Effects of trace metal pre-exposure on their bioaccumulation in marine bivalves

By

SHI Dalin

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SHI Dalin
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by

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This is to certify that I have examined the above MPhil thesis and have found that it is complete and satisfactory in all respects, and that any and all revisions required by the thesis examination committee have been made.

Dr. Wen-Xiong Wang, Supervisor

Prof. H. Benjamin Peng, Head of Department

Department of Biology

4 August, 2003
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Effects of trace metal pre-exposure on their bioaccumulation in marine bivalves

by SHI Dalin

Department of Biology
The Hong Kong University of Science and Technology

Abstract

Marine bivalves, such as mussels and clams, have been extensively used as biological monitors of metal pollution in the coastal waters worldwide. Previous studies showed that the past history of metal contamination in the ambient environments can affect subsequent metal bioaccumulation, due to the modification of the animals’ physiological or biochemical processes under metal stress conditions. The metal binding protein, metallothioneins (MTs), can be induced by exposures of many metals (e.g., Cd, Zn and Hg) in various marine invertebrates, and thereby has a close relationship with metal body burden and plays an important role in metal detoxification, storage, and homeostasis in the aquatic animals.

In this study, a series of metal pre-exposures were carried out with the green mussel *Perna viridis*. Additionally, the clams *Ruditapes philippinarum* and *Mactra veneriformis* were collected from both metal contaminated and relatively unpolluted sites. By means of the sensitive radiotracer techniques, the indices of metal bioavailability, namely, assimilation efficiency (AE), dissolved uptake rate and efflux rate, were quantified to examine the controls of metal body burden and MT level on
metal bioavailability in the bivalves pre-exposed to different metal stresses.

The results indicate that generally metal pre-exposure can influence subsequent metal bioaccumulation in the bivalves. The metal binding and storage strategies involved in this process are metal specific. The assimilation of Cd increases with the elevated Cd tissue burden, in correlation with an increase in the Cd associated with the MTLP fraction as well as a higher MT concentration. The increased AE and decreased dissolved uptake of Hg are likely caused by the induction of MT by Cd pre-exposure. The dietary uptake of Zn is enhanced when the MT is significantly induced by Cd. Contrarily, Ag bioavailability is mainly controlled by sulfide binding, which can increase Ag accumulation from both solution and food and decrease efflux of the metal. Information presented in this study is important in understanding biomonitoring data of metal pollution in marine environment.
Chapter 1
General introduction

1.1 Research background

1.1.1 Trace metal in marine environment

Heavy metals are defined as metallic elements of specific gravity more than 5 g cm$^{-3}$ (Martin and Coughtrey 1982). Many heavy metals are persistent in marine environment, and at elevated concentration they can be toxic effects to aquatic organisms. More importantly, heavy metals can be potentially transferred from a lower trophic level to a higher trophic level along the marine food chain, eventually resulting in adverse effects on human health due to the consumption of metal contaminated seafood. Over the past several decades, with the rapid development of heavy industrialization in coastal regions, metal contamination in marine systems becomes more and more serious in many parts of the world. The Minamata incident in consequence of the methylmercury pollution, which occurred in Japan in 1956, is one of the most famous cases in the world. Therefore, heavy metal pollution in marine ecosystem is a matter of increasing concern to both governmental authorities and publics around the world.

In this study, the term ‘heavy metal’ is used synonymously with the term ‘trace metal’, which refers to metal occurring in a small amount in nature. These two terms basically could be used equivalently to the same group of metals (Rainbow 1997). Although trace metals are typically less than 0.01% of mass of the
organisms, many of them as micronutrients are essential to living organisms (Rainbow 1985, 1997), for example zinc (Zn), selenium (Se), and copper (Cu), which are important components of enzymes and respiratory pigments and are maintained at constant level. On the other hand, some of the essential metals may cause toxic effects to organisms when their concentrations are beyond the threshold levels. Besides the essential metals, other trace metals, such as cadmium (Cd), silver (Ag), and mercury (Hg), are of no known biological function in organisms and can perform oppositely to essential metals, producing high toxicity even at low concentrations in animal’s tissue (Rainbow and Furness 1990; Rainbow 1995).

In addition to the category in terms of biological role of metals, metals can also be classified into class A, class B, or borderline metals based on their chemically binding affinity with different ligands (Nieboer and Richardson 1980). Class A metal ions are generally hard metal ions preferring to bind ligands with oxygen (O > N > S), while class B metal ions show the opposite preference order for binding atoms with sulfur ligands (S > N > O). Borderline metal ions exhibit intermediate characteristics between class A and class B metals.

In natural environment, metals are partitioned between the dissolved (i.e., ambient seawater) and particulate phases (i.e., food particles and resuspended sediments). For aquatic organisms, the metals in the large volume of seawater passed through the gills are important pools for bioaccumulation. On the other hand, metals from particles can also contribute considerably to their bioaccumulation in marine bivalves, as it has been shown that metals can be concentrated several
orders of magnitude higher in food particles from the ambient water (Eisler 1981; Luoma and Bryan 1982). Many studies have demonstrated that aquatic invertebrates can accumulate both aqueous and dietary metals (e.g., Langston and Spence 1995; Wang et al. 1996). Therefore, in order to better understand the impacts of metal pollution on organisms and to appropriately establish realistic water quality criteria, it is necessary to study both the fate and transport of aqueous metals and the trophic transfer of dietary metals.

The trace metals involved in this study include Ag, Cd, Hg, and Zn. Among them, Ag, Cd and Hg are non-essential metals and all belong to class B metals, therefore tending to form complexes with sulfur containing ligands, like sulphydryl (-SH), thioether (-SR), and disculphide (-S-S). On the contrary, Zn is an essential element to organisms and is defined as a transition metal. However, its chemical properties are similar to those of the class B metals. Thus, all the four metals chosen in this study commonly share a similarity of binding with sulfur containing ligands.

Ag, Cd, Hg and Zn were chosen in this study because their contamination in marine system is now a major problem worldwide, including China. For example, natural concentrations of Ag in seawater are very low (e.g., 4 – 17 pM in coastal waters), however, anthropogenic contamination can result in Ag concentrations in estuarine and marine waters that are 100 – 300 times higher than natural concentrations (Sanudo-Willhelmy and Fegal 1992). In Hong Kong, due to the dense population in its coastal area, the rapid growth of metal-related industries, and
the former lack of stringent controls on the release of agricultural and industrial
effluents like electroplating, production of paints, and battery making, Cd and Zn
pollution has been a inevitably serious issue, as reflected by the elevated metal levels
(2002) reported that the Cd and Zn concentrations in the sediments of an extremely
metal polluted bay in Northern China were as high as 1% and 0.06% of sediment dry
weight for Cd and Zn, respectively, and were presumably among the highest
recorded in the world. The best example of marine Hg contamination in the world
is the Minamata incident in 1956, the influences caused by which still exist today.

1.1.2 Marine bivalves in biological monitoring

Traditionally, the direct measurement of trace metal concentration in the
seawater is the main means by which the water quality criteria are set up accordingly.
However, the value of determination of ambient aquatic metal concentrations may be
limited in evaluating the potential effects of metal contamination, because the
quantified metal level in aquatic systems may not stand for the bioavailable fraction
of metals, which is the only form of metals that can influence organisms (Waldichuk
1985). Consequently, marine bivalves have been employed throughout the world as
biological monitors of heavy metal pollution in coastal waters, mainly due to the
following attributes: 1. their tissue metal burden directly reflects ambient
bioavailable metal concentration; 2. they process a large amount of metals due to
their extremely high filtration rate; 3. they are sessile, large, and easily collected from
field and maintained in laboratory; 4. they are abundant and distributed worldwide; 5. they are able to tolerate a wide range of environmental fluctuations.

The use of bivalve mollusks as biomonitor of trace metal bioavailability has been well developed in the past several decades. For example, the United States (US) Mussel Watch program began in 1976 utilizing species of mussels (Mytilus) and oysters (Ostrea or Crassostrea) as surveillance organisms of metal pollution in coastal regions (Goldberg et al. 1978, 1983). Since 1986, the US National Status and Trends (NS&T) Program has annually monitored trace element concentrations in mussels and oysters from coastal waters (O’Connor 1992, 1996). Now, there is a worldwide International Mussel Watch (IMW) project, providing an assessment of the status and trends of chemical contaminants in the world’s coastal waters (Tripp et al. 1992; Cantillo 1998).

The bivalve species selected in this study include the green mussel Perna viridis, the Manila clam Ruditapes philippinarum, and the clam Mactra veneriformis. The use of P. viridis (Phillips and Rainbow 1988; Rainbow and Phillips 1993) and R. philippinarum (Chong and Wang 2000, 2001) as biomonitor of heavy metal pollution in tropical and subtropical waters has been well established. Both of them are commercial edible bivalves and abundantly distributed in Hong Kong coastal waters. The other clam species M. veneriformis is one kind of largely consumed seafood in Northern China, bred on a large scale along coastal area (e.g., Bohai Bay, Liaoning Province). It has also been employed as biological monitor as shown in recent field or laboratory work due to its extremely high tolerance to heavy metal
pollution (Wang et al. 2002; Fan et al. 2002).

1.1.3 Metallothionein

Metallothionein (MT) was first discovered more than 40 years ago by Margoshes and Callee (1957), who isolated a Cd-binding protein responsible for the natural accumulation of cadmium from equine kidney cortex. Subsequently, more and more researches have discovered that MT and similar proteins compositionally are widely distributed in numerous organisms, from mammals to various invertebrates, higher plants, even protists, fungi and some prokaryotes (Roesijadi 1992; Engel and Roesijadi 1987). The high cysteine content, low molecular weight, heat stability and non-enzymatic nature, together with a strong affinity to bind class B metal cations such as Ag, Cd, Cu, and Hg enable MT to be differentiated from most other proteins.

The study of Olafson and Thompson (1974) on the marine fish *Sebastes seboides* was the first to describe the existence of low molecular weight, Cd-binding proteins in an aquatic organism. The oyster, *Crassostrea virginica*, was the first marine invertebrate in which metallothionein-like protein (MTLP) was found by Casterline and Yip in 1975. Subsequently, such proteins were reported to occur in other fish species (Roesijadi, 1992) and a large variety of invertebrates (Roesijadi 1981; Engel and Brouwer 1984; Geroge and Viarengo 1985), for example, the blue mussel *Mytilus edulis* (Roesijadi 1992).

It has been suggested that MT functions mainly in both the control of
intracellular availability of essential metals and the detoxification of excessive essential or pollutant metals. Additionally, MT can also be involved, indirectly, with elimination. For example, Hg is eliminated as Hg-MT from exposed mussels via gametes (Roesijadi et al. 1988). MT induction has been demonstrated in various marine bivalves (e.g., mussels, clams, and oysters) from polluted populations or following laboratory exposure to metals such as Cd, Cu, and Hg, and generally it varies in amount according to metal concentration in the animals (Roesijadi and Fellingham 1987; Langston et al. 1989; Mouneyrac et al. 2000; Hamza-Chaffai et al. 2000; Serafim and Bebianno 2001; Géret et al. 2002; Bebianno and Serafim 2003; Baudrimont et al. 2003; Riveros et al. 2003).

With all the characteristics described above, MT plays an important role in metal bioaccumulation in marine invertebrates. Therefore, MT has been regarded as a potential biological indicator of metal exposure, and thereby a tool to evaluate heavy metal contamination in marine environments (Langston et al. 1998).

1.2 Hypothesis and research objectives

1.2.1 Influences of metal exposure on metal bioaccumulation

An important and basic assumption underlying the biomonitoring program is that there is a simple and direct relationship between the ambient bioavailable metal and the metal concentrations in the soft tissue of bivalves. Previous studies have demonstrated that a variety of factors, such as salinity (Blackmore and Wang 2003), diatom chemical composition (Wang and Fisher 1996), and food abundance (Wang et
al. 1995), can affect metal bioaccumulation in marine mussels employed as biomonitors of metal pollution in coastal waters. Therefore, when utilizing the observed metal concentrations in the soft tissue of marine mussels to monitor heavy metal pollution in seawater, these factors need to be taken into account considerably.

In addition to the factors mentioned above, recently, the influences of the history of metal exposure and resulting metal body burden on metal bioaccumulation have received increasing attentions. For example, Selck et al. (1999) investigated the effects of Cd exposure on the metal assimilation efficiency by a deposit-feeding polychaete Capitella, and found that the assimilation of Cd decreased following Cd exposure. The clam Macoma balthica from the Ag and Hg contaminated estuary accumulated both two metals at lower rates than the population originating from the relatively uncontaminated estuary (Boisson et al. 1998). Contrarily, Rainbow et al. (1999) reported that the mean metal uptake rates of amphipods and crabs from a metal-enriched site were not lower than those of the same crustaceans from a control site. More recently, in the marine green mussel P. viridis, Blackmore and Wang (2002) demonstrated that the assimilation of Cd in the animal increased significantly, as a result of Cd pre-exposure and elevated Cd concentration in mussel’s soft tissue. All of these researches indicate the significance of potential effects of metal exposure history on metal bioaccumulation in marine invertebrates.

It has been suggested that the influences of metal pre-exposure on subsequent metal bioaccumulation in marine mussels are closely related to the modified biochemical or physiological processes induced by ambient metal stress. In this
case, the mechanisms of metal binding and storage of mussels under metal exposure situation are important for understanding the subsequent metal bioaccumulation in animals. Blackmore and Wang (2002) examined the metal uptake from both solution and food by the green mussel following laboratory Cd and Zn pre-exposures, and found the increase of Cd assimilation efficiency was very likely due to the increasing association of Cd with MTLP after Cd pre-exposure. This phenomenon was not observed in the uptake of Zn, which was shown to store mainly in the metal rich granule (MRG) when accumulated significantly. Besides forming complexes with MT or granule, metals such as Ag could also be bound with insoluble compounds like sulfur (George et al. 1986; Berthet et al. 1992). These studies therefore highlight that mussels may develop different strategies accordingly in response to different metal stresses, and metal partition in different subcellular pools of organism can play a role in the following metal accumulation.

1.2.2 Study hypothesis

The relationships between metal exposure and bioaccumulation as well as MT induction have been extensively studied in marine invertebrates (Langston et al. 1989; Bebianno and Langston 1995; Mouneyrac et al. 2000; Hamza-Chaffai et al. 2000; Serafim and Bebianno 2001; G^mret et al. 2002; Bebianno and Serafim 2003). However, few studies have been carried out so far to investigate the controls of metal pre-exposure, resulting metal body burden, and MT induction on subsequent metal bioaccumulation in marine bivalves. Therefore, the main hypothesis tested in
this study was whether or not the metal body burden, MT induction, and metal subcellular partitioning resulting from metal pre-exposure can affect the metal bioaccumulation in marine bivalves that are used as biological monitors of trace metal contamination in coastal waters.

1.2.3 Research objectives

Although previous studies have clearly demonstrated that biomonitoring organisms may potentially modify physiological or biochemical processes to adapt to ambient metal conditions consequently resulting in a variation of metal body concentration, several important aspects in understanding metal bioavailability and bioaccumulation in marine bivalves following metal exposure history are usually ignored or still remain relative unknown. Therefore, to test the hypothesis as described above, my study aims to further explore these aspects for better understanding the influences of metal pre-exposure upon metal bioaccumulation in biomonitors.

First, as showed by previous studies, metals from both the dissolved and the particulate (e.g., food) phases are potential sources for their accumulation in the aquatic invertebrates employed as biomonitors of metal pollution in nature seawaters (Langston and Spence 1995; Wang et al. 1996). Therefore, when considering the effects of metal pre-exposure on subsequent metal bioaccumulation, metal uptake from the food pathway should also be taken into account, particularly for the particle-reactive metals, such as Ag. Thus, in my study I will try to test the
influence of dissolved metal pre-exposure and dietary metal pre-exposure, separately, on subsequent metal bioavailability to marine bivalves, consequently comparing the relative importance of these two exposure pathways.

Second, laboratory studies of metal exposure are inevitably limited by the duration of metal pre-exposure. Within a relatively short period of exposure under laboratory conditions, in order to induce significantly enhanced metal tissue burden so that the modification in biochemical or physiological processes can be achieved in marine bivalves, many studies had to employ very high levels of metals in the exposures. These metal concentrations are, however, usually environmental unrealistic. As reviewed by Klerks and Weis (1987), these responses of animals to metal pollution may only represent a degree of tolerance obtained during a relatively short-time exposure (i.e., laboratory exposure), which is the result of physiological acclimation. They could be different from the genetically based resistance obtained by whole life-time exposure of animals to metal contamination in their living environments (i.e., field conditions), which results from adaptation. In order to examine whether or not the laboratory data can be extrapolated to field situation, in my study, the same species of bivalves from both serious metal-contaminated sites and relatively clean sites will be collected to compare the metal accumulation behaviors between the polluted and clean populations. The results will then be discussed and compared with those obtained in laboratory experiments.

Third, under natural conditions, there is a possibility that the comparable metal body burdens in different populations are the results of different metal pre-exposure
regimes. In other words, different metal concentrations in ambient seawaters and
different exposure durations may result in similar tissue metal concentrations.
Accordingly, a specific question addressed in my study is whether the history and
regimes of pre-exposure instead of the metal tissue body burden will lead to
physiological and biochemical modification and thus cause changes in metal
bioaccumulation. To our knowledge, this aspect remains essentially unknown at
present.

Fourth, marine bivalves are simultaneously exposed to a variety of metals in the
natural environment, thus the potential influences of one metal pre-exposure on the
biological uptake of other metals are inevitable. In the marine common mussel *M. edulis*, it has been demonstrated that pre-exposure of the bivalves to Cu or Cd can
increase the tolerance to subsequent inorganic Hg exposure (Roesijadi and
Fellingham 1987). Blackmore and Wang (2002) reported that when Cd
concentration in the soft tissue of the green mussel *P. viridis* elevated to a very high
level (e.g., 25.9 μg g⁻¹ dry weight), the assimilation efficiency of Zn increased by 1.4
times. Therefore, four metals, i.e., Ag, Cd, Hg and Zn, are selected in my study
because of their common characteristic of preferentially binding with sulfur
containing ligands. The green mussel *P. viridis* will be pre-exposed to Cd with
different concentrations and durations, and then the effects of Cd exposure on
subsequent Ag, Hg, and Zn bioaccumulation in the organism will be examined.

Following various metal pre-exposures as described above (i.e., different metals,
different concentrations, different exposure pathway and duration, laboratory
exposure and field collected populations, and different exposure regimes), in addition to quantifying metal body burden and MT concentration, metal bioavailability index including metal assimilation efficiency from food, influx rate (or influx rate constant) from the dissolved phase, and efflux rate constant will be determined. These three metal-specific physiological parameters can be incorporated to predict the metal bioaccumulation to marine invertebrates, according to a well developed bioenergetic-based kinetic model (Wang and Fisher 1997, 1999). According to this model, metal bioaccumulation in marine invertebrates can be described by the following first-order equation:

$$\frac{dC}{dt} = (AE \cdot IR \cdot C_t) + (k_u \cdot C_w) - (k_e + g) \cdot C$$

where $C$ is the metal concentration ($\mu g g^{-1}$) in the animals at time $t$ (d); $AE$ (%) is the metal assimilation efficiency from ingested particles; $IR$ is the ingestion rate (mg $g^{-1}$ d$^{-1}$); $C_t$ is the metal concentration in ingested particles ($\mu g mg^{-1}$); $k_u$ is the metal uptake rate constant from the dissolved phase (L g$^{-1}$ d$^{-1}$); $C_w$ is the metal concentration in the dissolved phase ($\mu g L^{-1}$); $k_e$ is the efflux rate constant (d$^{-1}$); and $g$ is the growth rate constant (d$^{-1}$).

With sensitive radiotracer technique, the three physiological parameters (i.e., AE, $k_u$ and $k_e$ can be quantified to predict the metal bioaccumulation in marine bivalves pre-exposed to different metal stresses. This can provide important information when using the observed metal body burden as the criteria of marine metal pollution.
1.3 References


Chapter 2

Effects of aqueous and dietary pre-exposure and resulting body burden on silver biokinetics in the green mussel *Perna viridis*

2.1 Abstract

To determine whether pre-exposure of green mussel *Perna viridis* to Ag influenced metal uptake kinetics we compared various physiological indicators of metal uptake kinetics between the control mussels and mussels pre-exposed to Ag in both diet and water at different levels (up to 5 weeks). In all pre-exposed mussels, the assimilation of Ag increased by $1.1 - 3.0 \times$ with increasing Ag body concentration (0.7 – 19.3 g⁻¹) as compared with the controls (Ag body concentration of 0.3 – 0.5 g⁻¹), whereas the efflux rate constants decreased by 45 – 88%. There was no significant increase in Ag associated with the metallothionein-like protein (MTLP) fraction following exposing the mussels to Ag through either the dissolved or food phase. The clearance rates were little affected or depressed by Ag pre-exposure, and the relationship between the Ag influx rate from the dissolved phase and the Ag pre-exposure was somewhat complicated. The influx rate decreased with increasing Ag body burden at < 2.5 g⁻¹, above which it increased with increasing Ag body burden. Our results indicate that the mussels may modify physiological processes to ambient chronic Ag exposure, consequently accumulating more Ag. Ag body concentration in these mussels may therefore increase disproportionally in response to increasing Ag concentration in the ambient environments. Ag pre-exposure and resulting body burden should be considered carefully when interpreting the observed Ag concentration in biomonitoring animals to evaluate the Ag pollution in seawater.
2.2 Introduction

Marine mussels are able to process a large amount of dissolved and particulate metals due to their extremely high filtration rate (Widdows et al. 1995), and thus accumulate a higher metal concentration in the soft tissue as compared with that in the ambient environment. Because the body metal concentrations generally reflect the ambient bioavailable metal levels, marine mussels have been employed as biological monitors of metal pollution in coastal waters worldwide (Goldberg et al. 1978, 1983). Previous studies have demonstrated that metal bioaccumulation can be affected by a variety of extrinsic conditions (Wang and Fisher 1996a, b; Blackmore and Wang 2003; Wang et al. 1995). Thus, factors influencing the metal accumulation in biomonitors should be carefully considered when interpreting biomonitoring data, especially when comparing the metal body burdens in the same species from different sites. Recently, there has been increasing attention on the effects of metal pre-exposure on subsequent metal bioaccumulation. For example, the overall Ag and Hg bioaccumulation potential of the clam *Macoma balthica* appears to be largely affected by their long-term exposure in the contaminated environment (Boisson et al. 1998). Selck et al. (1999) reported that Cd assimilation efficiency in the polychaete *Capitella* decreased following Cd pre-exposure. A recent study found that pre-exposure to Cd and subsequent induction of metallothionein-like proteins (MTLPs) affected Cd accumulation via ingested food but not Cd uptake from the dissolved phase in the green mussel *Perna viridis* (Blackmore and Wang 2002). These limited studies indicated that metal pre-exposure may cause potential physiological changes and subsequently affect metal uptake in the animals. However, these previous studies generally only focused on the influence of metal pre-exposure from the dissolved phase on
subsequent metal bioaccumulation, even though it has been demonstrated that food ingestion (i.e., dietary intake) can be a significant pathway for metal accumulation in aquatic animals (Bryan and Langston 1992; Wang et al. 1996).

Silver (Ag) is one of the most toxic metals to aquatic organisms (Bryan and Langston 1992; Ho et al. 1995). Due to the extensive use of Ag in the photographic and imaging industries, Ag discharge from such industrial applications into the aquatic environment may potentially cause contamination and thereby has received increasing attention in recent years (Purcell and Peters 1998; Smith and Flegal 1993). The high particle-reactivity enables Ag to be easily associated with the particles and, as a result, taken up by aquatic animals (e.g., filter-feeders) through the dietary pathway (Ho et al. 1995; Ettajani et al. 1992). The bioaccumulation of Ag from ingested food is therefore critical in understanding and modeling Ag trophic transfer in the marine food chain and its bioavailability to aquatic organisms. A previous modeling study predicted that 40 – 70% of Ag in the mussels *Mytilus edulis* from Long Island Sound and San Francisco Bay can indeed be accumulated from the dietary intake (Wang et al. 1996).

The form of metal storage in the animal’s tissue is closely related to metal detoxification and bioaccumulation. Blackmore and Wang (2002) reported that Cd assimilation increased significantly by pre-exposing the green mussel *Perna viridis* to Cd, as a result of the increase in Cd body burden and the association of Cd with MTLPs. In the same study, Zn was found in the granules of mussels following pre-exposure to Zn. In addition to metal binding with metallothionein (MT) and granules, metals may also be bound with insoluble compounds such as sulfide (Berthet et al. 1992; George et al. 1986). The subcellular distribution of metals may thus play a critical role in metal accumulation and detoxification process, but this
aspect has received little attention.

Several studies have examined the influences of different Ag uptake pathways (i.e., water and food) on the bioaccumulation and toxicity of Ag as well as the physiology of aquatic invertebrates (Berthet et al. 1992; Sanders et al. 1990; Metayer et al. 1990). There is, however, limited understanding on the effects of Ag pre-exposure on subsequent metal bioaccumulation in marine bivalves. This study attempts to illustrate the effects of both aqueous and dietary Ag pre-exposure on Ag uptake and loss in the green mussel *Perna viridis*. The use of this species as a biomonitor and model organism for bioenergetic-based kinetic modeling study in the subtropical and tropical waters has been well established (Phillips and Rainbow 1988; Chong and Wang 2001). Following previously exposing mussels to Ag through either the dissolved or food phase, we then measured the assimilation efficiency from the particulate phase, the clearance rate, the influx rate from the dissolved phase, the efflux rate constant, and the subcellular Ag distribution in the mussels. These physiological parameters are essential for determining the metal body burden in the bivalves. These measurements represented somewhat ‘short-term’ (maximum of 5 weeks exposure) physiological responses to elevated Ag exposure.

2.3 Materials and methods

2.3.1 Collection of mussels

Green mussels *Perna viridis* with 3.0 – 3.5 cm shell lengths were collected from Wu Kai Sha, Tolo Harbour, Hong Kong, regarded as relatively uncontaminated by Ag (Rainbow and Blackmore 2001). They were maintained in aerated artificial seawater (Instant Ocean, 30‰) at 23 °C throughout the experimental period. During the acclimation period, the mussels were fed the diatom *Thalassiosira*
*Pseudonana* (clone 3H) at a ration 1 – 2% of tissue dry weight per day.

### 2.3.2 Metal pre-exposure treatments

Three different experiments were performed by exposing groups of mussels separately to different concentrations of Ag (as AgNO₃) through either the aqueous or dietary phase. The exposure treatments are shown in Table 2-1. In the dissolved exposure treatments, the mussels were exposed to Ag spiked seawater for 18 h each day and fed the diatom *T. pseudonana* in unspiked seawater for the remaining 6 h. In the food exposure treatments, the animals were maintained in clean seawater and fed Ag-enriched *T. pseudonana* for 4 h each day. These diatoms had been previously exposed to Ag for 24 h (for concentrations, see Table 2-1) and then filtered from the growth medium, rinsed with clean seawater and resuspended in clean water before being fed to the mussels. There was minimal desorption of diatom Ag into the dissolved phase within the short feeding period (i.e., > 90 – 100% of the Ag remained in the particle phase, see also Fisher and Wang 1998), thus Ag accumulated in the mussels in these treatments was considered to be derived from the dietary phase. In the control treatments, no Ag was spiked into the seawater or the growth medium of the diatoms. Seawater for all groups was changed every two days to keep the nominal metal concentrations relatively constant and the water quality in good condition. In Expt. 1 and Expt. 2, the metal assimilation efficiency from the ingested food, the clearance rate, the metal influx rate from the dissolved phase, the efflux rate, the metal subcellular distribution, and the Ag soft tissue concentrations were determined following the pre-exposure. In Expt. 3, these measurements (except the efflux rate and metal subcellular distribution) were made after exposing the mussels for 1, 3, and 5 weeks, respectively.
2.3.3 Metal assimilation efficiency from diatoms

Metal assimilation efficiency (AE) was determined as the percentage of initial radioactivity retained in the mussels after 60 h of depuration, using an established pulse-chase feeding technique (Chong and Wang 2000). The diatom *T. pseudonana* was radiolabeled with the radioisotope $^{110m}$Ag for 24 h at a radioactivity addition of 185 kBq L$^{-1}$. These radiolabeled cells were collected onto a 3 μm polycarbonate membrane and resuspended in 0.22 μm filtered seawater before being added to the feeding beakers.

Mussels from each treatment (including the control) were individually placed in a polypropylene beaker containing 500 ml of glass fiber filtered (GF/C) seawater. When the mussels opened and pumped normally, the radiolabeled diatom cells were added to result in a cell density of $4 - 5 \times 10^4$ cells ml$^{-1}$. Further additions were made at 10 min intervals to maintain this density. After 30 min feeding, the mussels were rinsed with clean seawater and radioassayed. For each treatment, five mussels were then placed individually into polypropylene beakers (180 ml seawater) held within a 10 L enclosed recirculating flow-through aerated seawater aquarium. Nonradioactive *T. pseudonana* was fed twice daily at a ration of about 2% dry weight per day. The radioactivity retained in the mussels was measured over a 72 h depuration period at intervals from 3 to 12 h. To minimize the possibility that radiotracers may have desorbed from the fecal materials into the ambient water and subsequently be accumulated by the mussels, the pellets were collected frequently during the depuration period.

2.3.4 Clearance rate of the mussels

This experiment quantified the influence of Ag body burden on the physiological
pumping rate of the mussels. Eight mussels from each treatment were placed individually into 1.5 L GF/C seawater within a polypropylene beaker. After the mussel opened and pumped normally (usually within 10 min), diatoms *T. pseudonana* (filtered from their growth medium) were added to each beaker at a concentration of $10^4$ cell ml$^{-1}$. The algal suspension in each beaker was homogenized by a magnetic stirrer. Immediately after adding the algae, a 10 ml aliquot water sample was taken and the cell density was counted using a Coulter Counter. Further water samples were taken at 20 min and 40 min and the cell density was determined. The clearance rate was calculated using the following equation (Widdows et al. 1997):

$$CR = (\ln C_1 - \ln C_2) \times \frac{\text{Vol}}{T}$$  \(1\)

where CR is the clearance rate of the mussels (L h$^{-1}$), $C_1$ is the cell density (cell ml$^{-1}$) at time 1, $C_2$ is the cell density (cell ml$^{-1}$) at time 2, $T$ is the time interval of measurement ($T_2 - T_1$, in h) and Vol is the volume of water (L). The clearance rate of each individual was finally calculated from the mean of the two consecutive measurements at 20 min intervals.

### 2.3.5 Trace metal influx rate from the dissolved phase

The radiotracer $^{110m}$Ag (4.6 kBq L$^{-1}$, equivalent to 0.4 nM) was spiked into 0.22 µm filtered seawater and allowed to equilibrate overnight (Wang et al. 1996). Following this addition, microliter amounts of 0.5 N Suprapure NaOH were added to the seawater to maintain the pH. Short-term exposure (1 h) was employed in this study to determine Ag influx rate from the dissolved phase in order to avoid the
decline of mussel’s ventilating activity due to the absence of food particles, as well as the decrease in Ag ambient concentration. Eight replicated individuals from each treatment were placed individually in 200 ml seawater for 1 h, after which the mussels were dissected and the radioactivity of the soft tissues was measured. The tissues were then dried at 80 °C overnight and the dry weights were determined. The influx rate was calculated as the amount of metal accumulated by the soft tissue of the mussel and was standardized as per nanogram dry weight per hour (ng g\(^{-1}\) h\(^{-1}\)). During the 1 h exposure period, any feces egested by the mussels were removed immediately to minimize fecal scavenging of the metal.

### 2.3.6 Metal efflux from mussels

The mussels were radiolabeled with \(^{110m}\text{Ag}\) (with the addition of stable Ag to maintain different nominal Ag concentrations) during their pre-exposure to either the dissolved or the food phase (Table 2-1). In the control treatments (two controls in Expt. 1, one for the dissolved exposure, the other for the food exposure), only the radioisotope was used to label the diatom or seawater. Due to the lack of significant efflux difference in the food and dissolved control groups in Expt. 1 (see results), only one control (for both the dissolved exposure and food exposure) was used in Expt. 2. In Expt. 1, the radioactive addition was 0.74 kBq L\(^{-1}\) \(^{110m}\text{Ag}\) for both the dissolved and food exposures; in Expt. 2, the radioactivity addition was 1.48 kBq L\(^{-1}\) \(^{110m}\text{Ag}\). Following the exposure, ten mussels were removed and rinsed with clean seawater and their radioactivity was counted. They were then placed individually into 180 ml polypropylene beakers within an enclosed recirculating seawater aquarium, as described above, to depurate \(^{110m}\text{Ag}\). The aquarium water was changed twice weekly. Nonradioactive \(T.\ pseidonana\) was fed twice daily at a
ration of about 2% dry weight per day. Throughout the course of depuration, the radioactivity of each mussel was assayed periodically and fecal pellets were collected frequently to minimize the desorption of radiotracers from the fecal materials into the water. On day 0, 16 and the last day of depuration, 3 individuals from each treatment were dissected and the radioactivity in the shell, digestive gland and other soft tissues was counted.

2.3.7 Stable Ag tissue concentration

Following the pre-exposure, five mussels from each treatment were dissected, and their soft tissues were dried at 60 °C to a constant weight and then digested in concentrated nitric acid (HNO₃, Aristar grade BDH Ltd.). These digests were diluted with Nanopure water to make the Ag concentration in an appropriate range for analysis by Inductively Coupled Plasma-Mass Spectroscopy (ICP-MS) (Perkin Elmer, Elan 6000). The stable Ag tissue concentration was expressed as μg g⁻¹ dry weight.

2.3.8 Subcellular Ag distribution

Differences in the subcellular Ag distribution among different treatments after the exposure of mussels to stable metal and radioisotope were investigated by homogenizing the soft tissues of the mussels and subjecting the homogenate to differential centrifugation and tissue digestion procedures, using a modified method of Wallace et al. (1998).

Five mussels from each treatment were dissected and the soft tissues were homogenized in 6 ml Nanopure water. The homogenate was centrifuged at 1450 g (15 min at 4 °C) and the supernatants were spun at 100,000 g (1 h at 4 °C) to separate the cytosol and proteins from the intracellular pellets containing nuclear,
mitochondrial and microsomal fractions. The 100,000 g supernatants containing
cytosol and protein were further centrifuged at 50,000 g (10 min at 4 °C) after being
heated (10 min at 80 °C) and further ice-cooled (1 h). This separated the heat-stable
proteins or metallothionein-like proteins (MTLPs), which remained in the
supernatant, from the heat-sensitive proteins (HSPs) that were denatured by the heat
treatment and thus formed the pellet. The 1450 g pellet contained tissue fragments
and other cellular debris (i.e., membranes and metal-rich granules – MRG). MRG
was isolated from other cellular debris by resuspending the pellet in 1 ml Nanopure
water, heating at 100 °C for 2 min and subsequently adding an equal volume of 1 N
NaOH, followed by heating at 70 °C for 1 h. The differential centrifugation as well
as above treatments resulted in 2 supernatants (i.e., MTLPs and cellular debris) and 3
pellets (i.e., organelles, HSPs and MRG). All fractions were radioassayed for
\(^{110m}\)Ag to elucidate the subcellular Ag distribution.

2.3.9 Radioactivity measurements and statistical analysis

Radioactivity was measured using a Wallac 1480 NaI (T1) gamma detector
(Wallac Turku, Finland). All counts were related to standards and radioactive decay
was corrected. The gamma emission of \(^{110m}\)Ag was determined at 658 keV.
Counting times were adjusted to yield a propagated counting error < 5%. Statistical
analysis was carried out through analysis of variance (ANOVA) or t-test. Statistical
significant difference was accepted at \(p < 0.05\)

2.4 Results

2.4.1 Stable Ag body concentration

In Expt. 1, with one exception (food 0.5 \(\mu g\) L\(^{-1}\) treatment), the Ag tissue
concentrations of all exposed mussels (1.0 – 2.3 μg g⁻¹) were significantly higher (p < 0.05) than those of the controls (0.5 μg g⁻¹) after 14 d exposure (Table 2-2). A similar trend was observed in Expt. 2 that body Ag concentrations in exposed mussels (1.4 – 3.4 μg g⁻¹) increased as compared to the controls (0.581 μg g⁻¹) (Table 2-3). In Expt. 3, Ag soft tissue concentrations in the mussels from each exposed treatment increased significantly (p < 0.01) with increasing exposure time (Table 2-4). In the 0.5 μg L⁻¹ dissolved exposure treatment, soft tissue concentration increased from 0.7 μg g⁻¹ in week 1 to 1.1 μg g⁻¹ in week 3 and to 3.9 μg g⁻¹ in week 5. In the 5 μg L⁻¹ dissolved exposure treatment, the soft tissue concentrations in weeks 1, 3 and 5 were 3.1, 7.5 and 17.2 μg g⁻¹, respectively. Following 30 μg L⁻¹ food exposure, the Ag body concentrations of three time points were 2.3, 8.1 and 19.3 μg g⁻¹, respectively. The increase of Ag tissue concentration was the greatest (62 ×) following the food exposure to 30 μg L⁻¹ for 5 weeks.

2.4.2 Ag assimilation from ingested diatoms

Depuration of Ag in the mussels following pulse-chase radioactive feeding is shown in Fig. 2-1 to Fig. 2-3. Assimilation efficiencies (AEs) were calculated as the amount of radioactivity left in the mussels at 60 h divided by the amount of radioactivity ingested, measured following the 30 min pulse radioactive feeding. In Expt. 1 (Table 2-2), the Ag AEs were 2.1 × greater (p < 0.01) in those individuals that had been exposed to dissolved 5 μg L⁻¹ for 14 d as compared with those in the controls and other treatments. There was essentially no loss of Ag from the mussel body after 1 d of depuration in this treatment. In Expt. 2, following 7 d exposure, only the mussels from the 15 μg L⁻¹ dissolved exposure treatment assimilated Ag at a significantly higher level (p < 0.001) than the controls (Table 2-3). Similarly, there
was no apparent loss of Ag after the initial rapid loss (< 6 h). In Expt. 3, the Ag assimilation was gradually affected by increasing the time of exposure (Table 2-4). After 1-week exposure, no major difference ($p > 0.05$) of Ag AEs between the exposure treatments and the control was observed. Following two more weeks of exposure, the AEs in the dissolved 5 µg L$^{-1}$ and the food 30 µg L$^{-1}$ treatments increased significantly ($p < 0.01$) compared with those of the controls. After another two weeks of exposure, the mussels from all the exposure treatments had significantly higher ($p < 0.01$) AEs than the control. Generally, the depuration pattern of Ag following a pulse of radioactive feeding was characterized by a rapid initial egestion within the first 12 h, followed by a continuous, but slower, egestion during 24 to 72 h. Mussels that exhibited significantly higher AEs than the controls retained the Ag very efficiently after the initial stage of digestion. The much slower metal egestion replaced the second phase of continuous egestion as found in the control treatments, thereby resulting in the increase of Ag assimilation.

2.4.3 Clearance rate of mussels

Following exposure to Ag for 14 d (Expt. 1), only those mussels from the 5 µg L$^{-1}$ food exposure treatment exhibited a significantly lower clearance rate than the control ($p < 0.05$) (Table 2-2). In Expt. 2, the clearance rates in mussels exposed to dissolved 15 µg L$^{-1}$ and food 100 µg L$^{-1}$ were 26 – 38% higher compared to the controls but these differences were not statistically significant ($p > 0.05$) (Table 2-3). In Expt. 3, after one week of exposure, only mussels in the food 30 µg L$^{-1}$ treatment had a significant ($p < 0.01$) reduction in the clearance rate compared with the controls (Table 2-4). Following three weeks of exposure, the clearance rates of the exposed mussels were 9 – 26% lower than the controls, but the differences were not
significant \((p > 0.05)\). Additionally, following two more weeks of exposure, no significant difference between the exposed mussels and the controls was observed \((p > 0.05)\). Furthermore, with increasing the exposure time (from week 1 to week 5), the clearance rates in the pre-exposed and control mussels increased gradually and the clearance rates in the pre-exposed individuals approached those of the controls.

### 2.4.4 Ag uptake from the dissolved phase

The influx rate of Ag from the dissolved phase in the mussels in the three different experiments is shown in Table 2-2 to Table 2-4. In Expt. 1, the Ag influx into those individuals exposed to dissolved Ag for 14 d was 14 – 32% lower (33.8 – 42.6 ng g\(^{-1}\) h\(^{-1}\)) as compared with the controls (49.6 ng g\(^{-1}\) h\(^{-1}\)). This decrease was only significant \((p < 0.001)\) for the group that had been exposed to dissolved 5 μg Ag L\(^{-1}\). The influx of Ag into the mussels was similar between the food exposure treatment (52.3 – 53.0 ng g\(^{-1}\) h\(^{-1}\)) and the controls. After 7 d pre-exposure (Expt. 2), the influx rates in the mussels pre-exposed to dissolved 15 μg L\(^{-1}\), food 20 μg L\(^{-1}\) and food 100 μg L\(^{-1}\) were 6.5, 6.5 and 5.68 ng g\(^{-1}\) h\(^{-1}\), respectively, and were decreased by 8 – 21% compared with the control (7.1 ng g\(^{-1}\) h\(^{-1}\)). In Expt. 3, the influx rates following one week pre-exposure (7.5 – 9.4 ng g\(^{-1}\) h\(^{-1}\)) were somewhat lower than the control (9.7 ng g\(^{-1}\) h\(^{-1}\)) but the difference was not statistically significant \((p > 0.05)\). After two more weeks of pre-exposure, although there was still no significant difference \((p > 0.05)\) between all the pre-exposed mussels and the control, the influx rates (10.1 and 11.7 ng g\(^{-1}\) h\(^{-1}\), respectively) in the mussels from dissolved 5 μg L\(^{-1}\) and food 30 μg L\(^{-1}\) groups were 3 – 20% higher than the control (9.8 ng g\(^{-1}\) h\(^{-1}\)). By the end of the pre-exposure (week 5), the influx rates (14.0 and 15.3 ng g\(^{-1}\) h\(^{-1}\), respectively) in these two treatments increased significantly \((p < 0.01)\) as compared
with the control (9.7 ng g$^{-1}$ h$^{-1}$). In contrast, the influx rate in those mussels exposed to 0.5 µg L$^{-1}$ (9.0 ng g$^{-1}$ h$^{-1}$) was lower than that of the control treatment.

2.4.5 Ag efflux rate from mussels

In Expt. 1 and Expt. 2, the depuration of Ag in the mussels following exposure to both radioisotope and stable metal is shown in Fig. 2-4 and Fig. 2-5. The depuration pattern was characterized by an initial rapid loss of Ag during the first 5 – 6 d followed by a second slower loss for the remaining period (6 d onward). The efflux rate constants were calculated from the slope of the natural log of the percentage of Ag retained in the mussels and the time of depuration (between 6 and 24 d). In Expt. 1, the Ag efflux rate constant (0.002 d$^{-1}$) was significantly lower ($p < 0.001$) following 14 d exposure to dissolved 5 µg L$^{-1}$ than the control (0.016 d$^{-1}$). The efflux rate constant (0.013 d$^{-1}$) of the mussels exposed to dissolved 0.5 µg L$^{-1}$ was not significantly different ($p > 0.05$) from the control. Compared with the control (0.021 d$^{-1}$), the two food exposure treatments (0.027 and 0.015 d$^{-1}$, respectively) were not significantly different ($p > 0.05$). In Expt. 2, the efflux rate constants of the mussels exposed to dissolved 15 µg L$^{-1}$ (0.002 d$^{-1}$) and food 100 µg L$^{-1}$ (0.006 d$^{-1}$) for 7 d were significantly lower ($p < 0.001$) than the control (0.011 d$^{-1}$), which was not different from those individuals exposed to food 20 µg L$^{-1}$ ($p > 0.05$).

In Expt. 2, the distribution of Ag in the shells (including both surface sorption and incorporation), soft tissues and digestive glands during the depuration period is shown in Fig. 2-6. The control mussels contained negligible amounts of Ag in the shells (3 – 5%), and the majority of Ag was distributed in the soft tissues (35 – 45%) and digestive gland (> 49%). In general, following dissolved Ag exposure, a larger
fraction (38 – 46%) of Ag was found in the shell, while most (40 – 73%) of the Ag was distributed within the digestive gland of the mussels exposed to dietary Ag. The Ag distribution in each part of the mussels throughout the depuration period did not vary greatly.

2.4.6 Subcellular Ag distribution after pre-exposure

Subcellular Ag distributions in the exposed and control mussels of Expt. 1 and Expt. 2 are shown in Fig. 2-7 and Fig. 2-8, respectively. The control mussels in the two experiments had a similar Ag distribution, with about 25 – 32% being distributed in the metal-rich granule fraction (MRG), 52 – 56% in the other cellular debris (tissue fraction), < 5% in the intracellular fraction (organelles), and about 8 – 19% in the cytosol (100,000 g supernatants). For all the exposed mussels in both experiments, most of the Ag was associated with the debris (45 – 64%) and MRG (18 – 40%) fractions. When compared with the controls, however, there was no consistent pattern of subcellular Ag distribution in the exposed mussels in two experiments. Ag distribution in the metallothionein-like protein (MTLP) fraction either increased (i.e., dissolved 0.5 μg L\(^{-1}\) and food 5 μg L\(^{-1}\) groups in Expt. 1) or decreased (i.e., dissolved 5 μg L\(^{-1}\), food 0.5 μg L\(^{-1}\) and all exposed groups in Expt. 2) following pre-exposure, but the difference was not significant (\(p > 0.05\)). A similar pattern was also observed in the Ag distribution in the MRG, the debris as well as the heat-sensitive proteins (HSP) and the MTLP when comparing the exposed mussels with the controls.

2.5 Discussion

To investigate the effects of elevated body Ag concentrations on subsequent Ag
uptake and loss in the mussels, the Ag levels used in the pre-exposure were much higher than the typical Ag concentrations in coastal seawater. This is to ensure the elevation of Ag body burden in the mussels within a relatively short period (maximum of 5 weeks of exposure). The body Ag concentrations achieved from such pre-exposure were however environmentally realistic. For example, body Ag concentrations in the exposed treatments were lower than the highest concentrations (about 70 μg g⁻¹ dry weight) observed in the mussel *Mytilus californianus* (Martin et al. 1988). Moreover, Luoma and Phillips (1988) reported that Ag concentrations in bivalves living in contaminated environments exceeded 100 μg g⁻¹ dry weight, which are much greater than the highest body concentrations resulting from pre-exposure in our study. It should also be noted that the body burdens of Ag in the mussel tissues were achieved after 1-5 weeks exposure, and it is possible that the Ag biochemical fates in the mussels may have been different from those found in field collected individuals that have been exposed to Ag throughout their life-time. Extrapolation of the laboratory results to the field situation remains to be further tested.

In our study, there were some differences in the measured physiological parameters among the three different experiments in the controlled treatment, especially for Expt. 1 which had a much higher dissolved uptake rate constant as compared to the other two experiments. The AE and the clearance rate of the mussels were however comparable among the three different experiments. Reason for the abnormally high Ag influx rate from the dissolved phase is unknown, but may be due to the seasonal variation of protein metabolism in the mussels, as the three experiments were conducted at different seasons. In Expt. 3 which encompassed a 5 weeks exposure, however, the quantified Ag AE, influx rate, and clearance rate of the mussels were remarkably comparable in the control treatment.
following 1, 3, and 5 weeks pre-exposure, suggesting that these physiological processes were maintained rather constant within this period of exposure.

Furthermore, we included a control treatment in each experiment to contrast the biokinetics of Ag in the mussels pre-exposed to Ag. Any difference from the control treatment within each single experiment was therefore due to the Ag effect.

The present work demonstrated that no significant increase in Ag associated with the MTLP fraction was achieved following exposure to the metal through either the dissolved or food phase in the mussels. Preliminary experiments showed that there was also not significant difference in the metallothionein concentrations in mussels exposed to these concentrations of Ag (Shi et al., unpublished data). This suggests that Ag elevation in the soft tissues following pre-exposure is likely due to the metal binding with other ligands instead of metalloprotein. Indeed, up to 80% of Ag in marine bivalves may be bound with sulfides while only a small part may be associated with the protein (Fisher and Wang 1998). Whether the Ag-sulfide complex is associated with any the subcellular fractions (e.g., MRG) remained unknown in this study. Considering the extremely high toxicity of Ag (Bryan and Langston 1992; Ho et al. 1995), the accumulation of Ag mainly as an insoluble sulfide complex, which is very stable, may be regarded as a detoxification mechanism in the mussels. Such strong binding may also have implication for the potential trophic transfer of Ag to higher trophic levels, since earlier experimental studies indicated that metals bound strongly with metal-rich granules may have a lower bioavailability to higher animals (Wallace et al. 1998). In agreement, by exposing the mussel Mytilus edulis to dissolved 25 µg Ag L\(^{-1}\) for 1 year, George et al. (George et al. 1986) found that most of the Ag was associated with sulfide and stored along the basement membranes of the digestive gland and kidney.
Data presented herein show that Ag AE generally increased following pre-exposure to Ag. In Expt. 3, the AE in each exposed treatment increased with increasing pre-exposure period and the body Ag concentrations. A possible physiological mechanism underlying the increase of Ag AE may be described as the induction of sulfide complexes in the mussel. As a consequence, compared with the unaffected mussels, the exposed individuals may have more sulfide ligand for Ag binding. Meanwhile, more Ag binding complexes may have been induced when more Ag was accumulated, enabling mussels to further assimilate more Ag from the particulate source. In agreement, Calabrese et al. (1984) reported that the amount of yellowish-brown to black particulates in the basement membrane and connective tissue of the body organs in the blue mussel Mytilus edulis (e.g., digestive diverticula and kidney) increased with an increase in Ag exposure concentration. In a recent study, Blackmore and Wang (2002) found a similar phenomenon that due to the sequestration of Cd by MTLPs, Cd pre-exposure leads to an increase of its AE in the green mussels. These studies thus highlight the complicate mechanisms in metal sequestration that may affect metal subsequent assimilation from the dietary phase.

With a comparable Ag body concentration obtained from different exposure pathways (i.e., dissolved or dietary phase), the effect of different Ag sources on Ag AE can be compared. For example, following the 3 week pre-exposure (Expt. 3), the body Ag concentrations in the mussels exposed to the dissolved 5 μg L⁻¹ (7.5 μg g⁻¹) were similar to those in the individuals exposed to the food 30 μg L⁻¹ (8.1 μg g⁻¹). Concomitantly, there was no significant difference between the Ag AEs of these two treatments (28% and 24%, respectively). This result suggests that the level of accumulated Ag is important rather than the source and, therefore, both the dissolved and food pathways play a role on Ag bioavailability to the mussels.
Clearance rates have been utilized as good indicators reflecting the toxic effects of pollutants in organisms (Naimo 1995). For example, Krishnakumar et al. (1990) found that after a 2-week dissolved exposure to 25 μg L⁻¹ Cu and Hg, the clearance rates decreased significantly. In the zebra mussel Dreissena polymorpha, the clearance rates were affected by exposure to high concentrations of Pb or Zn (85 and 382 μg L⁻¹, respectively), but not by lower metal levels (Kraak et al. 1994). In that study, a different pattern of variance of the clearance rate with an increase in the metal body concentration was found between Pb and Zn. Our study show that, in Expt. 1 and 2, except for the 5 μg L⁻¹ (14 d) food exposure treatment, the clearance rates were not significantly affected by Ag pre-exposure as compared with the control, suggesting that the Ag pre-exposure treatments had little toxic effect on the mussels. It remains possible, however, that the concentration of the metal accumulated during the pre-exposure period was not sufficiently high to affect the clearance rate. This was further investigated in Expt. 3, in which the clearance rate was measured with increasing Ag body concentrations. The results show that although the clearance rates of pre-exposed mussels (except for food 30 μg L⁻¹ in week 1) were lower (not significant) than those of the controls, which might indicate some toxic effects of Ag pre-exposure, there was no significant relationship between the increased Ag accumulation in the soft tissues and the clearance rate. Moreover, the clearance rates of all mussels (both pre-exposed and the control) increased gradually over the experimental period. This phenomenon may be a reflection of the acclimatization of mussels to the laboratory conditions, and furthermore suggests that over the concentrations measured in the present study, Ag body burdens have little effect on the clearance rate.

Our results indicate that the relationship between Ag pre-exposure and dissolved
uptake rate was rather complex. With increasing the body Ag concentrations following pre-exposure, the influx rate either decreased or increased. In three independent experiments, the influx rate decreased with increasing body Ag concentration until it reached around 2.5 μg g⁻¹, above which this pattern was then reversed and the influx rate began to increase. It may be likely that the binding ligand such as sulfides had not yet been induced when the body Ag concentration was at a low level (< 2.5 μg g⁻¹), and the decrease in the influx rate may reflect a protection mechanism for the ambient toxic metal. With a continual accumulation of Ag to a high soft tissue concentration (> 2.5 μg g⁻¹), Ag complexing ligands may have been induced, and the uptake may therefore increase because of the strong affinity of Ag with these complexing ligands (Fisher and Wang 1998). Thus, mussels with higher body Ag concentrations may have more potential sulfide binding sites, resulting in a higher efficiency of Ag uptake from the dissolved phase. This is also consistent with our results of AE (see above).

The reduction of the Ag influx rate agreed with the phenomenon observed in the clam *Macoma balthica* by Boisson et al. (1998). Clams subjected to chronic Ag contamination accumulated Ag at a significantly lower rate than those originating from a clean estuary. In the mussel *P. viridis*, the Cd and Zn dissolved uptake reduced following Zn pre-exposure, although this pattern was not achieved following Cd pre-exposure (Blackmore and Wang 2002). Rainbow et al. (1999), however, reported that the mean metal uptake rates of amphipods and crabs from a metal-enriched site were not lower than those of the same crustaceans from a control site. Further study is therefore needed to fully understand the influence of Ag pre-exposure and resulting body burden on the dissolved uptake rate and to better illustrate the function of sulfides in this process.
It should be noted that we pre-exposed the mussels to different Ag concentrations for a maximum of 5 weeks, and thus our measurements represented relatively short-term physiological responses to Ag exposure (e.g., physiological acclimatization) and the influences of different Ag tissue body burdens on the biokinetics of Ag in the mussels. In the natural environments, the bivalve populations have been exposed to different metal levels throughout their life-times, and it is likely that they may have developed adaptations to metal enriched environments, as shown by Boisson et al. (1998). Thus, it is possible that the metal accumulation in the metal enriched environments may be due to the selective forces where offspring's tolerant of a metal enriched environment have been selected for, resulting a metal tolerant population. In a recent study, we compared the Cd biokinetics in mussels and clams following physiological acclimatization (e.g., 5 weeks exposure) and from a Cd contaminated bay. We found that the physiological responses following 5 weeks pre-exposure were consistent with those collected from the contaminated bay where the bivalves were exposed to Cd throughout their life-times (Shi and Wang, unpublished data).

Following pre-exposure, the efflux rate constants of Ag decreased with increasing body Ag concentrations. This effect can be similarly explained by the binding of Ag with sulfides induced by the chronic Ag pre-exposure. Such complexes are very stable and not easily depurated by the mussels, thus leading to a smaller efflux rate constant in those individuals with higher body Ag concentration. Our studies were also designed to compare the efflux rate constants in mussels pre-exposed to Ag through different routes. Generally, Ag efflux was slower in the mussels pre-exposed to dissolved Ag than in those pre-exposed to dietary Ag (e.g., Expt. 1). Furthermore, the results show that Ag uptake from seawater (the
dissolved phase) is mainly distributed in shells while following the food exposure Ag is found mostly in the digestive glands. These results agree well with results from the mussel Mytilus galloprovincialis (Fisher et al. 1996). In contrast, Berthet et al. (1992) found that the oyster Crassostrea gigas lost Ag accumulated from food slower than from the dissolved phase.

This study shows that Ag pre-exposure substantially affects its bioavailability to the green mussel P. viridis. Additionally, in contrast to Cd accumulation in the mussel Mytilus edulis (Riisgård et al. 1987) and Ag accumulation in the oyster Crassostrea gigas (Ettajani et al. 1992; Berthet et al. 1992; Sanders et al. 1990), the present study indicates that Ag uptake from the dietary phase can be important to its bioaccumulation in the mussel P. viridis and thus contribute to bioavailability. Following chronic exposure to Ag, mussels increased AE and the dissolved uptake rate (when the metal body burden was above certain level, see above) and decreased the efflux rate, resulting in accumulating more Ag as compared with the control individuals. One point to highlight is that Ag-sulfide complexes may play a significant role in the mechanism of Ag uptake and loss by the mussels. These results indicate that the body Ag concentration may not simply reflect the real bioavailable metal level in the environment. Mussels from Ag-enriched environments may have somewhat higher Ag body burden than anticipated due to the disproportionally higher uptake from both the aqueous and dietary pathways. In this case, the physiological process of acclimatization to ambient conditions appears to be of importance when interpreting Ag body concentrations in mussels used as biomonitors for metal pollution. Further study is, therefore, needed to investigate the detailed process of intracellular metal detoxification in order to better understand the acclimatization of the mussels to chronic Ag exposure from different pathways.
2.6 References


Chong, K., and Wang, W.-X. 2001. Comparative studies on the biokinetics of Cd, Cr,


Table 2-1 Ag concentrations (μg L⁻¹) and exposure durations (wks) used in different pre-exposure experiments with the green mussel *Perna viridis*. D: dissolved exposure, the mussels were directly exposed to Ag in the seawater; F: food exposure, the diatom food *Thalassiosira pseudonana* was exposed to the Ag concentration in the growth medium before being fed to the mussels.

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Duration (wks)</th>
<th>Ag concentration (μg L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>D0.5 D0.5 F0.5 F5</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>D15 F20 F100</td>
</tr>
<tr>
<td>3</td>
<td>1, 3, 5</td>
<td>D0.5 D5 F30</td>
</tr>
</tbody>
</table>

49
Table 2-2 Ag body burden (μg g⁻¹, mean ± SD; n = 5), Ag assimilation efficiency (AE) (%; mean ± SD; n = 5), Ag influx rate from the dissolved phase (ng g⁻¹ h⁻¹; mean ± SD; n = 8), Ag efflux rate constant (d⁻¹; mean ± SD; n = 8), and clearance rate (CR) (L g⁻¹ h⁻¹; mean ± SD; n = 8) in the green mussel *Perna viridis* following Ag pre-exposure in Expt. 1. *a* Control for dissolved exposure; *b* Control for food exposure. Significant difference from the control treatment was indicated by *: p < 0.05 and **: p < 0.01.

<table>
<thead>
<tr>
<th>Treatment (μg L⁻¹)</th>
<th>Ag (μg g⁻¹)</th>
<th>AE (%)</th>
<th>Influx rate (ng g⁻¹ h⁻¹)</th>
<th>Efflux rate constant (d⁻¹)</th>
<th>CR (L g⁻¹ h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>0.5 ± 0.1</td>
<td>21.1 ± 6.4</td>
<td>49.6 ± 5.9</td>
<td>0.016 ± 0.005&lt;sub&gt;a&lt;/sub&gt;</td>
<td>10.3 ± 2.3</td>
</tr>
<tr>
<td>D0.5</td>
<td>1.5 ± 0.3&lt;sup&gt;*&lt;/sup&gt;</td>
<td>29.2 ± 5.9</td>
<td>42.6 ± 10.6</td>
<td>0.013 ± 0.006</td>
<td>10.9 ± 2.1</td>
</tr>
<tr>
<td>D5</td>
<td>2.3 ± 1.6&lt;sup&gt;*&lt;/sup&gt;</td>
<td>43.4 ± 10.9&lt;sup&gt;**&lt;/sup&gt;</td>
<td>33.8 ± 3.8&lt;sup&gt;**&lt;/sup&gt;</td>
<td>0.002 ± 0.008&lt;sup&gt;**&lt;/sup&gt;</td>
<td>8.4 ± 2.4&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>F0.5</td>
<td>0.9 ± 0.3</td>
<td>25.0 ± 5.1</td>
<td>53.0 ± 10.8</td>
<td>0.027 ± 0.013</td>
<td>10.8 ± 1.7</td>
</tr>
<tr>
<td>F5</td>
<td>1.0 ± 0.2&lt;sup&gt;*&lt;/sup&gt;</td>
<td>25.4 ± 8.7</td>
<td>52.3 ± 13.2</td>
<td>0.015 ± 0.011</td>
<td>8.0 ± 1.7</td>
</tr>
</tbody>
</table>
Table 2-3 Ag body burden (µg g⁻¹, mean ± SD; n = 5), Ag assimilation efficiency (AE) (%), mean ± SD; n = 5), Ag influx rate from the dissolved phase (ng g⁻¹ h⁻¹, mean ± SD; n = 8), Ag efflux rate constant (d⁻¹, mean ± SD; n = 8), and clearance rate (CR) (L g⁻¹ h⁻¹, mean ± SD; n = 8) in the green mussel *Perna viridis* following Ag pre-exposure in Expt. 2. Significant difference from the control treatment was indicated by *: p < 0.05 and **: p < 0.01.

<table>
<thead>
<tr>
<th>Treatment (µg L⁻¹)</th>
<th>Ag (µg g⁻¹)</th>
<th>AE (%)</th>
<th>Influx rate (ng g⁻¹ h⁻¹)</th>
<th>Efflux rate constant (d⁻¹)</th>
<th>CR (L g⁻¹ h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>0.6 ± 0.1</td>
<td>18.7 ± 4.3</td>
<td>7.1 ± 3.0</td>
<td>0.011 ± 0.004</td>
<td>9.1 ± 1.6</td>
</tr>
<tr>
<td>D15</td>
<td>3.4 ± 0.2*</td>
<td>37.7 ± 5.8**</td>
<td>6.5 ± 1.6</td>
<td>0.002 ± 0.004**</td>
<td>12.6 ± 1.0</td>
</tr>
<tr>
<td>F20</td>
<td>1.4 ± 0.6*</td>
<td>16.3 ± 5.8</td>
<td>6.5 ± 1.7</td>
<td>0.011 ± 0.007</td>
<td>8.4 ± 1.8</td>
</tr>
<tr>
<td>F100</td>
<td>2.6 ± 0.5*</td>
<td>20.8 ± 4.6</td>
<td>5.6 ± 1.2</td>
<td>0.006 ± 0.008**</td>
<td>11.5 ± 2.7</td>
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</table>
Table 2-4 Ag body burden (µg g⁻¹, mean ± SD; n = 5), Ag assimilation efficiency (AE) (% mean ± SD; n = 5), Ag influx rate from the dissolved phase (ng g⁻¹ h⁻¹, mean ± SD; n = 8), and clearance rate (CR) (L g⁻¹ h⁻¹, mean ± SD; n = 8) in the green mussel *Perna viridis* following Ag pre-exposure in Expt. 3. Significant difference from the control treatment was indicated by *: p < 0.05 and **: p < 0.01.

<table>
<thead>
<tr>
<th>Duration (wks)</th>
<th>Treatment (µg L⁻¹)</th>
<th>Ag (µg g⁻¹)</th>
<th>AE (%)</th>
<th>Influx rate (ng g⁻¹ h⁻¹)</th>
<th>CR (L g⁻¹ h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C</td>
<td>0.3 ± 0.2</td>
<td>14.5 ± 6.5</td>
<td>9.7 ± 3.3</td>
<td>10.6 ± 1.9</td>
</tr>
<tr>
<td></td>
<td>D0.5</td>
<td>0.7 ± 0.2*</td>
<td>18.7 ± 6.5</td>
<td>9.4 ± 4.1</td>
<td>7.6 ± 2.3</td>
</tr>
<tr>
<td></td>
<td>D5</td>
<td>3.1 ± 1.1**</td>
<td>24.0 ± 5.2</td>
<td>7.6 ± 2.2</td>
<td>6.9 ± 1.7</td>
</tr>
<tr>
<td></td>
<td>F30</td>
<td>2.3 ± 0.5**</td>
<td>15.8 ± 4.5</td>
<td>7.5 ± 1.8</td>
<td>2.5 ± 1.0**</td>
</tr>
<tr>
<td>1</td>
<td>C</td>
<td>0.3 ± 0.2</td>
<td>12.4 ± 2.4</td>
<td>9.8 ± 2.9</td>
<td>17.6 ± 3.0</td>
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<tr>
<td></td>
<td>D0.5</td>
<td>1.1 ± 0.4**</td>
<td>14.4 ± 1.4</td>
<td>8.6 ± 2.3</td>
<td>16.0 ± 1.7</td>
</tr>
<tr>
<td></td>
<td>D5</td>
<td>7.5 ± 2.5**</td>
<td>28.4 ± 6.2**</td>
<td>10.1 ± 2.5</td>
<td>14.7 ± 3.3</td>
</tr>
<tr>
<td></td>
<td>F30</td>
<td>8.1 ± 2.1**</td>
<td>24.2 ± 7.1**</td>
<td>11.7 ± 4.4</td>
<td>13.0 ± 3.1</td>
</tr>
<tr>
<td>3</td>
<td>C</td>
<td>0.3 ± 0.2</td>
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<td>18.1 ± 6.4</td>
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<tr>
<td></td>
<td>D0.5</td>
<td>3.9 ± 1.9**</td>
<td>29.0 ± 8.9**</td>
<td>9.0 ± 2.4</td>
<td>16.6 ± 4.3</td>
</tr>
<tr>
<td></td>
<td>D5</td>
<td>17.2 ± 3.9**</td>
<td>39.9 ± 8.7**</td>
<td>14.0 ± 4.5**</td>
<td>17.4 ± 5.7</td>
</tr>
<tr>
<td></td>
<td>F30</td>
<td>19.3 ± 6.3**</td>
<td>25.9 ± 1.4**</td>
<td>15.3 ± 2.1**</td>
<td>17.4 ± 5.4</td>
</tr>
</tbody>
</table>
Fig. 2-1 Retention of Ag in the Ag-pre-exposed mussel *Perna viridis* following a pulse ingestion of the radiolabeled diatoms *Thalassiosira pseudonana* in Expt. 1. Data are mean ± SD (n = 5). C: control; D: dissolved exposure; F: food exposure. Numbers in the legends are the Ag concentrations used in the exposure treatment (see Table 2-1).
Fig. 2-2 Retention of Ag in the Ag-pre-exposed mussel *Perna viridis* following a pulse ingestion of the radiolabeled diatoms *Thalassiosira pseudonana* in Expt. 2. Data are mean ± SD (n = 5). C: control; D: dissolved exposure; F: food exposure. Numbers in the legends are the Ag concentrations used in the exposure treatment (see Table 2-1).
Fig. 2-3 Retention of Ag in the Ag-pre-exposed mussel *Perna viridis* following a pulse ingestion of the radiolabeled diatoms *Thalassiosira pseudonana* in Expt. 3. Data are mean + SD (n = 5). C: control; D: dissolved exposure; F: food exposure. Numbers in the legends are the Ag concentrations used in the exposure treatment (see Table 2-1).
Week 1

Week 3

Week 5

% retained in mussels

Time of depuration (h)
Fig. 2-4 Retention of Ag in the Ag-pre-exposed green mussel *Perna viridis* in Expt. 1. Data are mean ± SD (n = 8). CD: control after dissolved exposure; CF: control after food exposure; D: dissolved exposure; F: food exposure. Numbers in the symbol legends are the nominal Ag concentrations used in the exposure treatments (see Table 2-1).
Fig. 2-5 Retention of Ag in the Ag-pre-exposed green mussel *Perna viridis* in Expt. 2.

Data are mean ± SD (n = 8).  C: control; D: dissolved exposure; F: food exposure.

Numbers in the symbol legends are the nominal Ag concentrations used in the exposure treatments (see Table 2-1).
Fig. 2-6 Ag distribution in the digestive gland, other soft tissues and shell of the green mussel *Perna viridis* following a 7 d Ag pre-exposure (Day 0), after 16 d depuration (Day 16) and at the end of the depuration (Day 24) in Expt. 2. Data are mean + SD (n = 3). C: control; D: dissolved exposure; F: food exposure. Numbers in the symbol legends are the nominal Ag concentrations used in the exposure treatments (see Table 2-1).
Fig. 2-7 Subcellular distribution (%) of Ag in the Ag pre-exposed green mussel *Perna viridis* in Expt. 1. Subcellular fractions (organelles, debris, metal-rich granules-MRG, metallothionein-like protein-MTLP, and heat sensitive protein-HSP) were obtained through homogenization, differential centrifugation, heat treatment and tissue digestion procedures. Data are mean + SD (n = 5). CD: control after dissolved exposure; CF: control after food exposure; D: dissolved exposure; F: food exposure. Numbers in the symbol legends are the nominal Ag concentrations used in the exposure treatments (see Table 2-1).
Fig. 2-8 Subcellular distribution (%) of Ag in the Ag pre-exposed green mussel *Perna viridis* in Expt. 2. Subcellular fractions (organelles, debris, metal-rich granules-MRG, metallothionein-like protein-MTLP, and heat sensitive protein-HSP) were obtained through homogenization, differential centrifugation, heat treatment and tissue digestion procedures. Data are mean ± SD (n = 5). C: control; D: dissolved exposure; F: food exposure. Numbers in the symbol legends are the nominal Ag concentrations used in the exposure treatments (see Table 2-1).
Fig. 2-9 Relationships between Ag body burden and Ag assimilation efficiency index, clearance rate (CR) index, and Ag efflux rate constant following Ag pre-exposure. The index was calculated as the percentage of their respective control value in each treatment. Date are mean ± SD (n = 5 - 8).
Fig. 2-10 Relationship between Ag body burden and Ag influx rate index from the dissolved phase following Ag pre-exposure. The index was calculated as the percentage of their respective control value in each treatment. Data are mean ± SD (n = 5).
Chapter 3

Understanding the differences in Cd and Zn bioaccumulation and subcellular storage among different populations of marine clams

3.1 Abstract

The marine clams *Mactra veneriformis* were collected from three different locations in a contaminated bay in Northern China. Another species of clams *Ruditapes philippinarum* was collected from the same contaminated bay as well as from a relatively clean site in Hong Kong. The indices of Cd and Zn bioaccumulation (assimilation efficiency, dissolved uptake rate, and efflux rate), tissue concentration, subcellular distribution, metallothionein (MT) content, and clearance rates of the clams were subsequently quantified in these populations in the laboratory. In the two species of clams, the population with a higher Cd tissue concentration assimilated Cd and Zn more efficiently, in correlation with an increase in the Cd associated with the metallothionein-like protein (MTLP) fraction. The subcellular partitioning of Zn was similar among the different populations. The dissolved uptake rates of Cd and Zn were not influenced by the different tissue concentrations of metals in the clams. However, the clam *R. philippinarum* from the contaminated site reduced disproportionally their Zn uptake in response to increasing Zn concentrations in the water. Differences in Cd and Zn tissue concentrations had little influence on the metal efflux rate constant and the clams’ clearance rate. Our results indicate that the higher Cd and Zn tissue concentrations
observed in these two species may be partially caused by the high levels of metal assimilation. Populations living in contaminated environments may be able to modify their physiology and biochemistry in response to metal stress, which can subsequently alter trace metal bioaccumulation to aquatic animals. The relative significance of dietary uptake and the potential trophic transfer of metals in the contaminated areas may be substantially different from those in the clean environments.
3.2 Introduction

Marine bivalves have been employed successfully throughout the world as biological monitors of metal pollution in coastal waters. During the last several decades, numerous studies have investigated the potential factors affecting metal bioavailability and bioaccumulation in these bivalves (Phillips and Rainbow 1993; Wang and Fisher 1997; Wang and Dei 1999; Wang 2003). More recent field and laboratory studies have documented that the history of metal pre-exposure and tissue concentration can considerably affect metal bioaccumulation in these bivalves (Bossion et al. 1998; Blackmore and Wang 2002; Shi et al. 2003). These limited studies indicate that bivalves may potentially modify their physiological or biochemical processes under metal stress conditions, leading to ‘abnormal’ behaviors of metal uptake and loss and thus metal concentrations in the biomonitor (O’Connor 2002). The history of metal pre-exposure, soft tissue burden, and potential biological controls need to be carefully considered when the metal concentrations in these biomonitors are used to monitor metal pollution.

Trace metal bioaccumulation in aquatic organisms is controlled by numerous geochemical, ecological and physiological factors. Among the many factors affecting metal bioaccumulation, metallothioneins (MTs) have received relatively less attention (Couillard et al. 1993, 1995). MT as a low molecular weight, heat stable and cysteine-rich metal binding protein can be induced by many metals (e.g., Cd, Zn, Cu and Hg) in various marine invertebrates and fish (George and Olsson 1994). MTs have a close relationship with metal tissue concentration and play an
important role in metal detoxification, storage, and homeostasis in these aquatic animals (Roesijadi 1980, 1992). For example, there is a highly significant relationship between metallothionein-like protein (MTLP) and accumulated Cd concentration in the clam *Macoma balthica* following Cd exposure (Mouneyrac et al. 2000). George and Olsson (1994) observed that MTs in the common mussel *Mytilus edulis* can be induced by Cd, Cu and Hg, but not by Zn. In contrast, Serafin and Bebianno (2001) found a significant relationship between MT and Zn in the heat-treated cytosolic fraction (instead of total Zn) in the clam *R. decussatus*, but this relationship was less significant than with Cd or Cu. All these studies highlight that marine bivalves are able to develop various strategies in the detoxification and storage of metals under different metal stress conditions. Such diversity in metal handling strategies and subcellular fractionation has important implications for metal bioaccumulation and trophic transfer, but this aspect is just starting to be understood (Wang 2002; Wallace et al. 2003).

Under natural conditions, the animals are simultaneously exposed to different metals from different sources (e.g., aqueous and particulate metals) throughout their life times. Inevitably, the ‘natural’ exposure (e.g., life time exposure, different sources of exposure) can be difficult to achieve under laboratory conditions when studying metal bioaccumulation. Given the differences in metal exposure, the ‘speciation’ or cellular storage of metals may be different between the laboratory-exposed and the field-collected animals. Currently there is a considerable interest on metal storage in aquatic organisms and its influence on metal
biokinetics (Wallace et al. 2003). Klerks and Weis (1987) indicated that the responses of animals to metal pollution is the result of either physiological acclimation, representing a degree of tolerance obtained during a relatively short-time exposure, or adaptation, representing the genetically based resistance obtained by whole life-time exposure. Clearly, studies on field populations can offer the advantages of investigating the adaptations of marine animals to metal pollution, but they can be limited by the availability of polluted populations from the contaminated areas. Most bioaccumulation studies employed animals collected from single population in the field, and there are few studies examining the differences in metal bioaccumulation among different field populations and the underlying physiological and biochemical mechanisms (Blackmore and Wang 2003).

In this study, we collected two species of suspension-feeding clams from a highly metal contaminated bay in Northern China (Wang et al. 2002). Due to the severe sediment contamination, no organism in the benthic zones has been found close to the point sources of metal input. We also collected one species of clam from a clean reference site in Hong Kong to compare the difference in metal bioaccumulation between the polluted and the clean clam populations. All these clam populations had been exposed to metals throughout their life times. In addition to quantifying the metal tissue concentration and MT concentrations in the clams, we further quantified metal bioaccumulation index (metal assimilation efficiency from the food, influx rate from the dissolved phase, and efflux rate constant), subcellular fractionation, and the clearance rate of these clam populations.
Few studies have been conducted to examine the inter-relationships among metal
tissue concentration, MT levels, subcellular distribution, and metal bioaccumulation
in field-collected aquatic animals (Couillard et al. 1993, 1995).

3.3 Materials and methods

3.3.1 Collection of clams

Three native populations of the clam *Mactra veneriformis* (shell length of 3.0 cm,
tissue dry weight 0.32 – 0.62 g) were collected from three sites [Cuitun (CT),
Zhaotun (ZT) and Linhai (LH)] in Jinzhou Bay, Bohai, Northern China, in the
summer of 2002. The bay is highly polluted by metals due to the input from
numerous sources of industrial wastes. Selections of these three populations were
based on their availability from the bay and their distances from the point sources of
metal input (a Zn smelting factory). Metal concentrations in the sediments were
however not quantified in this study. Another native species of the clams,
*Ruditapes philippinarum* (shell length 2.0 – 2.5 cm, tissue dry weight 0.15 – 0.35 g)
was also found from Cuitun from the bay, but was not found in the other two
locations. To compare *R. philippinarum* from the contaminated bay, we therefore
collected the same species of clam from Wu Kai Sha, Tolo Harbor, Hong Kong (HK),
which is considered relatively unpolluted by metals. All the animals were collected
by hand from muddy sediment <10 cm depth, kept in the cool boxes at low
temperature, and transported immediately to the laboratory at HKUST, Hong Kong.
Throughout the whole experiment period, the clams were maintained in natural
coastal seawater (Clear Water Bay, Hong Kong) at 23 °C, and fed the diatom
*Thalassiosira pseudonana* (clone 3H) at a ration 1-2 % of tissue dry weight per day.

All clams were acclimated to laboratory conditions for one week before the
experiments described below.

The clam *M. veneriformis* was not found in Hong Kong area. Thus our
experimental designs allow us to compare the metal bioaccumulation among the 3
different populations of *M. veneriformis* from the same contaminated bay but with
different contamination levels, and between the two populations of *R. philippinarum*
from the contaminated bay and the clean reference sites (Hong Kong). Given the
difference in the sampling design, comparison between the two clam species was not
attempted in this study.

### 3.3.2 Metal body burden

Five individuals of clams from each site were dissected and the soft tissues were
individually placed into acid-cleaned glass tubes. The dry weights of the tissues
were determined by drying them at 80 °C to constant weights. Afterward,
concentrated Ultrapure HNO₃ (69 %, Aristar grade BDH Ltd) was added to digest the
dry tissues. The digests were subsequently diluted with Nanopure water and the
metal concentrations in an appropriate range were analyzed by Inductively Coupled
Plasma-Mass Spectroscopy (ICP-MS) (Perkin Elmer, Elan 6000). The stable metal
tissue concentrations were expressed as µg g⁻¹ dry weight.
3.3.3 Clearance rate

Clams were placed individually into polypropylene beakers containing 1.5 L of glass-fiber-filtered (GF/C) seawater. There were eight replicates in each experimental population. Diatoms *T. pseudonana* were filtered onto a 3 μm polycarbonate membrane to remove the growth medium and then resuspended in clean filtered seawater. When the clams opened and pumped normally, diatom cells were added into each beaker at a density of $10^4$ cell ml$^{-1}$. An aliquot of 10 ml of water sample was taken immediately after adding the algae, and the cell density was determined by a Coulter Counter. Further samplings were made at 30 min and 60 min and the cell density was counted. During the whole experimental period, the algal suspension was stirred by a magnetic stirrer and the beakers were covered by aluminum foil to avoid any disturbance or light that may affect the pumping activity of the clams. The clearance rate was calculated as:

$$CR = (\ln C_1 - \ln C_2) \times \frac{Vol}{T}$$

where, CR is the clearance rate of the clams (L h$^{-1}$), $C_1$ is the cell density (cell ml$^{-1}$) at time 1, $C_2$ is the cell density (cells ml$^{-1}$) at time 2, $T$ is the duration of measurement ($T_2 - T_1$, in h), and Vol is the volume of the water (L). After the measurements, the clams were dissected and their soft tissues were dried at 80 °C for determining the dry weights (g). The clearance rate of each individual was finally calculated from the mean of the two consecutive measurements at 30 min intervals.
and expressed as L g\(^{-1}\) h\(^{-1}\).

### 3.3.4 Metal uptake from the dissolved phase

Clams from each population of the two clam species were exposed to 0.5, 2, 10 and 50 μg L\(^{-1}\) Cd (as CdCl\(_2\)) and 2, 10, 50 and 100 μg L\(^{-1}\) Zn (as ZnCl\(_2\)) in 0.22 μm-filtered seawater for 1 h. This short-term (1 h) exposure minimizes the decline of the clams’ pumping activity caused by the absence of food particles, and avoids the decrease of metal concentration during exposure. Radiotracers \(^{109}\)Cd (3.7 kBq L\(^{-1}\)) and \(^{65}\)Zn (9.25 kBq L\(^{-1}\)) were added into the filtered seawater and allowed to equilibrate with the stable metals overnight before the experiments. For each treatment, eight clams were placed individually in beakers containing 250 ml of filtered seawater. All beakers were covered with aluminum foil during the 1 h exposure. After the exposure, the clams were dissected and the radioactivity of the soft tissues was measured. Finally the soft tissues were dried at 80 °C overnight and the dry weights were determined.

### 3.3.5 Metal assimilation efficiency

The diatom \(T.\ \text{pseudonana}\), grown in \(f/2\) nutrients for N, P, Si and vitamins, and \(f/20\) nutrients for trace elements minus Zn, Cu and EDTA, was radiolabeled with the isotopes \(^{109}\)Cd and \(^{65}\)Zn for 4 days prior to the experiment. The radioactivity additions were 185 kBq L\(^{-1}\) for \(^{109}\)Cd and 370 kBq L\(^{-1}\) for \(^{65}\)Zn. After the radiolabeling, these cells were collected onto a 3 μm polycarbonate membrane and
resuspended in 0.22 µm filtered seawater. The resuspension was performed twice to minimize the desorption of radiotracers from the diatoms to the ambient waters during the radioactive feeding period.

Clams were placed into 500 ml of GF/C seawater and the radiolabeled diatoms were added into each beaker giving a cell density of $4 \times 5 \times 10^4$ cells ml$^{-1}$ after the clams opened and pumped normally. This cell density was maintained by further additions at 10 min intervals. Following 30 min radioactive feeding, the clams were rinsed with clean seawater and assayed for their radioactivity. In each population, seven clams were placed separately into polypropylene beakers (180 ml seawater) held in a 10 L enclosed recirculating flow-through aerated seawater aquarium.

Nonradioactive *T. pseudonana* was fed twice daily at a ration of 1-2 % dry weight per day to depurate the initially ingested radioisotopes. The radioactivity retained in the clams was counted over a 48 h depuration period at intervals from 3 to 12 h. Metal assimilation efficiencies (AEs) were determined as the percentage of initial radioactivity retained in the animals after 36 h of depuration. Feces were collected frequently throughout the depuration period.

### 3.3.6 Metal efflux

Twelve clams from each population were maintained in clean seawater and fed $^{109}$Cd and $^{65}$Zn radiolabeled diatom *T. pseudonana* for 4 h each day. *M. veneriformis* from Cuitun was not examined in the efflux experiments because of the lack of sufficient numbers of individuals. These diatoms had been radiolabeled
with $^{109}$Cd and $^{65}$Zn for 4 d (as described above) and were filtered from the growth medium, rinsed and resuspended in clean seawater before being fed to the clams. The seawater was renewed every day after the radioactive feeding. Following 7 d feeding on radiolabeled food, the clams were radioassayed and then placed into an enclosed recirculating seawater aquarium (as described above) to depurate the $^{109}$Cd and $^{65}$Zn for 21 d. During the course of depuration, the aquarium seawater was changed every two days, and nonradioactive *T. pseudonana* was fed twice daily at a ration of about 2 % dry weight per day. The radioactivity retained in each individual was counted periodically and fecal pellets were collected frequently to minimize the desorption of radiotracers from the fecal materials into the water. On the first and the last day of depuration, three individuals from each population were dissected to determine the radioactivity in the shell and soft tissue.

3.3.7 MT quantification

The MT concentration in the digestive gland of the freshly collected clams was quantified using a modified (Leung and Furness 1999) silver saturation method (Scheuhammer and Cherain 1991). For each population, the digestive glands of three clams were dissected and the wet weights were determined. These tissues were then homogenized at 4 °C separately in 2 ml Eppendorf tubes containing 4x volume of cold 0.25 M sucrose. The homogenates were ultrasonicated and centrifuged at 20,000 g (20 min at 4 °C) to obtain the supernatants, which were then frozen at -80 °C until MT quantification. For each population, a 0.5 ml supernatant
was incubated with 0.3 ml of 0.5 M glycine buffer and 0.5 ml of 20 μg Ag ml⁻¹ for 10 min. This Ag solution was spiked with ¹¹⁰mAg at 3.7 kBq ml⁻¹. After MT binding sites were saturated with Ag within 10 min, excess Ag was removed by three additions of 0.1 ml rabbit red blood cell haemolysate, heating (5 min at 100 °C) and centrifuging (5 min at 1200 g). The supernatant was centrifuged again for 20 min at 20,000 g. The amount of Ag left in the final supernatant was determined following the radioassay for ¹¹⁰mAg. MT concentrations were calculated as 3.55× of the Ag concentrations and expressed as μg g⁻¹ wet weight of the digestive gland.

3.3.8 Metal subcellular fractionation

The subcellular partitioning of metals in the clams before (Day 0) and after the depuration (Day 21) was quantified using a modified method of Wallace et al. (1998). The subcellular fractionation of clam *R. philippinarum* on Day 0 of depuration was not quantified. Five clams from each population were dissected and the soft tissues were homogenized in 6 ml of Nanopure water. The homogenate was centrifuged at 1450 g for 15 min at 4 °C. The pellet was resuspended in 1 ml Nanopure water and heated at 100 °C for 2 min. Subsequently, an equal volume of 1 N NaOH was added, followed by heating at 70 °C for 1 h. A centrifugation at 5000 g (10 min at 20 °C) was then performed to separate the metal-rich granules (MRG) from other cellular debris. The 1450 g supernatant was further spun at 100,000 g (1 h at 4 °C) to separate the intracellular organelles (including the nuclear, mitochondrial and microsomal fractions) from the cytosol and proteins (supernatants). The 100,000 g
supernatants were then heated at 80 °C for 10 min, ice-cooled for 1 h, and finally centrifuged at 50,000 g (10 min at 4 °C). The pellet contained the heat-sensitive proteins (HSPs) and the supernatant contained the heat-stable proteins or metallothionein-like proteins (MTLPs). Thus there were a total of five fractions, including the cellular debris, MTLPs, organelles, MRG, and HSPs. Each fraction was radioassayed for $^{109}$Cd and $^{65}$Zn to calculate the subcellular fractionation of these two metals. The total radioactivity of the five fractions was compared with the initial radioactivity of the clam’s soft tissues before the fractionation. Results indicated that the amount of the radiotracer’s loss during each fractionation procedure was insignificant.

3.3.9 Radioactivity measurements and statistical analysis

Radioactivity was measured using a Wallac 1480 NaI (T1) gamma detector (Wallac Turku, Finland) or a Canberra gamma detector. All counts were related to standards and radioactive decay was corrected. The gamma emission of $^{109}$Cd was determined at 88 keV, $^{65}$Zn at 1115 keV, and $^{110m}$Ag at 658 keV. Counting times were adjusted to yield a propagated counting error < 5 %. All data were analyzed by $t$-test or analysis of variance (ANOVA). Statistically significant differences were accepted at $p < 0.05$.

3.4 Results

3.4.1 Metal body burden and MT concentration
The Cd and Zn tissue concentrations as well as the MT concentrations in the two species of clams collected from different contaminated sites and the clean site are shown in Table 3-1. In *M. veneriformis*, the Cd tissue concentration from LH was significantly ($p < 0.001$) higher than those of CT and ZT. There was, however, no significant ($p > 0.05$) difference in the Zn tissue concentration among these three populations. The MT concentrations in the digestive glands of *M. veneriformis* (about $64 \mu g \, g^{-1}$ wet weight) were not significantly different among the 3 sites ($p > 0.05$).

In *R. philippinarum*, both the Cd and Zn concentrations from the contaminated site (CT, Jinzhou Bay) were significantly higher (10.4 $\times$ and 2.8 $\times$ higher for Cd and Zn, respectively) than those concentrations from the clean site (Hong Kong). Similarly, the MT concentrations in the digestive glands were 3.6 $\times$ higher for the contaminated population than for the clean population ($p < 0.001$).

### 3.4.2 Clearance rate and dissolved uptake

In *M. veneriformis*, the population from LH exhibited a significantly higher (2.4 – 2.6 $\times$, $p < 0.001$) clearance rate compared with the two other populations (Table 3-2). The tissue dry weight of the LH population was 34% lower than the tissue dry weight of the CT and ZT populations. The difference in clearance rate between the two populations of *R. philippinarum* was not statistically significant ($p > 0.05$).

The influx rates of Cd and Zn into clams at different ambient dissolved metal concentrations are shown in Fig. 3-1. The influx rate is modeled as $k_u \times C_w^b$, where
$k_u$ is the uptake rate constant, $C_w$ is the dissolved metal concentration, and $b$ is the power coefficient. In general, there was a log-log linear relationship between the influx rate and the ambient dissolved metal concentration for both Cd and Zn in the two clam species. The power coefficient $b$ of this relationship was generally close to 1 for Cd, whereas the $b$ coefficients of Zn (0.64-0.82) were less than 1, suggesting that the uptake rate decreased disproportionately with increasing ambient Zn concentration (Table 3-2). This was especially true for *R. philippinarum* collected from the contaminated bay. The $k_u$ for Zn was therefore calculated directly using the linear regression between the influx rate and the dissolved concentration assuming that the intercept of the linear regression was zero (Table 3-2). In general, there was not major difference in the $k_u$ for Cd and Zn among the 3 different populations of *M. veneriformis*. The $k_u$ of Cd was similar between the two populations of *R. philippinarum*, whereas the $k_u$ of Zn was 1.7× higher for the clean population than for the contaminated population.

### 3.4.3 Metal assimilation efficiency

Depuration of Cd and Zn in the clams following 30 min pulse-chase radioactive feeding is shown in Fig. 3-2. Metal assimilation efficiencies (AEs) were calculated as the radioactivity retained in the clams at 36 h divided by the initial radioactivity measured immediately after 30 min of radioactive feeding (Table 3-3). Among the three populations of *M. veneriformis*, the individuals from LH egested metals at a slower rate than the other two populations, particularly after 24 h depuration,
resulting in the highest ($p < 0.01$) AEs of both Cd and Zn. There was no significant
difference in the AEs of Cd and Zn between the populations of ZT and CT.

For *R. philippinarum*, there was nearly no loss of both Cd and Zn after 12 h
depuration. The difference in metal assimilation between these two clam
populations was essentially due to the different egestion within the first 12 h. The
clams from the contaminated population retained metals more efficiently during the
initial stage of digestion, thereby assimilating Cd and Zn at a significantly higher
level ($p < 0.01$) than the individuals from the clean site.

### 3.4.4 Metal efflux rate

The depuration of Cd and Zn in the clams following 7 d ingestion of radiolabeled
food is shown in Fig. 3-3. Throughout the 21 d depuration period, there was
generally an initial rapid loss of metals within the first 4 – 5 d followed by a
relatively slower loss during the remaining period (5 – 21 d). The efflux rate
constants were therefore determined from the slope of the natural log of the
percentage of metals retained in the clams and the time of depuration (5 – 21 d).
The calculated efflux rate constants for Cd and Zn generally ranged between 0.02
and 0.05 d$^{-1}$ in the two species of the clams (Table 3-3). In each clam species, the
efflux rate constants of Cd and Zn were essentially identical between the two
different populations.

After 7 d of feeding on radiolabeled diatoms, Cd was distributed evenly between
the soft tissues (50 – 57%) and shells (43 – 50%) in the two populations of *M.*
veneriformis. This pattern was also observed in the Zn distribution in the ZT population (54% in soft tissues), while most Zn was found in the soft tissues (80%) of the clams from LH. By the end of 21 d depuration, a larger fraction of Cd (69 – 84%) and Zn (69 – 87%) was distributed in the soft tissues of the two populations. For R. philippinarum, the distribution of Cd and Zn between the soft tissues and shells did not vary greatly throughout the 21 d depuration period. The majority of Cd (88 – 97%) and Zn (75 – 86%) was distributed in the soft tissues.

3.4.5 Subcellular metal distribution

Subcellular Cd and Zn distributions in the two clam species are shown in Fig. 3-4 and Fig. 3-5. All populations of clams had similar patterns of Cd and Zn distributions in each fraction. Most Cd was distributed in the cellular debris (21 – 51%) and the metal-rich granule (MRG, up to 46%), and about 22 – 39% was in the cytosol (including heat sensitive and heat stable proteins) and 10% in the organelles (intracellular fraction). The organelles and MRG contained most Zn (42 – 52% and 32 – 43%, respectively), and only about 14 – 21% was associated with the cytosol and the cellular debris. A comparison of the different populations within each species showed that the clams M. veneriformis collected from LH accumulated significantly higher ($p < 0.05$) levels of Cd (21%) in the metallothionein-like protein (MTLP) fraction than the individuals from ZT (6%). For R. philippinarum, the Cd distribution within the MTLP fraction from the contaminated population was 5× higher than those from the clean population. There was however no significant
difference ($p > 0.05$) in the Zn distribution in each fraction between two different populations in each clam species.

### 3.4.6 Relationship between metal bioavailability and body burden

For *M. veneriformis*, there was generally a positive relationship between the metal AE and the Cd tissue concentration, although only 3 populations were examined (Fig. 3-6). No relationship was however found for the metal uptake rate constant or the efflux rate constant with the Cd tissue concentration in this clam species. Significant correlation between the Cd and Zn AEs or influx rates was also documented when all experimental individuals were considered together (Fig. 3-7 and Fig. 3-8). No such relationship was evident for the efflux rate constant between these two metals among the different individuals (Fig. 3-9).

### 3.5 Discussion

In our study, the three populations of *M. veneriformis* (CT, ZT, and LH) and one population of *R. philippinarum* (CT) were collected from a highly metal (mainly Cd and Zn) contaminated bay (Wang et al. 2002; Fan et al. 2002). These animals had been exposed to high levels of metals throughout their life-times. The differences of metal tissue concentration among the different populations of *M. veneriformis* were about $2 \times$ for Cd, and between the contaminated and the clean populations of *R. philippinarum* was $10 \times$ and $3 \times$ for Cd and Zn, respectively. Moreover, the contaminated population of *R. philippinarum* with higher Cd and Zn tissue
concentrations had significantly higher (3.5×) MT concentrations than the clean population had. These results agreed well with Hamza-Chaffai et al. (2000) who showed a positive and significant relationship between MT synthesis and Cd and Zn (either individual metal or combined) concentrations in the soluble fraction of the digestive glands of the clam *Ruditapes decussatus*. Similar results have also been obtained in freshwater clams (Couillard et al. 1993, 1995). For *M. veneriformis*, although the LH population had a significantly higher Cd tissue concentration (and a similar Zn tissue concentration) as compared with the other two populations, there was no notable difference in the MT concentrations in the digestive glands of the three populations. One possible reason was that the MT production was relatively independent of the Cd tissue concentration over this range (4.4-8.8 μg g⁻¹). It is unknown whether the MT production had been saturated over this Cd tissue concentration in this clam species, although previous studies indicate that MT concentration was proportional to Cd tissue concentration in clams when the MT : Cd ratio was larger than 1:7 (Hamza-Chaffai et al. 2000; Bebianno et al. 1993; Bebianno and Serafim 2003).

Our study demonstrated that the Cd AE was significantly higher in both species of the clams with higher Cd tissue concentrations. Concomitantly, within each clam species, the population with higher Cd tissue concentration and Cd AE had significantly more Cd associated with the MTLP fraction. These results implied that the MTLP may potentially play an important role in the Cd assimilation. The relationships among Cd tissue concentration, Cd AE and subcellular Cd distribution
were comparable to recent findings on the green mussel *Perna viridis* (Blackmore and Wang 2002). For example, following pre-exposure to Cd, Cd assimilation in the green mussels increased noticeably with an increase in the soft tissue Cd concentration and the association of the metal with MTLP. Gully and Mason (1993) also reported a similar mechanism for Cd and Cu uptake by the marine gastropod *Littorina littorea* after pre-exposing the animal to dissolved Cd.

In the natural environment, clams are exposed to Cd and Zn simultaneously, and the potential interactions between the two metals need to be considered when interpreting the metal kinetic uptake data. In the present study, the Zn tissue concentrations of the three populations of *M. veneriformis* were comparable and may not contribute to the different Cd AEs among these three populations. For *R. philippinarum*, the contaminated population not only had a higher Cd but also a much higher Zn tissue concentration than the clean population. Whether or not the body Zn concentration affected the Cd assimilation in this species is thus unknown. Blackmore and Wang (2002) indicated that only when the Zn concentration in the ambient water was sufficiently high (250 μg L\(^{-1}\)) could the Cd AE in the green mussels increase (from 32% to 50%) by Zn exposure.

The population of both clam species with a higher Cd AE generally exhibited a higher Zn AE. There appears to be a strong relationship between the Zn AE and the Cd tissue concentration. Previous studies indicated that the Zn AEs in the green mussels were not significantly impacted by elevated Cd tissue concentration until it reached a very high concentration (i.e., 26 μg Cd g\(^{-1}\)), after which the Zn AE
increased (Blackmore and Wang 2002). The MTLP was unlikely to play an important role because only a small fraction of Zn was bound with the MTLP and the majority was indeed associated with the organelles and MRG fractions. Since there was a strong relationship between the Cd AE and the Zn AE, we speculate that the high Zn AE from populations with higher Cd tissue concentration was modified by the gut physiology of the clams. Specifically, the efficient digestion and assimilation of dietary Cd by the clams with a high Cd tissue concentration also resulted in an efficient assimilation of dietary Zn. For the clam *M. veneriformis*, the Zn AEs were variable among the 3 different populations whereas the Zn tissue concentrations were somewhat comparable, which may imply that Zn tissue concentration had little control of its AE in this species. Zn is an essential metal for the clams and its accumulation may be carefully controlled. Whether the clams need to up-regulate the Zn AE by competing with other metals for absorption in the digestive tract in order to maintain sufficient Zn for essential function is unknown.

A direct relationship of Cd and Zn influx from the dissolved phase was also observed in both species of clams, similar to finding in other bivalve species (Wang and Dei 1999; Wang 2001). The estimated dissolved uptake rate constants ($k_u$) in the present study were comparable to those quantified in previous studies on the same (*R. philippinarum*) or other bivalve species (Wang and Dei 1999; Wang 2001; Chong and Wang 2001), and were higher for Zn than for Cd (Wang and Fisher 1997; Wang et al. 1996; Lee et al. 1998). The $b$ coefficient of the log-log relationship between the Zn influx rate and the ambient Zn dissolved concentration was
somewhat lower for *R. philippinarum* collected from the contaminated site than from the clean site, suggesting that this population may exhibit a higher potential to regulate their Zn uptake from the dissolved phase in response to increasing ambient Zn levels. Similarly, the dissolved uptake rate constant was 1.7× lower from the contaminated site than from the clean site. However, such regulation was of limited value because the influx rate still increased by 12× with an increase of dissolved Zn concentration by 50× (from 2 to 100 μg L⁻¹). The *b* coefficients of Cd were generally close to one among different populations with different Cd tissue concentrations, thus the clams were unable to regulate Cd uptake in response to increasing Cd concentration in the ambient waters.

Previous studies showed that Cd pre-exposure and Cd tissue concentration had little influence on the dissolved uptake of Cd and Zn in marine invertebrates (Blackmore and Wang 2002; Rainbow et al. 1999). In contrast, the uptake of both Cd and Zn by the green mussels decreased by 47% (for Cd) and 19% (for Zn) by pre-exposure to 250 μg L⁻¹ (Blackmore and Wang 2002). Therefore, the regulation of Zn dissolved uptake in *R. philippinarum* is likely due to their high Zn tissue concentration instead of the high Cd tissue concentration. Boisson et al. (1998) found that the clam *Macoma balthica* chronically exposed to Ag in contaminated seawater accumulated Ag at a significantly lower rate than did individuals from a clean site. For *R. philippinarum*, the higher body Zn burden from the contaminated site was mainly due to its higher intake of Zn from the dietary phase and the high Zn concentration in the contaminated bay, enabling the clams to obtain a higher level of
Zn tissue concentration.

Many studies have proposed clearance rates as indicators of toxic effects of contaminants in aquatic organisms (Naimo 1995; Krishnakumar et al. 1990; Kraak et al. 1994). In the green mussels, the clearance rate was depressed when the Ag tissue concentration was elevated to 2.5 µg g⁻¹, above which it began to recover due to the acclimatization of mussels to the Ag-enriched environment (Shi et al. 2003). Previous studies suggest that the individual clearance rate of the bivalves increases whereas the weight-specific clearance rate decreases with increasing body weight (Wang and Dei 1999; Chong and Wang 2001). In our study, the shell lengths of individual clams were comparable, whereas there were differences in the tissue dry weights among the different populations. The clean population of R. philippinarum had less body weight as compared to the contaminated population and, accordingly, the clearance rate was somewhat higher (1.6×, but not significantly different). For the three populations of M. veneriformis, both individual and weight-specific clearance rates were the greatest in the LH clams, which also had the smallest body weight, thus the difference of clearance rates between this population and the other two populations may be due to the inherent influence of body weight. In addition, the environments in which the clams were collected may also affect the clearance rate. Earlier studies by Green et al. (1989) and Hinch and Green (1989) demonstrated that the physical environments had a large source effect on the behavior of the freshwater mussels.

Our measurements of the Cd and Zn efflux rate constants in the two bivalves
showed no significantly difference between populations within each species and were comparable to those determined in the same or other bivalve species (0.01 – 0.03 d\(^{-1}\)) (Wang and Fisher 1997; Chong and Wang 2001; Fisher et al. 1996), except for the efflux rate constant of Zn in *M. veneriformis*. The relatively high efflux in this clam may reflect the Zn efflux from the soft tissue as well as the shell, because Zn was almost evenly distributed between the soft tissue and the shell at the beginning of the depuration, and was subsequently mostly distributed in the soft tissue by the end of the depuration. In *R. philippinarum*, the majority of Cd and Zn were found in the soft tissue throughout the depuration period, indicating that the measured efflux may be mainly due to the efflux from the soft tissue. The lack of major differences in Cd and Zn efflux rates from the clams in our study was similar to the findings on the green mussel *Perna viridis* (Blackmore and Wang 2002). Mussels pre-exposed to elevated levels of Cd and Zn did not show notable differences in the efflux rates unless the Zn concentration in the exposure was as high as 250 μg L\(^{-1}\).

We quantified the metal subcellular partitioning into 5 different biochemical fractions, including the cellular debris, MTLPs, organelles, MRG, and HSPs. Wallace et al. (2003) recently combined some of these fractions into metal sensitive fraction (organelles and HSPs), biologically detoxified metal fraction (MT and MRG), and trophically available metal fraction (organelles, HSPs and MT). They found that MT played an important role in Cd storage while organelles were the major site for Zn accumulation in the clam *Potamocorbula amurensis*. In contrast, MRG was important for Cd storage and MRG and MT were important for Zn storage.
in another species of clam *Macoma balthica*. Our fractionation results in the two clam species were generally consistent with the finding of Wallace et al. (2003). However, our data also indicated that within the biological detoxified metal fraction (MT and MRG) defined by Wallace et al. (2003), there was considerable variation among the different populations of clams for Cd. Thus there is a need to consider these different fractions individually for a better interpretation of the inter-population difference in metal storage.

Our study enabled the comparison of physiological responses among populations of *M. veneriformis* within a fairly uniformly contaminated environment (with similar exposure histories) and between populations of *R. philippinarum* with significantly different exposure histories. Due to the limits of the sampling sizes, it is difficult to assess the role of the exposure history on the physiological response. Despite that we found a major difference in the MT concentration between the polluted and clean clam populations, the concentration range of MT in affecting the metal bioaccumulation still need to be mechanistically understood. In *M. veneriformis*, there was no apparent relationship between the MT concentration and the metal AE, suggesting that the roles of MT in metal assimilation from clam populations with different exposure histories can be rather complex. Further carefully controlled laboratory studies are required to diagnose the relationship between MT turnover and metal bioaccumulation.

In summary, our study demonstrates that in both species of clams collected from a contaminated bay, there was a close relationship between the Cd AE, Cd tissue
concentration, and the Cd fraction associated with the MTLP. The Zn AE also increased in association with increasing Cd AE in populations more contaminated with Cd. The dissolved uptake rate of Cd was not influenced by the different tissue metal concentrations in the clams, but Zn uptake decreased disproportionately with increasing Zn ambient concentrations in populations collected from the contaminated site. On the contrary, the efflux rate of metals and the clearance rate of the clams were not significantly influenced by the metal tissue concentration. Therefore, in these two species of clams, populations with a higher Cd tissue concentration tend to accumulate more Cd and Zn from the dietary pathway. This suggests that the higher Cd and Zn tissue concentrations observed in *R. philippinarum* may be due to the high levels of metal assimilation as well as the high metal concentration in the water. Thus, the metal tissue concentration and biochemical fractionation need to be carefully considered when using the observed tissue concentration as biomonitoring data for metal pollution. Furthermore, the relative significance of dietary uptake and the potential trophic transfer of metals in the contaminated areas may have been different in the clean environments, but this requires further investigation (Wang 2002).
3.6 References


Ecotoxicol. 4: 341 – 362.


Table 3-1 Metal body burden (μg g⁻¹, mean ± SD, n = 5) and MT concentration (μg g⁻¹ wet wt, mean ± SD, n = 3) in the digestive glands of the clams (*M* : *Mactra veneriformis*; *R* : *Ruditapes philippinarum*) collected from metal contaminated or relatively clean sites (CT: Cuitun; ZT: Zhaotun; LH: Linhai; HK: Hong Kong).

Significant difference from the other populations within each species is indicated by ***: p < 0.001.

<table>
<thead>
<tr>
<th>Species</th>
<th>Population</th>
<th>Cd (μg g⁻¹)</th>
<th>Zn (μg g⁻¹)</th>
<th>MT (μg g⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M</em></td>
<td>CT</td>
<td>5.0 ± 1.7</td>
<td>65.4 ± 8.29</td>
<td>64.4 ± 13.0</td>
</tr>
<tr>
<td></td>
<td>ZT</td>
<td>4.4 ± 1.2</td>
<td>50.3 ± 12.7</td>
<td>61.9 ± 9.21</td>
</tr>
<tr>
<td></td>
<td>LH</td>
<td>8.8 ± 1.5</td>
<td>62.5 ± 11.1</td>
<td>67.4 ± 9.51</td>
</tr>
<tr>
<td><em>R</em></td>
<td>HK</td>
<td>0.5 ± 0.2</td>
<td>79.2 ± 10.7</td>
<td>13.0 ± 2.49</td>
</tr>
<tr>
<td></td>
<td>CT</td>
<td>5.1 ± 1.3***</td>
<td>222.0 ± 39.9***</td>
<td>46.2 ± 5.47***</td>
</tr>
</tbody>
</table>
Table 3-2 Clearance rate (CR, L g\(^{-1}\) h\(^{-1}\), mean ± SD; n = 8) and metal dissolved uptake rate constant \(k_u\) (L g\(^{-1}\) d\(^{-1}\)) in the clams (*M*: *Mactra veneriformis*; *R*: *Ruditapes philippinarum*) collected from metal contaminated or relatively clean sites (CT: Cuitun; ZT: Zhaotun; LH: Linhai; HK: Hong Kong). b: power coefficient of the relationship between metal influx rate and metal concentration in the dissolved phase. Significant difference from the other populations within each species is indicated by ***: \(p < 0.001\).

<table>
<thead>
<tr>
<th>Species</th>
<th>Population</th>
<th>CR (L g(^{-1}) h(^{-1}))</th>
<th>Influx</th>
<th>Influx</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Cd</td>
<td>Zn</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(r^2)</td>
<td>(k_u) (L g(^{-1}) d(^{-1}))</td>
</tr>
<tr>
<td><em>M</em></td>
<td>CT</td>
<td>0.8 ± 0.2</td>
<td>0.998</td>
<td>0.145</td>
</tr>
<tr>
<td></td>
<td>ZT</td>
<td>0.9 ± 0.3</td>
<td>0.999</td>
<td>0.129</td>
</tr>
<tr>
<td></td>
<td>LH</td>
<td>2.2 ± 0.6***</td>
<td>0.999</td>
<td>0.145</td>
</tr>
<tr>
<td><em>R</em></td>
<td>HK</td>
<td>1.1 ± 0.1</td>
<td>0.998</td>
<td>0.050</td>
</tr>
<tr>
<td></td>
<td>CT</td>
<td>1.8 ± 0.9</td>
<td>0.998</td>
<td>0.051</td>
</tr>
</tbody>
</table>
Table 3-3 Metal assimilation efficiency (AE) (%; mean ± SD, n = 6 or 7), metal efflux rate constant $k_e$ (d$^{-1}$; mean ± SD, n = 8) and the retention half-time $t_{1/2}$ (d, mean ± SD, n = 8) in the clams ($M$: Mactra veneriformis; $R$: Ruditapes philippinarum) collected from metal contaminated or relatively clean sites (CT: Cuitun; ZT: Zhaotun; LH: Linhai; HK: Hong Kong). Nd: not determined. Significant difference from the other populations within each species is indicated by *: $p < 0.05$ and **: $p < 0.01$.

<table>
<thead>
<tr>
<th>Species</th>
<th>Population</th>
<th>AE (%)</th>
<th>Efflux</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Cd</td>
<td>Zn</td>
</tr>
<tr>
<td>$M$</td>
<td>CT</td>
<td>18.5 ± 10.0</td>
<td>19.9 ± 15.0</td>
</tr>
<tr>
<td></td>
<td>ZT</td>
<td>12.7 ± 4.9</td>
<td>11.5 ± 9.1</td>
</tr>
<tr>
<td></td>
<td>LH</td>
<td>36.3 ± 7.0**</td>
<td>48.3 ± 11.0**</td>
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<tr>
<td>$R$</td>
<td>HK</td>
<td>42.7 ± 4.2</td>
<td>52.0 ± 14.0</td>
</tr>
<tr>
<td></td>
<td>CT</td>
<td>71.2 ± 9.3**</td>
<td>70.9 ± 7.6*</td>
</tr>
</tbody>
</table>
Fig. 3-1 Influx rate of Cd and Zn from the dissolved phase into the clams at different ambient concentrations. The legends show different sites where each clam population was collected (CT: Cuitun; ZT: Zhaotun; LH: Linhai; HK: Hong Kong). Data are mean ± SD (n = 8).
Fig. 3-2 Retention of Cd and Zn in the clams following pulse-chase feeding of radiolabeled diatoms (*Thalassiosira pseudonana*). The legends show different sites where each clam population was collected (CT: Cuitun; ZT: Zhaotun; LH: Linhai; HK: Hong Kong). Data are mean ± SD (n = 6 or 7).
Fig. 3-3 Retention of Cd and Zn in whole individual clams (soft tissue plus shell) following 7 d ingestion of radiolabeled diatoms *Thalassiosira pseudonana*. The legends show different sites where each clam population was collected (CT: Cuitun; ZT: Zhaotun; LH: Linhai; HK: Hong Kong). Data are mean ± SD (n = 8).
Fig. 3-4 Subcellular distribution of metals in the soft tissue of the clam *Mactra veneriformis* following 7 days ingestion of radiolabeled diatoms (Day 0) and after 21 days depuration in nonradioactive waters (Day 21). HSP: heat sensitive protein; MTLP: metallothionein-like protein; MRG: metal-rich granules-MRG. The legends show different sites where each clam population was collected (ZT: Zhaotun; LH: Linhai). Data are mean ± SD (n = 5). Significant difference between the two populations is indicated by * (p<0.05), ** (p<0.01), and *** (p < 0.001).
Fig. 3-5 Subcellular distribution of metals in the soft tissue of the clam *Ruditapes philippinarum* after 21 days depuration in nonradioactive waters (Day 21). HSP: heat sensitive protein; MTLP: metallothionein-like protein; MRG: metal-rich granules-MRG. The legends show different sites where each clam population was collected (CT: Cuitun; HK: Hong Kong). Data are mean + SD (n = 5). Significant difference between the two populations is indicated by * (p<0.05), ** (p<0.01), and *** (p < 0.001).
Fig. 3-6 Assimilation efficiency (AE) of Cd and Zn in relation to Cd tissue concentrations in the clam *Mactra veneriformis*. Data are mean ± SD (n = 5-8).
Fig. 3-7 Interrelationship of assimilation efficiency (AE) between Cd and Zn in the clams *Mactra veneriformis* and *Ruditapes philippinarum*. Each data point represents one individual clam.
Fig. 3-8 Interrelationship of influx rate between Cd and Zn in the clams *Mactra veneriformis* and *Ruditapes philippinarum*. Each data point represents one individual clam.
Fig. 3-9 Interrelationship of efflux rate constant between Cd and Zn in the clams *Mactra veneriformis* and *Ruditapes philippinarum*. Each data point represents one individual clam.
Chapter 4

Metal accumulation in Cd pre-exposed marine mussels *Perna viridis*

4.1 Abstract

The green mussels *Perna viridis* were pre-exposed to different levels of Cd (0.9-178 nM) for different durations (up to 5 weeks) from either the dissolved or dietary phases. The influences of different Cd pre-exposure regimes on the bioavailability of Cd, Ag, Hg, and Zn to the mussels were subsequently quantified by measuring the metal assimilation efficiency (AE), influx rate from the dissolved phase, Cd body burden, metallothionein (MT) concentration in the digestive gland and the mussel’s clearance rate. The Cd AEs increased following both the dissolved and the dietary Cd pre-exposure, in correlation with an elevated Cd body burden and a higher concentration of MT. Assimilation of Hg was also affected by Cd pre-exposure probably due to the binding of Hg with MT specifically induced by Cd. On the contrary, MT appeared to be of little significance in determining the AEs of Ag and Zn. Except for the dissolved uptake of Hg, which decreased significantly, Cd pre-exposure did not apparently affect the uptakes of other three metals from the solution, indicating that MT did not control their uptake in the mussels. Different routes of exposure did not significantly affect the metal uptake, suggesting that Cd body burden instead of the pre-exposure regime significantly affects the induction of MT, which subsequently affected metal accumulation to the mussels. Moreover, metal-metal interaction is likely to be affected by the specificity of MT induction.
Such factors need to be considered in interpreting metal body burdens in biomonitors.
4.2 Introduction

The influences of past history of metal exposure (i.e., pre-exposure) on metal bioaccumulation in marine invertebrates have received increasing attention (Boisson et al. 1998; Rainbow et al. 1999; Wang 2002). Past metal exposure may eventually impact the observed metal body burden in these marine animals, which are frequently used as biomonitors of metal contamination in coastal waters (Blackmore and Wang 2002; Shi et al. 2003). Recent laboratory and field studies both suggest that biochemical or physiological processes induced by ambient metal stress may lead to variations in metal concentrations in these monitoring organisms. For example, the induction of sulphide by the pre-exposure of the green mussel Perna viridis to Ag resulted in an elevation of Ag bioavailability (Shi et al. 2003). A higher level of metallothionein (MT), a low molecular weight and heat stable-metal binding protein (Roesijadi 1992; George and Olsson 1994), was found to correlate with a greater Cd tissue burden in the marine clams, Mactra veneriformis and Ruditapes philippinarum, collected from a Cd-contaminated bay (Shi and Wang submitted). Mouneyrac et al. (2000) also observed a significant relationship between Cd and metallothionein-like protein (MTLP) concentrations in the clam Macoma balthica originating from estuaries with and without industrial influences. These studies strongly suggest that metal pre-exposure and the corresponding biological responses should be cautiously taken into account when using biomonitoring data to evaluate metal pollution in seawater.

The relationships between Cd exposure and metal body burden as well as the
induction of MT have been extensively studied in marine invertebrates (Langston et al. 1989; Bebianno and Langston 1995; Mouneyrac et al. 2000; Hamza-Chaffai et al. 2000; Serafim and Bebianno 2001; Géret et al. 2002; Bebianno and Serafim 2003). Nevertheless, the link between Cd pre-exposure, resulting body burden and MT induction to subsequent metal bioavailability in aquatic organisms is just starting to be understood. In the green mussel _P. viridis_, Blackmore and Wang (2002) tested the Cd assimilation and influx from the dissolved phase by pre-exposure to different levels of dissolved Cd. Their results demonstrated that Cd pre-exposure significantly increased the Cd assimilation efficiency (AE) but not the dissolved uptake rate, accompanied by an increase in the Cd soft tissue concentration and an increasing percentage of Cd distributed in MTLP. Dietary Cd pre-exposure was not considered in that study, but dietary uptake and potential trophic transfer of metals has been highlighted in many recent studies (Wang 2002).

In natural environments, it is possible that the same metal tissue burdens are the result of different exposure regimes (different ambient metal concentrations and durations of exposure). Whether or not the history and regimes of pre-exposure instead of the metal body burden will lead to physiological and biochemical modification and thus to changes in metal bioavailability is rather speculative. Furthermore, aquatic animals are simultaneously exposed to a variety of metals under natural conditions. Thus, the influence of pre-exposure to one metal on the biological uptake of other metals is inevitable. This aspect remains essentially unstudied at present. Roesijadi and Fellingham (1987) found that the pre-exposure
of the mussel *Mytilus edulis* to Cd or Cu (but not Zn) resulted in an increased
tolerance to subsequent Hg (II) exposure because of the significant induction of gill
MT, which bound with Hg to decrease its toxicity. The bioavailability of Hg to the
mussels following the metal pre-exposures was not quantified in that study.

The green mussel *P. viridis* is an established and widely distributed biomonitor
of metal pollution in tropical and subtropical seawater (Phillips and Rainbow 1988;
Chong and Wang 2001). In the present study, a series of pre-exposure experiments
were conducted to address 1) if metal accumulation in the mussels was influenced by
Cd pre-exposure to dietary Cd; 2) if different exposure regimes resulting in
comparable Cd body burdens caused modification of metal bioavailability to the
mussels; 3) if Cd pre-exposure affected the bioavailability of other metals (Zn, Ag,
and Hg) to the mussels. The Cd concentrations used in this study ranged from
relatively low (typically coastal) concentrations to relatively high Cd concentrations.
We considered Cd, Zn, Ag, and Hg in this study, largely because of their similarity in
preferentially binding with sulfur-containing ligands. The induction of MT by the
exposure of these metals has been demonstrated in a variety of aquatic invertebrates
(Langston et al. 1998). Following different Cd pre-exposures, the metal AE, the
dissolved uptake rate, the clearance rate, the Cd body burden, and the MT
concentration were all determined to examine the inter-relationship among metal
tissue concentrations, MT levels and different physiological and biochemical
parameters of metals.
4.3 Materials and methods

4.3.1 Collection of mussels

The green mussel *Perna viridis* (shell length 3.0 – 3.5 cm, tissue dry weight ~0.15 g) was collected from Wu Kai Sha, Tolo Harbor (Expts. 1 and 3), the New Airport, Lantau Island (Expt. 2), and Sai Kung (Expt. 4), Hong Kong, at different times. The mussels were transported to the laboratory immediately after collection. Throughout the whole period of the experiments, they were maintained in natural coastal seawater (Clear Water Bay) at 23 °C and 30 psu and fed with the diatom *Thalassiosira pseudonana* (clone 3H) at a ration 1 – 2% of tissue dry weight per day.

4.3.2 Cd pre-exposure

Four independent experiments were conducted by exposing the mussels separately to different levels of dissolved or dietary Cd (as CdCl₂) for different durations (Table 4-1). In Expt. 1, we compared Cd pre-exposure through the aqueous and dietary phases. The dissolved Cd concentration during the aqueous pre-exposure was 10 μg L⁻¹. In the dietary exposure treatment, the diatom *T. pseudonana* was spiked with stable Cd at 100 μg L⁻¹ for four days, and the diatoms were collected from their exposed medium before being dosed to the mussels. The diatoms were spiked with a relatively high Cd concentration such that the tissue Cd burdens in the mussels were comparable between the aqueous and dietary treatments after 5 weeks of pre-exposure. Expt. 2 examined the influences of comparable Cd body burdens derived from different exposure regimes. The mussels were exposed
to dissolved Cd for different durations (1, 3, and 5 weeks) and at different dissolved concentrations, such that the final tissue body burdens were comparable among the different treatments before the bioavailability measurements. Exposure was conducted at different times such that the measurements were conducted simultaneously. Expt. 3 was performed at relatively lower Cd levels (0.1 – 5 µg L\(^{-1}\)) for 2 weeks of exposure. Expt. 4 was further designed to test whether Cd pre-exposure influenced the uptake of Zn, Ag, and Hg, all of which as well as Cd can preferentially bind with sulfur-containing ligands such as metallothionein (MT) in aquatic invertebrates.

For the dissolved pre-exposure, on the daily basis, the mussels were exposed to Cd spiked seawater for 18 h and fed the diatom *T. pseudonana* in unspiked seawater for the remaining 6 h. In the dietary exposure, the mussels were placed in clean seawater and fed Cd-enriched *T. pseudonana* for 4 h each day. These diatom cells had been spiked with Cd for 4 days and then filtered from the growth medium, rinsed with clean seawater and finally resuspended in clean water before being fed to the mussels. Our preliminary experiments indicated that within this short feeding period (4 h), most Cd (> 80%) remained in the diatoms. The Cd uptake by the mussels in the dietary exposure treatment was thus presumed to be derived from the food phase. In the control treatments, the seawater was not Cd-enriched. In order to keep the nominal metal concentrations relatively constant during the pre-exposure period and good water quality, the seawater in all exposure treatments was renewed every other day.
4.3.3 Metal assimilation efficiency from food

The pulse-chase feeding technique was employed to quantify the metal assimilation efficiency (AE), as described by Chong and Wang (2000). Briefly, the diatom *T. pseudonana*, cultured in f/2 levels for N, P, Si and vitamins, and f/20 levels for trace elements minus Zn, Cu and EDTA, was radiolabeled with the radiotracers $^{109}$Cd (in 0.1 N HCl), $^{65}$Zn (in 0.1 N HCl), $^{110m}$Ag (in 0.1 N HNO$_3$, in Expt. 4 only), and $^{203}$Hg (II) (in 0.1 N HCl, in Expt. 4 only) for 4 d. These radiolabeled diatoms were collected by filtering through a 3 μm polycarbonate membrane and then resuspending them in 0.22 μm filtered seawater. To minimize the desorption of loosely labeled radiotracers from the diatoms to the water during the subsequent feeding period, filtration and resuspension were repeated one more time. In each treatment, the mussels were placed in polypropylene beakers containing 500 ml of seawater and allowed to open and pump normally (within 10 min). Afterwards, the radiolabeled diatoms were added into the water giving a cell density of $4 - 5 \times 10^4$ cells ml$^{-1}$, which was maintained relatively constant by further additions at 10 min intervals. The mussels were removed from the feeding water after 30 min of feeding and then they were radioassayed after being rinsed with seawater. For each group, five individuals were then transferred into individual polypropylene beakers (180 ml seawater) held in a 10 L enclosed recirculating flow-through aerated seawater aquarium, to depurate the radiotracers that remained in the mussels for 72 h. During the depuration period, nonradioactive *T. pseudonana* was fed twice daily at a ration of 1 – 2% dry weight per day, and the radioactivity retained in the mussels was
assayed at intervals from 3 to 12 h. Feces were collected frequently throughout the depuration period.

4.3.4 Metal influx rate from the dissolved phase

Eight mussels from each group were carefully cleaned to remove any particles on the shells and placed in filtered seawater before the uptake rate measurements. The 0.22 μm filtered seawater was spiked with the radiotracers $^{109}$Cd, $^{65}$Zn, $^{110m}$Ag (in Expt. 4 only), and $^{203}$Hg (in Expt. 4 only), as well as stable metals Cd (as CdCl$_2$, 17.8 nM) and Zn (as ZnCl$_2$, 76.9 nM), and allowed to equilibrate for 24 h prior to the experiments. The radioisotope additions were 3.7 kBq L$^{-1}$ (corresponding to 0.3 nM) for $^{109}$Cd, 9.25 kBq L$^{-1}$ (corresponding to 0.06 nM) for $^{65}$Zn, 3.7 kBq L$^{-1}$ (corresponding to 0.3 nM) for $^{110m}$Ag (Expt. 4 only), 1.48 kBq L$^{-1}$ (corresponding to 0.3 nM) for $^{203}$Hg (II) (Expt. 4 only). The mussels were then put individually in 200 ml of the radioactive filtered seawater for 1 h. This short-term exposure avoided the possible decline of the mussels’ ventilating activity due to the absence of food particles and minimized the decrease of the metal concentrations in the seawater. Following the exposure, the mussels were dissected and the radioactivity of the soft tissues was assayed. The soft tissues were finally dried at 80 °C overnight and the dry weights were determined.

4.3.5 Clearance rate of the mussels

For each group, eight mussels were placed individually in polypropylene beakers
containing 1.5 L of glass-fiber-filtered (GF/C) seawater. After the animals pumped water normally, the diatom *T. pseudonana* was added into each beaker to generate a concentration of $10^4$ cell ml$^{-1}$. Immediately after this addition, 10 ml of water was sampled from each beaker and the cell density was measured using a particle analyzer (Z1, Coulter). Further samplings of water and measurements of cell density were performed at 20 and 40 min. The algal suspension was stirred by a magnetic stirrer throughout the experimental period. The clearance rate of the mussel was calculated as:

$$CR = (\ln C_1 - \ln C_2) \times \frac{Vol}{T}$$  \hspace{1cm} (1)

where CR is the clearance rate of the mussels (L h$^{-1}$), $C_1$ is the cell density (cell ml$^{-1}$) at time 1, $C_2$ is the cell density (cells ml$^{-1}$) at time 2, $T$ is the duration of measurement ($T_2 - T_1$, in h), and Vol is the volume of the water (L). After the experiments, the mussels were dissected and their soft tissues were dried at 80 °C to derive the dry weights (g). The clearance rate of each individual mussel was then calculated from the mean of the two consecutive measurements at 20 min intervals and expressed as L g$^{-1}$ h$^{-1}$.

**4.3.6 MT concentration in digestive gland**

A modified (Leung and Furness 1999) silver saturation method (Scheuhammer and Cherian 1986) was used to quantify the MT concentration in the digestive gland.
of the mussels following pre-exposure. There were three replicates for each group. Three digestive glands were separated from three individuals and the wet weights were determined. The tissues were then homogenized and ultrasonicated individually in 4 × volume of cold 0.25 M sucrose within 2 ml Eppendorf tubes at 4 °C, followed by centrifuging the homogenates at 20,000 g for 20 min at the same temperature. The supernatants were kept at -80 °C for the determination of MT concentration. For each replicate, 0.5 ml of the supernatant was incubated with 0.3 ml of 0.5 M glycine buffer and 0.5 ml of 20 μg Ag ml⁻¹ 0.5 M glycine buffer at room temperature. The Ag solution was spiked with radiotracer ¹¹⁰mAg with the activity of 3.7 kBq ml⁻¹. The MT was saturated with an excessive amount of Ag during the incubation. After 10 min incubation, the Ag not bound with MT was removed by adding 0.1 ml rabbit red blood cell haemolysate (Schuhammer and Cherian 1991), heating for 5 min at 100 °C and centrifuging for 5 min at 1,200 g. The addition of haemolysate, heating and centrifugation were repeated twice. The supernatant obtained by the last centrifugation was further centrifuged for 20 min at 20,000 g. The amount of Ag remaining in the final supernatant was determined following radioassay for the tracer ¹¹⁰mAg. The MT concentration was calculated as 3.55 times the Ag concentration and expressed as the μg g⁻¹ wet weight of the digestive gland.

**4.3.7 Stable metal concentration in soft tissues**

To determine the Cd body burden in the soft tissue of the mussels, the tissues
were dried in glass tubes at 80 °C until a constant weight was reached. There were five replicates for each group. All the dried samples were digested using concentrated Ultrapure HNO₃ (69%, Aristar grade BDH Ltd.). Afterwards, the digests were diluted with Nanopure water to bring the Cd concentration into an appropriate range for analysis by Inductively Coupled Plasma-Mass Spectroscopy (ICP-MS) (Perkin-Elmer, Elan 6000). Standard oyster tissues were concurrently digested and quantified for Cd concentrations. The Cd body burden was expressed as the μg g⁻¹ dry weight of the soft tissue.

4.3.8 Radioassay and statistical analysis

The radioactivity of the experimental samples was assayed by a Wallac 1480 NaI (T1) gamma detector (Wallac Turku, Finland). All counts were corrected for radioisotope decay, and the counting time was adjusted to yield a propagated counting error of less than 5%. The gamma emission of $^{109}$Cd was determined at 88 keV, $^{203}$Hg at 258 keV, $^{110m}$Ag at 658 keV, and $^{65}$Zn at 1115 keV. All data were analyzed by $t$-test or analysis of variance (ANOVA). Statistical significance was accepted at $p < 0.05$.

4.4 Results

4.4.1 Stable metal concentration in the soft tissues and MT concentration in the digestive gland

The Cd soft tissue concentrations increased in mussels previously exposed to Cd
as compared to the controls. In Expt. 1 (Table 4-2), the Cd body burdens from both the dissolved and the dietary exposure treatments were significantly higher ($p < 0.01$) than those of the controls. Cd concentrations in the mussels were comparable between the dissolved and dietary treatments after 5 weeks of exposure. In Expt. 2 (Table 4-3), although the Cd levels and the exposure durations were different among the treatments (i.e., dissolved 2 $\mu$g L$^{-1}$ for 5 weeks, 3.3 $\mu$g L$^{-1}$ for 3 weeks, and 10 $\mu$g L$^{-1}$ for 1 week), the body Cd concentrations detected in the mussels were rather similar ($p > 0.05$) and all were significantly higher ($p < 0.01$) than the control. In Expt. 3 (Table 4-4), the Cd body burdens in the pre-exposed individuals were 1.8 – 6.1 times higher than that of the control, but the differences were only significant ($p < 0.01$) in the two higher exposure concentration groups (i.e., dissolved 2 and 5 $\mu$g L$^{-1}$ groups). In Expt. 4 (Table 4-5), with one exception (dissolved 0.1 $\mu$g L$^{-1}$ treatment), the Cd tissue burdens of all the exposed mussels were significantly greater ($p < 0.01$) than that of the control. This increase was the largest ($37.5 \times$) following the exposure to 20 $\mu$g L$^{-1}$ for 5 weeks.

The mean MT concentrations in the digestive glands of the mussels exposed to Cd and the control mussels are shown in Table 4-2 to Table 4-5. In Expt. 1 (Table 4-2), following 1 – 5 weeks of pre-exposure to Cd from either the aqueous or the dietary phase, the MT concentrations in the digestive glands were 1.4 – 3.2 times higher than those of the control, and the increase at food 100 $\mu$g L$^{-1}$ for 5 weeks was significant ($p < 0.01$). All the pre-exposed mussels in Expt. 2 (Table 4-3) had higher (but not significantly, $p > 0.05$) MT levels in the digestive glands compared
with the control. Moreover, the differences among the three treatments were also insignificant ($p > 0.05$). In Expt. 3 (Table 4-4), the MT concentrations were 18 – 64% higher in the pre-exposed individuals than in the control, and a statistically significant increase ($p < 0.05$) was observed in the dissolved 2 μg L$^{-1}$ and 5 μg L$^{-1}$ treatments. In Expt. 4 (Table 4-5), after 5 weeks of exposure to dissolved 20 μg L$^{-1}$, the mussels had a significantly greater ($p < 0.01$) MT concentration in the digestive glands than the control had. At lower Cd concentrations, the MT levels were not significantly ($p > 0.05$) different from the controls.

4.4.2 Metal assimilation efficiency from the dietary phase

The retention of the four metals in the mussels during the 72 h depuration period is shown in Figs. 4-1 to Fig. 4-4. In all experiments, differences in the depuration pattern were observed among the four metals. Following the 0.5 h pulse radioactive feeding, Cd was rapidly egested within 12 h, after which there was much less loss from the mussels. In contrast, Zn and Ag were continuously lost throughout the whole period after the initial egestion, although the Zn depuration was relatively slower than that of Ag during this period. Hg (II) exhibited a totally different depuration pattern from the other three metals and was characterized by a rapid loss within the first 3 h, followed by a second period of almost no loss during 3 to 24 h, and a third period of continuous but more gradual loss after 24 h.

Metal AE was calculated as the percentage of the initial ingested metals remaining in the mussels following 60 h of depuration. Generally, in all
experiments, Cd AEs increased in the mussels pre-exposed to Cd, as a result of more efficient retention during the initial digestion period (within 12 h following feeding). In Expt. 1 (Table 4-2), after 1 week of pre-exposure, the Cd AE increased significantly ($p < 0.01$) only in the dissolved 10 $\mu$g L$^{-1}$ group (32%), but not in the food 100 $\mu$g L$^{-1}$ group (27.7%) as compared to the control (18.6%). After 3 and 5 weeks of the pre-exposure, the Cd AEs in these two Cd-treated groups were significantly higher ($p < 0.01$) than that of the control. In Expt. 2 (Table 4-3), the mussels from all the exposure treatments assimilated Cd at a significantly higher (1.9 $- 2.3 \times$, $p < 0.01$) efficiency than the control, but no significant difference ($p > 0.05$) was evident among the three Cd-exposed groups. In Expt. 3 (Table 4-4), 2 weeks of pre-exposure to dissolved Cd resulted in an increase in Cd AE in all treatments, among which only the 5 $\mu$g L$^{-1}$ group had a significantly higher ($p < 0.05$) Cd AE than the control. Similar results were also observed in Expt. 4 (Table 4-5), in which except for the dissolved 0.1 $\mu$g L$^{-1}$ group, the Cd assimilation from the other three exposure treatments was significantly more efficient ($p < 0.01$) than that of the control. Given the characteristic depuration pattern of Cd, the first stage of digestion (within 12 h) played an important role in determining the Cd AE.

Meanwhile, in these four separate experiments, the AEs of Hg, Ag and Zn in the mussels pre-exposed to Cd were not obviously affected by the pre-exposure (Figs. 4-1 to Fig. 4-4, Table 4-2 to Table 4-5). Although there were slight increases in the assimilations of Hg (by 10–16%) and Zn (by 12–28%) by the mussels in the Cd pre-exposed treatments, none of them was statistically significant ($p > 0.05$).
4.4.3 Metal influx rate from the dissolved phase

The influx rates of the metals from the dissolved phase are shown in Table 4-2 and Table 4-5. In Expt. 1 (Table 4-2), over the whole pre-exposure period from week 1 to week 5, there was no significant ($p > 0.05$) difference in the rate of Cd dissolved uptake between the control and the dissolved or food exposure treatments. Similarly, the Zn influx into the Cd pre-exposed mussels was similar ($p > 0.05$) with the control. In Expt. 4 (Table 4-5), after 5 weeks of pre-exposure to different levels of dissolved Cd, only the mussels in the dissolved 20 $\mu$g L$^{-1}$ group accumulated Cd at a significantly ($p < 0.05$) lower rate than the control. The influx rate of Hg decreased by 14 – 57% in the Cd pre-exposed treatments, among which the dissolved 5 and 20 $\mu$g L$^{-1}$ groups had a significantly lower rate of uptake ($p < 0.01$) than the control had. Compared with the control, the Zn dissolved uptake rate increased by 19% in the dissolved 1 $\mu$g L$^{-1}$ treatment and decreased by 18% in the dissolved 5 and 20 $\mu$g L$^{-1}$ groups, but these variations were not statistically significant, as found in Expt.1. For Ag, there was also no significant difference in the influx rate between the pre-exposed mussels and the control following 5 weeks of Cd pre-exposure.

4.4.4 Clearance rate of the mussels

For the two Cd-exposed treatments in Expt. 1, only the food 100 $\mu$g L$^{-1}$ group exhibited a significantly higher ($p < 0.05$) clearance rate than the control following 5 weeks of pre-exposure (Table 4-2). Similarly, no significant difference between the exposed mussels and the control was observed ($p > 0.05$) in Expt. 4 (Table 4-5).
4.4.5 Correlation between metal bioavailability with Cd tissue burdens and MT

In Expt. 1, a control treatment (without Cd pre-exposure) was included in all measurements conducted at different periods of pre-exposure (1, 3, and 5 weeks). We thus calculated the index of metal bioavailability (i.e., AE-index and influx rate-index) as the percentage of their respective control values during each measurement, to examine the inter-relationships between the metal bioavailability and the Cd body burden as well as the MT. For other experiments (Expts. 2, 3, and 4), metal bioavailability was directly correlated with the Cd body burden and the MT. In all experiments, a significant ($p < 0.05$) linear relationship was evident when the Cd AE (or Cd AE index) and the Cd body burden or the MT concentration (in the digestive gland of the mussels) was considered together (Fig. 4-5 to Fig. 4-8).

Additionally, there was a significant ($p < 0.05$) relationship between the Zn AE index and the Cd tissue concentration or MT in Expt. 1 (Fig. 4-5); such a relationship was however not found in the other three experiments (Fig. 4-6 to Fig. 4-8). The lack of a significant linear relationship was also documented for Ag and Hg AEs with either the Cd tissue burden or the MT level in the mussels (Fig. 4-8). Moreover, the dissolved influx rate (or index) of the metals appeared to have no clear relationship with the Cd body burden or MT concentration (Fig. 4-9 and Fig. 4-10), except for Hg, which decreased with increasing Cd tissue burden and MT level (Fig. 4-10). In Expt. 4, an obvious ($p < 0.001$) coupling between the AEs of any two elements of Zn, Ag, and Hg was observed when all experimental individuals were taken into account (Fig. 4-11). Although significant coupling between the Cd and Zn AEs was not
exhibited (Fig. 4-11), their dissolved uptake rates were significantly ($p < 0.0001$) coupled (Fig. 4-12).

4.5 Discussion

The measured Cd concentrations in the soft tissues of the control mussels were below 1.0 $\mu$g g$^{-1}$, except that in Expt. 2 (2.0 $\mu$g g$^{-1}$). The Cd body burdens achieved following Cd pre-exposure were generally 0.7 – 14.0 $\mu$g g$^{-1}$ with the highest concentrations (22.5 $\mu$g g$^{-1}$) observed in individuals pre-exposed to 20 $\mu$g L$^{-1}$ Cd for 5 weeks (Expt. 4). Such Cd body burdens are environmentally realistic, notwithstanding that the Cd concentrations applied in the pre-exposures are relatively higher than typical metal concentrations in unpolluted coastal seawater. For example, Chu et al. (1990) reported that the accumulated Cd tissue burden in *Perna viridis* collected from Outer Tolo Harbour, Hong Kong was as high as 9 $\mu$g g$^{-1}$ in 1986. The Cd tissue burden was up to 8.8 $\mu$g g$^{-1}$ in the marine clam *Mactra veneriformis* collected in an Cd-contaminated bay in Northern China (Wang et al. 2002; Shi and Wang submitted), where the Cd concentration in the sediment was as high as 488 mg kg$^{-1}$ (Fan et al. 2002). The dissolved Cd concentration measured in the same bay was 10 $\mu$g L$^{-1}$ (Chinese Environmental Protection Bureau Report 1999).

Based on the four independent experiments, our present study clearly showed that following Cd pre-exposure the assimilation efficiency (AE) of Cd increased linearly with the increasing Cd tissue burden in the green mussel *P. viridis*. 
Additionally, the significant linear relationship between Cd AE and metallothionein (MT) concentration in the digestive gland of the mussels indicated a close interrelationship among Cd body burden, MT level, and Cd AE. A higher MT induced by Cd pre-exposure tends to sequester more Cd from the dietary phase, resulting in a more efficient Cd assimilation in the mussels. In agreement, we recently found that the clams *M. veneriformis* and *Ruditapes philippinarum* collected from a Cd-contaminated bay had distinguishably higher Cd AE as compared with the populations from less contaminated or relatively clean sites, in correlation with a higher MT concentration in the digestive glands of the clams (Shi and Wang submitted). Therefore, Cd pre-exposure and elevated Cd tissue burden and MT concentration can positively influence Cd assimilation in the marine bivalves.

Numerous studies have demonstrated that metals from both the dissolved and the particulate (e.g., food) phases are the potential sources for their accumulation in the aquatic invertebrates (Langston and Spence 1995; Wang et al. 1996). It has been shown that dissolved Cd in ambient aquatic environments contributes much more than the dietary Cd to the overall Cd bioaccumulation in marine mussels (Borchardt 1983; Janssen and Scholz 1979; Riisgård et al. 1987). Nevertheless, in the present study (Expt. 1), following 3 or 5 weeks of pre-exposure to dietary Cd, the Cd AE increased substantially, suggesting that dietary Cd could become important in subsequent metal bioaccumulation. Thus, the relative importance of dietary vs. aqueous Cd uptake may be dependent on the history of the mussel’s exposure to Cd in the environment.
To the best of our knowledge, very few studies have considered the influences of chronic metal pre-exposure on metal bioavailability to marine invertebrates. Although a few studies have demonstrated that Cd pre-exposure that resulted in elevated Cd body burden and MT concentration can substantially affect Cd assimilation in marine bivalves (e.g., Blackmore and Wang 2002), whether dietary metal exposure modifies metal bioavailability remains unknown. Metal tissue burden is a result of exposure under different regimes in the natural environment. We deliberately carried out Expt. 2 to examine the influences of similar Cd body burdens derived from different exposure regimes on metal bioavailability. The lack of a significant difference in the AEs among the three groups of mussels pre-treated with different exposure regimes (i.e., dissolved 2 μg L\(^{-1}\) for 5 weeks, 3.3 μg L\(^{-1}\) for 3 weeks, and 10 μg L\(^{-1}\) for 1 week) and the similar resulting Cd body burdens imply that Cd assimilation by mussels may be mainly determined by the Cd body burden instead of the pre-exposure regime. In this case, when evaluating the influences of Cd pre-exposure on subsequent Cd accumulation in mussels, the observed Cd body burden can be directly taken into account without considering the pre-exposure regime. It should be realized, however, that our experiments were performed under laboratory conditions within a relatively short-term (1 to 5 weeks) pre-exposure, and it may be possible that Cd binding in biological tissues may not entirely reflect that under field environments, in which the animals are exposed to metals throughout their life times. To extrapolate the laboratory data to the field condition, the effects of longer pre-exposure durations are therefore of interest for further study.
Although Cd, Zn, Ag, and Hg are similar in terms of their preferential binding with sulfur-containing ligands, their bioavailability (i.e., uptakes from food and water) to Cd pre-exposed mussels are controlled by somewhat different and complicated factors, which could not be solely related to Cd pre-exposure or resulting induction of MT. Among the four metals, Cd and Hg generally show a closer relationship in terms of their uptake mechanisms, in which the Cd body burden and MT play an important role. The assimilations of both Cd and Hg are clearly related to the induction of MT by Cd pre-exposure, as shown in other studies that Cd- and Hg-binding metalloproteins are very similar (Roesijadi 1986; Frazier 1986). Therefore, it is very likely that the observed increase of Hg AE was a result of the enhanced level of Hg binding with the Cd-induced MT, which also increased the Cd AE. With regard to the dissolved uptake, it appears that Cd pre-exposure has little influence on its uptake from the dissolved phase, but decreases the influx of Hg. Our recent study also showed a decrease in Hg dissolved uptake following pre-exposure to Cu, another metal that has been identified as an MT-inducer in marine invertebrates (George and Olsson 1995; Langston et al. 1998). In a previous study, Roesijadi and Fellingham (1987) found that the pre-exposure of the mussel Mytilus edulis to Cu or Cd resulted in an enhanced tolerance to subsequent Hg (II) exposure due to the significant induction of gill MT, which could bind with Hg and decrease its toxicity. However, whether the increasing tolerance to Hg was a result of reduction in Hg uptake was not determined in that study. Hg (II) may be transported across the gills mainly by rapid diffusion, which may be different from
Cd and Zn transported by a facilitated process (Wang and Fisher 1999). The detailed mechanisms underlying the depression of Hg dissolved uptake following Cd pre-exposure remain undetermined at present.

Factors controlling Zn bioavailability to the mussels appeared to be different from Cd and Hg, although Zn and Cd are chemically similar. Despite the observed coupling of Cd with Zn in the uptake from solution, the Zn uptake rate was not considerably affected by the Cd pre-exposures, similar to Cd uptake. In *P. viridis*, Zn is not a strong MT inducer and it is mainly bound with metal-rich granules (MRGs) when accumulated by the mussels to a significant high level (Blackmore and Wang, 2002; Shi and Wang, submitted). However, whether or not the Cd body burden and MT can affect the dietary Zn uptake cannot be determined from this study. Since the binding affinity of Cd with MT is stronger than that of Zn, it may be difficult to discern any disturbance of Zn uptake caused by Cd-induced MT. We found that when the MT was induced by a much high Cd body burden (22.5 μg g⁻¹), the increase in Cd AE was also accompanied by an increase in Zn AE. This result agrees well with a previous study by Blackmore and Wang (2002), who found that when Cd body concentration reached a very high level (i.e., 25.9 μg g⁻¹), the Zn AE increased by 1.4 times in the green mussels. Thus, it remains possible that Zn may bind with MT with extra available binding sites, resulting in an increase in Zn assimilation.

In contrast to Cd, Hg and Zn, the bioaccumulation of Ag in *P. viridis* appeared to be completely unaffected by elevated Cd body burden and MT concentration.
Langston et al. (1998) documented that MT in marine mussels can be induced and bound by Ag. Our previous study suggested that the elevated Ag AE by the mussels after both aqueous and dietary Ag pre-exposure was a result of Ag binding with sulphide induced by Ag pre-exposure (Shi et al. 2003). This may explain the lack of influence of Cd pre-exposure on subsequent Ag uptake from ingested food in the present study.

The AE data (Expt. 4) presented here also show a linear positive relationship between the AEs of any two metals of Zn, Ag and Hg, whereas no such relationship was found with Cd. It has been demonstrated that Cd AE is highly dependent on its desorption from ingested food particles. For example, Wang and Fisher (1996) suggested that Cd desorption controlled its assimilation in the mussel *M. edulis* feeding on the diatom *Thalassiosira pseudonana* with different Cd concentrations. In contrast to Cd, desorption is not responsible for the variation in Zn AE, which may be a result of Zn regulation by the mussels (Wang and Fisher 1996). Similarly, no relationship between Hg assimilation and Hg desorption was evident in marine mussels (Gagnon and Fisher 1997). Although the Ag AE can be affected by its desorption, both intracellular and extracellular digestion can also influence its assimilation in marine bivalves (Fisher and Wang 1998). It is therefore likely that Cd AE may be controlled both by geochemical behavior as well as the MT induction, whereas the mussel's digestive physiology plays an important role in the assimilation of Zn, Hg and Ag. Another reason for the decoupling between the Cd assimilation and the assimilation of the other three metals is the control of specifically induced
MT on Cd assimilation, which did not significantly result in change in the assimilations of other metals.

The clearance rate, an indicator of metal toxicity in aquatic organisms (Naimo 1995; Krishnakumar et al. 1990; Kraak et al. 1994), was not significantly affected by the Cd pre-exposure. Blackmore and Wang (2002) found that the pumping activity of the mussel *P. viridis* was not significantly different between the control and the pre-exposed individuals with increasing Cd or Zn body burdens. We recently pre-exposed the green mussels to elevated levels of dissolved and dietary Ag for 5 weeks and found that the Ag tissue concentration had little effect on the mussels’ clearance rates, which increased gradually in both the pre-exposed and the control animals over the pre-exposure period, suggesting the acclimatization of the mussels to the laboratory conditions (Shi et al. 2003).

In conclusion, our data indicated that chronic Cd exposure and resulting Cd body burden and induced MT can play an important role in the assimilations of Cd and Hg and the dissolved uptake of Hg and potentially increase the Zn AE when the MT level reached a high level. In contrast, Cd and Zn uptake from the solution and Ag intake from both food and water were not affected by Cd pre-exposure. Different bioavailabilities of the metals to the Cd pre-exposed mussels may be closely related to their different biochemical bindings and storages in the animals (e.g., binding with MT and sulphide). Differences in the uptake and digestive mechanisms may also contribute to the differences in metal bioavailability following Cd exposure. In field situations, the metal body burden may be of more significance than the regime
of metal exposure history. Thus, when using the measured metal body burden as a criterion for metal contamination in seawater (e.g., with a biomonitor), the influences of dietary metal exposure as well as the pre-exposure to other metals need to be considered carefully, in addition to the pre-exposure history of that specific metal.
4.6 References


Chu, K.H., Cheung, W.M., and Lau, S.K. 1990. Trace metals in bivalves and
sediments from Tolo Harbour Hong Kong. Environ. Int. 16: 31 – 36.


Table 4-1 Cd concentrations (μg L⁻¹) and exposure durations (wks) used in different pre-exposure experiments with the green mussel *Perna viridis*. D: dissolved exposure, the mussels were directly exposed to Cd in the seawater; F: food exposure, the diatom food *Thalassiosira pseudonana* was exposed to the Cd concentration in the growth medium before being fed to the mussels.

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Duration (wks)</th>
<th>Cd concentration (μg L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1, 3, 5</td>
<td>D10</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>D2</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>D3.3</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>D10</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>D0.1</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>D0.1</td>
</tr>
</tbody>
</table>
Table 4-2 Cd body burden (µg g⁻¹, mean ± SD; n = 5), MT concentration (µg g⁻¹ wet weight, mean ± SD; n = 3) in the digestive glands, metal assimilation efficiency (AE) (%; mean ± SD; n = 5), metal influx rate from the dissolved phase (ng g⁻¹ h⁻¹, mean ± SD; n = 8), and clearance rate (CR) (L g⁻¹ h⁻¹, mean ± SD; n = 8) in the green mussel *Perna viridis* following Cd pre-exposures in Expt. 1. Significant difference from the control treatment was indicated by *: p < 0.05 and **: p < 0.01.

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Duration (wks)</th>
<th>Treatment</th>
<th>Cd (µg g⁻¹)</th>
<th>MT (µg g⁻¹)</th>
<th>AE (%)</th>
<th>Influx rate (ng g⁻¹ h⁻¹)</th>
<th>CR (L g⁻¹ h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td>Cd</td>
<td>Zn</td>
<td>Cd</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>C</td>
<td>0.5 ± 0.2</td>
<td>0.7 ± 0.1</td>
<td>18.6 ± 4.1</td>
<td>13.0 ± 1.9</td>
<td>23.0 ± 6.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>D10</td>
<td>2.8 ± 0.5**</td>
<td>1.0 ± 0.3</td>
<td>32.0 ± 5.5**</td>
<td>13.7 ± 2.1</td>
<td>23.4 ± 8.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>F100</td>
<td>1.1 ± 0.2**</td>
<td>1.1 ± 0.1</td>
<td>27.7 ± 8.1</td>
<td>12.1 ± 1.0</td>
<td>22.8 ± 6.0</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>C</td>
<td>0.5 ± 0.1</td>
<td>0.6 ± 0.1</td>
<td>20.4 ± 4.4</td>
<td>19.5 ± 1.3</td>
<td>27.2 ± 7.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>D10</td>
<td>7.5 ± 2.5**</td>
<td>1.1 ± 0.3</td>
<td>34.9 ± 3.2**</td>
<td>20.0 ± 3.1</td>
<td>32.5 ± 7.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>F100</td>
<td>4.0 ± 0.5**</td>
<td>1.0 ± 0.3</td>
<td>37.5 ± 11.7**</td>
<td>24.7 ± 6.5</td>
<td>31.0 ± 5.4</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>C</td>
<td>0.4 ± 0.1</td>
<td>0.6 ± 0.3</td>
<td>16.6 ± 11.7</td>
<td>19.7 ± 7.3</td>
<td>32.1 ± 11.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>D10</td>
<td>10.8 ± 2.3**</td>
<td>1.1 ± 0.4</td>
<td>42.1 ± 9.1**</td>
<td>26.4 ± 4.4</td>
<td>31.7 ± 2.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>F100</td>
<td>14.0 ± 3.6**</td>
<td>2.5 ± 0.5**</td>
<td>45.0 ± 9.7**</td>
<td>25.6 ± 2.5</td>
<td>25.2 ± 5.3</td>
</tr>
</tbody>
</table>
Table 4-3 Cd body burden (µg g⁻¹, mean ± SD; n = 5), MT concentration (µg g⁻¹ wet wt, mean ± SD; n = 3) in the digestive glands, and metal assimilation efficiency (AE) (%, mean ± SD; n = 5) in the green mussel *Perna viridis* following Cd pre-exposures in Expt. 2. Significant difference from the control treatment was indicated by *: p < 0.05 and **: p < 0.01.

<table>
<thead>
<tr>
<th>Duration (wks)</th>
<th>Treatment (µg L⁻¹)</th>
<th>Cd (µg g⁻¹)</th>
<th>MT (µg g⁻¹)</th>
<th>AE (%) Cd</th>
<th>AE (%) Zn</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>C</td>
<td>2.0 ± 0.4</td>
<td>1.1 ± 0.4</td>
<td>14.7 ± 2.7</td>
<td>18.4 ± 2.7</td>
</tr>
<tr>
<td>5</td>
<td>D2</td>
<td>5.2 ± 1.2**</td>
<td>1.7 ± 0.8</td>
<td>33.1 ± 4.6**</td>
<td>17.4 ± 5.0</td>
</tr>
<tr>
<td>3</td>
<td>D3.3</td>
<td>5.7 ± 1.3**</td>
<td>1.7 ± 0.3</td>
<td>30.2 ± 8.0**</td>
<td>17.1 ± 1.6</td>
</tr>
<tr>
<td>1</td>
<td>D10</td>
<td>4.6 ± 0.6**</td>
<td>1.5 ± 0.4</td>
<td>27.5 ± 3.0**</td>
<td>17.4 ± 2.2</td>
</tr>
</tbody>
</table>
Table 4-4 Cd body burden (µg g⁻¹, mean ± SD; n = 5), MT concentration (µg g⁻¹ wet wt, mean ± SD; n = 3) in the digestive glands, and metal assimilation efficiency (AE) (%) (mean ± SD; n = 5) in the green mussel *Perna viridis* following Cd pre-exposures in Expt. 3. Significant difference from the control treatment was indicated by *: p < 0.05 and **: p < 0.01.

<table>
<thead>
<tr>
<th>Treatment (µg L⁻¹)</th>
<th>Cd (µg g⁻¹)</th>
<th>MT (µg g⁻¹)</th>
<th>AE (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Cd</td>
</tr>
<tr>
<td>C</td>
<td>1.0 ± 0.3</td>
<td>1.1 ± 0.1</td>
<td>23.8 ± 6.9</td>
</tr>
<tr>
<td>D0.1</td>
<td>1.9 ± 0.4</td>
<td>1.4 ± 0.4</td>
<td>26.1 ± 11.3</td>
</tr>
<tr>
<td>D0.5</td>
<td>1.8 ± 0.5</td>
<td>1.3 ± 0.2</td>
<td>25.6 ± 6.8</td>
</tr>
<tr>
<td>D2</td>
<td>3.4 ± 0.7**</td>
<td>1.8 ± 0.3*</td>
<td>30.1 ± 6.0</td>
</tr>
<tr>
<td>D5</td>
<td>6.1 ± 1.7**</td>
<td>1.8 ± 0.2*</td>
<td>35.3 ± 6.8*</td>
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</table>
Table 4-5 Cd body burden (µg g\(^{-1}\), mean ± SD; n = 5), MT concentration (µg g\(^{-1}\) wet weight, mean ± SD; n = 3) in the digestive glands, metal assimilation efficiency (AE) (% , mean ± SD; n = 5), metal influx rate from the dissolved phase (ng g\(^{-1}\) h\(^{-1}\), mean ± SD; n = 8), and clearance rate (CR) (L g\(^{-1}\) h\(^{-1}\), mean ± SD; n = 8) in the green mussel *Perna viridis* following Cd pre-exposure in Expt. 4. Significant difference from the control treatment was indicated by *: p < 0.05 and **: p < 0.01.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cd (µg g(^{-1}))</th>
<th>MT (µg g(^{-1}))</th>
<th>AE (%)</th>
<th>Influx rate (ng g(^{-1}) h(^{-1}))</th>
<th>CR (L g(^{-1}) h(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Cd</td>
<td>Hg</td>
<td>Ag</td>
</tr>
<tr>
<td>C</td>
<td>0.6 ± 0.1</td>
<td>2.7 ± 0.2</td>
<td>22.7 ± 4.5</td>
<td>59.9 ± 5.3</td>
<td>15.6 ± 2.0</td>
</tr>
<tr>
<td>D0.1</td>
<td>0.7 ± 0.1</td>
<td>2.9 ± 0.6</td>
<td>31.1 ± 6.2</td>
<td>67.3 ± 4.1</td>
<td>16.0 ± 5.3</td>
</tr>
<tr>
<td>D1</td>
<td>2.0 ± 0.3**</td>
<td>3.0 ± 0.3</td>
<td>35.3 ± 5.3**</td>
<td>65.6 ± 3.6</td>
<td>12.5 ± 2.3</td>
</tr>
<tr>
<td>D5</td>
<td>7.4 ± 0.9**</td>
<td>3.3 ± 1.0</td>
<td>44.2 ± 8.4**</td>
<td>68.7 ± 8.1</td>
<td>14.4 ± 6.1</td>
</tr>
<tr>
<td>D20</td>
<td>22.5 ± 2.5**</td>
<td>5.7 ± 0.9**</td>
<td>58.4 ± 0.7**</td>
<td>69.7 ± 6.3</td>
<td>17.1 ± 6.3</td>
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</tbody>
</table>
Fig. 4-1 Retention of metals in the Cd-pre-exposed mussel *Perna viridis* following a pulse ingestion of the radiolabeled diatoms *Thalassiosira pseudonana* in Expt. 1. Data are mean ± SD (n = 5). C: control; D: dissolved exposure; F: food exposure. Numbers in the legends are the Cd concentrations used in the exposure treatment (see Table 4-1).
Fig. 4-2 Retention of metals in the Cd-pre-exposed mussel *Perna viridis* following a pulse ingestion of radiolabeled diatoms *Thalassiosira pseudonana* in Expt. 2. Data are mean ± SD (n = 5). C: control; D: dissolved exposure. Numbers in the legends are the Cd concentrations used in the exposure treatment (see Table 4-1).
Fig. 4-3 Retention of metals in the Cd-pre-exposed mussel *Perna viridis* following a pulse ingestion of radiolabeled diatoms *Thalassiosira pseudonana* in Expt. 3. Data are mean + SD (n = 5). C: control; D: dissolved exposure. Numbers in the legends are the Cd concentrations used in the exposure treatment (see Table 4-1).
Fig. 4-4 Retention of metals in the Cd-pre-exposed mussel *Perna viridis* following a pulse ingestion of radiolabeled diatoms *Thalassiosira pseudonana* in Expt. 4. Data are mean + SD (n = 5). C: control; D: dissolved exposure. Numbers in the legends are the Cd concentrations used in the exposure treatment (see Table 4-1).
The graphs show the percentage of metals retained in mussels over time during depuration. The x-axis represents the time of depuration in hours (0 to 80), and the y-axis represents the percentage retained in mussels (100 to 10). The graphs are labeled for Cd, Hg, Ag, and Zn.

- **Cd** graph: Shows the percentage of Cd retained in mussels over time. The data points are represented by different symbols for different treatments.
- **Hg** graph: Similar to Cd, but for Hg.
- **Ag** graph: Shows the percentage of Ag retained in mussels. The data points are also represented by different symbols for different treatments.
- **Zn** graph: Shows the percentage of Zn retained in mussels over time.

The legend indicates different treatments denoted by symbols C, D0.1, D1, D5, and D20.
Fig. 4-5 The relationship between the index of metal assimilation efficiency (AE) and the Cd body burden or MT concentration in the digestive gland of the mussel *Perna viridis* following Cd pre-exposure in Expt. 1. The AEs were determined in the Cd pre-exposed and controlled mussels following 1, 3, and 5 weeks of pre-exposure. The index was calculated as the percentage of their respective control values. Data are mean ± SD (n = 3 – 5).
Fig. 4-6 The relationship between the index of metal assimilation efficiency (AE) and the Cd body burden or MT concentration in the digestive gland of the mussel *Perna viridis* following Cd pre-exposure in Expt. 2. Data are mean ± SD (*n* = 3 – 8).
Fig. 4-7 The relationship between the index of metal assimilation efficiency (AE) and the Cd body burden or MT concentration in the digestive gland of the mussel *Perna viridis* following Cd pre-exposure in Expt. 3. Data are mean ± SD (n = 3 – 8).
Fig. 4-8 The relationship between the index of metal assimilation efficiency (AE) and the Cd body burden or MT concentration in the digestive gland of the mussel *Perna viridis* following Cd pre-exposure in Expt. 4. Data are mean ± SD (n = 3 – 5).
Fig. 4-9 The relationship between the index of metal influx rate and the Cd body burden or MT concentration in the digestive gland of the mussel *Perna viridis* following Cd pre-exposure in Expt. 1. The index was calculated as the percentage of their respective control values. Data are mean ± SD (n = 3 – 5).
Fig. 4-10 The relationship between the metal influx rate from the dissolved phase and the Cd body burden or MT concentration in the digestive gland of the mussel *Perna viridis* following Cd pre-exposure in Expt. 4. Data are mean ± SD (n = 3 – 8).
Fig. 4-11 Inter-relationship of the assimilation efficiency (AE) of the metals in the mussel *Perna viridis* following Cd pre-exposure in Expt. 4. Each data point represents 1 individual mussel.
Fig. 4-12 Inter-relationship of the influx rates of Cd and Zn from the dissolved phase in the mussel *Perna viridis* following Cd pre-exposure in Expt. 4. Each data point represents 1 individual mussel.
Influx rate (ng g\(^{-1}\) h\(^{-1}\))

\[ r^2 = 0.45, p < 0.0001 \]
Chapter 5

General conclusion

For the four heavy metals (i.e., Ag, Cd, Hg, and Zn) involved in this study, all the data presented herein generally suggest that the past history of metal exposure can cause modifications in biochemical or physiological processes by marine bivalves, subsequently affecting metal bioaccumulation in the aquatic biomonitoring organisms. The strategies of metal binding and storage developed by the bivalve species in response to ambient metal stress are crucial in determining metal bioavailability to the metal pre-exposed animals.

Among the four metals, Ag exhibits rather different patterns of assimilation, dissolved uptake and efflux from the other three metals. We demonstrated that in the green mussel *Perna viridis* all the three experimental determined metal bioavailability index, namely, assimilation efficiency (AE), influx rate from dissolved phase, and efflux rate constant, are closely related to the induction of sulfur by either aqueous or dietary Ag pre-exposure. Due to the extremely strong binding affinity of sulfur with Ag, induced sulfur tends to sequester more Ag from both water and food, and in the meantime there is no any apparent efflux of the metal from the animals. Additionally, our data of metal subcellular distribution showed that the fraction of Ag associated with metallothionein-like protein (MTLP) did not increase significantly with the increasing Ag tissue concentration, suggesting that the changes in Ag bioavailability to Ag pre-exposed mussels do not involve MTLP.
The most obvious and consistent inter-relationship among metal pre-exposure, resulting metal body burden, MT induction, and metal bioavailability was observed in Cd for both the laboratory Cd pre-exposed green mussels as well as field collected clams *Ruditapes philippinarum* and *Mactra veneriformis*. Those bivalves with a higher Cd body burden also assimilated Cd more efficiently, in correlation with an increase in the Cd associated with the MTLP fraction as well as a higher MT concentration in the digestive gland of the animals. Although these results highlight the significance of MT in Cd assimilation from food source and storage in the bivalves, Cd pre-exposure and elevated MT concentration seemed to have no direct relationship with the dissolved uptake and efflux rate constant of Cd, both of which remained relatively unchanged despite the enhanced Cd body burden following the metal exposure.

The increase in Hg AE following the pre-exposure of the green mussel to Cd suggests a close relationship between Cd and Hg in terms of their dietary uptake mechanisms, in which MT plays an important role. The enhanced Hg AE was likely a result of the elevated level of Hg binding with the Cd-induced MT which also increased the Cd AE. Moreover, different from the dissolved uptake of Cd, the influx of Hg from solution was also notably affected by Cd pre-exposure. Even though the detailed mechanism underlying the decrease of Hg influx from dissolved phase remained unknown at present, it is very likely that this depression is due to the induction of MT by Cd pre-exposure. This assumption could be supported by our recent findings that following pre-exposure to Cu, a strong MT inducer, the green
mussel decreased its Hg uptake from dissolved phase. Therefore, further study is needed to illuminate the role MT plays in Hg dissolved uptake.

The data presented in this study also indicate that the assimilation of Zn in the three bivalve species could be affected by the significantly increased MT concentration (achieved either by laboratory exposure or by field contamination), although accumulated Zn was mainly bound with granules. Due to the stronger binding affinity of Cd with MT than that of Zn, it may be difficult to observe any variation of Zn uptake from food particles by Cd-induced MT, unless MT is highly induced so that can provide extra binding sites for Zn besides its preferential binding with Cd. Additionally, for the two clam species, those individuals with higher metal body burden appeared to be able to regulate their Zn uptake from the dissolved phase in response to increasing ambient Zn levels.

This study also demonstrates that both dissolved and dietary metal pre-exposure can influence subsequent metal bioaccumulation in the bivalves. Compared with the exposure routes (i.e., aqueous and dietary pathways) and the regime of metal exposure history, the resulting metal body burden and MT concentration are of more significance in determining following metal bioavailability to the organisms. Moreover, the pre-exposure of one metal can produce effects on the bioaccumulation of other metals in the bivalves. Therefore, when employing marine bivalves as biological monitors of heavy metal pollution in marine environment, not only the extrinsic factors that may affect metal bioavailability to biomonitors, but also the intrinsic factors (e.g., modification in biochemical or physiological processes) caused
by chronic metal pre-exposure should also be considered thoroughly. In this case, studies on the influences of metal exposure can provide us a better idea on correctly interpreting biomonitoring data and appropriately establishing the criteria of seawater quality.