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Studies on Heavy Metal Accumulations in Green-Lipped Mussel *Perna viridis* from Malaysia by Using Multiple Linear Stepwise Regression Analysis

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ABSTRAK

Kepekatan Cd, Cu, Pb and Zn dalam tisu lembut kupang *Perna viridis* daripada 15 lokasi di Malaysia didapati rendah berbanding data di rantau ini. Namun demikian, sampel-sampel Kuala Perlis dan Kg. Pasir Puteh patut diberi lebih perhatian kerana lokasi-lokasi tersebut dikenal pasti menerima logam berat hasil daripada aktiviti manusia. Analisis regresi 'multiple linear stepwise' didapati berguna untuk mencari parameter yang paling berpengaruh dalam menentukan biopenimbunan logam berat dalam kupang *P. viridis.* Keputusan analisis tersebut menunjukkan bahawa parameter yang terpilih adalah lebih kurang konstan tidak kira pelbagai model yang digunakan dalam prosedur. Oleh yang demikian, analisis regresi 'multiple linear stepwise' boleh dicadangkan sebagai cara statistik alternatif dalam menentukan parameter yang paling berpengaruh yang menyebabkan biopenimbunan logam berat di dalam *P. viridis* dan invertebrata yang lain.

ABSTRACT

The concentrations of Cd, Cu, Pb and Zn in soft tissue of green-lipped mussel *Perna viridis* from 15 sampling sites in Malaysia were found to be not serious but low when compared to regional data. However, samples from Kuala Perlis and Kg. Pasir Puteh should be given more attention since these locations were identified to have received anthropogenic metals. The multiple linear stepwise regression analysis was found to be useful in finding the most influential statistical parameters affecting heavy metal accumulation in *P. viridis.* The regression analyses indicated that the parameters were rather constant regardless of different sets of models being included in the procedure. Therefore, the multiple linear stepwise regression analysis can be proposed as an alternative statistical method for determining the most important variables affecting heavy metal accumulations in *P. viridis* as well as in other invertebrates.

Keywords: *Perna viridis*, heavy metals, multiple linear stepwise regression analysis

INTRODUCTION

Statistical analysis in biological data has always been an important step in interpreting the data. There are a number of different ways as well as different statistical means in performing the analysis. Regression analysis is a statistical method to determine if there are relationships between the variables studied and it is also useful in the study of the shape of the curve of the relationship and allows researchers to look for the reasons for the relationships (Snedecor and Cochran 1980).

Multiple Linear Stepwise Regression Analysis (MLSRA) was applied here to find an empirical equation relating to the dependent variable(s) (metal concentration) that has a high value of r^2 (coefficient of determination) using only the most important independent variables (Bethea *et al.* 1985). Stepwise regression requires the assumption that there is only one best equation (Bethea *et al.* 1985) and that the procedure in the Statistical Analysis System (SAS) program will find it. The general forward selection procedure in SAS begins by using the independent variables one at a time, comparing the corresponding reduction in the error sum of squares with some pre-set criterion ($a = 0.15$ is the default for PROC STEPWISE) and then either retaining or rejecting the term (Bethea *et al.* 1985). STEPWISE procedure is a modified forward selection method in which the F-value for each term in the model is calculated, compared to the corresponding tabulated values of F and rejected if it is not significant for the model (Bethea *et al.* 1985). Then the next term is added to the model and the process is repeated. In the present study, MLSRA was carried out by using independent variables which included non-ratios and ratios. The non-ratio independent variables included shell length (LE), shell width (WI), shell height (HE), flesh dry weight (FW), shell dry weight (SW) and sex while the ratio independent variables included condition index (CI), width : height (WH), width : length (WL), length : height (LH) and shell thickness (ST). Ratios express relationships between quantitative variables and thereby aid in summarizing and interpreting statistical data (Whitmore *et al.* 1973).

In addition, a comparison between a procedure which included all independent variables and a procedure which included only independent ratios was discussed. These two models were compared based on all populations collected from 15 stations. They were termed as inter-station MLSRA. The third model which only included one population from one sampling site (Pasir Panjang) was termed as intra-station MLSRA which included 27 female and 29 male individuals. Inter-station and intra-station models were calculated in order to find out if similar independent variables were selected as the most influential parameters affecting metal accumulation in both flesh and shell of green-lipped mussel *P. viridis.* The comparison of these different models was made to examine if MLSRA can be recommended as a good statistical means in finding out the most important factors affecting the accumulations of Cd, Cu, Pb and Zn of *P. viridis.*

The dependent variables for the MLSRA included Cd, Cu, Pb and Zn concentrations in both flesh and shell of the mussel *P. viridis.*

MATERIALS AND METHODS

There were 15 sampling sites from Malaysian coastal waters (*Fig. 1*) in this study. All the samples were collected between May and September 1998. Information about reproductive cycles of *P. viridis* in the sampling sites revealed that in the Straits of Johore the natural spats could be found throughout the year, mainly from November to February and May to June (Shamsudin 1992; Aldon and Buendia 1998). Koh *et al.* (1991) reported that the settlement of pelagic *P. viridis* in the coastal waters of the Straits of Johore peaked during the inter-monsoon months of November and April.

The mussels at each sampling site were analysed according to sex, and shell lengths for each site and sex are given in Table 1. In the present study, between 20 and 32 individuals (male and female) were used to represent one station (Table 1) and this sample size was within what was usually reported in many studies using bivalves as biomonitors (Phillips and Rainbow 1993, Hung *et al.* 2001, Yap *et al.* 2002).

In general, the sampling sites could be classified depending on their surroundings. For instance, a site at Kuala Perlis could be categorized as a jetty, sites at Langkawi are fish culture areas whilst Kg. Pasir Puteh is a marina and near urban and industrial areas. Other areas are believed to have agricultural activities.

All individuals were measured for their LE, WI and HE with vernier calipers. Further, dried shell weight and dried flesh weight were determined for calculating

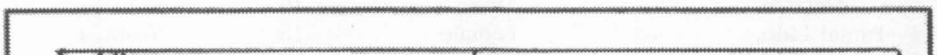

Fig. 1: Map showing sampling stations of green-lipped mussel Perna viridis

TABLE 1
Mean cm ± standard error (SE) of shell length, shell width, shell weight, fresh dry weight and shell dry weight per individual of mussel *P. viridis* from 15 sampling stations

	Sampling locations	Sex	N	Shell length
1	K. Perlis	Female	10	3.64 ± 0.17
	Perlis.	Male	12	3.40 ± 0.12
2	Tanjung Rhu,	Female	10	7.00 ± 0.06
	Langkawi.	Male	15	7.08 ± 0.07
3	Sangkar Ikan,	Female	9	7.12 ± 0.23
	Langkawi.	Male	11	6.87 ± 0.25
4	Tanjung Dawai,	Female	7	7.96 ± 0.52
	Kedah.	Male	13	8.25 ± 0.20
5	Penang Island,	Female	8	6.60 ± 0.52
	Penang.	Male	15	7.59 ± 0.19
6	Bagan Lalang,	Female	10	9.38 ± 0.18
	Selangor.	Male	15	8.93 ± 0.20
7	Lukut,	Female	14	9.53 ± 0.18
	N. Sembilan.	Male	11	9.23 ± 0.19
8	Pasir Panjang,	Female	12	8.40 ± 0.24
	N. Sembilan.	Male	13	9.18 ± 0.49
9	Pantai Kundor,	Female	16	8.89 ± 0.09
	Malacca.	Male	14	8.52 ± 0.11
10	Anjung Batu,	Female	13	9.31 ± 0.12
	Malacca.	Male	16	9.17 ± 0.14
11	Pantai Lido,	Female	18	6.06 ± 0.15
	Johore.	Male	14	5.80 ± 0.13
12	Kg. Pasir Puteh,	Female	15	6.89 ± 0.44
	Johore.	Male	16	5.92 ± 0.40
13	Kg. Tg. Batu,	Female	10	2.51 ± 0.04
	Pahang.	Male	12	2.57 ± 0.04
14	Trayong,	Female	8	10.97 ± 0.50
	Sabah.	Male	11	10.98 ± 0.42
15	Kuala Penyu,	Female	7	7.20 ± 0.15
	Sabah.	Male	15	6.66 ± 0.10

Note: Besides what is shown in Table 1, samples from Pasir Panjang were also analysed from 2.0 to 11.0 cm of shell lengths.

the allometric ratios with LE, WI and HE. The soft tissues and shells of *P. viridis* were analyzed according to sex. The whole soft body was carefully removed by deshelling the mussel with a stainless knife. The dry weight of the individual soft body was determined by drying the whole body tissues for 72 h at 105°C to a constant weight (Mo and Neilson 1994). At each location, every individual's inner nacreous shell layer was ground into a fine powder in an agate mortar and pestle, before being homogenised and weighed for digestion with an accuracy of 0.1 mg. The samples were digested in concentrated nitric acid and assayed for heavy metals by using a flame atomic absorption spectrophotometer (AAS) Perkin-Elmer Model 4100. The data are presented in mg/g dry weight basis. Mutiple-level calibration standards were analysed to generate calibration curves against which sample concentrations were calculated. Standard solutions were prepared from 1000 mg/l stock solution of each metal (MERCK Titrisol).

A quality control sample was routinely run through during the period of metal analysis. To avoid possible contamination, all glassware and equipment used were acid-washed and the accuracy of the analysis was checked with the standard addition testing method. Procedural blanks were prepared and the metal concentrations in the blanks were negligible. Percentages of recoveries were 95% for Cu, 92% for Zn, 110% for Cd and 92.5% for Pb.

The statistical analyses were done by using the Statistical Analysis System Version 6.0 (SAS Institute 1987) software package. Data transformation was carried out to stabilize the variance and the lack of normality to produce a frequency distribution that was nearer to normal distribution (Sheldon and Haick 1981). After all data were converted into the desired values, the data would be transformed to $\log_{10}$ (X) according to the procedure of Boulton and Lake (1990).

The models are as follows :-

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + \ldots + b_NX_N$$

where Y = dependent variable

X_1 - X_N = independent variable

b_0 = intercept

b_1 - b_N = slope for each independent variable

The allometric ratios calculated were WH, WL, LH, ST and CI. ST, considered to be an age measure in bivalves (Cossa *et al.* 1980; Frew *et al.* 1989), was calculated with the following formula :-

$$\text{ST (g/cm}^2\text{)} = \frac{\text{shell weight (g)}}{\text{shell length (cm)} \times \text{shell height (cm)}}$$

CI, regarded as an indication of the mussel's individual physiological state reflecting the amount of stored energy which may be considered as the animal's metabolic response to environmental conditions (Peddicord 1977), was calculated as shown below:-

$$\text{CI (g/cm}^3\text{)} = \frac{\text{total fresh dry weight (g)}}{\text{shell height (cm)} \times \text{shell length (cm)} \times \text{shell width (cm)}} \times 1000$$

RESULTS AND DISCUSSION

Heavy Metal Concentrations in Mussels

Since the international 'Mussel Watch' approach uses total soft tissue of marine mussels to monitor the heavy metal contamination in the coastal waters (Goldberg 1975; Phillips and Segar 1986; Phillips and Rainbow 1993), only heavy metals in the soft tissue of *P. viridis* were discussed in the present study. From the 15 locations, the ranges of metals varied from 0.19 to 1.56 μg/g dry weight for Cd, 5.78 to 15.14 μg/g dry weight for Cu, 1.17 to 8.27 μg/g dry weight for Pb and 46.78 to 145.95 μg/g dry weight for Zn *(Fig. 1)*. These ranges of Cd, Cu, Pb and Zn were all comparable and lower than those reported in *P. viridis* from Thailand (Sukasem and Tabucanon 1993), India (Senthilnathan *et al.* 1998) and Hong Kong (Wong *et al.* 2000).

Samples from Kuala Perlis were found to have high concentrations of Cd (1.56 mg/g dry weight) and Pb (8.27 mg/g dry weight). Kuala Perlis has a lot of boating activities and some port operations (Ismail *et al.* 1991). These have become the potential sources of heavy metal contamination in the area. To support our results, some crabs were reported to have elevated levels of heavy metal in Kuala Perlis by Ismail *et al.* (1991). Samples from Kg. Pasir Puteh were found to have the highest Cu concentration (15.14 μg/g dry weight). Since industries, shipping and urban sewage were considered the major sources of metal contamination, the elevated Cu level found in Kg. Pasir Puteh was an interesting result. Samples from Trayong of Sabah had high concentrations of Cd (1.18 μg/g dry weight) and Zn (121.79 μg/g dry weight). Only fish and mussel aquacultural activities were found in this area and the potential sources of this metal contamination are still unknown. Other sampling sites showed relatively low concentrations of heavy metal since most of these areas practised agricultural activities. However, these levels were not considered serious.

Finding the Most Influential Factors by Using Mutiple Linear Stepwise Regression Analysis

Inter-station Comparison

All populations from 15 stations (inter-station) were tested to determine the most important known factors affecting metal accumulation (Tables 2, 3). The regression equations observed appear to fall into four distinct categories. First, Cu, Pb and Zn concentrations in the flesh and Pb concentration in the shell were all significantly influenced by sex besides other variables (Table 2). The above-mentioned metals were higher in females, as indicated by the negative sign in sex (Lobel *et al.* 1989). Second, it was noted that CI was the most influential variable in both flesh (Cu and Zn) and shell (Cd, Pb and Zn). However, Zn-flesh was negatively influenced by CI.

Third, Cu and Pb in both flesh and shell were inconsistently influenced by ratios such as WH, WL and LH. It was somewhat surprising that ST was not found to influence the Cu and Pb concentrations. Probably, ST could be replaced by physiological terms such as growth rate (WL) and CI. Since CI is

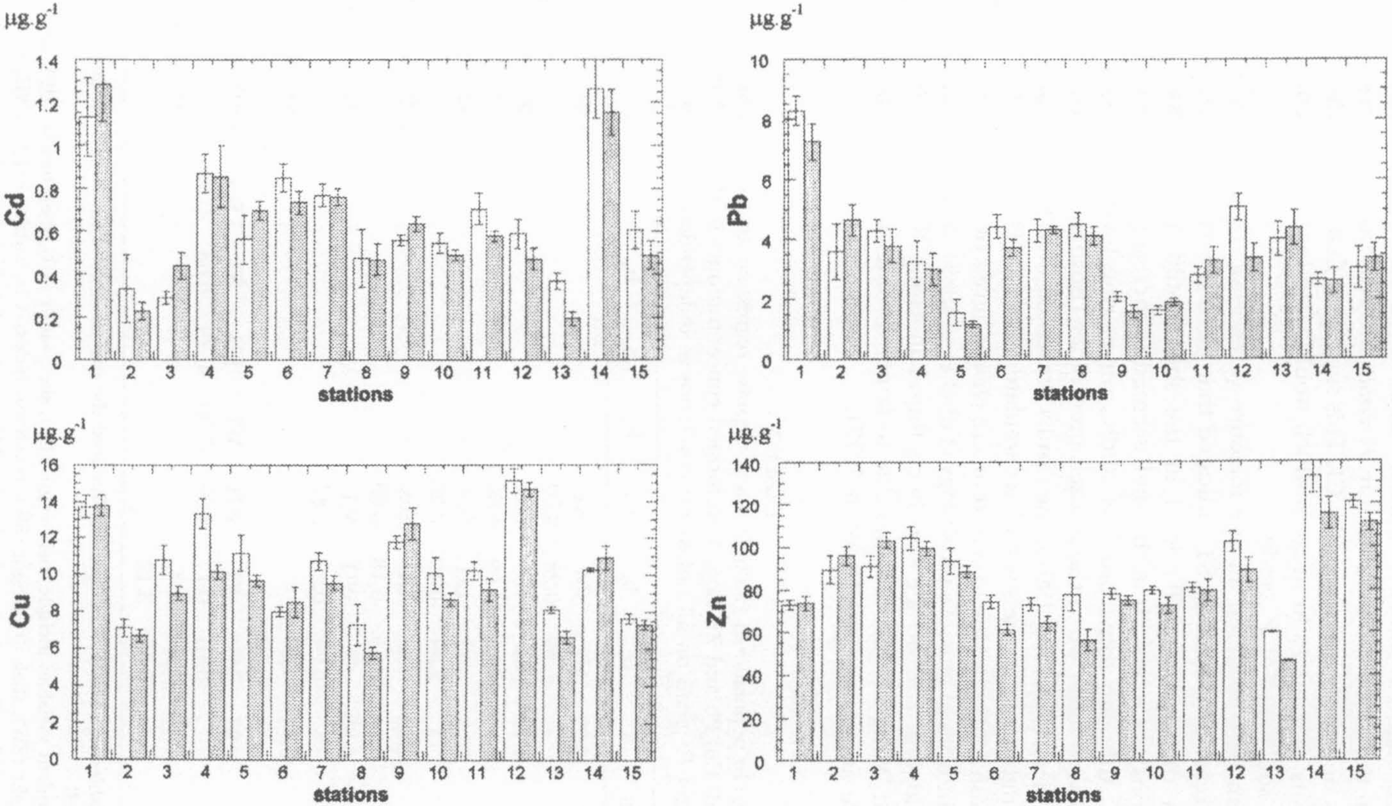

Fig.2: Concentrations of Cd, Cu, Pb and Zn in total soft body (mean mg/g dry weight ± standard error) of mussel P. viridis *sampled from 15 sampling stations (▭ : female; ■ : male).*

Note: Anjung Batu (St 10),Bagan Lalang (St 6), Kg. Pasir Puteh (St 12), Kg. Tg. Batu (St 13), Kuala Penyu(St 15), Kuala Perlis (St 1), Lukut (St 7), Pantai Kundor (St 9), Pantai Lido (St 11), Pasir Panjang (St 8), Penang (St 5), Sangkar Ikan (St 3), Tg. Dawai (St 4), Tg. Rhu (St 2), Trayong (St 14).

indicative of maturity status of marine mussels (Broman *et al.* 1991), its influence on the metal concentration in *P. viridis* was reasonable. Interestingly, Zn-flesh was negatively influenced by CI. This suggested that high CI indicated great amounts of gametes in mussels which would yield less Zn concentration in the total soft tissue of *P. viridis.*

As ST can be considered an age measure (Frew *et al.* 1989), Cd-flesh and Pb-shell increasing with rising ST indicated that when the mussels grew older, Cu level in the flesh and Pb level in the shell would also increase. The concentrations of Cu and Pb in the shell seemed to increase when the mussels grew older. Although some previous studies on marine bivalves showed that metal levels were lower when the mussels grew older (Boyden 1977; Cossa *et al.* 1980; Swaileh and Adelung 1994), the results presented in Table 2 showed that the older (higher ST) mussels would accumulate more Cd. This higher Cd level in mussel flesh might be due to different size classes, levels of exposure and site-dependent individual mussel's physiology (Lobel *et al.* 1989). A slightly increasing trend for Cd with size or age had been found in Hong Kong's *P. viridis* by Cheung and Wong (1992). This was due to larger or older mussels containing considerable amounts of Cd (Boyden 1977).

TABLE 2

Inter-station (15 populations) multiple linear stepwise regression analysis of dependent variables (Cd, Cu, Pb and Zn Log_{10} transformed concentrations) in flesh and shell of mussel *P. viridis* on all ratios and non-ratios as independent variables

Heavy Metals		a	b_1	b_2	b_3	b_4	b_5	b_6	b_7	r	F
Flesh	Cd	-0.27	HE -0.50	FW 0.28	ST 0.50					0.39	16.44
	Cu	1.19	CI 0.16	WL -1.09	Sex -0.06					0.36	15.74
	Pb	0.31	WL 2.09	LH -0.19	Sex -0.06					0.32	11.89
	Zn	2.59	CI -0.45	ST -0.18	Sex -0.03					0.62	66.83
Shell	Cd	-0.63	CI 0.29	WH 0.28	WL 0.82					0.39	17.99
	Cu	0.74	LE 0.10							0.33	39.43
	Pb	2.91	WI 0.15	CI 0.17	WH 2.29	WL -5.00	LH -0.79	ST 0.08	Sex -0.02	0.52	16.59
	Zn	-1.10	CI 0.37	WL 3.12						0.39	28.06

Note: All variables significantly ($P< 0.001$) influenced the metal concentrations in both the flesh and shell.
Independent variables include shell length (LE), shell width (WI), shell height (HE), flesh dry weight (FW), shell dry weight (SW), condition index (CI), width : height (WH) ratio, width : length (WL) ratio, length : height (LH) : ratio, shell thickness (ST) and sex.
r is the coefficient of correlation.

WL ratios seemed to influence most of the metals in this study, namely Cu and Zn in the flesh and Cd, Cu and Zn in the shell. This ratio is important as it relates to the growth rate and relative age of the mussel (Kautsky 1982). Seed (1968) suggested that mussels in a given environment reach a maximum shell length beyond which no further linear growth could occur. Owing to shell erosion, the shell would continue to thicken and expand resulting in a lowering of the CI. The expansion of the shell which is necessary to prevent the ST from being squashed, is in the form of increased shell width. Therefore, an increase in WL ratio indicated a mussel with increased growth rate and an increased relative physiological age.

Comparison Between Two Models: All Variables (Non-ratios and Ratios) and All Ratios

Two different sets of independent variables (Tables 2, 3) were investigated to evaluate the differences and how well the MLSRA models performed. Independent variables in Table 2 included all ratios and non-ratios as independent variables while Table 3 only included all ratios as independent variables. The results showed almost the same independent variables as the

TABLE 3

Inter-station (15 populations) multiple linear stepwise regression analysis of dependent variables (Cd, Cu, Pb and Zn Log_{10} transformed concentrations) in flesh and shell of mussel *P. viridis* on all ratios as independent variables

Heavy	Metals	a	b_1	b_2	b_3	b_4	b_5	b_6	b_7	r	F
Flesh	Cd	-0.97	CI	ST						0.35	22.16
			0.22	0.85							
	Cu	1.19	CI	WL	Sex					0.36	15.74
			0.16	-1.09	-0.06						
	Pb	0.31	WL	LH	Sex					0.32	11.89
			2.09	-0.19	-0.06						
	Zn	2.59	CI	ST	Sex					0.62	66.83
			-0.45	-0.18	-0.03						
Shell	Cd	-0.63	CI	WH	WL					0.39	17.99
			0.29	0.28	0.82						
	Cu	0.88	WL	ST						0.35	20.57
			-0.32	0.09							
	Pb	1.32	CI	LH	ST	Sex				0.45	18.86
			0.14	-0.05	0.22	-0.02					
	Zn	-1.10	CI	WL						0.39	28.06
			0.37	3.12							

Note: All variables significantly ($P< 0.001$) influenced the metal concentrations in both the flesh and shell.
Independent variables include condition index (CI), width : height (WH) ratio, width : (WL) length ratio, length : height (LH) ratio, shell thickness (ST) and sex.
r is the coefficient of correlation.

most important factors affecting Cu, Pb and Zn levels in the flesh and Cd and Zn levels in the shell, for both regression models each with its respective r value. For example, Zn-flesh was significantly affected by CI (negative), ST (negative) and sex (negative) with r= 0.62 in both regression models. Therefore, these almost similar results suggested that multiple linear stepwise regression analysis was performing as expected and that the independent factors selected were reliable. It is logical that some equations did not include the same influential factors. For instance, Cd in flesh and Cu and Pb in shell were found to have different significant independent variables. Flesh-Cd was significantly influenced by shell height, flesh dry weight and ST as shown in Table 2, whereas as shown in Table 2, flesh-Cd was significantly influenced by CI and ST.

In the two models, it was noted that the r values obtained were low which indicated that there may be other independent variables that had not been included in this study of what we think are the most important factors to influence metal accumulation in the mussel *P. viridis.*

Intra-station and Inter-station Comparisons

Mussels with a wide range of age and size (shell length: 2.0-11.0cm) in a single population at Pasir Panjang (station 5) were tested to find out the most important factors known to influence their heavy metal concentrations in the flesh and shell (Table 4). The results suggested that the factors which significantly influenced each metal accumulation in intra-station analysis were not similar to those found in inter-station analysis. This may be due to the different size classes of mussels in this intra-station population receiving the same amount of environmental inputs while inter-station mussels were receiving different environmental inputs and the maximum potential shell length at each station was not similar (Lobel *et al.* 1991). However, flesh-Cu and shell-Pb seemed to be influenced by the same known important factors for both intra-station and inter-station populations namely CI and sex. This may be indicative that CI and sex were the two important factors affecting the accumulation of flesh-Cu in the mussel *P. viridis* regardless of different statistical models provided that CI and sex were included in the stepwise procedures. Similarly, ST and sex were two important variables affecting the accumulation of Pb-shell in the three models (Tables 2, 3 and 4). These results agreed with an intra-station study by Lobel *et al.* (1991) who found that sex, FW, CI and WH ratios were four of the most important independent variables which influenced the concentration of metals in the flesh of the blue mussel *M. edulis.* In this study, ST and sex were also two important factors which influenced Pb accumulation in the shell of *P. viridis.*

CONCLUSION

The ranges of heavy metal concentrations in all sampling sites were found to be comparable and lower than the regional data in the soft tissue *P. viridis.* Kuala Perlis and Kg. Pasir Puteh were found to be potentially receiving anthropogenic metals. In the identification of the most influential known

TABLE 4
Intra-station (Population Pasir Panjang) multiple linear stepwise regression analysis of dependent variables (Cd, Cu, Pb and Zn Log_{10} transformed concentrations) in flesh and shell of mussel *P. viridis* on all ratios and non-ratios as independent variables

Heavy	Metals	a	b_1	b_2	b_3	b_4	b_5	b_6	b_7	r	F
Flesh	Cd	-0.06	FW -0.77							0.88	88.67
	Cu	1.62	CI 0.29	Sex -0.08						0.45	3.04
	Pb	0.36	HE -4.39	ST 2.28						0.70	11.85
	Zn	1.96	FW -0.20	Sex -0.07						0.84	30.06
Shell	Cd	1.45	WI -1.82	ST 1.58	Sex -0.11					0.68	6.86
	Cu	0.71	LH 0.44							0.77	38.94
	Pb	1.64	FW -0.23	ST 0.71	Sex 0.07					0.63	5.26
	Zn	1.84	HE -1.51	FW -0.21	ST 1.43					0.80	14.32

Note: All variables significantly ($P < 0.001$) influenced the metal concentrations in both the flesh and shell.
Independent variables include shell length (LE), shell width (WI), shell height (HE), flesh dry weight (FW), shell dry weight (SW), condition index (CI), width : height (WH) ratio, width : length (WL) ratio, length : height (LH) : ratio, shell thickness (ST) and sex.
r is the coefficient of correlation.

factors in the accumulation of metals, there may be other important factors, besides the known factors included in the present study, affecting metal accumulation in the tissues and shells of the mussels. The factors affecting heavy metal accumulation in mussels seemed to be variable whether they were ascertained as intra-station or inter-station. However, the same known variables ascertained in the three models indicated that MLSRA may be proposed as an alternative statistical approach to determine the most influential factors out of a number of factors which were known to affect metal accumulation in the green-lipped mussel *P. viridis* in particular and in other invertebrates in general.

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