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Original article

Metallothionein response of aninikad, *canarium labiatum* (Roding, 1798) to heavy metal concentrations in Balamban coastline, Cebu

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ABSTRACT

Heavy metals in the marine environment pose risk to mollusks and cause undesirable effects to the environment. This study is focused on the potential-threat of trace metals to locally available univalve in the coastline of Balamban, Cebu, Philippines. Sediment along with univalve mollusk samples were collected in the sites. The sediment sample was digested and analyzed for metal concentration by Flame Atomic Absorption Spectrophotometer (FAAS). Toxic effects to the univalve *Canarium labiatum* (*Aninikad*) posed by heavy metals were quantified in the whole tissue of *C. labiatum* using metallothionein (MT) assay.

The study revealed that the accumulation of metals in sediments had a general trend of Zn>Cu>Pb>Cr>Cd with no seasonal variation while mean concentrations of metals in the soft tissues of the mollusk have the general trend of Zn>Cu>Pb>Cd>Cr in both seasons. This shows that *C. labiatum* accumulates high concentration of metals when there is a higher level of metals in sediments. The results also showed statistically significant correlations between MT, Zn, Cu, Cd and Pb. This means that accumulation of these metals could influence the concentrations of MT in mollusks. Thus, MT can act as an effective biomarker for Cu accumulation in *C. labiatum* and it could be used as a valuable biomonitor for heavy-metal pollution. A more detailed study is recommended to understand the capacity of this univalve to induced MT as a response to heavy metal

KEYWORDS: *Metallothionein, Balamban coastline, Heavy metal, univalve, biomarkers*

1 INTRODUCTION

In recent years, heavy metal pollution of the aquatic environment has become a global problem. Toxic pollutants, such as heavy metals, originate from direct atmospheric deposition or geologic weathering or through the discharge of industrial waste products deposited in marine sediments as a sink. Due to their potential toxic effect and ability to bioaccumulate in aquatic ecosystems (Wang and Rainbow *et al.*, 2008), the investigation of the distribution and pollution degree of heavy metals in the coastal area has attracted more public concerns recently (Yang *et al*., 2012)

The potential sources of heavy metal pollution in the aquatic environment are industrial wastes and mining. Metals like arsenic, cadmium, chromium, mercury, nickel, and lead are often considered indicators of anthropogenic influence in marine environments and are themselves of potential risk to the natural environment (Chandurvelan *et al.*, 2015). Some of the heavy metals are essential to the life of the organisms (Suzuki et al., 2002; Ryu *et al.*, 2003) but some are extremely toxic even in small doses (Singh *et al.,* 2011). Exposure to metals can lead to physiological, biochemical, molecular, and behavioral changes in the organisms (Guanghua *et al.*, 2012). Additionally, these effects may significantly reduce the survival capacity of the organism by increasing susceptibility to diseases and damage (de Montaudouin *et al.*, 2010).

Balamban, Province of Cebu, Central Visayas, Philippines, became a first-class town from being a fourth-class municipality with a total annual income of P301.9 million, according to data from the Commission on Audit. Among the fast-growing barangays are Buanoy and Arpili, where West Cebu Industrial Park (WCIP) is located. WCIP is the economic zone that hosts Tsuneishi Heavy Industries, Inc. (THI) (Inquirer, P. (2018). How a shipbuilding firm jump-started Balamban's economy. [online] Business.inquirer.net. Available at: http://business.inquirer.net/238178/shipbuilding-firm-jump-started-balambans-economy [Accessed 3 Jun. 2018].). It is one of the leading medium sized shipbuilders in the world. Shipbuilding, ship repair and manufacturing of outfittings for ships and vessels constitute the main business of the company. Tsuneishi Heavy Industries, Inc. ships medium sized vessels to different parts of the world from the town of Balamban,

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Cebu in the central Philippines (Tsuneishi Heavy Industries (Cebu), Inc. (2018). Tsuneishi Heavy Industries (Cebu), Inc. [online] Available at: http://www.thici.com/ [Accessed 3 Jun. 2018].). To date, no published literature reports on how the mentioned activities affected the coastal waters in terms of trace metal levels and its aquatic organisms.

Biomarkers are functional measures reflecting changes in biological responses of organisms to certain stressors (Adams *et al.*, 2001). They indicate the biological effects of pollutants in contaminated environments (Van der Oost *et al.,* 2003) and predict the outcome or incidence of disease (WHO 2001). Metallothionein (MT) synthesis is one of the most important reactions as a response to metal exposure and therefore a potential biomarker for metal uptake (Frank *et al.*, 2008). It has been reported that metallothionein is induced in different tissues of aquatic mollusks following exposure to metals (Gillis *et al.*, 2014). Increase in MT concentration in tissues represents the typical response to metal contamination both in field or laboratory conditions (Geng *et al*., 2015).

In the Philippines, gathering of seafood or gleaning from shallow areas is carried out by locals of any age for food or subsistence for poor coastal communities. Small conch shells, locally known as aninikad are the most abundant gastropod providing high quality protein and nutrients among Filipino households (FAO, 2016, de Guzman *et. al,* 2019). Mollusks (univalves and bivalves) are susceptible to heavy metals from water and sediments due to their suspension feeding mechanism (Burkhardt III & Calci, 2000). Presence of heavy metals in edible aninikad poses threat to the public. Consumption of these seafoods exposes humans to heavy metals (Góngora-Gómez *et al.*, 2017).

To assess the degree of pollution in our environment and to minimize the hazardous effects to human and natural ecosystems, monitors of these heavy metals are very important. Living organisms are used as efficient indicators of environmental contamination (Avaler, 2000). Mollusks, a good sentinel organism, are commonly used in these monitoring programs as they can accumulate high levels of different metal contaminants in their body through particles filtered from seawater (Le *et al.*, 2011). With their long-life cycle, ease of collection, wide distribution and high bioaccumulation of metals they are useful and sensitive indicators of environmental contamination (Villnäs *et al*., 2011; Goldberg,1986; Ji *et al*., 2006).

The results of this study will serve as baseline data for the levels of trace metals in Balamban shipyard and the ominous threat it poses to mollusks (univalves and bivalves) and can be used in drafting policies for regulatory measures by the City government council for implementation by the respective local government unit (LGU).

2 MATERIALS AND METHODS

2.1 Sampling Site

The sediment and shell samples were collected along Arpili, Balamban coastline (GPS Address: 10.47141 N, 123.69051 E). The shipyard of Tsuneishi Heavy Industries (Cebu) Inc. (THICI) is located near the site and predominantly industrial effluents from the shipyard contaminate this part of the coastline as shown in Figure 1.

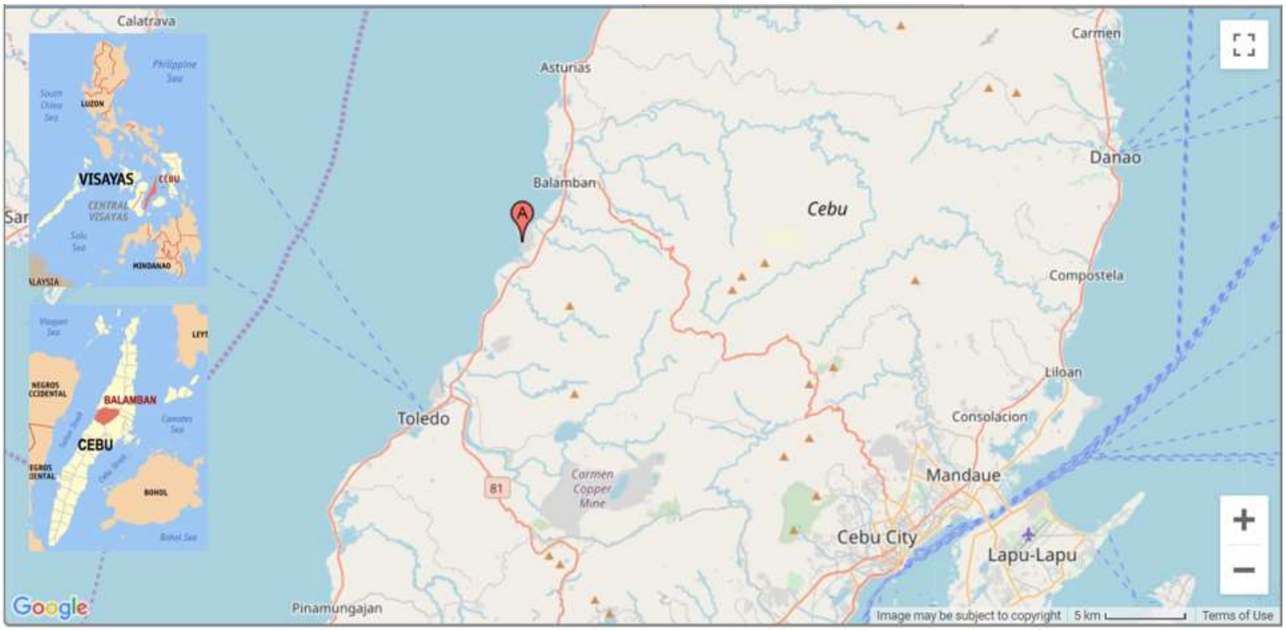

Figure 1. Location map of the sampling site in Arpili, Balamban Coastline

2.2 Sample collection

Prior to field sampling, the method of collection and handling the samples prior to analysis in this study was submitted to the Institutional Animal Care and Use Committee (IACUC) for approval in order to screen any unethical procedures that might be performed on the mollusk samples and to ensure that no ethical issue was violated by the research. It was only upon approval by IACUC that field sampling was conducted last April and November 2018 for sediment and mollusk samples corresponding to the dry and wet season, respectively.

The pH of the sediment was analyzed on site using the sediment pH meter. DO levels of the sea water were also determined *in situ*. Approximately 1 kg of the sediment was randomly collected in the sampling site within the stretch of the coastline using a plastic shovel into a polyethylene bag and was chilled during transport to the laboratory. Coning and quartering was the method used until the required size of the sample is obtained (Ronquillo *et al.,* 2014).

Mollusk samples shown in Figure 2 were randomly collected along the sampling station. The samples were washed then packed in plastic bag, labeled, and transported in ice boxes to the laboratory. The shell samples were washed again with distilled water, placed in aluminum foil and ziploc bag and were frozen to –10°C until further treatment. The sample were also submitted for identification to the Curator of the Marine Biological Collection, Department of Biology of the University of San Carlos.

2.2 Sample Preparation and Heavy Metal Analysis

2.2.1 Sediment sample preparation and analysis for heavy metal analysis. In the laboratory, the sediments were air dried until parched, sieved using a 180 µm mesh and homogenized further using a ball mill. The homogenized samples were oven dried at 110°C to constant weight.

The digestion method that was used to analyze heavy metals in sediment is the ISO 11466.3 Method. About 1.000 g of dried sediment sample was weighed and transferred into a 100-mL beaker and added with 10 mL of a (3:1) mixture of 12M HCl and 17M HNO_3, the slurry was mixed and covered with a watch glass. The mixture was heated to 95ºC without boiling for 10 – 15 minutes. Then it was cooled to room temperature until no more brown fumes were generated or until mixture is about 5 mL. If still brown fumes were produced, indicating incomplete oxidation of the sample, another 5 mL of aqua regia were added over and over until no brown fumes were given off by the sample. Then the solution was cooled down to room temperature. The digested sediment sample was then filtered using Whatman 42 filter paper and was diluted to 100 mL with distilled water in 100mL volumetric flasks.

The sediment samples were analyzed with Flame Atomic Absorption Spectroscopy (FAAS) using external calibration techniques. The analysis was done in triplicate and the recovery test was analyzed per batch of analysis. Excess samples were stored in the refrigerator at –10°C until further treatment. The concentration of the metals was then calculated based on the equation of the line from its respective calibration curves. Recovery test was also performed in triplicates and was calculated according to the equation:

$$\% \text{ recovery} = \frac{A_{sple+std} - A_{sple}}{A_{std}} \times 100$$

where:

$A_{sple+std}$ = absorbance of sample + metal standard
A_{sple} = absorbance of sample only
A_{std} = absorbance of metal standard only

2.2.2 Univalve sample preparation and heavy metal analysis. Univalve sample preparation and analysis were based on the method of Chandurvelan *et al.*, (2012). Three replicates were conducted with each replicate consisting of five pooled samples of the mollusk. After thawing, 0.5 g wet weight of the whole tissues dried to constant weight at 60 °C, transferred into acid-washed tubes, and were digested at 90 °C for 1 h using 5 mL of 50% HNO_3. It was diluted appropriately using 2% HNO_3 to achieve desired concentrations before being analyzed for metal content. Trace metal concentrations were analyzed using Flame Atomic Absorption Spectrophotometry (FAAS) via external calibration standard (ECM) and were expressed as mg/kg dry weight tissue. Recovery test per metal per batch of analysis was conducted in triplicate.

2.3 Metallothionein Assay

To quantify the metallothionein concentration in cells, the method of Linde and Garcia-Vazquez (2006) and Viarengo *et al.*, (1997) was adapted. Bivalve tissue was weighed and then homogenized with 3-mL of homogenization buffer consisting of 0.5 M sucrose, 20 mM Tris-HCl buffer, pH 8.6 and 0.01% β-mercapthol in plastic or glass tubes using a tissue homogenizer. The homogenates were centrifuged at 30,000 rpm per gram for 20 minutes to obtain a supernatant containing metallothionein. A measured volume of supernatant was quantitatively transferred to another microtube. Cold absolute ethanol (1.05-mL) and 80-μL of chloroform per 1 mL of the resulting supernatant were added and the samples were centrifuged again at 6,000 rpm per gram of sample for 10 minutes at 0 – 4°C. Three volumes of cold ethanol were added and stored at –20°C for one hour (preferably, overnight) before recentrifugation.

The resulting pellets were washed with cold (-20°C) ethanol: chloroform: homogenization buffer in 87:1:12 ratio. The mixture was centrifuged once more for 10 minutes at 6,000 rpm and was allowed to dry overnight on air. The dried pellets were resuspended in 300-μL of 0.25 M NaCl and subsequently, 150-μL 1N HCl containing 4 mM EDTA were added to the sample. A vortex shaker was used to completely dissolve the pellets. Then a volume of 4.2-mL 2 M NaCl containing 0.43 mM DTNB (5,5-dithiobis-2-nitrobenzoic acid) buffered with 0.2 M Na-phosphate, pH 8, were added to the sample and were left for 30 minutes at room temperature. The supernatant was read at 412 nm using a UV-Vis spectrophotometer.

The amount of metallothionein was estimated using reduced glutathione (GSH) as standard, assuming 1 mole of MT contains 20 moles of cysteine. External calibration method was used. Once concentration of the sample was calculated from the standard curve, the value was divided by 20 to determine the MT content.

2.4 Statistical Treatment

The means of triplicates and standard deviation was analyzed by Excel while the evaluation of significant differences between April and November 2018 were determined using two-way Analysis of Variance (ANOVA) utilizing the statistical software, Graph Pad Prism 6.0. Correlations between heavy metal accumulation and metallothionein response of bivalves were evaluated using Pearson's r coefficient.

3 RESULTS AND DISCUSSION

3.1 Heavy Metal Concentration in Sediments

Sediment samples were obtained to determine the environmental concentrations of Cd, Pb, Cu, Zn and Cr. Physico-chemical parameters of different areas ranged environmental concentrations of Cd, Pb, Cu, Zn and Cr. Physico-chemical parameters of different areas ranged between 7.87-8.10 and 27.2 – 28.4°C for pH and temperature, respectively. Table 1 shows that the metals detected in the Balamban Coastline follow the order Zn>Cu>Pb>Cr>Cd with no seasonal variation. All heavy metal concentrations in the sediment sample did not exceed the maximum limits set by the sediment Toxicity Reference Value (TRV). The level of Pb exceeded the World Health Organization (WHO) standards while the concentrations of Cd, Cu, Zn, and Cr in sediments for both dry and wet seasons were below the World Health Organization set limits for the survival of aquatic organisms.

Pb was the only metal that exceeded the WHO limits of 10.0 mg/kg for sediments with a concentration of 11.24 mg/kg and 13.74 mg/kg in dry and wet seasons, respectively. Compared with sediment TRV (46.70 mg/kg), Pb mean values did not exceed the permissible limit. Pb concentration in sediment may have been due to lead discharged from the battery waste and gasoline, engine oil, and used container carried by runoff in the sampling location (Nwadinigwe *et al*., 2014).

Figure 3 shows the bar graph of the metals' concentrations in sediments with statistical analysis. Results revealed a significant difference between the Zn concentrations from dry season (26.15 mg/L) and wet season (30.44 mg/L). This can be justified by the number of launched ship products on May – November higher than January – April (https://www.tsuneishi.co.jp/english/news/topics/2018/. Date accessed: May 5, 2019). Zinc, one of the most common trace metals prevalent in Data, is expressed in mg/kg. Different letters show significant differences ($p \leq 0.05$) in concentration of Zn between months. Runoff waters discharge from industrial sites (Line *et al*.,1997). The collection site of the study was near a shipyard, which is one of the most economic activities in the area, providing jobs to the community and nearby localities.

Table 1. Environmental concentrations of Cd, Pb, Cu, Zn and Cr of sediments in Balamban Coastline, Cebu

Sampling Month/Year	**Mean Metal concentrations, mg/kg**					**References**
	Cd	**Pb**	**Zn**	**Cu**	**Cr**	
Balamban Coast April 2018	0.47 ± 0.0096	11.23 ± 1.33	26.15 ± 1.63	20.88 ± 2.27	10.2 ± 0.38	This study
Balamban Coast November 2018	0.43 ± 0.039	13.74 ± 2.17	30.44 ± 1.13	22.24 ± 0.37	11.7 ± 0.11	
World Health Organization	6.00	10.0	25.0	123	25.0	WHO/USEPA (2002)
Toxicity Reference Values	1.20	46.7	34.0	150	81.0	USEPA, 1999

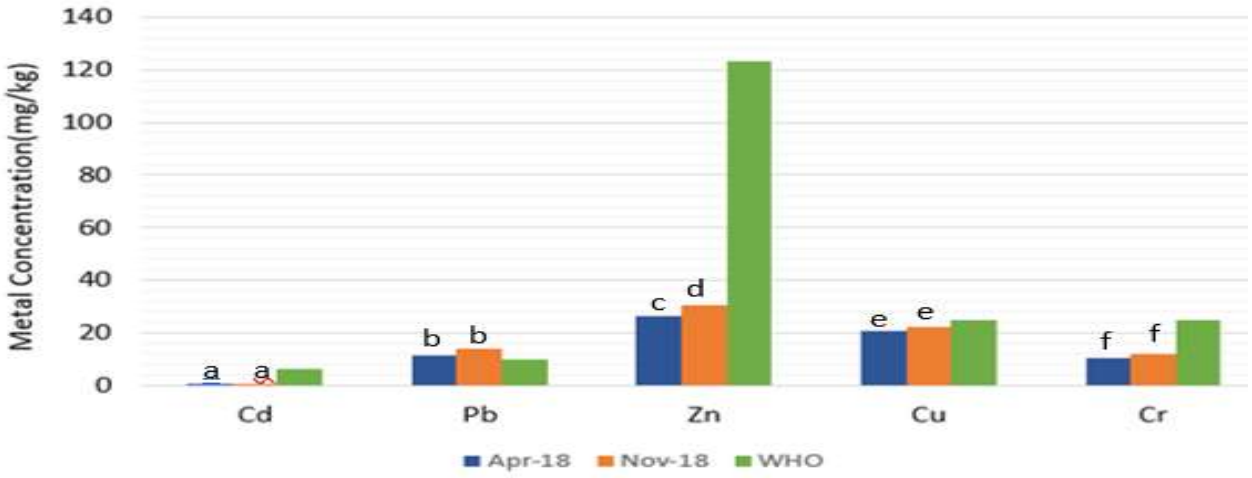

Figure 3. Heavy metal concentrations in the sediments.

Arpili, Balamban hosts the Tsuneishi Heavy Industries (Cebu) Inc. (THICI) and from the company's operations, hull washing and waste effluents may account for the high concentration of Zn (Gary *et al*., 2008).

Sediment samples collected during the dry season recorded lower heavy metal concentrations in this study except for Cd. The higher concentrations of heavy metals during the wet season could be attributed to heavy rainfall that speeds up the rate of corrosion of metals (Garnaud *et al*., 1999). These results were also in accordance with the study of Ntakirutimana *et al*. (2013) in which the majority of metal concentration increased during the rainy season. Heavy rainfall leads to high fluvial inputs into the coast carrying metals from industrial wastes responsible for these increased metal concentrations.

3.2 Heavy Metal Concentrations in Canarium labiatum

The univalve samples were analyzed for metal content, and the results, as shown in Figure 4, demonstrated significantly high concentration levels for Cu and Zn metals in both seasons. Mean values of metal contents measured in Canarium labiatum have generated Zn>Cu>Pb>Cd>Cr trend in both seasons.

Cadmium and lead were only detected during the wet season with mean concentrations of 0.426 mg/kg and 6.58 mg/kg for Cd and Pb, respectively. None detection of both metals in the dry season was consistent with the results in sediments. These values were higher than the study of Ziomek et al., (2018) in West Pomeranian, Poland which reported 0.34 mg/kg Cd and 0.12 mg/kg Pb in Helix pmatia or HP snails. Their study demonstrated that snails of this genus are macroconcentrators for Cd and microconcentrators for Pb, and their hepatopancreas is where these elements accumulate Ziomek et al. (2018). Cd levels in this study are relatively higher compared to Cd concentrations for both bivalves, A. maculosa and A. puerpera, with 0.03 mg/kg and for gastropods 0.06 mg/kg in C. urceus while 0.08 mg/kg in L. lambis from Pajuda Bay, Philippines. On the other hand, Pb concentrations in

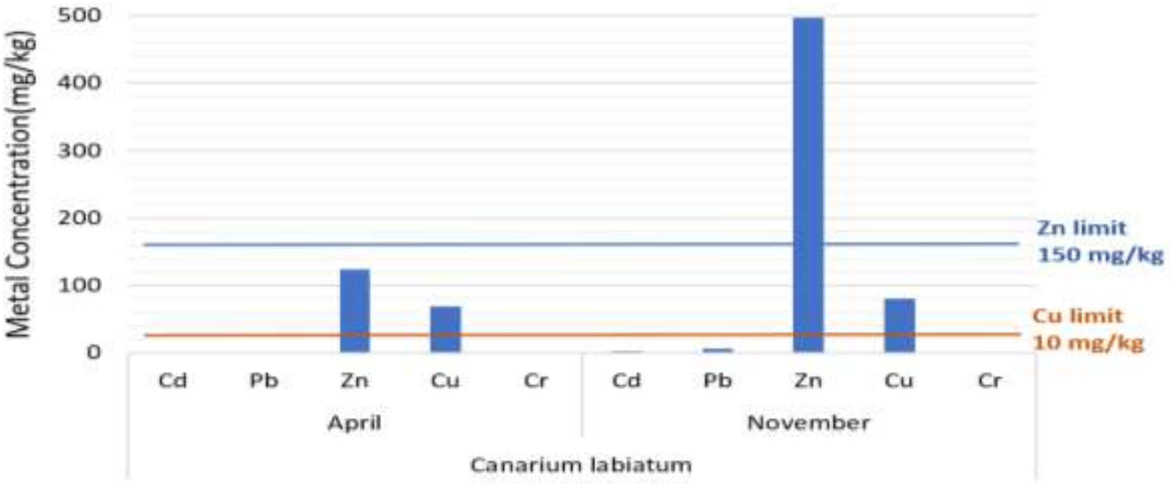

Figure 4. Heavy metal accumulation of *Canarium labiatum*. Data are expressed in mg/kg. Different letters show significant differences in the concentration of Zn between April and November.

both bivalves and gastropod mollusks were all equal 0.10 mg/kg (Tayone et al., 2020).

Sediment: mollusk ratios show that *C. labiatum* accumulates high concentration of metals when there is a higher level of metals in sediments. Further analysis is needed to confirm the behavior of metal accumulation in mollusks. Accumulation of metal in organisms are influenced by factors such as bioavailability of heavy metals, climate condition, season, species sexual characteristics and maturity (Boyden and Phillips, 1981). In this study, the concentrations of Cu and Zn in sediments and *C. labiatum* both exceeded the recommended standard by the WHO/FAO (1989).

3.2.1 Zinc. Zn is the second most abundant trace metal after Fe and is essential for metabolic processes in living organisms (Pan and Wang, 2012) so it is expected that Zn will accumulate more than the other metals. It tends to gather in the fatty tissues of aquatic organisms, including mollusks and affects their reproductive physiology (Graham *et al*., 2010). The Zn level recorded in *Canarium labiatum* has a mean concentration of 124.0 mg/kg and 497.9 mg/kg for dry and wet seasons, respectively. Statistical analysis showed that there was a significant difference in Zn levels between dry and wet season ($P<0.0001$). Zinc permissible limits according to WHO/FAO (1989) and Malaysian Food Regulation (1985) is 100 mg/kg, Brazilian Ministry of Health (ABIA, 1991) is 250 mg/kg, Ministry of Public Health, Thailand (MPHT, 1986) is 667 mg/kg and Australian Legal Requirements for Food Safety (NHMRC, 1987) is 750 mg/kg as shown in Table 2. Zn level exceeded the maximum permissible limit set by WHO/FAO and Malaysian Food Regulation standards during the dry and the wet season. Comparison with the Brazilian Ministry of Health (ABIA, 1991), Ministry of Public Health, Thailand (MPHT, 1986) and Australian Legal Requirements for Food Safety (NHMRC, 1987) demonstrated that the level of Zn obtained from this study is essential to the species since Zn levels are lower than the guidelines.

The results of this study showed that Zn accumulation in *C. labiatum* from the Balamban coastline were lower compared to the study of *M. strigata* and *C. corteziensis* by Ruelas-Insunza *et al*., (2000) in Southeast Gulf of California with Zn level of 200 mg/kg and 3500 mg/kg, respectively. A spectacular bioaccumulative response to Zn was also exhibited by the *Crassostrea* with 772 mg/kg, followed by the digestive gland of *Telescopium* (17 mg/kg) in coastal waters near a mining site in Paracale, Camarines Norte (Carino, *et al*., 1993). In southern gulf of California, *C. subrugosa* Zn concentration range from 64 to 1218 mg/kg (Paez-Osuna *et al*., 1993); and in *S. cucullata* from northern coast Qeshm Island, Persian Gulf, Iran with Zn level of 1395 mg/kg (Shirneshan *et al*., 2013).

3.2.2 Copper. Cu is an essential element required for different enzymes of living organisms but can be toxic at high concentrations. It is a common component in anti-biofouling paints applied on the surfaces of ships and in offshore engineering (Pan and Wang, 2012). Thus Cu is usually monitored in aquatic environments. The highest Cu levels were recorded during the wet season in *C. labiatum* with mean concentration of 80.88 mg/kg. Statistical analysis did not show any significant difference in Cu levels between seasons. The mean Cu concentration in both seasons in this study exceeded the permissible level recommended by the WHO/FAO (1989) for human consumption. These can be attributed to intense industrial activities and operations near the site.

The result also showed that although being an essential metal, Cu levels (69.4 and 80.9 mg/kg) in *C. labiatum* are beyond the maximum tolerable limits suggesting that the amount of Cu available may be lethal and could pose health risks to the locals in Balamban who used "*aninikad*" as a food. These findings were similar to the study conducted in *C. corteziensis* and *C. palmula* from Altata-Ensenada del Pabellon lagoon in Mexico with Cu levels at 82 mg/kg (Paez-Osuna *et al*., *1993*). Cu levels in this study are relatively higher compared to the study of Carino, *et al*., (1993) where Cu was highest in *Terebralia sulcatus* (16.5 mglkg); *M. squalida* with Cu levels of 8 mg/kg (Mendes et al., 2006); and in *M. galloprovincialis* collected near an industrial area of Moroccan coast with Cu mean concentration of 26.8

Table 2. Average concentrations of Cd, Pb, Zn, Cu and Cr in the univalve samples and the permissible limit of metals set by WHO/FAO (1989) and different countries; Malaysian Food Regulation (1985), Brazilian Ministry of Health (ABIA, 1991), Ministry of Public Health, Thailand (MPHT, 1986), and Australian Legal Requirements for Food Safety (NHMRC, 1987).

Countries	Metal Concentration, mg/kg			
	Cd	Cu	Pb	Zn
This study (April)	-	69.40	-	123.98
This study (November)	0.43	80.90	6.58	497.85
WHO/FAO (1989)	1.00	30.0	2.00	100.0
Malaysian Food Regulation (1985)	1.00	30.0	2.00	100.0
Brazilian Ministry of Health (ABIA, 1991)	5.00	150	10.0	250.0
Ministry of Public Health, Thailand (MPHT, 1986)	-	133	6.67	667.0
Australian Legal Requirements for Food Safety (NHMRC, 1987)	10.0	350	-	750.0

mg/kg (Maanan, 2007).

3.2.3 Cadmium. Cd is a non-essential element for aquatic organisms, except for its discovered biological role in marine diatoms (Lane and Morel 2000), and has been demonstrated to be highly toxic to both wildlife and humans. Cadmium concentration of *C. labiatum* during the dry season was below the detection limit. Cd level in *C. labiatum* falls under the acceptable limit set by WHO and FAO (1989).

3.3 Metallothionein Assay

Metallothionein (MT) response of the C. labiatum to the concentrations of heavy metals exposure was quantified using spectrophotometric assay via Ellman's reaction. Figure 5 shows that the highest MT levels were recorded in C. labiatum in November with a value of 88.13 µg/g. These results were comparable to the study of Oaten et al. in 2015 with MT values ranging from 60.72 – 74.23 µg/g in M. edulis.

This study showed that at Balamban, MTs and metal concentrations increased from April to November, indicating that the species were subjected to metal pollution. Marine coastal streams carrying industrial effluents with high Cd, Cu and Zn concentrations (Sarbaji, 1991; Illou, 1999) can explain this. This study also showed higher MT levels than in *R. philippinarium* with MT levels ranging from 15.86 – 25.65 µg/g (Oaten *et al.*, 2017). The ability to synthesize and accumulate MT in response to metal exposure varies among marine species. For example, in *Crassostrea gigas*, protein levels depend on the metal concentration in the sampling sites (Boutet *et al.*, 2002). In contrast, in the digestive gland of *M. galloprovincialis*, natural factors contribute more to MT content than sub-lethal Cd concentrations (Raspor *et al.*, 2004). In other bivalves, such as the scallop *Argopecten gibbus*, it was found that MT levels increase significantly in the gills in sites contaminated with municipal sewage (Quinn *et al.*, 2005).

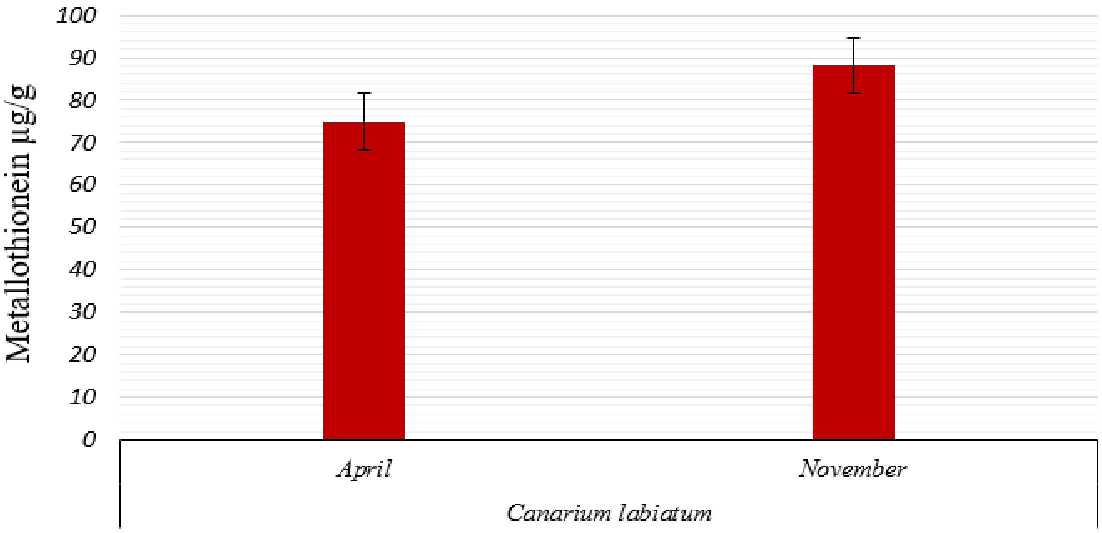

Figure 5. MT levels *Canarium labiatum*. Data are expressed in µg/g.

Comparison of metallothionein concentrations in *C. labiatum* between dry and wet season showed no significant difference (P>0.05) as shown in Figure 5. Seasonal fluctuations of MT in mollusk samples were reported by Falfushynska *et al.* (2010) and Leiniö and Lehtonen (2005). The highest value of MT recorded in November 2018 (88.1 ug/g) could be explained by the high heavy metal levels accumulated in sediment and *C. labiatum* in this month. Opposite trend was observed in April with an average MT concentration of 74.9 µg/g and may be due to the lower concentrations of heavy metals detected in the sediment and the univalve sample. These results suggested that accumulation of heavy metals during the wet and dry seasons could influence the concentrations of MT in mollusks.

3.4 Metal-Biomarker Correlations

The presence of many thiol groups (mainly cysteine) in the active center of the protein is the main distinguishing feature of the metallothionein structures and is responsible for metal complexation. Correlation analysis between metal and MT levels showed a significant correlation between Cu and MT for dry season with (R>0.98) at P<0.05. MT is suggested to be a Cu-regulating protein as it binds to Cu via Cu-thiolate clusters (Presta and Stillman, 1997). Since MT binding of Cu is thermodynamically and kinetically stable, excess Cu is sequestered by MT to mask Cu toxicity (Salgado and Stillman, 2004). Metal uptake must reach threshold levels for essential metals like Cu to have a significant effect on MT levels (Eroglu et al., 2005). Cu levels in the

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C labiatum tissues exceeded acceptable limits suggesting that metal uptake by the univalve may have exceeded tolerance levels attributing to the high levels of MT found in this study.

Further, this study showed lower MT levels compared to the study of Purina et al. in 2013 in heavy metal-exposed Anodonta spp. and Unio spp. with MT levels of 148.3 µg/g and 160 µg/g, respectively. Purina et al. (2013) attributed this significant variation of MT levels between species to the seasonal fluctuations of MT, increase of recreational activities and different response of each species towards stress and heavy metal accumulation.

Cu concentration was significantly correlated with MT response ($R>0.98$) at $P<0.05$. This implies that MT response was higher at high metal accumulation levels, thus supporting its usefulness in environmental monitoring in complex environments (Carvalho et al., 2012). MT concentration usually parallels metal concentrations. Contrary to the general hypothesis it showed the absence of induction in C. labiatum in November. A lack of significant trend in Zn accumulation and MT response in November emphasizes C. labiatum's ability to regulate this essential heavy metal. Table 3 shows the Pearson correlation analysis between heavy metal concentrations and biomarker response.

Table 3. Pearson correlation analysis between MT and heavy metals

April	MT	Cd	Pb	Zn	Cu	Cr
MT				0.9918	0.9973*	0.4521
Cd						
Pb						
Zn	0.9918				0.9797	0.3341
Cu	0.9973*			0.9797		0.5164
Cr	0.4521			0.3342	0.5164	
November	**MT**	**Cd**	**Pb**	**Zn**	**Cu**	**Cr**
MT		0.8898	0.9934	-0.1298	-0.7616	
Cd	0.8898		0.8315	0.3371	-0.9734	
Pb	0.9934	0.8315		-0.2427	-0.6823	
Zn	-0.1298	0.3371	-0.2427		-0.5437	
Cu	-0.7616	-0.9734	-0.6823	-0.5437		
Cr						

These findings agree with the results of Mazon *et al.* in 2002 in which Cu in tropical freshwater *Prochilodus scrofa* were significantly related to MT response. This means that higher heavy metal concentrations and MT levels prove the role of MT in metal homeostasis and detoxification. Thus, MT content can act as an effective biomarker for Cu stress in the mollusk. Generally, the MT levels can function and could be considered as a sensitive Cu indicator in ecotoxicological studies.

Zn and Cu are also components of metallothionein (MT). MT is characterized mainly by a high cysteine content (30%) and metal-binding capacity. The synthesis of MT is induced by metals such as Cd, Zn, and Cu, and also indirectly by non-metallic compounds such as glucocorticoids and hormones (Vilanova *et al*., 1997). The study of Tanguy *et. al*., 2003 quantified the induction of MT that allows the chelation and detoxification of heavy metals, particularly cadmium, but also the homeostasis of basic metals. In the present study, a correlation in Zn and Pb accumulation and MT response is also reflected. However, the correlation of MT and metal accumulation in November opposed to what was found in April. This may be due to aspects known to affect the accumulation of metal in organisms that were beyond the scope of our study such as the climate condition, species sexual characteristics and maturity (Boyden and Phillips, 1981).

4 CONCLUSIONS AND RECOMMENDATION

The data obtained in the findings indicated that the concentrations of all investigated heavy metals in sediment were within the permissible limit recommended by WHO (2002) except for Pb. On the other hand, Cu and Zn concentrations in *C. labiatum* exceeded the limit set by WHO (1989). This study indicated that Zn, and Cu (April) and Pb and Cd (November) levels have a significantly induced metallothionein response in the tissues of *Canarium labiatum*. These metals showed an increase in concentration in November, which is consistent with the results in MT. These results reflect the relationship between MTs and heavy metals and confirm the hypothesis of MT induction by metals. Moreover, the significant correlation between these metals and MT reinforces the hypothesis about the efficiency of MT as a biomarker of metal exposure, useful for biomonitoring

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purposes. The strong correlation between MT and accumulation of Cu, Zn, Cd and Pb makes MT a sensitive indicator for *C. labiatum* in environmental studies.

Further studies on the bio availability and mobility of heavy metals in the test organisms are also recommended. Accounting for the length, mass, and sex of the test organism is also recommended. Other organs such as the digestive gland, gills, food and mantle can be studied since other studies have shown how heavy metals and MT concentrations vary in different organs. Other biomarker parameters and their correlation with MT by the same pollutants can be investigated.

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