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Distributions of Cu and Zn in the Shell Lipped Part Periostracum and Soft Tissues of *Perna viridis*: The potential of Periostracum as a Biomonitoring Material for Cu Contamination

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ABSTRACT

Periostracum is the outer shell layer composes mainly of organic materials. In the present study, the green-lipped mussel *Perna viridis* was used to investigate the distributions of Cu and Zn in the periostracum and soft tissues of the *P. viridis* which were sampled from 17 geographical sites [23 populations] along the coastal waters of Peninsular Malaysia. The concentrations of Cu in the periostracum and the soft tissues of *P. viridis* were 7.41-42.63 μg/g dry weight and 3.49-31.1 μg/g dry weight, respectively. Meanwhile, the concentrations of Zn in the periostracum and soft tissues of *P. viridis* were 4.90-39.79 μg/g dry weight and 65.75-144.9 μg/g dry weight, respectively. The ratios of the metals in periostracum to soft tissues were 0.73-3.99 μg/g for Cu and 0.05-0.36 μg/g for Zn. These ratios indicated that the concentrations of Cu in the periostracum were generally greater than those in the soft tissues while the concentrations of Zn were generally higher in the soft tissues than those in the periostracum. The higher Cu levels in the soft tissues compared to that in the periostracum (Fig. 2) and the relatively close relationships of Cu between periostracum and sediment indicated that the periostracum was a good biomonitoring material for Cu, but periostracum was not a good biomonitoring material for Zn because it did not reflect the environmental contamination as reflected in the low correlation between the periostracum and sediment.

Keywords: Periostracum, *Perna viridis*, biomonitoring material, Cu and Zn

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INTRODUCTION

Soft tissues of marine bivalves have been frequently used in the biomonitoring studies of heavy metal pollutions in coastal waters. Several researchers used molluscs' shells as biomonitors of heavy metal pollution

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and their studies have been documented (Bertine & Goldberg, 1972; Koide *et al*., 1982; Bourgoin & Risk, 1987; Foster & Chacko, 1995; Ravera *et al*., 2001; Szefer *et al*., 2002; Brown *et al*., 2005, Cravo *et al*., 2002, 2005; Yap *et al*., 2003a, 2004). For example, Cravo *et al*. (2002) found that the shell of limpets *Patella aspera* is a tissue for potential use in environmental trace metal monitoring based on the marked and significantly higher levels of Fe and Mn in the contaminated site compared to the reference site. Brown *et al*. (2005) utilized the freshwater mussel shells to assess mercury (Hg) contamination in the North Fork Holston River. Foster and Cravo (2003) found *Nerita albicilla* as having the greatest potential as a biomonitoring tissue for trace metals, whereas Gillikin *et al*. (2005) assessed the use of clam shells (*Mercenaria mercenaria*) as a proxy of lead pollution.

The rationales of using bivalve shells in the study of heavy metal pollution were made based on several positive arguments and the characteristics of the shell formation. Each year, a mussel produces an incremental layer of its shell which is composed mainly of calcium carbonate and a small fraction of organic substance (Lindh *et al*., 1987). Many other elements are simultaneously deposited in these annual layers and are assumed to be essentially immobile after biodeposition into the crystalline lattices of the shell structure (Yap *et al*., 2003a). In the process of shell secretion, it is the mantle epithelium which secretes the extrapallial fluid. The fluid contains the components for biomineralization (Ca^{2+}, HCO^{3-}, organic molecules) and may also contain heavy metals if these are present in the outer medium (Watson *et al*., 1995). Any trace metals actively incorporated within the shell matrix during shell growth must have been assimilated by the organism (Wilbur & Saleuddin, 1983).

The shell of most bivalves is generally composed of an outer organic layer, the periostracum and two calcareous layers, namely the prismatic and nacreous (Gordon & Carriker, 1980). The periostracum layer is entirely organic and produced at the edge of mantle. The periostracum consists of proteins that have been sclerotized by quinone tanning to give the characteristic of horny texture (Gordon & Carriker, 1980). Several investigators of the periostracum in different species of bivalves have shown that it is composed of quinine tanned protein, mucopolysaccharides and lipids, and that some consist of several layers with different structure and staining properties. In addition, it is also thought that the two main functions of the periostracum are; firstly, to provide a waterproof covering for the shell, protecting it from acid dissolution and, secondly, to provide a substratum upon which calcium carbonate crystals can be deposited initially at the edge of the shell (Nakahara & Bevelander, 1971).

To our knowledge, little is known about the concentrations of Cu and Zn in the periostracum of green-lipped mussel *Perna viridis* in the literature. Therefore, the objective of this study was to determine the distributions and concentrations of Cu

and Zn between the periostracum and soft tissues of the *P. viridis* which had been collected from 17 geographical sampling sites in Peninsular Malaysia, including those collected from different environmental backgrounds.

MATERIALS AND METHODS

Mussels *P. viridis* were collected from 17 geographical sites (23 populations) along the coastal waters of Peninsular Malaysia (Fig.1). Surface sediments (0-10 cm) were also collected from 9 sampling sites (Table

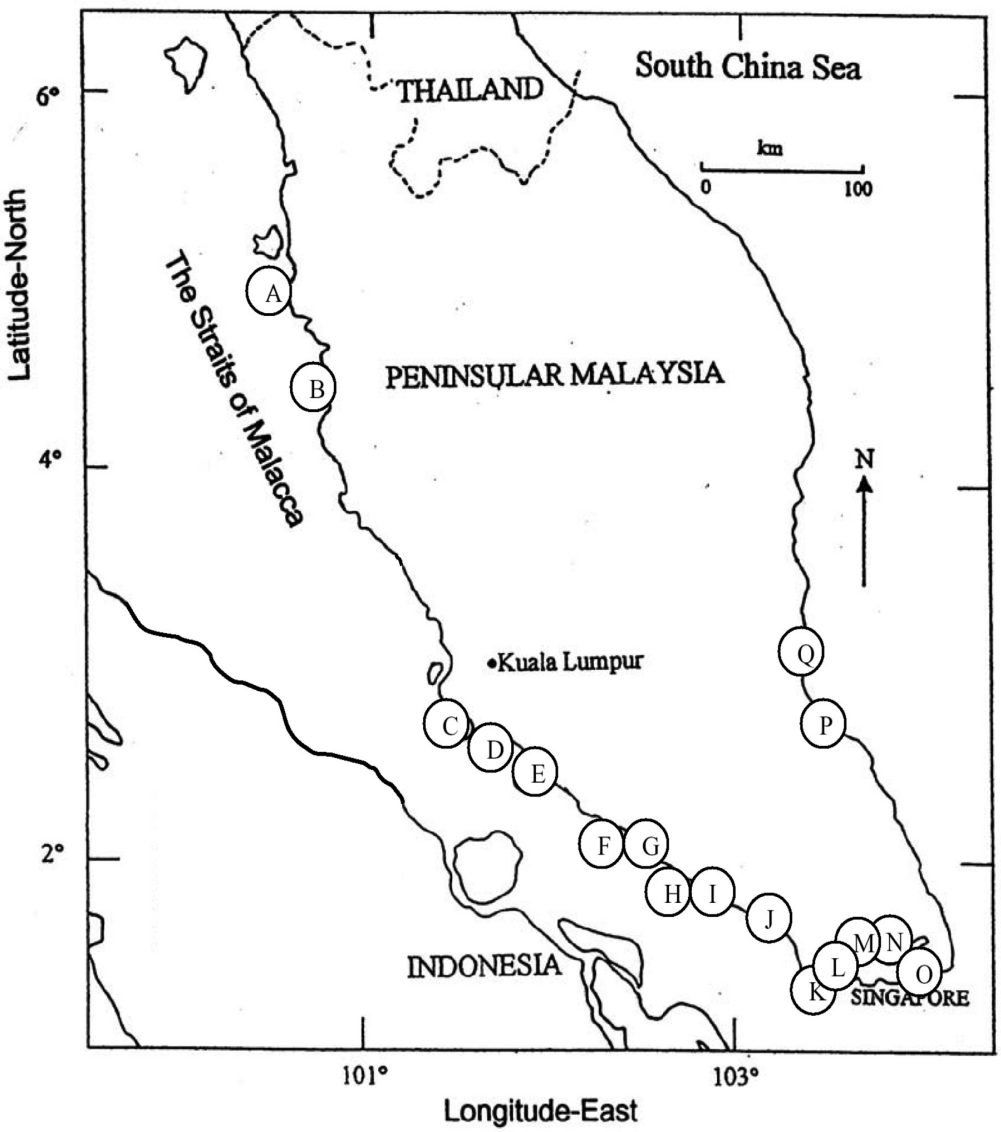

Fig.1: The sampling sites of the green-lipped mussel *Perna viridis* from the coastal waters of Peninsular Malaysia. Names of the sampling sites are represented by the alphabets as shown in Table 1

TABLE 1
Sampling dates and shell lengths (mean ± SE) of *Perna viridis* analyzed and descriptions of the sampling sites in the coastal waters of Peninsular Malaysia. Shell lengths are in mm. About 15-20 individuals were analysed in each sampling site

	Sampling sites	Sampling dates	Shell lengths	Descriptions of sampling site
A	*Pulau Aman (Penang)	11 Sep1999	91.50 ± 2.0	A fish aquacultural area
B	Bagan Tiang-1 (Perak)	01 Apr 2002	135.0 ± 6.0	An aquacultural area
	Bagan Tiang-2 (Perak)	20 Apr 2005	86.5 ± 3.0	An aquacultural area
C	*Bagan Lalang (Selangor)	08 Jun 1998	91.2 ± 4.5	A recreational and agricultural areas
D	*Lukut (Negeri Sembilan)	08 Aug 1998	93.9 ± 3.1	An aquacultural area
E	*Pasir Panjang (Negeri Sembilan)	22 Sep 1998	88.6 ± 2.6	A mussel aquacultural area
F	*Kuala Linggi (Negeri Sembilan)	21 Nov 2000	80.0 ± 1.2	A fish and mussel aquacultural area
G	Merlimau-1 (Malacca)	19 Apr 2002	68.55 ± 2.5	A mussel aquacultural area
	Merlimau-2 (Malacca)	09 Apr 2004	82.30 ± 3.8	A mussel aquacultural area
H	Telok Emas (Malacca)	09 Apr 2004	84.61 ± 4.8	A mussel aquacultural site
I	*Sebatu-1 (Malacca)	12 Aug 2000	85.4 ± 4.5	A mussel aquacultural area
	Sebatu-2 (Malacca)	19 Feb 2002	63.04 ± 3.8	A mussel aquacultural area
J	Minyak Beku (Johore)	18 Jan 2005	76.0 ± 1.0	Wild mussels found a recreational site.
K	Kukup (Johore)	18 Jan 2005	83.0 ± 3.0	Wild mussels found at a fish agricultural floating house and near a jetty.
L	*Tg. Kupang (Johore)	19 Jan 2000	83.6 ± 3.0	A port and an aquacultural area.
M	*Pantai Lido-1 (Johore)	23 Sep 1998	59.4 ± 2.7	Urban and restaurant areas.
	Pantai Lido-2 (Johore)	17 Apr 2002	89.08 ± 5.0	Urban and restaurant areas.
	Pantai Lido-3 (Johore)	19 Jan 2005	103.0 ± 6.8	Urban and restaurant areas.
N	*Kg. Pasir Puteh-1 (Johore)	19 Jan 2000	61.10 ± 2.1	Industrial and mooring activities and urban areas
	Kg. Pasir Puteh-2 (Johore)	17 Apr 2002	92.84 ± 3.3	Industrial and mooring activities and urban areas
O	Kuala Belungkor (Johore)	18 Apr 2002	64.94 ± 1.2	Pristine area and a fish aquacultural area.
P	Kuala Pontian (Pahang)	08 Apr 2004	70.11 ± 1.5	A mussel aquacultural site.
Q	Nenasi (Pahang)	08 Apr 2004	76.11 ± 2.3	A light house nearshore; pristine water.

Note:* indicated where the sediments were also collected in the mussel habitats.

TABLE 2
The Cu concentrations (mean μg/g dry weight) in periostracum (perios) and the total soft tissues (ST) of *Perna viridis* (N= 3)

	Sampling sites	Perios	ST	$\frac{Cu_{perios}}{Cu_{ST}}$
A	Pulau Aman	22.99	10.80	2.13
B	Bagan Tiang-1	13.24	14.14	0.94
	Bagan Tiang-2	7.41	9.86	0.75
C	Bagan Lalang	26.09	8.20	3.18
D	Lukut	24.50	10.22	2.40
E	Pasir Panjang	17.76	10.87	1.63
F	Kuala Linggi	24.03	9.14	2.63
G	Merlimau-1	15.63	11.07	1.41
	Merlimau-2	18.95	8.94	2.12
H	Telok Emas	12.97	3.49	3.72
I	Sebatu-1	14.99	11.16	1.34
	Sebatu-2	17.97	15.72	1.14
J	Minyak Beku	8.63	10.21	0.85
K	Kukup	13.22	11.68	1.13
L	Tg.Kupang	15.39	6.31	2.44
M	Pantai Lido-1	20.50	9.39	2.18
	Pantai Lido-2	18.88	14.27	1.32
	Pantai Lido-3	11.47	15.71	0.73
N	Kg.Pasir Puteh-1	29.49	20.10	1.47
	Kg.Pasir Puteh-2	42.63	31.09	1.37
O	Kuala Belungkor	16.67	7.96	2.09
P	Kuala Pontian	20.28	14.00	1.45
Q	Nenasi	18.53	4.64	3.99

1). All the samples were stored at -10°C until metal analysis. At the laboratory, the soft tissues were carefully separated from the shell and the byssus was discarded. The periostracums were pooled and triplicates were analyzed; 15-20 individuals of the total soft tissues of mussel *P. viridis* were analyzed in each sampling site (*see* Table 1).

After rinsing with double distilled water and 0.5% HCl, they were dried for 72 hours at 105°C to constant weights (Mo & Neilson, 1994). In order to separate the outermost layers (periostracum layers) of the shells, they were cooled at room temperature after heating at 105°C. While the shells were cooling, most of the outer layers cracked and fell off (Puente *et al*., 1996). The periostracum layers were weighed with an accuracy of 0.1 mg before acid digestion. The periostracum and soft tissues were digested in concentrated HNO_3 (AnalaR grade; BDH 69%).

The sediment samples were also dried at 105°C to constant weights. The dried sediment samples were crushed using a mortar and pestle and then sieved through a 63 μm aperture stainless steel sieve before they were shaken vigorously to produce homogeneity. For the analyses of the total Cu and Zn concentrations in the sediment samples, three replicates were analyzed using the direct aqua-regia method. About 1g of each dried sample was digested in a combination of concentrated HNO3 (AnalaR grade; BDH 69%) and HClO4 (AnalaR grade; BDH 60%) in the ratio of 4:1 (Yap *et al*., 2002a).

The periostracum, total soft tissues and sediment samples were put into a hot-block digester first at low temperature (40°C) for 1 hour and then were fully digested at 140°C for at least 3 hours. The digested samples were then diluted to a certain volume (40 ml) with double distilled water.

TABLE 3
The Zn concentrations (mean μg/g dry weight) in the periostracum (perios) and total soft tissues (ST) of *Perna viridis* (N= 3)

	Sampling sites	Perios	ST	$\frac{Zn_{perios}}{Zn_{ST}}$
A	Pulau Aman	19.92	109.6	0.18
B	Bagan Tiang-1	7.67	65.75	0.12
	Bagan Tiang-2	4.90	101.8	0.05
C	Bagan Lalang	6.25	96.36	0.06
D	Lukut	11.51	69.40	0.17
E	Pasir Panjang	8.15	98.92	0.08
F	Kuala Linggi	13.09	101.1	0.13
G	Merlimau-1	11.34	88.64	0.13
	Merlimau-2	39.79	111.8	0.36
H	Telok Emas	6.84	102.1	0.07
I	Sebatu-1	6.80	75.14	0.09
	Sebatu-2	14.59	105.4	0.14
J	Minyak Beku	11.58	139.4	0.08
K	Kukup	15.89	140.6	0.11
L	Tg.Kupang	13.82	88.58	0.16
M	Pantai Lido-1	13.23	80.49	0.16
	Pantai Lido-2	12.60	105.5	0.12
	Pantai Lido-3	20.95	135.8	0.15
N	Kg.Pasir Puteh-1	15.70	128.9	0.12
	Kg.Pasir Puteh-2	9.60	69.89	0.14
O	Kuala Belungkor	6.81	86.26	0.08
P	Kuala Pontian	24.00	140.3	0.17
Q	Nenasi	8.03	96.50	0.08

TABLE 4
Overall mean concentrations, minimum and maximum values of Cu and Zn in the periostracum (Perios) and total soft tissues (ST) and their ratios (Perios/ST) of *Perna viridis*. N= 23 populations

		Minimum	Maximum	Mean	SE
Zn	Perios	4.90	39.79	13.18	1.60
	ST	65.75	140.63	101.67	4.80
	Perios/ST	0.05	0.36	0.13	0.01
Cu	Perios	7.41	42.63	18.79	1.57
	ST	3.49	31.09	11.69	1.18
	Perios/ST	0.73	3.99	1.84	0.19

After filtration, the prepared samples were determined for Cu and Zn by using a flame atomic absorption spectrophotometer (AAS) Perkin-Elmer Model 4100. The data were presented in μg/g dry weight basis. To avoid possible contamination, all glassware and equipment used were acid-washed. In addition, our analytical procedures were checked with Certified Reference Materials for Dogfish liver (DOLT-3) and the recoveries were being satisfactory (Cu: certified = 31.20 μg/g dw; measured =32.0 μg/g dw and Zn: certified = 86.6 μg/g dw; measured = 100.3 μg/g dw).

RESULTS

From Tables 2 and 4, the concentrations of Cu in the periostracum and soft tissues of *P. viridis* were 7.41-42.63 μg/g dry weight and 3.49-31.1 μg/g dry weight, respectively. The ratios of Cu periostracum/soft tissues are between 0.73 and 3.99. Except for four populations (out of 23 populations), the Cu levels were higher in the periostracum than in those in the soft tissues, as indicated in Fig.2. The highest Cu concentration in the periostracum was found at Kg. Pasir Puteh-2 collected in 2002 (42.6 μg/g dry weight), followed by Kg. Pasir Puteh-1 (29.5 μg/g dry weight) which was sampled in 2000. These results were supported by the higher Cu levels in the soft tissues of the similar sampling sites and the high Cu levels in the sediments collected from Kg. Pasir Puteh (Yap *et al*., 2002a). Therefore, this good relationship had provided a basis for the reason why the periostracum could be used as a potential biomonitoring material for Cu pollution.

From Tables 3 and 4, the concentrations of Zn in the periostracum and soft tissues of *P. viridis* were 4.90-39.79 μg/g dry weight and 65.75-144.9 μg/g dry weight, respectively. The ratios of Zn periostracum/soft tissues are between 0.05 and 0.36. All the 23 populations showed higher levels of Zn in the soft tissues than in the periostracum, as indicated in Fig.2. Meanwhile, the highest Zn concentration in the periostracum was found at Merlimau-2 (39.8 μg/g dry weight), whereas the lowest one was found at Bagan Tiang (4.90 μg/g dry weight). However, these results are not supported by the Zn levels in the soft tissues of the similar sampling site.

The relationships of Cu and Zn between the periostracum or total soft tissues and the sediments are presented in Figure 3. It was found that the soft tissue-Cu is highly correlated (R= 0.83) with sediment-Cu while the soft tissue-Zn is also correlated well (R= 0.73) with sediment-Zn. The lower correlation in Zn as compared to than Cu indicated that Zn is most likely to be partially regulated (Yap *et al*., 2002b). However, the soft tissues of *P. viridis* are usually used for the biomonitoring of Cu and Zn in the tropical coastal waters. As shown in Figure 3 again, periostracum-Cu is found to be correlated (R= 0.61) with sediment-Cu, while periostracum-Zn is not well correlated (R= 0.33) with sediment-Zn. The insignificant correlation between periostracum-Zn vs. sediment-Zn could be due to lower Zn levels in the periostracum than those in the soft tissues (see Figure 2). The higher Cu levels in the soft tissues than in the periostracum (Figure 2) and the relatively close relationship of Cu between periostracum and sediment indicated that the periostracum was a good biomonitoring material for Cu, compared to periostracum which was not a good biomonitoring material for Zn because it did not reflect the environmental Zn contamination, as indicated by the low correlation between the periostracum and sediment.

DISCUSSION

The distribution or partitioning of Cu and Zn in the periostracum and the soft tissues of *P. viridis* indicated three important points from the biomonitoring points of view. First, the differences in the accumulations of Cu and Zn in the hard and soft tissues which could be due to the differences of the binding affinities to sites between the periostracum, which is mainly composed of organic materials, whereby the soft tissues are

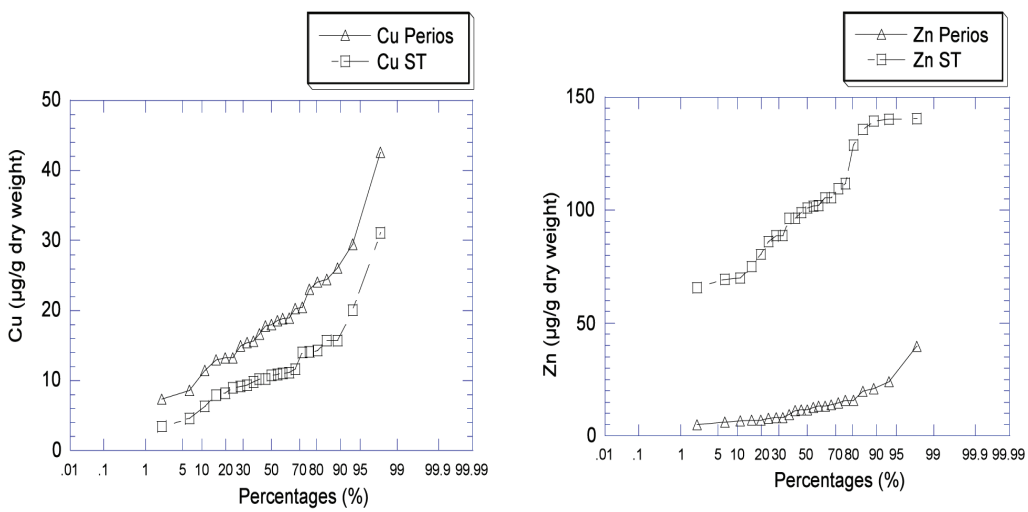

Fig.2: The probability of concentrations of Cu and Zn in the periostracum (Perios) and total soft tissues (ST) of *Perna viridis*. N= 23 populations

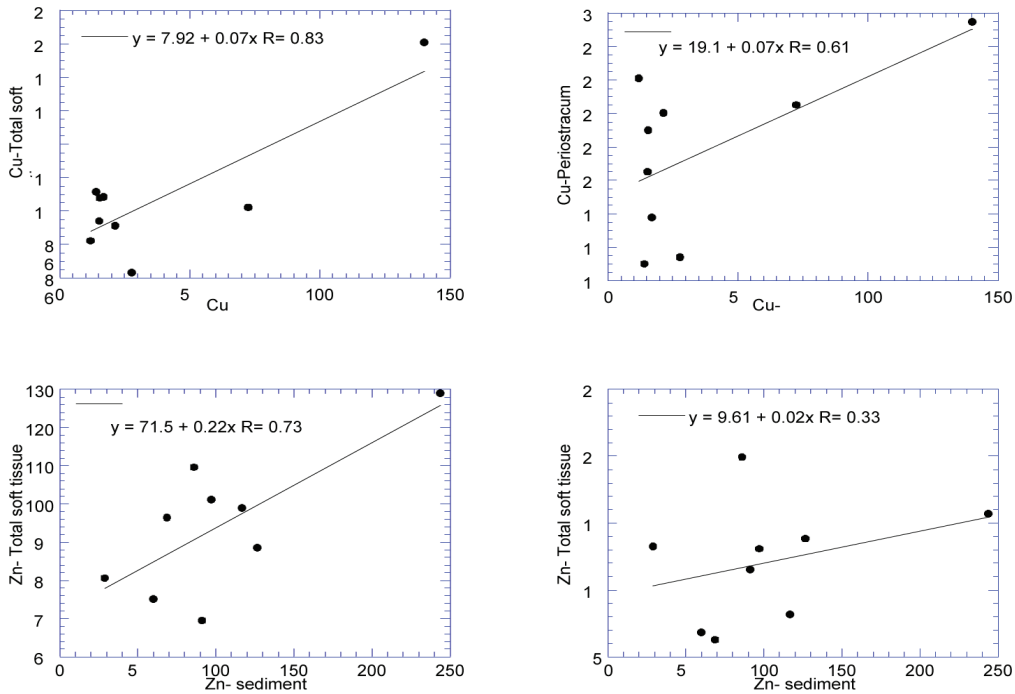

Fig.3: The relationships of Cu and Zn between the periostracum or total soft tissues and sediments. N= 9 populations

mainly composed of organic and inorganic materials. Second, the higher Cu levels in the periostracum than those in the soft tissues and the relatively close relationship of Cu between periostracum and sediment are the findings which support the use of periotracum as a potential biomonitoring material of Cu. In addition, the highest Cu concentration in the periostracum was found in the known Cu-contaminated site at Kg. Pasir Puteh (Yap *et al*., 2002a, 2003b). Since the periostracum is mainly composed of the green lipped part of the outer shell layer, the higher Cu levels in this shell layer is expected since the Cu concentration is responsible for the green colour of the lipped shell part (Yap *et al*., 2003a).

From the literature, the binding of Cu and Zn in the soft tissues of marine mussels has been shown to be due to the metallothionein synthesis for detoxification (Yap *et al*., 2006). However, the way in which the metals are incorporated into the shells of molluscs is due to the substitution of the calcium ion in the crystalline phase of the shell or associated with the organic matrix (Watson *et al.,* 1995). Moreover, the mineralogy and chemistry of the shell's material secreted by organisms can vary with the environment of growth (Dodd, 1963). Al-Dabbas *et al.* (1984) suggested that a shell's composition is sufficiently sensitive to environmental variation that the sub-environments can be distinguished within

a limited water system. This suggestion has further strengthened the hypothesis that the periostracum could be a potential biomonitoring material for Cu.

Although it has been reported that the periostracum shell layer is mainly composed of organic materials (Kennedy *et al*., 1969), the reason for the higher levels of Cu in the periostracum than those in the soft tissues is interesting from the biochemical point of view. Thus, 'how do the Cu levels bind to the organic materials of the shells?' should prompt further studies. However, the periostracum layer covers less than 5% of the total shell weight and therefore, a mussel shell mainly consists the two calcareous inorganic layers. This could be the reason why many researchers did not focus on the outer shell layer of the periostracum but instead examine on the inner or total shells of the mollusks.

The periostracum is a potential biomonitoring material for Cu because it has some advantages as a biomonitoring material. First, the shells themselves can also accumulate Cu to a considerable extent (Sturesson, 1976) and are higher than the soft tissues. Second, the comparison of metals between the newer shells and the material from collections or fossil shell, can also be made. Bourgoin and Risk (1987) studied the recent and fossil shells of *Mya truncata* (8200 a BP) and their surrounding sediments which were collected from three sites near Pangnirtung, Northwest Territories, in the eastern Canadian Arctic. The researchers found that the Pb levels in the fossil shells were approximately five times lower than those detected in the modern shells. Hence, the results of this study suggested that the determination of pollutant levels in the shells of bivalves might be an important and underutilized tool for environmental assessment.

Another advantage of using periostracum as a biomonitoring material is that it is easier to handle and to store; in fact, there is no need to preserve the shell materials under low temperature like the soft tissues. Furthermore, the problem of whether or not to depurate the animals before analyses is avoided (Koide *et al*., 1982). Brown *et al*. (2005) also proposed that the shell-based strategies based on freshwater mussels do not require sampling live specimens and might augment more standard strategies applied to environmental monitoring.

Thus, it is essential to choose a shell structural component that has not been exposed to either particulate or dissolved metals in the water column (Bourgoin, 1990) because these metals will also be incorporated by adsorption and cannot be distinguished from those metals which are truly assimilated (Phillips, 1980). The arguments and weaknesses of using the periostracum layer for the biomonitoring of trace metals are as follows:

1. The layer is exposed and therefore it is not the best bioavailability biomonitoring material;
2. It is the newly developed shell layer and therefore it does not provide an indicator of long-term exposure to heavy metal pollution in the coastal waters.

However, based on the findings of the

present study, the use of the periostracum as a good biomonitoring material of Cu is proposed because of three positive arguments listed below:

1. First, since it is exposed to environmental seawater, it is therefore a partially good indicator of environmental Cu in the seawater adsorbed onto the periostracum surfaces.
2. Second, it is a newly developed shell layer from the mantle edge of the mussels and therefore, the metals found in this layer contain biologically deposited Cu in the recent time.
3. Third, the relatively close relationship of Cu between periostracum and sediment positively supported that the periostracum of *P. viridis* was a good biomonitoring material for Cu since the Cu levels in the periostracum was found to be generally higher than those in the soft tissues. Therefore, the Cu levels of the periostracum could provide an index of Cu bioavailability, apart from reflecting the Cu contamination of the sampling site.

CONCLUSION

The higher Cu levels in the soft tissues compared to those in the periostracum (as indicated by the ratios of periostracum to soft tissues) and the relatively close relationship of Cu between the periostracum and sediment indicated that the periostracum was a good biomonitoring material for Cu but not for Zn. However, it is still unknown from the molecular point of view (DNA level) for reasons why the higher Cu levels have been found in the periostracum compared to the soft tissues, and on the other hand, a reverse pattern was found for Zn. All these should prompt more future studies on the use of periostracum and the inner shell layers of mussels as biomonitoring materials of heavy metal pollutions in coastal waters.

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