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Bioaccumulation of trace metals and their biochemical influence on the tissues of clam *Ruditapes philippinarum*

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바지락 *Ruditapes philippinarum* 기관별 미량 금속의 생체 축적 및 생화학적 변화

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by

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Ruditapes philippinarum

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Abstract

Marine sediments represent the final repository of most contaminants and elements carried to the oceans by rivers, but also play a key role in estuarine systems as potential sources and sinks for these substances. Clams have been selected as indicator organisms world wide because of their abundance, ubiquity, long life span, high filtration rate, and especially, because of concern in the sea. The clam *Ruditapes philippinarum* was a proposed as suitable biomonitors of metal contamination because of their wide distribution throughout in Korean costal regions. Cadmium was one of the major contaminants that was toxic to living organisms and was widely distributed in the marine environment. Lead was exerts toxic effects at lower concentrations than many other metallic contaminants. Therefore, the aims of the present study are (a) to evaluate the concentration of trace metal in seawater, sediment and clam at different clam-farm sites and (b) to investigate accumulated tissues and the effect of Cd and Pb exposure on the activities of protective antioxidant enzymes in the gill and digestive glands of the clam, *R. pillippinarum* in order to explain accumulation and elimination Cd and Pb in tissues.

Concentrations of trace metals (Cu, Cd, Pb, Cr, As, Se, Zn) determined in different clam farms seawater, sediment and clams. Each metal concentration, Cu, Pb, Zn, were appeared to be the highest the clam farm of Jinhae. For Cu, the mean value was recorded at 2.40 ± 0.69 μg/L and Pb of mean value was at 2.07 ± 0.48 μg/L. Zn of mean value concentration was at 6.83 ± 1.65 μg/L. The highest As of mean value concentrations measured in Sacheon clam farm. This mean value was 5.42 ± 5.55 μg/L. Cd, Cr , Se mean values concentrations

were similarity in all clam farms. As a whole, trace metal concentrations in seawater of Goheung clam farm were observed lower than the other clam farm. The concentrations of Cu range between 44.15 μg/g and 50.05 μg/g in the Jinhae clam farm, while the range 28.07 ~ 37.84 μg/g and 18.78 ~ 29.04 μg/g in the Sacheon, Goheung clam farm, respectively. The highest concentration of Cd (0.31 ± 0.03 μg/g dry wt.) was found at Jinhae clam farm. Pb was quite high (59.11 ~ 73.08 μg/g dry wt.) in the sediment clam farm of Jinhae. The highest concentrations of Cr range between 82.00 and 85.69 μg/g dry wt. in the Goheung clam farm. The concentrations Zn were very high (191.76 ~ 235.72 μg/g dry wt.) in the sediment clam farm of Jinhae. As a result, all trace metals concentrations of sediment except Cr were the highest the clam farm of Jinhae. Cu, Cr, As, Se, Zn concentrations in gill was higher than other tissues at all sampling site. The highest Cr concentrations in clam tissues were recorded 2.18 ± 1.80 μg/g, 2.00 ± 0.86 μg/g in gill at Jinhae and Sacheon, respectively. The highest As, Se concentrations in clam gill tissues were recorded for Sacheon (73.40 ± 12.91 As μg/g, 7.62 ± 1.89 Se μg/g) clam farm. The highest Zn concentrations in clam tissues were recorded for Jinhae (e. g. 114.24 ± 13.65 μg/g in gill) clam farm. In conclusion, trace metal concentrations in clam tissues of Goheung clam farm were observed lower than the other clam farm and were recorded highest trace metals concentrations of gill in the clam tissues.

Cd accumulation and depuration were assessed in the tissues of *R. philippinarum* in four experimental concentration (10, 20, 100, 200 μg/L) over eliminated period 1 week after exposed periods 2 weeks. Cd accumulated in the digestive glands, gills and residue tissues of the clam and the accumulation increased with the time of exposure (2 weeks) and concentration (over 100 μg/L), and the depuration decreased with below 100 μg/L concentration. At 2 weeks of Cd exposure, the order of Cd accumulation in tissues was gill > digestive glands > residue tissues. An inverse relationship was observed between the accumulation factors (AF) and the exposure level, but AF showed an increase with exposure time. During the depuration periods, Cd concentration in digestive glands, gill and residue tissues decreased immediately following the end of the exposure periods except 200 μg/L concentration. The order of Cd elimination rate in tissues were decreased digestive glands > gill > residue tissues during depuration periods. SOD activity no significant

differences in the digestive glands of the clam observed during total periods. And gill only significantly deceased at 200 μg/L concentration in 2 weeks. GPx activity was observed at 200 μg/L concentration in 2 week decreased significant in the digestive glands and observed over 100 μg/L concentration in 2 week decreased significant in the gill. GR activity significantly increased at 200 μg/L concentration for total periods in the gill, but no activity observed in the digestive glands. GST activity significantly increased over 100 μg/L concentration for 1 week in the gill, and no observed after 2, 3 weeks. The digestive glands were observed significant increased at 200 μg/L concentrations in 1 week, significant decreased at 200 μg/L concentration in 2, 3 week. GSH activity was observed significantly increased at 200 μg/L concentration in total periods in the digestive gland. In the gill, GSH activities were increased at 200 μg/L Cd exposure group during 1 week, and then significantly increased at the 100 and 200 μg/L Cd concentrations in 2 week. But, it was significantly decreased at 200 μg/L Cd concentration during the depuration period. In digestive gland and gill, Malondialdehyde activity of Cd exposed to clams were significantly increased at the 200 μg/L, and then were no significant difference each Cd concentration in 2, 3 week

Pb accumulation and depuration were assessed in the tissues of *R. philippinarum* in four experimental concentration (15, 30, 150, 300 μg/L) over eliminated period 1 week after exposed periods 2 weeks. Pb accumulated in the digestive glands, gills and residue tissues of the clam and the accumulation increased with the time of exposure (2 weeks) and concentration (over 150 μg/L), and the depuration decreased with above 150 μg/L concentration. At 2 weeks of Pb exposure, the order of Pb accumulation in tissues was gill > residue tissues > digestive glands. A direct proportion relationship was observed between the accumulation factor and the exposure concentrations at 1 week. Moreover the accumulation factor in over 150 μg/L increased with 2 week. During the depuration periods, Pb concentration in digestive glands, gill and other tissues decreased immediately following the end of the exposure periods above 150 and 300 μg/L concentrations. The order of Pb elimination rate in tissues were decreased digestive gill > digestive gland > residue tissues during depuration periods. SOD activity significantly decreased at 150 and 300 μg/L concentration in 2 week in the digestive gland, but no significantly decreased for depuration

period. And gill showed decreasing level after 3weeks (150 and 300 μg/L). Malondialdehyde activity of gill and digestive gland showed decreasing level after 2weeks and 3weeks (300 μg/L). Glutathione activities in digestive gland and gill were decreased for all experimental periods at 150 and 300 μg/L. Glutathione peroxidase activities were decreased all experimental concentrations except 15 μg/L at 2weeks. Glutathione S-transferase activity which is protecting against oxygen toxicity was increased in 300 μg/L at 2 weeks. Glutathione reductase activity in gill was elevated 150 and 300 μg/L at 1 week.

In conclusion, *R. philippinarum* can be considered a good bioindicator for Cd and Pb exposure. Antioxidant enzyme activity in the digestive glands and gill can be considered a potential biomarker of sub-lethal stress as a result of exposure to cadmium and lead. The choice of the tissues for determining the effect of trace metals also has advantages from the experimental point of view, because a smaller number of specimens will be necessary for analyzing enzyme activity. Although direct metal-enzyme interaction in the case of Cd and Pb cannot be eliminated as a possibility, the most probable cause of the response measured in this study is adaptive reaction to stress. Nevertheless, experiments carried out at environmentally relevant concentrations and field samples will be necessary to confirm the usefulness of antioxidant enzyme as a biomarker of stress by trace metal.

Chapter 1

General introduction

High metal concentrations in the environment are the result of both natural and anthropogenic sources. The accumulation of metals in waters and sediments affects various organisms in the environment, influencing their functions in several different ways (Duquesne *et al*., 1995; Regoli and Principato 1995; Temara *et al*., 1997). The pollution levels of the aquatic environment by trace metals can be estimated by analyzing water, sediments and marine organisms. The levels of trace metals in mollusks and other invertebrates are often considerably higher than in other constituents of the marine environment. Compared to sediments, seawater and mollusks exhibit greater spatial sensitivity and therefore, is the most reliable tool for identifying sources of biologically available trace metals contamination (Thomson *et al*., 1984; Szefer, 1986). The coastal region receives a large amount of metal pollution from coastal towns, industrial dumps and rivers. Pollution by trace metals is a serious problem due to their toxicity and their ability to accumulate in the biota (Islam and Tanaka, 2004). Therefore, a determination of metal concentrations in organisms should be part of any assessment and monitoring program in the coastal region. In the case of metals, this marked bioaccumulation potential reflects a number of factors: (ⅰ) ecological (e. g., close contact with sediments, known to constitute a major environmental sink for metals); (ⅱ) physiological (e. g., important filtering activity to satisfy respiratory and nutritional needs); and (ⅲ) biochemical (e. g., metal tolerance strategies that involve metal sequestration rather than metal exclusion or elimination).

The coastal part of this continental shelf is the most sensitive, as it receives large amounts of contaminants introduced by domestic, industrial and agricultural activities, directly or via rivers or through atmospheric deposition (Zoller and Hushan, 2000; Usero *et al*., 2005). In most cases, the impact and synergistic effects of contaminants on marine ecosystem are poorly known (Zoller, 2006). The early

identification of inorganic contaminants such as trace metals, which are all toxic above a specific threshold of bioavailable level (Kucuksezgin *et al*., 2006), is essential to avoid damage to marine biogenesis (Girotti *et al*., 2006).

The determination of trace metals concentrations in seawater may represent a useful tool to evaluate the quality of the marine environment and can elucidate the mechanisms of pollutant transfer among abiotic compartments and biota. The toxicological assessment of water contamination through sub-lethal bioassays becomes relevant because they allow the early detection of adverse effects on particular test organisms. Laboratory tests facilitate data interpretation and validate their responses as a monitoring index (Bourdou and Ribeyre, 1997). For the water quality management in aquatic ecosystems, it is important to be able to predict the impact of chemical and toxic effects on aquatic species. It must be taken into account that mussels are powerful filter feeders and each individual filters around 15 m^3 of water every year. This means that they can accumulate toxic substances dissolved in sea water or that is associated with material in suspension, and because of this they are used as bioindicators to monitor marine contamination (Besada *et al*., 2002). Active bio-monitoring is now commonly used to evaluate sea water quality using bio-markers measured in transplanted mussels (Roméo *et al*., 2003 a, b; Andral *et al*., 2004). Some studies have shown significant amounts of trace metals in the soft tissues of mussels (Otero and Fernández-Sanjurjo, 2000). These metals become incorporated into the sediment after release from the mussels themselves or through the faecal matter that they produce.

Marine sediments are the ultimate sink of trace elements in the marine environment. Sediment-incorporated trace elements do not stay fixed in the solid phase but they can be partly remobilized in pore-water by physical, chemical and biological processes. Sediments are not only a reservoir for contaminants, but also a source of toxicants for marine animals (Long *et al*., 1996; Fichet *et al*., 1998). Thus, sediment bioassays constitute an important step in the assessment of the marine environment quality, providing an integrated measure of toxicity, and they are becoming widely used tools in monitoring programs, permissions for dumping

dredging material, and other regulatory activities. Sediment analysis is a sound tool for the assessment of marine pollution as settled particles on the seafloor with different chemical constituents reflects long-term changes in the marine ecosystems. In addition, concentrations of trace metals in the sediments are much higher than overlying water, permitting us to determine the interested metals accurately in sediment phase (Libes, 1992).

Molluscs are mainly consumers of the first order in the food chain and can accumulate a high amount of trace metals without exhibiting marked visible physiological effects. Bivalves are considered excellent candidates for use as biomonitors of coastal contamination, but the rates and routes of metal uptake in these biomonitors are not well understood. Thus, they are able to bioaccumulate environmental pollutants, reflecting the degree of pollution of their ecosystem. In natural environments, bivalves can accumulate metals from both ambient seawater and ingested food. Also, they can easily be transferred from control to contaminated areas, which make them quite appropriate for studying the impact of pollution sources in field studies. There are even fewer studies which have considered the biokinetics of metal uptake in bivalves from tropical or subtropical waters (e.g. the black mussel *Septifer virgatus*, Wang and Dei, 1999a).

Cadmium (Cd) is a non-essential element that has severe toxic effects on aquatic animals when present in excessive amounts (Sorensen, 1991). Several studies indicate that cadmium accumulation in mollusks was both dose and time dependent (Badino *et al*., 1991; Roesijadi and Unger, 1993; Wang and Evans, 1993). Accumulation of cadmium by aquatic animals can occur either via direct uptake across body membranes or via indirect uptake by absorption from digested food in the gut tract, and the relative importance of these mechanisms probably varies among animals (Abel and Barlocher, 1988). Of the two uptake patterns, direct uptake involves the transport of ionic or complexed cadmium to receptor sites, followed by transfer through membrane of an animal. In most cases, only the free metal ion is expected to interact with the binding site and the extent of binding would depend on ion activity.

While various sources of lead (Pb) (e.g. mining, refining, coal burning and gasoline) are important, dust deposition today remains a significant component of the elevated lead flux. With respect to lead contamination in the oceans, domestic effluents and industrial activity have been identified as significant sources, but the primary one is the atmospheric input (Brown and Depledge, 1998). Besides provoking acute toxicity effects which may arise at high lead concentration, a variety of sub-lethal toxic responses can occur in organisms at much lower levels of the contaminant in marine waters (Luoma and Carter, 1991; Depledge *et al*., 1995). In case of lead contamination, a proportion of the Pb in seawater occurs in a soluble form; therefore direct uptake from solution should be a quantitatively important process in marine organisms (Schulz-Baldes, 1974). Lead is of concern in some coastal waters as a contaminant derived principally from industrial pollution, and it is therefore periodically measured in large national and international monitoring programs (O'Connor, 1992). The accumulation pattern in animals is dependent not only on uptake but also on the depuration rate which was reported in some mussels to be an exponential function of exposure time (Han *et al*., 1993).

Antioxidant systems are efficient protective mechanisms against chemical reactive species produced by endogeneous metabolism or by the biotransformation of xenobiotics. The activity of these systems may be induced of inhibited after chemical stress. An induction can be considered an adaptation, allowing the biological systems to partially or totally overcome stress resulting from exposure to an unsafe environment. Thus, the parameters of antioxidant systems could be useful biomarkers reflecting not only exposure to contaminants, but also toxicity. The parameters studied include antioxidant enzymes such as superoxide dismutases (SOD), glutathione peroxidase (GPx), glutathione reductase (GR), glutathione S-transferases (GST) and reduced glutathione (GSH). SOD metabolises superoxide anion into less reactive species, molecular oxygen and hydrogen peroxide (H_2O_2). GPx is an antioxidant enzyme that reduces organic peroxides, by means of GSH, which is oxidized in GSSG at the end of the reaction. GSH can be regenerated by

glutathione reductase which reduces GSSG in the presence of NADPH. GST catalyse the conjugation of the sulphur atom of glutathione with a large variety of electrophilic compounds of both endobiotic and xenobiotic origin. And, cytotoxicity as reflected by lipid peroxidation (LPO) was noted in the cases where the antioxidant defenses were the lowest, which provided support for the use of these parameters as biomarkers for toxicity. Oxidative damage, by means of lipid peroxidation, must be evaluated in order to establish the possible relationship between cytotoxicity and antioxidant system disturbances. These biochemical parameters had already been compared in mussels from the clean and polluted sites, providing some useful indications on their potential as biomarkers of contaminant-mediated oxidative stress (Regoli and Principato, 1995).

Chapter 2.

Trace metal concentrations in the aquatic environment

2.1. Introduction

For the maximum utilization of Korea's abundant marine resources in coastal waters, it is necessary to protect the water quality. Since trace metals, being persistent, are accumulated in sediments and biota in the marine environment, they comprise an essential class of the critical pollutants to be monitored (Lee *et al.*, 1998). In coastal bay, metals can enter surface waters principally through atmospheric deposition, industrial effluent discharge and streams. Wastewater discharges from various locations are the major sources of organic and inorganic pollutant in the coastal water ecosystems. Urban and industrial activities in coastal areas introduce significant amounts of pollutants (including trace metals) into the marine environment. Unfortunately, monitoring data are lacking in most regions where anthropogenic activities are well developed, and especially in the Korean a clam farm.

There is currently a great deal of interest in using living organisms as pollution biomonitors in aquatic ecosystems, given that the method that has been used traditionally-chemical analysis of the water-does not provide information on the bioavailability of metal concentrations in water often lies near or below the detection limit of instruments and they fluctuate drastically, depending on water flow and intermittence of discharge (Rainbow, 1995). Marine sediments represent the final repository of most contaminants and elements carried to the oceans by rivers, but also play a key role in estuarine systems as potential sources and sinks for these substances. The fate of these substances in sediments is related to the nature and extent of biogeochemical transformations as well as their relative mobility or degradation under varying redox conditions. In addition, adjective

processes such as resuspension and bioturbation, can return these substances to the water column where they may become available for uptake by the pelagic biota. Processes which contribute to sediment remobilization and redistribution include shear stresses imposed by tidal currents, disturbance by wave action and bioturbation by burrowing marine animals (Jago *et al*., 1993). With respect to contaminated sediment, bioavailability may be defined as the maximum amount of a contaminant which is available, or solubilized, in the environment of an organism. Bioavailability is, therefore, a complex concept since it is species-, sediment- and chemical-specific, and is not necessarily equivalent to the amount of contaminant which is absorbed into the tissue of an organism. Nevertheless, a general understanding of the mechanisms and extent to which contaminants are solubilized in the environment is essential in order to improve our ability to predict the potential environmental and ecotoxicological impacts arising from contaminated sediment.

The overall range of concentrations in the coastal waters of the central Mediterranean is 0.01 ~ 0.62 μg/L for cadmium (Manfra and Accornero, 2005). Typically, the concentration of dissolved lead in low-moderately anthropogenically impacted waters is < 10 μg/L, with levels reaching > 200 μg/L in highly contaminated locales (Dassenakis *et al*., 1997). Under normal conditions, waterborne lead falls within the range of 0.0006-0.12 mg/L (Demayo *et al*., 1982) through concentrations as high as 0.89 mg/L have been reported (Research Triangle Institute, 1999). Upon occupational chronic exposure to inorganic forms of lead (Pb) and cadmium (Cd), nephropathy may be occurred which usually starts insidiously. A cascade of events may develop leading from initial dysfunction and focal damage to a clinically detectable disease. The kidneys are usually the most critically affected organs in occupational exposure to Cd, known to adversely interfere with the renal handling of plasma derived proteins (Mueller *et al*., 1998). Tubular proteinuria needs also to be considered as an adverse effect attributed to Cd exposure, because it can lead to irreversible renal damage associated with an exacerbation rate and a decrease in the filtration reserve capacity (Satarug *et al*.,

2003). Chronic massive exposure to Pb produces hematological, cardiovascular, neurological, and renal adverse effects; notably progressive tubulointerstitial nephropathy that develop insidiously and leads to kidney failure.

Of the wide variety of organisms which can be used for biomonitoring, shellfish are favored as they meet the general requirements of biomonitors (Phillips and Rainbow, 1992), and in particular because of their often widespread distribution, ecological importance, sedentary nature, relatively high tolerance to pollutants, bioaccumulation of chemicals, and ability to be transplanted and held in cages (Farrington *et al*., 1987). Clams have been selected as indicator organisms world wide because of their abundance, ubiquity, long life span, high filtration rate, and especially, because of concern in the sea. In Korea, cultivation of the clam (*Ruditapes philippinarum*) is the important activity, with an annual production of 14,327 metric tones, which represents 4% of shellfish aquaculture production in Korea (Statistical Year Book of Maritime Affairs & Fisheries, 2006). Therefore, the aims of the present study were (a) to evaluate the concentration of trace metals in seawater, sediment and clam at different clam-farm sites and (b) to evaluate the relationship between metal contents of seawater, sediment and clam tissue concentrations.

2.2. Material and methods

2.2.1 Sampling areas

Samples were collected in three stations, Jinhae, Sacheon and Goheung, located on the south shore of the Korean (Fig. 2-1). On July, August, December of 2004 and January of 2005 we sampled three sites. Jinhae is located at the southern tip of Korea peninsula, 128°50′34″ of east longitude and 35°10′34″ of north latitude. This region is the center of machine industry in Korea, and beside to Busan that is the second largest city in Korea. Sacheon is strategic transportation point with-developed air, sea and land transportation network. This region is hub of high-tech aerospace industry, such as Korea Aerospace Industries Ltd., and other foreign companies. Goheung, located in the southern most extremity of the Korean peninsula, has the depth on the coast is low and the spacious reclaimed land spreads the coast so that it has become adequate for growing various kinds of shellfish and seaweeds.

Seawater samples were collected at the three clam-farms using the water sampler, and then were kept in 1-L acid washed polyethylene bottles and were later filtered through the 0.45-μm membrane filter and acidified with concentrated nitric acid to a pH of 2. The acidified samples were kept at 4°C for metal analysis. Sediment samples were taken using Van-Veen Grab from surface sediments. The top 20 cm of the bottom sediment was obtained using this sampling equipment. The sediment was kept in a polyethylene bottle and later freezes dried in the laboratory. The dried sediment samples were kept in desiccators for further analysis. Clams were collected from the sediment where they usually dwelled. These were commonly found fine sediment that consisted of silt, fine sand and organic materials such as vegetation debris. At each site one-hundred samples of surface sediment were collected. All specimens collected were segregated according to the sampling station. After collection, the clams were allowed to flush out undigested matter in filtered seawater from the sampling sites for 24 h.

Figure 2-1. location of the sampling sites.

2.2.2. Metal analysis

Acidified seawater (80 ml) was placed in a screw cap polypropylene separating funnel (Nalgene 125 ml) using a 100-mL measuring cylinder. The separating funnel was mounted in a Lab-Line Multi-Wrist Shaker. The pH was adjusted to 4.5 with buffer (0.73 ml), as determined on a separate representative sample that was not extracted. Finally, 100 μL of 0.5% each APDC/NaDDC complexing agent and 5 ml of DIBK were added. The samples were shaken at 1000 rpm with 1-cm amplitude for 10 min and allowed to stand for 5 min for phase separation. The lower seawater phase was drained and retained for Mn analysis (if required). Using an adjustable pipette, fitted with a long tip, 4.5 ml of the DIBK phase was withdrawn and placed in a 10 ml screw-cap polypropylene, tapered, centrifuge tube. One ml of Hg solution (100 ppm) was added and the tube was shaken by hand for 2 min for back-extraction. After phase separation, 3 ml of the upper DIBK was removed by adjustable pipette, and 0.9 ml of the lower aqueous phase was transferred to an autosampler cup for ICP-MS (Elan 6000, Perkin-elmer) analysis. Blanks were determined by extraction of 80 ml of acidified ultra-pure water.

Dried sediment samples were ground to a size of 60 meshes, weighed to approximately 0.5 g into the digestion vessels. The sediments were digested with 10 ml of concentrated nitric acid and 1 ml of concentrated hydrochloric acid using the microwave. The digested contents were filtered, diluted to 100 ml in volumetric flasks. Heavy metal contents were analyzed using ICP-MS.

The clam soft tissues of 10 individuals from each location were carefully removed by shelling the clams with a plastic knife; they were then freeze-dried and ground to a fine powder in mortar before analysis. The resulting powder underwent microwave acid (HNO_3 Suprapur) digestion. Following acid digestion, all samples were analyzed using ICP-MS.

2.2.3. Biochemical analysis

Eight clams were removed from each station region. Four clams were measured and the gill, digestive glands dissected, weighed, frozen stored at -80℃ until analyses of antioxidant enzyme activities, MDA, GSH. Tissues were homogenized in 0.5 M sucrose and 0.15 M NaCl in 0.02 M Tris-HCl (pH 7.6). The homogenates were centrifuged at 500g for 15 min at 4℃ and the resulting supernatant at 12,000×g for 30 min at 4℃. SOD, GR, GST, GPx activities were measured in the supernatant. MDA activities were measured pre-centrifuged homogenated tissues. Reduced glutathione (GSH) activity measured the other four clams. Tissues were dissected, homogenized 10% $HClO_4$, and followed centrifuged 5000g for 15 min. The resulting supernatants measured GSH determination.

SOD activity was evaluated with the xanthine oxidase-cytochrome c method as described by Flohe and Otting (1984). The cytochrome c reduction by superoxide anion radicals generated by the xanthine oxidase-hypoxanthine reaction was monitored at 550 nm. One unit of SOD activity is defined as the amount of sample causing 50% inhibition of cytochrome c reduction under the assay conditions. The SOD activity in the tissues of the clams was expressed as U mg/ total protein.

Gutathione reductase (GR; EC 1.6.4.2) activity was assayed according to Carlberg (1985) in 1 unit of glutathione reductase contained 100 mM potassium phosphate buffer (pH 7.5) 100 *u*L, 2 mM oxidized glutathione 500 *u*L, 3 mM DTNB 250 *u*L, 2 mM NADPH 50 *u*L, enzyme sample 100 *u*L. The decrease in absorbance caused by total volume 1 ml was measured at 412 nm.

Glutathione peroxidase (GPx; EC 1.11.1.9) was measured as described by Paglia and Valentine (1967) against hydorgen peroxide (H_2O_2) or cumene hydro-peroxide (cumOOH). Briefly, 0.1 ml of material was added to obtain 1 ml of a final mixture containing 0.05 M Tris-HCl buffer (pH 8.0), 0.5 mM EDTA, 10mM sodium azide, 50 mM reduced glutathione, 10 IU of glutathione reductase, 5 mM NADPH, and the reaction was started with either 6 mM H_2O_2 or 12 mM cumOOH (in relation to the final concentration). Activity was calculated on the basis of the decrease in

absorbance at 340 nm after the subtraction of a rate of non-enzymatic NADPH oxidation.

Glutathione-S-transferase (GST; EC 2.5.1.18) activity was estimated from the increase in absorbance at 340 nm in 1,5 ml of 33 mM Tris-HCl buffer, pH 6.0, with 7.5 mM reduced glutathione (GSH) including 0.5 ml of active material. The reaction was initiated by 10 *u*L of 15 mM 1-chloro-2, 4-dinitrobenzene (CDNB) to obtain its final concentration of 1 mM (Habig and Jacoby, 1981a, b).

Lipid peroxidation (polyunsaturated fatty acid peroxides generate malondialdehyde; MDA) activity was according to John and Steven (1978) method. MDA activity was 1 ml homogenized tissues were added 15% w/v trichloroacetic acid, 0.375% thiobarburic acid, 0.25N hydrochloric acid contained TCA-TBA-HCl reagent. The mixture was heated in at water-bath 100℃ for 15 min. After cooling, supernatants centrifuged 1000g for 10 min, and then measured absorbance at 535 nm.

Reduced glutathione (GSH) content was determinated according to Richardson and Murphy (1975). The reaction mixture contained 0.01M 5, 5'-dithiobis, 2-nitrobenzoix acid (DTNB), 0.1M PBS buffer (pH 8.0). GSH standard curve performed 10nM reduced glutathione standard solution. The linear increase in absorbance was recorded at 412 nm.

Total protein concentrations according to Bradford (1976) by using bovine serum albumin (BSA) as standard.

2.2.4. Statistical analysis

Data are expressed as mean ± standard error (S.E.). All statistical analysis was performed using SPSS/Pc+ statistical package. Prior to analysis, all data were tested for homogeneity of variances among groups using the Barttlett test. Comparisons in normalized data between control and treatments group were made by one-way analysis of variance (ANOVA) followed by Duncan's multiple comparisons test of mean values if significant differences were found ($P < 0.05$).

2.3. Results

2.3.1. Trace metal concentration in the seawater and sediment

Concentrations of trace metals (Copper: Cu, Cadmium: Cd, Lead: Pb, Chromium: Cr, Arsenic: As, Selenium: Se, Zinc: Zn) determined in the three clam farm seawater are presented in Table 2-1, 2-2, 2-3. The highest Cu, Pb, Zn concentrations of trace metals found in Jinhae clam farm. For Cu, the mean value was recorded at 2.40 ± 0.69 μg/L and Pb of mean value was at 2.07 ± 0.48 μg/L. Zn of mean value concentration was at 6.83 ± 1.65 μg/L. The highest As of mean value concentrations measured in Sacheon clam farm. This mean value was 5.42 ± 5.55 μg/L. Cd, Cr and Se mean values concentrations were similarity in all clam farm. As a whole, trace metal concentrations in seawater of Goheung clam farm were observed lower than the other clam farm.

Average sediment metals concentrations and ranges for the three clam farms are presented in Table 2-4, 2-5, 2-6. The concentrations of Cu range between 44.15 μg/g and 50.05 μg/g in the Jinhae clam farm, while the range 28.07 ~ 37.84 μg/g and 18.78~29.04 μg/g in the Sacheon, Goheung clam farm, respectively. The Cu concentrations at Jinhae found higher than the other sampling regions. The highest concentration of Cd (0.31 ± 0.03 μg/g dry wt.) was found at Jinhae clam farm. The concentrations of Cd measured in sediment were ranged from 0.17 to 0.19 μg/g in the Sacheon clam farm and 0.08 to 0.13 μg/g in Goheung clam farm. Pb was quite high (59.11 ~ 73.08 μg/g dry wt.) in the sediment of Jinhae clam farm. The concentration of Pb ranges between 36.35 and 47.05 μg/g in the Sacheon clam farm. Low levels of Pb (31.63 ~ 48.34 μg/g dry wt.) were measured in Goheung clam farm. The highest concentrations of Cr range between 82.00 and 85.69 μg/g dry wt. in the Goheung clam farm. The low Cr values were observed in the Jinhae and Sacheon, ranging from 55.97 to 64.07 μg/g and 65.47 to 69.06 μg/g, respectively. The concentrations Zn were very high (191.76 ~ 235.72 μg/g dry wt.) in the sediment of Jinhae clam farm. The concentrations of As, Se in three clam

farm were generally similarity. As a result, all trace metal concentrations of sediment except Cr were the highest in the Jinhae clam farm.

Table 2-1. Concentrations of trace metals (μg/L) in seawater of the different clam farms for summer.

Trace metal	Site		
	Jinhae	**Sacheon**	**Goheung**
Cu	1.75 ~ 4.48 (2.62 ± 1.10)	1.55 ~ 3.70 (2.28 ± 0.76)	0.05 ~ 3.03 (2.03 ± 1.06)
Cd	0.05 ~ 0.10 (0.07 ± 0.01)	0.03 ~ 0.06 (0.05 ± 0.01)	0.03 ~0.09 (0.07 ± 0.02)
Pb	1.69 ~ 3.36 (2.37 ± 0.73)	1.24 ~ 3.20 (1.88 ± 0.69)	0.36 ~ 2.62 (1.38 ± 0.85)
Cr	0.08 ~ 0.14 (0.11 ± 0.02)	0.10 ~ 0.15 (0.12 ± 0.02)	0.09 ~ 0.11 (0.10 ± 0.01)
As	1.35 ~ 7.72 (3.75 ± 2.39)	1.09 ~ 6.77 (3.68 ± 2.67)	1.20 ~ 7.32 (3.54 ± 2.74)
Se	0.01 ~ 0.05 (0.03 ± 0.02)	0.01 ~ 0.02 (0.01 ± 0.00)	0.01 ~ 0.05 (0.03 ± 0.02)
Zn	4.22 ~ 7.28 (5.31 ± 1.20)	2.28 ~ 7.12 (4.18 ± 2.19)	1.99 ~ 3.67 (2.75 ± 0.66)

Table 2-2. Concentrations of trace metals (μg/L) in seawater of the different clam farms for winter.

Trace metal	Site		
	Jinhae	**Sacheon**	**Goheung**
Cu	1.37 ~ 4.10 (2.19 ± 1.09)	0.49 ~ 1.65 (1.05 ± 0.49)	0.45 ~ 2.30 (1.36 ± 0.93)
Cd	0.04 ~ 0.09 (0.05 ± 0.02)	0.03 ~ 0.04 (0.03 ± 0.01)	0.02 ~ 0.03 (0.03 ± 0.01)
Pb	1.08 ~ 2.38 (1.70 ± 0.42)	0.39 ~ 2.49 (1.05 ± 0.82)	0.59 ~ 1.91 (1.27 ± 0.50)
Cr	0.10 ~ 0.21 (0.16 ± 0.05)	0.10 ~ 0.22 (0.16 ± 0.05)	0.21 ~ 0.25 (0.23 ± 0.02)
As	1.11 ~ 4.12 (2.10 ± 1.36)	1.01 ~ 1.60 (1.27 ± 0.21)	0.96 ~ 3.23 (1.91 ± 0.88)
Se	0.01 ~ 0.02 (0.01 ± 0.00)	0.01 ~ 0.03 (0.02 ± 0.01)	0.02 ~ 0.04 (0.03 ± 0.01)
Zn	3.42 ~ 8.44 (6.68 ± 2.15)	1.39 ~ 3.23 (2.13 ± 0.64)	0.55 ~ 6.04 (3.27 ± 2.66)

Table 2-3. Concentrations of trace metals (μg/L) in seawater of the different clam farms for month 7, 8, 12 and 1 at 2004-2005.

Trace metal	Site		
	Jinhae	**Sacheon**	**Goheung**
Cu	1.37 ~ 4.48 (2.40 ± 1.07)	0.49 ~ 3.70 (1.66 ± 0.88)	0.05 ~ 3.03 (1.70 ± 1.01)
Cd	0.04 ~ 0.10 (0.06 ± 0.02)	0.03 ~ 0.06 (0.04 ± 0.01)	0.02 ~ 0.09 (0.05 ± 0.03)
Pb	1.08 ~ 3.36 (2.03 ± 0.66)	0.39 ~ 3.20 (1.47 ± 0.84)	0.36 ~ 3.62 (1.32 ± 0.67)
Cr	0.08 ~ 0.21 (0.13 ± 0.04)	0.10 ~ 0.22 (0.14 ± 0.04)	0.09 ~ 0.25 (0.17 ± 0.07)
As	1.11 ~ 7.72 (2.92 ± 2.04)	1.01 ~ 6.77 (2.48 ± 2.20)	0.96 ~ 7.32 (2.93 ± 2.21)
Se	0.01 ~ 0.05 (0.02 ± 0.01)	0.01 ~ 0.03 (0.02 ± 0.01)	0.01 ~ 0.05 (0.03 ± 0.01)
Zn	3.42 ~ 8.44 (5.99 ± 1.81)	1.39 ~ 7.12 (3.15 ± 1.87)	0.55 ~ 6.04 (3.01 ± 1.86)

Table 2-4. Concentrations of trace metals (μg/L) in sediment of the different clam farms for summer.

Trace metal	Site		
	Jinhae	**Sacheon**	**Goheung**
Cu	39.20 ~68.30 (49.04 ± 10.24)	30.69 ~ 46.49 (34.81 ± 5.88)	20.27 ~ 34.62 (24.59 ± 5.45)
Cd	0.24 ~ 0.39 (0.33 ± 0.05)	0.15 ~ 0.20 (0.17 ± 0.02)	0.07 ~ 0.14 (0.09 ± 0.03)
Pb	58.17 ~ 85.33 (71.08 ± 9.41)	35.19 ~ 51.93 (41.86 ± 7.84)	29.34 ~ 41.25 (33.73 ± 4.67)
Cr	50.57 ~ 64.55 (56.28 ± 5.21)	64.24 ~ 71.18 (67.02 ± 2.83)	76.31 ~ 87.88 (82.76 ± 4.30)
As	13.07 ~ 14.11 (13.45 ± 0.38)	9.55 ~ 10.69 (10.17 ± 0.40)	8.62 ~ 10.88 (9.71 ± 0.88)
Se	0.05 ~ 0.30 (0.19 ± 0.09)	0.11 ~ 0.59 (0.29 ± 0.16)	0.05 ~ 0.17 (0.12 ± 0.04)
Zn	173.84 ~ 256.10 (225.20 ± 29.37)	142.44 ~ 172.66 (156.52 ± 10.02)	115.07 ~ 134.35 (122.86 ± 6.94)

Table 2-5. Concentrations of trace metals (μg/L) in sediment of the different clam farms for winter.

Trace metal	Site		
	Jinhae	**Sacheon**	**Goheung**
Cu	39.45 ~ 49.20 (44.43 ± 4.04)	20.71 ~ 35.82 (29.31 ± 5.71)	18.41 ~ 34.15 (23.91 ± 6.63)
Cd	0.25 ~ 0.32 (0.29 ± 0.03)	0.14 ~ 0.26 (0.18 ± 0.05)	0.05 ~ 0.25 (0.10 ± 0.07)
Pb	53.25 ~ 65.83 (60.92 ± 4.44)	31.60 ~ 43.53 (36.83 ± 4.45)	31.35 ~ 67.69 (39.99 ± 14.22)
Cr	57.30 ~ 66.42 (63.32 ± 3.54)	64.39 ~ 73.66 (68.81 ± 3.35)	80.87 ~ 87.48 (84.05 ± 2.23)
As	12.95 ~ 15.60 (14.23 ± 0.99)	9.04 ~ 11.56 (9.94 ± 1.02)	9.86 ~ 10.69 (10.30 ± 0.08)
Se	0.19 ~ 0.55 (0.35 ± 0.12)	0.10 ~ 0.45 (0.25 ± 0.14)	0.02 ~ 0.25 (0.17 ± 0.08)
Zn	171.35 ~ 210.16 (192.73 ± 13.00)	135.60 ~ 171.31 (155.36 ± 14.64)	115.80 ~ 122.81 (120.25 ± 2.76)

Table 2-6. Concentrations of trace metals (μg/L) in sediment of the different clam farms for month 7, 8, 12 and 1 at 2004-2005.

Trace metal	Site		
	Jinhae	**Sacheon**	**Goheung**
Cu	39.20 ~ 68.30 (46.73 ± 7.80)	20.71 ~ 46.49 (32.06 ± 6.22)	18.41 ~ 34.62 (24.25 ± 5.80)
Cd	0.24 ~ 0.39 (0.31 ± 0.04)	0.14 ~ 0.26 (0.18 ± 0.04)	0.05 ~ 0.25 (0.09 ± 0.05)
Pb	53.25 ~ 85.33 (66.00 ± 8.80)	31.60 ~ 51.93 (39.35 ± 6.62)	29.34 ~ 67.69 (36.86 ± 10.61)
Cr	50.57 ~ 66.42 (59.80 ± 5.62)	64.24 ~ 73.66 (67.92 ± 3.10)	76.31 ~ 87.88 (83.41 ± 3.33)
As	12.95 ~ 15.60 (13.84 ± 0.82)	9.04 ~ 11.56 (10.05 ± 0.75)	8.62 ~ 10.88 (10.01 ± 0.70)
Se	0.05 ~ 0.55 (0.27 ± 0.13)	0.10 ~ 0.59 (0.27 ± 0.14)	0.02 ~ 0.25 (0.15 ± 0.07)
Zn	171.35 ~ 256.10 (208.96 ± 27.51)	135.60 ~ 172.66 (155.94 ± 11.98)	115.07 ~ 134.35 (121.56 ± 5.22)

2.3.2. Trace metal concentration in the tissues of clam

Table 2-7, 2-8, 2-9 show the levels of trace metals found in clam each tissues, expressed in μg/g dry weight, in different sampling regions during July, August, December and January, 2004-2005, respectively. Cu, Cr, As, Se, Zn concentrations in gill was higher than other tissues at all sampling site. Cu concentrations value of clam tissues in the order was gill ≈ digestive glands > siphon > foot > adduct muscle at all site. The highest and lowest Cu concentrations in clam gill tissues were recorded for Sacheon (12.26 ± 2.63 μg/g) and Goheung (7.90 ± 2.23 μg/g) clam farm, respectively. Also, the highest Cr concentrations in clam tissues were recorded 2.18 ± 1.80 μg/g, 2.00 ± 0.86 μg/g in gill at Jinhae and Sacheon, respectively. The highest and lowest As, Se concentrations in clam gill tissues were recorded for Sacheon (73.40 ± 12.91 As μg/g, 7.62 ± 1.89 Se μg/g) and Goheung (32.33 ± 5.06 As μg/g), Jinhae (4.48 ± 1.09 Se μg/g) clam farm. The most abundant elements in clam tissues were Zn. The highest and lowest Zn concentrations in clam tissues were recorded for Jinhae (e. g. 114.24 ± 13.65 μg/g in gill) and Goheung (e. g. 55.10 ± 6.69 μg/g in adduct muscle) clam farm.

Cd and Pb concentrations in digestive glands were higher than other tissues, except for Goheung region. Cd concentrations value of clam tissues in the order was digestive glands > gill > siphon > foot ≈ adduct muscle at Jinhae and Sacheon. Cd concentrations clam tissues at Goheung in the order was gill >digestive glands > siphon > foot > adduct muscle, and was recorded lowest Cd concentration in clam tissues. Pb concentrations value of clam tissues order was digestive glands > gill > adduct muscle > foot ≈ siphon at Jinhae and Sacheon. The highest and lowest Pb concentrations in clam tissues were recorded for Jinhae (e. g. 2.14 ± 0.89 μg/g in digestive glands) and Goheung (e. g. 0.73 ± 0.68 μg/g in digestive glands) clam farm. Trace metal concentrations in clam tissues of Goheung clam farm were observed lower than the other clam farm and were recorded highest trace metals concentrations of gill in the clam tissues.

Table 2-7. Concentrations of trace metals (μg/L) in the clams of the different clam farms for summer. (D.G: Digestive gland; G: Gill; Ft: Foot; Am: Adduct muscle; Sp: Siphon)

Trace metal	Clam tissues	Site		
		Jinhae	Sacheon	Goheung
Cu	D. G.	10.10 ± 0.44	11.30 ± 0.20	6.58 ± 2.29
	G.	11.28 ± 3.39	12.66 ± 1.69	9.24 ± 0.16
	Ft.	6.35 ± 3.41	8.09 ± 1.21	5.75 ± 1.93
	Am.	4.68 ± 2.58	6.77 ± 3.36	3.66 ± 0.83
	Sp.	7.53 ± 1.79	13.34 ± 1.59	6.25 ± 1.68
Cd	D. G.	1.45 ± 0.20	1.93 ± 1.43	1.78 ± 1.08
	G.	1.67 ± 0.64	1.72 ± 0.04	2.57 ± 0.65
	Ft.	0.09 ± 0.01	0.23 ± 0.16	0.24 ± 0.05
	Am.	0.28 ± 0.08	0.26 ± 0.09	0.18± 0.08
	Sp.	0.32 ± 0.02	0.38 ± 0.04	0.47 ± 0.20
Pb	D. G.	1.63 ± 0.79	2.34 ± 2.54	0.98 ± 0.92
	G.	1.38 ± 0.50	1.74 ± 1.09	1.28 ± 0.11
	Ft.	0.46 ± 0.01	0.79 ± 0.34	0.66 ± 0.39
	Am.	0.52 ± 0.10	0.96 ± 0.09	0.61 ± 0.42
	Sp.	0.40 ± 0.05	0.73 ± 0.50	0.47 ± 0.31
Cr	D. G.	1.78 ± 1.38	1.51 ± 0.55	0.75 ± 0.05
	G.	2.99 ± 2.65	2.25 ± 1.40	1.35 ± 0.65
	Ft.	2.55 ± 2.80	1.46 ± 1.64	0.65 ± 0.49
	Am.	2.68 ± 1.74	2.77 ± 0.10	1.18 ± 0.37
	Sp.	2.26 ± 1.93	1.46 ± 0.67	1.04 ± 0.22
As	D. G.	25.96 ± 1.13	47.34 ± 15.35	20.40 ± 6.35
	G.	44.84 ± 0.75	64.41 ± 8.19	34.63 ± 2.12
	Ft.	16.66 ± 4.19	29.05 ± 1.71	11.38 ± 0.65
	Am.	9.43 ± 1.73	20.37 ± 3.39	6.78 ± 0.04
	Sp.	26.74 ± 0.62	38.99 ± 8.21	18.47 ± 3.92
Se	D. G.	2.22± 0.50	3.13 ± 0.68	2.36 ± 5.95
	G.	4.11 ± 0.60	7.28 ± 3.07	5.95 ± 1.89
	Ft.	055 ± 0.03	2.49 ± 2.10	1.55 ± 0.69
	Am.	0.51 ± 0.06	2.16 ± 1.79	1.50 ± 0.55
	Sp.	1.24 ± 0.12	2.00 ± 1.00	1.55 ± 0.29
Zn	D. G.	79.70 ± 0.41	76.64 ± 12.41	56.65 ± 12.42
	G.	124.79 ± 8.51	110.94 ± 0.64	113.74 ± 23.61
	Ft.	75.17 ± 14.77	82.23 ± 9.41	70.87 ± 8.21
	Am.	59.34 ± 9.20	74.24 ± 0.76	59.03 ± 1.46
	Sp.	79.82 ± 0.12	86.03 ± 23.41	66.28 ± 7.16

Table 2-8. Concentrations of trace metals (μg/L) in the clams of the different clam farms for winter.

Trace metal	Clam tissues	Site		
		Jinhae	Sacheon	Goheung
Cu	D. G.	8.58 ± 1.13	13.21 ± 3.07	6.71 ± 4.71
	G.	7.58 ± 1.15	11.87 ± 4.15	6.56 ± 2.76
	Ft.	3.83 ± 0.47	10.51 ± 3.79	3.92 ± 2.07
	Am.	3.51 ± 1.01	7.49 ± 5.27	2.06 ± 1.23
	Sp.	7.25 ± 1.26	10.92 ± 3.26	5.84 ± 1.64
Cd	D. G.	3.07 ± 0.26	2.97 ± 0.48	1.73 ± 1.25
	G.	1.60 ± 0.73	2.19 ± 0.92	3.30 ± 0.27
	Ft.	0.15 ± 0.03	0.23 ± 0.09	0.29 ± 0.15
	Am.	0.17 ± 0.02	0.19 ± 0.21	0.30 ± 0.04
	Sp.	0.33 ± 0.01	0.51 ± 0.04	0.58 ± 0.10
Pb	D. G.	2.65 ± 0.86	1.33 ± 0.61	0.49 ± 0.53
	G.	1.75 ± 0.94	0.64 ± 0.60	0.72 ± 0.44
	Ft.	0.20 ± 0.04	0.20 ± 0.02	0.20 ± 0.23
	Am.	0.21 ± 0.07	0.22 ± 0.06	0.08 ± 0.03
	Sp.	0.29 ± 0.05	0.12 ± 0.11	0.10 ± 0.02
Cr	D. G.	1.35 ± 0.34	2.05 ± 0.88	1.41 ± 0.98
	G.	1.37 ± 0.30	1.75 ± 0.12	1.54 ± 0.72
	Ft.	1.11 ± 0.71	1.04 ± 0.20	1.24 ± 0.73
	Am.	0.93 ± 0.41	1.50 ± 0.06	1.19 ± 0.77
	Sp.	1.32 ± 0.03	1.19 ± 0.45	1.28 ± 0.21
As	D. G.	49.32 ± 0.33	75.46 ± 7.87	23.45 ± 8.42
	G.	52.15 ± 4.62	82.39 ± 10.45	30.03 ± 7.15
	Ft.	23.53 ± 3.48	43.13 ± 1.89	11.32 ± 3.47
	Am.	12.91 ± 2.61	30.72 ± 8.40	5.29 ± 1.65
	Sp.	33.47 ± 0.19	48.64 ± 6.43	21.80 ± 1.58
Se	D. G.	3.45 ± 0.20	5.07 ± 1.01	3.14 ± 1.84
	G.	5.64 ± 0.94	7.96 ± 0.89	5.23 ± 2.22
	Ft.	1.29 ± 0.07	2.20 ± 0.82	1.40 ± 0.19
	Am.	1.22 ± 0.30	1.86 ± 0.59	1.21 ± 0.13
	Sp.	1.62 ± 0.04	2.20 ± 0.14	1.75 ± 0.3
Zn	D. G.	102.60 ± 7.07	97.86 ± 4.57	65.17 ± 9.03
	G.	103.69 ± 6.48	100.35 ± 5.89	98.89 ± 1.40
	Ft.	76.79 ± 1.50	88.93 ± 7.88	68.17 ± 9.58
	Am.	57.72 ± 2.60	68.20 ± 7.79	51.17 ± 8.39
	Sp.	73.80 ± 2.50	73.12 ± 0.81	79.72 ± 22.61

Table 2-9. Concentrations of trace metals (μg/L) in the clams of the different clam farms for month 7, 8, 12 and 1 at 2004-2005.

Trace metal	Clam tissues	Site		
		Jinhae	Sacheon	Goheung
Cu	D. G.	9.34 ± 1.12	12.26 ± 2.09	6.64 ± 3.03
	G.	9.43 ± 2.97	12.26 ± 2.63	7.90 ± 2.23
	Ft.	5.09 ± 2.46	9.30 ± 2.69	4.84 ± 1.95
	Am.	4.09 ± 1.74	7.13 ± 3.63	2.86 ± 1.26
	Sp.	7.39 ± 1.27	12.13 ± 2.52	6.05 ± 1.37
Cd	D. G.	2.26 ± 0.95	2.45 ± 1.06	1.75 ± 0.95
	G.	1.63 ± 0.56	1.95 ± 0.60	2.93 ± 0.58
	Ft.	0.12 ± 0.04	0.23 ± 0.11	0.26 ± 0.10
	Am.	0.22 ± 0.08	0.22 ± 0.14	0.24 ± 0.08
	Sp.	0.33 ± 0.01	0.44 ± 0.08	0.53 ± 0.14
Pb	D. G.	2.14 ± 0.89	1.83 ± 1.62	0.73 ± 0.68
	G.	1.57 ± 0.65	1.19 ± 0.96	1.00 ± 0.42
	Ft.	0.33 ± 0.15	0.50 ± 0.39	0.43 ± 0.37
	Am.	0.36 ± 0.20	0.59 ± 0.43	0.35 ± 0.39
	Sp.	0.34 ± 0.07	0.42 ± 0.46	0.29 ± 0.28
Cr	D. G.	1.56 ± 0.86	1.78 ± 0.68	1.08 ± 0.68
	G.	2.18 ± 1.80	2.00 ± 0.86	1.44 ± 0.53
	Ft.	1.83 ± 1.86	1.25 ± 0.99	0.94 ± 0.61
	Am.	1.81 ± 1.44	2.14 ± 0.74	1.18 ± 0.49
	Sp.	1.79 ± 1.24	1.32 ± 0.49	1.16 ± 0.22
As	D. G.	37.64 ± 13.51	61.40 ± 19.05	21.93 ± 6.34
	G.	48.50 ± 5.01	73.40 ± 12.91	32.33 ± 5.06
	Ft.	20.09 ± 5.06	36.09 ± 8.27	11.35 ± 2.04
	Am.	11.17 ± 2.70	25.54 ± 7.94	6.03 ± 1.29
	Sp.	30.10 ± 3.90	43.82 ± 8.20	20.13 ± 3.11
Se	D. G.	2.83 ± 0.78	4.10 ± 1.32	2.75 ± 1.15
	G.	4.88 ± 1.09	7.62 ± 1.89	5.59 ± 1.74
	Ft.	0.92 ± 0.43	2.35 ± 1.31	1.47 ± 0.42
	Am.	0.86 ± 0.45	2.01 ± 1.10	1.36 ± 0.37
	Sp.	1.43 ± 0.23	2.10 ± 0.59	1.65 ± 0.20
Zn	D. G.	91.15 ± 13.84	87.25 ± 14.44	60.91 ± 10.14
	G.	114.24 ± 13.65	105.65 ± 7.01	106.31 ± 16.12
	Ft.	75.98 ± 8.62	85.58 ± 8.07	69.52 ± 7.45
	Am.	58.53 ± 5.60	71.22 ± 5.71	55.10 ± 6.69
	Sp.	76.81 ± 3.76	79.58 ± 15.44	73.00 ± 15.74

2.3.3. Correlation analysis of trace metals in seawater, sediment and clam

There is a significant correlation ($p < 0.05$) for concentrations of trace metals in clam tissues relative to their concentrations in seawater (Table 2-10). The correlation coefficient obtained for Zn was positive in Sacheon clam (siphon). Cd and Se in seawater correlated negatively with foot and digestive gland in clam, respectively, at Jinhae. Inverse correlations were also found Cd and adduct muscle in clam at Goheung. Out of 105 correlations calculated, only 4 were significant (3.8%).

The correlation coefficients between trace metals contents of clam tissues and farm sediment are listed in Table 2-11. The most significant correlations were demonstrated for clam tissues (gill, foot, adduct muscle) of trace metals (Pb, As and Se) in sediment at Jinhae. It is positively correlated with the sediment values, which were out of 35 correlations, 4 were significant (11.4%) only at Jinhae region. Other regions were no found correlations coefficients.

Table 2-10. Correlation of trace metals in the tissues of clam and seawater in different clam farms. (* = $p < 0.05$) are denoted in bold face.

Site	Clam tissues	Trace metal						
		Cu	**Cd**	**Pb**	**Cr**	**As**	**Se**	**Zn**
Jinhae	D.G.	0.861	-0.877	-0.330	-0.018	-0.342	**-0.963***	0.104
	G.	0.639	0.568	-0.692	-0.092	0.130	-0.843	-0.356
	Ft.	0.531	**-0.968***	0.797	-0.127	0.258	-0.834	-0.691
	Am.	0.678	0.883	0.705	-0.306	0.338	-0.799	-0.523
	Sp.	0.780	0.039	0.903	0.034	-0.308	-0.949	0.333
Sacheon	D.G.	-0.130	0.119	0.528	-0.500	-0.493	0.849	-0.184
	G.	0.399	0.096	0.526	-0.489	-0.500	0.343	0.429
	Ft.	-0.203	-0.704	0.783	-0.293	-0.787	-0.369	-0.904
	Am.	0.068	-0.442	0.654	-0.496	-0.780	-0.346	0.199
	Sp.	0.894	-0.642	0.717	-0.806	-0.285	0.222	**0.965***
Goheung	D.G.	0.388	0.308	0.307	0.643	0.880	0.166	0.498
	G.	0.816	-0.925	0.673	0.328	0.681	0.854	0.069
	Ft.	0.331	-0.404	0.612	0.658	0.929	0.743	0.649
	Am.	0.480	**-0.952***	0.727	0.131	0.773	0.856	0.626
	Sp.	0.164	-0.030	0.718	0.700	-0.019	-0.040	-0.788

Table 2-11. Correlation of trace metals in the tissues of clam and sediment in different clam farms. (* = $p < 0.05$) are denoted in bold face.

Site	Clam tissues	Trace metal						
		Cu	**Cd**	**Pb**	**Cr**	**As**	**Se**	**Zn**
Jinhae	D.G.	0.858	-0.774	-0.879	-0.380	0.694	0.905	-0.872
	G.	0.897	0.244	-0.226	-0.580	**0.958***	**0.969***	0.671
	Ft.	0.808	-0.724	**0.954***	-0.526	0.925	0.835	-0.513
	Am.	0.654	0.944	0.847	-0.757	**0.982***	0.638	-0.254
	Sp.	0.410	0.250	0.565	-0.491	0.711	0.819	0.817
Sacheon	D.G.	-0.161	0.924	-0.560	0.028	-0.485	-0.048	-0.046
	G.	0.578	0.744	-0.191	-0.919	-0.422	-0.460	0.531
	Ft.	-0.044	-0.665	0.073	-0.909	-0.843	-0.840	0.722
	Am.	0.436	-0.824	0.458	-0.694	-0.923	-0.807	0.806
	Sp.	0.391	0.642	-0.096	-0.735	-0.231	-0.575	-0.229
Goheung	D.G.	0.936	0.868	0.131	-0.443	0.562	-0.325	0.518
	G.	0.769	-0.187	0.148	-0.762	-0.555	-0.672	-0.598
	Ft.	0.720	0.788	-0.074	-0.482	0.106	-0.320	0.712
	Am.	0.667	0.316	-0.382	-0.891	-0.536	-0.473	0.400
	Sp.	0.775	0.777	-0.377	-0.381	0.909	0.487	0.022

2.3.4. Value of biochemical contents in the tissues of clam

At Jinhae clam, the responses were greater in the gill than in digestive glands and were similar MDA contents. The contents of SOD, GSH, MDA in the tissues at Sacheon and Goheung clam were greater in the gill than in digestive glands. In contrast, the contents of GR, GPx, GST in the digestive glands were higher than in gill (Table 2-12, 2-13, 2-14).

In digestive gland, SOD, GR, GST values were similar at all sampling site, but that had been little in contents at Goheung clam. GPx values were highest contents (427.88 ± 93.68) at Jinhae clam, other site contents means (289.71 ± 46.33, 254.98 ± 13.54) were similar. At Sacheon, the values of GSH were higher (1306.96 ± 346.53) than the contents of Jinhae (1053.25 ± 313.84) and Goheung (745.62 ± 170.75). MDA values were highest in clam at Jinhae (13.07 ± 5.24) and in the order Sacheon (10.20 ± 2.73) and Goheung (7.77 ± 1.94). In gill, SOD values were similar at all clam farms and then were low contents at Goheung clam. GR, GPx, GST values were far the higher contents of Jinhae clam (54.61 ± 21.74, 539.60 ± 183.16, 188.88 ± 125.63, respectively). In case of GSH values, Sacheon clam contents were highest than other site clam. MDA values were lowest contents at Goheung clam.

SOD values were higher in the gill than in the digestive glands at all sites the difference in up to approximately 2, 3 fold. GR, GST, MDA values were higher in the digestive glands than in the gill at Sacheon and Goheung clam in up to approximately 1.5 fold, except Jinhae clam. GPx values were higher in the gill than in the digestive glands at Jinhae clam in up to little, but other site was similar. GSH contents were always higher in the gill than in the digestive glands in all three sampling sites and the difference in up to approximately 2-fold.

Table 2-12. Values of the biochemical in the clam for summer in different clam farms. SOD (Superoxide dismutase, U/min/mg protein), GR (Glutathione reductas, nmol/min/mg protein), GPx (Glutathione peroxidase, nmol/min/mg protein), GST (Glutathione-S-transferase, nmol/min/mg protein), GSH (Reduced glutathione, μM/g protein), MDA (malondialdehyde, μM/mg protein)

	Jinhae		Sacheon		Goheung	
	D.G.	G.	D.G.	G.	D.G.	G.
SOD	40.04 ± 0.19	123.30 ± 7.54	45.49 ± 1.62	99.35 ± 35.37	42.55 ± 3.52	102.06 ± 23.29
GR	44.14 ± 29.86	56.69 ± 30.32	55.61 ± 38.54	33.52 ± 6.86	40.16 ± 31.83	28.42 ± 20.83
GPx	338.46 ± 32.78	402.60 ± 10.58	263.70 ± 30.30	342.34 ± 14.01	250.24 ± 20.93	223.55 ± 4.01
GST	147.70 ± 12.80	119.14 ± 28.68	119.47 ± 0.43	89.80 ± 10.52	127.08 ± 38.87	114.60 ± 35.10
GSH	1096.06 ± 179.27	2356.03 ± 244.75	1495.72 ± 334.36	2657.76 ± 768.56	880.59 ± 74.28	2162.36 ± 194.65
MDA	10.43 ± 3.97	12.62 ± 0.86	8.70 ± 1.20	9.24 ± 1.00	10.33 ± 3.51	8.67 ± 0.79

Table 2-13. Values of the biochemical in the clam for winter in different clam farms.

	Jinhae		Sacheon		Goheung	
	D.G.	G.	D.G.	G.	D.G.	G.
SOD	64.99 ± 21.78	155.99 ± 5.14	59.09 ± 4.17	165.58 ± 22.94	51.70 ± 8.16	155.33 ± 9.90
GR	59.27 ± 33.44	52.34 ± 2.67	36.74 ± 9.85	29.20 ± 6.06	47.78 ± 12.36	27.22 ± 3.59
GPx	455.85 ± 54.13	616.28 ± 74.72	313.43 ± 30.29	253.34 ± 5.10	259.73 ± 4.66	246.06 20.33
GST	201.11 ± 13.03	233.90 ± 139.31	213.81 ± 87.44	115.87 ± 3.96	174.71 ± 50.36	75.21 ± 14.80
GSH	1010.44 ± 505.99	2127.94 ± 525.65	1118.20 ± 325.48	2166.39 ± 145.73	610.65 ± 95.14	1345.60 ± 396.42
MDA	15.80 ± 3.20	10.98 ± 0.54	12.12 ± 1.52	9.63 ± 0.37	7.73 ± 2.75	8.49 ± 0.01

Table 2-14. Values of the biochemical in the clam for month 7, 8, 12 and 1 at 2004-2005 in different clam farms.

	Jinhae		Sacheon		Goheung	
	D.G.	G.	D.G.	G.	D.G.	G.
SOD	52.52 ± 19.12	135.41 ± 21.64	52.29 ± 8.27	139.09 ± 43.49	48.04 ± 7.84	128.69 ± 34.05
GR	47.19 ± 31.58	54.61 ± 21.74	46.18 ± 25.42	30.93 ± 5.98	43.97 ± 20.20	27.70 ± 10.74
GPx	427.88 ± 93.68	539.60 ± 183.16	289.71 ± 46.33	279.70 ± 45.81	254.98 ± 13.54	235.85 ± 21.48
GST	162.43 ± 27.07	188.88 ± 125.63	166.64 ± 74.26	109.66 ± 11.11	150.90 ± 45.88	104.96 ± 29.92
GSH	1053.25 ± 313.84	2241.99 ± 359.73	1306.96 ± 346.53	2528.32 ± 587.89	745.62 ± 170.75	1753.98 ± 536.07
MDA	13.07 ± 5.24	11.80 ± 1.12	10.20 ± 2.73	9.43 ± 0.65	7.77 ± 1.94	8.58 ± 0.46

2.4. Discussion

The metals considered toxic and which are of concern have been restricted largely, but not exclusively, to the ten which appear to be most poisonous to marine life. These include, in order of decreasing toxicity (Davies, 1978): mercury, cadmium, silver, nickel, selenium, lead, copper, chromium, arsenic and zinc. Goldberg (1995) reviewed different sources of trace metal inputs into the sea and their possible role in ecosystems. Trace metals are non-degradable elements naturally occurring in coastal seas. They are not particularly toxic as the condensed free elements but they are dangerous to living organisms in the form of cations with capacity to bind with short carbon chains. In this form, they were bioaccumulated in marine organisms and concentrated year after year. Our results support the hypothesis that the coastal area of the Korean south has significant basal contamination levels, which, however, do not reach those of clearly contaminated areas. In any case, the expression “background levels” must be used with some caution, as its meaning differs according to the local geochemical or hydrodynamic conditions of the body of water under examination. Our work shows the importance of using several possible biomonitors when studying the quality of ecosystems, such as bivalve mollusks (filtering organisms). Each biomonitor responds to a particular metal fraction of the water body: clams to the fraction present in particulates, particle matter in sediment to metals in solution.

When trace metal levels from different areas are compared, two regions (Jinhae and Sacheon) musts are quite wary. The highest concentrations of metals were found in the Jinhae’ clam sediments where intensely industrialized compared to Sacheon and Goheung. Sediments are preferred to water as environmental matrix for both chemical and biological monitoring because pollutant concentrations in sediments are much higher and less variable in time and space (e. g. Beiras *et al*., 2002). A comparison with our sampling regions, unpolluted sediments of this system (Ruiz *et al*., 1998) shows that the concentration of trace metals may have the following provenance: industrial discharges (70-80%), acid-mine drainage (20-

30%) and minor contributions from urban effluents. The results indicated that the accumulation of trace metals is predominant in sediments than in mollusks. This can be interpreted as sediments act as reservoir for all the contaminants and dead organic matter descending from the ecosystem above. As well as from solution, marine organisms have the potential to take up metals that are adsorbed onto inorganic particles and absorbed onto organic matter. Digestion following ingestion of suspended particles will release at least some of the metals contained within them. The metals are then available for absorption across the wall of the alimentary tract. There is potential for different organisms to respond to different sources, correlating with different feeding types and prey taken, and it is difficult to ascertain the relative importance of each of the major routes of uptake.

Of the metals analyzed, Cd is the least abundant, geologically, in fine sediments, and hence concentrations are comparatively low. Nevertheless, Cd-enrichment in Jinhae sediments is evident when compared with those of Sacheon and Goheung. The source of any anthropogenic Cd at Jinhae is probably industry close to the lagoon. Bearing in mind the filter-feeding habit of clam, it is likely that body tissues a contribution from dissolved Cd, as well as easily solubilised particulate forms. Indeed, Bryan and Langston (1992) have suggested that Cd solubilised from sediment, rather than the solid-phase itself, may be the main source of Cd to a number of benthic organisms. The prediction of precise contributions from sediment will depend on site-specific conditions that influence partitioning. Thus, following exposure of another filter-feeding mollusc *Mercenaria mercenaria* to an organic-rich, heavily Cd-contaminated sediment from New Port Harbor (5-38 μg Cd/g), Rubinstein *et al*. (1983) were unable to detect any Cd uptake, which they attributed to high levels of sulphide complexation. The high sedimentary Pb content may be due to the precipitation of decomposed organic matter. The present concentration of Pb in sediment of the Jinhae clam-farm was higher than other farms. Bidet *et al*. (1997) had both previously attributed low Pb concentration to the fact that Pb is relatively rare in the neighboring soils. Apparent enhancement of sediment-bound Pb may therefore be linked to recent human factors such as: (1)

development of a motorway and increased traffic skirting the lagoon, (2) extension of modern intensive agriculture, using heavy machinery, and (3) an increase in the number of motorized fishing boats. The impact of all these factors is likely to be magnified by the capacity of fine sediments to retain contaminants, coupled with the sheltered depositional habitat of lagoon systems. Zn is the element that presents the highest concentrations in digestive glands of clam. Walker *et al*. (1975) identified metal-rich granules around the mid-gut of *Balanus balanoides* and showed that this species has the ability to accumulate large quantities of Zn. A high level of Zn in comparison with other elements is typical in other living organisms habitually used as pollution biomonitors (Castro *et al*., 1999; Jeng *et al*., 2000; Wong *et al*., 2000). Mean Zn concentrations in clam ranged from 55.10 μg/g (adduct muscle) to 114.24 μg/g, in all sampling regions.

Chemical constituents bound to suspended particles impact on biological systems through their chemical or biological solubilization and consequent entry into the food chain. Solubilization of meterial in the water column, via chemical or microbiological processes, increases the mobility and general bioavailability of the chemical constituent, while solubilization of material in the gastro-intestinal environment of organisms affords a more direct route for chemical uptake by suspension and deposit feeders. Thus, phase partitioning (or the distribution coefficient) and speciation and their dependence on environmental parameters critically affect the biological pathways of chemicals in the aquatic environment (Wang *et al*., 1999; Roditi *et al*., 2000). The following discussion centers on the role of suspension-feeding bivalve molluscs (clams, mussels, oysters) in utilizing and modifying suspended sediments and associated chemicals in estuaries and coastal waters. Because of their high rates of water filtration and particle processing, such organisms are particularly significant in the transformation of the physical and chemical properties of suspended particles and to the translocation of this material to the substratum (Jaramillo *et al*., 1992; Allison *et al*., 1998). Moreover, dense populations have been reported to exert the principal control on

phytoplankton biomass under certain conditions (Cloern, 1982; Carlton *et al*., 1990).

Correlation coefficients showed that clam may represent useful bioindicators of metal-polluted seawater and sediments. In contrast, the deposit- and filter-feeder *Viviparus* sp. showed several significantly positive correlations with the suspended matter concentrations or filtrate contents. Significantly positive correlations demonstrate that fluctuating metal contents of the regarded species are closely related to the fluctuations of the environmental compartments. Two conclusions may be drawn: (1) animals reflect the metal contamination of sampling sites; and (2) positive correlation between body concentrations and particles of a particular grain size indicate a preferential uptake of that grain size, thus implying a relationship between feeding behaviors and metal bioaccumulations (Gundacker, 2000). Coefficients close to zero or negative were found several trace metals, which indicate that the amount in the seawater and sediment is not directly reflected in the tissues of clam. This can be explained if we consider that concentrations of trace metals are low in sediments, probably lower than the threshold below which these organisms are able to regulate the accumulation of metals in their bodies (Usero *et al*., 2005). No correlation was found between trace metal concentrations in clam tissues and ambient compartments (seawater and sediment) and it was concluded that the use of clam as biological indicators will be of greater assistance in detecting chronic pollution. It was reported that *R. philippinarum* could accumulate more trace metals in digestive glands and gill than the remaining tissue. Metal sub-cellular partitioning clearly differs between the gills and the digestive glands. In feeding, bivalves pass a large amount of water over modified gill and ingest potentially metal-rich particles. They have a high potential, therefore, to accumulate metals. Clam, in particular, accumulated high concentrations of copper, arsenic and zinc. Overall, these results suggest than the gills may be more sensitive to metal-induced oxidative stress than the digestive gland. In a biomonitoring context, the gill would thus be more responsive to the external exposure gradient than would the digestive glands.

Chapter 3

Accumulation of cadmium in the clam

3.1. Introduction

Aquatic systems are contaminated by different pollutants, including metal, as a result of man's activities. Metal contamination in coastal waters has received increasing attention due to their potential bioaccumulation in and toxicity to many aquatic organisms. Cadmium (Cd) is a metal that is toxic to living organisms and is widely distributed in the marine environment. It presents a serious hazard to public health and is a threat to most life forms (Breakman *et al*., 1997). Cd concentrations in suspension-feeder invertebrates, and especially in clams, increase with the time of exposure (Bebianno and Langston, 1998). Experimental studies have proved that Cd can cause various deleterious biochemical alterations in mollusks tissues such as decreased activities of some enzymes (Evtushenko *et al*., 1986), enhanced lipid peroxidation (Chelomin and Belcheva, 1992).

Bivalve molluscs are known to accumulate high concentrations of trace metals in their tissue and are widely used as bioindicators for pollution in marine environments (Regoli and Orlando, 1994; Geret *et al*., 2003). Bivalve molluscs are filter-feeding organisms which, consequently, may be exposed to large amounts of chemical pollutants even if these are present in fairly dilute concentration. They are also capable of bioconcentrating xenobiotics to many thousands times background which can facilitate chemical analysis (Sheehan D, *et al*., 1995). Manila clam *Ruditapes philippinarum* was proposed as suitable biomonitors of metal contamination because of their wide distribution throughout in Korean costal regions.

Marine animals can bioaccumulate metals from seawater, suspended particles, sediment and through food chains (Luoma, 1983). Elimination may occur as a result of excretion through a permeable membrane, *e.g*. kidney or gill membranes,

the exocytosis of metal rich material, such as granules, into the digestive tract, or adsorption from the whole body (Langston *et al*., 1998). In many organisms the body metal concentration is correlated with tissue weight. Such correlations have been reported upon for many animals, for example, bivalves (Wang and Fisher, 1997) and barnacles (Phillips and Rainbow, 1988). Growth rates vary between individuals and species with consequent effects on body metal concentration (Phillips and Rainbow, 1993).

Antioxidant systems have been studied for some years in fish and bivalves exposed experimentally to chemicals or collected from polluted areas (Di Giulio *et al*., 1989; Winston and Di Giulio, 1991; Stegeman *et al*., 1992). The usefulness of bioindicators is greatly increased when chemical analyses are integrated with data on the biological effects of pollutants (Bayne *et al*., 1988). In this respect, oxidative stress is a common pathway of toxicity induced by several classed of pollutants (Winston and Di Giulio, 1991) by which production of reactive oxygen species is enhanced. Protection against toxicity of oxyradicals toward cellular targets is afforded by a complex defense system consisting of both low-molecular-weight scavengers and antioxidant enzymes. Variations of antioxidant defenses, such as the content of glutathione (one of the most important antioxidant agents), the activity of glutathione-dependent, and antioxidant enzymes, have often been proposed as biomarkers of contaminant-mediated oxidative stress in several marine organisms (Di Giulio *et al*., 1989; Livingstone 1993; Winston and Di Giulio, 1991). The gills and digestive glands are also the main target organs for several pollutants, these tissues were chosen to compare the different effect of metal concentration. The same organs were also used for investigating the principal antioxidant defenses of the bivalves. These included the concentration of total glutathione and the activity of several glutathione-dependent and antioxidant enzymes. Therefore, the aims of present study were to investigate Cd accumulation and elimination in clam of sub-chronic water-born Cd exposure, and that were to evaluated effect of Cd exposure on the activities of protective antioxidant enzymes in the gill and

digestive glands of the manila clam, *R. pillippinarum* in order to explain the cytotoxic and tissue damaging effect of Cd exposure.

3.2. Material and Methods

3.2.1. Clam conditions

Manila clams, *R. philippinarum* were collected in July 2005 a clam farm in Go-heung country Jeon-nam, Korea. The clams were acclimatized for 5days in semi-static system. After acclimatization, clam (shell length: 35.81 ± 2.51 mm, body weight: 10.40 ± 2.16 g) were selected for the experiments. Fifty clams were separated to 50 L control and test tanks.

3.2.2. Exposure and depuration system

The animals in the test tank were exposed to the different concentrations from 10, 20, 100 and 200 ㎍/L of cadmium sulfate ($CdCl_2$, Sigma Chemical, and St. Louis, MO) under sub-lethal concentration for 2 weeks. After that was transferred clean sea-water for 1 week. Sea-water was changed every 48 hours. Animals were held healthy under the laboratory condition in 12:12h light/dark cycle for further studies. Seawater quality was measured every 1 week during the experiment periods (Table 3-1). Four clams were sampled every 1 week for 3 weeks from each group for chemistries determined. The condition index (CI) was calculated (CI = fresh flesh weight (g) × 100/shell weight (g), derived from Duquesne *et al* (2004).

Table 3-1. The chemical components of seawater and experimental condition used in the experiments

Item	Value
Temperature(℃)	22.0±1
pH	8.14±0.7
Salinity(‰)	33.5±0.8
Dissolved oxygen (mg L^{-1})	7.2±0.2
COD (μg L^{-1})	1.14±0.1
Ammonia (μg L^{-1})	12.52±0.8
Nitrite (μg L^{-1})	1.28±0.2
Nitrate (μg L^{-1})	11.55±1.1
Cadmium	Not detected

3.2.3. Cadmium analysis

The gill, digestive glands, residue tissues were sampled every 1 week for analysis of metal concentration. Four clams were removed each test concentration and control. Tissue samples were dried at 65℃ and kept in desiccators until digestion. Dry tissue was digested with 1:1 HNO_3 (Suprapur grade, Merck, Germany) and samples were fumed to near dryness on a hot plate at 120℃ for overnight. After digestion, the residue was dissolved in 20 mL of 0.2N HNO_3 and kept in a refrigerator until analysis for trace metal. Cd concentration of tissue was measured using an ICP-MS (Elan 6000, Perkin-elmer). Cd concentration in the tissues of clam was expressed as μg/g dry wt.

3.2.4. Biochemical analysis

Eight clams were removed from each tank every week during the 3 week of the experiment. Weight and total length were recorded for each individual. Four clams were dissected gill and digestive glands and then weighted. Tissues were homogenized in 0.5 M sucrose and 0.15 M NaCl in 0.02 M Tris-HCl (pH 7.6). The

homogenates were centrifuged at 500g for 15 min at 4℃ and the resulting supernatant at 12,000×g for 30 min at 4℃. Antioxidant enzyme activities in the tissues were measured with a temperature-controlled spectrophotometer (DR/4000U, Germany). The assays were run in duplicate or triplicate. Polyunsaturated fatty acid peroxides generate malondialdehyde (MDA) activities were measured pre-centrifuged homogenated tissues according to John and Steven (1978) method. The post-centrifuged supernatants were measured Superoxide dismutase (SOD), Glutathione reductase (GR), Glutathione-S-transferase (GST), Glutathione peroxidase (GPx). SOD activity was evaluated with the xanthine oxidase-cytochrome c method as described by Flohe and Otting (1984), GR was Carlberg (1985), GPx was Paglia and Valentine (1967), and GST was Habig and Jacoby (1981a, b). Reduced glutathione (GSH) activity measured the other four clams. Tissues were dissected, homogenized 10% $HClO_4$, and followed centrifuged 5000g for 15 min. The resulting supernatants measured GSH determination. GSH content was determinate according to Richardson and Murphy (1975). The reaction mixture contained 0.01M 5, 5'-dithiobis, 2-nitrobenzoix acid (DTNB), 0.1M PBS buffer (pH 8.0). GSH standard curve performed 10nM reduced glutathione standard solution. The linear increase in absorbance was recorded at 412 nm. Total protein concentrations according to Bradford (1976) by using bovine serum albumin (BSA) as standard.

3.2.5. Statistical analysis

Data are expressed as mean ± standard error (S.E.). All statistical analysis was performed using SPSS/Pc^+ statistical package. Prior to analysis, all data were tested for homogeneity of variances among groups using the Barttlett test. Comparisons in normalized data between control and treatments group were made by one-way analysis of variance (ANOVA) followed by Duncan's multiple comparisons test of mean values if significant differences were found ($P < 0.05$).

3.3. Results

3.3.1. Condition index

Exposure to Cd induced significant in Cd concentration in the clam. The CI (Condition Index) not significantly in response to increased to Cd exposure (Figure 3-1).

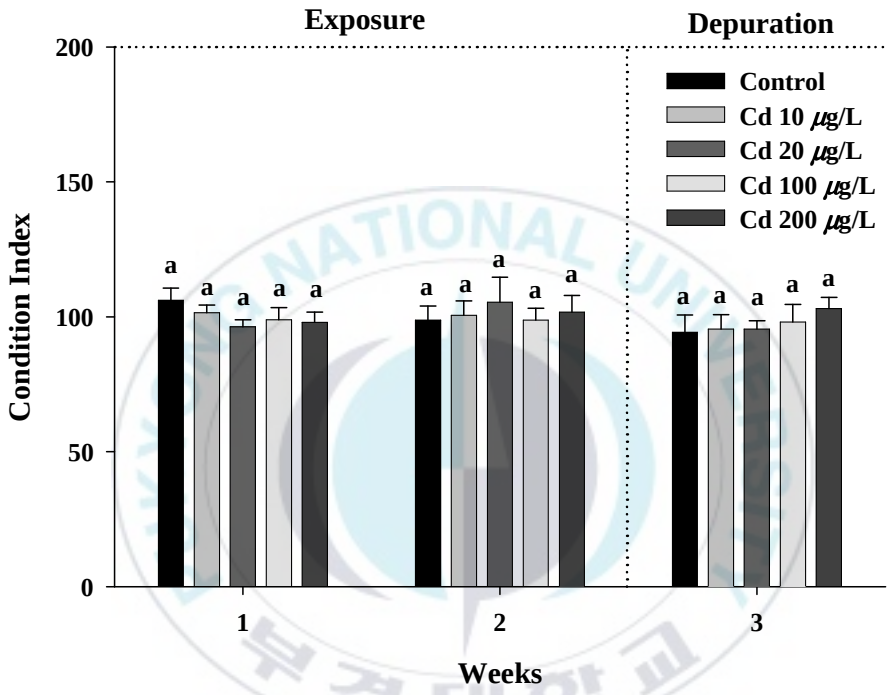

Figure 3-1. Changes of condition index (CI). Clam, *R. philippinarum* exposed to 10, 20, 100, 200 μg/L Cd for 3 weeks. Vertical bar presented standard error (Mean ± S.E., n=12).

3.3.2. Cadmium accumulation

The mean levels of total Cd accumulated in the digestive glands, gill, residue tissues of *R. pillippinarum* exposed to 10, 20, 100, 200 μg/L Cd for 3 weeks are presented in figure 3-2. Cd accumulation in digestive glands was significantly increased for 2 week at 100 μg/L and 200 μg/L. After 1 week of exposure, Cd accumulation values were 9.10±3.60 μg/g and 14.22±3.53 μg/g and were approximately 18-fold and 30-fold higher than in the control at 100 μg/L and 200 μg/L–Cd exposures, respectively. After 2 week of exposure, Cd accumulation values were 16.45 ± 8.50 μg/g and 26.17 ± 5.49 μg/g and were approximately 8-fold and 12-fold higher than in the control at 100 μg/L and 200 μg/L–Cd exposures, respectively. No significant Cd accumulation occurred in the digestive glands exposed to at 10 μg/L and 20 μg/L. For gill, Cd accumulation was significantly increased for 2 week at 100 μg/L and 200 μg/L. During the first 1 week, Cd concentration values were 41.82 ± 26.99 μg/g and 88.93 ± 25.88 μg/g and were approximately 6-fold and 13-fold higher than in the control at 100 and 200 μg/L, respectively. At the end of the 2 week exposure, Cd concentration values were 103.21 ± 26.25 μg/g and 186.78 ± 106.08 μg/g and were approximately 17-fold and 32-fold higher than in the control at 100 μg/L and 200 μg/L–Cd exposures, respectively. No significant Cd accumulation occurred in the gill exposed to at 10 μg/L and 20 μg/L. Cd accumulation in residue tissues (adduct muscle, foot, siphon) were significantly increased with exposure periods and above 100 μg/L for 2 week. During 1 week of Cd exposure, Cd concentration values were 16.46 ± 8.86 μg/g and 18.14 ± 6.95 μg/g and were approximately 3-fold higher than in the control at 100 μg/L and 200 μg/L–Cd exposures, respectively. After 2 week of Cd exposure, Cd concentration values were 30.27 ± 7.36 μg/g and 44.36 ± 17.53 μg/g and were approximately 12-fold and 18-fold higher than in the control at 100 μg/ L and 200 μg/L–Cd exposures, respectively. No significant Cd accumulation occurred in residue tissues exposed to at 10 μg/L and 20 μg/L. After 2 week of Cd exposure, the order of Cd accumulation in organs was gill > digestive glands > residue tissues. The accumulation factors are presented for digestive glands, gill and residue tissues

at 10, 20, 100, 200 μg/L Cd exposure in figure 3-3. The accumulation factors were increased with the exposure period in digestive glands, gill and residue tissues. An inverse relationship was observed between the accumulation factor and the exposure concentrations at 1 week. Although the accumulation factor in over 100 μg/L increased with 2 week, it did not increase with exposure concentrations.

3.3.3. Cadmium elimination

Cd depuration in digestive glands, gill, other tissues of *R. pillippinarum* exposed to 10, 20, 100, 200 μg/L Cd for 3 weeks are presented in figure 3-2. During the depuration phase, Cd concentration in digestive glands, gill and residue tissues decreased slowly following at 100 μg/L, but increased at 200 μg/L the end of depuration periods. The elimination rates in digestive glands, gill and residue tissues at the end of depuration periods were 41.22%, 17.39% and 15.92% for 100 μg/L, respectively. Cd concentration values in digestive glands, gill and residue tissues were 35.24 ± 15.79 μg/g, 319.50 ± 87.61 μg/g and 51.28 ± 14.36 μg/g were approximately 95-fold, 66-fold and 27-fold higher than in the control at 200 μg/L–Cd exposure, respectively. The order of Cd elimination in organs during the depuration period was digestive glands > gill > residue tissues.

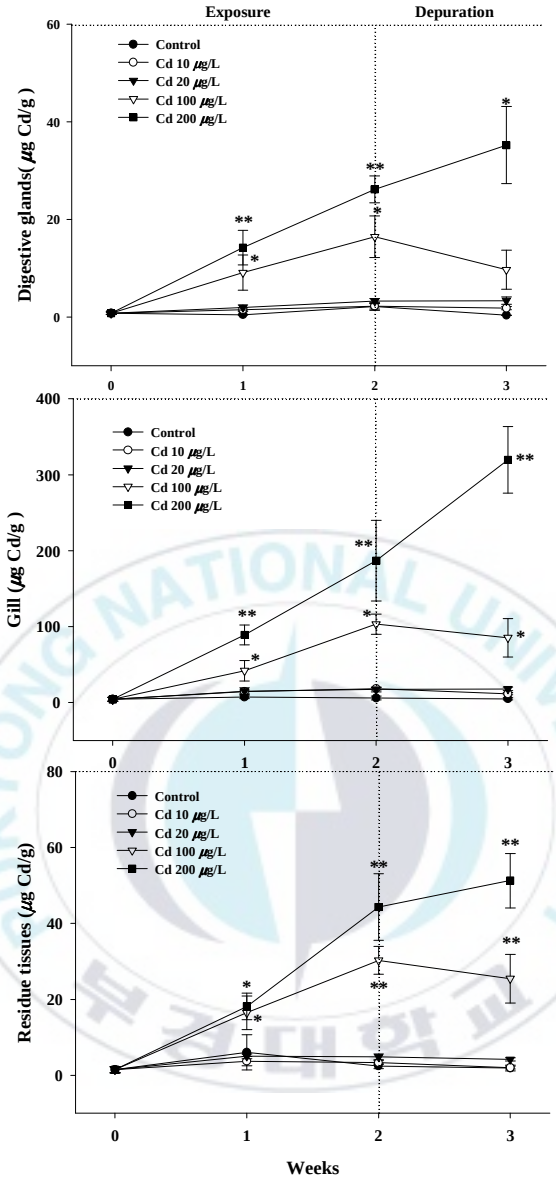

Figure 3-2. Accumulation and elimination of Cd in digestive glands, gill and residue tissues of clam. (* indicates significant difference from control value, $P<0.05$ as determined with Duncan post hoc test.)

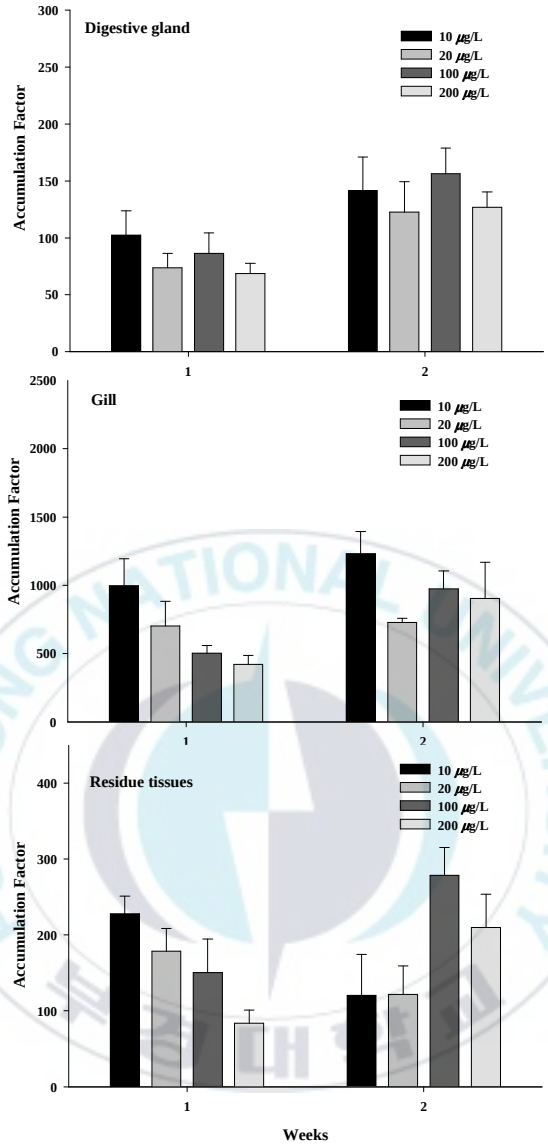

Figure 3-3. Determination of the accumulation factors (AF) in digestive glands, gill and residue tissues of clam *R. philippinarum*.

3.3.4. Value of biochemical contents

SOD activities in the digestive glands and gill of *R. pillippinarum* those exposed to Cd (control, 10, 20, 100, 200 μg/L) are presented as a function of exposure time and exposure concentrations are shown in Fig. 3-4. There were no significant differences in the digestive glands of the clam observed during the 2 week of exposure, and then after the end of Cd exposure, SOD activity in the digestive glands were no significant during the depuration period. SOD activities in the gill of the clam showed significantly increase at 200 μg/L Cd for 2 week of Cd exposure, but those were significantly reduced during depuration periods.

Change of GPx activity in the digestive gland observed significantly decrease at 200 μg/L Cd compared to control group during 2 weeks of Cd exposure, but it was recovery after 3 weeks in the 200 μg/L exposure group. GPx activities in the gill decreased significant difference over 100 μg/L Cd concentrations in 2 week, and then GPx activities levels are recovery at the 100 and 200 μg/L Cd exposure group during depuration period (Fig. 3-5).

Values of GR activities levels in the clam tissues are presented in Fig. 3-6. Although GR activities in the digestive glands showed a slight decrease with Cd concentration, the difference was not significantly different compared to control group at overall experiment periods. Gill GR activity in high exposure concentration (at 200 μg/L) was significantly elevated above GR activity in the control group at overall experiment periods.

Values of GST activities levels in clam tissues are presented in Fig. 3-7. GST activities in the digestive glands were significantly increased during 1 week at 200 μg/L Cd concentration. After that, GST activities at the 200 μg/L Cd were no significant during the 2 week exposure and depuration periods. At the 100 and 200 μg/L Cd exposure group, GST activities in the gill were significantly increased. After that, GST activity did not vary significant compared to control for 2, 3 week.

In digestive gland, GSH activities were showed similar patterns of GR (in the gill) activities during exposure periods, and it were significantly increased at 200 μg/L Cd exposure group compared to control group during the exposure and

depuration periods (Fig. 3-8). In gill, GSH activities were increased at the 200 μg/L Cd exposure group for 1 week, and then significantly increased at the 100 and 200 μg/L Cd concentrations in 2 week. After that, GSH activity decreased significantly at 200 μg/L Cd during the depuration period.

In digestive gland and gill, MDA level of Cd exposed to clams were significantly increased at the 200 μg/L Cd for 1 week, and then were no significant difference each Cd concentration in 2, 3 week (Fig. 3-9).

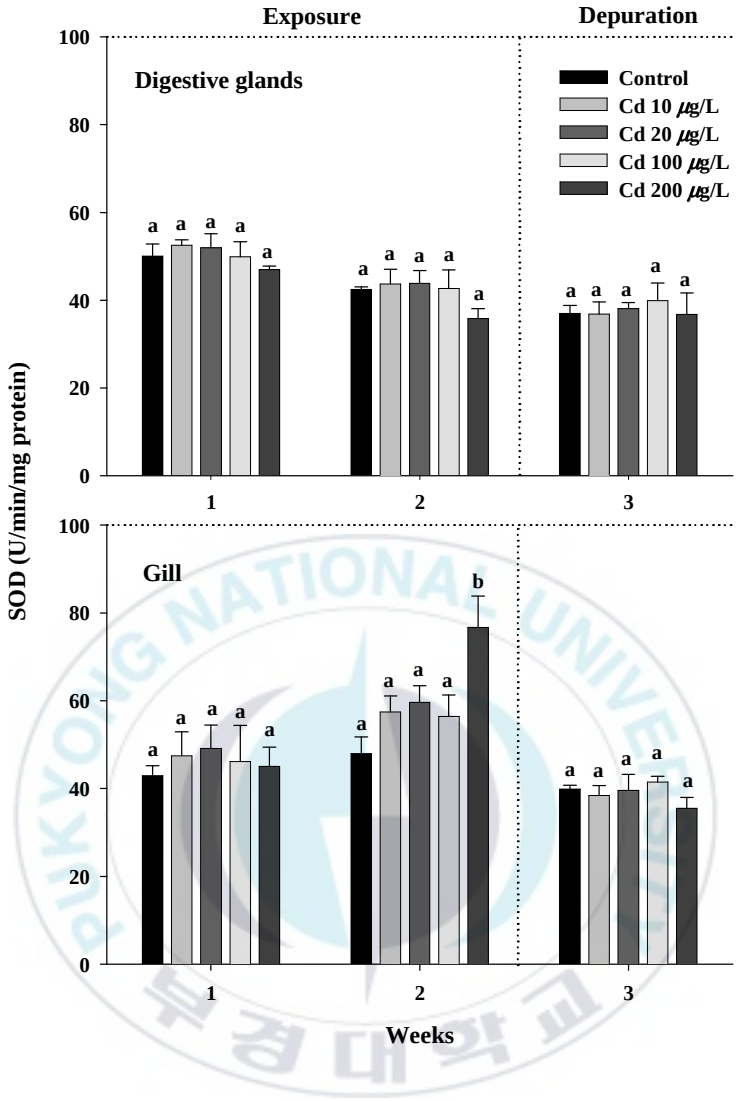

Figure 3-4. Variation of SOD activities in the digestive glands and gill of clam *R. philippinarum* depend upon the exposure of Cd. (^{b} indicates significant difference from control value, $P<0.05$ as determined with Duncan post hoc test.)

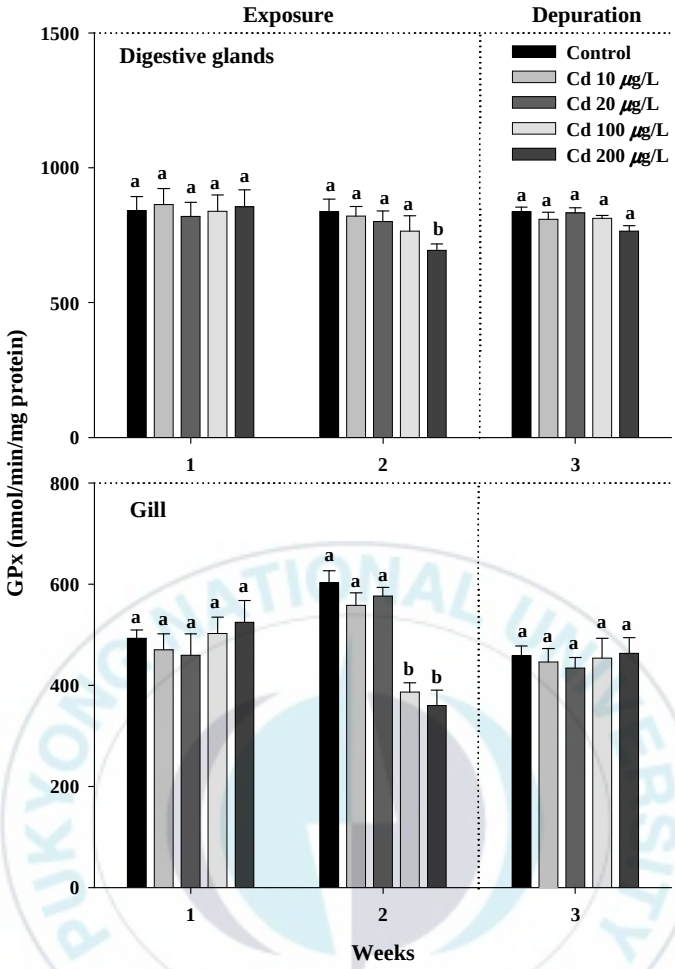

Figure 3-5. Variation of GPx activities in the digestive glands and gill of clam *R. philippinarum* depend upon the exposure of Cd. (^{b} indicates significant difference from control value, $P<0.05$ as determined with Duncan post hoc test.)

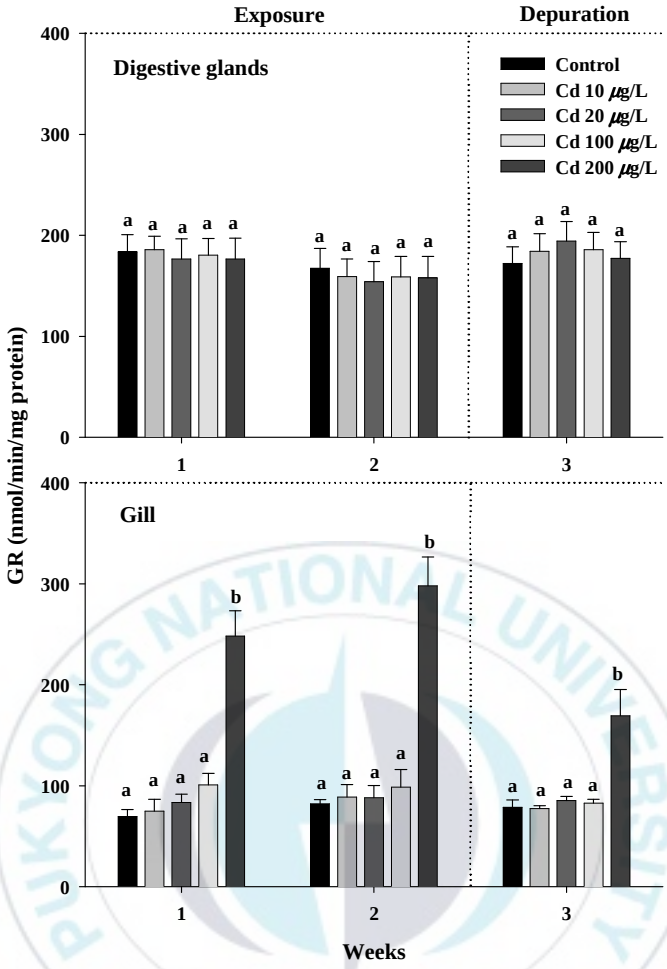

Figure 3-6. Variation of GR activities in the digestive glands and gill of clam *R. philippinarum* depend upon the exposure of Cd. (^{b} indicates significant difference from control value, $P<0.05$ as determined with Duncan post hoc test.)

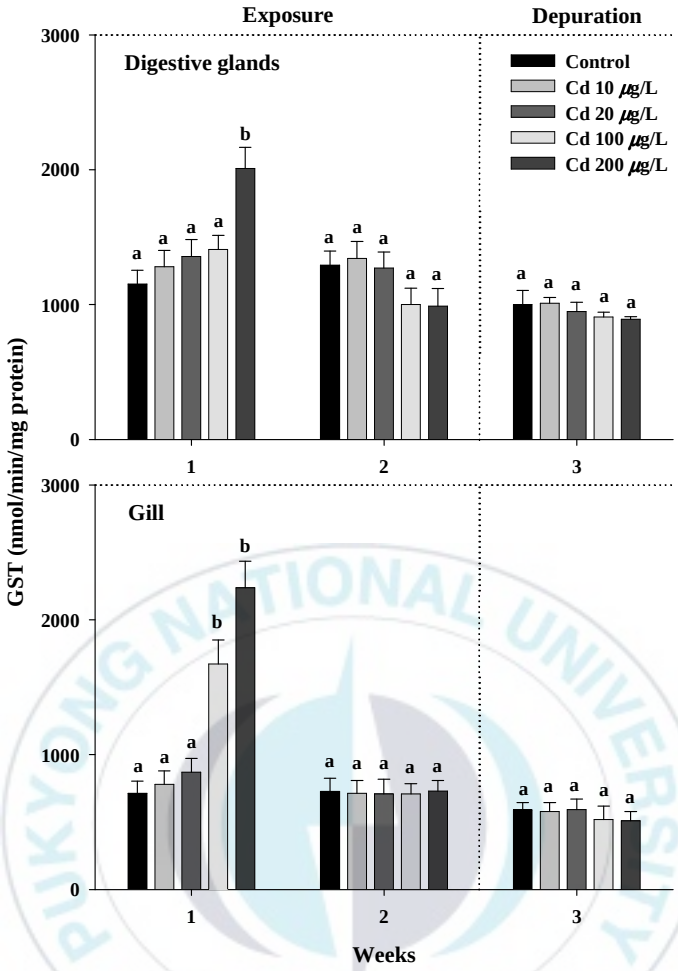

Figure 3-7. Variation of GST activities in the digestive glands and gill of clam *R. philippinarum* depend upon the exposure of Cd. (^{b} indicates significant difference from control value, $P<0.05$ as determined with Duncan post hoc test.)

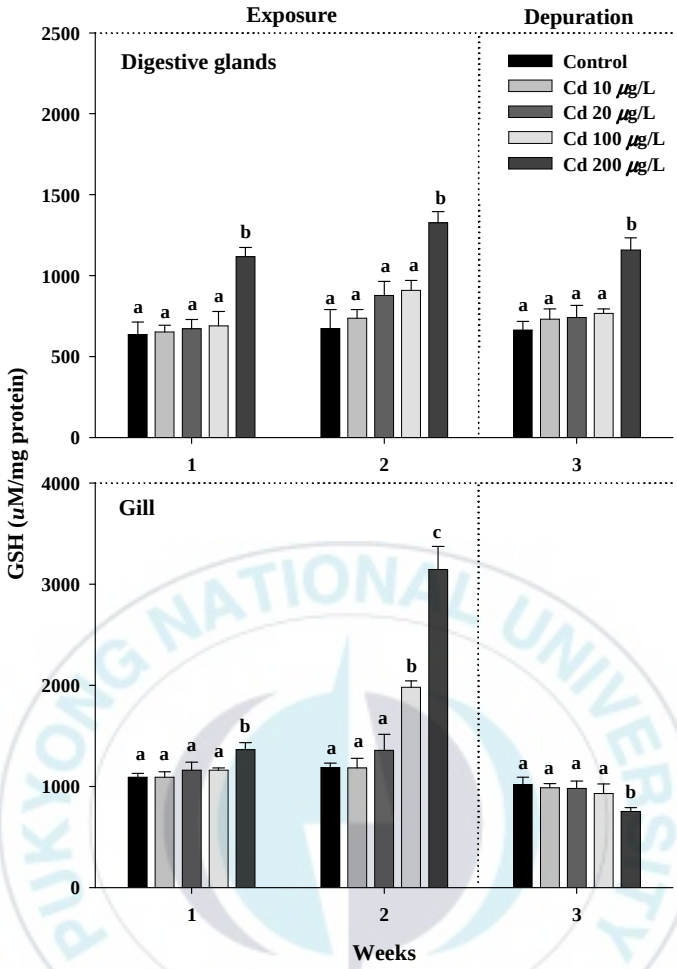

Figure 3-8. Variation of GSH activities in the digestive glands and gill of clam *R. philippinarum* depend upon the exposure of Cd. (^{b} indicates significant difference from control value, $P<0.05$ as determined with Duncan post hoc test.)

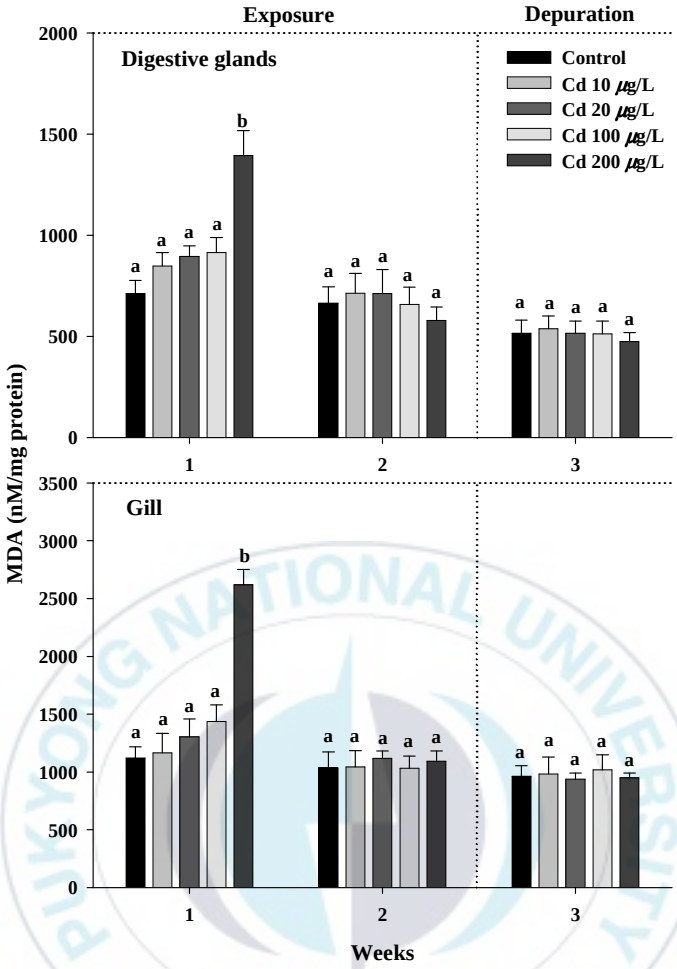

Figure 3-9. Variation of MDA activities in the digestive glands and gill of clam *R. philippinarum* depend upon the exposure of Cd. ([b] indicates significant difference from control value, $P<0.05$ as determined with Duncan post hoc test.)

3.4. Discussion

The CI, an indicator of stress with low specificity, also declined significantly in response to increased Cd exposure. Moreover, the difference between the CI of control animals and those exposed to 300 ppb was more marked for small than for large individuals (Sabine *et al*., 2004). In this study, CI resulted in no significantly in response to increased Cd exposure. The CI was generally higher in smaller individuals, but shell lengths and weights were recorded large in our results (shell length: 35.81 ± 2.51 mm, body weight: 10.40 ± 2.16 g).

Cd accumulated in the digestive glands, gills and residue tissues of the clam and the accumulation increased with the time of exposure and concentration in the water (Figure 3-2). The gill of the clams are in direct contact with the water, and are very sensitive to oxidative stress, at least during the 2 weeks of Cd exposure. It is widely accepted that marine bivalve molluscs can accumulate large amounts of cadmium in their tissues (Ray, 1984; Skul'sky *et al*., 1989). The ability of various species of Pectinidae to accumulate Cd in soft tissues to a higher degree than other bivalve molluscs in nature and under laboratory conditions is well documented (Gould *et al*., 1985; Skul'sky *et al*., 1989; Evtushenko *et al*., 1990). Metals may be taken up bound to particulate material or in soluble form. Both the digestive glands and gill are involved in these mechanisms. In any case the major site of metal deposition is species-specific regardless of the metal source. To study metal accumulation, many studies have also examined the total soft tissue of bivalves (Cantillo 1998; O'Connor 1998; Jeng *et al*., 2000). It is possible that cadmium uptake in the test animal was mainly by absorption, and was facilitated by diffusion of $CdCl_2$ across the gills or by some type of complexation with a high molecular weight compound present on the gill surface. The biochemical processes as well as passive diffusion and adsorption were important ways of cadmium uptake via water (Everaarts, 1990; Roesijadi and Unger, 1993). In *M. edulis*, the accumulation of cadmium from seawater showed a significant equilibrium relationship between total recoverable cadmium in seawater and its concentration in mussel (Talbot,

1985). It appears that cadmium uptake by different tissues was not from a single source; while the gill uptake was from water through direct absorption, uptake by digestive glands were from hemolymph implying no common pattern of cadmium uptake in different tissues examined.

Determination of accumulation factors in various tissues suggested the highest accumulation ability for gill followed by digestive glands and lowest for residue tissues. Distribution of specific tissue cadmium relative to total body cadmium also led to this hypothesis. For freshwater mussel, gill is proposed as the major site for metal uptake from solution, because of their large surface area and the immediate accumulation of any administered dose (Holwerda *et al*., 1989; Everaarts, 1990). Shell may act as safe storage matrix for toxic contaminants resistant to soft tissue detoxification mechanisms (Walsh *et al*., 1995).

In this study, the order of Cd elimination in tissues of bivalve during depuration period was digestive glands > gill > residue tissues. The rate of cadmium release by clam was strongly influenced by the amount of initial cadmium accumulation in the tissue. It is possible that the release of cadmium was perhaps mediated through diffusion processes following a well defined potential gradient from tissue to water, possibly involving the same mechanism as demonstrated in the marine bivalve, *M. edulis* (George and Viarongo, 1985) and in the marine oyster, *C. virginica* (Roesijadi and Klerks, 1989).

Laboratory studies are a useful tool to evaluate the impact of trace metals on antioxidant enzyme systems and to explain the intervention and relationship of the different antioxidant enzyme mechanisms as a function of metal exposure. Antioxidant enzymes such as glutathione peroxidase (GPx), glutathione reductase (GR), glutathione-S-transferase (GST), reduced glutathione (GSH), lipid peroxidation (LPO), superoxide dismutase (SOD) were carried out to evaluate the impact of exposure waterborne Cd. GPx catalyze the reduction of hydorgen peroxide into water or organic peroxide to their corresponding stable alcohols by oxidizing the reduced glutathione (GSH) into its oxidized form (GSSG), while glutathione reductase (GR) in regenerated GSH by catalyzing the reduction of

GSSG into GSH. GR is thus of utmost importance, its inhibition being a factor of sensitivity to chemical stress (Regoli and Principato, 1995; Cossu *et al.*, 1997; Doyotte *et al*., 1997). GST, which catalyzes conjugation reaction with the tripeptide glutathione, is quantitatively the most important phase Ⅱ enzymes (Mannervik and Danielson, 1988). These enzymes also play a role in protection against oxidative stress by catalyzing a selenium-independent glutathione peroxides activity (Prohaska, 1980). The tripeptide glutathione (GSH) is one of the most intensively studied intracellular solutes due to the critical role that it plays in cell biochemistry and physiology. GSH is protection against oxidative damage, detoxification of endogenous and exogenous reactive metals and electorphiles, storage and transport of cysteine, as well as for protein and DNA synthesis, cell cycle regulation and cell differentiation (Meister and Anderson, 1983; Meister, 1984; Wang and Ballatori, 1998; DeLeve and Kaplowitz, 1990).

SOD is oxido-reductases, which catalyze the dismutation of the superoxide anion into molecular oxygen and hydrogen peroxide (Fridovich, 1989). SOD result was the observation of a change in gill of clam heavily dose Cd 200 μg/L, and not only at the low dose under Cd 100 μg/L. Again, these changes were more pronounced in gill than in digestive glands. A change in SOD activity was found in clam after Cd treatment, especially Cd 200 μg/L content at after 2 weeks. This suggested that Cd causes oxidative damage in clam, possibly by generating reactive-oxygen stress in the body. This is seemed particularly stable as noted in *M. glllloprovincialis* (Livingstone *et al*, 1995). Cd has a similar ionic radius to that of calcium, and the interference of Cd with calcium homeostasis is well documented and may play an important role in Cd toxicity (Yang *et al*., 2000). The interaction of Cd with calcium is especially important with regard to antioxidant enzyme activities. Cd can replace calcium in the conversion of xanthine dehydrogenase to xanthine oxidase by calcain, a calcium-dependent protease (Stark *et al*., 1989). Xanthine oxidase then catalyses the oxidation of xanthine, producing O_2^-. The increase in Cd accumulated in the gill stimulates this reaction, and the increase in O_2^- along

with an induction of SOD from the results in the transformation of the superoxide anion radical to hydrogen peroxide (Geret *et al*., 2002).

GPx activity may play a protective role for gill tissues against oxidative stress when the activity of antioxidant enzyme is lowered (Power and Sheehan, 1996; Sheehan and Power, 1999). In this study, one of the most striking effects induced by Cd in the gill of clams was the decrease in GPx activity in the 2 weeks of Cd exposure, with the leveling off of these enzymes after that period. Moreover, digestive glands were decreased GPx activity of at 200 μg/L Cd for 2 weeks, but GPx activity was recovery Cd concentration 200 μg/L of depuration periods. GPx is considered an efficient protective enzyme against lipid peroxidation (Winston and Di Giulio, 1991). Its reduced activity has been linked to lipid peroxidation in *Micropogonias undulatus* treated with Cd or Aroclor in long-term experiments (Thomas and Wofford, 1993). In some other studies where the induction of GPx activity was recorded, this increase was not efficient enough to prevent oxidative damage (Di Giulio *et al*., 1993). GPx catalyze the reduction of hydorgen peroxide into water or organic peroxide to their corresponding stable alcohols by oxidizing the reduced glutathione (GSH) into its oxidized form (GSSG), while glutathione reductase (GR) in regenerated GSH by catalyzing the reduction of GSSG into GSH. An increase of glutathione level accompanied with a decrease of "GSH-consuming" GPx was noted by Mateo *et al*. (2003) in liver of mallards. Sivaprasad *et al*. (2002) showed a decrease of thiol capacity as well as a decrease of antioxidant enzyme levels in liver of rats administered lead. Inhibition of GPx activities has also been reported in the gills of Cd-contaminated marine clams *R. decussatus* concomitant with an increase in MDA levels (Geret *et al*., 2002).

In our results, GR activities exhibited higher levels in gill. The higher sensitivity of gill to oxidative stress was also indicated by the inhibition of antioxidant parameters, often more pronounced in this tissue than in the digestive glands. It was concluded that the gill was more sensitive than the digestive glands because lipid peroxidation was found exclusively in the gill (Cossu *et al*., 1997). GR activity is rarely investigated and few authors have studied this enzyme in field

experiments on oxidative stress of aquatic species (Hasspieler *et al*., 1994 a, b; Di Giulio *et al*., 1995; Regoli and Principato, 1995).

Induction of GST had been noted in *Mugil* sp. (Rodriguez-Ariza *et al*., 1993) and *M. edulis* (Suteau *et al*., 1988) collected from polluted coasts. The detoxification enzyme GST exhibited maximum activity in Cd concentration 200 μg/L during 1 week in digestive glands and gill tissues. GST are widely distributed in nature, their expression induction could make the basis for the early detection of stress responses in organisms exposed to toxicants. The enzyme activity has been assessed as a potential indicator of exposure to chemical pollutants in both Cork Harbor and Venice lagoon using the closely related species *M. galloprovincilais* (Buetler and Eaton, 1992).

GSH is an essential non-protein antioxidant that acts either directly as a reductant or as a substrate for enzymes such as glutathione peroxidases and glutathione transferases. GSH ensures the reduction of oxidants, the quenching of free radicals, the neutralization of organic peroxides, and the elimination of hydro carbons by conjugation. It can also bind directly to metals. Low GSH levels made the cells more sensitive to prooxidants and were found to be associated with increased MDA levels. Much lower GSH concentrations have also been measured in *M. galloprovincialis* living in polluted compared with unpolluted areas and in mussels transplanted from clean to contaminated sites (Regoli and Principato, 1995). A decline in GSH levels seemed to be a valuable indicator of exposure to Cd. Consequences on toxicity will depend on the capacity of cells to maintain sufficient turnover of GSH and/or to synthesize new pools of reduced glutathione.

MDA is considered to be an important feature in cellular injury, and largely results from free radical reactions in membranes, which are rich in polyunsaturated fatty acids. The MDA result demonstrated that exposure to sub-lethal Cd concentrations stimulates lipid peroxidation in the tissues of clams after 1 weeks of exposure. An increase in lipid peroxidation was also observed in *in vitro* studies with the giant fresh water prawn *Macrobrachium rasenbergii* after exposure of the crude homogenate to 500 *u*M $CdCl_2$ for 30 min (Dandapat *et al*., 1999). An

increase in basal peroxidation was also observed when the superantant of gill of the clam *R. decussatus* was in cubated *in vitro* with 500 μg/L Cd for 20 min (Romeo and Gnassia-Barelli, 1997). However, no modification of the levels of lipid peroxidation in the mussels *M. galloprovincialis* and *Perna viridis* was observed after short-term Cd exposure (Viarengo *et al*., 1990, Arasu and Reddy 1995). MDA levels deficiency of aquatic species had already been reported by several authors, whether the deficiency was caused by environmental contaminants (Livingstrone *et al*., 1993), seasonal factors (Viarengo *et al*., 1991a, b), or spawning (Ribera *et al*., 1989; Solé *et al*., 1995b). These results suggest that digestive glands appeared more susceptible to oxidative stress than gill and a relationship may exist between the degree of deficiency of antioxidant defenses, lipid peroxidation, and toxicity in clams.

Chapter 4

Accumulation of lead in the clam

4.1. Introduction

Lead (Pb) enters aquatic environments by a number of pathways. The earth's crust, geologic weathering phenomena, and volcanic activity account for natural sources, but most waterborne lead derives from human activities such as mining and smelting, coal burning, cement manufacturing, and use in gasoline, batteries, and paint (World Health Organization, 1995). As a common contaminant in industrially impacted waters, waterborne lead exerts toxic effects in fish through disturbance of ionoregulatory mechanisms, evident in the disruption of Ca^{2+} balance and the interference with Na^{+} and Cl^{-} regulation (Sorensen, 1991; Rogers *et al*., 2003, 2005; Rogers and Wood, 2004). Typically, the concentration of dissolved lead in low-moderately anthropogenically impacted waters is < 10 μg/L, with levels reaching > 200 μg/L in highly contaminated locales (Dassenakis *et al*., 1997). Lead is a non-essential metal, is accumulated rather than regulated by most aquatic taxa (Amiard *et al*., 1986), and exerts toxic effects at lower concentrations than many other metallic contaminants (MacFarlane *et al*., 2000). Bivalve mollusks are net accumulators of most metals (Phillips and Rainbow, 1993), accumulating metals to several orders of magnitude greater than ambient aquatic concentrations. Furthermore, tissue concentrations are known to equilibrate with environmental levels over time (Naimo, 1995). Thus, bivalves have been used extensively as successful biomonitors of aquatic metallic pollutant levels. Indeed the measurement of Pb in oyster tissue has been advocated as an appropriate monitor of urbanization effects in estuarine catchments (Scanes and Roach, 1999). Pearl oysters have also been employed as biomonitors of trace metals, including Pb (Fowler *et al*., 1993; Al-Sayed *et al*., 1994; Bou-Olayan *et al*., 1995).

Reactive oxygen species (ROS) production is usually associated with detoxification processes, requiring defense systems to counteract the deleterious effects of the damaging molecules. The major enzymatic defenses are superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR) and glutathione S-transferase (GST), inactivating superoxide anion and peroxides, respectively. An experimental approach to identify whether the cells are under oxidative stress is to measure GSSG formation and GSH/GSSG ratio. Under oxidative challenge, peptide thiols, like glutathione, can participate as reducing buffers, leading to a decrease in protein thiols or an increase in the levels of protein-mixed disulfide formation. Organisms can adapt to increasing ROS production by up-regulating antioxidant defences, such as the activities of antioxidant enzymes. Failure of antioxidant defences to detoxify excess ROS production can lead to significant oxidative damage including enzyme inactivation, protein degradation, DNA damage and lipid peroxidation (Halliwell and Gutteridge, 1999). In particular, lipid peroxidation is considered to be a major mechanism by which oxyradicals can cause tissue damage, leading to impaired cellular function and alterations in physicochemical properties of cell membranes, which in turn disrupt vital functions (Rikans and Hornbrook, 1997). The formation of metal complexes constitutes an important source of ROS in biological systems since many metals are required as nutrients and are ubiquitous in the cells. Metals widely distributed in the natural environment such as lead (Pb), can exacerbate the levels of ROS and, as a consequence, promote oxidative cell damage. Under conditions of environmental stress, organisms are able to alter their metabolism to minimize the strain imposed by the adverse condition. Since several stress factors including exposure to toxic metals can lead to an oxidative stress state, which is responsible for most cellular injuries, the induction of antioxidant enzymes is of great importance. Recently, the role of the enzymes superoxide dismutase (SOD) during adverse environmental conditions has received much attention. SOD is an antioxidant enzyme widely distributed among prokaryotes and eukaryotes and it can be located in mitochondria, chloroplasts, cytosol, and in the extracellular space,

depending on the organism. To minimize oxidative damage to cellular components, organisms have developed antioxidant defenses. Important antioxidant enzymes are the enzymes SOD and GPx. Production of cysteine-rich binding of free metal ions to glutathione (GSH) has been suggested to play a cooperative protective role against metal toxicity and in this way to prevent any detrimental metabolic reaction in cells. GSH seems to be a first defence against metal toxicity (Chan and Cherian, 1992). GSH in involved in a variety of reactions: synthesis, reduction, oxidation, conjugation and other. GSH functions as an antioxidant itself, and as a component of the detoxifying enzyme system containing oxidase and reductase (Valencia *et al*., 2001). This ubiquitous predominant molecule acts as a reducing agent and antioxidant in a variety of biochemical reactions, serves as a reservoir for cysteine, protects cells from lipid peroxidation, and detoxifies endogenous and exogenous compounds (including reactive oxygen species and trace metals). Two enzymes, glutathione peroxidase (GPx) and glutathione reductase (GR) are closely involved in GSH enzymatic change. The form of glutathione dominant in the cell depends on the activity of those enzymes. GSH and activity of glutathione-related antioxidative enzymes are regarded as fast and prognostic indicators of individual reaction to stress but most of investigation is carried out on laboratory animals exposure to stress under laboratory condition, and moreover effects of high metal doses on animals are tested, and a great number o investigations is performed on invertebrates (Cossu *et al*., 1997; Geret *et al*., 2002; Company *et al*., 2004; Wilczek *et al*., 2004). Among phase Π enzymes, the expression of glutathione S-transferase (GST) is a crucial and inducible factor in determining the sensitivity of cells to a broad spectrum of toxic chemicals (Gadagbui and James, 2000). GST is cytosolic or microsomal enzymes that catalyze the conjugation of electrophilc xenobiotics to GSH. A large body of literature indicates that the level of expression of GST is a crucial factor in determining the sensitivity of cells to a broad spectrum of toxic chemicals (reviewed in Jakoby, 1978; Armstrong, 1997) and that GST induction is part of an adaptive response mechanism to chemical stress that is widely distributed in nature.

Manila clam *Ruditapes philippinarum* was proposed as suitable biomonitors of metal contamination because of their wide distribution throughout in Korean costal regions. Despite its importance, relatively little information is available on the effect of Pb, particularly through waterborn exposure. Therefore, the aims of present study were to investigate Pb accumulation and elimination in clam of sub-chronic waterborn Pb exposure. Also, in the present study, we used clams to test the effects of 2 week exposure and 1 week depuration to the classical oxidative stress inducing lead (Pb). Protective enzymes SOD, GPx, GR, GST, GSH, MDA, as well as, several indicators of disturbance in the thiol/disulfide status were measured. We wanted to know if metal levels present in the environment have effects on enzyme activity and to how extent.

4.2. Material and Methods

4.2.1. Clam conditions

Manila clams, *R. philippinarum* were collected in July 2005 a clam farm in Go-heung country Jeon-nam, Korea. The clams were acclimatized for 5days in semi-static system. After acclimatization, clam (shell length: 33.98 ± 3.00 mm, body weight: 8.51 ± 1.35 g) were selected for the experiments. Fifty clams were separated to 50 L control and test tanks.

4.2.2. Exposure and depuration system

The clams in the test tank were exposed to the different concentrations from 15, 30, 150 and 300 μg/L of lead nitrate ($PbNO_3$, Sigma Aldrich Co.) under sub-lethal concentration for 2 weeks. After that was transferred clean sea-water for 1 week. Sea-water was changed every 48 hours. Animals were held healthy under the laboratory condition in 12:12h light/dark cycle for further studies. Four clams were sampled every 1 week for 3 weeks from each group for chemistries determined. Seawater quality was measured every 1 week during the experiment periods (Table 4-1). The condition index (CI) was calculated (CI = fresh flesh weight (g) × 100/shell weight (g), derived from Duquesne *et al* (2004).

4.2.3. Lead analysis

The gill, digestive glands, residue tissues were sampled every 1 week for analysis of metal concentration. Four clams were removed each test concentration and control. Tissue samples were dried at 65℃ and kept in desiccators until digestion. Dry tissue was digested with 1:1 HNO_3 (Suprapur grade, Merck, Germany) and samples were fumed to near dryness on a hot plate at 120℃ for overnight. After digestion, the residue was dissolved in 20 mL of 0.2N HNO_3 and kept in a refrigerator until analysis for trace metal. Pb concentration of tissue was measured using an ICP-MS (Elan 6000, Perkin-elmer). Pb concentration in the tissues of clam was expressed as μg/g dry wt.

4.2.4. Biochemical analysis

Eight clams were removed from each tank every week during the 3 week of the experiment. Weight and total length were recorded for each individual. Four clams were dissected gill and digestive glands and then weighted. Tissues were homogenized in 0.5 M sucrose and 0.15 M NaCl in 0.02 M Tris-HCl (pH 7.6). The homogenates were centrifuged at 500g for 15 min at 4℃ and the resulting supernatant at 12,000×g for 30 min at 4℃. Antioxidant enzyme activities in the tissues were measured with a temperature-controlled spectrophotometer (DR/4000U, Germany). The assays were run in duplicate or triplicate. Polyunsaturated fatty acid peroxides generate malondialdehyde (MDA) activities were measured pre-centrifuged homogenated tissues according to John and Steven (1978) method. The post-centrifuged supernatants were measured Superoxide dismutase (SOD), Glutathione reductase (GR), Glutathione-S-transferase (GST), Glutathione peroxidase (GPx). SOD activity was evaluated with the xanthine oxidase-cytochrome c method as described by Flohe and Otting (1984), GR was Carlberg (1985), GPx was Paglia and Valentine (1967), and GST was Habig and Jacoby (1981a, b). Reduced glutathione (GSH) activity measured the other four clams. Tissues were dissected, homogenized 10% $HClO_4$, and followed centrifuged 5000g for 15 min. The resulting supernatants measured GSH determination. GSH content was determinate according to Richardson and Murphy (1975). The reaction mixture contained 0.01M 5, 5'-dithiobis, 2-nitrobenzoix acid (DTNB), 0.1M PBS buffer (pH 8.0). GSH standard curve performed 10nM reduced glutathione standard solution. The linear increase in absorbance was recorded at 412 nm. Total protein concentrations according to Bradford (1976) by using bovine serum albumin (BSA) as standard.

4.2.5. Statistical analysis

Data are expressed as mean ± standard error (S.E). All statistical analysis was performed using SPSS/Pc^+ statistical package. Prior to analysis, all data were tested

for homogeneity of variances among groups using the Barttlett test. Comparisons in normalized data between control and treatments group were made by one-way analysis of variance (ANOVA) followed by Duncan's multiple comparisons test of mean values if significant differences were found ($P < 0.05$).

4.3. Results

4.3.1. Condition index

After 1 week of exposure, CI was no significantly in response at all concentration. CI was significantly decreased at 300 μg/L–Pb exposure with 2 week (Figure 4-1). For depuration periods, CI recovered at 300 μg/L and no significant CI occurred in the clam at all concentration.

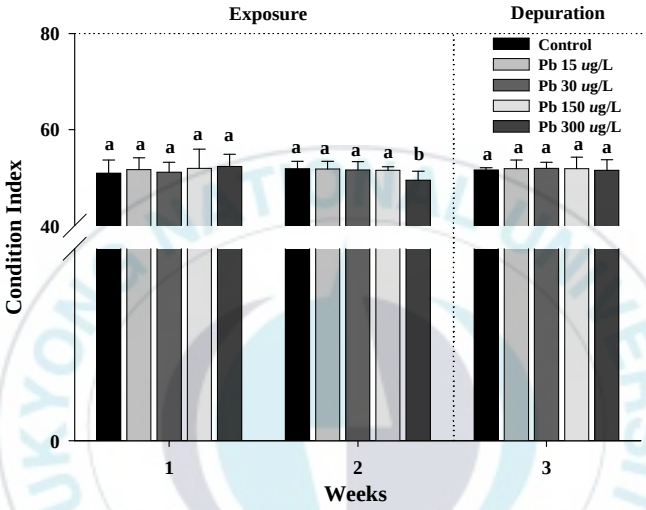

Figure 4-1. Changes of condition index (CI). Clam, *R. philippinarum* exposed to 15, 30, 150 and 300 μg/L Pb for 2 weeks and then transferred to Pb free water for 1 week.

4.3.2. Lead accumulation

The mean levels of total Pb accumulated in the digestive glands, gill, residue tissues of *R. pillippinarum* exposed to 15, 30, 150, 300 μg/L Pb for 3 weeks are presented in figure 4-2. Pb accumulations in digestive glands were significantly increased for 2 week at 150 and 300 μg/L. After 1 week of exposure, Pb accumulation values were 10.67 ± 5.24 μg/g and 13.99 ± 3.04 μg/g and were approximately 37-fold and 48-fold higher than in the control at 150 μg/L and 300 μg/L–Pb exposures, respectively. After 2 week of exposure, Pb accumulation values were 11.92 ± 4.72 μg/g and 19.51 ± 3.72 μg/g and were approximately 13-fold and 21-fold higher than in the control at 150 μg/L and 300 μg/L–Pb exposures, respectively. No significant Pb accumulation occurred in the digestive glands exposed to at 15 and 30 μg/L. For gill, Pb accumulation was significantly increased for 2 week at 150 and 300 μg/L. During the first 1 week, Pb concentration values were 67.87 ± 26.27 μg/g and 256.51 ± 52.53 μg/g and were approximately 38-fold and 145-fold higher than in the control at 150 and 300 μg/L, respectively. At the end of the 2 week exposure, Pb concentration values were 144.45 ± 26.35 μg/g and 282.00 ± 49.95 μg/g and were approximately 54-fold and 107-fold higher than in the control at 150 μg/L and 300 μg/L–Pb exposures, respectively. No significant Pb accumulation occurred in the gill exposed to at 15 μg/L and 30 μg/L. Pb accumulation in residue tissues (adduct muscle, foot, siphon) were significantly increased with exposure periods and above 150 μg/L for 2 week. During 1 week of Pb exposure, Pb concentration values were 39.92 ± 10.42 μg/g and 83.46 ± 19.57 μg/g and were approximately 69-fold higher and 144-fold higher than in the control at 150 μg/L and 300 μg/L–Pb exposure, respectively. After 2 week of Pb exposure, Pb concentration values were 57.73 ± 6.17 μg/g and 123.57 ± 42.32 μg/g and were approximately 144-fold and 309-fold higher than in the control at 150 μg/L and 300 μg/L–Pb exposures, respectively. No significant Pb accumulation occurred in other tissues exposed to at 15 and 30 μg/L. After 2 week of Pb exposure, the order of Pb accumulation in organs was gill > residue tissues > digestive glands. The accumulation factors are presented for digestive glands, gill and residue tissues at

15, 30, 150, 300 μg/L Pb exposures in figure 4-3. The accumulation factors were increased with the exposure period in digestive glands, gill and residue tissues. A direct proportion relationship was observed between the accumulation factor and the exposure concentrations at 1 week. Moreover the accumulation factor above 150 μg/L increased with 2 week.

4.3.3. Lead elimination

Pb depuration in digestive glands, gill, other tissues of *R. pillippinarum* exposed to 15, 30, 150, 300 μg/L Pb for 3 weeks are presented in figure 4-2. During the depuration phase, Pb concentration in digestive glands, gill and residue tissues decreased quickly following at 150 and 300 μg/L the end of depuration periods. The elimination rates in digestive glands, gill and other tissues at the end of depuration periods were 78.27%, 63.56% and 92.64% for 150 μg/L, respectively. Pb concentration values in digestive glands, gill and residue tissues were 7.08±2.69 μg/g, 13.36 ± 6.13 μg/g and 12.51 ± 5.15 μg/g were approximately 23-fold, 11-fold and 54-fold higher than in the control at 300 μg/L–Pb exposure, respectively. The order of Pb elimination in organs during the depuration period was gill > digestive glands > residue tissues.

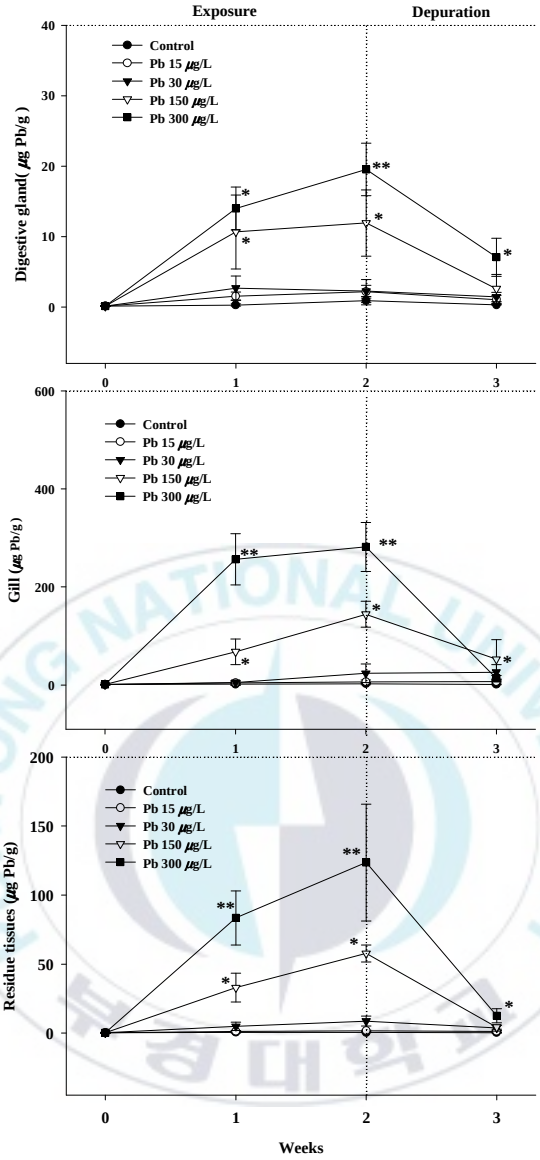

Figure 4-2. Accumulation and elimination of Pb in digestive glands, gill and residue tissues of clam. (* indicates significant difference from control value, $P<0.05$ as determined with Duncan post hoc test.)

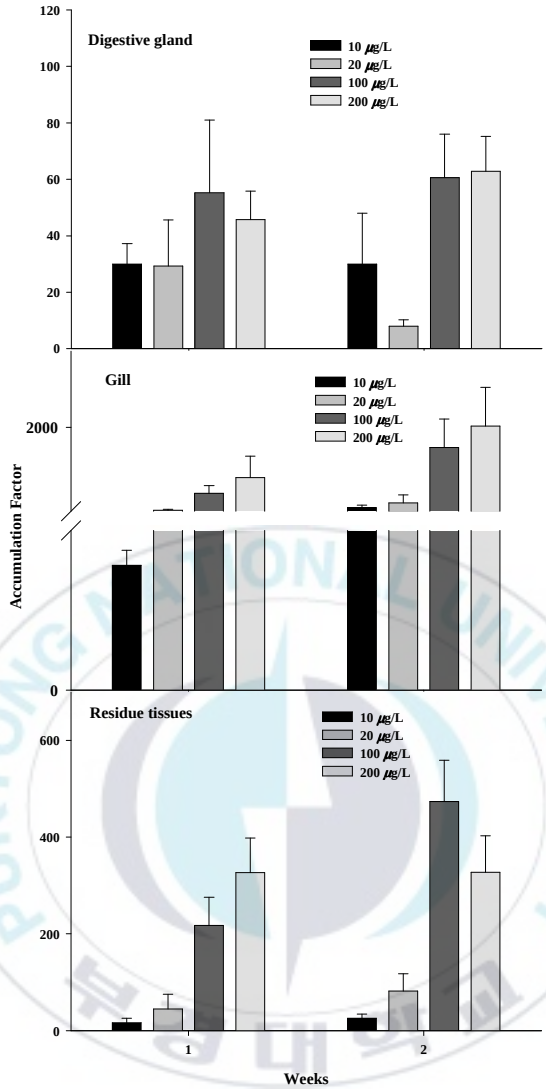

Figure 4-3. Determination of the accumulation factors (AF) in digestive glands, gill and residue tissues of clam *R. philippinarum*.

4.3.4. Value of biochemical contents

SOD activities in the digestive glands and gill of *R. pillippinarum* those exposed to Pb (control, 15, 30, 150, 300 μg/L) are presented as a function of exposure time and exposure concentrations are shown in Fig. 4-4. There were no significant differences in the digestive glands of the clam observed during the 2 week of exposure, and then after the end of Pb exposure, SOD activity in the digestive glands were no significant during the depuration period. SOD activities in the gill of the clam showed significantly increase above 150 μg/L Pb for 2 week of Pb exposure, but those were significantly reduced during depuration periods.

Change of GPx activity in the digestive gland observed significantly decrease above 30 μg/L Pb compared to control group during 2 weeks of Pb exposure, but it was recovery after 3 weeks in the above 30 μg/L exposure group. GPx activities in the gill decreased significant difference over 30 μg/L Pb concentrations in 2 week, and then GPx activities levels are recovery at the 30, 150 and 300 μg/L Pb exposure group during depuration period (Fig. 4-5).

Values of GR activities levels in the clam tissues are presented in Fig. 4-6. There were no significant differences in the digestive glands of the clam observed during the 1 week of exposure, those observed significantly increase above 30 μg/L Pb compared to control group during 2 weeks of Pb exposure, and then GR activities in the digestive glands showed a increase above 15 μg/L Pb (except 300 μg/L Pb) compared to control group during 3 weeks with depuration periods. Gill GR activity in high exposure concentration (at 300 μg/L) was significantly increased compared to control group during 1 and 2 week. And then those recovered at 300 μg/L Pb during the depuration periods.

Values of GST activities levels in clam tissues are presented in Fig. 4-7. GST activities in the digestive glands and gill were significantly increased in 2 week at 300 μg/L Pb concentration. After that, GST activities at the 300 μg/L Pb were no significant during the 3 week in the depuration periods.

In digestive gland, GSH activities were showed significantly decrease above 300 μg/L Pb compared to control group during 1 weeks of Pb exposure, and those were

significantly decreased at the above 150 μg/L Pb exposure group compared to control group during the 2 week (Fig. 4-8). After that, GSH activities at the above 150 μg/L Pb were no significant during the 3 week in the depuration periods. In gill, GSH activities were decreased at the 300 μg/L Pb exposure group during 2 week, after that were GSH activity no significant during the 3 week in the depuration period.

In digestive glands and gill, MDA level of Pb exposed to clams were no significantly difference for 1 week, and then were significantly increased at 30, 150 and 300 μg/L Pb exposure with compared control group in gill for 2 week (Fig. 4-9). And then MDA levels were no significant during the 3 week in the depuration periods. But, in the digestive glands, those were no significantly difference although slightly increased to the compared control group in 2 week. After that, MDA levels at the above 30 μg/L Pb were significantly increased in compared control group during the 3 week in the depuration periods.

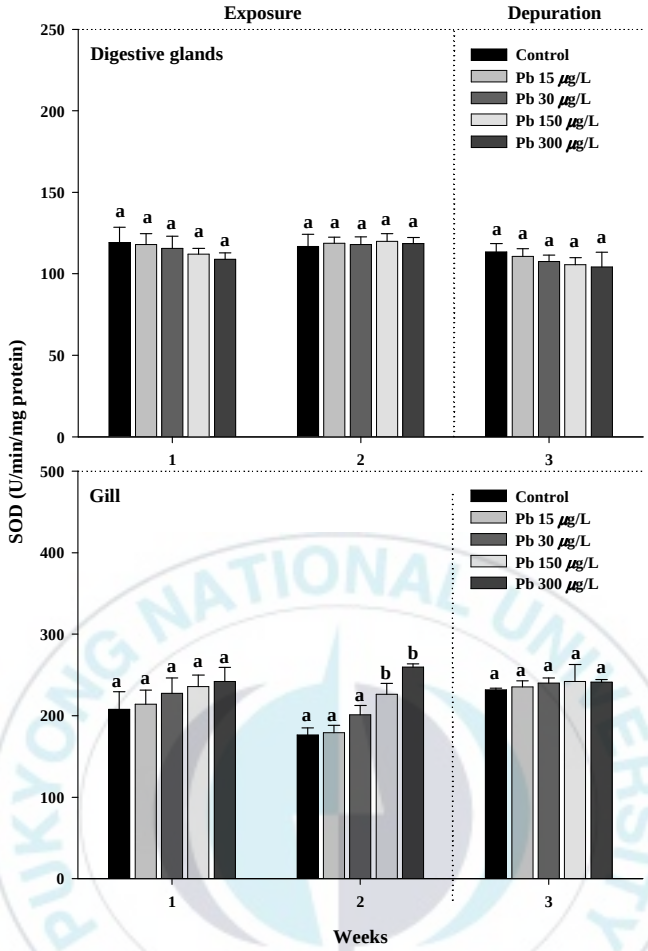

Figure 4-4. Variation of SOD activities in the digestive glands and gill of clam *R. philippinarum* depend upon the exposure of Pb. (^{b} indicates significant difference from control value, $P<0.05$ as determined with Duncan post hoc test.)

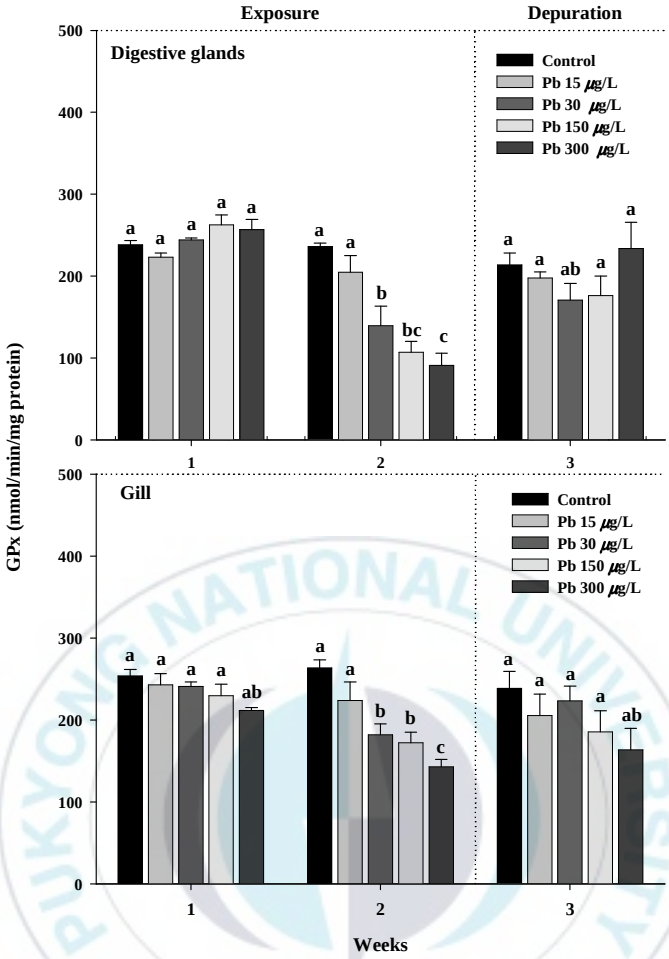

Figure 4-5. Variation of GPx activities in the digestive glands and gill of clam *R. philippinarum* depend upon the exposure of Pb. ([b] indicates significant difference from control value, P<0.05 as determined with Duncan post hoc test.)

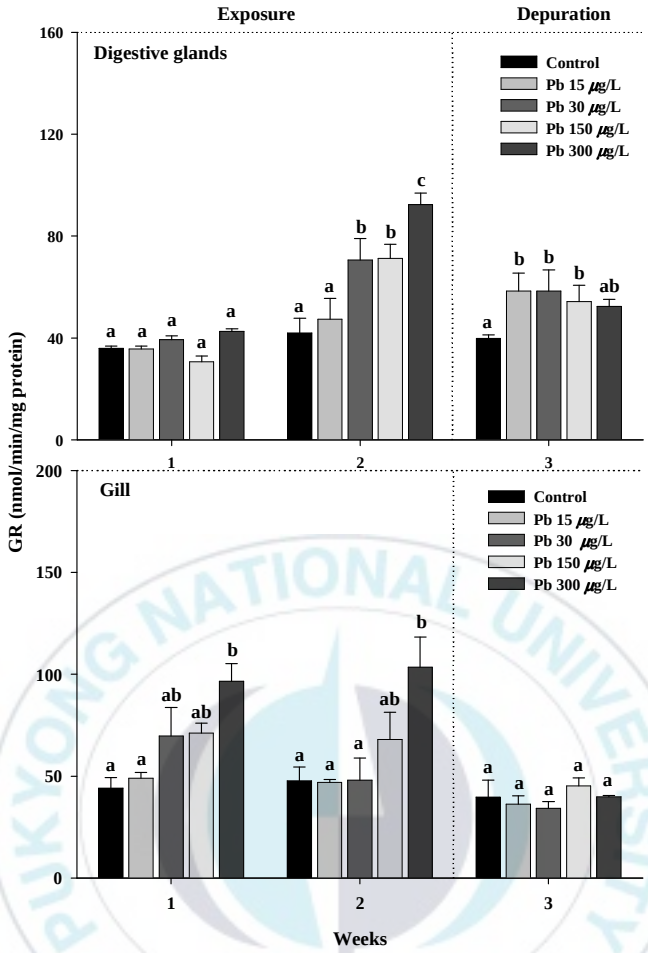

Figure 4-6. Variation of GR activities in the digestive glands and gill of clam *R. philippinarum* depend upon the exposure of Pb. (^{b} indicates significant difference from control value, $P<0.05$ as determined with Duncan post hoc test.)

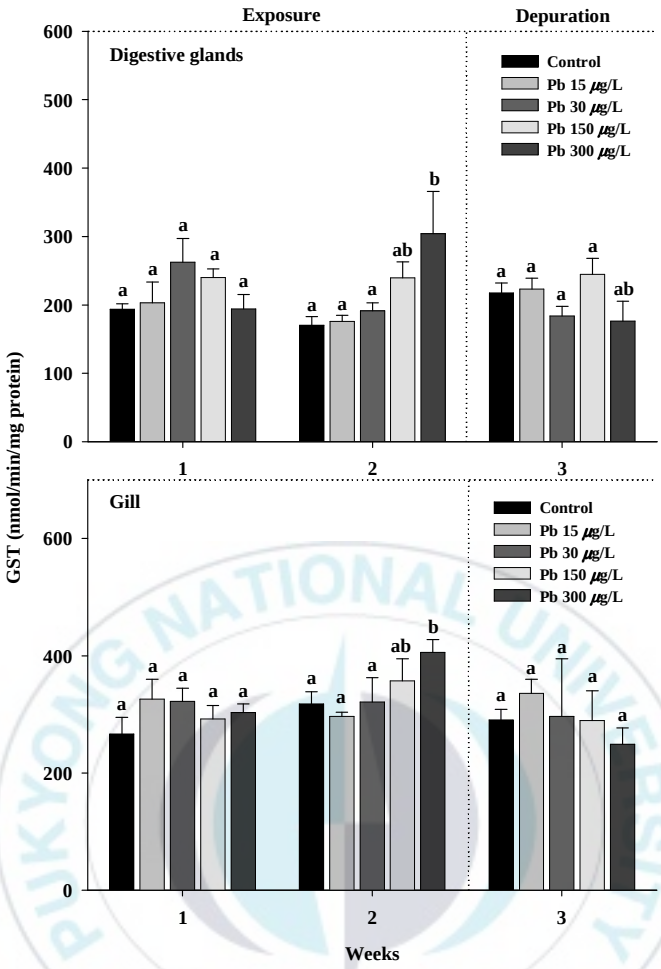

Figure 4-7. Variation of GST activities in the digestive glands and gill of clam *R. philippinarum* depend upon the exposure of Pb. (^{b} indicates significant difference from control value, $P<0.05$ as determined with Duncan post hoc test.)

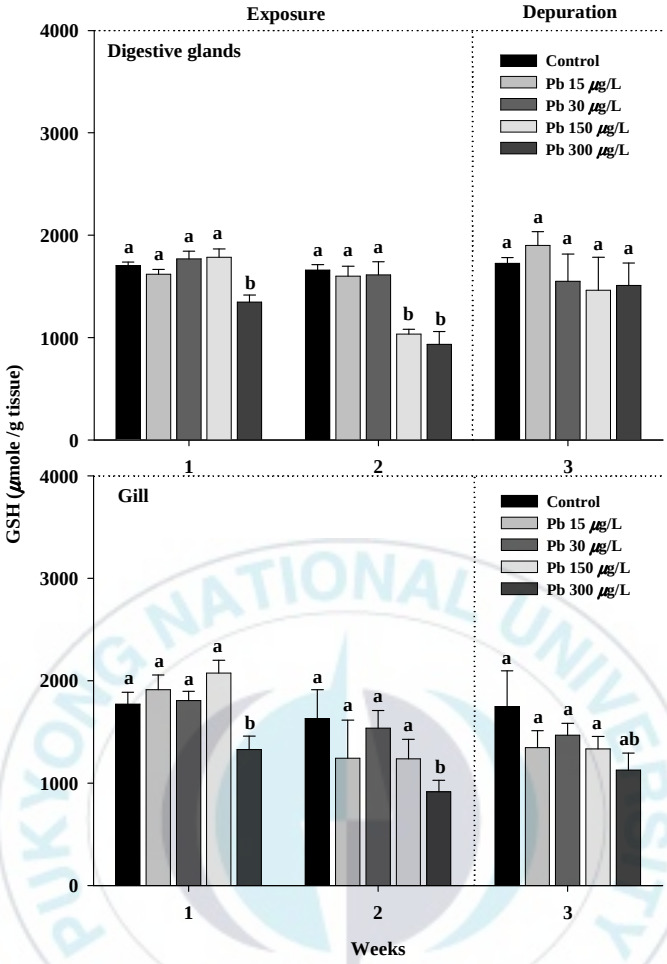

Figure 4-8. Variation of GSH activities in the digestive glands and gill of clam *R. philippinarum* depend upon the exposure of Pb. (^{b} indicates significant difference from control value, $P<0.05$ as determined with Duncan post hoc test.)

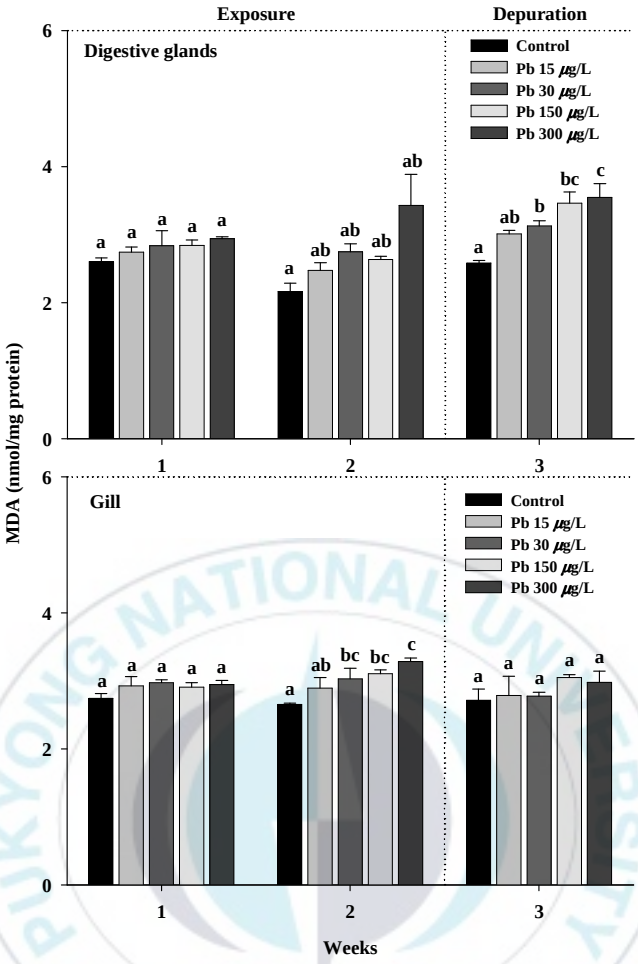

Figure 4-9. Variation of MDA activities in the digestive glands and gill of clam *R. philippinarum* depend upon the exposure of Pb. ([b] indicates significant difference from control value, $P<0.05$ as determined with Duncan post hoc test.)

4.4. Discussion

In this study, CI was significantly decreased at 300 μg/L–Pb exposure with 2 week. Trace metals inhibit growth in a variety of marine bivalves (Manley *et al*., 1984; Wikfors *et al*., 1994; Din and Ahamad, 1995; Keppler and Ringwood, 2001). Shell growth may thus provide an integrative morphological response to contaminant exposure over weeks/months, but dose not allow discrimination of temporal changes in pollutant exposure within these time frames, nor dose it provide any information on the underlying physiological mechanisms responsible for changes in growth (Widdows, 1985).

Pb accumulated in the digestive glands, gill and residue tissues of the clam and the accumulation increased with the time of exposure and concentration in the water. Lead is not essential and tends to be detoxified by metallothioneins or phosphatic granules and stored permancently in tissues (Rainbow, 1997). Lead accumulates in *R. philippinarum* in high quantities, as occurs with other bivalve mollusks (Simpson, 1979; Talbot, 1987). This species also possesses a low capacity for regulation, as seen from the observation that concentrations did not reach stationary values at either of the two exposure concentrations tested, even after 7 days. In the present study, the distribution of lead in the tissues analyzed shows that the highest concentration is reached in the gill. This tissue has been reported by various authors as that in which the greatest accumulation of metal occurs, at least during the initial period of exposure, owing to its large surface area and the chemical properties of the mucous covering the gill (Amiard-Triquet *et al*., 1986; Preston, 1971; Schulz-Baldes, 1974). This mucous has been indicated as responsible for the capture of metals in dissolved and particulate form present in water flowing through the cavity of the mantle (Cunningham, 1979). Tissue accumulation data point to the gill as the probable primary site for the acute toxic action of Pb based on the high Pb-burden measured here compared with digestive glands and residue tissues sampled. Chronically exposed clam also show elevated gill-Pb concentrations relative to digestive glands and residue tissues. It is clear

that Pb also crossed the gill and entered the clam as significant accumulation occurred in digestive glands and residue tissues. In our experiment, Pb accumulation levels in tissues elevated for 2 weeks exposure to 150 and 300 μg/L. Similar bioaccumulation equilibria of Pb in soft tissues have been reported for laboratory studies with the mussel, *Mytilus galloprovincialis*, with levels reaching a steady state after three weeks exposure to 220 μg/L Pb in seawater (Boisson *et al*., 1998). But, Pb accumulated by tissues in the hard clam, *Mercenaria mercenaria*, reached equilibria with the exposure environment within five days, and was not significantly ($p<0.05$) different to Pb concentration in tissues after 15 days exposure (Alcutt and Pinto, 1994). Since in the present study experiments were performed for four different exposure concentration (15, 30, 150, 300 μg/L), information is available on the relationship between the change in Pb tissue concentration with increasing Pb water concentration. Lead may be available for oyster tissue uptake via two major pathways, namely (a) the soluble/dissolved form (i.e. free hydrated and ionized hydroxyl species) and/or (b) phytoplankton and/or adsorbed onto inorganic particles or fragments of biogenic origin which may be subsequently filtered and potentially consumed (Boisson *et al*., 1998). The bioavail-ability of metals such as Pb is highly dependent on the speciation, or physicochemical form, of the metal in seawater. Lead uptake at the gill surface and/or the digestive glands may occur via a number of possible pathways including passive diffusion, active transport as an analogue of Ca (Markich and Jeffree, 1994), receptor-mediated transport (Brown and Markich, 2000), endocytosis or Pb reacting with phosphate groups of the lipid bilayer and subsequent binding with intracellular ligands (Viarengo, 1985). Establishing metal (pseudo) equilibria between mollusks tissues with the aquatic medium is well documented (Jeffree *et al*., 1995) and thought to be mediated via occupation of metal species at membrane receptor sites (Brown and Markich, 2000). Therefore, this suggested that gill of clam was an important accumulation organ for waterborne Pb. Determination of accumulation factors in various tissues suggested the highest accumulation ability for gill followed by digestive glands and lowest for residue tissues. The calculated

accumulation factor has two major purposes: first, to measure how much Pb is accumulating with respect to aqueous exposure concentration; second, to find the finite limit in the ability of clam to accumulate metals (Sorensen, 1991). In our study, a direct proportion relationship was observed between the accumulation factor and the exposure concentrations for exposure periods.

Depuration of metals from tissues is essentially a passive process and most aquatic molluscan studies suggest both incomplete depuration of Pb, and uptake rates which far exceed the rate of depuration. In this study, the order of Pb elimination in tissues of clam during depuration period was gill > digestive glands > residue tissues. In clam exposed to Pb concentration of 150 and 300 μg/L, the tissues that showed the fasted elimination of Pb were gill. Indeed, after three years, mussels (*Mytilus edulis*) transplanted from a contaminated location to a clean environment were only able to depurate approximately 50% of original tissue Pb levels (Riget *et al*., 1997). Approximately 62% of accumulated Pb was retained after depuration for three weeks for the same species under laboratory conditions (Boisson *et al*., 1998). Thus, it can be concluded that the target tissues for Pb elimination was gill.

For SOD, the presence of high activities in gill of clam may have allowed detoxification of excess reactive oxygen species (i. e. O_2^- and hydroperoxides), hence preventing the induction of this enzyme systems. This is in agreement with Okamoto and Colepicolo (1998), who found that Pb exposure, elevated SOD activity. The observed increase in the SOD activities in clam with Pb-induced hypertension may represent a compensatory response to oxidative stress. The highest GR activity which was observed in clam at 300 μg/L Pb exposure characterized by the lowest activity of GPx, may imply a kind of compensatory mechanism between the activity of GPx and GR. This mechanism may rely on intensification of turnover between reduced and oxidized glutathione under the conditions, which cause increased consumption of this peptide for the synthesis of trace metal-binding proteins, like metallothioneins. The detoxification enzyme GST exhibited maximum activity in Pb concentration 300 μg/L during 2 week in

digestive glands and gill tissues. The level of GST was found to be higher in mussel tissues and difference in the specific activities of this enzyme was also observed within the mussel tissues (Osman and van Noort, 2007). As found in clam tissues, a high activity of GST in gill compared to digestive glands was also previously described that was a higher expression of GST enzymes in the gill compared to digestive glands was also reported for *Mytilus edulis* (Fitzpatrick and Sheehan, 1993). Thiol chelating agents in association to antioxidants can be used in Pb poisoning (Gurer and Ercal, 2000; Tandon *et al*., 2002; Flora *et al*., 2003; Gurer-Orhan *et al*., 2004), which is justified by the oxidative stress induced by Pb and the high affinity of Pb towards thiol groups. Antioxidant, GSSG-reductase is possible targets for Pb-mediated inhibition. In our result, this inhibition was observed in clam exposed for 2 week to Pb, which may be related to the concentration and exposure period used. Pb also disturbs the cellular thiol status of the cell, binding to thiol groups with high affinity, and decreasing GSH/GSSG ratio. However, the effective increase in the GSH content in the gill and digestive glands of the green mussel *Perna virdis* after 2-3 weeks of exposure to Pb is of particular interest (Yan *et al*., 1997). MDA was accompanied by xenotoxicity incoming and damage of tissues although one out of process commonly appeared in death of cells as pathological / physiological phenomenon. In this study, oxidative gill damage was evaluated determining lipid peroxidation measured as MDA level of Pb exposed to clams were significantly increased at 30, 150 and 300 μg/L Pb exposure with compared control group in gill for 2 week. However, MDA levels in digestive glands were no significantly different for Pb exposure periods, but those were increased MDA levels at above 30 μg/L Pb exposure for depuration periods. This was meaning continuously arising of lipid perixidation which was increased MDA of digestive glands because only few time a half periods of MDA (Demling and Lalonde, 1990). It was showed maintain of effects at Pb depuration periods. Therefore, we guess lack of enzyme secretion compared with detoxification in accumulation of Pb, because it was increased MDA although antioxidants increasing.

Chapter 5.

Overall discussion

This field study was carried out a Cu, Cd, Pb, Cr, As, Se, Zn environmental gradient. Metal accumulation and metal sub-cellular partitioning were compared in the tissues of clam. Variations in tissue metal concentrations along the gradient increased in the order Cd < Pb < Se < Cr < Cu <As < Zn. In target tissues, total tissue trace metal responds in a concentration-dependent manner to changes in ambient metal levels. At the sub-cellular level, the differences between the gill and the digestive glands are even more pronounced. Our study demonstrates that the sub-cellular distribution of Cd, Pb or the essential metals Cu, Zn is linked to the metabolic orientation of the tissue and to the relative affinity of the various cellular ligands for each metal. High temporal variability of dissolved and suspended metal concentrations in near-bottom waters in coastal areas are to be expected, especially with non-point and/or sporadic metal input, and good water exchange. This circumstance makes it difficult to obtain reliable time integrated data on dissolved and suspended metal concentrations. In turn, it is difficult to use established kinetic models (Wang *et al.*, 1996) to estimate metal uptake and accumulation by mussels. We contend that the concentration of easily leach able metals in sediments is a time integrated proxy of metal contamination of near-bottom water and suspended matter. We compared metal concentrations in clams with those in ambient sediments, because some fraction of the sediment-bound metals should be available to suspension feeding mollusks. Trace metal levels were significantly higher at sites in Jinhae clam-farm than other sites. Jinhae region had the highest metal and nutrient loadings in the Jinhae catchment, perhaps due to its proximity to urban runoff and sewage treatment plant outfalls. Trace metals exhibited a high positive correlation with the percentage of silt and clay in the sediment, suggesting metals tend to bind and accumulate in fine sediments.

The tendency for shellfish to uptake metals in solute form (Borchardt, 1985; Riisgaard *et al*., 1987; Wang *et al*., 1997) and homogeneous levels in the water column seem to have little impact on variations in levels between marine stations. Under these conditions, levels of metals measured in natural populations sampled on the coast are nearly identical to those obtained from transplants of mussels immersed in the open sea. For nickel, chrome, and arsenic, the results available in the bibliography (Chiffoleau and Bonneau, 1994) point to the same conclusion. In this paper we are suggesting that the concentrations of trace metals in clams should be used to determine ecological impacts directly, and that should be used to identify which areas have a potential for ecological disruption as a consequence of increased aquatic concentrations of bioavailable metals. The link between exposure, measured by bioaccumulation and impact on organisms was discussed by Chapman (1997). His argument was centered on an analysis of the potential for predicting the level of impact on the organism in which the levels of tissue contamination were determined. He concluded that there is potential for concentrations of contaminants in tissues to be used as a predictor of impacts but the state of knowledge about relationships between toxic concentrations and body burdens is still too poor to allow useful predictions. There is very little likelihood, given the current level of knowledge, which valid predictions could be made in the foreseeable future about the effects on higher trophic levels resulting from consumption of contaminated prey. This is critical as Underwood and Peterson (1988) argue that it is the effects at ecosystem level that are the most important to determining long-term ecological viability. For the mollusks studied, cadmium was more readily concentrated in the tissues than lead. The combination of a high uptake rate coupled with a slow depuration rate for cadmium in the clams may explain this difference. Uptake of contaminants may result from processes at the body surface (e. g. gill) or in the digestive glands. We suppose that the gill was the principal absorption site. It could be possible that gill has a higher discriminating potential for cadmium absorption. Hence, cadmium will be found in a larger concentration in the tissues of the clams, probably because the gills' capacity to discriminate is lower for this metal. Under

an ambient lead exposure representative of natural contamination conditions including both dissolved and food sources, the shell compartment was shown to be the main contributor to the total lead accumulated by clams even if the lead concentration was higher in the soft parts than in the shell at the end of the exposure period. Therefore, clams may best be considered as bioindicators of lead contamination from the dissolved phase rather than from food sources. Furthermore, the low and gradual rate of uptake coupled with the long biological half-life for release of lead limits the usefulness of whole clams to trace short-term variation of lead in the ambient waters. This fact should be taken into consideration when designing the appropriate sampling frequency in biomonitoring programs involving lead.

The present results suggest that the components of antioxidant systems in epibenthic invertebrates, such as GSH, GPx, GR, GST, SOD, MDA, can provide sensitively biomarkers to control the exposure of these species to pollutants. These biomarkers had some potential in the prediction of toxicity, a deficiency expressing impaired capacity to overcome a chemical stress. Yet, further studies are needed to increase knowledge of the relationships between the degree of deficiency of antioxidant systems and cell injury. The use of species transplanted from unpolluted to study areas was found to offer a valuable means for detecting disturbances of natural environments. The results of such transplant studies do not always preclude adaptation or compensation mechanisms that may occur in native species in the long term. This kind of study represents a useful tool for environmental biomonitoring and ensuring environmental safety. The present work confirms the utility in measuring several biomarkers in the clams. Amiard-Triquet *et al*. (1998) noted variations in three biomarkers: catalase, GST, AChE activities and TBARS levels in mussels translocated for three months from a non-polluted area to the Gironde estuary (France). In these multiparametric studies, principal component analysis may be useful, for instance in a much larger area of NW Mediterranean sea from Marseilles to Corsica, biomarkers (EROD, GST and AChE activities) as well as trace metal concentrations were measured in the wild fish

Serramus cabrilla (Roméo *et al*., 2001). The observed alterations of enzyme activity in clams exposed to a gradient of pollution probably reflect short-term developmental response, which covers the range of tolerance for the species, rather than the effect of long term selection under the environmental pressure. Analysis of the tissues accumulation of metals as well as the range of enzyme activity variations, suggest that clam species manifest more apparent reactions to environmental stressors. Observed hormetic-like effects of enzyme activity in animals over the gradient of environmental pollution may indicate the borderline between the area endangered by an adverse effect and the area in which compensatory responses may balance the adverse action of pollutants. Phase Π of detoxification involves a range of enzyme activities which conjugate xenobiotics to endogenous substrates. The conjugate thus formed is usually more water-soluble than the xenobiotic and this facilitates its exclusion from the cell. GSH is known to bind trace metal cations through it's SH group, giving rise to metal –SG complexes and it is generally considered to act as a physiological defense against trace metal cytotoxicity. The data obtained in vitro demonstrate that hexokinase from clam digestive glands and gill are susceptible to inactivation by trace metals and suggest a role for GSH in the protection against the effects of trace metals, cadmium and lead in particular; moreover, the results indicate that the enzyme can be inhibited by metal-mediated oxyradical production. The results from the in induced by trace metal exposure was associated with a decrease in GSH content; although the decrease in hexokinase activity can be mainly due to a direct effect of the metal, the Cd, Pb-induced reduction in tissue GSH levels and consequent imbalance between pro-oxidant and antioxidant processes may contribute, to some extent, to the enzyme inhibition. Although some Phase Π activities are associated with the microsomes, the glutathione S-transferase (GSTs), the most abundant Phase Π activity in mammalian species, are principally located in the cytosol. In *M. edulis*, a number of GSTs are present. In common with mammalian GSTs, these proteins also are capable of non-catalytic binding of a wide range of xenobiotics. GSTs are most abundant in the gill and digestive glands tissues of *M. edulis*. The highest GR

activity which was observed in earthworms form site Π characterized by the lowest activity of GPx and GST, may imply a kind of compensatory mechanism between the activity of GPx and GR, as was postulated earlier in the paper of Laszczyca (1999). This mechanism may rely on intensification of turnover between reduced and oxidized glutathione under the conditions, which cause increased consumption of this peptide for the synthesis of heavy metal-binding proteins, like metallothioneins.

Although the species we studied in the present work and suggested as biomonitors present numerous advantages, more information and more studies are necessary to clarify accumulation patterns. It is important, as well, not to overlook the possible existence of regulating and/or competition mechanisms of metals inside tissues. Also, the most recent developments in the field of analytical techniques can significantly contribute to our knowledge in this field. It is quite important to take into account the knowledge derived from the study of metal speciation in seawater, along with traditional biomonitoring investigations, for use in environmental protection policies of the future. Metal partition coefficient was more important than metal assimilation efficiency and ingestion rate of clams in controlling the relative importance of dissolved uptake vs. dietary ingetion in the overall metal accumulation in clams. Considering the complex mixture of contaminants in aquatic ecosystems, antioxidant parameters represent biomarkers of interest due to the aspecific character of their response. Nevertheless, further research is required to clarify fundamental processes, specificity, dose-response and mechanistic studies. Transfer studies offer the advantage of being independent of natural variables (seasonal variation, temperature, salinity, etc.) since the organisms used were obtained from the same sampling site. Such studies are quite appropriate for evaluating the impact of pollution on benthic species. Antioxidant enzymes such as GR and GPx, as well as GSH levels may be promising indicators of an oxidant impact on aquatic organisms. Biomarkers should not be applied alone, but integrated into ecotoxicological research programmes including the chemical

analyses of contaminants, physiological studies of animal condition and the quality of the ecosystem.

In conclusion, *R. philippinarum* can be considered a good bioindicator for Cd and Pb exposure. Antioxidant enzyme activity in the digestive glands and gill can be considered a potential biomarker of sub-lethal stress as a result of exposure to cadmium and lead. The choice of the tissues for determining the effect of trace metals also has advantages from the experimental point of view, because a smaller number of specimens will be necessary for analyzing enzyme activity. Although direct metal-enzyme interaction in the case of Cd and Pb cannot be eliminated as a possibility, the most probable cause of the response measured in this study is adaptive reaction to stress. Nevertheless, experiments carried out at environmentally relevant concentrations and field samples will be necessary to confirm the usefulness of antioxidant enzyme as a biomarker of stress by trace metal.

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바지락 *Ruditapes philippinarum* 기관별 미량금속의 생체축적 및 생화학적 변화

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요약

해양 퇴적물은 금속의 저장 장소로 생태계에서의 해결책 역할 뿐만 아니라 대부분 오염의 최종 저장소이며, 이러한 금속은 강을 통해 해양으로 유입되고 있다. 패류는 풍부하게 분포하고 있으며 바다에서 서식하는 이유로 세계적으로 지표생물로 이용되어 왔다. 이러한 패류 중 바지락은 우리나라 연안에 널리 분포하고 있으며 금속 오염의 생체지표로 적당할 수 있다. 카드뮴은 해양 환경에 널리 분포하고 있으며 생물체에서 독성을 일으키는 주요 오염물질이다. 납은 비 필수 금속으로 해양 생태계에서 많은 다른 금속 오염보다 더 낮은 농도에서 독성 영향이 미칠 수 있다. 그러므로 이번 연구의 목적은 (a) 바지락 양식장에서의 해수, 퇴적물, 바지락의 금속 농도를 평가하고 (b) 카드뮴과 납 노출에 따른 바지락에서의 항산화 효소의 활성으로 카드뮴과 납의 축적과 제거를 설명하는 것이 목적이다.

바지락 양식장에서 해수, 퇴적물과 바지락의 금속 (Cu, Cd, Pb, Cr, As, Se, Zn)을 측정하였다. 진해 지역의 바지락 양식장에서 해수 농도에서 구리, 납, 아연이 가장 높았다. 구리에서는 2.40 ± 0.69 ㎍/L, 납에서는 2.07 ± 0.48 ㎍/L, 아연에서는 6.83 ± 1.65 ㎍/L 으로 나타났다. 비소는 사천지역에서 가장 높은 값 5.42 ± 5.55 ㎍/L 로 나타났다. 카드뮴과 크롬, 셀레늄은 모든 양식장에서 비슷한 값을 보였다. 전체적으로 고흥 지역의 바지락 양식장 해수에서 가장 낮은 금속 농도가 조사되었다. 진해 지역의 양식장 퇴적물 구리 농도는 44.15 ~ 50.05 ㎍/g 으로 나타나 가장 높은 농도를 보였으며, 카드뮴, 납과 아연도 다른 지역의

농도보다 높았다. 그러나 크롬은 고흥 지역의 퇴적물에서 가장 높은 농도로 나타났다. 결과적으로 크롬을 제외한 퇴적물 내 금속 농도는 진해 지역에서 가장 높게 조사되었다. 모든 조사 지역의 바지락 조직 내 구리, 크롬, 비소, 셀레늄, 아연 농도가 아가미에서 다른 조직보다 높았으며, 바지락 조직에서의 금속 농도는 고흥 지역에서 가장 낮게 조사되었다.

카드뮴 및 납에 대한 실내 노출실험 결과, 카드뮴 축적은 노출 시간에 따라 증가하였으며, 배출은 100 ppb 이하의 농도에서 감소되는 양상으로 나타났다. 2 주 동안 카드뮴 축적의 조직 순서는 아가미 > 소화선 > 나머지 조직으로 나타났으며, 배출 기간에서 200 ppb 를 제외한 농도에서 감소되는 카드뮴 농도로 나타나고 있으며, 배출 순서는 소화선 > 아가미 > 나머지 조직 순으로 나타난다. SOD, GST, GR, GSH, MDA 활성은 200 ppb 농도 구에서 증가된 유의성이 나타난다. GPx 활성에서 200 ppb 에서 감소한 유의성이 나타나고 있다. 납 축적 실험에서의 납 축적은 시간에 따라 증가되고 150 ppb 이상에서 축적되고 배출 시에도 150 ppb 농도 구에서 제거가 이루어지고 있다. 2 주 동안의 납 노출 기간 동안 조직에서의 납 축적 순서는 아가미 > 나머지 조직 > 소화선으로 나타나며, 배출 기간에서의 납 농도는 증가된 150 ppb 이상의 농도 구에서 즉각적인 제거가 이루어지고 있으며, 납 제거 순서는 아가미 > 소화선 > 나머지 조직으로 나타난다. SOD, GPx, GSH 활성은 150 ppb 이상의 농도에서 유의성이 나타나고 있다. GR 활성은 300 ppb 농도에서 유의성이 나타나며, GST 활성에서 300 ppb 에서 증가된 유의성이 나타난다. MDA 변화에서 30 ppb 이상에서 유의성이 나타나고 있다.

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