

THE DIFFERENT CAPABILITY OF METAL UPTAKE IN THE SHELL OF *Perna viridis* COMPARED TO THE DIFFERENT SOFT TISSUES: A STATISTICAL APPROACH

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Abstract: Distribution of metals in bivalves is usually examined based on different metal concentrations in the different tissues of the bivalves. Patterns of metal distribution between the shells and the different soft tissues of two geographical populations of the green-lipped mussel *Perna viridis* were investigated. The correlation analysis showed insignificant correlation between the shells and the different soft tissues. The multiple linear stepwise regression analysis showed that the shells were not influential in metal accumulation in the total soft tissues. The cluster analysis showed that the shells were clustered differently, as a single entity, from the rest of the different soft tissues. All these findings indicate that the capability of metal uptake by the shell of *Perna viridis* differs.

KEYWORDS: Green-lipped mussel, cluster analysis, different parts, multivariate analysis

Introduction

Phillips and Rainbow (1993) noted that among the common approaches used to survey environmental contamination, the use of biomonitor species has proven to be a valuable and informative tool. Bivalve, like *Perna viridis* is a filter-feeder, that accumulates heavy metals found in food, water and also from the ingestion of inorganic particulate materials (Huang et al. 2007). Hence, it is a good biomonitor. Organisms which are sedentary, widespread and have long life span are well established as biomonitor for monitoring the concentrations of heavy metals in the coastal areas (Farrington et al. 1983; Giusti et al. 1999). Yap et al. (2003a, 2003b, 2004, 2006a, 2006b) had established the use of green-lipped mussel, *P. viridis* as a biomonitor of heavy metal pollution in the coastal waters of Peninsular Malaysia.

On the other hand, monitoring of heavy metal contamination using different parts of bivalve is still new. The use of different tissue can overcome the inaccuracy incurred in determining heavy metal levels in the total soft tissues of bivalve. The spawning season of the bivalve, biotic and abiotic environmental factors, may contribute to the wide variability of heavy metal concentrations in the total soft tissues. Several researchers have reported the use of different parts of mollusks, Chou et al. (2003) reported the use the digestive glands, residuals and the adductor muscles of the horse mussel *Modiolus modiolus* to monitor metal contamination. Sato et al. (1997) studied the distribution of heavy metals in different soft tissues such as mantle, muscle, ligament, foot, remainder, gill and digestive gland of mussels. Yap et al. (2003a, 2003b, 2004, 2006a, 2006b) used different parts of *P. viridis* to monitor the metal bioavailabilities in the coastal water of Peninsular Malaysia. A study on the gill and hepatopancreases of the eastern oyster *Crassostrea virginica* was conducted by Sokolova et al. (2005).

In this study, the focus is on the application of different parts of *Perna viridis*. The use of mussel shell to monitor the contaminant in marine environments is not a new idea. The shell is used

since it may record environmental pollution events more accurately because contaminants in the shells are not continually exposed to metabolic and detoxification processes and may therefore have longer biological half-lives (Lee and Koide, 1987). The use of molluscs shells as biomonitoring materials had been employed by numerous researchers (Foster and Chacko, 1995, Walsh et al., 1995 and Szefer et al., 2002). In Malaysia, the study on the shell of *P. viridis* as a biomonitoring material was conducted by Yap et al. (2003d).

The application of multivariate statistical techniques, such as cluster analysis (CA) is useful in the interpretation of complex data matrices to better understand the heavy metals and ecological status of the systems studied. These techniques allow the identification of possible factors and sources that might influence heavy metals and can offer a valuable tool for reliable management as well as rapid solution to pollution problems (Vega et al., 1998; Adams et al., 2001; Reghunath et al., 2002; Simeonov et al., 2004; Habes and Nugem 2006). The use of multivariate analysis, statistically, could also assist in determining the potential biomonitor accurately, by referring to cluster groups of their different parts. Moreover, multivariate methods are recommended for the use in monitoring studies since they can help reduce the costs of carrying out further environmental surveys (Szefer, 2003; Szefer et al., 2006).

This study determines the possible differences of heavy metal concentrations between the shell and different soft tissues of *P. viridis* using correlation, cluster and multiple stepwise linear regression analyses.

Materials and Methods

The samples were collected from an area that stretched from Telok Mas (Malacca) to Pantai Lido (Johore Bharu), the west coast of Peninsular Malaysia. The description of the sampling site is shown in Table 1. The collected mussels were immediately placed in an ice compartment and transported to the laboratory. Prior to metal analysis, twenty (n=20) individuals of mussels with shell length of 81.8-109 mm were carefully dissected into different soft tissues (remainder, mantle, muscle, gills, gonad, foot and byssus). Then, the shell and each category of the tissues were placed in aluminium foil. They were dried in an oven for 72 hours at 60°C to constant dry weights. Dried samples were then stored in clean plastic bags.

Triplicates of each dried category of bivalve tissues were digested in 10 mL concentrated HNO₃ (Analar grade, BDH 69 %). They were first placed in a hot-block digester at a low temperature of 40°C for 1 hour and were then fully digested at a high temperature (140°C) for at least 3 hours (Yap et al. 2003a). The digested samples were diluted to 40 mL with double-distilled water (DDW). After filtration, the heavy metal concentrations were determined using an air-acetylene flame Atomic Absorption Spectrophotometer (AAS) Perkin-Elmer Model AAnalyst 800. The data are presented in µg/g dry weight.

To avoid possible contamination, all the glassware and equipment used were washed with acid. Procedural blanks and quality control samples made from standard solutions for Cadmium (Cd), Copper (Cu), Nickel (Ni), Iron (Fe), Lead (Pb) and Zinc (Zn) were analyzed after every 5 - 10 samples in order to check accuracy. The percentage of recoveries for the heavy metal analyses was acceptable at 80 - 110 %. The analytical procedures for the bivalves were checked with the Certified Reference Material (CRM) for dogfish liver (DOLT-3, National Research Council Canada). The recoveries of all the metals were satisfactory (Table 2).

The distributions of heavy metals in the different parts were examined using multivariate analysis. All data were log₁₀ (X + 1) transformed prior to the statistical analysis. The relationships

between heavy metal concentrations in the different parts were analyzed using Spearman's correlation coefficient. Multiple stepwise linear regression analysis was used to determine the influence of heavy metals in the different parts to those in the total soft tissues. Meanwhile, heavy metal concentrations in the different parts of the *P. viridis* were analyzed using the Post-hoc test (Student-Newman-Keuls) to know if there was any significant difference at $P < 0.05$. Correlation and multiple linear stepwise regression analyses was done using SPSS 12.0 while STATISTICA 99 edition was used to conduct cluster analysis.

Results

Distribution of heavy metal concentrations in different parts of *P. viridis* from both locations are shown in Table 3. Relatively higher concentrations of Fe and Zn were found in the different soft tissues of the *P. viridis* of both sites when compared to the shell. In the different soft tissues, Fe concentrations ranged from 42.9-1795 $\mu\text{g/g dw}$ in Pantai Lido, while, Fe concentrations ranged from 85.0-4895 $\mu\text{g/g dw}$ in Telok Mas. On the other hand, Zn concentrations in the different soft tissues ranged from 47.6-89.8 $\mu\text{g/g dw}$ in Pantai Lido, while in Telok Mas, the concentrations ranged from 48.7-78.9 $\mu\text{g/g dw}$. For Cu, the muscle contained low concentrations of Cu when compared to other different parts. The Cu in the different soft tissues ranged from 5.16-13.6 $\mu\text{g/g dw}$ in Pantai Lido while its ranged from 5.51-28.4 $\mu\text{g/g dw}$ in Telok Mas. As for Cd, Pb and Ni, significant higher ($P < 0.05$) concentrations of these metal were found in the shell when compared to the different soft tissues.

Spearman's correlation coefficient between the shell, total soft tissues and different soft tissues of *P. viridis* collected from Pantai Lido and Telok Mas are shown in Tables 4 and 5. Two clear patterns can be observed from Tables 4 and 5. First, all the different soft tissues including byssus are positively and significantly ($P < 0.05$) correlated. Second, only the hard tissue shells are not significantly ($P > 0.05$) correlated with all the different soft tissues, total soft tissues and byssus.

Table 6 shows the multiple linear stepwise regression between the total soft tissues and the different soft tissues and shells. From the regression equation, it was found that the heavy metals found in the total soft tissues of *P. viridis* of both populations are significantly ($P < 0.05$) influenced by the metals accumulated in the byssus, foot, gill and the gonad.

Figure 2 shows the cluster analysis (Euclidean Distances) of general accumulation of heavy metals (Cu, Cd, Fe, Ni, Pb and Zn) by the different parts of *P. viridis*. In general, major differences of heavy metal concentrations were found between the mussel's shell and the different soft tissues. The different parts of the *P. viridis* were clustered into five groups. However, each cluster group consists of different member except for the first, second and the fifth groups where it consists of the shell in the first group; the remainder and gill in the second group; and the byssus in the fifth group for the two populations. As for the *P. viridis* from Pantai Lido, the third group consists of the muscle; while the fourth group consists of the mantle, gonad and foot. For the population from Telok Mas, the third group consists of the muscle and foot; and the mantle and gonad in the fourth group.

Discussion

From the observation of the metal concentrations in the different parts, there was no significant difference between the two populations. This could be due to relatively low metal bioavailabilities to *P. viridis* at Telok Mas and Pantai Lido. Similarly, based on cluster analysis (Figure 2), besides the shell, the byssus is distinctly clustered from the rest of different soft tissues, indicating the level of metal bioavailabilities to both populations. In addition, evidences of metal redistribution due

to metal contamination in both population (Yap et al., 2006a) based on the clustering pattern was absent.

Based on the three multivariate analyses, three major patterns were observed. First, there was insignificant correlation between the shells and different soft tissues while strong positive and significant correlation was found among the different soft tissues. Second, multiple linear stepwise regression analysis showed that the shells were not selected as an influential factor in affecting the metal accumulation in the total soft tissues. Third, the cluster analysis showed that the shells were clustered differently, as a single entity, from the rest of the different soft tissues.

The three above patterns indicate that the difference between soft tissues and shells is due to the fact that the accumulation of heavy metals in the shell differs from the soft tissues. Metals, sequestered into mussel tissues are translocated to the mantle, the tissue surrounding the animals' soft body. From the outside epidermis of the mantle the elements are released into the extrapallial fluid, located between mantle and mussel shell. From this extrapallial fluid the ions are incorporated into the inner surface of the newly forming calcium carbonate skeleton (Tynan et al., 2005; Dick et al., 2007). On the other hand, the strong positive and significant ($P < 0.05$) correlations between the heavy metals in the total tissues and the different soft tissues indicated that heavy metal concentrations in the total soft tissues and in a specific organ(s) would affect or is influenced by the metal accumulation in each of the soft tissues.

Strong correlation matrices of the different soft tissues in the mussel are due to their ability to accumulate the metals taken up from all sources including from water and food and offering integrated measures of supply of trace metals available to them in an environment (Phillip and Rainbow, 1993; Rainbow, 1995; Rainbow and Blackmore 2001). These findings were confirmed by Wang and Fisher (1999) who mentioned that metals dissolved in water may be accumulated by direct adsorption to body surface while particulate metals can be accumulated by organisms following ingestion and digestion of food.

Simkiss and Taylor (1995) also hypothesized that the pathways of trace metal transport across the biological membrane are through the passive diffusion of neutral metal species across the membrane, facilitated diffusion of metals via membrane transporter proteins, access through major ion channels (e.g., Ca channels) perhaps in association with active ion pumps and endocytosis. Environmental and biological factors including dissolved metal interactions (Amiard-Triquet and Amiard, 1998) have been considered for their influence on trace metal uptake and bioaccumulation.

It is well known that aquatic invertebrates possess very diverse strategies in the handling and storage of accumulated metals (Rainbow, 1998, 2002) which included in the forms of metal-rich granules and metallothioneins (MT) or metallothionein-like proteins (George, 1982; Mason and Jenkins, 1995). The existence of the three types of MT in the molluscs may influence the metal distribution in the different soft tissues. From the literatures, the Cd accumulation which included the induction of MT has been extensively studied in marine invertebrates (Bebianno and Langston, 1995; Bebianno and Serafim, 2003). For Cu, the accumulation was associated with both the soluble cytosolic and the insoluble particulate cell fractions as reported by Choi et al. (2003) in the different soft tissues of bivalve *Laternula elliptica*. Low-molecular-weight was the major Cu-binding ligands in the cytosol of the bivalve's different soft tissues. However for Zn, the exact mechanism of the regulation of Zn tissue concentration is not well known. Zn was believed to be taken up from the solute phase was proportional to the ambient Zn concentrations (Wang et al., 1996; Chong and Wang, 2001).

The metals found in the shells could be explained by the fact that some trace metals are incorporated into the shells of the *P. viridis* through substitution of calcium ion in the crystalline

phase of the shell or are associated with the organic matrix of the shell (Foster and Chacko, 1995; Yap et al., 2003c). At first, the metals could be distributed in different soft tissues before they are biodeposited in the shell (Yap et al., 2003c) instead of induction of MT as found in the soft tissues.

Conclusion

The multivariate analyses indicated that the accumulation of metals by the shell was significantly different from the different soft tissues of *P. viridis*. The present findings provided strong evidence that a shell has different capability of heavy metals uptake compared to the different soft tissues of *P. viridis*.

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Table 1: The descriptions of the sampling locations

No	Location	Sampling date	GPS reading	Site description
1.	Pantai Lido, Johore	30 th April 2006	N 01° 28' 06.8" E 103° 43' 35.7"	1. A reacreational area 2. Semi-enclosed intertidal area
2.	Telok Mas-2, Malacca	28 th April 2006	N 02° 09' 35.0 E 102° 19' 02.1	1. Seafood restaurant found.

Table 2: Analytical results for the Certified Reference Material (CRM) and its certified values for each metal ($\mu\text{g/g}$ dry weight).

Metal	Sample	CRM values	Measured values	Percentage of recovery
Cd	DOLT-3 Dogfish-liver	19.4 ± 0.6	20.5 ± 0.4	106.0 ± 2.3
Cu	DOLT-3 Dogfish-liver	31.2 ± 1.0	26.5 ± 2.6	85.0 ± 8.3
Fe	DOLT-3 Dogfish-liver	1484.0 ± 57.0	1070.0 ± 0.0	72.1 ± 0.0
Ni	DOLT-3 Dogfish-liver	2.72 ± 0.4	2.77 ± 0.7	102.0 ± 27.2
Zn	DOLT-3 Dogfish-liver	86.6 ± 2.4	80.9 ± 1.9	93.4 ± 2.2

NA: Pb value is not available

Table 3: Heavy metal concentrations in the different parts of *Perna viridis* collected from Pantai Lido (Johore) and Telok Mas (Malacca).

Site	Different part	Cu $\pm$ SE	Cd $\pm$ SE	Zn $\pm$ SE	Pb $\pm$ SE	Ni $\pm$ SE	Fe $\pm$ SE
Pantai Lido	Byssus	13.6 $\pm$ 0.0	2.6 $\pm$ 0.0	52.1 $\pm$ 0.0	46.5 $\pm$ 0.0	23.6 $\pm$ 0.0	1795.0 $\pm$ 0.0
		D	C	B	F	C	C
	Foot	8.8 $\pm$ 0.0	1.2 $\pm$ 0.0	49.3 $\pm$ 0.0	35.2 $\pm$ 0.0	7.3 $\pm$ 0.0	57.0 $\pm$ 0.0
		B	A, B	B	E	A	A
	Gill	11.0 $\pm$ 0.0	1.5 $\pm$ 0.0	89.8 $\pm$ 0.0	28.6 $\pm$ 0.0	9.8 $\pm$ 0.0	372.0 $\pm$ 0.0
		C	B	C	C, D	B	B
	Gonad	9.3 $\pm$ 0.1	1.3 $\pm$ 0.0	59.6 $\pm$ 2.9	31.1 $\pm$ 1.8	7.0 $\pm$ 0.4	102.0 $\pm$ 36.6
		B, C	A, B	B	D, E	A	A
	Mantle	12.0 $\pm$ 0.3	1.3 $\pm$ 0.1	47.6 $\pm$ 1.5	23.1 $\pm$ 0.7	5.9 $\pm$ 0.3	91.1 $\pm$ 0.8
		D	A, B	B	C	A	A
	Muscle	5.2 $\pm$ 0.2	0.859 $\pm$ 0.151	67.5 $\pm$ 0.4	12.4 $\pm$ 2.8	6.3 $\pm$ 0.4	42.9 $\pm$ 2.4
		A	A	B	B	A	A
Telok Mas	Remainder	12.3 $\pm$ 0.7	0.925 $\pm$ 0.175	92.8 $\pm$ 11.0	6.5 $\pm$ 0.9	9.6 $\pm$ 0.7	332.0 $\pm$ 71.6
		D	A	C	A	B	B
	Shell	8.4 $\pm$ 0.1	6.7 $\pm$ 0.0	6.0 $\pm$ 0.1	59.4 $\pm$ 0.1	40.4 $\pm$ 0.3	48.3 $\pm$ 0.4
		B	D	A	F	D	A
	Byssus	28.4 $\pm$ 1.7	2.4 $\pm$ 0.2	77.3 $\pm$ 1.4	11.5 $\pm$ 1.8	18.5 $\pm$ 1.7	4895.0 $\pm$ 75.0
		E	D	B	C	C	C
	Foot	5.9 $\pm$ 0.0	1.3 $\pm$ 0.0	51.6 $\pm$ 0.0	0.673 $\pm$ 0.000	6.6 $\pm$ 0.0	85.0 $\pm$ 0.0
		A	A, B	B	A	A	A
	Gill	11.7 $\pm$ 0.0	1.8 $\pm$ 0.0	78.9 $\pm$ 0.0	2.8 $\pm$ 0.0	10.8 $\pm$ 0.0	953.0 $\pm$ 0.0
		C	C	B	A	B	B
	Gonad	8.8 $\pm$ 0.0	1.5 $\pm$ 0.0	58.5 $\pm$ 0.0	0.558 $\pm$ 0.000	7.2 $\pm$ 0.0	262.0 $\pm$ 0.0
		B	B, C	B	A	A	A
	Mantle	6.7 $\pm$ 0.2	1.2 $\pm$ 0.1	48.7 $\pm$ 2.0	1.34 $\pm$ 0.0	6.5 $\pm$ 0.8	299.0 $\pm$ 12.5
		A, B	A, B	B	A	A	A
	Muscle	5.5 $\pm$ 0.4	0.998 $\pm$ 0.059	61.3 $\pm$ 0.6	2.4 $\pm$ 0.4	5.3 $\pm$ 0.2	140.0 $\pm$ 6.5
		A	A	B	A	A	A
	Remainder	13.6 $\pm$ 0.8	0.942 $\pm$ 0.085	61.3 $\pm$ 14.0	9.1 $\pm$ 0.6	6.6 $\pm$ 0.4	895.0 $\pm$ 137.0
		D	A	B	B	A	B
	Shell	8.0 $\pm$ 0.1	6.7 $\pm$ 0.1	5.5 $\pm$ 0.2	60.9 $\pm$ 0.2	40.6 $\pm$ 0.5	45.7 $\pm$ 1.8
		A, B	E	A	D	D	A

Note: Metal concentrations of different parts sharing a common letter in the post hoc column are not significantly different ($P > 0.05$)

Table 4: Spearman's correlation coefficient between the different parts of *Perna viridis* (Pantai Lido) [$\text{Log}_{10} x + 1$], based on the concentrations of Cd, Cu, Fe, Ni, Pb and Zn.

	Byssus	Foot	Gill	Gonad	Mantle	Muscle	Remainder	Shell	Total soft tissue
Byssus	1.000								
Foot	0.944**	1.000							
Gill	0.944**	1.000**	1.000						
Gonad	0.928**	0.983**	0.983**	1.000					
Mantle	0.911**	0.967**	0.967**	0.979**	1.000				
Muscle	0.928**	0.872**	0.872**	0.860**	0.856**	1.000			
Remainder	0.756**	0.812**	0.812**	0.800**	0.796**	0.719**	1.000		
Shell	0.309	0.253	0.253	0.240	0.249	0.086	-0.024	1.000	
Total soft tissue	0.777**	0.845**	0.845**	0.837**	0.862**	0.715**	0.936**	0.040	1.000

Note: ** Correlation is significant at the 0.01 level (2-tailed); N=20

Values without ** are not significantly ($P > 0.05$) correlated.

Table 5: Spearman's correlation coefficient between the different parts of *Perna viridis* (Telok Mas) [$\text{Log}_{10} x + 1$], based on the concentrations of Cd, Cu, Fe, Ni, Pb and Zn.

	Byssus	Foot	Gill	Gonad	Mantle	Muscle	Remainder	Shell	Total soft tissue
Byssus	1.000								
Foot	0.880**	1.000							
Gill	0.992**	0.889**	1.000						
Gonad	0.920**	0.928**	0.928**	1.000					
Mantle	0.925**	0.923**	0.929**	0.929**	1.000				
Muscle	0.946**	0.893**	0.955**	0.891**	0.924**	1.000			
Remainder	0.907**	0.688**	0.911**	0.804**	0.807**	0.868**	1.000		
Shell	-0.026	-0.197	-0.030	-0.265	-0.080	-0.005	0.090	1.000	
Total soft tissue	0.841**	0.655**	0.866**	0.699**	0.745**	0.816**	0.915**	0.183	1.000

Note: ** Correlation is significant at the 0.01 level (2-tailed); N=20.

Values without ** are not significantly ($P > 0.05$) correlated.

Table 6: Multiple linear stepwise regression analysis between the total soft tissues and the shells and the different soft tissues of *Perna viridis* based on the concentrations of Cu, Cd, Fe, Ni, Pb and Zn.

Sites	Stepwise linear regression equation
Pantai Lido	Total soft tissue = $0.861 - 0.779 (\text{Byssus}) - 0.559 (\text{Foot}) + 2.808 (\text{Gill}) - 1.006 (\text{Gonad})$; $R=0.988$, $R^2=0.976$
Telok Mas	Total soft tissue = $0.531 - 0.608 (\text{Byssus}) + 0.180 (\text{Foot}) + 3.464 (\text{Gill}) - 2.399 (\text{Gonad})$; $R=0.967$, $R^2=0.936$

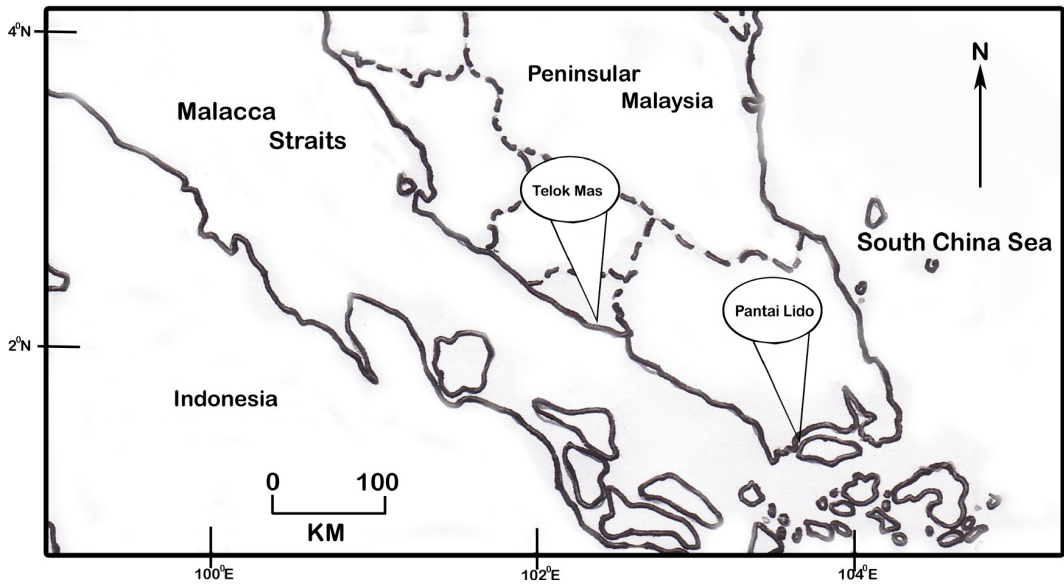


Figure 1: The sampling locations of *Perna viridis* in Peninsular Malaysia.

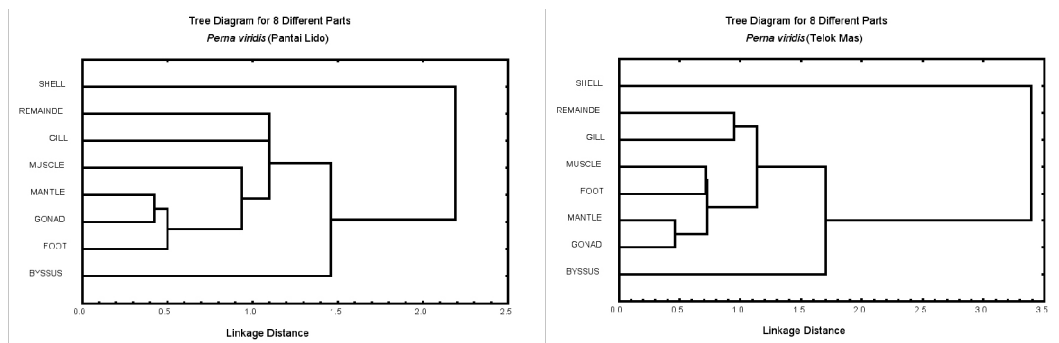


Figure 2: Hierarchical Cluster Analysis of the different tissues of *Perna viridis* based on heavy metal (Cd, Cu, Fe, Ni, Pb and Zn) concentrations [$\text{Log}_{10}(x + 1)$].