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Metal-Binding Protein in the Pacific Oyster, *Crassostrea gigas:* Assessment of the Protein as a Biochemical Environmental Indicator

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In this paper the determination of metal-binding proteins (MBP) in the Pacific Oyster (Crassostrea gigas) is reported. The objectives of this study were to employ a simple, cost-effective method for quantifying MBP and to assess this parameter for possible use as an indicator of identifiable sources of metal input to biological systems.

Abnormally high quantities of zinc had been found previously in C. gigas growing in waters adjacent to the Kraft pulp mill at Crofton, British Columbia (Ellis et al. 1981). From 1971 to 1973 oysters near the effluent outfalls were found to have body-burden zinc ranging from 14 to 20 g kg^{-1}. These were six to ten times the zinc concentrations found in reference specimens. Zinc dithionite was used in the pulping process at this mill until 1973 (Thompson et al., 1976). Subsequent to a change to sodium dithionite, concentrations of zinc in oysters decreased steadily. Ellis et al. (1981) described two numerical models for this decline and predicted control concentrations of zinc should have been attained at the outfall site by 1984.

A second potential source of contamination is sited directly south of the pulp mill. In this case, leaching of copper and zinc from smelter slag into Osborn Bay has been identified (Figure 1). A study in 1979 (D. Goyette, *pers. comm.*) indicated that the slag contained elevated quantities of zinc (>1%), copper (>0.3%) and lead (145 mg kg^{-1}), among others. Oysters in the same area were found to contain elevated concentrations of Cu (1800 mg kg^{-1} dry weight), Zn (up to 1300 mg kg^{-1}) and Cd (up to 15 mg kg^{-1}). In the case of Cu and Zn these concentrations were six times and four times reference sample concentrations, respectively. Cadmium concentrations were only slightly elevated.

MATERIALS AND METHODS

Samples of oysters were collected on the same day from four locations in the Crofton area on Vancouver Island, British Columbia (Figure 1).

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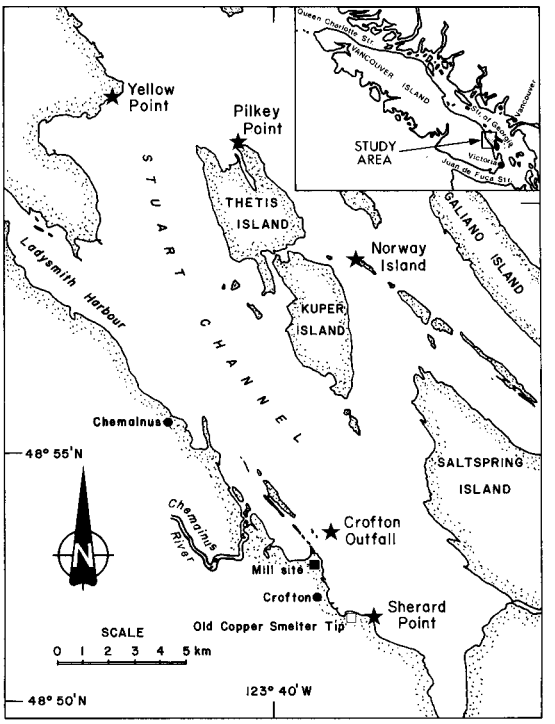

Figure 1. Map of a portion of Southern Vancouver I., British Columbia. Stars indicate sampling sites in this study.

Samples were selected for similar shell size ranging from approximately 10 cm to 15 cm in length, at each of the four locations (shown in Figure 1) and placed into polyethylene bags. They were live-frozen (-20^{o}C) within six hours of collection.

Dissections were performed on partially thawed specimens on a bed of ice and were completed within 15 minutes of shucking. Digestive glands were weighed and immersed in 9 vol chilled buffer containing 50 mM TRIS-HCl (pH 8.6), 10 mM 2-mercaptoethanol, 100 µm phenylmethylsulfonyl fluoride and 0.02% sodium azide. Homogenization (5^{o}C; Polytron) was followed by centrifugation (34,860 xg, 5^{o}C) for one hour. Cytosol supernatants were decanted and filtered through 0.2 um membranes in preparation for gel permeation chromatography (GPC).

Chromatograms were developed on a Sephadex G-75 (2.6 x 100 cm) jacketed (5^{o}C) column which had been calibrated previously with materials of known molecular weight.

Cytosol aliquots were applied to the column equilibrated with 10 mM TRIS (pH 8.6) buffer containing 2 mM mercaptoethanol and 0.02% NaN_3. Elution with deaerated buffer was controlled at 0.62 mL min^{-1}.

Fractions (5 mL) were collected and absorbance and transmittance were monitored at 280 nm. Cytosols were denatured by addition of an equal volume of 95% ethanol. After overnight incubation at 5°C the cytosol/ethanol mixtures were centrifuged at 34860 x g to remove precipitated protein. Denatured cytosols were chromatographed as described above.

Thiolic proteins were determined using the differential pulse polarographic procedure reported by Thompson and Cosson (1984) with the following modifications. Cell temperature was reduced to 7°C and the concentration of $Co(NH_3)_6^{3+}$ in the supporting electrolyte was doubled to 320 mg L^{-1}. These modifications permitted a limit of detection of less than 10 µg of standard protein per liter of the cell solution. The instrumentation employed consisted of a PARC Model 174 polarographic analyzer, an EG&G/PARC Model 303 static mercury drop electrode assembly and a Bausch and Lomb/Houston Model 2000 X-Y recorder. The jacketed cell (EG&G/PARC Model G0192) was supplied with cooling water at 7°C. Three replicate determinations were carried out on crude and denatured cytosols and precisions of better than 10% were obtained. Calibrations curves were generated using purified Cd-thionein isolated from the crab Scylla serrata (Olafson et al. 1979).

Selected fractions from the GPC separations were analyzed for copper, zinc, and cadmium by flameless atomic absorption spectrometry.

Data for each group of C. gigas digestive gland samples were first examined for outliers (Bauer, 1971). Rejection was made at $p \leq 0.05$ in all cases. In this study six values were rejected using this criterion (Table 1).

One way, completely randomized analysis of variance and the Student-Newman-Keuls' (S.N.K.) procedure were applied to test for statistical differences ($p = 0.05$) among means for the four group data sets.

RESULTS AND DISCUSSION

The concentration of metal binding proteins and Cu, Cd and Zn from digestive gland tissue of some of the sample oysters are represented in Table 1, together with concentrations of polarographically active protein fractions for all samples studied. Protein determined in digestive gland cytosols denatured with 95% ethanol ranged from 7.18 g kg^{-1} (dry weight) in the Crofton outfall (CO) sample to 5.02 g kg^{-1} at Yellow Point (YP), one of two reference sites.

The protein was determined in both crude and ethanol-denatured cytosols. The effect of the denaturation is shown in the G-75 profiles depicted in Figure 2.

Table 1. Concentration of Protein and Metal Contained in Cytosolic Fractions of *Crassostrea gigas* Digestive Gland

Sample	Cu(mg kg^{-1})[a] Crude	Cu(mg kg^{-1})[a] EtOH[b]	Cd(mg kg^{-1})[a] Crude	Cd(mg kg^{-1})[a] EtOH[b]	Zn(mg kg^{-1})[a] Crude	Zn(mg kg^{-1})[a] EtOH[b]	MBP(g kg^{-1})[a] Crude	MBP(g kg^{-1})[a] EtOH[b]
YP-1	-	-	-	-	-	-	13.3	4.70
YP-2	19.7	11.1	15.7	13.4	173	16	16.6	4.21
YP-3	79.1	36.6	19.7	6.6	2870	277	19.1	4.76
YP-4	-	-	-	-	-	-	12.9	4.62
YP-5	46.2	25.1	11.9	7.0	854	105	21.5	6.13
YP-6	-	-	-	-	-	-	24.7	6.16
YP-7	49.2	19.3	26.2	9.6	2480	288	13.4	3.63
YP-8	-	-	-	-	-	-	12.8	6.55
YP-9	57.6	39.7	28.2	12.0	2730	486	32.4	5.28
YP-10	-	-	-	-	-	-	16.7	7.58*
YP-11	-	-	-	-	-	-	17.9	4.13
NI-1	51.9	20.3	13.0	2.7	778	-	23.2	7.00
NI-2	26.2	10.9	5.4	1.4	444	23	14.1	6.08
NI-3	31.9	15.8	14.3	2.7	338	-	30.0	9.70*
NI-4	-	-	-	-	-	-	12.1	4.58
NI-5	46.8	11.9	13.1	0.8	917	21	15.1	4.94
NI-6	-	-	-	-	-	-	6.55	2.45*
NI-7	-	-	-	-	-	-	14.7	4.73
NI-8	16.2	9.8	13.6	3.0	226	-	12.1	3.69
SP-1	96.4	40.1	6.9	0.9	957	-	13.8	7.78
SP-2	-	-	-	-	-	-	12.7	7.52
SP-3	-	-	-	-	-	-	8.6	4.51*
SP-4	105.0	35.6	11.1	0.9	1090	-	18.5	7.26
SP-5	69.9	15.5	4.2	-	1050	-	15.9	5.44
SP-6	-	-	-	-	-	-	11.7	4.93*
SP-7	-	-	-	-	-	-	10.4	4.03*
SP-8	-	-	-	-	-	-	13.7	6.24
SP-9	77.7	28.0	6.8	1.0	887	23	14.7	6.45
SP-10	45.3	17.7	14.1	3.0	873	-	16.1	6.74
SP-11	-	-	-	-	-	-	17.0	7.76
CO-1	11.7	44.6	8.8	3.3	1020	75	26.4	7.90
CO-2	-	-	-	-	-	-	21.8	7.31
CO-3	-	-	-	-	-	-	17.3	6.29
CO-4	-	-	-	-	-	-	25.2	8.98
CO-5	-	-	-	-	-	-	26.5	8.30
CO-6	51.8	27.3	7.6	3.7	812	65	21.4	6.97
CO-7	36.0	20.8	6.2	3.3	778	65	23.6	7.66
CO-8	12.5	25.8	4.5	1.6	1030	94	17.5	5.47
CO-9	-	-	-	-	-	-	21.9	8.31
CO-10	96.8	90.1	6.2	2.3	1650	188	15.7	5.38
CO-11	-	-	-	-	-	-	17.4	6.36

[a] Dry-weight basis
[b] Ethanol-soluble fraction
*Rejected as outliers; p = 0.05 (Bauer, 1971)

Table 2. *Crassostrea gigas*. Summary of Statistical Analyses of Digestive Gland Data.

A. ANOVA - Cytosolic Components

Cytosol Status	Analyte	Site	X	S(1)	N	F
Denatured	MBP	CO	7.18[a]	1.19	11	10.1 (<0.001
		SP	6.90	0.83	8	(3, 31df
		NI	5.17	1.18	6	
		YP	5.02	0.98	10	Reject Ho[b]
Crude	Cu	CO	72.8[c]	32.3	5	4.18 (<0.025
		SP	75.2	18.9	8	(3, 19d
		NI	34.6	14.7	5	
		YP	50.4	21.4	5	Reject Ho
Crude	Cd	CO	6.65	1.65	5	
		SP	8.84	3.14	9	12.5 (<0.001
		NI	11.9	3.7	5	(3, 20d
		YP	20.3	6.9	5	Reject Ho
Crude	Zn	CO	1058	351	5	44.4 (p<0.001
		SP	752	279	9	
		NI	541	295	5	(3, 20df)
		YP	1821	1226	5	Reject Ho

B. Student-Newman-Keuls Test for Significant Differences[d]

MBP:	YP	NI	SP	CO	p = 0.01
Cu :	NI	YP	CO	SP	p = 0.05
Cd :	CO	SP	NI	YP	p = 0.05
Zn :	NI	SP	CO	YP	p = 0.05

Increasing ⟶ Concentration

[a] In units of g kg^{-1}, [b] Ho = no significant differences among groups, [c] In units of mg kg^{-1} [d] Underlining signifies no statistical differences between means.

Various methods for denaturation prior to MBP determination have been reported (Olafson and Sim 1979; Lobel and Payne 1984; Thompson *et al.* 1986) and methods appear to be tissue dependent. For *C. gigas* digestive gland, the addition of an equal weight of ethanol was found to be the most effective procedure for removing polarographically active materials of molecular weight greater than 60,000 Da.

Statistical treatment of these data using ANOVA and the SNK test for differences of means indicated significant differences were established at the p = 0.01 level between the impacted (CO, SP) and reference (YP, NI) sites.

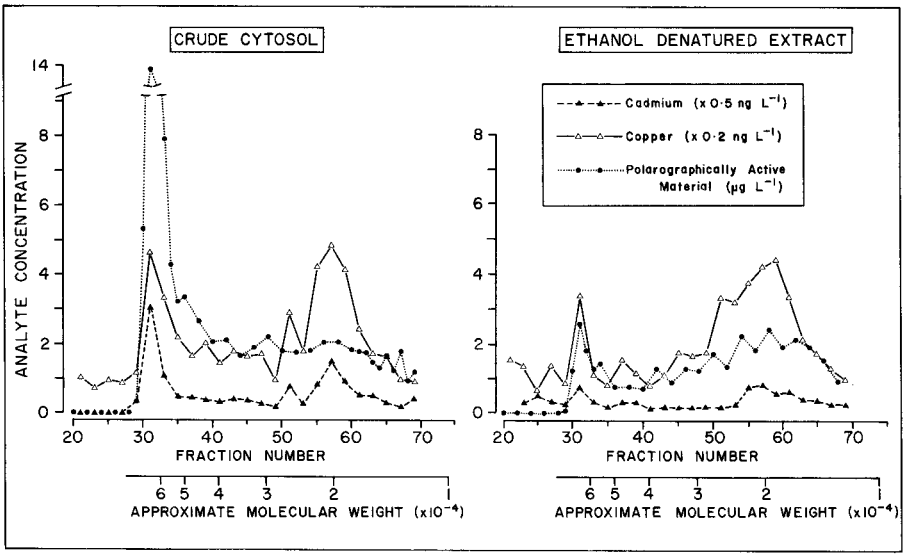

Figure 2. Gel-permeation chromatograms of Crassostrea gigas digestive gland cytosol. Profiles of copper, cadmium and metal-binding protein are shown.

Significant differences were also found among means for cytosolic metals (Table 2B). However a similar trend to higher concentrations was observed only for Cu. Interestingly, trends for Cd and Zn tended to the inverse, YP oysters having significantly higher amounts in both cases. Reasons for high and widely variant Zn in YP samples are not apparent at this time.

Analysis of fractions from G-75 gel-permeation chromatography of crude and denatured cytosols (Figure 2) indicated that copper and, to a lesser extent, cadmium are associated with a polarographically active protein fraction at about 20,000 Da. There are also smaller peaks indicating protein-metal associations around 10,000 Da. These observations suggest that the major metal-binding component is a copper protein. The molecular weight assignment indicates structure possibly similar to a dimeric metallothionein.

The salient feature of this study is the relationship between the MBP or cytosolic metal concentrations and the sites of collection. While it is possible that other environmental factors such as salinity and bed-substrate could be invoked to explain these statistically significant differences, the data can be more readily interpreted to reflect difference in environmental exposure to copper. Both negative and positive correlations between various environmental factors (including salinity and sediment loading) and metal burdens in C. virginica have been reported (Huggett et al. 1975; Frazier 1976). In our study area salinities of >25 °/oo are normal (Waldichuk 1964) thus making

salinity fluctuations likely insignificant. Substrate effects would also not appear to be important as three of the four sites were rocky beach.

Frazier and George (1983) compared proteins induced in the oysters C. gigas and Ostrea edulis and found that there was marked difference in the molecular masses of Cd proteins isolated from the two species. In C. gigas the molecular weight from G-75 chromatography was 20,400 Da. In O. edulis, two protein components were identified at about 12,000 and 7,500 Da. respectively. In this respect their findings with C. gigas were similar to those reported herein. Frazier and George (1983) also reported the presence of very low molecular weight components in C. gigas containing both Cu and Cd. In the interpretation of very low molecular weight peaks, problems of contamination from buffers and non-specific metal complexes must be considered.

The data obtained for C. gigas in this study suggest that the MBP is a copper-binding protein, possibly a dimer of a metallothionein-like molecule. However, without further purification and sequencing studies, this assignment can be only tentative.

In order to make this procedure more applicable to monitoring situations, it is necessary to increase the precision of the procedure. This can in part be achieved by improved chemical detection, but more important is the relation of metal binding protein concentration to bioavailable metal fraction. It has been proposed (Roesijadi, *pers.comm.*) that MBP increase in response to the free metal ion within the cell. Other cell constituents that change in concentration, that also bind metals, will affect the quantity of MBP production. Thus the correlation of such cell constituents to MBP may well normalize increases of MBP from a given population exposed to the same biologically available metal. Accordingly, work in continuing in this area and lipid and high molecular weight bound metals are also being investigated.

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