放送大学審査学位論文（博士）

Evaluation of the environmental heavy metals and their circulation in the Hiroshima Regional Urban Area

（広島広域都市圏における、環境重金属とその循環の評価）

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Element symbols used are
aluminum : Al; arsenic: As; boron: B ; cadmium: Cd; chromium: Cr; copper: Cu; cobalt: Co; fluorene: F; iodine: I; lead: Pb; manganese; Mn; molybdenum: Mo; nickel: Ni; selenium: Se; silicon: Si; tin: Sn; vanadium: V; zinc: Zn; cyan compounds: CN.

Abbreviations used are
Atomic Absorption Spectrometry: AAS; Inductively Coupled Plasma Atomic Emission Spectrometry: ICP-AES; Codex Alimentarius Commission: Codex; European Union: EU ; Mean plus minus standard deviation of the mean: mean±SD; World Health Organization: WHO.

CONTENTS

I. GENERAL INTRODUCTION

I.-1 Heavy metals in foods

I.-1-1 Heavy metals as essential trace elements

Organisms are made up of raw elements (Yasugi *et al.*, 2013). Mammals obtain trace elements, B, F, Al, Si, V, Cr, Mn, Co, Ni, Cu, Zn, As, Se, Mo, Sn, and I, from foods for normal life activities. Eight of the trace elements, V, Cr, Mn, Co, Ni, Cu, Zn, and Se, form chelates with enzyme proteins or other compounds and play an important role in biochemical reactions *in vivo*, whereas six of them, Cr, Mn, Co, Ni, Cu, and Zn, are the heavy metals essential for mammalian nutrition.

I.-1-2 Heavy metals as hazardous substances

Foods should not be hazardous to human health (*Food sanitation act on 1947*). However, some foods contain hazardous substances such as chemical components artificially added for food production (Nitta *et al.*, 1991b; Nitta *et al.*, 1994), environmental pollutants (Nitta *et al.*, 1991a; Niwa *et al.*, 1995), radioactive substances (Nitta *et al.*, 2001), viruses (Yamamoto *et al.*, 2019), detrimental microorganisms, natural toxins, and others.

Heavy metals in foods, irrespective of whether they are the essential trace elements, are hazardous and become toxic to mammals on chronic or heavy exposure. Cr is carcinogenic (Shrivastava et al., 2002); Mn (Peres *et al.*, 2016), Co (Danzeisen *et al.*, 2020), Zn (Komai M, 2020), and Mo (American Society for Nutrition, 2018) cause acute or chronic toxicity; and Ni (Schrenk *et al.*, 2020) and Cu (Tchounwou *et al.*, 2012) are detrimental to the bone marrow.

Non-essential heavy metals such as Cd and Hg accumulated in the tissues or organs to cause serious damages through food (Burger *et al.*, 2009; Nordberg GF, 2009). Itai-Itai disease (Järup *et al.*, 1998) and Minamata disease (Nishijo *et al.*, 2017) are caused due to food poisoning via the two metals.

Some chemical substances that are detrimental to humans and the environment have been specified with the promulgation of the law, *the understanding and managing the amount of specified chemical substances released into the environment act on 1999*. Cd and Hg are registered in the Class II specified hazardous substances by the Ministry of the Environment (*Soil contamination countermeasures act on 2002*) (**Appendix 1**).

I.-2 Heavy metals in organisms

I.-2-1 Heavy metals in marine organisms

Heavy metals from the aquatic environment accumulate in marine organisms. High concentrations of the Class II specific hazardous substances have been found in seaweeds (Kikuchi *et al.*, 2002), bivalve mollusks (Wang and Lu, 2017), and fishes (Sow *et al.*, 2019), when collected or captured as foodstuff. High concentrations of heavy metals have been reported in whales (Wise Jr *et al.*, 2019). To reduce the human exposure levels of Cd from seafoods, the concentration limit of Cd in bivalve mollusks, <2 mg/kg, has been presented by Codex (**Appendix 1**). Pacific oyster (*Crassostrea gigas*) (oyster) is excluded from this regulation.

Oyster is a salt-water bivalve mollusk well adapted to the highly variable intertidal environment (Clark *et al,* 2013; Takatsuki T, 1949; Zhang *et al.*, 2012). Aqua-cultured oysters contain high concentrations of Zn (Tanaka *et al.*, 1971). Intracellular Zn ions work as the metal component of enzymes to adapt to the stressful culturing environment (Meng *et al*, 2021). The Zn in oyster meat leads to food poisoning in humans (Motooka *et al.*, 2016) by reducing the amount of Cu transporter in the small intestinal epithelium (Kaplan *et al.*, 2016). High concentrations of Cd in meat (Kikuchi *et al.*, 2002) can be used to monitor the perspective of human health and the oyster-culturing environment.

I.-2-2 Heavy metals in terrestrial organisms

Natural field plants absorb heavy metals from terrestrial environment (Chen *et al*, 2009). Heavy metals accumulate in the body of wild boar inhabiting the natural environment (Danieli *et al.*, 2012; Mulero *et al.*, 2016). Hheavy metals in game meat are of great concern in the EU countries owing to its high consumption (Ferri *et al.*, 2017; Filippini *et al.*, 2018; Lanocha *et al.*, 2012; Marín *et al.*, 2018). In Japan, the Ministry of the Health, Labour and Welfares promotes the consumption of game meat (*Protection and control of wild birds and mammals and hunting management act on 2002*) as the by-product of pest control. However, there is no regulation on the quality of game meat and concentration of heavy metals.

Agricultural farm plants, including banana (Romero-Estevez *et al*, 2019), cacao (Argullo *et al*, 2019; Pereira de Araújo *et al.*, 2017), radish (Shan *et al.*, 2016), or any other vegetables (Hamid, *et al.*, 2016), absorb heavy metals from soil and water (Alengebawy *et al.*, 2021). A high concentration of heavy metals accumulates in the bodies of fielded ruminants through consumption of grass in farm pastures (Lane *et al.*, 2015; López-Alonso *et al.*, 2016). Cd is accumulated in humans mainly from staple food crops such as rice in Japan (Horiguchi *et al.*, 2013).

Cd in the crops is transferred to the human food chain. Cd accumulated in the human body does not turnover but stays within the tubular epithelium of kidneys with a biological half-life of 30 years (Ishizaki *et al.*, 2015). The WHO set a provisional limit for the dietary intake of Cd, <1 μ g/kg of body weight/day, in 1978. The Ministry of Health, Labour and Welfares has set the reference value for Cd in rice, <0.4 mg/kg, since 2006.

I.-3 Heavy metals in wastes

I.-3-1 Heavy metals in sludge

Sludge is a muddy solid waste formed via agglomeration of organic final products generated from homes and factories. Most of the heavy metals taken and excreted by humans are accumulated in the sludge. The sludge is landfilled or fertilized, estimated ratios of which are 87.2% or 12.1%, respectively, in Japan (Nakanishi *et al.*, 2008).

Detrimental effects of the application of sludge on the agricultural farm soil on human health and the subsequent transfer of the heavy metals to food chain are unlikely (Smith SR, 2009). This may be owing to appropriate controls. The EU has protected contamination by limiting the concentration of Cd in sludge-treated agricultural farm as <3 mg/kg (EU, 2002).

I.-3-2 Heavy metals in oyster shell waste

Oyster shells are the industrial by-products (https://www.env.go.jp/recycle/post_55/mat01_1-1-1.pdf). They are partly recycled as specific fertilizer, feed for egg-laying hens (Lee *et al.*, 2021), and soil conditioner (Chang YT, 2013). However, unlike sludge, effects of the heavy metals in the specific fertilizers made from oyster shells in humans and their subsequent transfer from the agricultural farm soil to the food-chain have not been reported. It is possible that some amounts of Cd in oyster shells gets ingested into fruits and vegetables and the rest remains in the soil.

I.-4 Flows of heavy metals in the ecosystem

I.-4-1 Heavy metals from heavy industries in the ecosystem

Industrially emitted heavy metals from smelting or product manufacturing are released into air and water (Ono *et al.*, 2005; Matsuno *et al.*, 2012) (**Fig. I-1**). In marine ecosystems, the heavy metals dissolved in seawater are fixed by benthos. Metals in the benthos' body are transferred into their predators including humans. Humans excrete most of the heavy metals obtained from benthos, and the excreta is accumulated as sludge on land. Other metal ions partly become a part of the sediment by activities of marine microorganisms.

I.-4-2 Heavy metals from food industry in the ecosystem

Waste disposal of food is completed in Japan. Wastes of household food are incinerated, while the industrially manufactured food wastes are recycled into feed and compost (https://www.env.go.jp/recycle/R02_houkokusyo.pdf).

Wastes from seafoods produced via the aquaculture-type, shell, hard skeleton, and other soft tissues, are not processed effectively in southeast Asian countries (Yan *et al.*, 2015). The by-produced oyster shell wastes are dumped in landfills or the sea (Seesanong *et al.*, 2021). Oyster shell waste is potentially used in medicine, chemical industry, and agriculture, e.g., the conversion of oyster shell waste into pharmaceuticals (Ravi *et al.*, 2021), chemicals (Seesaning *et al.*, 2021), water purification materials (Alves *et al.*, 2021), and agricultural fertilizers (Chilakala *et al.*, 2019).

Only a few studies have investigated the presence of heavy metals in agriculturally recycled oyster shell wastes and their effects on the local ecosystem.

I.-4-3 Evaluation of heavy metals in the ecosystem

The concentrations of heavy metals and other hazardous substances in wastes should be measured. Systematic investigations following any fixed approach of ecotoxicology will be helpful to understand the effects of the wastes on agricultural farms in the local ecosystem at the level of biological community unit (Schwarzenbach *et al.*, 2006). To visualize the dynamics of heavy metals in ecosystem, a research method of Community Modules' Approach in ecotoxicology can be adequate (Hold RD, 2002), where the subjects of the toxicity to resident organisms, the relationship between the exposure conditions and toxicity, and effects on different levels of the ecosystem, individual, population, biological community unit, and landscape scale, can be investigated (Noda T, 2016).

Log-normal concentration distributions are ubiquitously assumed in environmental sciences (Andersson A, 2021). This is explained that because any environmental system is governed by a multitude of different processes, occurring simultaneously. However, it is unclear whether this assumption is valid for every ecotoxicological study, in which not only inanimate but also plants and animals make up the target ecosystem.

I.-5 Questions to be clarified in this study

I.-5-1 Effects of the fertilizers made from oyster shell waste

Heavy metals in general fertilizers are toxic, and they affect the agricultural ecosystem as well as human health (Alengebawy *et al.*, 2021). The concentrations of specified hazardous substances in domestic and overseas fertilizers have been investigated to evaluate their safety for use in agricultural farms (Roberts TL, 2014). The concentrations of Zn and the Class II specified hazardous substances in agricultural farm soil are regulated (*Soil contamination countermeasures act on 2002*); however, those in special fertilizers (*Fertilizer Regulation act on 1950*), to which oyster shell fertilizer belongs, are not.

The local government has promoted industrial plants for recycling oyster shell waste into specific fertilizers (https://hiroshimaforpeace.com/wp-content/uploads/2019/12/sdgsbook2_1210.pdf) to solve the industrial waste disposal problem. In the Hiroshima Bay, the resumed oyster culturing in the late 1940s has grown into an aqua-industrial system. At the same time, as the amount of oyster meat accounted to 65% of Japan's total production in 2011, the industrial waste disposal problem became serious.

Whether the system to recycle oyster shells from aquacultural waste to agricultural fertilizer might transfer Cd from sea to land is an inconvenient issue (**Fig. I-1**), as any kinds of organic wastes should not be hazardous or pose risks to the ecosystem during re-use. Human activities to recycle fishery aquaculture wastes may add to the burden of hazardous substances on terrestrial environment. Another important issue is that whether the landscape including the oyster aqua-culturing area, can produce high-quality oyster meat, which would better sustain human health and improve preventive medicine, under the law enacted in 2015 (*Revision of the Law Concerning Special Measures for Conservation of the Environment of the Seto Inland Sea and Modification of the Basic Plan for the Conservation of the Environment of the Seto Inland Sea)*. One of the basic principles of this law is that the value and function of the Seto Inland Sea should be maximized, in order to ensure biodiversity and productivity.

In line with the law, the concept of Hiroshima Regional Urban Area (https://www.city.hiroshima.lg.jp/site/kouiki/list846.html) has been put into action since 2015. Agricultural landscapes disappeared in the late 20th century, and depopulation has accelerated the abandonment of agricultural landscape in the 21st century. In such time, revitalization of the local economy and maintenance of biodiversity of the landscape are essential (Berglund *et al.*, 2014; Katoh *et al.*, 2009).

I.-5-2 Environmental impact of heavy metals on oyster shells

This study hypothesized that a type of aqua-industrial waste transfers heavy metals from the sea to land, and that the transferred heavy metals affect the terrestrial ecosystem on land (**Fig. I-1**).

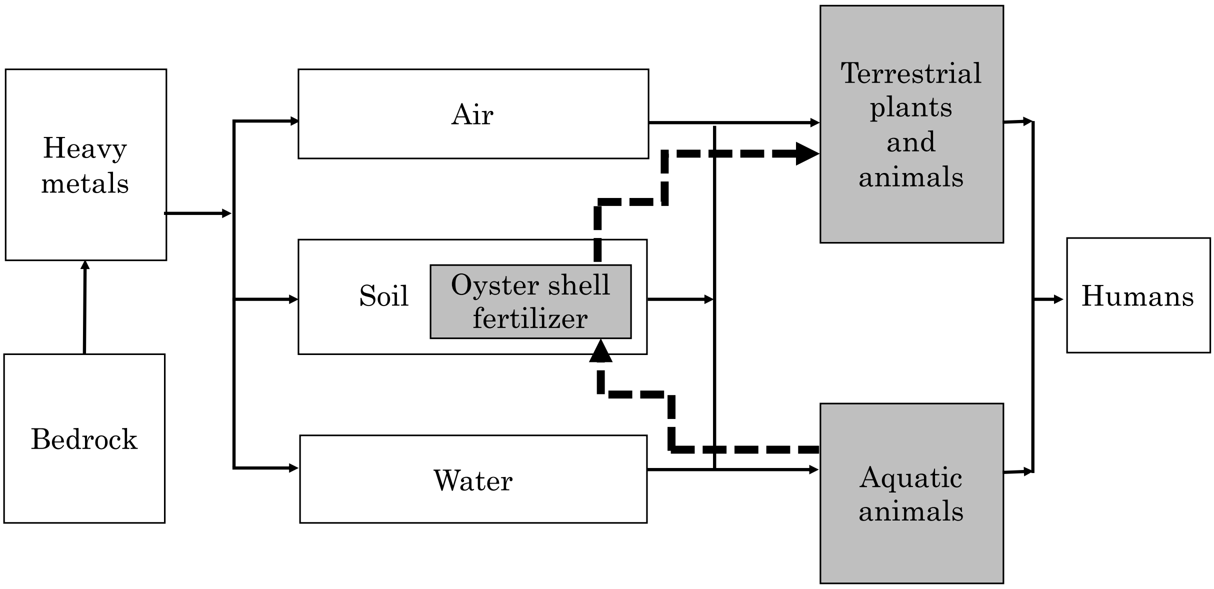

Fig. I-1. Hypothesis of the Cd flow with the mediator of oyster shell fertilizer. Both sea and land areas were examined in this study. The examined sea elements were sediment and benthos whereas the examined land elements were river sediments, soils, natural as well as cultivated plants, and wildlife. ☐ and arrows indicate the well-established flows of heavy metals. ■ and hatched arrows are the hypothesized flows of heavy metals from the sea to land mediated by oyster shell fertilizers.

Cd is ubiquitous in rocks with certain ratios of Cd to Zn, using which, each rock and geological stratum is to be characterized (Dharma-Wardana MWC, 2018). Sources of Cd in soils (Fishbein L, 1981; Hamid *et al.*, 2016; Kubicka *et al.*, 2015; Zhu *et al*, 2013), terrestrial plants (Ikeda *et al*, 2006), sediments (Soliman *et al.*, 2015), and seagrass beds (Bouchon *et al.*, 2016) are Zn mining, metal smelting, battery production, or cement factories (Smith SR, 2009; WHO Regional Office for Europe, Copenhagen, Denmark, 2000).

The estimated total amount of Cd discarded into air and water was 130 t/year in Japan. The ratio of Cd to Zn, Cd/Zn, has been estimated to be 0.0053 and 0.0045 in mine soils (Ono *et al.*, 2005) and galvanized materials (Matsuno *et al.*, 2012), respectively. Considering their toxic effects on human health, WHO (2006) and EU (2002) have specified the maximum limit values of Cd/Zn in soil as 0.0133 and 0.0100, respectively.

An early-warning system can transform people's activity to prevent the exposure to hazardous substances. In wildlife symbiotic with people, certain amounts of Cd and Zn are concentrated in the bodies of animals, and the origin and flows of the anthropogenic heavy metals should be investigated, since exposure of such wildlife to the local ecosystem is inevitable during their lifetime. The best wildlife for ecotoxicological investigation would be the medium-sized mammals widely distributed within the target area (O'Brien *et al.*, 1993).

To prove this hypothesis, the dynamics of the Cd from marine organism to terrestrial organisms through oyster shell fertilizers was challenged to be shown numerically. Geological maps of Yamaguchi (Nishimura *et al,* 2012) and Hiroshima (Geospatial Information Authority of Japan, 1963) prefectures will show geological bedrock information. Heavy metal concentrations surrounding the Seto Inland Sea area (https://gbank.gsj.jp/geochemmap/) information on the industrial environment of cities (Imai T, 2001). Cd and Zn concentrations in granitoids of the Sanyo Belt (Ishihara *et al.*, 2006) and the distribution map of wild fern plants in Hiroshima prefecture (Yoshino Y, 2016) will provide information on the local natural environment.

I.-6 Structure of the paper

This paper comprises six chapters. **Chapter I** is the introduction, in which the background of the study, what has been known, and what will be clarified are described.

Results are described in **Chapters II** to **V**. In **Chapter II**, concentrations of Cd and Zn concentrations in the sediment, oyster, and the fertilizers made from oyster shells provided within the Hiroshima Regional Urban Area were screened, and the total amount of Cd fixed in the oyster shells was estimated.

In **Chapter III**, the distribution of Cd and Zn with the ratio of Cd/Zn in the Hiroshima Regional Urban Area was referenced, and the concentrations of Cd and Zn in commercially available game meat within the area were screened. Further, the distribution of Cd and Zn in the body of wild boars privately captured within the area was investigated. Risks to humans being exposed to Cd through game meat consumption were estimated.

In **Chapter IV**, the accumulation of Cd and Zn in wildlife symbiotic with residents was examined in the local towns of Hiroshima Regional Urban Area. Pteridophytes, agricultural farm plants, wild boars and raccoon dogs were selected as the elements in the terrestrial ecosystem.

In **Chapter V**, transition of Cd from oyster shells into plants was investigated. Farm-, pot-, and hydroponic cultivation with two kinds of plants were performed with two kinds of plants. One of functions of oyster shells as a transporter of Cd from sea to land was shown by the index of concentration factor (CF), which was calculated using the following equation: *CF = (value of foliage) / (value of pot soil)*.

General discussions are provided in **Chapter VI**. Cd and Zn concentrations in the organisms from their natural habitat were compared using CF (Petersen *et al.*, 2019).

For evaluation of Cd and Zn data, the acceptable maximum levels of the metals in sediment, soil, and food were referenced (**Appendix 1**). Materials and methods used for the measurements of Cd and Zn in all the samples, including wildlife, are explained in **Appendix 2**.

Log-normal and normal distributions were assumed for the comparison of metal concentrations in the target ecosystem. The log-normal distribution model suited acute accumulations of Cd and Zn in plants, whereas, the normal distribution models were more suited than log-normal distribution models to explain the chronic exposure of the metals in mammals, which has been experimentally proven in *Fischer 344* rats using radioactive iodine, an essential trace element (Nitta *et al.*, 2001).

In studies dealing with wild mammals, the animal welfare and ethics were the highest priority (**Appendix 2**).

This study will reveal a cross examination of Cd in the Hiroshima Regional Urban Area (**Fig. I-1**, **Table I-1**). Among the pollutants, Cd was used as the indicator. Oyster shell is a mediator that transports hazardous substances from the marine to terrestrial ecosystem. Raccoon dogs are the sentinel animal indicating the amount of exposure to Cd at a landscape in the Hiroshima Regional Urban Area. The data will contribute for the evaluation of present biological environment and for the promotion of human health.

Table I-1. Zn and Cd in aquatic area, fertilizer, and terrestrial areas.

		Zn (mg/kg)		Cd (mg/kg)	
		Nakanishi, *et al*., Zn, 2008[a]	Hiroshima Regional Urban Area (2018~2021)	Nakanishi, *et a*l., Cd, 2008[b]	Hiroshima Regional Urban Area (2018~2021)
Aquatic area	Culturing sea water	< 0.01*	-	< 0.005*	-
	Sea area	< 0.02*	-	< 0.02*	-
	Sediment	< 150	-	0.03 - 1.1	-
	Clams	-	-	< 2	-
	Benthos	-	-	0.56	-
	Oyster	33	-	0.1 - 0.68	-
	Hiroshima oyster (Tanaka *et al*., 1974) [c]	139.0 - 272.0	-	0.12 - 0.32	-
Fertilizer	Sewage sludge	-	-	0.9	-
	Sewage sludge fertilizer	-	-	5	-
	Oyster shell fertilizer	-	-	-	-
	General fertilizer	-	-	0.02 - 5.5	-
Terrestrial area	Pedosphere	-	-	2	-
	Soil	-	-	0.1 - 1000	-
	Pteridophyta	-	-	-	-
	Meat	-	-	0.001 - 0.03	-
	Wild boar kidney	-	-	-	-
	Raccoon dog kidney	-	-	-	-
	Human (Komai, Kambe, 2013) [d]	8.5 - 15.9 (blood) *	-	2.0 (kidney)	-

[a]: Nakanishi J, Naito W, Kamoh M. Risk Assessment Documents Vol 20 Zn. eds New Energy and Industrial Technology Development Organization, The Research Institute of Science for Safety and Sustainability, National Institute of Advanced Industrial Science and Technology, Maruzen, Tokyo. 2008 209p. (in Japanese)

[b]: Nakanishi J, Ono K, Kamoh M, Miyamoto K. Risk Assessment Documents Vol 13 Cd. eds New Energy and Industrial Technology Development Organization, The Research Institute of Science for Safety and Sustainability, National Institute of Advanced Industrial Science and Technology, Maruzen, Tokyo. 2008 369p. (in Japanese)

[c]: Tanaka Y, Ikebe K, Tanaka R, Sonoda S. Contents of heavy metals in foods (III), Food Hygiene Saf Sci. 1974 15: 390-393. (in Japanese)

[d]: Komai M, Kambe D. Zinc function and health. Kenpakusha, Tokyo. 2013 233p. (in Japanese)

*: mg/L

Zn and Cd are measured simultaneously in this research because of the following reasons (Kawaguchi *et al*., 2020). (1) Both Zn and Cd are ubiquitous in rocks with the ratio of Cd to Zn, Cd/Zn, which characterizes each bedrock, soil, terrestrial plant, sediment, and seagrass bed. (2) High concentrations of Zn and Cd have been reported in benthos for decades in the Hiroshima Bay. (3) A group of Zn transporters transport Cd *in vivo*. (4) Zn concentrations in kidney indicate physiological condition of individual mammal, while Cd concentrations in kidney represent its lifelong exposure.

II. Cd IN THE OYSTER CUTURING ENVIRONMENT OF HIROSHIMA BAY

Abstract: To investigate the distribution of Zn and Cd in the Hiroshima Bay, their concentrations in the sediment, whole oyster, oyster meat, and oyster shell were evaluated. The sediment at the coast of Hiroshima Bay contained 144.3±95.8 mg/kg of Zn and 0.34±0.19 mg/kg of Cd. Oysters attached on the coast sediment contained 340±173.2 mg/kg of Zn and 0.19±0.14 mg/kg of Cd as a whole, sediment below the culturing rafts contained 160.0±39.2 mg/kg of Zn and 0.38±0.06 mg/kg of Cd. Cultured oysters contained 183.9±120.1mg/kg of Zn and 0.22±0.12 mg/kg of Cd in meat, and oyster shell wastes contained 24.4±23.2 mg/kg of Zn and 0.05±0.05 mg/kg of Cd. Considering the total amount of oysters produced in the area, 5.5~6.3 kg/year of Cd was fixed in oyster shells.

II.-1 Purpose

Cd and Zn are ubiquitous in rocks with the ratios of Cd to Zn, Cd/Zn, which characterizes each bedrock (Dharma-Wardana MWC, 2018), soil (Fishbein L, 1981; Hamid *et al.*, 2016; Kubicka *et al.*, 2015), terrestrial plant (Ikeda *et al.*, 2006), sediment (Soliman *et al.*, 2015), and seagrass bed (Bouchon *et al.*, 2016). The sources of Cd in soils were Zn mining, battery production plant, metal smelting, or cement factories (Smith RS, 2009; WHO Regional Office for Europe, Copenhagen, Denmark, 2000). The Cd/Zn in Japan was estimated as 0.0053 in soil (Ono *et al.*, 2005) and 0.0045 in galvanized materials (Matsuno *et al.*, 2012). The total amount of Cd discarded into air and water was estimated as 130 t/year (Nakanishi *et al.*, 2008). Owing to the toxic effects on human health, WHO has determined the maximum acceptable limits of Cd and Zn in soil as 4 mg/kg and 300 mg/kg, respectively (WHO, 2006). According to EU, the maximum limit values are 3 mg/kg for Cd and 300 mg/kg for Zn (EU, 2002). The maximum limit values of Cd/Zn in soil set by WHO and EU are 0.0133 and 0.0100, respectively.

Cd pollution poses hazards to food safety, and its artificial contaminations are of the greatest concern. Application of phosphate fertilizers, whose concentration of Cd is 130 mg/kg in average (Roberts T, 2014), is the major inputs of Cd into agricultural soils. The usage of oyster shell waste as a fertilizer may play an important role in increasing the Cd consumption of humans, no matter how the total metal content, pH, soil organic matter, cation exchange capacity, or cray content controls the metal bioavailability of soil (McComb *et al.*, 2014).

The objective of this part of the study is to screen current concentrations of Cd in the pacific oyster (*Crassostrea gigas*) (oyster) and the oyster culturing environment of Hiroshima Bay facing the Hiroshima Regional Urban Area (https://www.city.hiroshima.lg.jp/site/kouiki/list846.html). Cd concentrations in the sediments, benthos, oyster meat, and fertilizers made from oyster shell waste were measured. These data would be helpful to explain a food chain hypothesis that high concentrations of Cd in wildlife could be caused by taking an excess amount of Cd through the agricultural farm plants given the fertilizers blended with oyster shells (**Fig. I-1**).

II.-2 Materials and methods

Study area: The Hiroshima Regional Urban Area is an economic unit comprising of 23 cities and towns of Hiroshima and Yamaguchi prefectures, the population of which is 2 million (**Fig. II-1-A**). The total area is 6302.8km^2. Oyster culturing was systematically performed at Hiroshima Bay facing the Area (**Fig. II-1-B**).

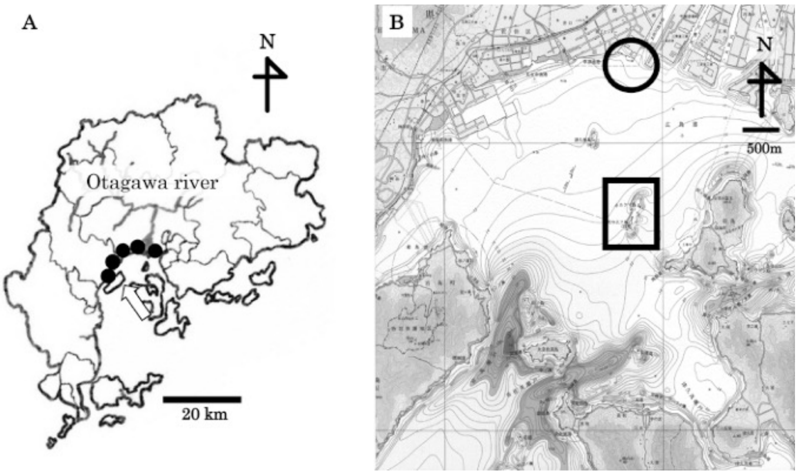

Fig. II-1. The Hiroshima Regional Urban Area and Hiroshima Bay. A: A rough map of the Hiroshima Regional Urban Area. Oysters were purchased at the five marketplaces (●). Arrow indicates the Itsukushima Island. **B:** A nautical map of Hiroshima Bay. Sediments were collected at the mouth of Otagawa river (○) and around the iles of Kakuma-jima (N34°31' 33.21, E 132°40' 17.83) (□). Arrow indicates the Itsukushima Island. The original map (BATHYMETRIC CHART No 6386[3], the Japan Coast Guard, 2007) was modified.

Sediment, benthos, and oyster: Oysters were purchased or collected (**Fig. II-1-A** and **-B**). Sediments and benthos were collected (**Fig. II-1-B**). Original samples were prepared for testing (**Appendix 2 - Table 2**).

Oysters were purchased at marketplaces in the area or collected at the mouth of Otagawa river, when the tide was low, with wet weight of more than 1 kg per place. Fresh oysters with their shells were dried and grounded using a mill. Fresh oyster meat was separated from the shell. The shells were cleaned with running water, dried, grinded via a mill, and sieved through a 2-mm screen to remove impurities. All the original samples were store stored at -20 ℃ until use.

Sediments and benthos were collected using a specific sampler (Ekuman-Birge, RIGO Co. Ltd., Tokyo, Japan) (Fukuhara *et al.*, 1987). Sediments at the mouth of the river and streams were collected when the tide was low. Sediments under the oyster rafts were collected at a depth of less than 10 cm from the surface, with wet weight of more than 0.3kg each scoop. Depth was measured using a water depth detector.

Oyster shell fertilizer: Commercially available oyster shells sold as fertilizers were used as the commercially available fertilizers in the present study.

Oyster shells collected from the Hiroshima Bay (**Fig. II-1-A**) were subjected to a series of steps, including cleaning, drying, grinding, and sieving. The obtained powders were used as the handmade fertilizer in the present study.

***Measurements of Zn, Cd, and other Class II hazardous substances*:** The concentrations of Zn, As, Cd, Cr, Pb, B, CN, and F were measured (**Appendix 2 - Table 1** and **2**). Sediments, benthos, oyster shells, and oyster meat were prepared as powdered samples for tests. Concentrations of Cd, Zn, and Pb in the sediment, benthos, and oyster shell were determined via AAS, and those of As, Cr, B, CN, and F were via absorptiometry. Concentrations of Cd and Zn in oyster meat were determined via ICP-AES.

Statistical analysis: Student's *t*-test was used to assess the significance of the observed differences among Cd, Zn and Cd/Zn. KaleidaGraph software version 3.6 (HULINKS, Tokyo, Japan) was used for statistical analysis.

II.-3 Results

Cd and Zn in the sediment and benthos: The sediments of riverbed at the mouth of Otagawa river and the oysters attached on the stones of the riverbed contained Cd (**Table II-1**). The Cd values were smaller than that of the environmental standard. The average value of Zn in the benthos was more than twice as high as the upper limit of environmental standard.

Table II-1. Concentrations of Cd and Zn in the sediment and benthos.

	Sediment	Benthos	Sediment standard *
Number	3	3	-
Cd (mg/kg, mean±SD)	0.34 ± 0.19	0.19 ± 0.14	<1
Zn (mg/kg, mean±SD)	144.3 ± 95.8	340.0 ± 173.2	<150
Cd/Zn (x100)	0.249 ± 0.138	0.052 ± 0.019	-

Samples were collected in January from 2018 to 2020. Wild oysters attached on the sediment were used as the representative of benthos. Cd and Zn concentrations in whole oyster and meat plus shell were measured. *: **Appendix 1**.

The meat of both cultured and wild oysters contained Cd, and its concentration was not statistically different between the two (**Table II-2**). The oyster meat available in Hiroshima Bay (*n*=8) contained Cd and Zn with concentrations of 0.21±0.12 mg/kg and 254.9±173.9 mg/kg, respectively, and the value of Cd/Zn (x100) was 0.114±0.086 (mean±SD).

Table II-2. Concentrations of Cd and Zn in oyster meat.

	Purchased (Cultured)	Collected (Wild)	Tolerable intake level *
Number	5	3	-
Cd (mg/kg, mean±SD)	0.22 ± 0.12	0.20 ± 0.13	<0.007 mg/kg body weight/week
Zn (mg/kg, mean±SD)	183.9 ± 120.1	373.3 ± 208.2	<60 mg/day
Cd/Zn (x100)	0.153 ± 0.089	0.050 ± 0.008	-

Samples were purchased or collected between 2018 and 2020. *: **Appendix 1**.

Cd concentration in the sediments below oyster rafts: Sediments below the oyster raft contained Cd (**Table II-3**). Concentrations of Cd were under the sediment standard level, whereas those of Zn were above the upper standard limit.

Table II-3. Concentrations of Zn, Cd,
and the other Class II hazardous substances in the sediment below oyster rafts.

			Kakuma (2012)	Kakuma (2021)	Sediment *
Number			4	4	-
Zn (mg/kg)			277.5±32.0	160.0±39.2	<150
Specified hazardous substances, Class II (mg/kg)	Heavy metals	As	6.8	1.7±0.3	-
		Cd	0.44±0.12	0.38±0.06	<1
		Cr	78.9	16.0±7.6	<80
		Pb	48.5	20.4±3.0	<46.7
	Others	B	NT	27.3±9.4	-
		CN	NT	<3	-
		F	NT	90.0±9.4	-
Cd/Zn (x100)			0.15±0.06	0.24±0.03	-

Sediments around the lies of Kakuma-jima were collected in 2012 and 2021 (**Fig. II-1-B**).
The water depth was between 19m and 21m at high tide. *: **Appendix 1**; NT: not tested.

Cd concentration in oyster shell fertilizer: Cd concentrations in the fertilizer made from oyster shell waste are shown in **Table II-4**. B concentrations in the handmade fertilizer were higher than those in the commercially available fertilizer ($p<0.05$).

Table II-4. Concentrations of Zn, Cd,
and the other Class II hazardous substances in oyster shell.

			Oyster shell fertilizer (mg/kg) a)		Sediment (mg/kg) b)	Agricultural soil c)
			Commercially available	Handmade		
Number			4	5	-	-
Zn (mg/kg)			23.3±6.4	24.4±23.2	<150	≦120
Specified hazardous substances, Class II	Heavy metals	As	<0.5	<0.5	-	≦0.01
		Cd	0.04±0.04	0.05±0.05	<1	≦0.003
		Cr	27.5±4.4	18.6±12.1	<80	-
		Pb	0.73±0.17	0.48±0.24	<46.7	≦0.01
	Others	B	17.2±3.1	23.8±2.3 *	-	≦1
		CN	< 3	< 3	-	undetected
		F	50.0±24.5	28.0±14.8	-	≦0.8
Cd/Zn (x100)			0.17±0.03	0.24±0.26	-	-

a): Prepared in 2019. The oyster shell powder is classified as a special fertilizer, for which no standard value of hazardous substance has been set (**Appendix 1**);

b): Determined by the Ministry of Land, Infrastructure, Transport and Tourism (**Appendix 1**);

c): Determined by the Ministry of the Environment (**Appendix 1**). Units for Zn and the Class II specified hazardous substances are mg/kg and mg/L, respectively.

*: p<0.05 when compared to the commercially available oyster shell fertilizers by t-test.

II.-4 Discussion

Cd and Zn concentrations in the marine ecosystem of Hiroshima Regional Urban Area are included in **Table II-5**. The total amounts of Cd in meat and shells of the oysters from Hiroshima Bay were 4.1 kg/year and 5.5~6.3 kg/year, respectively. The average concentration of Cd in oyster shells was 0.05 mg/kg. The agricultural field occupies 128.54 km^2 (2.04 %) of the total area of Hiroshima Regional Urban Area, 6302.8 km^2 (https://www.pref.hiroshima.lg.jp/site/toukei/nouringyocensus.html#r2, https://www.pref.yamaguchi.lg.jp/cms/a12500/nourin_census/2020-sokuhou.html). When the entire oyster shell waste was re-used as a fertilizer, Cd in the fertilizer could cover the entire agricultural farm with concentrations of 0.043~0.049 mg/m^2/year.

High concentrations of Zn pose a threat to food safety and human health (Maares *et al.*, 2020; Maret *et al.*, 2006; Motooka *et al.*, 2016). Zn concentrations in oyster meat were as high as those reported 50 years ago (Tanaka *et al.*, 1974). Since the Ministry of Health, Labour and Welfares issued the <60 mg/day of Zn intake as health disorder prevention level, consumers would cross this intake level by eating 10 pieces of oyster meat. The mean weight of commercially available oyster meat was 21.4 g (**Appendix 2 - Table 3**).

In Japan, 8.5% (44 out of 519) of the foods contain a Cd concentration of more than 0.1mg/kg (Kikuchi *et al.*, 2002). The Cd concentration in oyster meat, 0.2 mg/kg, was not too low, no matter how the upper limit of Cd concentration to oyster meat was not determined by the Ministry. On consumption of 200 g of oyster meat in a week, the total amount of Cd reaches to the upper limit of the Provisional Weekly Intake Level, <0.007 mg/kg body weight/week (**Appendix 1**).

Cd in oyster shells poses a threat to the local terrestrial ecosystem when recycled as a fertilizer material (*Fertilizer Regulation act on 1950*). The concentration of Cd in fertilizers made from oyster shell waste were obtained by the Sediment Inspection Methods (https://www.env.go.jp/water/teishitsu-chousa/00_full.pdf) (**Appendix 2 - Table 1** and **2**). On the other hand, dilution ratios to scatter them on agricultural farms, which are important for users to be informed, are available only when elution experiments were performed for individual Class II hazardous substances (https://www.env.go.jp/kijun/dt1-1.html).

Origins of B, CN and F contained in the oyster shells would be anthropogenic. B in the agricultural soil decreased the yields of crops (Nable *et al.*, 1997; Nielsen FH, 1998). F reduces photosynthesis and causes leaf necrosis (Choudhary *et al.*, 2019). This information obtained by the present study is essential for residents who fertilize their agricultural farm with oyster shell powder.

The average concentration of Cd in oyster shell, 0.05 mg/kg (**Table II-4**), was at the lowest level, when compared to previous data (Nitta Y, 2019). This could be due to three factors, the collection site, time, and oyster size used for measurement. The collection sites were scattered along the coast of Hiroshima Bay and straddled Hiroshima and Hatsukaichi cities. The collection time, which spanned from March to July, was in the rest period of oyster culturing. The size of oysters collected in spring and summer was smaller than that in winter, and the size of 8-month-old oyster shells obtained in the Nishi-ward of Hiroshima city was the smallest (**Appendix 2 - Table 4**). The Zn concentration in meat peaked in December, and it gradually declined after January (Nitta Y, 2018).

Table II-5. Zn and Cd at the sea area facing Hiroshima Regional Urban Area.

Material			Amount	Cd (mg/kg)	Zn (mg/kg)	Cd/Zn (x100)	References
Ore	Total	import (2019)	859,943 (t/year)	-	-	-	https://www.kogyo-kyokai.gr.jp
	Cd	produt (2003)	2,500 (t/year)	-	-	0.526	Ono *et al.*, 2005
		waste into air + water (2000)	130 (t/year)	-	-	-	
		waste into air (2003~2008)	demand * (t/year)	-	-	0.189～0.296 x10^{-4}	Matsuno *et al.*, 2012
		waste into water (2003~2008)	(t/year)	-	-	0.308 x10^{-4}	
Sediment	Cd	at the mouth of Otagawa river (2018-2020)	-	0.34±0.19	144.3±95.8	0.25±0.14	present study
		Hiroshima Bay (2021)	-	0.38±0.06	160.0±39.2	0.24±0.03	
Oyster	Total	meat in Japan (2019)	30,278 (t/year)	-	-	-	https://www.pref.hiroshima.lg.jp/soshiki/88/syukkasisin.html
		meat in Hiroshima (2019)	18,809 (t/year)	-	-	-	
		estimated shell waste in Japan (meat x 4)	121,112 (t/year)	-	-	-	present study
		estimated shell waste in Hiroshima (meat x 5.8~6.7) **	109,092~ 126,020 (t/year)	-	-	-	
	Cd	meat in Hiroshima	4.1 (kg/year)	0.20±0.13	373.3±208.2	0.05±0.08	present study
		shell in Hiroshima	5.5~6.3 (kg/year)	0.05±0.05	24.4±23.2	0.24±0.26	

*: For meeting the demand of Zn, 415,007 t/year in 2019, the total amount of Cd distributed in the environment was 0.08~0.12 t/year into air and 130 t/year into water.

**: Dry weights of the oyster shell were 87.5±1.7% (mean ± SD) of those wet, when examined three times using the wild oysters collected from three different sites of the Hiroshima Bay.

III. Cd IN LOCAL FOODSTUFFS IN THE HIROSHIMA REGIONAL URBAN AREA

Abstract: Cd contamination in foods is unavoidable. The Cd concentrations in game meat were screened with the criterion of <0.05 mg/kg in the Hiroshima Regional Urban Area, where no specific spot showing high Cd levels in the sediment was reported. On screening two kinds of commercially available game meat, it was observed that all wild boar meat contained less than 0.05 mg/kg of Cd, however, 28.6 % of sika deer meat contained Cd over the criterion limit. On screening of wild boars privately captured in two narrow districts, the Cd concentrations found to be were <0.05 mg/kg in muscle tissues and 1.97±1.67 mg/kg in the kidneys, which was more than 40 times higher than that in muscles. Thus, screening of the kidneys of wildlife can be useful to assess Cd contamination in every ecosystem.

III.-1 Purpose

Cd circulating in the environment is concentrated in the soil, rice, and humans, and its toxic effects on humans have been shown as Itai-Itai disease (Nishijo *et al.*, 2017) (**Table I-1**). Epidemiological studies came to the following consensus: the Cd concentration in soil and rice are the intake indicators of external exposure, and those in the urine or kidney are indicators of internal exposure (Horiguchi *et al.*, 2013; Pastorelli *et al.*, 2012; Sawada *et al.*, 2012).

A high consumption of Cd from foodstuffs has been reported in patients with chronic kidney disease (CKD) (Ishizaki *et al.*, 2015; Nogawa *et al.*, 1983). Experimental exposures proved the dose-dependent accumulation of Cd in monkey kidneys (*Macaca fascicularis*) (Kurata *et al.*, 2014). However, the Cd-related effects on humans and experimental animals were different depending on the combination of external factors, such as exposed duration and doses, and internal factors, such as ages, health condition and genetic background. For example, CKD was inducible by Cd in diabetic rodents, irrespective to whether streptozotocin-induced diabetes alone caused any CKD-related histopathological change in their kidneys (Bernard *et al.*, 1991; Nitta *et al.*, 2009).

Cd concentrations in ordinally consumed foods indicated that seaweeds contained higher amounts of Cd followed by oyster and rice while most of the examined fruits and vegetables in Japan (Kikuchi *et al.*, 2002), Spain (López-Alonso *et al.*, 2016), and Ireland (Lane *et al.*, 2015) had a low Cd concentration. The average Cd concentrations in commercially available meat of fielded ruminants were <0.2 mg/kg and 0.0281mg/kg in Japan and Spain, respectively (Kikuchi, et al., 2002; Marín *et al.*, 2018). The maximum officially acceptable levels of Cd for farm animal meat have not been determined in Japan, while <0.05mg/kg and <1mg/kg values have been designated for muscle meat and kidneys, respectively, in an EU member country (Food safety authority of Ireland, 2009).

The Cd concentration in game meat was analyzed in EU countries owing to the high consumption of wild game meat. The Cd concentrations in wild boar muscles were 0.02 mg/kg or 0.08 mg/kg in Italy (Chiari *et al.*, 2015; Danieli *et al.*, 2012) and 0.006~0.018 mg/kg in Poland (Rudy M, 2010) whereas those in wild boar kidneys were

3.72 mg/kg and 0.112~5.4 mg/kg in Italy and Spain, respectively (Chiari *et al.*, 2015; Dharma-Wardana MWC, 2018). Further, Cd concentrations in the muscles and kidneys of red deer (*Cervus elaphus*) were 0.006 mg/kg and 1.02 mg/kg of Cd, respectively, in Italy (Chiari *et al.*, 2015).

The objective of this part is to screen the current concentrations of Cd in game meat in the habitat of large-sized wildlife in the Hiroshima Regional Urban Area since the Cd concentrations in game meat is not regulated by the Ministry of Health, Labour, and Welfares (**Appendix 1**). Cd concentrations in commercially or privately available game meat commercially or privately available were screened. Cd concentrations in the muscle of wild boar were screened, and they were compared between wild boar muscles and kidneys (Horiguchi *et al.*, 2013; Ishizaki *et al.*, 2015; Nogawa *et al.*, 1983; Pastorelli *et al.*, 2012; Sawada *et al.*, 2012).

III.-2 Materials and methods

Study area: Game meat and wildlife were available in the Hiroshima Regional Urban Area (**Fig. III-1**).

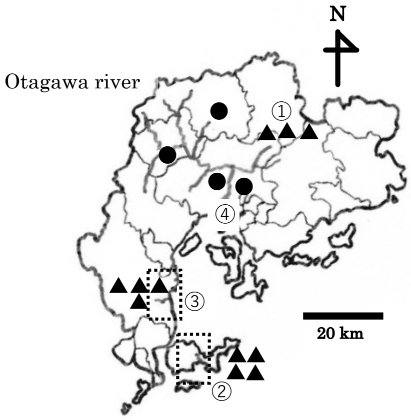

Fig. III-1. Hiroshima Regional Urban Area and places wildlife captured. Markets were placed at Akitakada (①) and SuouOshima (②). Sediments were collected at the mouth of two streams and one river, the Tachibanagawa (②), Ejirigawa (③), and Otagwa (④). Other sediments collected were at the points of Otagawa river (●) and in the three areas (▲), total points of which were 36 (http://gbank.gsj.jp/geochemmap). Wild boars were captured by hunters at the two areas (⬚).

***Game meat*:** Two kinds of meat, wild boar, and sika deer muscles, were purchased at the two marketplaces of Akitakada and SuouOshima from April to July in 2017 (**Fig. III-1**).

***Wildlife*:** Wild boars were captured at Oogi and SuouOshima districts, with an area of less than 4 km^2 each, during routine hunt from November 2017 to February 2018 (**Fig. III-1**). They were wire-trapped by two registered hunters. The hind limb muscle, kidney, and macroscopically abnormal tissues were obtained from the carcasses, and they were immediately fixed in 10% phosphate buffered formalin. More than 0.5kg of the muscle and another kidney samples were stored at -20℃ until further examinations.

Ectoparasites on the skin and endoparasites of skeletal muscles, such myocardium, lungs and renal hilus, were examined macroscopically at autopsy to exclude severely infected animals from further experiments. A total of 16 wild boars were used in this study.

***Pathology*:** The tissues fixed in formalin were processed for the preparation of paraffin sections. The thin-sliced sections were stained with hematoxylin and eosin or with other special staining methods for microscopic examination.

***Animal welfare and ethics*:** Wildlife were treated along with the guidelines of the Health Management of Wild Birds and Beasts in Yamaguchi prefecture and obeying the code of ethics and The Oath of Veterinarians-declaration '95 (**Appendix 2**).

***Sediment*:** Sediments were collected in January 2018 at the mouths of Ejirigawa stream and the Tachibanagawa streams flowing in Oogi and Agenosho areas, respectively (**Fig. III-1**). Sediments at the mouth of Otagawa river, Otagawahousuiro, were collected in every January from 2014 to 2018. The sampling depth was 0 to 10 cm, and the sampling time was once per site per year. All materials were stored at -20 ℃ until use.

***Measurement of Cd and Zn*:** The hind limb muscle, kidneys, and sediments were used for measurement (**Appendix 2 - Table 2**). The meat, muscle, and kidney samples were washed properly with deionized water to remove superficial blood, oven-dried, and pulverized. The sediment was air-dried, milled, and sieved through a 2-mm screen to remove impurities.

Two grams of each sample dried using a hot plate was digested in 2 mL of 61% HNO_3 solution in the vessel, and they were then dried on a hot plate at 500 ℃. Concentrations of Cd and Zn in meat, muscle and kidney were determined using ICP-AES. The measuring limit for the metal concentrations was 0.05 mg/kg. The concentration of the metals in sediments was determined via AAS.

***Statistical analysis*:** Student's *t*-test, *F*-test and χ^2-test were performed to compare the concentrations of Cd and Zn in the wildlife and sediment. Pearson's coefficient of correlation was used to examine correlations among the indices of body weight, Cd, and Zn. Kaleida Graph (HULINKS, Tokyo, Japan) software was used.

III.-3 Results

***Cd contamination in sediments*:** Cd concentrations in the sediment was measured to estimate the external exposure of wildlife (**Fig. III-1**, **Table III-1**). Using the information of 36-collection-points in the Hiroshima Regional Urban Area, the concentrations of Cd, Zn and Cd/Zn(x100) were 0.21±0.09 mg/kg, 110.3±31.1 mg/kg and 0.19±0.05, respectively (http://gbank.gsj.jp/geochemmap/). The average value of Cd at the mouth of Otagawa river was 0.50±0.18 mg/kg.

Table III-1. Concentrations of Cd and Zn in sediments.

		Iwakuni		SuouOshima		Otagawa system			Hiroshima Regional Urban Area b)
		Ejirigawa a)	Iwakuni b)	Tachibanagawa c)	SuouOshima d)	Akitakada b)	Otagwa-housuiro b)	Otagwa river b)	
Number		1	4	1	4	3	5	4	36
Cd (mg/kg)	mean ± SD	0.18	0.27±0.17	<0.05 e)	0.08±0.06	0.46±0.26	0.50±0.18 f)	0.18±0.02	0.21±0.09
	low - high		0.09 - 0.48		0.02 - 0.15	0.35 - 0.76	0.26 - 0.73	0.16 - 0.20	0.09 - 0.48
Zn (mg/kg)	mean ± SD	52.0	131.2±30.4	9.0	103.3±32.3	200.8±95.1	156.8±77.3	101.6±5.5	110.3± 31.1
	low - high		99.8 - 171.5		71.6 - 136.9	102.9 - 292.9	84.0 - 230.0	94.0 - 107.0	51.8 -171.5
Cd/Zn (x100)		0.35	0.21	< 0.56	0.08	0.23	0.32	0.18	0.19

a): Flowing at Oogi in Iwakuni city. Collected in January, 2018;

b): Average values of the points (http://gbank.gsj.jp/geochemmap/);

c): Flowing at Agenosho in SuouOahima island. Collected in January, 2018;

d): Flowing at Nishi-word, Hiroshima city. Collected in January from 2014 to 2018;

e): Measuring limit was 0.05 mg/kg;

f): Correlation coefficient to Zn was 0.90 (*p*<0.05).

***Cd contamination in commercially available game meat*:** To estimate the risk of exposure of Cd to consumers, its concentrations in 14 game meats were screened (**Table III-2**). Wild boar meat was available at Akitakada and SuouOshima (**Fig. III-1**). All the wild boar meat showed Cd concentrations of <0.05mg/kg.

Sika deer meat was available in Akitakada, but not in SuouOshima (**Fig. III-1**). Two out of the seven sika deer meat (28.6%) contained 0.07 mg/kg and 0.08 mg/kg of Cd and 52.4 mg/kg and 80.6 mg/kg of Zn, respectively. The other five meat showed <0.05 mg/kg of Cd and the 23.6 mg/kg of Zn with the range of 15.9~40.4 mg/kg.

Table III-2. Contaminations of Cd and Zn in the commercially available thigh meat.

Animal species		Wild boar		Sika deer
(Purchased area)		(Akitakada)	(SuouOshima)	(Akitakada)
Number		3	4	7
Cd (mg/kg)	mean ± SD	-	-	-
	range (low - high)	<0.05	<0.05	≦0.08 a)
Zn (mg/kg)	mean ± SD	25.1 ± 15.5	22.6 ± 5.1	35.9±23.9 b)
	range (low - high)	11.4 - 41.9	18.6 - 29.4	15.9 - 80.6

a): Concentrations were 0.07 mg/kg and 0.08 mg/kg for the two, while <0.05 mg/kg for the others;

b): The mean concentration was 66.6 mg/kg for the two samples with high Cd, and 23.6 mg/kg with the range of 15.9~40.4 mg/kg for the others.

***Cd contamination in captured wild boar*:** To estimate the distribution of Cd in bodies, Cd concentrations in the muscles and kidneys of wildlife were measured (**Table III-3**). Wild boar was captured at Iwakuni and SuouOshima during the hunting period (**Fig. III-1**). All the nine animals showed Cd concentrations of less than 0.05 mg/kg in the muscles and 1.97±1.67 mg/kg with a range of 0.73~6.03 mg/kg in the kidneys. Zn concentrations in the muscles were as high as those in kidneys, and the average concentration ratio of kidney to muscle was 39.4±33.5 mg/kg. Sex difference in the metal concentrations was not obvious nor any histopathological change was observed.

Table III-3. Distribution of Cd and Zn within the body of wild boars captured at Oogi and Agenoshou.

No.	Captured area	Sex	Body weight (kg)	Muscle (hind limb)		Kidney		Kidney/Muscle	
				Cd (mg/kg)	Zn (mg/kg)	Cd (mg/kg)	Zn (mg/kg)	Cd	Zn
1	Oogi (Iwakuni)	Female	25	<0.05	12.9	2.33	23.2	46.6	1.80
2		Female	35	<0.05	16.0	6.03	25.9	120.6	1.62
3		Male	30	<0.05	18.0	1.63	23.1	32.6	1.28
4		Male	35	<0.05	33.4	1.60	20.5	32.0	0.01
5		Male	38	<0.05	19.0	1.48	19.1	29.6	1.01
6	Agenoshou (SuouOshima)	Female	90	<0.05	41.3	2.56	17.4	51.2	0.42
7		Male	25	<0.05	17.2	0.89	23.7	17.8	1.38
8		Male	31	<0.05	30.6	0.47	16.1	9.4	0.53
9		Male	43	<0.05	37.7	0.73	25.1	14.6	0.07
	mean±SD		39.1±20.0	<0.05	25.1±10.6	1.97±1.67	21.6±3.45	39.4±33.5	1.03±0.51

The value, 0.05, was used as the concentration of Cd in muscles for calculation.

The geographical effect was examined (**Table III-4**). No statistically significant difference was observed in the body weight and concentration of the two metals on comparison of the concentration values of captured wild boars between the two areas (**Fig. III-1**).

Table III-4. Accumulation of Cd in the kidney of wild boars captured at Oogi and Agenoshou.

Captured area	Number	Body weight (kg)		Cd (mg/kg)		Zn (mg/kg)	
		mean ± SD	range (low - high)	mean ± SD	range (low - high)	mean ± SD	range (low - high)
Oogi (Iwakuni)	11[a]	38.4 ± 21.3	13 - 70	1.61 ± 1.59	0.22 - 6.03	22.1 ± 3.3	11.4 - 41.9
Agenoshou (SuouOshima)	5[b]	40.8±29.3[c]	15 - 90	1.11 ± 0.83	0.47 - 2.56	21.6 ± 4.5	18.6 - 29.4
Oogi + Agenoshou	16	39.1 ± 23.1	13 - 90	1.46 ± 1.39	0.22 - 6.03	21.9 ± 3.6	11.4 - 41.9

[a]: Five animals were the same individual of **Table III-3**;

[b]: Four animals were the same individual of **Table III-3**;

[c]: Correlation coefficient to the Cd of Agenoshou was 0.88 ($p<0.05$).

III.-4 Discussion

Cd and Zn concentrations in the terrestrial ecosystem of Hiroshima Regional Urban Area are shown in **Table III-5**. The sika deer meat with Cd contamination was obtained from Akitakada; however, no specific point showing high metal concentrations was mentioned (http://gbank.gsj.jp/geochemmap/).

To minimize the exposure to Cd through foods, screening of Cd in the game meat with a criterion dose of 0.05 mg/kg is reasonable. Considering the Provisional Tolerable Weekly Intake, <0.007mg/kg of body weight/week (**Appendix 1**), when a person with a body weight of 60 kg, eats 1 kg of game meat containing 0.05 mg/kg of Cd per week, the total amount of Cd intake is 0.04 mg/60 kg of body weight/week, which is as high as the Provisional Tolerable Weekly Intake, <0.042 mg/60kg/week. Cd concentrations in 28.6% of the sika deer meat sold in this area were high as compared to the levels of ordinary meat in Japan (Kikuchi *et al.*, 2002) and those of game meat in Italy (Chiari *et al.*, 2015). Accordingly, some source of Cd contamination is present. To inform the exposure risk of Cd should be performed for every consumer of game meat.

To make the contamination of Cd visible, it is essential to choose good sentinels. The ideal sentinels could be widely distributed within the area, so that the levels of Cd in the organism are representative of the entire area of concern (O'Brien *et al.*, 1993). Multiple species are adequate to monitor the environmental quality, as simultaneous exposure in a similarly contaminated environment may help to elaborate subtle influences on human health. The sentinels should be at the top position of the food chain in the environment, migrate to less than 5 km of radius during their lives, and be logistically available for screening (Ferri *et al.*, 2017). Wildlife inhabiting in the agricultural landscape are good candidates for sentinels sharing the same conditions of exposures to Cd with residents.

Wild boars captured at Oogi, had baited the vegetables and fruits cultivated in the agricultural farm. The accumulated Cd in their kidneys would contribute in finding the source of Cd contamination in the area. One possibility is the plants cultivated in the agricultural farm, where the Cd containing oyster shell fertilizer was sprinkled (Horiguchi *et al.*, 2013; Ikeda *et al.*, 2006; Yamanaka *et al.*, 1998).

Table III-5. Cd and Zn in game meat and sediment at the Otagawa river system.

Material		Number	Cd (mg/kg)	Zn (mg/kg)	Cd/Zn (x100)	References
Sediment	Hiroshima Bay (2021)	4	0.38±0.06	160.0±39.2	0.24±0.03	Table II-3
	Hiroshima Regional Urban Area (1999)	36	0.21±0.09	110.3±31.1	0.19±0.05	http://gbank.gsj.jp/geochemmap/
	Hiroshima (northern part) (1999)	9	0.46±0.26	200.8±95.12	0.23±0.58	
	at the mouth of Otagawa river (2014-2018)	5	0.50±0.18	156.8±77.3	0.32±0.06	present study
Wildlife	Deer game	7	≦ 0.08	15.0~80.6	0.20±0.08	present study
	Wild boar game	16	< 0.05	11.4~41.9	0.23±0.09	

The value, 0.05, was used as the concentration value of Cd in muscle for calculation. Measurement of Zn was used to assess the habitat environment and the physiological condition of the wildlife.

IV. ORIGIN OF Cd AND Zn IN WILDLIFE CAPTURED AT THE SOUTHERN PART OF THE HIROSHIMA REGIONAL URBAN AREA

Abstract: Information on anthropogenic Cd in local areas is important for public health, food hygiene and ecosystem. Bioaccumulation of Cd and Zn in wild pteridophytes, harvested farm plants and wildlife were measured in a local area of the southern part of Hiroshima Regional Urban Area for 3 years from 2017 to 2019. Wild pteridophytes showed mean values of 1.083±1.899 mg/kg for Cd and 68.2±71.3 mg/kg for Zn with a strong correlation between the Cd concentrations of the two metals. Farm plants, contained 0.0063±0.0056 mg/kg for Cd, which were not correlated with the concentration of Zn, 14.7±21.8mg/kg. Farm plants and wild boar muscles, which are used as foodstuffs, contained <0.025 mg/kg of Cd and <89.0 mg/kg of Zn. The kidneys of female wild boars and raccoon dogs contained Cd, and the concentration was correlated to that of Zn. The accumulation of Cd correlated to Zn among the examined elements indicated that female raccoon dogs are the primary element for investigating Cd in the local ecosystem. These data could be useful for risk communication on the food safety among residents, hunters, and researchers.

IV.-1 Purpose

Anthropogenic environmental pollutants have been cleaned up for decades, however, heavy metal contamination has resulted from improper disposal or specific chemicals released in and around cities. Pollutants piled up at the mouth of river over years accumulate in benthonic organisms via the corrosion chain (Bouchon *et al.*, 2016; Fishbein, L., 1981; Jeffree *et al.*, 2014; Rodney *et al.*, 2007). Cd and Zn are the heavy metal contaminants affecting plant and animal life.

During the evaluation of the nutrition beneficial for consumers, the meat of pacific oyster (*Crassostrea gigas*) (oyster), a local special foodstuff cultivated in Hiroshima Bay, showed high concentrations of Zn (Nitta *et al.*, 2017), which were high as compared to those of a previous report in early 1970s (Tanaka *et al.*, 1974). Further studies indicated that Cd as well as Zn are present in the oyster shell and sediments at the mouth of Otagawa river, where the oysters were cultivated (Nitta *et al.*, 2019; Nitta Y, 2019). The Food Safety Commission of Japan does not regulate Cd contamination in oyster meat. No attention has been paid to the Cd concentrated in shells (Lee *et al.*, 2010) nor the flow of Cd from benthic organisms to terrestrial organisms. Meanwhile, several plants for recycling oyster shell waste as fertilizers operate in the Hiroshima Regional Urban Area.

The main pathway of transport of the environmental Cd into mammals is via foodstuffs grown on the soil sowed with fertilizers containing Cd (Silvia *et al.,* 2014). To reduce human exposure from farm plants, determination of the critical levels of Cd in agricultural soils (Fadigas *et al.,* 2006), development of rice mutants accumulating lower concentration of Cd (Shimo *et al.*, 2011; Treesubsuntorn *et al.*, 2019), and examinations of phytoremediation techniques to remove Cd by pteridophyta, poaceae or fabaceae (Kubicka *et al.*, 2015; Rahman *et al.*, 2018; Zhu *et al.*, 2013), have been performed. However, from the viewpoint of ecology, any anthropogenic activity to change the original vegetation in the target area should be performed carefully. This is because the geological status, agricultural intensification, and other visible and invisible resident activities compose parts of the landscape, where wildlife functions as one of the main elements in the rural biome (Saito and Koike, 2013).

Cd and Zn widespread ubiquitously with the Cd/Zn ratio of one hundredth in rocks, soil, and seagrass beds (Bouchon *et al.*, 2016; Fishbein L., 1981; Kubicka *et al.*, 2015; Zhu *et al.*, 2013). Although an optimal Zn concentration is essential for organisms (Black e*t al.*, 1988; Fosmire, GJ, 1990), the tolerable upper intake level was determined (**Appendix 1**). Cd is a non-essential element for mammals, and it is known to cause health problems to humans on chronic exposure (Horiguchi *et al.,* 2013; Nishijo *et al.,* 2017; Nogawa *et al.,* 1983). Zn promotes metallothionein induction to detoxify Cd in organisms (Bourdineaud *et al.*, 2006; Coyle *et al.*, 2002; Piano, *et al.*, 2004) and to resist bacterial infections in the mammals chronically exposed to Cd (Simonyte *et al.*, 2003). Increased ratios of Cd/Zn in the kidneys indicate chronic exposures to Cd due to its long biological half-life in humans (Ishizaki *et al.*, 2015). Absolute levels of Cd in kidneys indicate the status of individual contamination, and the concentration ratios, Cd/Zn, indicates the functional ability of each organ or tissue of animals. Therefore, no additional contamination of Cd is imaginable in the animal colonies when constant correlations were observed between the two metals.

Exposure to environmental Cd has been assessed in local aquatic and terrestrial organisms (Casado *et al.*, 2008; Jankowski *et al.*, 2019; Jokanovic *et al.*, 2013; Lane *et al.*, 2015; Retamal-Salgado *et al.*, 2017). Some piscivorous birds contain Cd in eggs (Burger *et al.*, 2015; Kitowski *et al.,* 2018) while other mammalian game showed Cd in their muscles and other tissues (Chiari *et al.,* 2015; Mulero *et al.,* 2016). These data were referred from the perspective of public health (Danieli *et al.,* 2012; Ferri *et al.,* 2017; Hamid *et al.,* 2016), food hygiene (Filippini *et al.,* 2018; Marin *et al.,* 2018) and the ecosystem including humans (Katoh *et al.,* 2009; Sedláková *et al.,* 2019).

The objective of this part is to make the flow of Cd in organisms of the Hiroshima Regional Urban Area visible. Assuming the saturation model for metal accumulation (Nitta *et al.*, 2001), pteridophytes, farm plants, and wildlife were examined. Evaluation of the environment for residents' health was challenged by measuring Cd and Zn in agricultural farm plants and the mammals.

IV.-2 Materials and methods

***Study area*:** A hunting area in the southern part of the Hiroshima Regional Urban Area (**Fig IV-1**), which met the requirements of constant wildlife population and oyster shell usage in agricultural farms, was selected. A large wildlife population was witnessed in the three towns, Fujyu (FJ), Hirata (HI), and Oogi (OG). The vegetation in their mountainous part is mainly bamboo and deciduous hardwoods, and the topsoil is humus. A few streams flow along the natural pathways, and the residential parts comprised of traditional agricultural landscape, human habitats, vegetable farms, and the seashore.

Geologically, most of the mountainous areas are composed of a Jurassic stratum while areas of human habitat correspond to a Cretaceous stratum (Nishimura *et al.*, 2012). The Ryouke metamorphic rocks and the Hiroshima granites are mainly exposed in each stratum, respectively.

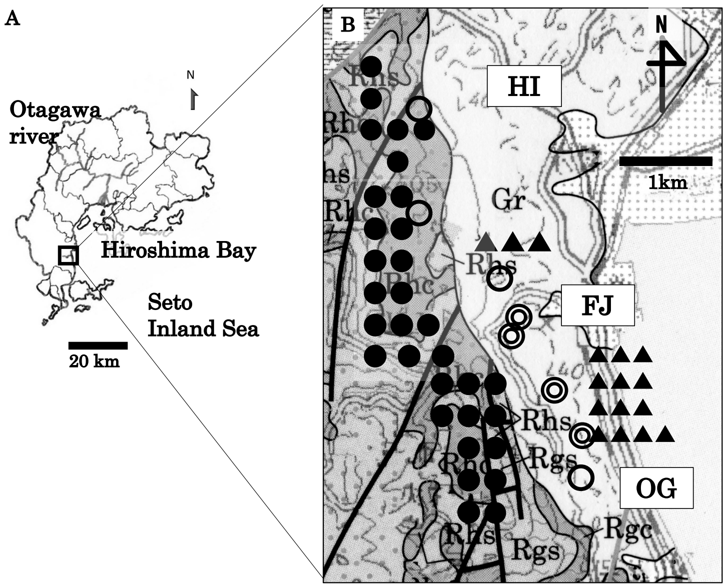

Fig. IV-1. Hunting area. A: A rough map of the Hiroshima Regional Urban Area. The square indicates the main hunting area determined by local hunters. **B**: Geographical stratum of the hunting area. The original geological map of Nishimura *et al.* (2012) was modified with permission. An applicable local area map (Approvable number: 2011-423 from the Geographical Survey Institute) was overlayed on the original geographical map. Rocks composing the strata were Ryoke metamorphic rocks (Rgc, Rgs, Rhc, Rhs) and Hiroshima granites (Gr). Sampling sites of the elements are shown with symbols, ○: Pteridophytes; ◎: Farm plants; ●: Wild boar; ▲: Raccoon dog. Most of the wildlife (32 out of the 38 wild boar and all raccoon dogs) were captured in Fujyu (FJ), Hirata (HI), and Oogi (OG) towns.

Pteridophytes: The concentrations of Cd and Zn were measured for wild pteridophytes in the hunting area (**Fig. IV-1**). The species met all the requirements, and they were sufficiently abundant (Matsumura *et al.*, 2016). Pteridophytes directly reflect the concentrations of metals in soil (Samecka-Cymerman *et al.*, 2012). *Athyrium yokoscense* (*A. yokoscense)* is known for its hyper-tolerable property against heavy metals (Van *et al.*, 2006; Yoshihara *et al.*, 2014).

Four species of pteridophyte were collected in winter seasons between 2017 and 2019. *Dicranopteris linearis* (*D. linearis*) grows widely in all the three towns. *A. yokosense* and *Plagioria euphlebia* (*P. euphlebia*) grow in the mountainous areas of FU and HI, while *Gleichenia japonica* (*G. japonica*) is observed in the residence areas.

The ground parts of each pteridophyte were used for measurement. The length of fronds and fresh weight were >15 cm and >1 kg per individual sampling, respectively.

Farm plants: Cd and Zn concentrations were measured for five species of farm plants provided by the residents in the winter seasons between 2017 and 2019 (**Fig IV-1**). Three species, orange (*Citrus unshu*), persimmon (*Diospyros kaki*), pumpkin (*Cucurbita moshcata*), were obtained from farms experiencing wildlife damage as species preferred by wildlife, and two other species, orange (*Citrus hassaku*) and perilla, were obtained from farms unaffected by wildlife.

Wild animals: Wildlife was captured between 2017 and 2019 according to their disruptive behavior of eating fruits or trampling farms, as witnessed by the residents (**Fig IV-1**). They were wire-trapped by registered hunters. Two species of wildlife, wild boars (*n*=38) and raccoon dogs (*n*=16) were selected in the study to obtain enough samples for statistical analyses.

Artificial hunting was performed in necessity of residents. Carcasses of the wild boars were prepared at their respective sites of capture. The raccoon dogs were submitted to an autopsy center, where they were anesthetized with diethyl ether until their last breath followed by autopsy.

After gross observation, one kidney of the animals was stored at -20 ℃ until use. Macroscopically abnormal tissues and the other kidneys were immediately placed in 10 % phosphate-buffered formalin. Seventeen out of the 38 wild boar quadriceps, which have been supplied as foodstuffs for residents, were provided as the muscle specimen to compare metal concentrations in the kidneys.

Animal welfare and ethics**:** Wildlife were treated according to the guideline of the Health Management of Wild Birds and Beasts in Yamaguchi prefecture, obeying the code of ethics and the oath of veterinarians-declaration '95 (**Appendix 2**).

Cd and Zn measurements: Cd and Zn concentrations were measured (**Appendix 2 - Table 2**). All materials except the kidneys were dried in a drying device overnight, ground using a mill, and stored at -20 ℃ until use. The drying ratios for muscles (*n*=17), farm plants (*n*=16), and pteridophytes (*n*=18) were 5.4±0.8, 4.5±2.7, and 2.6±0.6 times, respectively. The concentrations of metals were determined using ICP-AES. Using the pretreatment of drying and increasing the levels of solubility for the materials, the lower detection limit could be brought to 0.0005 mg/kg. The average value of the duplicated measurements was used as a representative for each sample.

Statistical analysis: Student's *t*-test was performed to compare the concentrations of Cd, Zn and Cd/Zn for the wildlife body weights, kidneys, and muscles of wild boars assuming normal distribution.

For comparison of the metal concentrations of pteridophytes and farm plants, log-normal distribution was assumed. After calculating the logarithm of individual data, their statistical differences were compared. *F*-test was used for the comparison of homoscedasticity among the variances of the farm plants.

Pearson's coefficient of correlation was used for comparisons of Cd, Zn and Cd/Zn values among the elements; and among sub-species within pteridophytes, farm plants, wild boar kidneys, and raccoon dog kidneys; as well as between the kidneys and muscles of wild boar groups. KaleidaGraph software version 3.6 (HULINKS, Tokyo, Japan) was used.

IV.-3 Results

Cd and Zn concentrations in pteridophytes: Bioconcentrations of Cd and Zn in the four species of pteridophytes are shown in **Table IV-1**. The Cd values of *A. yokoscense* (group 1) were higher than those of the other two species (group 3, 4 and 5) ($p<0.05$). The Zn values of *A. yokoscense* (group 1) were higher than those of the other two species (group 2 by $p<0.05$, group 3 and 4 by $p<0.01$). The higher concentration of the two metals were found in the Jurassic stratum groups than in the Cretaceous groups ($p<0.01$).

The Cd values were exponentially correlated with those of Zn in pteridophytes irrespective with those of *A. yokosense* ($r=0.89$, $p<0.01$) or without ($r=0.77$, $p<0.01$) (**Fig. IV-2-A**).

Table IV-1. Cd and Zn in pteridophytes.

Groups	Species	Number	Cd (mean ± SD, mg/kg)	Zn (mean ± SD, mg/kg)	Cd/Zn (x100) (mean ± SD)		Geological stratum of self-growing area a)
1	*Athyrium yokoscense*	5	3.12±2.76	171.8±29.2	1.72±1.38	1.50±0.96	Jurassic (Rgc, Rgs, Rhc, Rhs)
2	*Plagiogyria euphlebia*	4	0.76±0.57	48.3±37.5 c)	1.57±0.73		
3	*Dicranopteris linearis*	3	0.17±0.06 b)	16.6±5.6 d)	1.05±0.23		
4	*Dicranopteris linearis*	3	0.08±0.05 b)	11.0±4.5 d)	0.66±0.17	0.39±0.17 e)	Cretaceous (Gr)
5	*Gleichenia japonica*	3	0.55±0.03	30.8±25.7	0.39±0.23		
6 (1+2+3+4+5)	-	18	1.08±1.90	68.2±71.3	1.06±0.79		-

a): Rocks composing the strata were Ryoke metamorphic rocks (Rgc, Rgs, Rhc, Rhs) of the Jurassic stratum and Hiroshima granites (Gr) of the Cretaceous stratum in **Fig. IV- 1-B**;

b): $p<0.01$ when compared to the Cd values of group 1;

c): $p<0.05$ when compared to the Zn values of group 1;

d): $p<0.01$ when compared to the Zn values of group 1;

e): $p<0.01$ when compared to the Cd/Zn values of self-growing pteridophytes on the Jurassic stratum.

Cd and Zn concentrations in farm plants: The bioconcentration of Cd and Zn in the farm plants are shown in **Table IV-2**. The Cd values of *Citrus unshu* (group 1) were lower than those of persimmon (group 2) ($p<0.01$ by t-test). The Cd values of persimmon (group 2) were lower than those of perilla (group 5) ($p<0.05$ by t-test). The concentration values of Cd in persimmon (group 2) were different from those in *Citrus hassaku* (group 4) ($p<0.05$ by F-test). The Zn values of *Citrus unshu* (group 1) were lower than those of pumpkin and perilla (group 3 and 5) ($p<0.05$ by t-test). The Zn values of persimmon (group 2) were lower than those of perilla (group 5) ($p<0.01$ by t-test).

Table IV-2. Cd and Zn in agricultural farm plants.

Groups	Species	Number	Cd (mean±SD, μg/kg)	Zn (mean±SD, mg/kg)	Cd/Zn (mean±SD) (x100)
1	Orange (*Citrus unshu*) [a]	3	1.93±1.02 [d]	0.41±0.32 [f]	2.22±3.44
2	Persimmon (*Diopsyros kaki*) [b]	3	4.09±0.68 [e]	1.66±1.29 [g]	0.50±0.52
3	Pumpkin (*Cucurbita maxima*) [a]	4	7.53±2.88	21.63±23.73	0.07±0.07
4	Orange (*Citrus hassaku*) [a]	3	2.39±2.34	3.36±4.54 [h]	0.03±0.03
5	Perilla [c]	3	11.23±8.82	50.87±13.90	0.03±0.03
6 (1+2+3+4+5)	-	16	6.35±5.61	14.73±21.75	0.40±0.78

[a]: Fruits and vegetables were used;

[b]: non-astringent fruits were used;

[c]: Three subspecies, *Perilla furutescense*, *Perilla furutescense* var. *crispa,* and *Perilla citriodora*, were included. Seeds were used;

[d]: $p<0.01$ when compared to the Cd values of group 2 by t-test;

[e]: $p<0.05$ when compared to the Cd values of group 5 by t-test. $p<0.05$ when compared to those of group 4 by F-test;

[f]: $p<0.05$ when compared to the Zn values of group 3 and 5 by t-test;

[g]: $p<0.05$ when compared to the Zn values of group 5 by t-test;

[h]: $p<0.05$ when compared to the Zn values of group 5 by t-test.

Cd accumulation in wildlife kidneys: The two wildlife species bioaccumulated Cd and Zn in their kidneys (**Table IV-3**). The mean values of Cd and Cd/Zn were higher in raccoon dogs (group 6) than in wild boars (group 3) ($p<0.05$ and $p<0.01$, respectively).

Cd accumulation in female kidneys was linearly expressed as a function of Zn (**Fig. IV-2-C** and **-D**). Correlation coefficients between the two metals were high in groups of 1 ($r=0.50$, $p<0.05$), 4 ($r=0.74$, $p<0.05$) and 6 ($r=0.56$, $p<0.05$).

Table IV-3. Cd and Zn in wildlife kidneys.

Groups	Species	Sex	Number	Body weight (mean ± SD, kg)	Kidney (mean ± SD) Cd (mg/kg)	Zn (mg/kg)	Cd/Zn (x100)	Correlation coefficient of Cd to Zn
1	Wild boar	Female	19	47.0±16.5	1.74±1.20	20.7±2.6	8.2±4.7	0.50 ($p<0.05$)
2		Male	19	40.8±19.7	1.29±0.48	21.4±2.5	6.2±3.7	ns [c]
3 (1+2)		-	38	43.9±18.2	1.51±0.99	21.1±2.5	11.2±19.7	ns
4	Raccoon dog	Female	7	3.5±1.1	6.13±7.32	24.3±4.1	22.9±5.8	0.74 ($p<0.05$)
5		Male	9	4.0±1.0	3.05±7.09	21.8±4.8	13.5±11.9	ns
6 (4+5)		-	16	3.8±1.1	4.40±5.26 [a]	22.9±4.5	17.7±18.2 [b]	0.56 ($p<0.05$)

[a]: $p<0.05$ when compared to the Cd values of group 3;

[b]: $p<0.01$ when compared to the Cd/Zn values of group 3;

[c]: not statistically significant.

Cd and Zn distribution within the body of wild boar: Cd and Zn concentrations in muscles were compared to those in the kidneys for wild boars (**Table IV-4**). Cd values in the muscle were lower than those in the kidneys for the three groups (group 1, 2 and 3) (p<0.01). Zn values in the muscles were higher than those in the kidneys for the three groups (group 1, 2 and 3) (p<0.01). The Cd/Zn values were lower in the muscles than in the kidneys for the two groups (group2: p<0.01; group3: p<0.05).

No statistically significant correlation was observed between the values of Cd and Zn in the muscle and kidney.

Table IV-4. Cd and Zn in the muscles and kidneys of wild boars.

Groups	Sex	Number	Body weight (mean±SD, kg) [a]	Kidney (mean±SD)			Muscle (mean±SD)		
				Cd (mg/kg)	Zn (mg/kg)	Cd/Zn (x100)	Cd (mg/kg) [b]	Zn (mg/kg)	Cd/Zn (x100)
1	Female	7	42.1±12.5	1.45±7.83	19.9±2.5	7.4±4.2	0.004±0.005 [c]	39.5±36.9 [d]	0.011±0.011 [e]
2	Male	10	34.2±13.3	1.55±0.77	20.8±2.1	7.6±4.2	0.005±0.005 [c]	35.0±22.3 [d]	0.024±0.036 [e]
3(1+2)	-	17	37.5±13.2	1.51±0.75	20.4±2.2	7.5±4.0	0.004±0.005 [c]	36.9±23.6 [d]	0.017±0.030 [f]

a): No statistically significant difference was observed between the corresponding data of groups 1, 2 and 3 in **Table IV-3**;

b): Values below the lower limit were calculated as 0.0001 mg/kg;

c): p<0.01 when compared to the Cd values of kidney;

d): p<0.01 when compared to the Zn values of kidney;

e): p<0.01 when compared to the Cd/Zn values of kidney;

f): p<0.05 when compared to the Cd/Zn values of kidney.

Cd and Zn distribution in the wildlife habitat: The Cd values in pteridophytes, farm plants and wildlife are shown in **Fig. IV-2-D** as a function of Zn values. All Cd values in farm plants and wild boar muscles, which are used as foodstuff for residents, were <0.025 mg/kg. The values of Zn in these groups varied within three digits, and the highest value was <89.0 mg/kg.

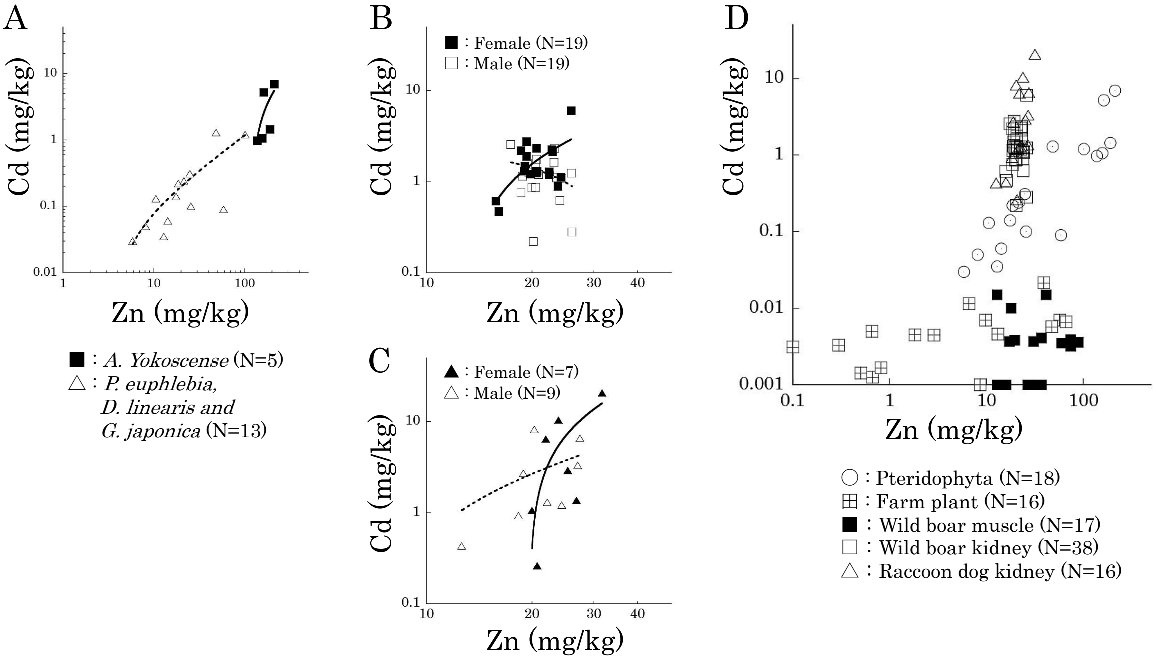

Fig. IV-2. Concentrations of Cd and Zn in the wildlife and plants. Raw data are plotted on the figures. **A**: Concentrations of the two metals in pteridophytes. Regression curves for *A. yokoscense* (*n*=5) and for the other three species (*n*=13) are given by the following equations: $y=0.061x - 7.305$ (r=0.64) (solid line) and $y=0.012x - 0.044$ (r=0.76) (dotted line), respectively. **B**: Concentrations of the two metals in the wild boar kidney. Regression curves for females and males are given by the following equations: $y=0.230x - 3.027$ (r=0.50) (solid line) and $y=0.088x + 3.147$ (r=0.31) (dotted line), respectively. **C**: Concentrations of the two metals in raccoon dog kidneys. Regression curves for females and males are given by the following equations: $y=1.262x - 24.401$ (r=0.74) (solid line) and $y=0.162x - 0.954$ (r=0.31) (dotted line), respectively. **D**: Comparison of the values of Cd and Zn among the elements.

Bioaccumulated Cd and Zn by environmental elements at the southern part of Hiroshima Regional Urban Area are shown in **Table IV-5**. Raccoon dogs accumulated Cd in the kidneys with a Cd/Zn value (x100) of 17.7, which was high by double figures as compared to that of pteridophytes and agricultural farm plants.

Table IV-5. Cd and Zn in terrestrial organisms.

	Number	Cd (mg/kg)	Zn (mg/kg)	Cd/Zn (x100)
Pteridophyte [a)]	18	1.08±1.90	59.6±64.2	1.06
Agricultural farm plant [b)]	16	0.006±0.006	14.7±21.8	0.51
Wild boar muscle	38	0.004±0.005	39.5±26.3	1.7
Wild boar kidney	17	1.51±0.99	21.1±2.5	7.2
Raccoon dog kidney	16	4.40±5.26	22.9±4.5	17.7

[a)]: Ground parts were used for measurement. [b)]: Edible parts were used for measurement.

IV.-4 Discussion

Both plants and mammals accumulated Cd exponentially to the concentration values of Zn with high correlation coefficient between the two metals, however, the concentration range was wide in plants but narrow in mammals. One possible reason for these results is the lifespan of organisms in this study, which was less than 6 months for the plants and over 6 months to 8 years for the wildlife (Helle *et al.*, 1993). The log-normal distribution model suited acute accumulations of Cd and Zn in the plants, whereas, a normal distribution model was used to explain the chronic exposure of the metals in the mammals. The latter model was experimentally proven in *Fischer 344* rats with iodine, another essential element for mammals (Nitta *et al.*, 2001). The narrow range of Zn concentrations in wildlife indicated the homeostatic mechanism to keep Zn at constant levels in their bodies. They accumulated Cd in kidney throughout their lives without any specific metabolic pathway to exclude from the body.

Wild raccoon dogs have shared with humans the exposure to Cd locally, and this represented the environmental status of Cd in their kidney, since they migrate within narrow areas, such as a 5-km radius (Helle *et al.*, 1993). Wild boar, too, migrated within narrow areas, which was 9 km for most of the females (Truvé *et al.*, 2003). Therefore, the values of Cd in female kidneys showed an endpoint profile of lifelong exposures from their habitats. Female wildlife is found feasible in biomonitoring the presence of anthropogenic Cd in satoyama (O'Brien *et al.*, 1993).

Age is a factor for bioaccumulation of Cd in mammals. Accumulated Cd levels in the two kinds of female wildlife kidneys were as high as those of the people living in a contaminated area for more than 10 years (Garcı´a *et al.*, 2001). The volume of the kidney medulla is another matter for the comparison of the Cd concentrations between the wildlife and humans, since the whole kidney was used for measuring in wildlife, while, only the cortex of kidney is used in humans. Cd is accumulated in the renal tubular epithelia of cortex. Concentrations of Cd in the cortex of wildlife kidney are estimated to be higher than those obtained in this study. Considering the lifespan of wild raccoon dogs, less than 8 years (Helle *et al.* 1993), the average Cd level in wildlife kidney was high.

Sex is another factor to characterize the accumulation of Cd, and more highly in females than males of the two species of wildlife in this study. Experimentally, a higher accumulation of Cd was observed in the kidney, liver, and thyroid glands of female mice (Yamanobe *et al.*, 2015). Ovariectomy accelerated the accumulation of Cd in the kidney of monkey (*Macaca fascicularis*) (Kurata *et al.*, 2014). Epidemiologically, high levels of Cd in urine (Nagata *et al.*, 2016) and in mandibular bone (Browar *et al.*, 2019) are associated with decreased levels of testosterone and estradiol in women. Cd levels in the kidneys are higher in female calves (Miranda *et al.*, 2000), while, no difference was found between the two sexes in red fox (Binkowski *et al.*, 2016; Lanocha *et al.*, 2012).

The arithmetic means of Cd/Zn for the pteridophytes growing on the Jurassic stratum were higher than those on the Cretaceous stratum in the present study. Wild pteridophytes just outside the agricultural farms showed strong correlation between the two metals; in contrast, no plants in the agricultural farm showed any correlation between the concentration values of Cd and Zn, no matter how the examined sample numbers for each species were small. Any anthropogenic factor could work for the inconsistent concentrations, and fertilizers would be a candidate source of Cd in agricultural soils in satoyama.

The autopsy of wildlife is worthy for the assessment of environmental metal status and subsequent transmission of information to the residents in a timely manner (Camizuli *et al.*, 2018; O'Brein *et al.*, 1993). When compared to the Provisional Tolerable Weekly Intake levels of Cd (<0.007 mg/kg of body weight/week) and Zn (<60 mg/day) (**Appendix 1**), the locally available examined foodstuffs contained the metals in low ranges, <0.025 mg/kg for Cd and <89.0 mg/kg for Zn. The data obtained from the elements comprising the terrestrial ecosystem could be useful for the risk communication between the residents and researchers.

V. EXPERIMENTAL TRANSFER OF Cd FROM OYSTER SHELLS TO PLANTS

Abstract: To investigate the transfer of Cd from fertilizers to farm plants, farm-, pot-, and hydroponic cultivation were performed to the two species of plants, perilla (*Perilla citriodora*) and bean sprout (*Pisum sativum* L.) conditioned with the fertilizer made from oyster shell waste. Farm-cultivated perilla concentrated Cd and Zn from soil with the concentration factor (CF) of 0.14 and 0.27, respectively. The transition of Zn from soil to plant increased via the pot-cultivation. Four weeks of hydroponically cultivated bean sprouts accumulated Zn in their foliage time dependently by a conditioning with oyster shell powder. Cd and Zn concentrations increased by 5.5 and 2.2 times, respectively. Amounts of the metals were estimated as 0.5 μg/foliage/brick and 0.77 mg/foliage/brick for Cd and Zn, respectively.

V.-1 Purpose

Cd and Zn are the contaminants negatively affecting organism's lives. Environmental contamination of these metals has resulted to be accumulated in organisms via the food-chain. Transfer of Cd from benthic organisms to terrestrial organisms has not been extensively investigated, when the industrial system to recycle oyster shell waste as specific fertilizer is in operation in the Hiroshima Regional Urban Area (https://hiroshimaforpeace.com/wp-content/uploads/2019/12/sdgsbook2_1210.pdf).

It was likely that any anthropogenic condition can influence the inconsistency of the Cd and Zn accumulation in the area (Nitta *et al.*, 2019, Nitta *et al.*, 2020), because that there was no concentration correlation between the concentrations of the two metals in the plants grown in the agricultural farm, and there was a high concentration correlation between the two metals in the wild pteridophytes just outside the farm. Phosphate fertilizers are the major source of Cd in agricultural soils (Dharma-Wardana MWC, 2018; Zhu *et al.*, 2018), no matter how the transfer coefficient of each metal in every plant depends on the conditions of individual agricultural soil (Argullo *et al.*, 2019; Lin *et al.*, 2018; McComb *et al.*, 2014).

In this part of the study, transfer of Cd from the oyster (*Crassostrea gigas*) (oyster) shell to plants was examined by culturing with three types of cultivation: at the farm, in pots and hydroponically, using a wild perilla and a cultivated bean sprout highly adapted hydroponics.

Perilla is a member of the *Lamiaceae* family, and it is cultivated all over Japan. Its leaves and seeds have been used as a foodstuff or natural medicine (Niwano *et al.*, 2010). *Perilla citriodora* (*P. citriodora*) grows naturally in the western parts of Japan (Makino T., 1989; Nitta, *et al.*, 2021). The perilla cultivated in agricultural farms was found to contain certain amounts of Cd in their foliage (**Table IV-2**) (Nitta *et al.*, 2020).

Bean sprout (*Pisum sativum* L.) is a nutritious vegetable consumed in China, Korea, and Japan (Lee *et al.*, 2018). Hydroponic cultivation enables us to enrich its nutritional value and allows to manipulate water-soluble substances for the vegetable (Nitta Y., 2020; Xu *et al.*, 2019).

V.-2 Materials and methods

Study area: The farm-, pot- and hydroponic cultivation experiments were performed in the Hiroshima Regional Urban Area (**Fig. V-1**).

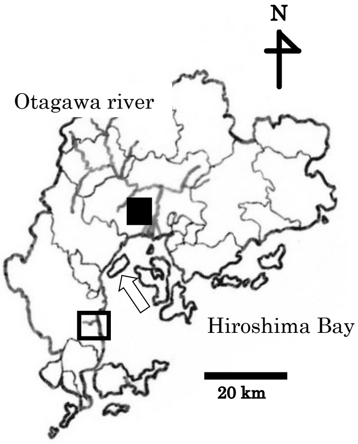

Fig. V-1. A rough map of the Hiroshima Regional Urban Area. Pot- and hydroponic cultivations were performed at Ozuka (□), N34°44’ 04.28, E 132°41’ 10.46. Farm cultivation was performed at Oogi (■), N34°19’ 35.65, E 132°19’ 43.04. Arrow indicates the Itsukushima Island.

***Plants*:** Two species of plants, perilla and bean sprout, were used. The above-ground parts of perilla and whole foliage of bean sprout were collected, dried and ground via a mill. The drying ratios (mean ± SD) for bean sprouts and perilla were 10.2±2.2 (*n*=20) and 3.7±1.7 (*n*=6) times, respectively. The seeds of perilla were manually collected from ears. All samples were stored at -20 ℃ until use.

***Soils*:** Agricultural farm soils were collected from the experimental farm at Oogi (**Fig. V-1**). The sampling depth of soil at the experimental farm was from 0 to 10 cm. The whole soil per pot was mixed up and ground by mill.

A cocktail soil was prepared for pot-cultivation at Ozuka (**Fig. V-1**). The cocktail soil comprised 5 g of chemical fertilizer, 2 g of $CaCO_3$ (Fuji Film Wako Ltd., Osaka, Japan) and 2 kg of andosols per pot (φ:21cm; depth: 18cm) (Muramatsu *et al.*, 2002). The pot filled with the cocktail soil was covered with or without the 100 g of oyster shell powder (**Appendix 2 - Table 4**).

***Measurements of Cd and Zn*:** Cd and Zn concentrations were determined using ICP-AES for plants, and those for soils and oyster shells were determined using AAS (**Appendix 2 - Table 1, 2** and **4**).

***Farm-cultivation experiment*:** Perilla and bean sprout were farm-cultivated.

Three subspecies of perilla: *Perilla frutescens*, *Perilla frutescens* ver. *crispa*, and *P. citriodora*, were farm-cultivated from May to October in 2019 and 2020. *P. citriodora* seeds were prepared by collecting them from the Itsukushima Island (**Fig. V-1**) (Nitta *et al.*, 2021). Commercially available perilla: *Perilla frutescens* and *Perilla frutescens* ver. *crispa*, were used for comparison. Oyster shell powder (1 kg/m^2) was scattered on the experimental agricultural farm in March, 2020, and the concentrations of Cd and Zn in this lot of oyster shell powder were not measured (**Appendix 2 - Table 4**). No other specific treatment was performed on the soil till the end of the experiment.

Pot-cultivation experiment: The *P. citriodora* was pot-cultivated from June to October in 2020. The seeds were sown directly on andosol in June. The germinated perilla grown up to 20 cm of height was transplanted to pot and cultivated outdoor for 60 days from August to October in 2020. The pot-cultivation was conditioned with or without the oyster shell powder (**Appendix 2 - Table 4**).

Hydroponic cultivation experiment: Bean sprout was used for hydroponic cultivation. Each brick was placed in a 6.5L plastic tank of polysulfone (CL-0123-3, CLEA Japan Inc., Tokyo, Japan) conditioned with or without the 100g of oyster shell powder (**Appendix 2 - Table 4**) (Nitta Y., 2020). Deionized water (0.5 L) was supplied every morning for 0, 1, 2, or 4 weeks in a laboratory room under the condition of natural light and temperature.

The first experiment was performed from December in 2018 to March in 2019. Room temperatures were between 11 and 20 ℃. The second experiment was performed from February to April in 2021 with room temperatures between 16 to 22 ℃.

Statistical analysis: For the comparison of the measurements of Cd, Zn and Cd/Zn of perilla and bean sprout, the log-normal distribution was assumed. After calculating the logarithm of individual data, their statistical differences were compared.

To investigate the transition of Cd and Zn from soil to plant, the concentration factor (CF) was calculated using the formula: *CF = (value of foliage) / (value of soil)* (Sasaki *et al.*, 2013). For the comparison of the ratio of Cd to Zn, Cd/Zn, the concentration rate was calculates using the formula *Concentration rate = (Cd/Zn of foliage) / (Cd/Zn of soil)*.

Student's *t*-test was used to assess the significance of the observed differences among Cd, Zn, Cd/Zn, CF, and concentration rate. KaleidaGraph software version 3.6 (HULINKS, Tokyo, Japan) was used.

V.-3 Results

Cd and Zn concentrations in the farm-cultivated perilla: Cd and Zn concentrations in the perilla are in **Table V-1**. The *Perilla frutescens* (Group 1) concentrated Cd with a high correlation coefficient to Zn (p<0.05). No statistically significant difference was found in the transitional coefficients relating to Cd and Zn from soil to foliage among the three subspecies of perilla. CF of Zn was twice as high as that of Cd without any statistical significance (group 4).

Table V-1. Cd and Zn concentrations in the farm-cultivated perilla.

Groups	Species	Number	Foliage			Soil (n=1)			Transition (foliage/soil)		
			Cd (mg/kg)	Zn (mg/kg)	Cd/Zn (x100)	Cd (mg/kg)	Zn (mg/kg)	Cd/Zn (x100)	CF (Cd)	CF (Zn)	Concentration rate (Cd/Zn)
1	*Perilla frutescens*	8	0.020±0.013 [a]	37.8±13.4	0.051±0.002	0.17	180	0.094	0.12±0.07	0.21±0.07	0.005±0.002
2	*Perilla frutescens* var. *crispa*	8	0.025±0.018 [b]	48.2±13.5	0.052±0.003				0.15±0.11	0.27±0.08	0.005±0.003
3	*Perilla citriodora*	8	0.027±0.019	57.6±22.6	0.077±0.101				0.16±0.11	0.32±0.13	0.008±0.011
4 (1+2+3)		24	0.024±0.016 [c]	47.9±18.2 [d]	0.060±0.061				0.14±0.10	0.27±0.10	0.006±0.006

Four samples of perilla per year (2019 and 2020) were measured for 2 years. Cd and Zn concentrations in soil were measured at the end of the experiment in October 2020. Wet density (ρt) of the soil, 1.45±0.04 g/cm^3, was obtained using the equation: $\rho t = m / V$, where m: weight of soil; V: volume of soil. Water saturation rate (Sr) of the soil was 42.7±4.8 %, which was given by the equation: $Sr = 100 x Vw / (Va + Vw)$, where Vw: volume of water and Va: volume of soil particle. CF was obtained by the following equation: *CF = (value of foliage) / (value of soil)*. Concentration rate (Cd/Zn) was defined as the value of (Cd/Zn of foliage) / (Cd/Zn of soil).

[a] Correlation coefficient to Zn was 0.73 (p<0.05) in group 1;

[b] Correlation coefficient to Cd/Zn(x100) was 0.84 (p<0.01) in group 2;

[c] Correlation coefficient to Cd/Zn(x100) was 0.57 (p<0.01) in group 4;

[d] Correlation coefficient to Cd/Zn(x100) was 0.43 (p<0.05) in group 4.

Cd and Zn concentrations in the pot-cultivated P. citriodora: Any specific increase in Cd or Zn concentration was not detectable in the foliage when compared the values between the two groups, conditioned with oyster shell powder (group 2) and not-conditioned (group 1) (**Table V-2**).

Table V-2. Cd and Zn concentrations in the pot-cultivated *P. citriodora*.

Group	Oyster shell [a]	Number	Foliage (mean±SD)			Pot soil (mean±SD) [b]			Transition (foliage/pot soil)		
			Cd (mg/kg)	Zn (mg/kg)	Cd/Zn (x100)	Cd (mg/kg)	Zn (mg/kg)	Cd/Zn (x100)	CF (Cd)	CF (Zn)	Concentration rate (Cd/Zn)
1	not conditioned	3	0.017±0.006	63.2±45.7	0.034±0.021	0.13±0.02	55.0±7.9	0.23±0.03	0.13±0.03	1.22±1.00	0.15±0.10
2	conditioned	3	0.023±0.015 [c]	43.6±3.62 [d]	0.052±0.030	0.17±0.04	60.3±28.0	0.32±0.18	0.16±0.15	0.83±0.33	0.22±0.17
3 (1+2)	-	6	0.02±0.01	53.4±30.9	0.04±0.03	0.15±0.04	57.7±18.7	0.28±0.13	0.15±0.10	1.02±0.70	0.18±0.13

P. citriodora was pot-cultivated from June to October in 2020. Cd and Zn concentrations in pot soil were measured at the end of the experiment. CF was obtained using the equation: *CF = (value of foliage) / (value of pot soil)*. Concentration rate (Cd/Zn) was defined as the value of (Cd/Zn of foliage) / (Cd/Zn of pot soil).

[a] Treated at the beginning of experiment. This lot contained 0.18 mg/kg and 19.0 mg/kg of Cd and Zn, respectively (**Appendix 2 - Table 4**);

[b] Cd and Zn concentrations were measured at the end of the experiment;

[c] Correlated to Cd/Zn of group 2 (r=0.99, p<0.05);

[d] p<0.05 when compared to Zn of group1 by F-test.

The average value of CF of pot-cultivation, 0.15, was as high as those of farm-cultivation, 0.14, however, the average value of Zn of pot-cultivation, 1.02, was 3.8 times higher than that of farm-cultivation, 0.27.

***Time course of Cd and Zn concentrations in hydroponically cultivated bean sprouts*:** In the first experiment, the time dependency of the Cd and Zn concentrations in a hydroponic cultivation was examined. The cultivating process was monitored via the growth curve of the foliage part of bean sprout and pH of water in the cultivation tank.

The rate of weight increase reached the highest value at 1 week, and it gradually decreased to the end of the observation period in the two groups, treated or non-treated oyster shells (**Fig. V-2-A**). The relative weights between the two groups were different at the point of 2 weeks, and they were higher in the oyster shell treated group than in the non-treated group (p<0.05). pH of a representative culturing tank shown in **Fig. V-2-B** was constant throughout the experimental periods.

Bean sprouts accumulated Cd and Zn in their foliage by the end of the observation period (**Fig. V-2-C** and **-D**). Cd concentrations in the foliage reached measurable levels at 4 weeks, while Zn concentrations in the foliage increased linearly with a time dependent manner.

The average weights of the 52 bricks of bean sprout, foliage plus roots, were 360.0±35.4 g (mean ± SD).

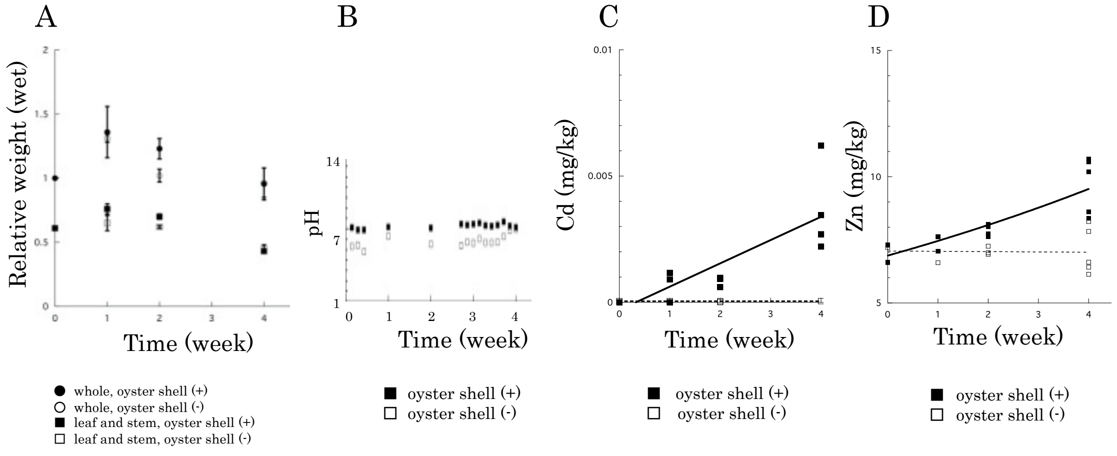

Fig. V-2. Hydroponic cultivation of the bean sprout. A: Weight changes of the bean sprout treated with or without oyster shell powder. The powder contained 0.18 and 19.0 mg/kg of Cd and Zn respectively (**Appendix 2 - Table 4**). Circle symbols (●, ○) for root plus foliage, and square symbols (■, □) for foliage. Vertical bars indicate standard deviation. **B**: pH of the cultivation solutions with or without oyster shell powder. **C**: Cd concentrations in the bean sprout. Solid line is expressed by the formula: $y=-0.00036+0.00091x$ (r=0.80, p<0.001). **D**: Zn concentration in the bean sprout. Solid line is expressed by the formula: $y=6.80+0.69\,x$ (r=0.85, p<0.001).

In the second experiment, the concentrated amount of Cd and Zn concentrated in the foliage part of bean sprouts was measured. The cultivating condition was judged using the weight of bean sprout foliage per brick.

Each brick increased their foliage weights by culturing with (group 3) or without (group 2) oyster shell at the end of the 4-week-cultivation, when compared to the weights of 0-week-cultivation (p<0.01) (**Table V-4**). The oyster shell conditioning increased the foliage weight by 3.5 times at the mean level.

Average Cd and Zn concentrations in group 3 were 5.5 and 2.2 times higher than those in group 1, respectively. Consequently, the concentration ratios of Cd to Zn, Cd/Zn, increased 2.5 times higher than that of group 1 with mean levels. The amount of metals were estimated as 0.5 μg/foliage/brick and 0.77 mg/foliage/brick for Cd and Zn, respectively, with mean levels.

Whole weights of the 59 bricks of bean sprout and foliage plus roots, were 320.8±40.4 g (mean ± SD).

Table V-3. Growth of bean sprouts using hydroponic cultivation.

Group	Cultivation			Number of bricks	Weight (foliage g/brick)	Foliage growth ratio	Cd (μg/foliage/blick)	Zn (mg/foliage/blick)	Cd/Zn (x100)
	Duration (week)	Deionized water	Oyster shell [a]						
1	0	-	-	8	89.6 ±11.7	1.0 ±0.1	0.11 ± 0.01	0.63 ± 0.08	0.018±0.02
2	4	+	-	3	188.8 ±12.7 [b]	2.1±1.0	NT [b]	NT	-
3	4	+	+	4	310.0 ±31.8 [b]	3.5±0.4	0.61 ±0.31 [c]	1.40 ± 0.14 [d]	0.045±0.002 [c]

[a]: This lot of oyster shell powder contained 0.18 mg/kg and 19.0 mg/kg of Cd and Zn, respectively (**Appendix 2 - Table IV**);

[b]: Not tested;

[c]: p<0.05 when compared to the 0-week group by t-test;

[d]: p<0.01 when compared to the 0-week group by t-test.

V.-4 Discussion

The two types of experiments of hydroponic cultivation with bean sprouts indicated that the Cd in oyster shells was transferred to plants, since no source of Cd except oyster shell powder was available in this experimental system. A time-dependent accumulation of Zn into foliage may represent the capability of some metal ions provided by oyster shell powder to be absorbed into plant through water. Statistically significant differences of the CF values between Cd and Zn observed in the transfer experiment with *P. citriodora* may suggest the difference of transition coefficient between Cd and Zn in the perilla. The transition phenomena of Cd into foliage shown by the hydroponic and farm-cultivations support the hypothesis of Cd flow from benthic organism to terrestrial organism using the fertilizer made from oyster shell.

The flow of Cd from Hiroshima Bay to Hiroshima Regional Urban Area by the mediator of oyster shells could be estimated quantitatively (**Fig. I-1**). When a handmade oyster shell powder was scattered with a dose of 1 kg/m^2 on the agricultural farm, the added Cd on the soil was 0.05 mg/m^2 (**Table II-3**). The total area of the agricultural farm is 128.54 km^2, which occupies 2.04% of the Hiroshima Regional Urban Area （https://www.pref.hiroshima.lg.jp/site/toukei/nouringyocensus.html#r2, https://www.pref.yamaguchi.lg.jp/cms/a12500/nourin_census/2020-sokuhou.html). The total amount of Cd fixed in oyster shells from the Hiroshima Bay was 5.5~6.3 kg/year (**Table II-5**). Therefore, the Cd in the oyster shell powder could cover the agricultural farm area with concentrations of 0.043~0.049 mg/m^2/year. On application of the value of wet density, 1.45 g/cm^3, for the entire agricultural soil in the Hiroshima Regional Urban Area (**Table V-1**), the oyster shell powder increases the concentration of Cd by 0.0031~0.0035 µg/kg of agricultural soil per year.

Cd and Zn concentrations in agricultural soil were obtained by the Sediment Inspection Method (Morita *et al.*, 2014) in this study, which was common in other countries (McComb *et al.*, 2014). The obtained mean measures, 0.15 mg/kg and 57.7 mg/kg for Cd and Zn, respectively, were in the acceptable ranges determined by WHO and EU. The maximum acceptable limits of Cd and Zn in soil were 4 mg/kg and 300 mg/kg, respectively for WHO (2006), and 3 mg/kg and 300 mg/kg, respectively for EU (2002).

Zn concentration was under the upper limit of the standard in Japan, while that of Cd was incomparable (**Appendix 1 - Table 1**). Expressions of the standard upper limit levels of Cd and Zn for agricultural soils were double standards: <0.001mg/L and ≦ 120mg/kg, respectively. This indicates that two types of measurements are required to evaluate Cd and Zn concentrations in the agricultural soils of Japan: a dissolution method for Cd (https://www.env.go.jp/kijun/dt1-1.html) and the Sediment Inspection Method doe Zn (https://www.env.go.jp/water/teishitsu-chousa/00_full.pdf).

It seems irrational to use oyster shell waste as a fertilizer in viewpoint of the Cd concentrations in soil, since the more fertilizer we use, the more Cd accumulate, no matter how the bio-accumulation of Cd by agricultural farm plants varies depending on soil conditions, such as pH (Naito *et al.*, 2007), Zn concentration as a micronutrient (Karna *et al.*, 2017), and salinity.

No specific increase in Cd or Zn concentration was detected in the perilla foliage using this way of conditioning with oyster shell powder. One reason might be the concentrations of Cd and Zn in the original pot soil. The ranges were 0.11~0.19 mg/kg of Cd and 49~64 mg/kg of Zn. Their ratios of Cd to Zn, 0.0017~0.0039, exceeded the maximum standard limits of WHO and EU, however, they were less than the concentrations of Cd and Zn of the decomposed granite soil at the bedrock of the Area, which were 0.35mg/kg for Cd, 64.0 mg/kg for Zn, and 0.0055 for Cd/Zn in average (unpublished observations).

Another reason might be the soil conditioning effect of oyster shells (Chang YT, 2013): an effectiveness of oyster shells to immobilize the Cd in soil.

P. citriodora is a different species from the commercially available perillas, *P. furutescense* and *P. furutescense* var. *crispa* (Ito *et al.*, 2000). The chromosome numbers are *2n=20* and *2n=40* for *P. citriodora* and the other two subspecies, respectively. Genomic diploidization of *P. furutescense* var. *crispa* from *P. citriodora* has been reported (Zhang *et al.*, 2021). However, no data has been reported for *P. citriodora* on the capability of Cd transition from soil (Hasegawa I, 2002). Climate conditions and soil characteristics of the western part of Japan enabled the cultivation of *P. citriodora* (Nitta *et al.*, 2021). A small number of seeds of *P. citriodora* managed to germinate, and very small numbers of the strains have adapted to the cultivation, grown, flowered, and fruited. Further experiments are required to evaluate Cd concentrated in the leaves and seeds, the highest among the farm plants (Nitta *et al.*, 2020) but less than the level of provisional tolerable weekly intake (**Appendix 1**), when we use *P. citriodora* was used as a foodstuff.

VI. GENERAL DISCUSSIONS

VI-1. Heavy metals in the Hiroshima Regional Urban Area

Information on natural as well as anthropogenic heavy metals in the local area is essential to ensure the health of residents. Well-studied sources of Cd in agricultural farm soils are phosphate fertilizers (Roberts TL, 2014), sludge (Smith SR, 2009) or sewage (Wang J, 2021). In this study, oyster shells as an agricultural fertilizer made from the aquacultural waste were investigated. The contribution of the oyster shell waste to increase the amount of Cd in the agricultural farm soil was shown numerically.

Measurement of Zn and Cd in the sediment, oyster shell, and organism at the Hiroshima Regional Urban Area were compared with those of previous studies (**Table VI-1**). The concentration relationship between sediment and benthos in the marine ecosystem and between terrestrial wildlife and humans were comparable. Commercially available oysters contained Zn in meat with as high levels as those reported 50 years ago. The oyster shells contained Zn as one tenth levels of and Cd as high levels as those in sediment. Wild boar and raccoon dogs accumulated high concentrations of Cd in the kidneys considering their short lifespan as compared to that of humans.

Table VI-1. Zn and Cd in the aquatic area, fertilizer, and terrestrial area

		Zn (mg/kg)		Cd (mg/kg)	
		Nakanishi, *et al*., Zn, 2008[a]	Hiroshima Regional Urban Area (2018~2021)	Nakanishi, *et a*l., Cd, 2008[b]	Hiroshima Regional Urban Area (2018~2021)
Aquatic area	Culturing sea water	< 0.01*	-	< 0.005*	-
	Sea area water	< 0.02*	-	< 0.02*	-
	Sediment	< 150	120 - 210	0.03 - 1.1	0.31 - 0.44
	Clams	-	-	< 2	-
	Benthos	-	140 - 370	0.56	0.06 - 0.41
	Oyster	33	33 - 540	0.1 - 0.68	0.09 - 0.32
	Hiroshima oyster (Tanaka *et al*., 1974) [c]	139.0 - 272.0	-	0.12 - 0.32	-
Fertilizer	Sewage sludge	-	-	0.9	-
	Sewage sludge fertilizer	-	-	5	-
	Oyster shell fertilizer	-	8 - 65	-	0.005 - 0.13
	General fertilizer	-	-	0.02 - 5.5	-
Terrestrial area	Pedosphere	-	-	2	-
	Granite (Ishihara et al., 2006) [d]	89 - 5438	-	0.5 - 66	-
	Granite	-	92 - 120	-	0.35 - 0.39
	Soil	-	75 - 180	0.1 - 1000	0.05 - 0.32
	Pteridophyta	-	5.85 - 212.0	-	0.03 - 6.92
	Farm plant	-	0.5 - 67.8		0.0005 - 0.0217
	Game (deer)	-	15.9 - 88.9	0.001 - 0.03	≦ 0.08
	Game (wild boar)	-	11.4 - 41.9		< 0.05
	Wild boar kidney	-	16.11 - 25.9		0.43 - 6.03
	Raccoon dog kidney	-	12.6 - 31.7	-	0.26 - 20.60
	Human (Komai, Kambe, 2013) [e]	8.5~15.9 (blood) *	-	2 (kidney)	-

*: mg/L.

a): Nakanishi J, Naito W, Kamoh M. Risk Assessment Documents Vol 20 Zn. eds New Energy and Industrial Technology Development Organization, The Research Institute of Science for Safety and Sustainability, National Institute of Advanced Industrial Science and Technology, Maruzen, Tokyo. 2008 209p. (in Japanese)

b): Nakanishi J, Ono K, Kamoh M, Miyamoto K. Risk Assessment Documents Vol 13 Cd. eds New Energy and Industrial Technology Development Organization, The Research Institute of Science for Safety and Sustainability, National Institute of Advanced Industrial Science and Technology, Maruzen, Tokyo. 2008 369p. (in Japanese)

c): Tanaka Y, Ikebe K, Tanaka R, Sonoda S. Contents of heavy metals in foods (III). Food Hygiene Saf Sci. 1974 15: 390-93. (in Japanese);

d): Ishihara S, Murakami H. Characteristics of REE distribution in granitoids of SW Japan. Bull Geol Surv Japan. 57(3/4): 89-103. (in Japanese);

e): Komai M, Kambe D. Zinc function and health, Kenpakusha Tokyo 2013 230p. (in Japanese).

Sediment of Hiroshima Bay contained Zn with exceeded the level of maximum limit acceptable. Commercially available oysters contained Zn in meat with as high levels as those in sediments. Fertilizer made from oyster shell waste contained one tenth levels of the sediment Zn. All agricultural farm plants contained <0.1 mg/kg of Cd. Wild boar and raccoon dogs accumulated high concentrations of Cd in the kidney as compared to humans.

Coefficients of CF of Cd and Zn in the marine and terrestrial ecosystems around Hiroshima Regional Urban Area are shown in **Table VI-2**. Female mammals symbiotic with humans could be the sentinel for the evaluation of biological environment locally, since its high sensitivity to environmental Cd was expressed by CF.

Table VI-2. Concentration factor (CF) of Cd and Zn in organisms.

		Number	Cd (mg/kg)	Zn (mg/kg)	Cd/Zn (x100)	Concentration factor (CF) [a]						Concentration rate of Cd/Zn [b]		
						Cd			Zn					
Sediment	Hiroshima Bay	4	0.38±0.06	160.0±39.2	0.24	1	-	-	1	-	-	1	-	-
Soil	Agricultural farm	3	0.18±0.14	110.3±60.3	0.19	-	1	-	-	1	-	-	1	-
Organism	Pteridophytes	18	1.08±1.90	59.6±64.2	1.06	-	-	1	-	-	1	-	-	1
	Cultured oyster meat	5	0.22±0.12	183.9±120.1	0.15	0.58	-	-	1.15	-	-	1.60	-	-
	Agricultural farm plants	16	0.006±0.006	14.7±21.8	0.51	-	0.033	-	-	0.13	-	-	2.11	-
	Wild boar muscle	38	0.004±0.005	39.5±26.3	1.7	-	-	0.004	-	-	0.66	-	-	1.55
	Wild boar kidney	17	1.51±0.99	21.1±2.5	7.2	-	-	1.398	-	-	0.35	-	-	6.55
	Raccoon dog kidney	16	4.40±5.26	22.9±4.5	17.7	-	-	4.074	-	-	0.38	-	-	16.09

a): Obtained using the equation: *CF = (value of organism) / (value of ambient)* (Sasaki *et al.*, 2013);

b): Defined as the value of (Cd/Zn of organism) / (Cd/Zn of ambient).

Oyster meat fixed Cd and Zn from sea water into body by 0.58 and 1.15 of CFs, respectively. Edible parts of agricultural farm plants contained <0.1mg/kg of Cd. Wildlife contained Zn in kidney with narrow ranges of concentrations. Cd accumulation in the kidneys was obvious, as shown by the concentration rate of Cd/Zn, 6.5 and 16.1 of for wild boar and raccoon dogs, respectively.

VI-2. Effects of the Cd in oyster shell waste on agricultural farm

The fertilizer made from oyster shell waste contained Zn and some Class II specific hazardous substances (**Table II-4**). Cd in the fertilizer could possibly get transferred into plants.

The total amount of Cd in oyster shell was 5.5~6.3 kg/year in the Hiroshima prefecture. This annual supply of Cd from sea to land would increase 0.0031~0.0035 μg of Cd/kg of agricultural soil, if the fertilizer was consumed locally.

Hydroponic cultivation of bean sprouts contained 0.5 μg/foliage/brick of Cd into foliage from the oyster shell powder. Farm cultivation confirmed the transfer of Cd from soil to foliage.

These results might indicate the possibility that the Cd in agricultural farm plants in the Area was partly originated from the fertilizer made from oyster shell waste; however, the origin has not been examined (Wang *et al*, 2021). Systematic examinations concerning the Cd dynamics in agricultural soil should be performed.

VI-3. Effects of the Cd in agricultural farm on the landscape ecosystem

Oyster shells are the anthropogenically by-produced wastes. The oyster shells contain Zn and some of the Class II specified hazardous substances from the sediment of Hiroshima Bay (**Table II-4**). The substances, when sprinkled on the agricultural farms as contaminants of fertilizer, are transferred from sea to land of the Hiroshima Regional Urban Area. Annual use of the oyster shell fertilizer could affect landscape, which includes Hiroshima Bay and satoyama areas surrounding the Hiroshima City, due to of the persistence of substances in the environment (**Fig. I.-1**).

The accumulation of Cd in wildlife kidneys is the result of lifelong exposure to Cd. High concentrations of Cd are found in the kidneys of some raccoon dogs. They are captured due to disrupting praying the plants of agricultural farms, no matter how much amounts of plants they have eaten, the origin of Cd in kidneys or the level of function of each kidney has not been identified (Wang *et al.*, 2021).

Cd flow in the Hiroshima Regional Urban Area (**Fig. VI-1)** is characteristic to show the flows from inanimate to organism and from aquatic area to terrestrial area of the target ecosystem in view point of environmental and human health. This flow is different from that at the Kamioka Mine, in which the flows between inanimate objects, industrial factories, is shown (https://www.pref.toyama.jp/1291/kurashi/kenkou/iryou/1291/disease-en/05-en.html), which is based on the Cd measurements in bedrock, soils, springs, and factory drainage for 50 years at the Jinzu river in view point of the soil pollution problem (Hata A, 2021). After closing the mine in 2001, vegetation experiments aiming at restoration of natural vegetation have been performed to solve the problem of agricultural soil restoration (Kaji M. 2012). Surface covering, fertilization, sowing and planting of young trees have been conducted. Exposures to Cd in organisms would be measured, and a new flow of Cd will be shown between inanimate objects and organisms composing the ecosystem.

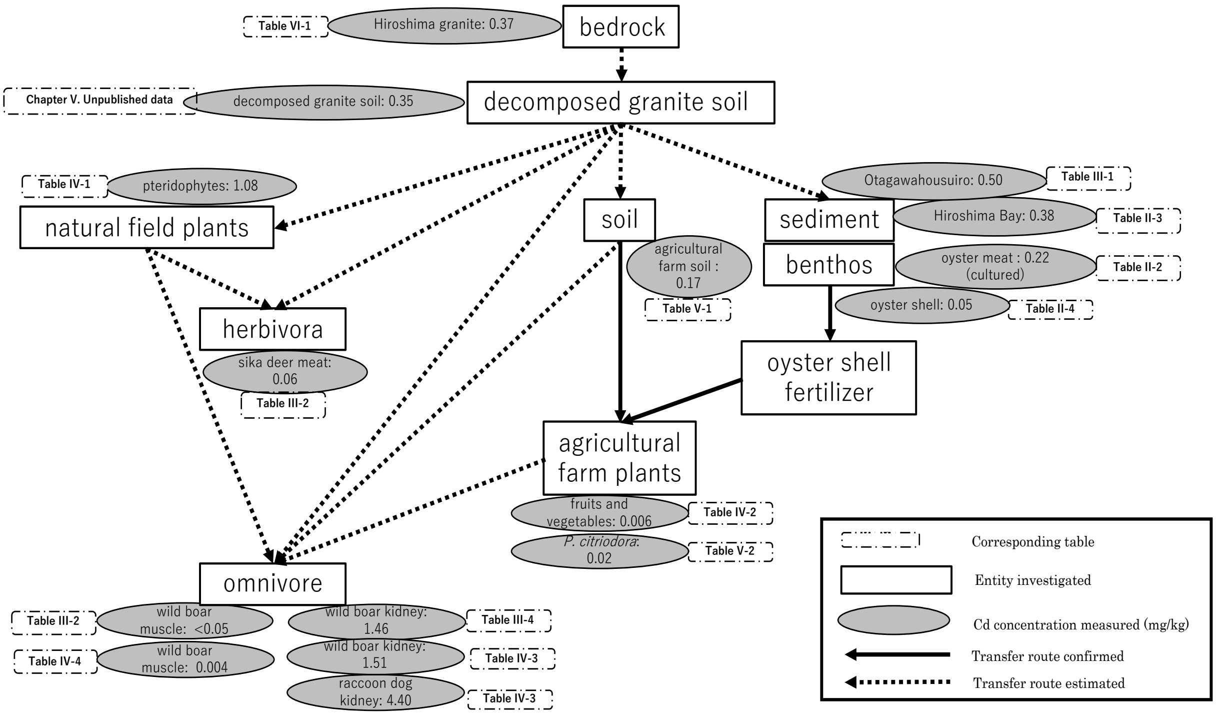

Fig. VI-1. Cd flow in the Hiroshima Regional Urban Area. Cd measurements for individual elements (**Table VI-2**) are shown.

The sediment below oyster culturing rafts of Hiroshima Bay was examined using Cd concentrations. The mean value of Cd was as high as that of 9 years ago. The amount of Cd from sea to land mediated by oyster shells was 5.5~6.3 kg/year (**Table II-5**). Concentrations of Cd in game meat did not exceed 0.05 mg/kg except sika deer meat, which had 28.6 % of risk for consumers to reach the Provisional Tolerable Weekly Intake of Cd, 7μg/kg body weight/week. Wild plants, four kinds of pteridophytes, accumulated Cd in their foliage with a correlation to Zn with the value of Cd/Zn, 0.012±0.001, which partly reflected geologic background of the Area. Concentrations of Cd in the edible parts of the agricultural farm plants were <0.1mg/kg. Wildlife inhabiting satoyama accumulated Cd in their kidney with a positive correlation to Zn accumulation.

VI-4. Conclusion

There is a route for Cd to circulate in the human environment with the mediator of oyster shell waste as a specific fertilizer at the Hiroshima Regional Urban Area in the western part of Seto Inland Sea Area. The amount of Cd in oyster shell, 5.5~6.3 kg/year, is an identifier of the burden added to the terrestrial environment added by the human activities based on only one system of recycling oyster shell waste.

Cd concentration in foodstuffs available in this Area was lesser than the Provisional Tolerable Weekly Intake level except oyster meat and game meat. However, wildlife symbiotic with humans are the primary mammals with high concentrations of Cd accumulated in the kidneys.

Detrimental substance investigation is required to promote the oyster shell recycling as a kind of specific fertilizers. Additional burden of Cd on agricultural farm soil cannot be ignored, when the oyster shell fertilizer is used annually. For the investigation, female raccoon dogs are the sentinel mammals to provide valuable data related to Cd in the terrestrial environment.

The conversion of oyster shell waste into pharmaceuticals and chemicals is practiced on a large scale in southeast Asian countries. Oyster shell wastes are recycled to improve water quality in Japan, although on a small scale. The fundamental solution would be to regain the sediment of lesser concentrations of Zn and Class II specified hazardous substances, which would restore the healthy Hiroshima Bay for oyster culturing, decrease viral infections through oyster meat, and conserve the ecosystem with landscape level.

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II. Cd IN THE OYSTER CULTURING ENVIRONMENT OF HIROSHIMA BAY FACING THE HIROSHIMA REGIONAL URBAN AERA

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IV. ORIGIN OF Cd AND Zn IN WILDLIFE CAPTURED AT THE SOUTHERN PART OF HIROSHIMA REGIONAL URBAN AREA

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V. EXPERIMENTAL TRANSFER OF Cd FROM OYSTER SHELLS TO PLANTS

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VI. GENERAL CONCLUSIONS

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Appendix 1. STANDARD VALUES OF Cd AND Zn

The standard values determined by each government were used to evaluate the concentrations of Cd and Zn (**Appendix 1-Table 1**). No upper limit has been determined for the pollutants of special fertilizers, to which oyster shell powder belongs, by the Ministry of Agriculture, Forestry and Fisheries. No intake limit of Cd was been determined for oyster meat by the Ministry of Health, Labour, and Welfare.

Appendix 1 - Table 1. Standard values of Cd and Zn in the sediments, fertilizer, soil, and foodstuff.

Target sample			Sediment	Special fertilizer	Agricultural farm soil	Foodstuff	
Ministry of decision			Land, Infrastructure, Transport and Tourism	Agriculture, Forestry and Fishery	the Environment	Codex	Health, Labour and Welfare
Zn			<150 mg/kg	-	≦120 mg/kg	-	<60 mg/day *
Specified hazardous substances, Class II (Ministry of the Environment)	Heavy metals	As	-	-	<0.01 mg/L	Undetected	-
		Cd	<1 mg/kg	-	<0.003 mg/L	<0.05 mg/kg for fruit vegetables, <2 mg/kg for marine bivalve and cephalopods	<0.007 mg/kg body weight/week **
		Cr	<80 mg/kg	-	-	-	-
		Pb	<46.7 mg/kg	-	<0.01 mg/L	-	-
	except heavy metals	B	-	-	<1 mg/L	-	-
		CN	-	-	Undetected	-	-
		F	-	-	<0.8 mg/L	-	-
Reference			https://www.pari.go.jp/search-pdf/no1174.pdf	https://www.maff.go.jp/j/syouan/nouan/hiryou/pdf/hikaku.pdf	https://www.env.go.jp/hourei/06/000049.html	https://www.mhlw.go.jp/topics/idenshi/codex/06/dl/codex_stan193.pdf	cho20150020001_020.pdf
				https://www.maff.go.jp/j/syouan/nouan/kome/k_hiryo/attach/pdf/hiryobse-11.pdf	https://www.env.go.jp/kijun/dt1.html	http://www.fao.org/fao-who-codexalimentarius/sh-proxy/en/?lnk=1&url=https%253A%252F%252Fworkspace.fao.org%252Fsites%252Fcodex%252FStandards%252FCXS%2B193-1995%252FCXS_193e.pdf	https://www.maff.go.jp/j/syouan/nouan/kome/k_cd/04_kijyun/02_nat.html

*: 10 mg/day from daily foods plus 50 mg/day from supplements.

**: Provisional Tolerable Weekly Intake.

Appendix 2. MATERIALS AND METHODS FOR THE MEASUREMENTS OF Cd AND Zn

Solid samples for the measurements of Zn and Cd were prepared as described below. Sediment and benthos were collected using a specific sampler (Ekuman-Birge, RIGO Co. Ltd., Tokyo, Japan) (Fukuhara *et al.*, 1987,) and they were stored at -20 ℃ until use. Sediments at the mouth of the river and streams were collected during low tide. Sediments under the oyster rafts were collected by measuring the depth using a water depth detector. All the sediments were collected at a depth of less than 10 cm from the surface, and the wet weight was more than 0.3 kg for each scoop.

Soils were collected in the farm and pot. Agricultural soil was scooped at a height of less than 10cm with the wet weight of more than 0.3 kg for each scoop. Ten scoops of agricultural soil were mixed in a bucket, and the soil in each pot was stored at -20 ℃ until use.

Oysters were purchased at the market places in the Hiroshima Regional Urban Area or they were collected at the mouth of Otagawa river during low tide, with wet weight of more than 1 kg per place. The fresh oysters with their shells were dried in a drying device (KM-300V, AS ONE, Osaka, Japan) overnight, ground using a stainless ball mill and stored at -20℃. Fresh oyster meat separated from the shells was stored at -20 ℃. The left shell was washed with running water, dried, and ground using a stainless ball mill. The shell powder was sieved through a 2-mm screen to remove impurities, and it was stored at -20 ℃.

The purchased frozen game meats of thigh with weights of more than 0.5kg each were stored at -20 ℃ until use. The fresh quadriceps of the slaughtered wild boar were washed properly with deionized water to remove superficial blood, and they were stored at -20 ℃ until use. The fresh side of the kidney of wild animals was stored at -20 ℃ until use.

The foliage parts of each type of pteridophytes, the length of whose fronds was more than 15cm for each species with the fresh weight of more than 1.0 kg each, were collected and stored at -20 ℃ until use. Edible parts of farm plants and foliage part of the bean sprout were dried, ground using a mill, and stored at -20 ℃ until use. The ear, stem, and leaf parts of perilla were collected, dried, ground using mill, and stored at -20 ℃ until use. Perilla seeds were directly stored at -20 ℃ until use.

Concentrations of Zn and the Class II specific hazardous substances were measured using the Sediment Inspection Method recommended by the Ministry of the Environment (https://www.env.go.jp/water/teishitsu-chousa/00_full.pdf) (**Appendix 2 - Table 1** and **2**). An AAS instrument (AA-6200, SHIMADU, Kyoto, Japan) was used of the determination of Cd in sediments, benthos, oyster shell, and soil. The standard food analysis method (https://www.city.hiroshima.lg.jp/site/eiken-news/225.html) was used for the measurement of Cd and Zn in organisms. The instrument, ICP-AES (ICP-OES 730-ES, Agilent, Tokyo, Japan) was used for measurement (https://www.mlit.go.jp/river/shishin_guideline/kasen/suishitsu/pdf/s06.pdf).

For studies on wild animals, the animal welfare and ethics was given the highest priority. The code of ethics, The Oath of Veterinarians-Declaration ’95, was obeyed throughout throughout the study（http://nichiju.lin.gr.jp/about/pdf/chikai.pdf）. The wildlife was treated following the guidelines of the Health Management of Wild Birds and Beasts in Yamaguchi prefecture (https://www.pref.yamaguchi.lg.jp/cms/a15300/syoku/yasei-chouju.html). Carcasses of the wild boars were prepared at their respective sites of capture. The raccoon dogs were captured because of their disruptive behaviors, such as eating farm plant, or trampling farms, witnessed by residents. They were submitted to an autopsy center, anesthetized with diethyl ether until their last breath and then autopsied. Grossly abnormal tissues and organs were stored in 10 % phosphate buffered formalin for histopathological observation.

Seeds of *Perilla citriodora*, one of native wild species in Japan, were collected with permission from the Itsukushima Island, a world cultural heritage, and they were artificially cultivated in an experimental farm to obtain seeds for further studies.

All the experiments were performed following the study regulation of ethics determined by the committee of Hiroshima Shudo University.

Appendix 2 - Table 1. Sediment Inspection Method to measure Zn, Cd and other specified hazardous substances in the sediment, benthos, oyster shell and soil.

Substance			Method	Reference *
Zn			Frea atomic absorption spectrometry	II-5.4.1
Specified hazardous substances, Class II	Heavy metal	As	absorptiometry	II-5.9.2
		Cd	Frea atomic absorption spectrometry	II-5.1.1
		Cr	absorptiometry	II-5.12.2
		Pb	Frea atomic absorption spectrometry	II-5.2
	Others	B	absorptiometry	II-5.13.1
		CN	absorptiometry	II-4.11
		F	absorptiometry	II-4.12

a): Morita M, Komori Y, Sasaki Y, Fukushima T, Fukushima M, Matsumura C, Yoshinaga J. Sediment inspection method. Division of water, soil and ground environment, Ministry of the Environment Government of Japan 2014 (https://www.env.go.jp/water/teishitsu-chousa/00_full.pdf).

Appendix 2 - Table 2. Samples and methods for the measurements of Cd and Zn.

		Original solid samples	
		sediment, benthos, oyster shell, soil	pteridophyta, farm plant, oyster meat, game, wildlife muscle and kidney
Sample collection	Main seasons	From October to March	From October to February
	Point	Hiroshima Regional Urban Area	Hiroshima Regional Urban Area
	Method	Sediment and benthos of less than 10cm of the surface were collected by a specific sampler (Ekuman-Birge) a).	Fresh or frozen samples purchased, kindly provided by residents, collected or hunted were used.
Sample preparation	At the time of collection	Original samples were kept in polyethylene bag and stored at -70～-20℃ until use.	Original plant samples were brought to laboratory, washed with running water to remove soil particles and kept in vinyl bag and stored at -70℃ until use. Others were directly kept in vinyl bag and stored at -70～-20℃ until use.
	Dried sample preparation	(1) The original sediment and soil samples were air-dried. The original benthos and oyster shell samples were dried in a drying oven at 110℃ for several hours and grounded into powder. (2) All the samples were sifted to a 2mm sieve made of synthetic resin to be dried in a drying oven at 110℃ for 2 hours. (3) The obtained powder was used as the dried sample.	(1) Thin-sliced original samples (<10mm of thickness) were prepared. (2) The samples were dried in a drying oven at 110℃ overnight. (3) The dried samples were grounded into powder and used as the dried samples.
	Test sample preparation	Wet decomposition method was used. (1) Weigh 0.1~5g of the dried sample. (2) Add 10ml of nitric acid and 20ml of hydrochloric acid. (3) Heating at 200℃ adding 10ml of nitric acid. (4) Cooling. (5) Add 20ml of nitric acid and 5 ml of perchloric acid. (6) Heating at 200℃. (7) Evaporative solidification. (8) Add 2ml of nitric acidand 50ml of deionized water. (9) Heating at 100℃. (10) Cooling. (11) Filtering using quaritative filter paper. (12) The obtained filtration was used as the test sample.	Pressure vessel method was used b). (1) Weigh 0.1~0.5g of the powderd sample. (2) Add 5ml of nitric acid and 2ml of hydrochloric acid. (3) Heating at 180℃. (4) Cooling. (5) Heat at 200℃. (6) Add 2ml of nitric acidand 50ml of deionized water. (7) Heating at 100℃. (8) Cooling. (9) Filtering using quaritative filter paper. (10) The obtained filtration was used as the test sample.
Determination of Cd and Zn	Instrument	Frame atomic absorption spectrometer (AA-6200, SHIMADZU, Kyoto, Japan)	Inductively coupled plasma emission spectrometer (ICP-OES730-ES, Agilent, Tokyo, Japan) c).
	Wave length	Wave lengths of the resonance line were set at 228.8 and 213.9nm for Cd and Zn, respectively.	Wave lengths of the emission line were 214.4 and 213.9nm for Cd and Zn, respectively.
	Thermal media	air-acetylene	argon
	Analytical quality control	(1) All the reagents used were of analytical grade. (2) Deionized water was used for all dilutions. (3) The procedure was repeated 3 times.	(1) All the reagents used were of analytical grade. (2) Deionized water was used for all dilutions. (3) The procedure was repeated 3 times.
	Obtaining quantitative value	Calibration curve method was used. (1) Reference standard solutions were purcased. The working standard solutions were prepared by apprpriate dilution with deionized water. (2) The blank sample was prepared by deionized water.	Calibration curve method was used. (1) Reference standard solutions were purcased. The working standard solutions were prepared by apprpriate dilution with deionized water. (2) The blank sample was prepared by deionized water.
	Concentration	Calculated on a dry weight basis.	Calculated on a wet weight basis.

a): Fukuhara H, Sakamoto M. An improved Ekman-Birge Grab for sampling an undisturbed bottom sediment core sample, Jpn J Limnol 1987 48: 127-32 (in Japanese).

b): Yamaki A. A simple simultaneous analytical method for multi-components including heavy metals in agricultural products using 1M nitric acid extraction-ICP-AES (ICP-OES 730-ES, Agilent, Tokyo, Japan). Annual research bulletin of the Chiba Prefectural Agriculture and Forestry Research Center 2011 3: 56-60.

c): Ikebe K, Nishimune T, Sueki K. Contents of 17 metals in food determined by inductively coupled plasma atomic emission spectrometry -meat and meat products- Food Hygiene Saf Sci. 1994 35: 323-27. (in Japanese)

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Appendix 2 - Table 3. Sizes of oysters cultured and wild.

	Cultured		Wild
	Immature (8 months)	Mature (over 20 months)	
N	37	55	60
Shell length (mm)	47.15 ± 9.89*	96.58 ± 20.27*	62.05 ± 10.92
Meat weight (g)	4.17 ± 2.29*	21.39 ± 10.33*	7.45 ± 3.56

Ages of the cultured oysters, immature and mature, are 8 months and more than 20 months, respectively. *: $p<0.01$ when compared to the wild oyster group by *t*-test.

Appendix 2 - Table 4. Concentrations of Cd and Zn in the oyster shells of cultured and wild.

Groups	Collected prefecture	Examined samples	Cd (mg/kg)			Zn (mg/kg)		
			low	-	high	low	-	high
1	Hiroshima (cultured)	4	0.07	-	0.24	9.8	-	19.0
2	Except Hiroshima (cultured)	5	0.10	-	0.17	2.0	-	6.3
3	Hiroshima (wild)	6	0.05	-	0.25	3.3	-	40.0
4	Okayama (wild)	8	0.10	-	0.18	11.0	-	88.0
5	Kagoshima (wild)	6	0.09	-	0.22	8.0	-	31.0
6 (1+2+3+4+5)		29	0.05	-	0.25	2.0	-	88.0

Oysters were collected from 2014 to 2019. Cd and Zn concentrations in their shells were measured following the procedure in **Appendix 2 - Table 1** and **2**. Five samples were added to the original 24 samples: Nitta, Y. Oysters (*Crassostrea gigas*) in the Hiroshima wide area urban districts concentrate cadmium (Cd) in their shells. Studies in the Health Sci. 2019 3 (1): 1-8.