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# Level of Heavy Metals in the Onsan Bay in Korea and Involvement of Metal Binding Protein in the Accumulation of Cadmium in *Littorina brevicula*

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ABSTRACT: The heavy metal concentrations in seawater and winkles (*Littorina brevicula*) collected from the Onsan bay area in southeast of Korea were analyzed. The heavy metal concentrations in the seawater obtained from the most polluted site showed approximately 189, 205, and 110 fold higher cadmium, copper, and zinc concentrations, respectively, than the uncontaminated control site. The contamination levels of these metals in winkles were 11.08 - 2.35, 334.5 - 212.5, and 426.0 - 499.2 $\mu$g per gram dry body weight, respectively. The concentrations of all three metals in both the seawater and winkles decreased gradually with increasing distance from Daejeong stream, suggesting the stream being the major source of heavy metal input into the bay. Among the four body parts of digestive gland and gonad, gill, kidney, and remaining tissue in contaminated winkles, kidney showed the highest accumulation level of cadmium; copper and zinc, however, were more or less distributed among the four body parts. Upon gel filtration chromatography of the cytosol from the kidney of cadmium induced winkles, one cadmium peak corresponded to the elution peak of horse kidney metallothionein.

*Key Words*: Accumulation, Cadmium, Copper, Heavy metal, *Littorina brevicula*, Zinc.

## INTRODUCTION

Since the establishment of non-ferrous and petrochemical based industrial plant complex along the upstream of Daejeong stream, cadmium (Cd), copper (Cu), and zinc (Zn) contamination level of the seawater in nearby Onsan bay was rising 5 to 10 fold between 1987 (KORDI 1987) and 1988 (Cho *et al.* 1988). The contamination level in sediments collected in this area in 1996 (Song *et al.* 1997) showed a maximum of 12280, 9975, and 14144 fold higher concentrations of Cu, Pb (lead), and Zn, respectively, than the unpolluted natural seawater level determined by USEPA (United States Environmental Protection Agency) (Giesy and Hoke 1990). This calls a need for a constant monitoring system for heavy metal disposal in this area.

Certain marine invertebrates are useful bioindicators for biomonitoring because they fail to match rates of heavy metal excretion to rates of metal uptake, and necessarily be net accumulators of heavy metals (Rainbow 1990). In such cases, metal accumulation level in these animals is directly proportional to the available heavy metals in the environment (Langston and Zhou 1986, Paek and Lee 1998). Since all heavy metals are toxic at a threshold level, such accumulation will usually lead to detoxification of the metal to render it unavailable metabolically and minimize detrimental effects (Rainbow 1990). One such mechanism is the involvement of cystein-rich small molecular weight proteins of metallothioneins in sequestering metals which might otherwise bind to sensitive cellular sites and exert toxic effects (Mason and Nott 1981, George 1990, Roesijadi 1994). Consequently metallothioneins have been suggested as specific bioindicators and possible early warning markers for the detection of toxic effects caused by metal exposure (Olafson *et al.* 1979, Pavicic *et al.* 1987).

Among the marine invertebrates found along the coastline on the Korean peninsula, winkles (*Littorina brevicula*) are useful for biomonitoring purpose because they are widely distributed geographically, sessile, and therefore easy to collect. They are also known to show highly resistant nature for a wide range of metal concentrations and have high survival rate in the laboratory (Paek 1997). These characteristics enable the researchers to compare the data collected from field studies with those from artificial exposure experiments conducted in the laboratory.

This paper provides an assessment of the heavy metal input into the Onsan bay and the effect of increased metal loads of Cd, Cu, and Zn on winkles, *Littorina brevicula*. In the field studies, the level of metal contamination in seawater was more or less correlated to the

bioaccumulation levels in winkles. This was further supported by artificial cadmium exposure experiments, where accumulated cadmium content in winkles was directly related to the duration of the exposure and the available metal content in the environment. Both data, from field studies and those obtained through laboratory exposure procedures suggest the suitability of the *Littorina brevicula* as bioindicators of environmental heavy metal contaminations.

## MATERIALS AND METHODS

### Sample collection

Seawater samples were collected in February of 1997 from the Onsan coastal area located southeast of Korea (Fig. 1). Samples were collected 20-30 cm below the seawater level at 11 different stations (Fig. 1, 1-11) at and away from Daejeong stream, from a 17-ton size ship sailing 1-2 knots. A 500 ml high density polyethylene bottles were suspended from the ship using a tygon tubing. Winkle samples from 6 coastal sites (Fig. 1, A-F) were collected in boxes containing seawater and kept alive from the sampling sites to the laboratory. A temperature of 10℃ was maintained throughout the transportation. The winkles were then frozen in the laboratory at -70℃ until use.

### Analyses of metal content

The concentrations of cadmium, copper, and zinc in the seawater were analyzed with high resolution ICP-MS (Fision Plasma Trace) at Korea Basic Science Institute (Daeduk, Korea) using the extraction methods published by Danielsson *et al.* (1978, 1982) and KORDI (1988). Certified reference materials (CASS-3) were run with each batch of the samples to assess the daily performance. To determine the total metal concentrations in tissue samples, 100 animals were deshelled and dried in a freeze-dryer (Il-Sin Engineering Co., Seoul, Korea) for 3 days. After homogenation of the dried tissue, 250 mg was mixed with 5 ml of 65% $HNO_3$ in a digestion vessel and let sit in room temperature for 1 hour. Digestion of the samples was carried out by the microwave acid digestion program described by MacCarthy and Ellis (1991) using MDS-2000 microwave digestion system (CEM Corp., Matthews, NC, U.S.A.). The digested samples were subsequently diluted with double distilled water to a final volume of 25 ml. The concentrations of cadmium (Cd), copper (Cu), and zinc (Zn) in samples were measured with flame atomic absorption spectrophotometer (AAS: Perkin Elmer Analyst 100, USA). For determining the accumulated metal levels in different body parts, animals were dissected and the soft tissue was separated into four parts: digestive gland and gonad, gill, kidney, and remaining tissue. Tissue parts were homogenized with a hand-held homogenizer in 100 mM Tris-acetate buffer (pH 8.1) containing 5 mM mercaptoethanol and 0.1 mM phenylmethylsulfonyl fluoride. For the digestive gland and gonad, gill, and remaining tissue fractions, 3 tissue-volumes of the buffer was used for the homogenization: a 20 ml volume was used for the homogenization of the kidney fraction. Metal concentrations in the homogenate of each the body part and the cytosol were analyzed by the methods described above.

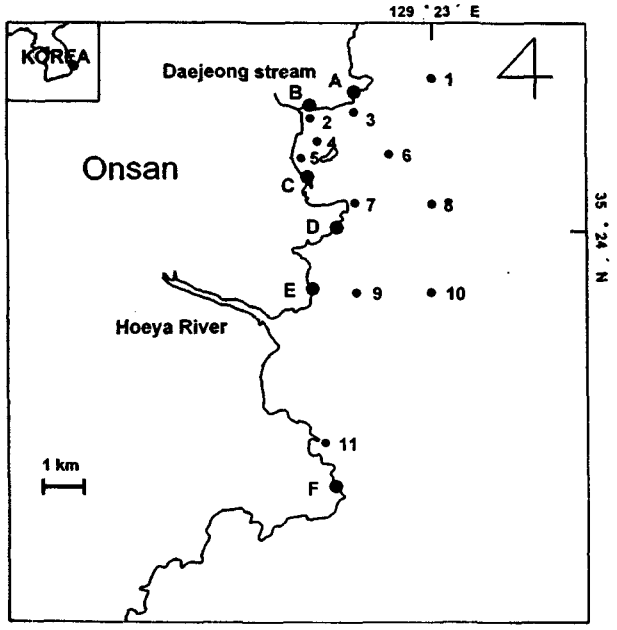



**Fig. 1.** The sampling stations for seawater (1-11) and *Littorina brevicula* (A-F).

### Cadmium exposure experiments of *L. brevicula*

Winkles collected at station F were acclimatized in the laboratory in aerated seawater (salinity, 34 ‰) at 10℃ for one week prior to the exposure. Groups of 200 animals were held in submerged cages and exposed to 4, 40, and 400 $\mu$g of cadmium (Cd) per 1 liter of seawater for up to 66 days. Control groups of winkles were maintained in clean seawater throughout the experiment. Seawater in each cage was changed weekly and Cd dosing was repeated at each change.

### Partitioning of metal content

After 10, 29, 50, and 66 days of exposure, groups of 40 animals were sacrificed and the

homogenates of the soft tissue were centrifuged at 15,000 x g for 15 min and subsequently at 100,000 x g for 1 hour. The supernatant fraction was denoted cytosol, and the pellet fraction was denoted membrane fraction. Metal contents in the homogenate, the cytosol, and the membrane fraction were analyzed with flame atomic absorption spectrophotometer following the methods described above.

**Gel filtration chromatography**

An aliquot of 200 $\mu$l of the cytosol was fractionated using 1 × 34 cm superose 12 column (preparative grade, Pharmacia) as published (Kaland *et al.* 1991). The column was equilibrated with 100 mM Tris-acetate (pH 8.1) containing 5 mM $NaN_3$ and 2 mM DTT at 4℃ and also used for the running phase of the column. Fractions were collected as 2 ml volumes and the cadmium contents of the eluents were measured directly by graphite furnace AAS (Unicam 989) without prior digestion of the samples at Korea Basic Science Institute (Daeduk, Korea). The column was calibrated for molecular weight estimation with blue dextran (200 kDa), bovine serum albumin (66 kDa), $\alpha$-chymotrypsinogen A (25 kDa), horse kidney metallothionein (6.5 kDa), and glutathione (620 Da) as standard markers.

## RESULTS AND DISCUSSION

**Metal concentrations in seawater**

To assess the heavy metal contamination in Onsan bay, 11 sampling stations were selected starting at the exit point and away from Daejong stream. Station number 11 was selected as a control spot (Fig. 1).

Station 2, which is immediately located at the exit point of the Daejong stream, showed the highest level of metal concentrations, being 1.705, 24.55, and 35.12 $\mu$g/L for Cd, Cu, and Zn, respectively (Table 1). These are approximately 189, 205, and 110 fold higher Cd, Cu, and Zn concentrations, respectively, when compared to those analyzed for control station number 11. Overall, the three metal levels in samples from stations 2, 3, 4, and 5 were considerably higher than in samples collected at stations 8, 9, and 10 (Table 1). This shows a pattern of decreasing metal concentrations in seawater with increasing distance from Daejeong stream. This in turn suggests that the stream contribute to the heavy metal input to the Onsan bay as incated by other researchers (Cho *et al.* 1988, Song *et al.* 1997). Samples at station 1, however, had a relatively high level of Cd, Cu, and Zn concentrations in spite of the similar distance as station 6. This is probably due to the water current forming northwest that carried the metals from the coastal area to this sampling site.

Table 1. Metal concentrations[1] in seawater samples from the Onsan bay area.

| Station | Cd | Cu | Zn |
|---|---|---|---|
| 1 | 0.072 | 0.50 | 3.00 |
| 2 | 1.705 | 24.55 | 35.12 |
| 3 | 0.037 | 0.24 | 3.11 |
| 4 | 0.059 | 0.22 | 3.09 |
| 5 | 0.311 | 0.40 | 10.35 |
| 6 | 0.026 | 0.20 | 2.16 |
| 7 | 0.015 | 0.12 | 0.52 |
| 8 | 0.010 | 0.08 | 0.21 |
| 9 | 0.013 | 0.10 | 0.41 |
| 10 | 0.009 | 0.09 | 0.23 |
| 11 | 0.009 | 0.12 | 0.32 |

[1] Expressed in $\mu$g/L.

The contaminated levels of Cd, Cu, and Zn at the highest point in Onsan bay area were 0.767, 10.00, and 68.75 $\mu$g/L, respectively in 1987 (KORDI 1987) and 4.08, 32.9, and 320.0 $\mu$g/L, respectively, in 1988 (Cho *et al.* 1988). If the three metal concentrations in 1988 are compared to those obtained for station number 2 in this study (Table 1), there seems to be approxitely 2.5 and 10 fold reduction in input of Cd and Zn, respectively. This reduction might be due to constant surveillance during the past 9 years for the heavy metal disposal in the Onsan bay. Nevertheless, the level of Cd, Cu, and Zn at the exit point of Daejeong stream (station 2) is still 30, 24, and 6 fold higher, respectively, than the natural seawater metal concentrations reported by Bruland and corkers (Bruland and Frank 1983, Bruland *et al.* 1991). If the metal pollution levels are not regulated, the heavy metal loads on coastal water is expected to cause serious destruction of the ecosystem in this area. Moreover, since Onsan coastal area is a well-known grounds for edible seaweed culture and frequent fishery business, metal pollution is evidently expected to cause negative effects on humans through the food web. This calls for constant monitoring system to regulate the metal disposal in this area.

**Metal content in soft body tissue of *L. brevicula***

Among the winkles collected at 6 different coastal areas (Fig. 1, A-F), animals from stations E and F had relatively low Cd, Cu, and Zn levels in their soft tissue (Table 2). In samples from stations B, C, and D, however, Cd, Cu,

able 2. Metal concentrations[1] in soft body tissue of *ittorina brevicula* collected from the Onsan coastal area.

| Station | Cd | Cu | Zn |
|---|---|---|---|
| A | 3.87 | 238.9 | 86.2 |
| B | 11.08 | 334.5 | 426.0 |
| C | 0.92 | 362.5 | 412.2 |
| D | 2.35 | 212.5 | 499.2 |
| E | 1.23 | 99.3 | 61.2 |
| F | 1.17 | 74.9 | 53.1 |

[1] Expressed in $\mu$g/g dry weight.

and Zn concentrations were 11.08 - 2.35, 334.5 - 212.5, and 426.0 - 499.2 $\mu$g per gram dry body weight, respectively. These levels are 9.5 fold higher value for Cd, 4.8 fold for Cu, and 9.4 fold for Zn, than samples from station F (Table 2). Except for the Cd level in samples collected at station C, the result in Table 2 suggests that all three metal concentrations in the environment are more or less correlated to the contaminated levels in winkles investigated (compare Tables 1 and 2). Langston and Zhou (1986), however, working with *Littorina littorea*, found similar results for Cd, but saw little variation in the accumulated Cu and Zn content in animals collected at different sites with various environmental contamination levels in England. Bryan *et al.* (1983) also reported the unsuitability of *Littorina littorea* as indicator of Cu and Zn contamination for a similar reason. It was suggested that cellular level of essential metals such as Cu and Zn might be regulated in these animals to maintain certain levels (Langston and Zhou 1986). It is unknown at this point why a different species of winkles show different behavior, but it is possible that different species of *Littorina* could have different level of regulations for Cu and Zn, or have different level of tolerance for specific metals. Nevertheless, findings in this study suggest that *Littorina brevicula* might serve as good bioindicator for both essential and nonessential metals investigated in assessing the level of environmental contamination.

Table 3 shows the distribution of contaminated Cd, Cu, and Zn among the four body parts in winkles collected at station B (Fig. 1). More than 55% of the total contaminated Cd in winkles were connected with kidney, whereas Cu and Zn showed somewhat equal distribution among the body parts investigated. Copper is an essential component of hemocyanin, the respiratory pigment of winkles, and hence significant amount of Cu detected in different body parts might not reflect the true accumulated metal level (Mason and Simkiss 1983). Variations

Table 3. Subcellular distribution of metals in *Littorina brevicula* collected from station B.

| Tissue | Cd | Cu | Zn |
|---|---|---|---|
| Digestive gland and gonad | 14.8% | 21.3% | 25.6% |
| Gill | 15.6% | 21.9% | 24.4% |
| Kidney | 55.2% | 34.0% | 32.3% |
| Remaining tissue | 14.4% | 22.8% | 17.7% |

in the Cu content of the soft tissues in animals in response to changes in environmental metal, therefore, could be due to fluctuations in the Cu content of hemocyanin. Zinc is another essential metal for animals that is tightly regulated to maintain a certain level (Langston and Zhou 1986). For this reason, the equal distribution pattern shown in Table 3 may reflect the total necessary Zn content the various body parts can tolerate. However, at elevated available Zn concentration, the stomach and visceral complexes are reported to play a prominent role in accumulating the metal (Mason 1988), and this seems to be the reason in somewhat elevated Zn level in the digestive gland and gonad part (compare the metal levels in Table 3).

To investigate the direct correlation between the Cd content in the animals and the available Cd in the environment, artificial exposure experiment was conducted. As indicated in Fig. 2A, there was a dosage and time dependent correlation of the Cd accumulated in the exposed animals. The accumulated level in animals exposed to 400 $\mu$g/L of Cd did not reach equilibrium even after 66 days, suggesting more than 640 $\mu$g of Cd can be accumulated per gram of dry body weight (Fig. 2A). Among the four body parts, digestive gland and gonad, gill, kidney, and remaining tissue, more than 40% of the total accumulated Cd was connected with the kidney (Fig. 2B). This agrees well with the field data shown in Table 3. Both results support that Cd is primarily transferred to the kidney for possible excretion in both vertebrates and invertebrates (George *et al.* 1980, Piotroski *et al.* 1973).

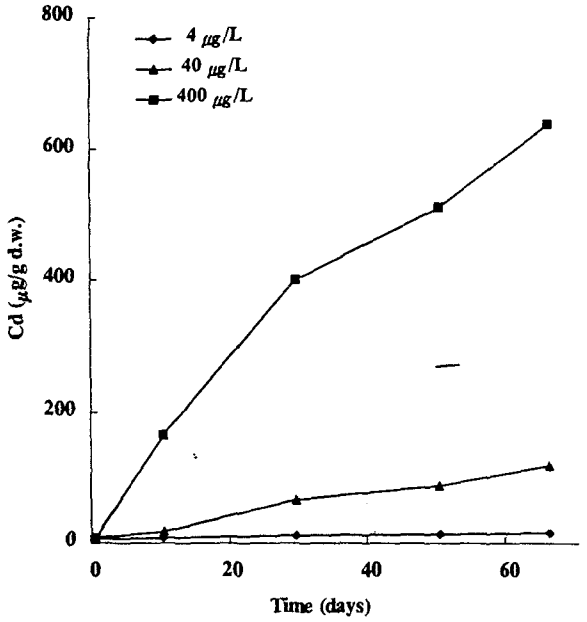
Partitioning of the Cd between the cytosol and membrane fractions was investigated for each of the body parts investigated. From the tissue homogenate obtained from animals exposed to 400 $\mu$g of Cd per 1 liter of seawater for 66 days, more than 85% of the metal was detected in the cytosol of the digestive gland and gonad, gill, and kidney (results not shown). A comparably high proportion of soluble Cd appears to be a consistent feature in marine organisms (Langston and Zhou 1986, Paek and Lee 1998), which suggests the presence of a

metal binding proteinlike molecule in sequestering free metals in the tissue.

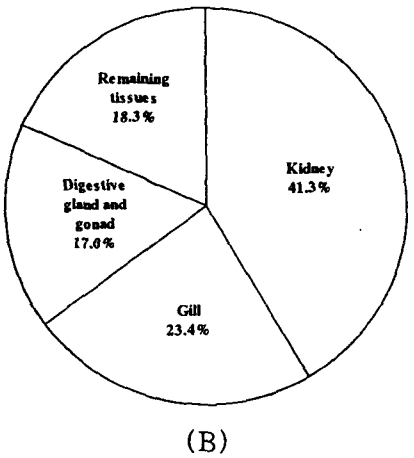
### Gel filtration chromatography

In order to identify the molecule involved in binding and sequestering Cd in the cytosol, gel filtration column chromatography was performed with the cytosol of kidney (see materials and meods). Elution profile showed only one peak eluting at fraction numbers 11-12 (Fig. 3). This Cd peak was also detected in the cytosol of other body parts such as gill, digestive gland and gonad (results not shown). Since the Cd peak in Fig. 3 corresponded to the elution peak of horse kidney metallothionein (molecular mass, 6.5 kDa (results not shown)), the result suggests an involvement of metal binding protein-like molecule in the Cd accumulation in *Littorina brevicula*.

Langston and Zhou (1986) were able to locate two different sizes of metallothionein-like proteins in *Littorina littorea*, having apparent molecular masses of 20 kDa (Cd-binding protein I) and 10 kDa (Cd-binding protein II). There seems to be, however, only one major peak connected to the protein size range of metallothionein-like fraction in *Littorina brevicula*. Furthor purification such as ion-exchange column chromatography is currently being employed to show if *Littorina brevicula* has more than one species of metallothionein-like protein in binding Cd.




(A)




(B)

Fig. 2. Cadmium contents in soft body tissue of *Littorina brevicula* artificially exposed in the laboratory. A. Time course of cadmuim accumulation in the total soft tissue. Accumulated cadmium concentration is expressed in μg per total dry tissue weight (μg/g d.w.). B. Distribution of accumulated cadmium content among four body parts in animals exposed to 40 μg/L of cadmium for 66 days.

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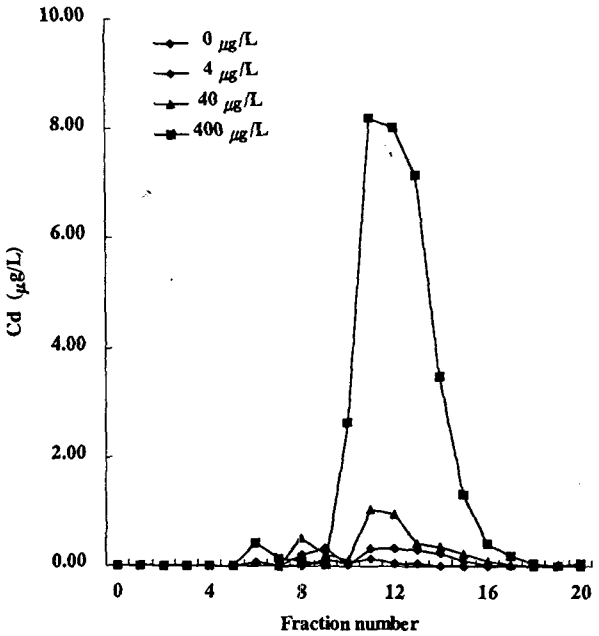


Fig. 3. Elution profile of gel filtration chromatography of the cytosol of kidney from *Littorina brevicula*. Animals were exposed to 400 μg/L of cadmium for 66 days. Horse kidney metallothionein eluted at fraction number 12 (results not shown). Metal concentration in each fraction was measured with graphite furnace AAS.

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