiked with an aerator with clean distilled water, and transported to the laboratory for weight measurement to the nearest gram (g) using a digital balance (±0.1g). The length, width, and height were measured to the nearest millimeter (mm) using a Vernier caliper (± 0.1mm). The length was measured at a maximum distance along the long axis of the valve. The height was measured at a maximum distance from the hinge to the ventral margin and the width/depth/thickness at a maximum distance between the outer edges of the two valves.

One gram of soft tissue from each mussel was obtained for the determination of total copper concentration. The soft tissues were processed by acid digestion with concentrated nitric acid (Merck). The acid-digested tissue samples were filtered using Whatman filter paper (no. 42) into acid-washed plastic bottles. The total concentration was determined using an Atomic Absorption Spectrophotometer Shimadzu AA-6300 (Shimadzu Scientific Instruments, Inc., Kyoto, Japan). The concentrations for each sample were measured in parts per million (ppm) and were read in triplicates. Calibration curves and standard solutions were prepared before analysis.

The hemolymph was collected from the posterior adductor muscle sinus of each mussel. Hemolymph collection was performed using a hypodermic syringe containing 0.2 ml of PBS buffer. The aspirated hemolymph samples were transferred into microtubes and stored at 4°C before slide preparation.

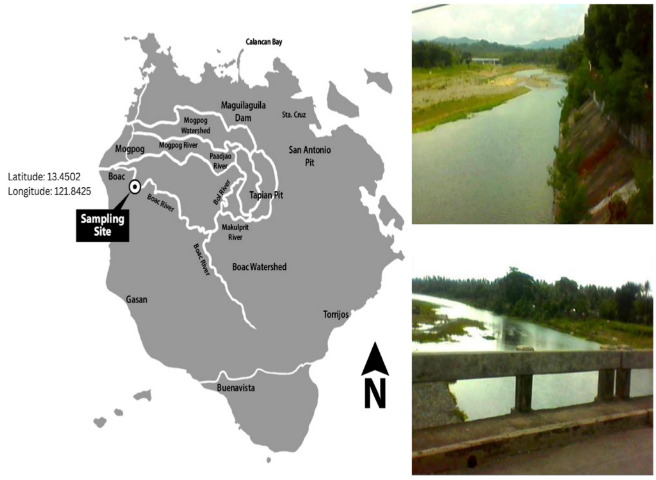

FIGURE 2 Map of Municipality of Boac, Marinduque, Philippines, and the surrounding localities showing the sampling site (Boac River) where the *Pseudodon* sp. Mussels were collected

Hemocyte slides were prepared following the micronucleus cytome assay of Bolognesi and Fenech (2012). Approximately 100 µl of the hemolymph was dropped onto a glass slide placed in a humid chamber for 15 minutes and air-dried for 10 minutes at room temperature. Cells were subsequently fixed by immersing the slides in 100% methanol for 10 minutes and air-dried for another 10 minutes at room temperature. The fixed slides were stained with 3% (vol/vol) Giemsa solution for 5 minutes and rinsed with distilled water for another 5 minutes before it was examined under the microscope at 1000× magnification. The frequencies of cells classified as granulated, agranulated, necrotic, apoptotic, micronuclei, binucleated, and nuclear buds were counted from among 1,000 cells per slide following the guide provided by Bolognesi and Fenech (2012).

Statistical Analysis

The mean copper concentration, mean frequencies of each nuclear abnormality, and corresponding standard error of the mean for all 30 samples were computed using descriptive statistics. Linear regression was used to test for the significant relationship between the mean total copper concentrations of the soft tissues and the mean frequencies of each hemocyte nuclear abnormalities set at $p<0.05$. All statistical analyses were performed using the GNU PSPP software.

Results and Discussion

The incurred mean (+SEM) measurements were 70.11 mm (+1.43) for length, 33.60 mm (+0.615) for width, and 14.17 mm (+0.46) for height. The total copper concentrations of the mussels are shown in Table 1. The mean +SEM total copper concentrations of the mussels *Pseudodon* sp. was 0.0550 (+0.0003) mg/kg. In the genotoxicity assessment, the mean + SEM frequencies of the granulated, agranulated, necrotic, apoptotic cells, micronuclei, binucleated, and nuclear buds were accounted for (Table 2).

The most frequent cells observed were the micronuclei cells. Ranked second in frequency of occurrence were the binucleated and nuclear buds' cells while the least were the apoptotic, necrotic, and agranular cells. The hemocyte cells and nuclear abnormalities observed in the *Pseudodon* sp. are shown in Figure 3.

The mean copper concentration in the soft tissues was significantly related ($r=0.366$; $P=0.047$) with micronuclei abnormality (Figure 4). All the other nuclear abnormalities did not show significant relationship with the copper concentration (Table 3 and Figure 5).

The Boac River, classified as a Class C water system, is suitable for the propagation and growth of aquatic resources, recreational purposes like boating, and manufacturing processes (Department of Environment and Natural Resources - Environmental Management Bureau 2011). The same government agency reported that the Boac River had an average of 0.02 mg/L (ppm) dissolved copper and hence qualified for the 0.05 mg/L (ppm) limit for dissolved copper set for Class C freshwater bodies. However, this study shows that the mean copper concentration in mussels (0.0550 + 0.0003 ppm) is higher than the reported dissolved copper level in the river waters. It also shows that the *Pseudodon* sp. used as an aquatic fauna indicator is an efficient copper accumulator.

The mean copper concentration found in the mussels (0.0550 + 0.0003 ppm) is below the 10 ppm maximum permissible level of the Food and Agriculture Organizations of the United Nations (2011). However, the accumulated copper concentrations in the mussel samples exceeded the range of normal daily intake of copper of 0.014-0.019 ppm (US Environmental Protection Agency 2004). The metal analysis of the mussels exceeded the standards for the daily intake of mussels for the copper concentrations.

The paper's results show that the genotoxic effects in the hemocyte cells have a significant relationship with the copper accumulated in the tissues. The occurrence of micronuclei frequencies in the bivalves provided an index on the accumulated genetic damage in the cells (Bolognesi et al. 1995) of the organism. This result is corroborated in a study by Lloyd and Philipps (1999), where they indicated that higher copper concentrations in the tissue could induce reactive oxygen species (ROS) production, which may cause Deoxyribonucleic (DNA) acid strand breaks.

The recorded high mean frequency of the micronuclei conforms with other previous studies (Fernando et al. 2007; Mersch et al. 1996; Dailianis et al. 2003; Dailianis 2010) where the authors

TABLE 1 The total copper concentrations of the mussels, *Pseudodon* sp. collected from Boac River, Marinduque Island, Philippines

Specimen	Concentrations (ppm)
1	0.0636
2	0.0384
3	0.0818
4	0.1706
5	0.0492
6	0.0395
7	0.0380
8	0.0785
9	0.0412
10	0.0441
11	0.0381
12	0.0501
13	0.0449
14	0.0360
15	0.0608
16	0.0581
17	0.0823
18	0.0577
19	0.0512
20	0.0612
21	0.0383
22	0.0454
23	0.0575
24	0.0401
25	0.0451
26	0.0441
27	0.0457
28	0.0513
29	0.0526
30	0.0446

TABLE 2 Mean + SEM frequency of the hemocyte nuclear abnormalities in *Pseudodon* sp. collected from Boac River, Marinduque Island, Philippines

Nuclear abnormalities	Mean + SEM frequency/1000 cells examined
Agranular	0.450 + 0.120
Apoptotic	1.333 + 0.271
Binucleated	6.550 + 0.649
Micronuclei	10.257+0.793
Necrotic	1.000 + 0.218
Nuclear buds	2.283 + 0.344

indicated that high micronuclei occurrences in contaminated zones affected by discharges from industry, heavy metals, and other organic pollutants are likely to happen. The study found a significant relationship between the total copper concentrations in the soft tissues and the micronuclei frequencies in the hemocytes. A study by Bolognesi et al. (1999) indicated that this occurrence is likely as copper may have genotoxic potential. This is likewise corroborated by Gomes et al. (2013) as they indicated that copper, in the form of a Copper oxide (CuO), may be capable of inducing DNA damage on the hemolymph cells of Mytilus galloprovincialis. The micronuclei formed during the cell division may have been induced as a result of the DNA damage event during the organism's cell cycle kinetics (Bolognesi and Hayashi 2011) where the accumulation of DNA-damaging free radicals may trigger the clastogenic and aneugenic processes in the bodies of the aquatic organisms (Barsiene et al. 2006). Burkhead and Lutsenko (2013) indicated that copper as a redox heavy metal could cause the peroxidation of lipids and alter the nuclear redox environment, which may impair the activity of the nuclear factors. Jakimska et al. (2011) also presented that copper can form DNA adducts in the bivalves, which results in the clastogenic effects and spindle disturbances manifested by the nuclear anomaly.

These nuclear abnormalities at different mean frequencies were also observed in the *Pseudodon* sp. The resulting differences in the nuclear abnormality frequencies may have been attributed to the mussel's inherent ecological characteristic to actively absorb, accumulate, assimilate, and eliminate substances (Jha 2004; Graberkiewicz and Davis 2008). It is also likely that factors such as the organism's efficiency in DNA repair and cell removal kinetics may have contributed to the variations in the micronuclei and nuclear abnormality frequencies observed (Bolognesi and Hayashi 2011).

Although not all of the nuclear abnormalities exhibited a significant correlation to the copper accumulated in the tissues, the presence of the abnormalities in the hemocyte cells indicate that *Pseudodon* sp. farmed in the river is an efficient copper accumulator and good aquatic fauna model for genotoxicity screening and environmental monitoring of freshwater environments as all of the nuclear abnormalities were observed within the organism.

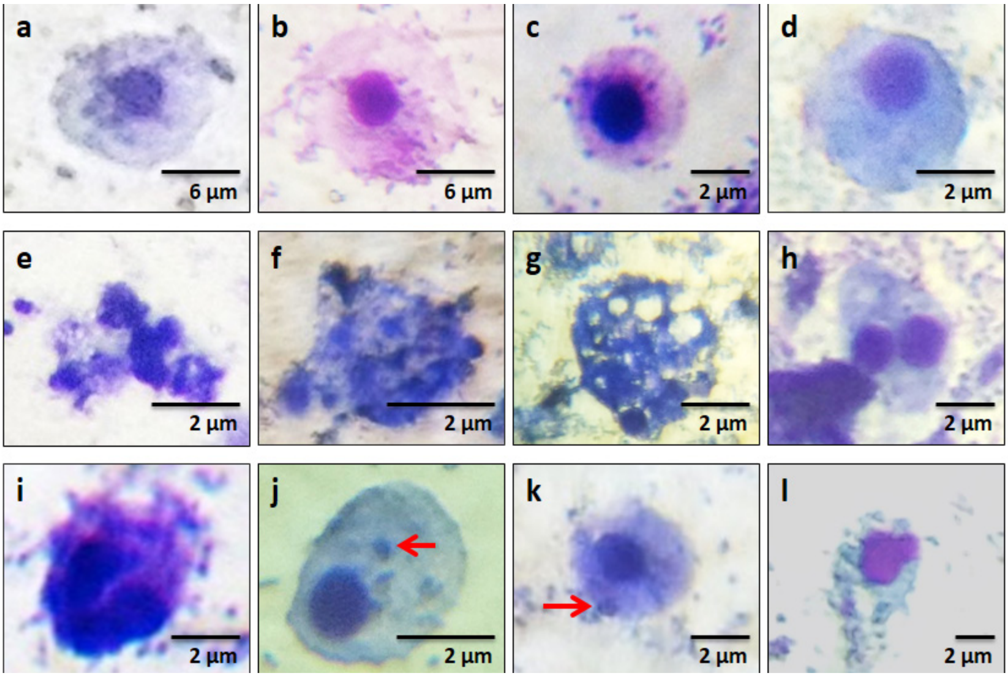

FIGURE 3 Nuclear abnormalities in *Pseudodon* sp. obtained in the tributaries of Boac River. The red arrow points at the micronuclei: (a, b) Normal granular hemocyte, (c, d) Agranular hemocyte, (e, f) Apoptotic hemocyte, (g) Necrotic hemocyte, (h, i) Binucleated hemocyte, (j, k) Micronuclei, and (l) Hemocytes with nuclear buds.

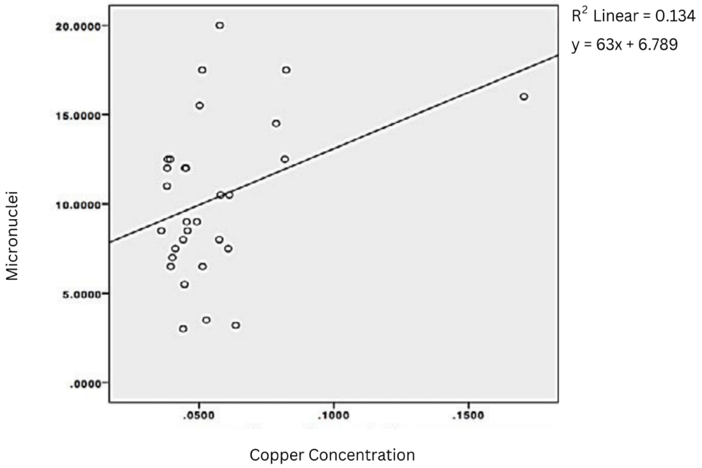

FIGURE 4 Relationship of hemocyte micronucleus with copper concentrations in the soft tissues of *Pseudodon* sp.

TABLE 3 Relationship of hemocyte nuclear abnormalities with the copper concentrations in the soft tissues of *Pseudodon* sp.

Nuclear abnormalities	Person's colleration coefficient (r)	Coefficient of determination (r2)	p
Agranular	0.078	0.006	0.682
Apoptotic	0.121	0.015	0.523
Binucleated	0.005	2.500 x 10-5	0.977
Micronuclei	0.366	0.134	0.047*
Necrotic	0.056	0.003	0.400
Nuclear buds	-0.159	0.025	0.523

*Significant at $P<0.05$

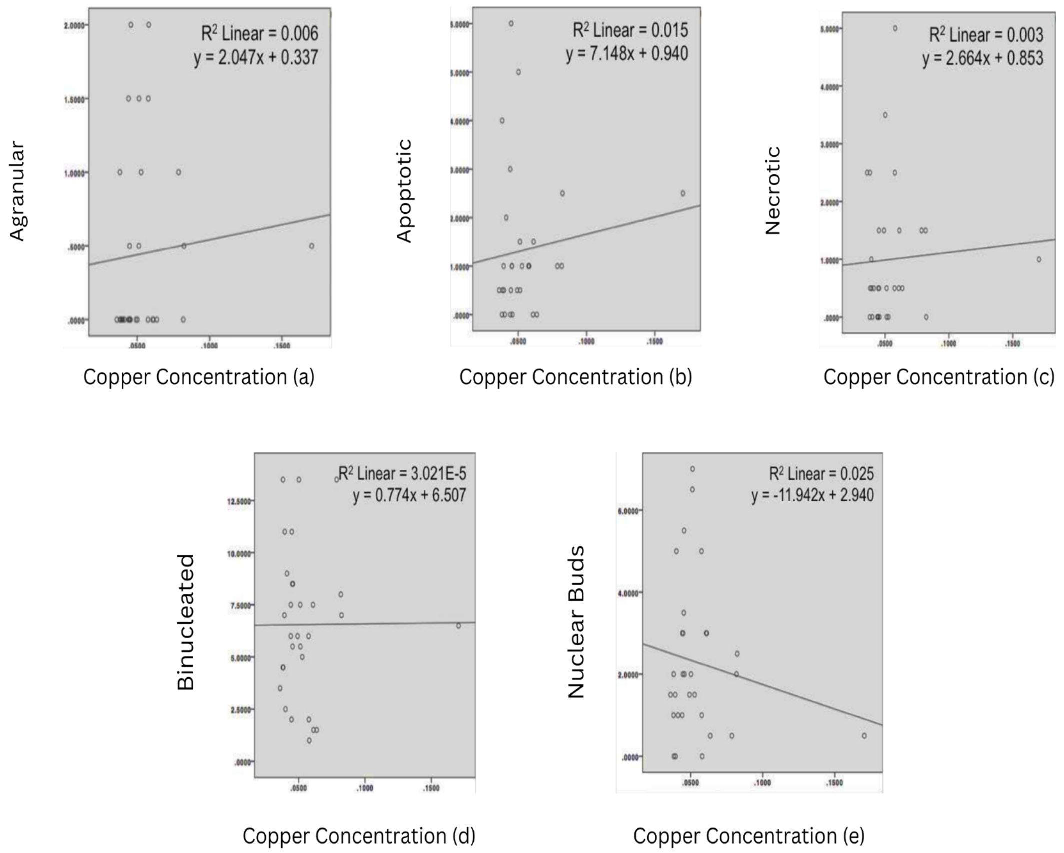

FIGURE 5 Relationship of other nuclear abnormalities with the copper concentrations in the *Pseudodon* sp.: (a) Agranular, (b) Apoptotic, (c) Necrotic, (d) Binucleated, and (e) Nuclear Buds.

Conclusion and Recommendations

Copper was detected in the edible tissues of *Pseudodon* sp. obtained from the tributaries of Boac River in Marinduque Island, Philippines. The copper concentrations in the edible tissues were below the maximum permissible level of copper in shellfish but exceeds the normal daily intake of copper. The total copper concentrations in the tissues of the *Pseudodon* sp. and the occurrence of micronuclei frequency identified as one of the indicators of genotoxicity show a significant statistical relationship. Other nuclear abnormalities that are indicators of genotoxic alterations were likewise observed on the hemocyte cells of the *Pseudodon* sp. although no significant relationships with the total copper concentrations were observed. It is recommended that a study on mussels obtained from non-polluted areas be conducted and compared with those from mine-tailed polluted waters. Continuous biomonitoring of mining-affected areas is also necessary.

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