博士論文

Soil Ecological Risk Assessment of Heavy Metal Pollution on a Floodplain in Japan

重金属汚染地における土壌生態リスク評価

－渡良瀬遊水地をモデル地域とした土壌生態学的評価手法の検討－

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Chapter 1

General introduction

1. Soil pollution and ecological assessment

Humans have changed ecosystems more rapidly and extensively over the past 50 years compared to any period in human history, resulting in degradation of many ecosystem services, increased risk of abrupt and harmful changes in ecological communities, and harm to humans themselves (Millennium Ecosystem Assessment, 2005). Soil pollution by hazardous chemicals has become a serious problem in many countries, impeding the use of land. Management of polluted land is generally based on human health risks, and ecological risk does not have a strong position in such assessments (Solomon and Sibley, 2002; Van Straalen, 2002). However, some advanced countries began to legislate for ecological assessment of soil contamination and remediation containing soil organisms. For example, the U.S. Environmental Protection Agency (USEPA) launched an effort to set Ecological Soil Screening Levels (Eco-SSLs) that could be used in the initial stages of site assessments under the Superfund Program (http://www.epa.gov/oswer/ riskassessment/risk_superfund.htm). In the procedure, soil invertebrates are used as ecological receptors of concern, and as foods to calculate biomagnification to birds and mammals by terrestrial food web models. In the Netherlands, the concept of Environmental Quality Standard is based on how much of a contaminant can be tolerated by the sensitive species exposed to contaminants. Maximum permissible concentrations are calculated by using the hazardous concentration (HC) for 5 % of the species, using species sensitive distribution model (Römbke et al., 2005a). Soil organisms are taken into account as members of terrestrial ecosystems in these criteria.

In Japan, the Agricultural Land Soil Pollution Prevention Law was established for

some heavy metals in 1970. Recently, since serious soil contaminations have been revealed mainly at vacant lots of factories, the Ministry of Environment established the Soil Contamination Countermeasures Law in 2002. However, the main purpose of this law is to protect human health, and the effects on terrestrial ecosystems are hardly noticed. The reasons for the littleness of attentions paid to ecological aspects would be partly the lack of basic knowledge of soil organisms and their functions, and toxicological effects on them. For soil ecological risk assessment in Japan, it is necessary to investigate the effect of soil pollution on domestic soil ecosystems. This thesis deals with the effects of heavy metal pollution on soil ecosystems, especially to soil invertebrates, in Japan.

In general, environmental risk assessment is the process of estimating the probability of occurrence of an undesired event and the probable magnitude of adverse effects in the environment. For ecological risk assessment of soil pollution, there are many basic questions: What should be protected; species, structure, or function? What scale; local, domestic, or world wide? What level; individuals, populations, communities, or ecosystems? To answer these questions, we should learn from case studies of soil quality evaluation.

2. Effect of heavy metal on soil invertebrate

Some heavy metals called ‘trace elements’ including Cu and Zn are definitely required for normal growth and reproduction in higher vertebrates, while very few laboratory experiments have been carried out to determine the trace metal requirements of terrestrial invertebrates (Hopkin, 1989). Metals exert toxic effects on animals if they

enter into internal biochemical reactions in which they are not normally involved. Some soil invertebrates can control metal concentrations in the body and reduce the damage of reactive forms of essential and non-essential metals (Vijver et al., 2004). For example, terrestrial isopods can accumulate large amounts of heavy metals in their cells of hepatopancreas (Hopkin, 1990; Paoletti and Hassall, 1999). Earthworms can accumulate heavy metals in granules in chloragogenous tissue, which is a loose assemblage of cells surrounding the gut, and discharge into coelomic fluid (Hopkin, 1989; Morgan et al., 2002). Collembora can store most of heavy metals in the midgut epithelium and excrete them during the next molt when the entire lining of the gut is shed (Fountain and Hopkin, 2001). Thus an increased exposure of heavy metals to organisms does not always result in effects. However, if exposure is too high, toxicants can affect the physiology of organisms, which can lead to a change in the growth, reproduction and/or survival of individuals. Such effects on life cycle parameters again have consequences on the population level, leading to degradation in community and ecosystem levels.

The ecotoxicological impacts of metals on earthworms have been often studied, because the toxicity of heavy metal especially Cu to earthworms were recognized earlier (Paoletti et al., 1988). The most serious problems seem to have occurred by use of Cu-amended pig fodder, leading to Cu-rich manure, and in orchards where Cu fungicides have completely wiped out earthworm populations (Van Straalen, 2004). The recent literature data on Cu concentrations in soil, which showed significant changes in lethal or sublethal endpoints, are listed in Table 1.

Table 1. Literature data on Cu concentrations in soil (mg/kg dry soil) that causing 50 % (sub)lethal effect on earthworm species

Endpoint	Earthworm species	EC_{50}/LC_{50} (mg/kg)	test medium	test period (day)	Source*
mortality	*Aporrectodea caliginosa*	640	field soil	56	1
	Aporrectodea tuberculata	134	artificial soil	14	2
		333	field soil	14	2
	Eisenia fetida	249	artificial soil	14	2
		519	artificial soil	28	3
	Eisenia foelide	626.7	field soil	14	4
	Lumbricus rubellus	325	commercial soil	28	5
	Lumbricus rubellus (juvenile)	398	commercial soil	296	6
cocoon production	*Aporrectodea caliginosa*	185.8	field soil	56	1
	Aporrectodea tuberculata	122	field soil	56	2
	Lumbricus rubellus	329	commercial soil	42	5
growth	*Aporrectodea caliginosa*	51.5	field soil	42	7
	Aporrectodea tuberculata	150	field soil	56	2
avoidance response	*Aporrectodea tuberculata*	56	field soil	2	2
	Eisenia andrei	181.1	commercial and field soil	2	8
	Eisenia fetida	56-236	artificial and field soil	2	9

* 1= Khalil et al. (1996b); 2= Lukkari et al. (2005); 3= Maboeta et al. (2004); 4= Liang and Zhou (2003); 5= Spurgeon et al. (2004a); 6= Spurgeon et al. (2004b); 7= Khalil et al. (1996a); 8= Loureiro et al. (2005); 9= Zwieten et al. (2004)

3. Triad approach

In the sediment system, Chapman (1986) had proposed a conceptual model of the sediment quality triad, which combines data from chemistry, bioassay and in situ studies (Fig. 1). Three facets to this approach would provide the strongest data for determining numerical sediment criteria. Similar approaches have been proposed for soil evaluation: chemical measurement, ecological surveys and bioassays (Van Straalen, 2004). In The

Netherlands, site-specific ecological risk assessment of soil contamination is directed to the application of biological tests, like bioassays and biological field observation (Römbke et al. 2005a). Kamitani et al. (2004) have evaluated the effect of bioremediation such as aeration and compost application to oil polluted soil by oil concentration, toxicity test with collembolan (*Folsomia candida*) and ecological survey (macrofauna, microarthropods and bacterial community using BIOLOG® Eco Plate). Their studies have shown that the abundance of microarthropods were much more sensitive to oil pollution and remediation process, and a battery of these studies showed that the recovery of soil ecosystem was more advanced in the aerated soil than the composted soil, although oil concentrations were equivalent between the aerated and composted soil. Thus, a triad approach would make it possible to assess the effects of pollutants on whole soil ecosystems, which is not clarified only by the concentration of contaminants.

Each facet in triad approach contains various targets for measurements. A description to each analysis is needed to set up my thesis.

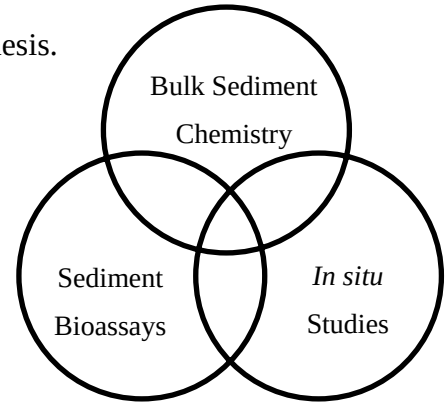

Fig.1 Conceptual model of the sediment quality triad. Areas where the three facets of the triad show the overlap provide the strongest data for determining numerical sediment criteria (Chapman, 1986).

3.1 Chemical analysis

Soils are a tremendously heterogeneous environmental matrix with varying spatial and temporal gradients of chemical and physical properties. Many researchers have

studied and suggested that heavy metals in soils are present in various forms due to interactions with various soil physico-chemical properties, e.g. soil pH, organic matter, clay content and so on (Yonebayashi et al., 1994; Adamo et al., 2003; Tipping et al., 2003; Zhang and Xu, 2003; François et al., 2004; Pietrzak and McPhail, 2004). In the scientific world it is common knowledge now that total concentrations in soil are insufficient to explain effects of contaminants (Peijnenburg and Jager, 2003). The bioavailable fraction of the pollutant is considered to be more strongly related to the free concentration in the pore water than to the total concentration in the soil (Van Straalen, 2002). On the other hand, authorities are much stronger focused on the total concentrations, since they are easier to handle and chemical laboratories have supplied a tremendous amount of data. In addition, the scientific basis for the adequate use of "bioavailability" in the assessment of ecological and human risk is weak (Peijnenburg and Jager, 2003).

The concept and experimental methods of bioavailability were explicated in several studies (e.g. Maiz et al., 2000; Belfroid et al., 1996; Hund-Rinke and Kördel, 2003; Rensing and Maier, 2003; Lanno et al., 2004). Lanno et al. (2004) have used three terms of availability: environmental availability, environmental bioavailability, and toxicological bioavailability (Fig. 2). The portion of total chemical in the soil that is not sequestered may be referred to as the "environmentally available" fraction. The "environmentally bioavailable" fraction is dependent on the physiology and behavior of the target and route of exposure. For example, earthworms would encounter and interact with specific fraction in soil through gut condition as well as the environmentally available chemicals through direct dermal contact. Toxicological bioavailability refers to that portion of absorbed chemical that reaches and interacts with the site of toxic

action in organism.

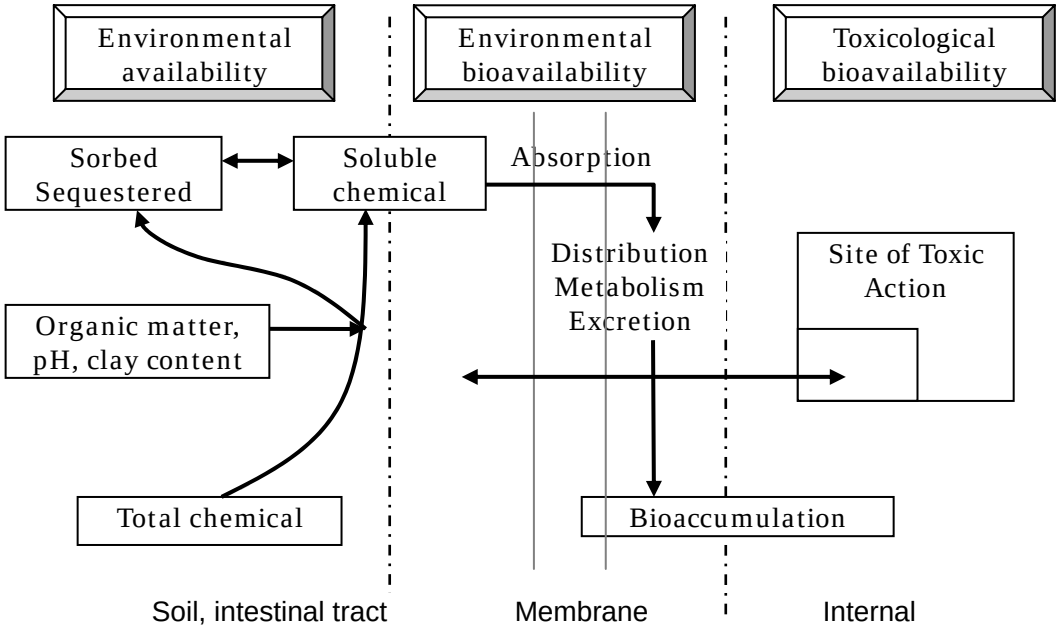

Fig. 2. Schematic model of bioavailability (after Lanno et al. 2004)

Chemical extractions represent an intermediate amount between total digestions and pore water samples. Peijnenburg and Jager (2003) have identified six types of extractions: weak salt, reductive extractions, weak acid, chelate agents, dilute strong acid and combined extracts. The concept of using a chemical extraction to assess the bioavailability is limited in environmental availability and partly in environmental bioavailability. Since the ideal extraction reagents will depend on target chemicals as well as target organisms, a certain extractant may provide a good estimate of chemical uptake by a certain species, whereas another extractant may provide a better estimate for a different species.

Thus, the concept of bioavailability should not be limited to the biological facets of the soil quality triad. Chemical measurement should include not only the total

concentration of a contaminant, but also adequate bioavailable fraction and soil properties affecting bioavailability. Such chemical availability relates strongly to physical and chemical conditions of soil, and ageing periods (Sijm et al., 2000).

3.2 Ecological surveys

There are several reports to the effects of soil pollution on soil organisms (e.g. Cortet et al., 1999; Van Straalen, 2004). The diminishment of soil organisms will be the most remarkable problem in anthropogenic change by soil pollution. It leads to decrease in soil biodiversity and change in community structure, leading to decrease in stability of energetic food web (de Ruiter et al., 2002). However, with many of soil microorganisms and soil invertebrates yet described, it is impossible to affix a quantitative value to losses of these members of the biota by soil pollution. In addition, since soil organisms are not recognized for their individual value, like whales and pandas, it is impossible to protect at the individual or population level. On the other hand, a number of bio-indicator systems were proposed using soil invertebrates in community level. For example, Spurgeon et al. (1996) suggested that earthworm, spider and isopod were suitable indicator taxa for monitoring pollutant impact, and also mentioned the necessity of 'Soil Invertebrate Prediction and Classification System (SIVPACS)'. Since earthworms are recognized as the most important soil invertebrates in terms of both biomass and activity, the structure of earthworm community, as well as their abundance and biomass were used as endpoints in determining the influence of anthropogenic stresses (Römbke et al., 2005b). Nematode maturity index, based on a classification of nematodes according to "colonizers" and "persisters", were well-known

and can be also applied to heavy metal polluted soil (Bongers, 1990; Korthals et al., 1996; Yeates and Bongers, 1999; Korthals et al., 2000). Microarthropods, including collembola and oribatid mites, have been studied profoundly for species richness and characters in polluted soils (Van Straalen and Verhoef, 1997; Van Straalen, 1998), so they will be good indicators for soil quality (Gardi et al., 2002).

Soil ecological functions: e.g. cycling of nutrients, litter decomposition, soil structure modification and primary productivity are related to soil biodiversity and species composition. Ecological functions of a soil are one of vulnerable functions to a pollution (Van Straalen, 2002). Therefore, it is reasonable that the impacts of soil pollution can be evaluated as decrease in such a functional performance. The loss of biodiversity can have significant impacts on ecosystem functioning. Lawton (1994) has proposed three alternative hypotheses that summarize the possible general responses of ecosystem processes to reductions in species richness. 1) The redundant species hypothesis suggests that there is a minimal diversity necessary for proper ecosystem functioning, and most species are redundant in their roles. 2) The rivet hypothesis suggests that all species make a contribution to ecosystem performance, and functioning will be impaired as species fall out. 3) The idiosyncratic hypothesis suggests that ecosystem function changes when diversity changes, but the magnitude and direction of change is unpredictable, because the roles of individual species are complex and varied. The general feeling among soil ecologists is that functional redundancy indeed plays a role in soil communities (Bradford et al., 2002; Laakso and Setälä, 1999; Van Straalen, 2002), and there is consensus that at least some minimum number of species is essential for ecosystem functioning under constant conditions and that a larger number of species is probably essential for maintaining the stability of ecosystem process in changing

environment (Loreau et al., 2001). Soil organisms are often classified to three functional categories, micropredator, litter transformer and ecosystem engineer (Lavelle et al, 1997; Wardle, 2002). To evaluate the effects of diminishing species richness on ecosystem processes, we should look at the change in functional groups of organisms defined according to ecosystem processes. Biodiversity effects on ecosystem functioning can be predicted by the degree of functional differences among species (Heemsbergen et al., 2004).

In addition, macrofauna like earthworms serve as food for a variety of vertebrate predators, therefore the presence of contaminants in earthworms poses a serious risk of secondary poisoning of predators due to biomagnification (Ma and Talmage, 2001; Reinecke and Reinecke, 2004), even if the contaminant does not extend to a critical tissue concentration for the organisms. It leads to migration of soil contaminants in terrestrial ecosystems and spread to wide range. So, the concentration in earthworm tissue should be an indicator of potential risk for toxicant diffusion, incorporating to the predator sensitive value.

3.3. Bioassays and laboratory test

Ecotoxicological effects of soil pollution have been assessed by use of single species tests, such as acute and chronic laboratory tests. The test methods of such species have been established [e.g. enchytraeid *Enchytraeus albidus* (OECD 220), earthworm *Eisenia fetida* / *Eisenia andrei* (OECD 207, 222), springtail *Folsomia candida* (ISO 11267)], and such a testing based approach of ecotoxicology greatly benefited environmental regulation for deriving maximum acceptable chemical concentrations

(Van Straalen, 2003). However, since studies have focused on only a few and single species in homogenized conditions, it is also recognized that laboratory tests cannot predict effects that critically depend on the interaction among species in field conditions (Cortet et al., 1999; Van Straalen, 2002). On the contrary, in situ assessments are useful for characterizing differences between locations, but field manipulation experiments can not control environmental conditions; e.g. temperature and moisture variations, resulting in a low repeatability and leading to difficulties with the interpretation of results. Therefore, simplified laboratory 'microcosm' tests have been constructed as a surrogate for the complicated field situation (Beyer and Odum, 1993; Verhoef, 1996). Studies in microcosms could allow for observations on the effect of pollutants on soil biota and function under complex interaction, controlled condition and replication (Parmelee et al., 1997; Vink and van Straalen, 1999; Martikainen et al., 1998). Sheppard (1997) has reviewed the design of microcosms and their use in ecotoxicological test.

Recently, using a type of soil microcosm, called Terrestrial Model Ecosystem (TME), has been regarded as a valid method by soil ecologists and especially, soil ecotoxicologists (Van Straalen 2002). TME approach uses undisturbed soil columns taken from field and includes living vegetation growing on the soil allowing for interactions between soil-living organisms and plant roots. In international programs sponsored by the EU, significant progress has been made in the standardization and field validation of TME, which was published as a series of papers in Ecotoxicology vol. 13 (Knacker et al. 2004).

In Japan, there are few basic toxicological studies concerning native soil animals, which have probably different toxicological characteristics from European species dealt in most ecotoxicological studies. Moreover, Japan has a large diversity of soil types due

to a variety of base materials, climates and landscapes. Therefore, an assessment system taking a local area into account would be appropriate to Japanese soil. The TMEs would be useful for such a site specific assessment.

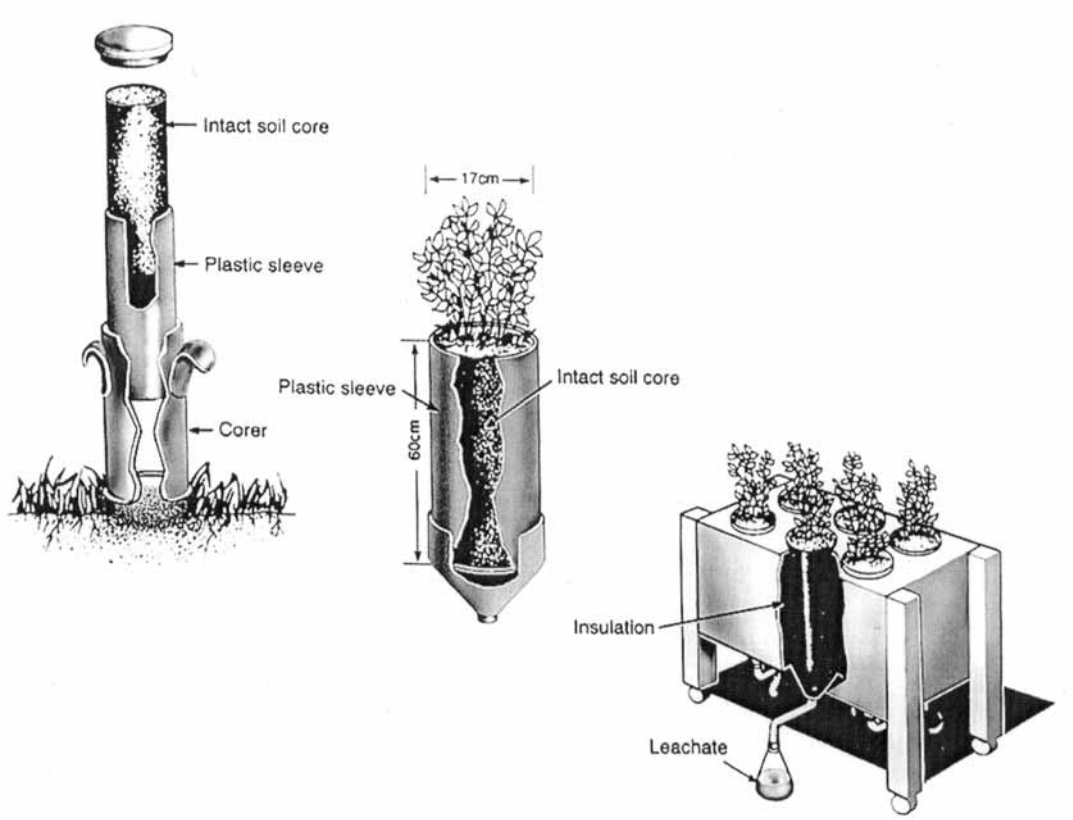

Fig. 3. General design of a Terrestrial Model Ecosystem (Sheppard, 1997).

4. Objectives of the thesis

The answers for questions ‘what should be protected?’ are discussed here. We should investigate species, community structure and ecological function, because they are important respectively, and because we do not have enough knowledge about the relationship among them. Soil ecological risk assessment should be discussed at community and ecosystem levels rather than population level, because it is difficult to

find out rare or endangered species for population level protection, and soil functions would be maintained at community and ecosystem levels. The scale of study should be limited in local area, because of a large diversity of soil types in Japan.

As mentioned above, little data are available in Japan on the effects of soil pollution on a domestic soil ecosystem. Therefore, the general objectives of this thesis were set to determine the effects of heavy metal pollution on a soil ecosystem, including soil biota, ecological function and bioaccumulation and to evaluate the validity of semi-field test using TMEs for soil ecological assessment in Japan. The research was proceeded on the line of the triad approach mentioned above: chemical analysis, ecological research in situ, and laboratory tests with field soil.

5. Watarase Retarding basin

Watarase Retarding basin (36^{o}14'N, 36^{o}14'E, 3,300ha) was used as a research area in this thesis. The study area locates downstream of Watarase River in Tochigi, central Japan, which is designed and operated to provide temporary storage and thus reduce the peak flood flows of a current downstream. This site gradually became contaminated with heavy metals caused by mining activity from Ashio Copper Mine at the headwaters. Since 1877, the mining activity rapidly enlarged and contamination from the mine caused severe damage in the Watarase River basin (Morishita, 1981). Residential house in Yanaka village, which had located in the present retarding basin, were dismantled under government compulsion due to severe damage of heavy metal pollution and flooding. Efforts have been made to improve the water quality from the mine until it was closed in 1973. However, the heavy metal concentrations in the sediment on

Watarase retarding basin remain until today (Ministry of Land, Infrastructure and Transport Government of Japan, 1976; 1991).

The study area is dominated by tall grasses (reed; *Phragmites australis* and *Miscanthus sacchariflorus*). The reeds are used for making traditional blinds, and once a year in March, the grasses are burned off to ensure high quality reeds and pest control, also reducing litter layers on the surface soil.

6. Outline of the research

Chapter 2 of this thesis describes the heavy metal concentration and soil properties in Watarase Retarding basin, and also focuses on the effects of heavy metals on microbial biomass and microbial functional community. Chapter 3 focuses on the effects of heavy metals on soil microarthropods, macrofauna and ecological functions in the study site. Chapter 4 presents the heavy metal concentrations in Japanese earthworms living in the study area and discusses on the exposure routes. Chapter 5 introduces a controlled environmental facility ‘Earthtron’ and its necessity for belowground manipulation study. Chapter 6 focuses on the impact of Cu on terrestrial compartment at ecosystem level by using TMEs in the Earthtron, and describes the applicability of ecotoxicological high-tier methods to the assessment for heavy metal pollution on Japanese soil. Finally, Chapter 7 is a summary of the studies presented in Chapter 2 to 6 and ends with general conclusions.

Chapter 2

Microbial biomass and tolerance of microbial community on an aged heavy metal polluted floodplain in Japan

Kamitani T, H Oba and N Kaneko

Abstract: The objective of the present study was to increase understanding of the effects of heavy metal pollution and soil properties on microorganisms in relation to the biomass and microbial functional community. Soil samples were collected from aged polluted and reference sites on a floodplain. The soil Cu, Zn and Pb total concentrations were much higher at the polluted sites (average 231.6-309.9 mg kg^{-1}, 195.7-233.0 mg kg^{-1}, and 72.4-86.0 mg kg^{-1}, respectively) than at the reference site (average 33.3-44.0 mg kg^{-1}, 76.7-98.0 mg kg^{-1}, and 30.8-41.6 mg kg^{-1}, respectively), while the available heavy metal concentrations in $CaCl_2$ extraction were similar in all sites. Small seasonal variations in the size of microbial biomass were observed. Ambient soil properties (e.g. total C, N, pH, moisture content, and CEC) affected the soil microbial biomass more than the heavy metal pollution. However, the aged pollution tended to impact on the composition of the microbial community. PICT (pollution-induced community tolerance) test using BIOLOG Ecoplates showed enhanced tolerance of the microbial community to Cu stress in the polluted site. In non polluted but low nutrient, low pH and low moisture soil, the microbial biomass was lower and the microbial community was more vulnerable to Cu stress. In spite of the low heavy metal availability due to ageing, the BIOLOG technique provided sensitive detection of microbial community level changes in PICT analysis.

Keywords: heavy metal pollution; microbial biomass; microbial functional community; PICT; soil property

1. Introduction

Soil pollution has become a serious problem in many countries, impeding the use of land. Management of polluted land is based on several considerations including human health risks, however, ecological risk does not have a strong position in such assessments (Solomon and Sibley, 2002; Van Straalen, 2002). Polluted soils are usually characterized by complex chemical mixtures rather than by single chemicals. To determine the environmental impact associated with complex contaminants, a toxicity-based approach, rather than a chemically-based approach, should be adopted in risk assessment and relevant biological information needs to be incorporated into the toxicity and risk assessment procedures (Haimi, 2000).

Decomposer organisms dwelling in soil are recognized to be essential for the functioning of terrestrial ecosystems; they mineralize carbon and nutrients that are bound to dead organic matter and provide resources for primary producers (Mikola et al., 2002; Wardle, 2002). Soil microorganisms are the most dominant group in terms of numbers and biomass, and they are the most important contributors to soil processes: the cycling of materials, energy and nutrients (de Ruiter et al., 2002). Adverse impacts of soil pollution on microorganisms would be a critical problem for the soil process at system level interaction.

Numerous studies have demonstrated the effects of heavy metal pollution on soil microorganisms; not only microbial biomass and population, but also community structure and microbial activities (Welp and Brummer, 1997; Dumestre et al., 1999; Kelly et al., 1999; Kandeler et al., 2000; Chander et al., 2001; Klumpp et al., 2003). However, the effects reported in the literature are contradictory. A major complicating factor in soil ecotoxicity is that most of the polluting substances are bound to the solid

phase of soil, while the bioavailable fraction of the pollutant is usually more strongly related to the free concentration in the pore water than to the total concentration in the soil (Van Straalen, 2002). Because heavy metals in soils are present in various forms due to interactions with various soil properties (Yonebayashi et al., 1994, Adamo et al., 2003; Tipping et al., 2003; Zhang and Xu, 2003; François et al., 2004; Pietrzak and McPhail, 2004), total heavy metal concentrations in soils cannot provide a precise evaluation of their influence on soil microorganisms (Kunito et al., 1999). Although bioavailability can be used to assess hazard or risk, natural and anthropogenic changes can change this value over time (Solomon and Sibley, 2002). Well designed studies, including seasonal observations, are needed to estimate the tangible effect of pollutants in field conditions.

Another important consideration is the adaptation of a microbial community to pollutants (Kelly et al., 1999; Turpeinen et al., 2004). Pollution-induced community tolerance (PICT) might be a useful approach for estimation as it is based on the phenomenon that the average resistance of a community to a stress factor increases as sensitive species become less dominant or disappear (Van Straalen, 2003).

The objective of the present study was to increase understanding of the effects of heavy metal pollution and soil properties on microorganisms in relation to the biomass and microbial functional community structure under field conditions of the heavy metals present at the polluted site. Since Cu was at the highest concentration, and it is highly toxic to microorganisms (Pennanen et al., 1996; Aoyama and Nagumo, 1997), we also assessed the Cu tolerance of the microbial community.

2. Material and methods

2.1. Field site and sampling design

The study area is at Watarase retarding basin on Watarase River in Tochigi, central Japan, which is designed and operated to provide temporary storage and thus reduce the peak flood flows of a current downstream. This field is partly contaminated with heavy metals caused by mining activity from Ashio Copper Mine at the headwaters. Since 1877, the mining activity rapidly enlarged and contamination from the mine caused severe damage to water and paddy in the Watarase River basin (Morishita, 1981). Efforts were made to improve the water quality from the mine until it closed in 1973.

The study area is dominated by tall grasses (reed; *Phragmites australis* and *Miscanthus sacchariflorus*). The reeds are harvested for making traditional blinds, and once a year in March, the grasses are burned off to ensure high quality reeds and control pests. Soil was sampled from two polluted sites (PA and PB, at a distance within 20 m) and two non polluted sites (RA and RS, at a distance within 50 m), each site measured 10 m wide by 30 m long. All the sampling sites are located within about 2 km distance. The difference in heavy metal concentrations resulted from the distance of the past channel of the Watarase River. To separate the influences of heavy metals from various soil properties, we selected one reference site (RA) carefully to obtain soil samples which showed similar properties to the polluted site except for their heavy metal loads. Another no polluted site (RS), which was rather sandy compared to the other sites, was selected to investigate the effects of soil properties on microorganisms without taking heavy metals into account. Five replicate soil samples were taken from the top soil (5 cm deep) of all four sites at three times: May, August and November in 2003. The samples were divided into subsamples for soil physico-chemical and microbial analyses. Soil samples for dry bulk density were also collected with a 100 cm^3 core sampler. All

sampled soils were stored in a cooling box, brought to the laboratory and treated in each analysis. Soil (5cm depth) and air temperatures were measured from April to November 2003 at each site using automatic data recorders (DS1921L Thermochron iButton, Maxim Integrated Products, Inc., CA).

2.2. Soil properties

The soil subsamples for physico-chemical analyses were dried at 60 ^{o}C for 72 h and sieved through a 2mm mesh. To estimate total amount of heavy metals (Cu, Zn, and Pb), 1 g dry soil was heated with 10 mL concentrated HNO_3, starting at 80 ^{o}C (Bruus Pedersen and van Gestel, 2001). The samples were then dried at 135 ^{o}C, redissolved in 40 mL 0.1 M HNO_3, centrifuged and filtered. The available fraction of heavy metals was measured by extraction with 0.01 M $CaCl_2$ (Novozamsky et al., 1993). Four grams of dry soil was shaken with 20 mL $CaCl_2$ solution for 20 h, and then centrifuged and filtered. Heavy metals were analyzed with ICP-AES (ICPS-8000E, SHIMADZU Co., Kyoto, Japan).

Soil pH was measured at a ratio of soil-to-solution of 1:2.5 in an aqueous suspension of soil. Exchangeable cations (Na, K, Ca and Mg) and cation exchange capacity (CEC) were determined by saturation with 1 M CH_3COONH_4 (pH 7.0), washing with 0.05 M CH_3COONH_4, and replacement with 1 M KCl (Nakano et al., 1995). Exchangeable cations and NH_4 for determining CEC were analyzed with AAS (AA-660, SHIMADZU Co., Kyoto, Japan), and an auto-analyzer (INTEGRAL FUTURA, ALLIANCE Instruments, Frépillon, France), respectively. Total C and total N were measured with an NC-analyzer (Sumigraph NC-95A, Sumika Chemical Analysis Service, Ltd., Osaka, Japan). Particle-size analysis was conducted by pipet method after dispersion with

chemical (Na-hexametaphosphate) and physical (shaking and stirring) methods (Gee and Or, 2002). Loss on ignition (LOI) was analyzed by igniting the dry soil for 2 h at 750 ^{o}C. Dry bulk density was measured by drying the soil subsamples collected with the 100 cm^3 core sampler.

2.3. Microbial biomass carbon

The microbial biomass C (C_{mic}) was determined by a chloroform fumigation-extraction method (Vance et al., 1987). Soils were fumigated soon in the laboratory: ten grams of fresh soil was fumigated with chloroform for 5 days at 25^{o}C in darkness and extracted by shaking with 10 % KCl for 30 min. Reference samples were analyzed at day 1. The amounts of organic C in the extracts were measured using an organic carbon analyzer (TOC-5000, SHIMADZU Co., Kyoto, Japan). C_{mic} was calculated from the equation (Inubushi, 1997), as follows:

$$C_{mic} = 2.04 * (C_{fum} - C_{ref})$$

where C_{fum} is the amount of C extracted by 10 % KCl from fumigated soil and C_{ref} is that from nonfumigated soil. C_{mic} to soil total C ratio (C_{mic}/total-C) was also calculated.

2.4. Functional diversity of microbial community

Microbial community-level physiological profiles were determined by using BIOLOG® Ecoplates (Biolog Inc., Hayward, CA, USA). Ten grams of soil, sampled in May and August, was shaken in 90 g sterile water with reciprocal shaker set at 180 oscillations min^{-1} for 10 min, and 1 mL supernatant was diluted with 99 mL sterile water.

Ecoplates were inoculated with 150 μL of the 10^{-3} diluted soil suspension to each well. The plates were incubated at 22.5°C for 7 days and absorbance of 590 nm (A_{590}) was measured every 24 h for 7 days with a microplate reader (Multiskan JX, Thermo Labsystems, Helsinki, Finland). Wells were considered to be positive in terms of substrate utilization where the value of A_{590} in wells was 0.25 units greater than that in control wells (Toyota et al., 2000). The values of average well color development (AWCD) were calculated for each plate. The data was standardized by subtracting the mean value of the control wells from all well color values and then dividing by AWCD (Garland and Mills, 1991).

The sample in November was used for the PICT approach to detect tolerance of microbial functional community to Cu. The PICT approach is based on the phenomenon that communities may show shifts towards greater tolerance when a toxicant stress is present. PICT is estimated by sampling communities from polluted and reference locations, exposing them to the same range of concentrations of the toxicants, and assessing the differences of sensitivity between communities (Boivin et al., 2002). In the present study, each soil sample was suspended with $CuSO_4$ at increasing concentration (corresponding to 0, 10, 100, 300, and 1000 μM g^{-1} fresh soil) instead of distilled water. To prevent the effect of different concentrations of anion, $MgSO_4$ was also added to the dilution at the same molar level, 1000 μM g^{-1} SO_4. The replicates were three for each treatment.

2.5. Statistical Analyses

Two-way ANOVA was carried out for C_{mic} and C_{mic}/total-C over the sampling season using Statview for Windows Version 5.0. One-way ANOVA was conducted for

all soil properties in each sampling season and the number of positive wells in BIOLOG test, and Post hoc test (Tukey-Kramer) was performed. The data from the BIOLOG test, based on equivalent AWCD values of about 1.0 for each plate of May and August soils, and 0.5 for November soil plates, were subjected to a principal component analysis (PCA) by Canoco for Windows Version 4.5.

3. Results

3.1. Soil properties

The total concentrations of Cu, Zn and Pb in HNO_3 extractions were significantly higher in the polluted sites (average ranges 231.6-309.9 mg kg^{-1}, 195.7-233.0 mg kg^{-1}, and 72.4-86.0 mg kg^{-1}, respectively) than the reference site RA (average ranges 33.3-44.0 mg kg^{-1}, 76.7-98.0 mg kg^{-1}, and 30.8-41.6 mg kg^{-1}, respectively) regardless of sampling month, as shown in Table I. RS soil had lower total heavy metal concentrations than RA in each core. However, the available concentrations in $CaCl_2$ extractions were similar and at low level in all samples.

The physico-chemical properties of soil showed different patterns among sampling sites (Table II). PA, PB, and RA soils were similar, while RS soil was different from the other sites. Because RS soil was composed of much more sand and less silt fractions than the other sites, many properties also significantly changed: higher in dry bulk density, and lower in moisture content, pH, total C and N, CN ratio, LOI, CEC and exchangeable cations.

Table I Microbial biomass and heavy metal concentrations of soils in the study sites

	total heavy metal (mg kg^{-1})			exchangeable heavy metal (mg kg^{-1})			C_{mic}	C_{mic}/total-C
May	Cu	Zn	Pb	Cu	Zn	Pb	(mg g^{-1})	(mgC_{mic} g^{-1}total-C)
PA	231.6^{a}	195.7^{a}	76.0^{a}	0.03	0.08	0.01	0.81^{a}	15.8
PB	265.6^{a}	202.4^{a}	72.4^{a}	0.02	0.11	<0.01	0.66^{ab}	13.7
RA	37.8^{b}	84.0^{b}	32.8^{b}	0.03	0.06	<0.01	0.66^{ab}	12.5
RS	19.9^{b}	53.0^{c}	22.2^{b}	0.03	0.16	<0.01	0.44^{b}	16.9
August								
PA	309.9^{a}	226.9^{a}	83.2^{a}	0.03	0.22^{ab}	<0.01	0.65	12.9^{a}
PB	292.6^{a}	231.7^{a}	80.2^{a}	0.03	0.32^{a}	<0.01	0.68	12.7^{a}
RA	33.3^{b}	76.7^{b}	30.8^{b}	0.03	0.12^{b}	<0.01	0.73	16.6^{ab}
RS	22.5^{b}	58.3^{b}	22.3^{c}	0.02	0.26^{ab}	<0.01	0.56	19.2^{b}
November								
PA	273.9^{a}	233.0^{a}	86.0^{a}	0.02	0.24	0.02	0.89^{a}	17.3
PB	284.2^{a}	228.7^{a}	80.5^{a}	0.02	0.26	0.01	0.74^{ab}	15.3
RA	44.0^{b}	98.0^{b}	41.6^{b}	0.01	0.11	0.02	0.52^{b}	11.4
RS	27.3^{b}	73.4^{c}	34.9^{b}	0.01	0.21	0.02	0.39^{b}	15.9

Values in a column with the same or no letter are not significantly different (Tukey-Kramer test, $P<0.05$) in each sampling time. Each value represents mean of replicates (n = 5). C_{mic}: microbial biomass carbon

3.2. Microbial biomass carbon

The soil and air temperatures did not differ among sites; mean soil temperatures a month before sampling times in May, August, and November were 17.0 ^{o}C, 21.1 ^{o}C, and 13.5 ^{o}C respectively, and mean air temperatures were 18.0 ^{o}C, 21.7 ^{o}C, and 13.3 ^{o}C respectively. Sampling site had a significant impact on C_{mic} and C_{mic}/total-C ($P<0.001$, two-way ANOVA). However, sampling month alone did not appear to affect these parameters ($P>0.05$). Interaction between sampling month and site was significant ($P<0.05$). The highest level of C_{mic} was found in PA soil in May and November, which was significantly different from RS in May, and RA and RS in November (Table I). No significant difference was observed in August. Conversely, C_{mic}/total-C showed no

significant change in May and November, and C_{mic}/total-C of RS soil was significantly higher than the polluted site in August.

Table II

Physico-chemical properties of soils in the study sites

	MC	BD	particle size distribution (%)			pH(H_2O)	total C	total N	CN ratio	LOI	CEC	exchangeable cation (cmol kg^{-1})			
May	(%)	(g cm^{-3})	clay	silt	sand		(%)	(%)		(%)	(cmol kg^{-1})	Na	K	Ca	Mg
PA	35.3^{a}	0.44^{a}	20.2^{ab}	63.8^{a}	16.0^{a}	6.2^{a}	5.3^{a}	0.39^{a}	13.4^{a}	13.3^{a}	23.4^{a}	0.07^{a}	1.10^{ab}	18.5^{a}	3.15^{a}
PB	34.6^{a}	0.48^{a}	22.5^{a}	65.8^{a}	11.8^{a}	6.3^{a}	4.9^{a}	0.35^{a}	14.0^{a}	12.4^{a}	22.7^{a}	0.07^{ab}	0.99^{b}	17.6^{a}	3.07^{a}
RA	32.8^{a}	0.44^{ab}	23.2^{a}	63.5^{a}	13.3^{a}	6.1^{a}	5.0^{a}	0.37^{a}	13.6^{a}	13.4^{a}	23.2^{a}	0.06^{b}	1.33^{a}	17.4^{a}	3.65^{a}
RS	22.8^{b}	0.56^{b}	18.6^{b}	31.8^{b}	49.6^{b}	5.7^{b}	2.6^{b}	0.22^{b}	11.5^{b}	8.0^{b}	11.9^{b}	0.04^{c}	0.98^{b}	7.9^{b}	1.44^{b}
August															
PA	35.8^{ab}	0.43^{a}	24.5^{a}	68.8^{a}	6.8^{a}	6.1^{a}	5.0^{a}	0.38^{a}	13.2^{b}	12.5^{a}	21.8^{a}	0.10^{a}	0.94	16.6^{a}	2.63^{a}
PB	40.4^{ab}	0.43^{a}	24.3^{a}	68.3^{a}	7.4^{a}	6.1^{a}	5.3^{a}	0.37^{a}	14.3^{a}	13.0^{a}	21.3^{a}	0.09^{ab}	0.98	17.7^{a}	2.86^{a}
RA	41.8^{a}	0.46^{a}	21.3^{ab}	63.0^{a}	15.7^{a}	6.0^{a}	4.4^{a}	0.33^{ab}	13.3^{b}	12.6^{a}	19.3^{a}	0.08^{bc}	1.12	14.3^{a}	2.91^{a}
RS	32.2^{b}	0.55^{b}	19.0^{b}	40.9^{b}	40.0^{b}	5.8^{b}	3.0^{b}	0.25^{b}	11.9^{c}	8.6^{b}	11.5^{b}	0.06^{c}	0.94	8.9^{b}	1.71^{b}
November															
PA	45.6^{a}	0.40^{a}	22.3^{a}	68.1^{a}	9.6^{a}	6.2^{ab}	5.2^{a}	0.38^{a}	13.5^{a}	11.1^{a}	24.0^{a}	0.08^{a}	1.17	19.1^{ab}	3.03^{a}
PB	46.4^{a}	0.43^{ab}	23.0^{a}	68.6^{a}	8.4^{a}	6.3^{a}	4.8^{a}	0.34^{a}	14.1^{a}	9.5^{a}	23.0^{a}	0.06^{b}	1.12	19.6^{a}	3.26^{a}
RA	41.7^{b}	0.46^{bc}	22.4^{a}	66.8^{a}	10.8^{a}	6.1^{b}	4.6^{a}	0.34^{a}	13.6^{a}	12.2^{a}	21.7^{a}	0.06^{b}	1.19	15.7^{b}	3.32^{a}
RS	31.0^{c}	0.53^{c}	18.4^{b}	35.3^{b}	46.2^{b}	5.8^{c}	2.7^{b}	0.22^{b}	12.2^{b}	8.3^{b}	13.4^{b}	0.05^{b}	1.03	9.2^{c}	1.81^{b}

Values in a column with the same letter are not significantly different (Tukey-Kramer test, $P<0.05$) in each sampling time. Each value represents mean of replicates ($n = 5$).

MC: moisture content, BD: dry bulk density, LOI: loss on ignition, CEC: cation exchangeable capacity

3.3. Functional diversity of microbial community

Ability of substrate utilization expressed by the number of positive wells in the BIOLOG test was significantly higher in RA soil than in the other soils at 2 days after incubation in May and August (Table III). The statistical analysis by PCA showed that the polluted soils had a similar utilization pattern to the RS soil, and the RA soil was separated from them in May (Figure 1(a)) and August (Figure 1(b)).

Another BIOLOG test showed that the number of positive wells decreased with gradual Cu additions in all extracts (Table IV). Although the number of replicates was small (three), the C utilization ability of RS soil tended to become lower than the other soils at the high Cu concentration treatments (100, 300 and 1000 μM g^{-1}). The result of PCA enabled us to differentiate the treatments of high Cu concentration (100 and 300 μM g^{-1}) from the control and the treatment of low Cu concentration (10 μM g^{-1}) by PC 1 (Figure 2). At the treatments of high Cu concentration, PB soil seemed to be separated from the reference soil by PC 2. The positive side of PC 2 was influenced mainly by a substrate, D-Cellobiose.

Table III Ability of C substrate utilization (BIOLOG® Ecoplate) by soil microbial community with water dilution

days of incubation	2	4	6
May			
PA	19.7 ± 0.80 [a]	29.3 ± 0.38 [a]	30.3 ± 0.18 [ab]
PB	21.0 ± 0.32 [a]	29.7 ± 0.18 [ab]	30.7 ± 0.12 [a]
RA	23.7 ± 0.28 [b]	30.5 ± 0.19 [b]	30.7 ± 0.16 [ab]
RS	19.6 ± 0.68 [a]	28.9 ± 0.35 [a]	30.1 ± 0.17 [b]
August			
PA	22.9 ± 0.54 [a]	29.9 ± 0.25 [a]	30.7 ± 0.13
PB	21.5 ± 0.50 [a]	29.8 ± 0.36 [a]	30.7 ± 0.13
RA	25.2 ± 0.28 [b]	30.9 ± 0.09 [b]	31.0 ± 0.00
RS	23.1 ± 0.45 [a]	30.1 ± 0.22 [ab]	31.0 ± 0.00

Values in a column with the same or no letter are not significantly different (Tukey-Kramer test, $P<0.05$) in each sampling time. Each value is expressed as the mean number of positive wells ± SE (n = 15).

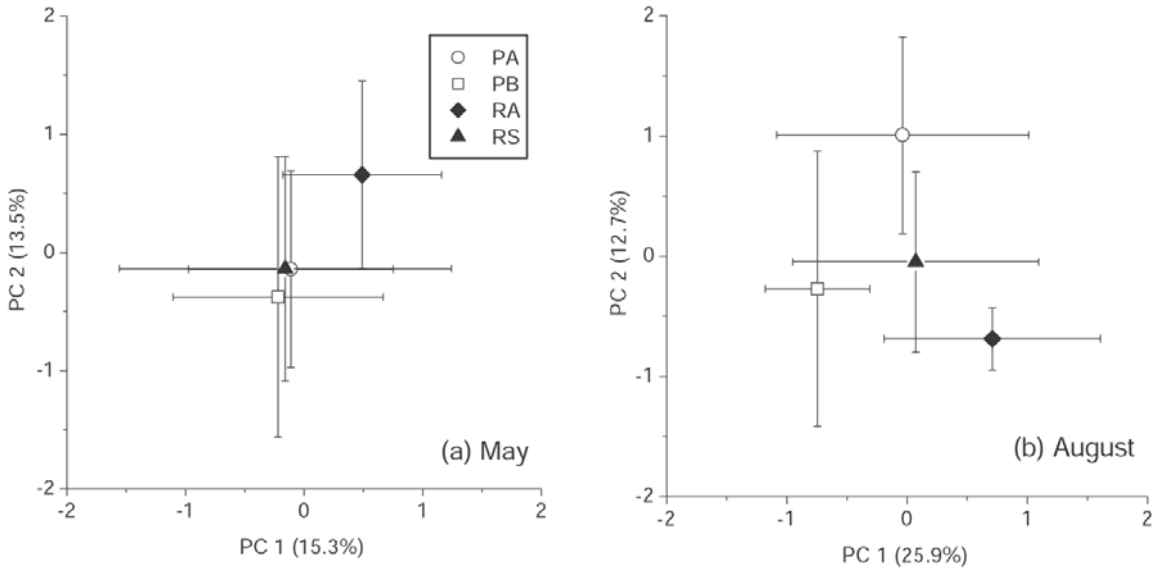

Figure 1

Two-dimensional principal component diagram of C substrate utilization profiles. Data based on AWCD values of ca. 1.0 in May (a) and August (b). Each value represents mean ± SD (n = 15).

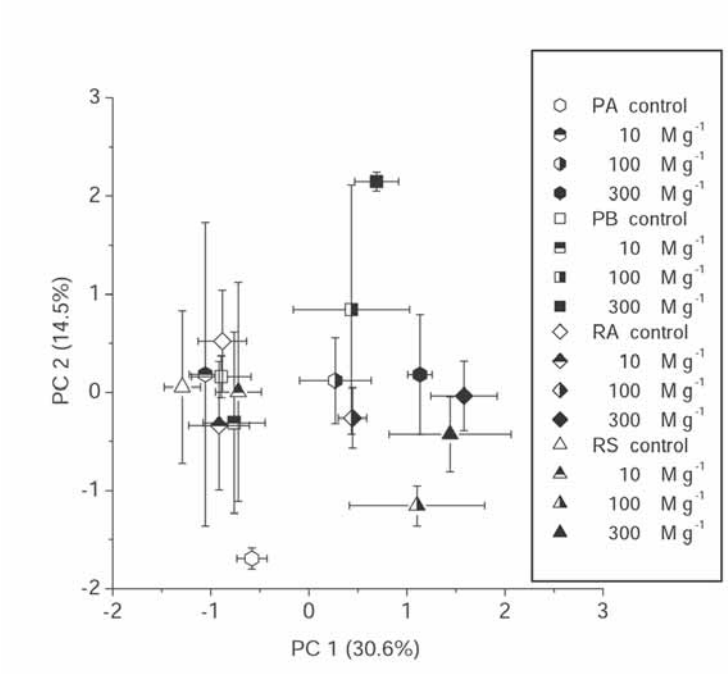

Figure 2

Two-dimensional principal component diagram of C substrate utilization profiles in the treatment of Cu addition. Data based on AWCD values of ca. 0.5. Because the data of 1000 μM g^{-1} Cu treatment did not reach AWCD values of 0.5 within the incubation period, they were excluded. Each value represents mean ± SD (n = 3).

Table IV Ability of C substrate utilization (BIOLOG Ecoplate) by soil microbial community with the treatment of gradual addition of Cu concentrations.

days of incubation	2	4	6
no copper addition (control)			
PA	18.7 ± 1.20 [a]	29.7 ± 0.33 [ab]	30.3 ± 0.33 [ab]
PB	20.3 ± 0.33 [ab]	28.3 ± 0.33 [b]	29.3 ± 0.33 [a]
RA	22.7 ± 0.33 [b]	30.3 ± 0.33 [a]	31.0 ± 0.00 [b]
RS	21.3 ± 0.88 [ab]	30.0 ± 0.58 [ab]	31.0 ± 0.00 [b]
10 μM g^{-1} copper concentration			
PA	19.0 ± 1.00	29.3 ± 0.88	30.7 ± 0.33
PB	19.0 ± 0.58	29.3 ± 0.33	29.7 ± 0.33
RA	21.3 ± 0.88	30.0 ± 0.00	31.0 ± 0.00
RS	21.0 ± 0.58	28.7 ± 1.20	30.0 ± 0.58
100 μM g^{-1} copper concentration			
PA	12.7 ± 0.33	25.7 ± 0.67 [ab]	29.3 ± 0.67 [ab]
PB	12.0 ± 1.16	25.3 ± 0.33 [ab]	28.3 ± 0.67 [b]
RA	11.0 ± 0.58	28.0 ± 1.16 [a]	31.0 ± 0.00 [a]
RS	6.0 ± 0.58	22.0 ± 1.53 [b]	27.7 ± 0.67 [b]
300 μM g^{-1} copper concentration			
PA	7.0 ± 0.58 [a]	20.7 ± 0.33	25.0 ± 0.00 [a]
PB	7.7 ± 0.67 [a]	20.0 ± 2.08	25.3 ± 0.33 [a]
RA	6.3 ± 0.33 [a]	18.7 ± 0.88	24.7 ± 0.33 [ab]
RS	3.0 ± 0.00 [b]	14.0 ± 2.31	22.0 ± 1.16 [b]
1000 μM g^{-1} copper concentration			
PA	2.0 ± 0.00 [a]	7.7 ± 0.67 [ab]	14.7 ± 0.88 [a]
PB	1.3 ± 0.33 [ab]	10.7 ± 1.20 [a]	17.3 ± 0.88 [a]
RA	2.3 ± 0.33 [a]	10.3 ± 0.67 [a]	15.3 ± 0.33 [a]
RS	0.7 ± 0.33 [b]	5.0 ± 0.00 [b]	8.3 ± 0.67 [b]

Values in a column with the same or no letter are not significantly different (Tukey-Kramer test, $P<0.05$) in each sampling time. Each value is expressed as the mean number of positive wells ± SE ($n = 3$).

4. Discussion

Because heavy metals in the soils are present in various forms, we measured both the total concentration (in HNO_3 extraction) and the available fraction (in $CaCl_2$

extraction) to try to clarify the effects of heavy metal pollution on microorganisms. The study site was complexly polluted with heavy metals. The available fraction of heavy metals were at low levels even in the polluted site. This resulted partly from a long aging period, which increased the binding of metals to soil (Van Gestel, 1997) and leached the soluble fractions.

Generally, the soil microbial biomass has been shown to have a high degree of seasonality (Bardgett et al., 1999; Chen et al., 2003a), driven by seasonal fluctuations in environmental condition (e.g. rainfall, temperature and soil moisture) (Chen et al., 2003b). Conversely, little seasonal fluctuations in microbial biomass have been reported (Rogers and Tate, 2001; Blume et al., 2002). In the present study, although soil temperature fluctuated during the study period, small seasonal variations in the size of C_{mic} were found, probably because of a static condition of soil properties as a function of season (Table II).

The soil properties between RA and the polluted sites (PA and PB) were similar except for heavy metals (Tables I and II). Therefore without considering soil properties, we could easily compare them to evaluate the effects of heavy metals. In the literature, the effects of heavy metals on microbial biomass have been shown to be adverse (Kandeler et al., 2000; Klumpp et al., 2003) or idiosyncratic (Kunito et al., 1999; Chander et al., 2001). In the present study, C_{mic} was similar among PA, PB and RA soils. C_{mic} tended to be significantly lower in RS soil, probably because RS soil was composed of much more sand and less silt fractions, resulting in low nutrient, low pH and low moisture properties. Chander et al. (2001) have shown that C_{mic} does not necessarily decrease with increasing heavy metal content, reflecting the importance of other environmental factors, e.g. differences in C input. Because microbial biomass is strongly connected to the soil carbon, we also used C_{mic}/total-C as an indicator of the

heavy metal impact (Table I). C_{mic}/total-C was similar among all sites including RS, indicating no effect of heavy metal pollution due to the low available concentration of heavy metal in the polluted site. However in August, C_{mic}/total-C of RS was significantly higher than the polluted sites. Efficient utilization of carbon by microorganisms would occur in the carbon-restricted condition in RS.

Despite its relative ease of use, the BIOLOG analysis approach is noted to have limitations for studying microbial functional diversity (Pennanen, 2001; Wardle, 2002). Wardle (2002) mentioned that it uses relatively simple substrates while many soil organisms use much more complex ones, and only a small subset of a microbial community can be assessed using this technique. We could not evaluate whole microbial functional diversity. However, BIOLOG analysis has shown changes in functional diversity of soils caused by heavy metal stress in some papers (Knight et al., 1997; Dobler et al., 2000). In the present study, the BIOLOG test separated RA soil from the polluted soils by the number of positive wells 2 days after incubation with water dilution (Table III), indicating higher ability of C substrates utilization in RA soil. The statistical analysis by PCA also supports the difference in microbial functional diversity of RA from the polluted sites in May and August. Because RS soil was similar to the polluted sites, the C substrate utilization of microorganisms in RS and polluted sites seemed to be suppressed compared to RA. In respect to microbial biomass, RS soil showed efficient soil C utilization by microorganisms in August. However, BIOLOG analysis indicated lower C utilization activity of microbial community in RS.

We conducted another BIOLOG test using the gradual addition of Cu concentrations to the soil suspension. Kelly and Tate (1998) have reported that Zn concentrations of >50 mg L^{-1} in the inocula at BIOLOG tests resulted in false positive readings due to production of a precipitate in the well. In the present study, no precipitate was observed

in any well inoculated with solution of 25 mg L^{-1} Cu concentration, corresponding to 1000 μg g^{-1} dry soil. In this way, we could successfully measure the tolerance of the microbial community to Cu stress with the BIOLOG test. The PICT analysis showed that the number of positive wells was lower in RS soil through an incubation period at the high Cu concentration treatments (Table IV). The results suggest that the microbial functional community in RS soil was vulnerable to Cu stress. Conversely, RA soil at high Cu concentration treatments decreased the ability of C substrate utilization but did not fall to the level of the RS soil. RA soil had a more active microbial population compared to RS soil, probably because of its nutrient-rich, high pH and moisture conditions. These physico-chemical properties enhance the biomass and diversity of microorganisms, and the functional resilience of the microbial community to Cu stress. The microbial community of the polluted soil, which has a lower ability for C substrate utilization than RA soil when inoculated with water dilutions, maintained the same C substrate utilization as RA soil at Cu addition treatments. The microbial community in the polluted soil might have developed tolerance to Cu during the long period of heavy metal pollution. In many studies, the tolerance of microbial communities has been considered to be developed under heavy metal exposure (Kelly et al., 1999; Kunito et al., 1999; Rasmussen and Sørensen, 2001). Kelly et al. (1999) have reported that after 420 day-incubation in Zn amended systems, the microbial biomass recovered from a significant decrease at 15 days, although the BIOLOG profile continued to show differences in the structure of the Zn treated and control microbial communities. The PCA showed a change in C substrate utilization patterns in high Cu concentration treatments (Figure 2), suggesting that the active members of the microbial community under Cu stress were different from those under no such stress. At high Cu concentration treatments, the different pattern of C utilization in the polluted soil,

situated on the active side of PC 2, seemed to result from PICT. The substrate D-Cellobiose might be a good indicator to PICT to Cu, which was also suggested by Niklińska et al. (in press).

Kunito et al. (1999) have concluded that Cu tolerance levels of the bacterial community was positively correlated with the soluble and exchangeable Cu (ranging from <0.001 to 12 mg kg^{-1}, extracted with 0.05 M $CaCl_2$ solution) in soil. They used a dilution plate-count technique: a diluted TSB-agar plate was supplemented with Cu at appropriate concentrations, and a decrease of 50 % from the control plate in colony forming units (IC_{50}) was determined. In the present study, we could not investigate the correlation between the available fraction of Cu and PICT due to the similar and low Cu concentrations in $CaCl_2$ extracts in all sites (< 0.03 mg kg^{-1}). However, some tendencies of PICT in heavy metal polluted sites were observed. The BIOLOG plate technique would be more sensitive to detect the community level changes in the environment compared to the dilution plate-count technique, because various C sources in BIOLOG plates can provide extra information. Boivin et al. (2005) have also suggested the advantage of using multiwell-plates to identify PICT for increasing the chance to detect small effects. Pennanen (2001) has indicated that the BIOLOG method reflects more the structure of the bacterial component, not the whole microbial community. We consider the exposure of bacteria to heavy metal at the microscopic level has induced the shift to a tolerant community in the aged polluted site, because bacteria are mainly associated with silt and clay, substrate where heavy metals bind (Kandeler et al., 2000). Further investigation into the microbial community structure, including fungi, will be needed to determine the full PICT of polluted soil.

4. Conclusion

In the heavy metal polluted area, the long aging period increased the binding of heavy metals to soil, and decreased the available fractions. Ambient soil properties affected the soil microbial biomass more than the heavy metal pollution because of low metal bioavailability. However, heavy metal pollution tended to impact on the microbial functional composition: the tolerance of the microbial community to Cu stress in the polluted site was enhanced. Results from this study suggest that if heavy metal pollution occurred in soil with low microbial biomass due to low nutrient, low pH and low moisture, a high ecological risk would be predicted because the inherent microbial community in such an area will be vulnerable to metal pollutants. In spite of the low heavy metal availability due to ageing, the BIOLOG technique could provide sensitive detection of microbial community level changes under PICT analysis.

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Chapter 3

Pollution by heavy metals demonstrates functional redundancy in a soil decomposer community

Kamitani T, N Kaneko, M Hasegawa, R Itoh and H Oba

Abstract: The effects of aged heavy metal pollution on soil macrofauna, microarthropod, and ecological functions were observed on a floodplain in Japan. Geophagous earthworms notably changed in biomass and community structure: low biomass and dominance of moniligastrid worms in the polluted sites, while high biomass and dominance of megascolecid worms in the reference site. In the predator group of macrofauna, Geophilomorpha was found mainly in the polluted sites, while Lithobiomorpha were found in the reference site. Collembolan community structure did not change conspicuously, probably due to low available heavy metal fraction. However, Oribatida was affected for their community structure. The total abundance of microarthropod was larger in the polluted sites, which would result from low toxicity and low earthworm biomass: small adverse effects of ecosystem engineer. Thus, soil fauna was not affected seriously, but complementally replaced in part by other sets of species in the same functional groups in such a moderately polluted site. Analysis of fungal based food web model showed in similar or lower N mineralization rates in the polluted sites compared to reference sites, indicating low N turnover rate in spite of high biomass of microarthropods at the polluted sites. Directly measured ecological function: organic matter decomposition, soil aggregates and primary production was not significantly influenced by heavy metal pollution. These results suggested that the ecological functions have redundancy at the pollution levels of this study, which would be maintained by alternative species in the same functional group.

Keywords: earthworm; ecological function; food web model; heavy metal pollution; microarthropod

1. Introduction

Soil fauna is involved in many aspects of organic matter decomposition, partial regulation of microbial activities, nutrient cycling and crumb structure (Cortet et al., 1999). Soil biodiversity have been recognized to be conserved for the maintenance of such ecological services (Hågvar, 1998). Heavy metals have been found to cause reductions in the abundance and diversity of decomposer animals (Hopkin, 1989; Haimi and Siira-Pietikainen, 1996). The change of communities under soil pollution results from several reasons: differential sensitivity among species, the priorities in life processes set by organisms, and indirect interactions acting through food-web or via physical changes in the habitat (Van Straalen, 2004). In the field, the impacts of soil pollution can be studied by analysis of species composition, relative abundance and biodiversity of soil invertebrate communities, in comparison with those in unpolluted biotopes. In functional respect, decrease in such ecological functional performance can be also evaluated as the impacts of soil pollution, because ecological functions of a soil are one of vulnerable functions to pollution (Van Straalen, 2002).

Soil organisms are often classified to three functional categories, micrograzer in microfood webs, litter transformer and ecosystem engineer (Lavelle et al, 1997; Wardle, 2002). Microfood webs involve nematodes, protozoa, and those animals that directly feed on them and each other. Litter transformers convert organic matter into fecal pellets, including saprophagous microarthropods and some macrofauna. Collembola and oribatid mites are the most abundant animals in the soil-litter subsystem, belonging to micrograzers and litter transformers. These microarthropods represent a wide range of feeding habits and contribute to the decomposition process, but they were often

categorized to fungivores in food web models (Hunt et al., 1987; de Ruiter et al., 1993a). Although several field studies concerning the effects of heavy metal pollution on Collembola and oribatid mites have been done in Europe (e.g. Bruus Pedersen et al., 1999; Zaitsev and van Straalen, 2001), no work was conducted in Japan. Earthworms play important roles in soil ecosystems, and they are often categorized to ecosystem engineer for their performance of excavation of burrows and production of casts (Lee, 1985; Edwards and Bohlen, 1996). The subjects of previous ecotoxicological studies about earthworms have converged on lumbricid earthworms, predominantly in Europe (Römbke et al. 2005b). Earthworms dwelling in Japan are mostly the families of Megascolecidae (69% of species), Lumbricidae (14%) and Moniligastridae (10%) (Blakemore, 2003). Knowledge of the effects of soil pollution on such important species is needed for ecological risk assessment in Japan.

The objective of the present study was to increase understanding of the effects of heavy metal pollution on soil fauna and ecological function at field conditions. We focused on the dynamics of earthworms and microarthropods because of their importance for ecological functions in soil.

2. Material and methods

2.1. Field site

The study area is at Watarase retarding basin on Watarase River in Tochigi, central Japan. This field is complicatedly contaminated with heavy metals caused by mining activity from Ashio Copper Mine at the headwaters (Kamitani et al., in press). Since

1877, the mining activity rapidly enlarged and contamination from the mine caused severe damage to water and paddy fields n the Watarase River basin (Morishita, 1981). Efforts have been made to improve the water quality from the mine until it was closed in 1973.

The study area is dominated by tall grasses (reed; *Phragmites australis* and *Miscanthus sacchariflorus*). The reeds are used for making traditional blinds, and once a year in March, the grasses are burned off to ensure high quality reeds and pest control. Soil was sampled from two polluted sites (PA and PB, at the distance within 20 m) and two non polluted sites (RA and RS, at the distance within 50 m), each site measured 10 m wide by 30 m long. Physico-chemical soil properties are documented in Chapter 2 of this thesis (Kamitani et al., in press). The soil properties between RA and the polluted sites (PA and PB) were similar except for heavy metals. The available fractions of heavy metals were at low levels even in the polluted site due to aging, which increased the binding of metals to soil (Van Gestel, 1997) and leached the soluble fractions.

2.2. Soil fauna sampling

Soil macrofauna was sampled at three times: April, August and November in 2003, except for earthworm which was sampled at every month from April to November. For macrofauna collection, five soil cores (15.5 cm diameter, 20 cm deep) were sampled at random in each site. The soil was separated into 0-5 cm and 5-20 cm deep. Macrofauna in 0-5 cm soil was hand-sorted in situ, followed by extraction from soil using Tullgren apparatus for three days. Macrofauna in 5-20 cm was hand-sorted in situ. Earthworms were only hand-sorted. All specimens were preserved in 80 % ethyl alcohol, identified

at order or class level using a stereomicroscope, and weighed. Soil microarthropods were also sampled at three times: May, August and November in 2003. For microarthropod collection, 20 soil cores (25cm^2, 4 cm deep) were sampled at each site. Microarthropods (collembolan and oribatid mites) were extracted in picric acid dilution by Macfadyen apparatus for 6 days. Specimens were preserved in 80 % ethyl alcohol, put on glass slide, and then identified to species level using an optical microscope.

2.3. Food web model

We studied nitrogen (N) mineralization rate by modeling the community food web. The model derives N mineralization by organisms by splitting the rate at which organisms take material from an energy resource into a rate at which feces or prey remains are added to detritus, a rate at which elements are incorporated into biomass, and a rate at which elements are released by organisms as inorganic compounds (Hunt et al., 1987). We did not study soil fauna entirely: especially lacking in microfauna (e.g. nematodes, protozoa, enchytraeids) which belongs mainly to bacterivores and omnivores, therefore we constructed only fungal-based food web model including microarthropods and their predators (Fig. 1). The description of the food web model is given in Appendix 1.

We need the average annual population size B_j, expressed as biomass C of a group j, and some physiological parameters: the specific death rate d_j; the loss of biomass due to predation M_j; assimilation efficiency a_j; production efficiency p_j. Biomass of functional groups were the average of three study seasons in the top 5 cm deep. Soil

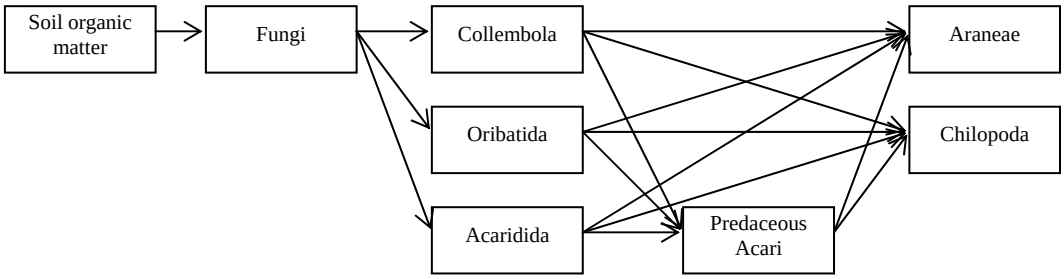

Fig. 1 Diagram of the soil food web composed of microarthropods. Boxes represent the functional groups of organisms, the vectors the flow of energy or nutrient between them.

organic matter and biomass of fungi in top 5 cm soil in each study site were estimated based on the data after Kamitani et al. (in press, chapter 2). The fungal fraction of microbial C was calculated with fungi/bacteria weight ratio 0.056 after de Ruiter et al. (1993), the values at an integrated winter wheat field. Biomass of Collembola was calculated by multiplying estimated individual number by typical average individual weight 2.7 µg (Petersen and Luxton, 1982). Biomass of predaceous mites (Gamasida and Actinedida) and Acaridida were calculated by multiplying estimated individual number by estimated average individual weights derived in Chapter 6: 5.5 µg, 2.9 µg and 0.69 µg, respectively. The length and width of oribatid mite were measured and the corresponding dry weight was calculated based on regression equation of length and width on dry weight (Engelmann, 1961):

$log\ w = 1.32 \times log\ (L \times W) - 5.87$

where, w was the weight of an oribatid mite (µg), L and W were length and width of the oribatid mite (µm), respectively.

For Chilopoda (Geophilomorpha and Lithobiomorpha) and Araneae, we chose a correction factor of 0.3 for the conversion of estimated fresh weight to the dry weight. Conversion to biomass C was based on a dry weight C content of 47.7% for Collembola

and Acari, 50.6% for Araneae (Berg et al., 2001), and 57.0% for Chilopoda (Pokarzhevskii et al., 2003). The values for the physiological parameters were chosen mainly from the parameters listed by Berg et al. (2001) (Table 1). Physiological parameter values for Chilopoda were assumed to be equal to the values used for Araneae.

Table1 Physiological parameters values for the functional groups in the food web (mainly after Berg et al. 2001; Pokarzhevskii et al. 2003). d_j: death rate; a_j: assimilation efficiency; p_j: production efficiency.

Functional groups	d_j (yr^{-1})	a_j	p_j	C/N_j ratio
Soil organic matter	0	1	1	13.4/14.1/13.5/11.9[a]
Fungi	0.63	1	0.45	10
Collembola	1.37	0.5	0.35	8
Oribatida	0.89	0.5	0.4	8
Acaridida	0.89	0.5	0.4	8
Predaceous Acari	1.37	0.6	0.35	8
Chilopoda	0.74	0.6	0.35	4.9
Araneae	0.74	0.6	0.35	4.2

[a] the values refer to the C/N ratio of the study sites: PA, PB, RA and RS, respectively.

2.4. Soil ecological functions

To determine the potential of cellulose decomposition in the study soils, Whatman Benchcote sheet (Whatman Ltd., Brentford, Middlesex, UK), which is a polyethylene backed absorbent cellulose paper, was used. Before burying, the papers were dried in a desiccator and weighted. Five replicate plots were assessed in each site, and five papers were buried at 5 cm depth in each plot in April. One paper was removed from each plot at 28, 63, 91, 111 and 146 days. The papers were dried (18h at 60^{o}C) after loose soil

particles and debris had been removed. The final weight of paper was determined as a weight loss after ignition (1.5 h at 750 ^{o}C) of the dried paper.

The cellulose decomposition rate was expressed by the percentage of dry weight of cellulose after an incubated period prior to burial, as follows:

% weight = $(w_0 - w_t)/(w_0 - w_p)$

where, w_0 and w_t were the weight of the benchcote sheet at time 0 and t, respectively. w_p is the weight of polyethylene, which is 21 % of the benchcote sheet weight. The partial loss of back polyethylene in soil was regarded as the effect of feeding activity of megafauna, therefore we subtracted the weight of lost area from each parameter to avoid the activity of megafauna.

To evaluate the difference of soil structure among sites, the amounts of soil aggregates were determined. Five soil samples were taken from the top soil (5 cm deep) of all four sites. A wet-aggregate size distribution was determined with a nest of sieves with openings of 2.00, 1.00, 0.50, 0.25, and 0.10 mm, raised and lowered 2 cm through the water 32 times per min for 40 min (Nimmo and Perkins, 2002). Mean weight diameter (MWD) was represented as follows:

$$\text{MWD} = \sum_{i=1}^{n} \bar{x}_i w_i$$

where $\bar{x}_i$ is the mean diameter of each size fraction and w_i is the proportion of total sample weight occurring in the corresponding size fraction. The percent effective aggregate (EA) was represented as follows, based on aggregate stability (Nimmo and Perkins, 2002):

EA (%) = $100 \times (w_a - w_s)/w_{sample}$

where w_a is the weight of aggregates including sand particles > 0.25 mm, w_s is that of sand > 0.25 mm diameter and w_{sample} is that of soil sample.

2.5. Statistical Analyses

Mann-Whitney U tests were carried out for the comparison of densities of macrofauna in each season. The Shannon-Wiener index was calculated with respect to the species data of microarthropods (Collembola and oribatid mites). One-way ANOVA was conducted for MWD and EA in each season and cellulose decomposition, and post hoc test (Tukey-Kramer) was performed using Statview for Windows Version 5.0.

3. Results

3.1. Macrofauna

Macrofauna communities comprised 12 morphotypes (Table 2). The density showed high seasonal fluctuations and variability in the same site. Ants (Formicidae) showed heterogeneous and concentrated distribution in all sites. Hemiptera (mostly aphids and scale insects) showed high numbers in all seasons, especially in PA soil, and clustered around plant root. Predators consisted of spiders (Araneae) and centipedes (Lithobiomorpha and Geophilomorpha). Lithobiomorpha was found mainly in RA soil in August and November, while Geophilomorpha was found in the polluted sites in April and August. Adults of Coleoptera were less abundant in RS in all season. Milllipedes (Diplopoda) seemed to decrease in the polluted sites in April and August.

Earthworms (Haplotaxida), millipedes and coleopteran larvae dominated the macrofauna biomass (Fig. 2). The biomass was higher in RA due to a large number of

earthworms observed in April, and due to millipedes and Coleoptera (mostly scarab) in August. Earthworm community composition was different between polluted and reference sites (Fig. 3). In polluted sites, earthworms consisted of two families (Megascolecidae and Moniligastridae) and Moniligastridae dominated the biomass, while three families (Megascolecidae, Moniligastridae and Lumbricidae) were found in reference sites. and Megascolecidae dominated in biomass. The RA showed higher earthworm biomass through the study period.

Table 2. Density (individuals /m^2) of macrofauna in the study sites, in April (a), August (b) and November (c). Each value in the table is the average number of animals (± SE) in 5 repricates. Values in a row with the same or no letter are not significantly different (Mann-Whitney U test, $P<0.05$). A between parentheses means adult individuals and L between parentheses means larvae individuals.

(a) April	PA	PB	RA	RS
Araneae	33.9 (13.8)	22.6 (22.6)	67.8 (21.1)	45.2 (21.1)
Lithobiomorpha	0.0 (0.0)	11.3 (11.3)	0.0 (0.0)	0.0 (0.0)
Geophilomorpha	79.1 (42.3)	45.2 (32.9)	0.0 (0.0)	0.0 (0.0)
Symphyla	802.3 (142.3) [a]	542.4 (65.9) [ab]	293.8 (80.7) [bc]	146.9 (72.8) [c]
Dermaptera	0.0 (0.0)	0.0 (0.0)	11.3 (11.3)	0.0 (0.0)
Coleoptera (A)	960.5 (96.2) [a]	802.3 (209.4) [a]	881.4 (205.0) [a]	180.8 (82.6) [b]
Coleoptera (L)	316.4 (137.9)	440.7 (146.7)	158.2 (65.4)	271.2 (104.8)
Hemiptera	2689.3 (848.7) [a]	282.5 (81.9) [b]	169.5 (56.5) [bc]	22.6 (13.8) [c]
Diptera (L)	678.0 (102.6) [a]	395.5 (113.0) [ab]	192.1 (38.3) [b]	339.0 (105.7) [ab]
Diplopoda	158.2 (90.0) [a]	169.5 (107.2) [a]	757.1 (144.7) [b]	463.3 (271.8) [ab]
Haplotaxida	45.2 (32.9) [ab]	67.8 (27.7) [ab]	180.8 (57.6) [a]	33.9 (13.8) [b]
Hymenoptera	101.7 (101.7) [a]	33.9 (22.6) [a]	553.7 (225.6) [b]	4339.0 (4296.7) [ab]

(continued on next page)

(b) August	PA	PB	RA	RS
Araneae	316.4 (124.6) [a]	79.1 (38.3) [bc]	169.5 (50.5) [ab]	45.2 (21.1) [c]
Lithobiomorpha	79.1 (28.8)	33.9 (13.8)	248.6 (115.2)	67.8 (32.9)
Geophilomorpha	79.1 (28.8) [a]	90.4 (49.3) [a]	0.0 (0.0) [b]	11.3 (11.3) [ab]
Symphyla	937.9 (211.1) [a]	440.7 (88.3) [b]	824.9 (184.5) [ab]	598.9 (86.8) [ab]
Dermaptera	11.3 (11.3)	45.2 (21.1)	0.0 (0.0)	0.0 (0.0)
Coleoptera (A)	678.0 (59.3) [a]	576.3 (82.6) [ab]	610.2 (203.2) [ab]	316.4 (88.6) [b]
Coleoptera (L)	598.9 (206.5) [ab]	576.3 (100.1) [a]	1005.6 (86.4) [b]	1209.0 (526.8) [ab]
Hemiptera	8508.5 (2207.2) [a]	293.8 (180.8) [b]	1491.5 (258.0) [ac]	1378.5 (585.7) [bc]
Diptera (L)	305.1 (124.6) [ab]	508.5 (123.8) [a]	339.0 (128.8) [ab]	180.8 (60.3) [b]
Diplopoda	519.8 (212.4)	565.0 (187.4)	1288.1 (572.7)	1333.3 (430.5)
Haplotaxida	56.5 (30.9)	45.2 (21.1)	90.4 (65.9)	90.4 (22.6)
Hymenoptera	113.0 (73.79) [a]	1028.2 (636.5) [b]	1581.9 (754.2) [b]	305.1 (105.1) [ab]

(c) November	PA	PB	RA	RS
Araneae	90.4 (28.8)	180.8 (32.9)	305.1 (142.5)	101.7 (37.5)
Lithobiomorpha	22.6 (13.8) [a]	22.6 (13.8) [a]	226.0 (114.4) [b]	33.9 (33.9) [ab]
Geophilomorpha	33.9 (13.8)	22.6 (22.6)	0.0 (0.0)	11.3 (11.3)
Symphyla	418.1 (97.2)	226.0 (114.4)	316.4 (109.6)	192.1 (72.8)
Dermaptera	0.0 (0.0)	22.6 (13.8)	0.0 (0.0)	11.3 (11.3)
Coleoptera (A)	644.1 (136.8) [a]	632.8 (231.8) [a]	350.3 (159.2) [ab]	146.9 (28.8) [b]
Coleoptera (L)	135.6 (52.4)	22.6 (13.8)	90.4 (52.4)	113.0 (47.3)
Hemiptera	2011.3 (763.2)	1163.8 (352.6)	3073.4 (1438.8)	1706.2 (652.1)
Diptera (L)	316.4 (128.3) [ab]	700.6 (294.4) [a]	237.3 (74.5) [ab]	237.3 (45.2) [b]
Diplopoda	734.5 (216.6)	305.1 (58.2)	429.4 (72.8)	542.4 (102.0)
Haplotaxida	56.5 (17.9)	33.9 (22.6)	56.5 (43.8)	56.5 (25.3)
Hymenoptera	79.1 (28.8)	146.9 (81.1)	372.9 (289.0)	135.6 (58.2)

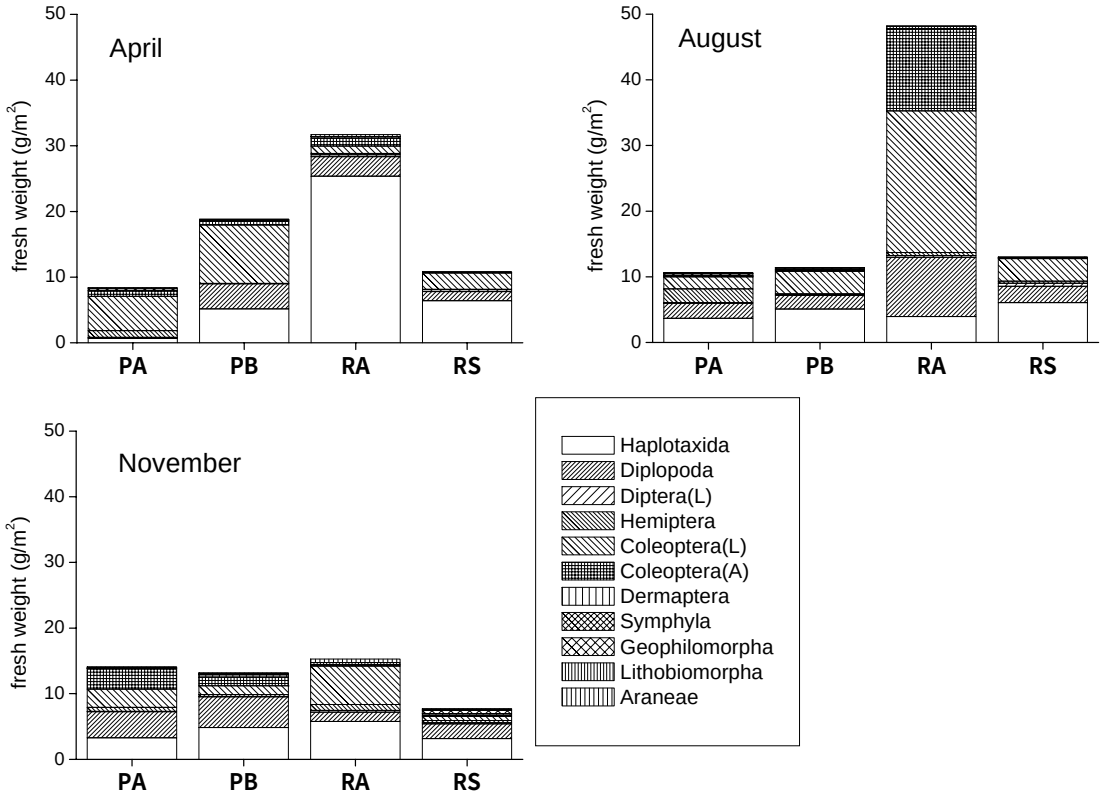

Fig. 2. Comparison of total biomass of macrofauna among study sites in each season

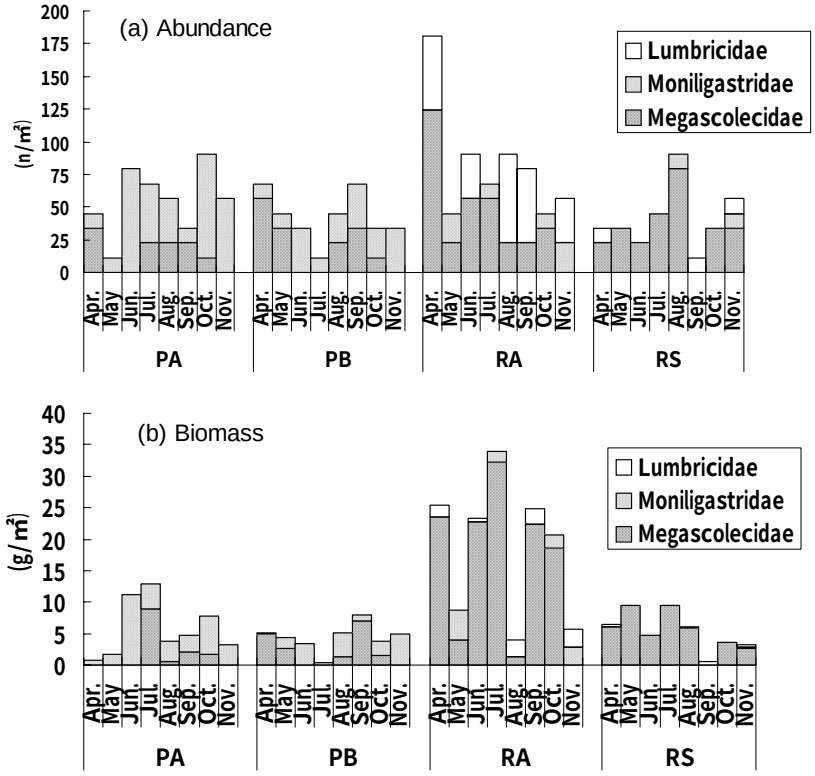

Fig. 3. Number and biomass of the family of earthworms in the study sites in each month.

3.2. Microarthropods

The species of Collembola found at the field sites were listed in Appendix 2. Total number of Collembola was higher in the polluted sites than in the reference sites in May (Table 3). RA showed lower densities compared to the polluted sites in all seasons. Collembolan species diversity, quantified as Shannon-Wiener index, was slightly lower in the polluted sites (Table 3). The relative annual average abundance curve of collembolan community was presented in Fig. 4(a). The top three species, including *Isotomiella minor* and *Onychiurus yodai*, made up more than 50% of collembolan community in PA, PB and RA.

The species of oribatid mites and groups of other mites found at the field sites are listed in Appendix 3. PA had the highest total density of oribatid mites in comparison with the others (Table 3). The lowest density was observed in the reference sites on all three sampling times. The Shannon-Wiener index was slightly higher in RA, despite the fact that the numbers of species were not the largest at all sampling months (Table 3). The relative annual average abundance curve of oribatid mite community is presented in Fig. 4(b). *Ischeloribates lanceolalus* and *Oppiella nova* were included in the top three species at all sites. Some species, *Cosmohermannia frondosa*, *Masthermannia hirsute* and *Nippohermannia* sp., which are all members of the same family, Nanhermanniidae, tended to be found in larger numbers in the polluted sites compared to the reference sites.

Table 3. Density (individual m^{-2}) of microarthropods found in the study sites. Each value in the table is the average number in 20 samples (19 samples at RA in May of oribatid mite), number of species, and Shannon-Wiener index (nat).

	May				August				November			
	PA	PB	RA	RS	PA	PB	RA	RS	PA	PB	RA	RS
Collembola												
Total no. individuals (No. per m^2)	8,080	5,800	3,500	2,780	7,740	6,020	4,760	6,360	4,040	3,940	2,360	5,020
No. of species	17	15	16	13	18	19	15	19	14	17	15	17
Shannon-Wiener index	1.95	1.85	1.99	2.07	2.03	2.23	2.25	2.40	1.86	1.77	2.18	1.90
Oribatid mite (adult)												
Total no. individuals (No. per m^2)	8,420	6,520	4,253	5,000	9,660	7,760	8,400	3,760	13,220	6,480	4,540	6,940
No. of species	19	22	23	26	25	26	33	24	23	20	24	19
Shannon-Wiener index	2.33	2.21	2.53	2.17	2.64	2.72	2.73	2.56	2.22	2.47	2.56	2.28

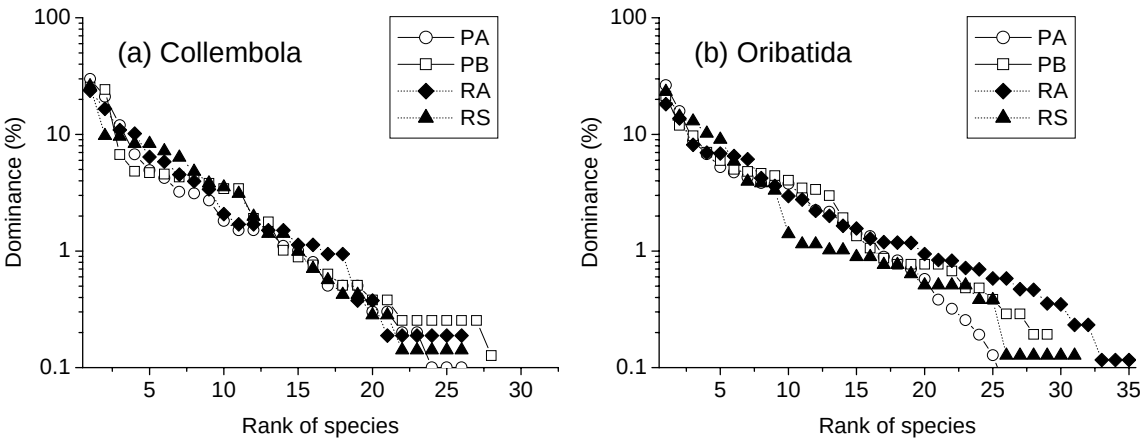

Fig. 4 Relative abundance of Collembola (a) and Oribatida (b) species expressed as a percent of the annual mean abundance.

3.3. Model estimates of N mineralization

The biomass estimates for functional groups for the four study sites are given in Table 4. All groups of organisms listed in Fig. 1 were present in each site. However, food webs differed in their structure quantitatively. The fungal biomass was larger in the polluted sites compared to the reference sites, resulting in a large biomass of

microarthropods in the polluted sites. Simulated N mineralization rates through the fungal based food webs showed no remarkable difference between PA and RA, but were larger in RS and smaller in PB (Table 5). The amount of N mineralized by fungi was approximately more than 70 % of the total mineralization at each site.

Table 4 Average annual biomass estimates (mg C/m^2) for the functional groups in the food webs.

Functional groups	PA	PB	RA	RB
Soil organic matter	1092000	1125000	1057500	770000
Fungi	871.2	826.4	744.5	676.4
Collembola	8.5	6.8	4.6	6.1
Oribatida	38.2	42.8	19.2	11.9
Acaridida	0.03	0.08	0.02	0.04
Predaceous Acari	4.6	3.2	4.8	4.9
Chilopoda	43.4	37.5	35.8	40.2
Araneae	6.3	7.6	13.6	5.7

Table 5 Simulated N mineralization rates (mg N/m^2/yr) for the food web.

	PA	PB	RA	RB
Fungi	141	108	142	192
Collembola	9.6	6.2	10.7	18.8
Oribatida	31.2	28.1	33.9	28.0
Acaridida	0.02	0.05	0.03	0.09
Predaceous Acari	5.1	3.3	8.2	9.5
Chilopoda	4.9	4.2	4.0	4.5
Araneae	0.5	0.7	1.2	0.5
Total	192	150	200	253

3.4. Soil ecological functions

The time course of cellulose decomposition in the polluted and reference sites

gradually increased for 63 days (Fig. 5). The mean weight of remaining cellulose reached less than 10 % in the reference sites after 63 days, while 16.3% in PA and 31.3% in PB, although no significant difference was observed. A high variability was found in the polluted sites, which reduces the probability of determining significant differences by statistical evaluation of the data.

Mean weight diameter of aggregate was not different among sites (Table 6). However, the effective aggregate size, from which large sand particle were excluded for estimation of meaningful aggregate stability, was significantly lower in RS soil compared to other sites (Table 6).

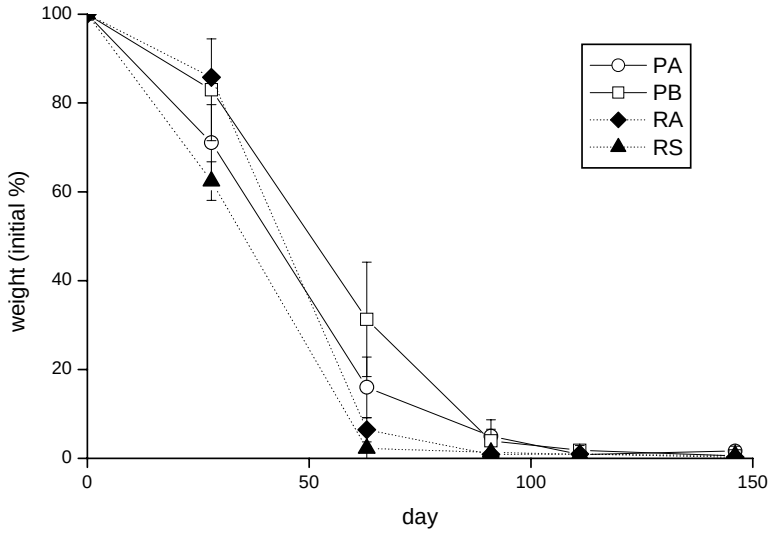

Fig. 5. The remaining weight of cellulose buried in the soil over time. Values are not significantly different (P>0.05). Each value represents mean ± SE.

Table 6. Aggregate size distribution. Values in a column with the same or no letter are not significantly different (Tukey-Kramer, $P<0.05$) in each sampling month. Each value represents mean of 5 replicates. MWD: mean weight diameter, EA: effective aggregate.

	May		August		November	
	MWD (g mm)	EA (%)	MWD (g mm)	EA (%)	MWD (g mm)	EA (%)
PA	1.67	81.6[a]	2.02	88.0[a]	1.92	86.1[a]
PB	1.68	80.6[a]	1.87	87.2[a]	1.75	84.1[a]
RA	1.82	81.0[a]	1.87	83.2[ab]	1.49	82.7[a]
RB	1.36	59.6[b]	1.70	72.9[b]	1.58	71.7[b]

4. Discussion

4.1. Macrofauna

In the present study, the soil Cu, Zn and Pb total concentrations were much higher at the polluted sites than at the reference site RA, while the available heavy metal concentrations in $CaCl_2$ extraction were similar in all sites (Kamitani et al., in press, Chapter 2). Heavy metal pollution drastically affected macrofauna biomass, especially earthworms (Fig. 3). Because the collected earthworms were found in mineral soil horizon and mainly fed on soil, we can regard them as endogeic species (Kamitani and Kaneko, in press a, chapter 4). The high biomass of earthworms in RA resulted from the predominance of megascolecid worms, which had large body as adult compared to Moniligastridae and Lumbricidae. Kamitani and Kaneko (in press a) have shown that Moniligastridae species could accumulate heavy metals higher than the other families. The difference of metal accumulation strategies might affect the distribution of these species. Nahmani and Lavelle (2002) have observed a decrease in earthworm density and increase in coleopteran larvae density with increase in Zn concentrations (more than

330 mg kg^{-1}). They supposed that the organic layer, which was formed by the decrease in decomposition rate due to depression of earthworm and/or microorganism activities in polluted soil, protected coleopteran larvae from the cold in winter. In the present study, coleopteran larval density did not notably change among sites and large seasonal variability was observed in biomass. No organic matter layer and litter transformer in the study site would not allow for the effect suggested by Nahmani and Lavelle (2002). Among the saprophagous macrofauna, Isopoda were not observed, probably due to the lack of litter layer, while millipedes (Diplopoda) were one of the dominant invertebrates in the study site. Large number of edaphic scale insects was found in PA, probably because the amount of *M. sacchariflorus* fine root was larger in PA compared to RA (Oba et al., unpublished data). Migliorini et al. (2004) have suggested that Symphyla were sensitive to heavy metal pollution in their study site of a shooting range (Pb > 1576 mg g^{-1}). In the present study, no adverse effects were observed on Symphyla, probably due to moderate contamination level.

Predators are generally exposed to soil toxicants mainly through their diet. In the category of predators, Lithobiomorpha occurred in RA while Geophilomorpha were found in the polluted site. These species have a different way of life: the former was hemi-edaphic, the latter was endogeneous (Grelle et al. 2000), the palatable foods might be different. Blackburn et al. (2002) have shown that the Geophilomorpha were markedly synanthropic whereas the Lithobiomorpha were not. In the present study, Lithobiomorpha seemed to be sensitive to heavy metals, showing that they can be considered useful indicators among the predatory group.

4.2. Microarthropods

In the present study, an increase of microarthropod abundance at the polluted sites was observed. Bruus Pedersen et al. (1999) have suggested that high microarthropod abundance appeared at intermediate Cu concentrations (300-1300 mg Cu/kg). However, the effects of ecosystem engineers like earthworms should be taken into account for analysis of microarthropod abundance. Irrespective of heavy metal pollution level, the density of microarthropods showed a positive relationship with the biomass of earthworms (Fig. 6). Earthworms might disturb the habitat for microarthropods, and feed on soil including the egg, larva and even adult individual of microarthropods, affecting the abundance of them.

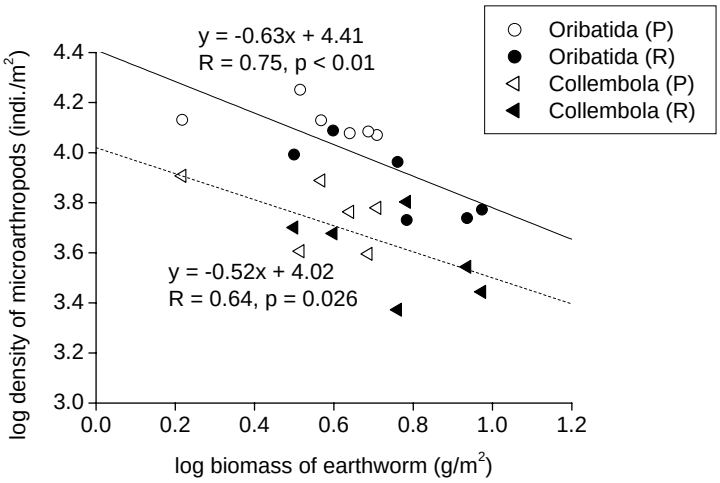

Fig. 6 The relation between abundance of microarthropods and biomass of earthworm. P between parentheses means microarthropods in the polluted sites and R between parentheses means microarthropods in the reference sites.

Bruus Pedersen et al. (1999) have shown that the Shannon-Wiener index of collembolan biodiversity decreased linearly with increasing soil copper concentrations, which agrees with our result that collembolan species diversity decreased slightly in the

polluted sites. However, a notable change in collembolan species composition was not observed. Collembola can store heavy metals in midgut intracellular mineral concretions that are eliminated at each moulting interval by intestinal exfoliation (Fountain and Hopkin, 2001). As a representation of the bioavailable amount of heavy metal in soil, Peijnenburg and Jager (2003) have identified six types of extractions: weak salt, reductive extractions, weak acid, chelate agents, dilute strong acid and combined extracts. Lock et al. (2003) have suggested that $CaCl_2$ extracted fraction might be a good indicator of bioavailable heavy metal (Zn) for Collembola. Van Gestel and Hensbergen (1997) have presented estimates of EC_{50} for reproduction of *Folsomia candida* as 14 mg $CaCl_2$-extractive Zn/kg. In the present study, the moderate heavy metal concentration and low $CaCl_2$ extracted fractions even in the polluted sites, would be smaller than the tolerance level of Collembola.

A comparison of sensitivity on the basis of sublethal effects has shown that oribatid mites were more sensitive to heavy metal than collembola (Crommentuijn et al., 1995). In the vicinity of a metallurgical factory in Russia, Van Straalen et al. (2001) have studied heavy metal concentrations in soil invertebrates and suggested that oribatid mites showed higher internal concentration than Collembola. Their study site has showed low bioavailability of pollution just as our sites, and the community structure and species diversity of oribatid mite were not seriously affected by metal contamination (Zaitsev and van Straalen, 2001). In the present study, an effect of heavy metal pollution on oribatid mite communities was not revealed, however, some species in family Nanhermanniidae, *C. frondosa*, *M. hirsute* and *Nippohermannia* sp. tended to be found in larger number on the polluted sites compared to the reference sites. Zaitsev and van Straalen (2001) have indicated that microphytophagous species, feeding

exclusively on fungi, accumulated Zn in higher concentrations. Prinzing et al. (2002) have also argued that species with short life cycles and species feeding on fresh macrophyte detritus might have more tolerance to pesticide than the species feeding on fungal microphytes. Siepel (1995) showed that life history strategies were valuable for assessing the impact of heavy metals on oribatids: the proportion of thelytokous species increased after disturbance. Further works for life cycle and feeding habitat of the species in the family Nanhermanniidae would be needed to recognize their specific distribution in the polluted sites.

4.3. Food web structures and N mineralization

To connect community structure to ecosystem processes, we conducted food web model analysis, regarding each functional group in soil food webs as a component in the cycling of nutrients. Heavy metal pollution might affect the biomass of the fungi and bacteria because bacteria are mainly associated with silt and clay, substrate where heavy metals tend to bind (Kandeler et al., 2000). In the present study area, high fungi/bacteria ratio was observed in the polluted site compared to the reference site (Oba et al., unpublished data). Therefore the biomass of the fungal and bacterial energy channels may also change. We constructed only fungal-based food web model including microarthropods and their predators (Fig. 1). The estimated fungal biomass were the same order to those in the wheat field conducted by de Ruiter et al. (1993), and larger in the polluted sites. Fungivorous microarthropods, especially Oribatida, also showed larger biomass in the polluted sites, because of large abundance and dominance of large size species (the family Nanhermanniidae). In spite of the quantitative difference in

food web structure, N mineralization rates did not notably differ among sites. The higher feeding rate of predaceous Acari in the reference sites supposed to be enhanced the feeding activity of fangivorous microarthropods and high turnover of fungi, resulting in the same N mineralization level to the polluted sites in which large biomass of fungi and fungivorous fauna were estimated. Thus, the fungal based food web model analysis indicated that the change in food web structure between polluted and reference sites does not affect the total amount of nutrient cycling.

In the present study, the bacterial channel including microfauna was not considered in food web diagram. Hunt et al. (1987) have indicated that bacteria are estimated to mineralize most of the N in their food web model analysis. Among soil fauna, amoebae and bacterivorous nematodes have been recognized as the most important contributors to N mineralization (Hunt et al. 1987; de Ruiter et al., 1993a). Earthworms may also act as a key factor for N cycling due to their large biomass. Although earthworms are often treated as detritivores in soil food web models (de Ruiter et al. 1993b), earthworms would consume communities of the lower levels in detrital food webs as a whole (Pokarzhevskii et al., 2003), and may preferentially feed microbes, which has lower body C/N ratio than detritus (Whalen and Parmelee, 1999). Together with the complete data set including bacterial channel, accurate biomass and physiological parameter values will improve and complete the performance of food web model (Berg et al. 2001).

4.4. Soil ecological functions

In addition to the simulated food web, we directly measured the effect of soil biota

on ecological functions: organic matter decomposition and soil structure. The cellulose decomposition rates tended to be slower in the polluted site than in the reference site, but not significant (Fig. 5). Chew et al. (2001) have showed that the metal-contaminated (Pb, Cu and Zn) soils had lower cellulose decomposition rates than uncontaminated soils by using a cotton strip assay. However, they have also proved that higher CEC, readily oxidisable material and loss on ignition values were the major factors in higher decomposition rates. In the present study, conversely, RS soil which was characterized by low CEC, total C and N, LOI, and especially low C_{mic} (Kamitani et al., in press, Chapter 2) had the same decomposition rate as other sites. We inserted cellulose paper directly in the soil without any protection, allowing for direct contact with the soil and unlimited access to all soil organisms. Therefore the decomposition of organic matter which is the most integrating process within the soil ecosystem was not affected significantly by heavy metal pollution in situ.

Soil aggregation has a great influence on the physical characteristics of the soil. In the present study, the mean weight diameter of water stable aggregates did not significantly change between the polluted and reference sites (Table 6). However, the rate of effective aggregates was significantly small in RS soil due to large fraction of coarse sand particles. Thus, the aggregation size distribution and stability were not affected by heavy metal pollution. Earthworm activity has been supposed to affect significantly on the distribution of water stable aggregates, resulting in a high proportion of large macroaggregates (Bossuyt et al., 2005). Heavy metal pollution may caused disappearance of earthworms, resulting in the decrease of aggregates. In the present study, the presence of earthworm in the polluted sites would maintain the soil aggregate structure, although the earthworm community structure was changed. We did

not estimate the quality of aggregate structure. Jongmans et al. (2003) have suggested that angular and prismatic aggregates were formed in soil devoid of earthworm due to heavy metal pollution, while granular and subangular blocky aggregates were formed in earthworm present soil. To estimate the possibility of aggregate as an indicator of soil heavy metal pollution, further works including the shape of aggregates and the physicochemical effects of heavy metal on cohesion of soil particles were needed.

Plant productivity had been also studied in PA and RA. Although the individual shoot biomass and shoot/root ratio of *M. sacchariflorus* were lower in the polluted site, primary production of plants did not show significant difference among the sites (Oba et al., unpublished data). The low shoot/root ratio of *M. sacchariflorus* suggests that the individual plants at the polluted site allocated more energy to stock and root. The primary production in the polluted site would be sustained by whole soil ecosystems, including soil biota and soil ecological processes.

5. Conclusion

We compared soil fauna in the polluted sites with the reference site of similar soil properties. Geophagous earthworms notably changed in biomass and community structure: low biomass and dominance of monilligastrid worms in the polluted sites, while a high biomass and dominance of megascolecid worms was observed in the reference site. In predator group of macrofauna, Geophilomorpha was found mainly in the polluted sites, while Lithobiomorpha dominated in the reference site. Collembolan community structure did not change conspicuously, probably due to low available heavy metal fraction. However, Oribatida would be affected for their community structure:

large numbers in a certain family at the polluted site. The total abundance of microarthropods was larger in the polluted sites, which partly results from the low earthworm biomass and small adverse effects of this ecosystem engineer. Thus, soil fauna was not affected seriously, but complementally replaced in part by other sets of species in the same functional groups in such a moderately polluted site.

Analysis of fungal based food web model resulted in similar N mineralization rates among sites, indicating low N turnover rate in spite of high biomass of microarthropods at the polluted sites. Directly measured ecological functions, such as organic matter decomposition, soil aggregates and primary production, were not significantly influenced by heavy metal pollution. These results suggest that the ecological functions are characterized by redundancy at the pollution levels of this study, which would be maintained by replaced species in the same functional group.

Appendix 1 Description of the food web model (cf. Hunt et al., 1987; Berg et al., 2001)

The model derives N mineralization from feeding rates of organisms following a scheme in which consumption, biomass production, excretion of organic material, and excretion of inorganic material are related to each other. Organisms within the same taxon, with similar diets or food processing abilities, and shared predators were classified into functional groups. The annual feeding rates of a functional group of organisms are calculated by assuming that the annual average production of the organisms balances the rate of loss through natural death and predation:

$$a_j p_j F_j = d_j B_j + M_j \qquad (1)$$

where F_j is the feeding rate of the jth group, d_j the specific death rate, B_j the average annual population size, expressed as biomass C, of a group j, M_j the loss of biomass due to predation, a_j assimilation efficiency, and p_j production efficiency.

For polyphagous predators, the feeding rate per food type (F_{ij}) depends on the relative abundance of their food types and on their food preference:

$$F_{ij} = \frac{w_{ij} B_i}{\sum_{k=1}^{n} w_{kj} B_k} F_j \qquad (2)$$

where w_{ij} is a weighting factor, measuring the preference of group j for food type i over its other food types, and k is the index for the summation over all (n) trophic groups on which j feeds. In our case, however, the preference for specific food types was not determined in this study. Therefore, w_{ij} was set to 1, and consumption depended on relative abundance of food types. Calculations of feeding rates begin with the top predators, which are assumed to suffer only from natural death, and this routine is worked backward to the lowest trophic levels, the primary consumers. The total C

mineralization by the food web can then be calculated as

$$C_{min} = \sum_{j=1}^{n} a_j (1 - p_j) F_j \qquad (3)$$

Given the fluxes of C through the web, the fluxes of N are derived by taking the C/N ratio of a trophic group into account. As C/N ratios, unlike C concentrations, show considerable differences between groups, the mineralization of N by a consumer depends also on the C/N ratio of its food, relative to its own C/N ratio. The total N mineralization by the food web is thus calculated as

$$N_{min} = \sum_{j=1}^{n} \sum_{k=j}^{n} a_j \left\{ \frac{1}{C/N_i} - \frac{p_j}{C/N_j} \right\} F_{ij} \qquad (4)$$

where C/N_i and C/N_j denote the C/N ratio of food and consumer. Fluxes of C and N through the web were calculated for the trophic interactions presented in Fig. 1 (Chapter 3).

Appendix 2. Density (individuals m^{-2}) of collembolan species found in the study sites. Each value in the table is the average numbers in 20 samples (19 samples at RA in May) and number of species.

	No. per m^2												
	May				August				November				total
	PA	PB	RA	RS	PA	PB	RA	RS	PA	PB	RA	RS	average
Isotomiella minor	3,000	2,640	1,360	520	2,900	1,160	1,100	820	40	20	60	40	1,138
Onychiurus yodai	600	80	120	580	1,860	1,800	680	1,140	1,720	2,120	360	1,960	1,085
Folsomides parvulus	500	960	460	340	120	100	220	1,000	0	0	0	20	310
Folsomina onychiurina	1,880	120	140	60	500	560	220	220	0	0	0	0	308
Sinella umesaoi	0	100	320	120	180	100	940	360	120	400	500	420	297
Tullbergia yosii	300	40	80	100	380	340	300	940	660	160	40	140	290
Megalothorax minimus	640	460	560	200	340	160	480	200	0	40	40	40	263
Isotomodes fiscus	300	60	0	640	340	700	160	380	0	0	0	0	215
Xenyllodes armatus	100	440	20	100	300	260	60	580	440	20	20	0	195
Homidia sauteri	0	0	0	20	140	100	40	80	80	60	580	1,080	182
Sinella dubiosa	20	0	160	0	220	320	260	0	60	220	60	20	112
Lepidocyrtus sp.	0	340	0	0	100	40	0	0	440	360	20	0	108
Pseudachorutes sp.	40	60	40	60	40	20	140	120	20	0	0	320	72
Hypogastrura gracilis	0	0	0	0	0	0	0	0	40	60	220	540	72
Freisia japonica	0	0	0	0	20	100	0	0	180	180	100	140	60
Arrhopalites minutus	160	0	60	0	140	40	60	20	0	0	0	0	40
Sphaeridia tunicata	60	20	20	20	40	60	0	100	0	40	0	80	37
Sminthurides sp.	0	300	0	0	100	0	0	40	0	0	0	0	37
Folsomia candida	0	0	0	0	0	80	0	0	160	0	180	0	35
Isotoma pinnata	360	0	0	0	0	40	0	0	0	0	0	0	33
Sphyroteca multifasciata	0	140	80	0	20	0	80	20	0	0	0	0	28
Willemia anophthalma	20	0	0	0	0	0	0	0	40	60	120	60	25
Hypogastrura sp.	20	0	40	0	0	0	0	200	0	0	0	0	22
Homidia socia	0	0	0	0	0	0	0	0	40	100	20	20	15
Hypogastrura communis	0	0	0	0	0	0	0	0	0	40	40	100	15
Hypogastrura sp. (cf. *schoetella*)	0	40	0	0	0	0	0	80	0	0	0	0	10
Isotomurus prasinus	60	0	0	0	0	0	0	40	0	0	0	0	8
Pseudachorutes longisetis	20	0	0	20	0	0	0	20	0	0	0	20	7
Aracma sp.	0	0	0	0	0	40	0	0	0	0	0	0	3
Desoria sp.	0	0	0	0	0	0	0	0	0	40	0	0	3
Arrhopalites habei	0	0	0	0	0	0	0	0	0	20	0	0	2
Ptenothrix sp.	0	0	0	0	0	0	20	0	0	0	0	0	2
Entomobrya sp.1	0	0	20	0	0	0	0	0	0	0	0	0	2
Entomobrya sp.2	0	0	20	0	0	0	0	0	0	0	0	0	2
Pranura sp.	0	0	0	0	0	0	0	0	0	0	0	20	2
Total no. individuals	8,080	5,800	3,500	2,780	7,740	6,020	4,760	6,360	4,040	3,940	2,360	5,020	5,033
No. of species	17	15	16	13	18	19	15	19	14	17	15	17	35

Appendix 3. Density (individuals m^{-2}) of oribatid mite species found in the study sites. Each value in the table is the average numbers in 20 samples (19 samples at RA in May) and number of species.

	No. per m^2												
	May				August				November				total
	PA	PB	RA	RS	PA	PB	RA	RS	PA	PB	RA	RS	average
Gamasida	1,480	680	1,116	960	1,940	1,260	2,120	2,400	800	1,040	1,380	1,280	1,371
Actinedida	200	220	484	540	1,080	280	660	580	620	880	420	600	547
Tarsonemida	40	20	21	40	100	40	140	100	180	100	60	80	77
Acaridida	20	40	0	80	200	200	100	80	40	520	80	180	128
Total Oribatida	13,520	11,960	5,474	5,920	13,460	11,760	12,240	5,380	17,840	12,140	9,180	9,820	10,724
Oribatida (adult)													
Ischeloribates lanceolatus	2,080	540	1,200	2,200	1,160	320	480	280	5,040	1,620	680	1,180	1,398
Oppiella nova	760	140	421	120	2,040	1,280	1,900	980	2,160	600	800	940	1,012
Cosmohermannia frondosa	1,520	2,480	295	80	860	1,260	700	0	380	680	60	0	693
Scheloribates latipes	120	100	400	380	260	260	180	220	800	1,100	820	1,640	523
Brachychthoniidae sp.	300	200	84	300	800	440	780	480	1,020	200	260	640	459
Tectocepheus velatus	400	200	400	220	840	800	400	320	240	240	380	380	402
Eremulus sp.	1,020	740	189	560	320	60	180	220	300	120	140	820	389
Quadroppia quadricarinata	420	120	105	240	460	560	480	200	300	280	140	160	289
Masthermannia hirsuta	460	660	42	60	280	240	540	80	600	140	40	0	262
Suctobelbidae sp-1	120	0	21	200	600	160	1,120	280	220	60	60	40	240
Arcoppia viperea	60	20	168	20	420	640	80	60	720	340	20	40	216
Fosseremus quadripertitus	560	180	295	140	40	0	160	80	80	0	20	0	130
Hypochthonius rufulus	280	460	63	20	200	180	120	60	60	80	20	0	129
Nippohermannia sp.	60	280	0	20	320	220	60	60	320	200	0	0	128
Galumniidae sp-2	40	20	42	120	20	60	20	40	120	80	140	460	97
Rhysotritia ardua	140	20	42	40	80	0	0	40	200	120	100	80	72
Oppiidae sp-6	0	0	0	0	420	160	100	120	20	0	20	20	72
Oppiidae sp-2	40	20	63	20	0	480	40	0	0	120	20	0	67
Xylobates sp.	0	20	42	20	0	80	20	40	20	300	100	0	54
Oppiidae sp-3	0	40	0	20	80	120	220	80	20	0	0	60	53
Eremobelba japonica	0	180	84	40	0	100	60	0	0	0	0	140	50
Ceratozetes sp.	0	0	0	20	20	20	20	0	240	80	80	100	48
Galumniidae sp-3	0	0	0	60	0	0	80	20	0	0	300	100	47
Eohypochthonius magnus	0	0	84	0	0	0	60	0	0	0	200	20	30
Galumniidae sp-1	0	0	63	20	0	0	120	20	0	0	100	40	30
Oppiidae sp-4	0	0	0	20	280	0	40	0	0	0	0	0	28
Nothrus silvestris	0	0	0	0	40	80	20	0	180	0	0	0	27
Atropacarus striculus	0	60	0	20	0	0	20	0	0	100	20	80	25
Suctobelbila tuberculata	0	0	21	40	0	100	60	20	0	0	0	0	20
Palaeacarus hystricinus	0	0	105	0	0	0	100	0	0	0	0	0	17
Oppiidae sp-1	20	20	0	0	0	40	80	0	0	0	0	0	13
Peloribates sp.	0	0	0	0	20	20	0	0	100	20	0	0	13
Suctobelbidae sp-2	0	20	21	0	0	40	40	0	0	0	0	0	10
Eporibates sakamorii	0	0	0	0	20	0	100	0	0	0	0	0	10
Nothrus palustris	0	0	0	0	0	0	0	0	80	0	0	0	7
Oppiidae sp-7	0	0	0	0	0	40	0	20	0	0	0	0	5
Prionoribatella sp.	0	0	0	0	60	0	0	0	0	0	0	0	5
Dolicheremaeus elongatus	0	0	0	0	0	0	0	20	0	0	20	0	3
Steganacarus sp.	0	0	0	0	0	0	20	0	0	0	0	0	2
Oppiidae sp-5	0	0	0	0	20	0	0	0	0	0	0	0	2
Oppiidae sp-8	0	0	0	0	0	0	0	20	0	0	0	0	2
Scheloribates sp.	20	0	0	0	0	0	0	0	0	0	0	0	2
Total no. individuals (adult)	8,420	6,520	4,253	5,000	9,660	7,760	8,400	3,760	13,220	6,480	4,540	6,940	7,079
No. of species	19	22	23	26	25	26	33	24	23	20	24	19	42

Chapter 4

Species-specific heavy metal accumulation patterns of earthworms on a floodplain in Japan

Kamitani T and N Kaneko

Abstract: We identified all earthworm species found on a floodplain contaminated by heavy metals (Cu, Zn, Cd and Pb) from an old mine in central Japan, and compared their abundance, biomass and heavy metal concentrations in tissue. There were six species, belong to three families: Megascolecidae; Moniligastridae and Lumbricidae. Earthworm community structure seemed to be mostly influenced by soil properties, especially pH and clay fraction. Despite the same endogeic categories, species-specific patterns of heavy metal accumulation were observed: species of Megascolecidae and Lumbricidae had relatively lower concentrations compared to those of Moniligastridae. Even in the same family of Moniligastridae, *Drawida* sp. accumulated Cu and Pb markedly higher than *Drawida japonica*. Based on heavy metal concentrations in extracts of $CaCl_2$ and DTPA, the ageing caused remarkable low concentrations in pore water, indicating low availability by dermal uptake, therefore the different patterns of heavy metal accumulation among species must partly result from species specific gut process.

Keywords: heavy metals; earthworm; ecological category; bioavailability; biomagnification

1. Introduction

Earthworms play important roles in soil ecosystems, since they contribute to organic matter incorporation and decomposition, excavation of burrows and production of casts (Lee, 1985; Edwards and Bohlen, 1996). Decrease in earthworm abundance caused by soil heavy metal contamination may diminish these ecological functions. For evaluation of ecological risks of soil contamination, earthworms have undergone considerable lethal and sublethal testing (Cortet et al., 1999; Spurgeon et al., 2003). However, single-species tests have often been criticized for their lack of realism in terms of their modes of exposure and for difficulties in extrapolating such results to field conditions (Edwards, 2002).

In field studies, the limited mobility of earthworms makes them very suitable for monitoring the impact of contaminants (Paoletti, 1999). Generally, earthworms can be used as an indicator of soil impairment because their abundance and biomass decrease with soil contamination level (Belotti, 1998; Spurgeon and Hopkin, 1999a). The critical body residues (CBR) in relation to toxicity endpoints also have been suggested to provide a better estimate of ecological risk than soil concentration of contaminant (Ma, 2005), since the toxic effect of a chemical on an organism results from uptake relative to critical tissue concentration (Van Gestel, 1992). However, Vijver et al. (2004) claimed that the CBR approach does not work accurately for metals because of the specific internal compartmentation of metals in organisms and its consequences for toxicity. The presence of contaminants in earthworms itself poses a serious risk of secondary poisoning of vertebrate predators due to biomagnification, since earthworms serve as food for a variety of predators (Ma and Talmage, 2001; Reinecke and Reinecke, 2004).

The amount of contaminants in tissue of earthworms results from the net inward flux of contaminants from the soil due to the balance between uptake and depuration processes (Lanno et al., 2004). To determine bioavailability indirectly, aqueous extracts (e.g. in weak salts, weak acids and chelating agents) have been used for estimating contaminant concentrations in the soil solution (Peijnenburg and Jager, 2003). Such values relate strongly to physical and chemical conditions of soil, and ageing, which binds strongly to soil (Sijm et al., 2000). However, only organisms can determine which available fraction is bioavailable. In the case of earthworms, there are two pathways by which a contaminant enters the earthworm body; via dermal and/or intestinal uptake (Lanno et al., 2004). The soluble contaminants at the conditions of soil and intestine can be thought as environmentally available fractions, which relate to bioaccumulation in earthworm. The contaminants residing in pore water are available to earthworms for dermal uptake, which is supposed to be important for uptake of metals (Vijver et al., 2003). In the earthworm gut, since ingested materials are buffered to near neutral pH (Laverack, 1963; Oste et al., 2001), a different fraction of the total contaminants would be available. The difference in dietary intake of heavy metals should be one of the important factors in contributing to different bioavailability between different earthworm species. Earthworms can be divided according to ecological categories such as epigeic, endogeic and anecic, linking feeding strategies (Bouché, 1977), which have been also confirmed for Japanese species (Uchida et al., 2004). When contaminants are concentrated and bound to soil, epigeic species, feeding mainly on plant litter, have lower concentrations of heavy metals than endogeic species, feeding on soil (Morgan and Morgan, 1999; Dai et al., 2004). Attention should be paid to the ecological categories of earthworm, including feeding mode and habitat use, when the effects of

soil contamination on the concentration in organism are evaluated.

The subjects of previous studies about earthworms in contaminated soils have converged on lumbricid earthworms, predominantly in Europe (Ma, 2004), and other families have rarely been investigated, e.g. Megascolecidae (Guo et al., 1998; Fang et al., 1999) and Moniligastridae (Panda et al., 1999). Few data in previous literature have compared the effects of heavy metals among different earthworm families living in the same contaminated field. The aim of this study was to estimate the effect of heavy metal contamination on earthworms in Japan, focused on their abundance, biomass and the heavy metal concentrations in their body tissue. Earthworms dwelling in Japan belong mostly to the families of Megascolecidae (Easton, 1981; Blakemore, 2003), Moniligastridae and Lumbricidae. Family Megascolecidae distributes in Oriental, Oceanian, Australian regions and North and Central America. Moniligastridae also distributes in most of Oriental region, especially India (Blakemore, 2003). Knowledge of the effects of soil contaminations on such species is needed for ecological risk assessment in these areas.

We also investigated indirect chemical availability of soil heavy metals with extracts of $CaCl_2$ and diethylenetriaminepentaacetic acid (DTPA) to explore the relationship between chemical availability and concentration in tissue.

2. Materials and Methods

2.1. Field site and sampling design

The study area is at Watarase retarding basin ($36^{o}14'$N, $36^{o}14'$E) in the Watarase

River in Tochigi, central Japan, which is designed and operated to provide temporary storage and thus reduce the peak flood flows of a current downstream. This site gradually became contaminated with heavy metals caused by mining activity from Ashio Copper Mine at the headwaters. Since 1877, the mining activity rapidly enlarged and contamination from the mine caused severe damage in the Watarase River basin (Morishita, 1981). Efforts have been made to improve the water quality from the mine until it was closed in 1973. Since the retarding basin has been flooded only ten times during last 30 years, it is assumed that few materials from the river have been sedimented after 1973. The study area is dominated by tall grasses (reed; *Phragmites australis* and *Miscanthus sacchariflorus*). The reeds are used for making traditional blinds, and once a year in March, the grasses are burned off to ensure high quality reeds and pest control, also reducing litter layers on the surface soil.

We set up six line transects, 80 m long, within approximately 3 km across the study area to get a wide range of soil heavy metal concentration. Before setting the transects, we consulted some reports on the distribution of past copper concentration in this area (Ministry of Land, Infrastructure and Transport Government of Japan, 1976; 1991). Because of small differences in heavy metal concentrations along each transect, five sampling plots were selected along a transect at a distance of 20 m for random sampling. The difference in heavy metal concentrations resulted from the distance of the past channel of the Watarase River. Earthworms were sampled from each plot with five replicates (25 cm x 25 cm quadrat to a depth of 20 cm) in May, 2004. Earthworms were taken by digging and hand-sorting. Soil samples taken at the same time were homogenized and pooled for each sampling plot.

2.2. Soil heavy metal and soil properties

The soil samples were dried at 60^{o}C for 72 hours and sieved through a 2 mm mesh. To estimate total heavy metal contents (Cu, Zn, Cd and Pb), one gram dry soil was heated with 10 mL concentrated HNO_3, starting at 80^{o}C (Bruus Pedersen and van Gestel, 2001). The residues were then dried at 135^{o}C, redissolved in 40 mL 0.1 M HNO_3, centrifuged and filtered. Because heavy metals in the soils are present in various forms, we observed two available fractions; mobile (extracted by $CaCl_2$) and mobilisable (extracted by DTPA) (Maiz et al., 2000). Pore water heavy metal concentrations are thought to correlate with $CaCl_2$-extractive fraction (Peijnenburg et al., 1999b). Four grams of dry soil was shaken with 20 mL of 0.01 M $CaCl_2$ solution for 20 h. The chelating agent DTPA has been used for assessing bioavailability of non-essential trace metals (Khodadoust et al., 2005). As materials in earthworm gut are buffered to near neutral pH (Oste et al., 2001), we evaluated DTPA-extractive soil heavy metals at pH 7.2, assuming this to be the condition in the gut (Laverack, 1963). Another dry soil sample (4 g) was shaken with 20 mL of a mixture of 0.005 M DTPA and 0.01 M $CaCl_2$, adjusted to pH 7.2 with triethanolamine for 20 h. After filtering, heavy metals were analyzed with ICPAES (ICPS-8000E, SHIMADZU Co., Kyoto, Japan) for Cu, Zn, and Pb, and AAS (AA-660, SHIMADZU Co., Kyoto, Japan) for Cd.

Soil pH was measured at a ratio of soil-to-solution of 1:2.5 in an aqueous suspension of soil. Loss on ignition (LOI) was analyzed by igniting the dry soil for 2 h at 750^{o}C. Total C and total N were measured with an NC-analyzer (Sumigraph NC-95A, Sumika Chemical Analysis Service, Ltd., Osaka, Japan). Particle-size analysis was conducted by pipet method after chemical dispersion (Na-hexametaphosphate) and physical (shaking

and stirring) methods (Gee and Or, 2002). Exchangeable cations (Na, K, Ca and Mg) and cation exchange capacity (CEC) were determined by saturation with 1 M CH_3COONH_4 (pH 7.0), washing with 0.05 M CH_3COONH_4, and replacement with 1 M KCl. Exchangeable cations and NH_4 for determining CEC were analyzed with AAS, and an auto-analyzer (INTEGRAL FUTURA, ALLIANCE Instruments, Frépillon, France), respectively. Degree of base saturation, representing amount of absorbed cations (Na, K, Ca and Mg) on exchangeable site of soil, was estimated by dividing the sum of exchangeable cations by CEC.

2.3. Earthworms

Earthworms were weighed, counted and identified to species after being carefully rinsed in water. Adult, semi-adult and some juvenile specimens (> 0.15 g wet mass) were kept on moist filter papers in plastic Petri dishes (1 animal per dish) in a climate chamber (15^{o}C, darkness) for three days to empty their guts. The filter papers were changed once a day. Finally, the worms were stored at -30^{o}C and then freeze-dried. Gut contents remaining in some worms were removed manually. Dried and weighed earthworms were heated with 2 mL concentrated HNO_3, starting at 80^{o}C. The solutions were then concentrated at 135^{o}C, made up to 10 mL with deionized water. The heavy metal contents (Cu, Zn, and Pb) were determined by ICPAES and Cd by AAS as for the soil samples.

2.4. Statistical analyses

Pearson linear correlations among soil heavy metals and soil properties were computed with SPSS for Windows, version 11.5. Canonical correspondence analysis (CCA) was performed with Canoco for Windows, version 4.5. CCA is a robust eigenvalue analysis used to relate the distribution of multispecies assemblages to environmental factors (ter Braak, 1986). Here, it was performed to relate the selected environmental variables of soil to the distribution of earthworms. In the analysis of CCA, the species data were (*log* x + 1) transformed. A sampling plot in which no worms were detected was excluded from this analysis. Environmental variables were tested using forward selection of variables along with a Monte Carlo test with 299 unrestricted permutations ($P < 0.05$). Stepwise regression analysis was conducted for heavy metal concentrations in some earthworm species relating soil heavy metals and selected soil properties with SPSS. Significance was considered at 0.01 probability level.

3. Results

3.1. Soil heavy metals and soil properties

Summary statistics for soil heavy metal concentrations and soil properties at thirty sampling plots are shown in Table 1. A complex contamination was observed in the study area; mean total Cu concentration was highest among metals because this is the main product of mining, followed by Zn and Pb. Total Cd concentration was much lower. The concentrations of total heavy metals showed positive correlations to each other ($r > 0.55$, $P < 0.01$). A positive relationship was also shown between total and DTPA-extractive heavy metal concentrations (Fig. 1). The $CaCl_2$-extractive Cd and Pb

Table 1. Summary statistics for soil heavy metal concentrations and soil properties (n=30)

Variable*		Minimum	Maximum	Mean	SD**
Cu_{total}	mg/kg	14.8	386.7	183.6	121.9
Zn_{total}	mg/kg	45.3	260.6	159.5	62.4
Cd_{total}	mg/kg	0.58	2.51	1.40	0.57
Pb_{total}	mg/kg	17.2	95.1	60.2	23.4
Cu_{DTPA}	mg/kg	3.0	98.2	45.2	29.3
Zn_{DTPA}	mg/kg	2.9	24.9	10.9	5.8
Cd_{DTPA}	mg/kg	0.01	1.06	0.51	0.32
Pb_{DTPA}	mg/kg	1.6	37.3	14.3	10.0
Cu_{CaCl2}	mg/kg	0.03	0.15	0.07	0.03
Zn_{CaCl2}	mg/kg	0.11	1.07	0.48	0.28
Cd_{CaCl2}	mg/kg	<0.03	0.09	-	-
Pb_{CaCl2}	mg/kg	<0.03	0.08	-	-
pH(H_2O)		5.39	6.09	5.77	0.23
WC	%	19.8	46.9	36.8	6.1
LOI	%	5.1	13.7	9.8	2.3
C	%	1.65	5.85	3.69	0.98
N	%	0.14	0.41	0.29	0.07
CN ratio		10.9	14.4	12.8	0.8
sand	%	1.5	74.2	24.3	22.8
silt	%	14.9	70.0	54.2	18.4
clay	%	10.9	29.2	21.5	5.1
CEC	cmol/kg	8.4	29.4	20.3	5.3
Na	cmol/kg	0.05	0.21	0.10	0.04
K	cmol/kg	0.4	1.3	0.8	0.2
Ca	cmol/kg	6.0	19.0	12.1	3.2
Mg	cmol/kg	0.9	3.1	2.0	0.6
BS	%	45.9	100.0	75.6	15.1

* Me_{total}: concentration of total soil metal; Me_{DTPA}: concentration of DTPA-extractive soil metal; Me_{CaCl2}: concentration of $CaCl_2$-extractive soil metal; WC: water content (water/wet soil); LOI: loss on ignition; CEC: cation exchangeable capacity; Na, K, Ca, Mg: exchangeable cations; BS: degree of base saturation

** SD: standard deviation

were too low to be detected at every sampling plot. The concentration of

$CaCl_2$-extractive Cu showed a positive relationship with total Cu, while that of Zn was higher at intermediate levels of total concentration. Soil texture ranged from sandy to clayey. The amounts of silt and clay were positively correlated with water content, soil organic matter (LOI, total C and N) and CEC, negatively with degree of base saturation. Exchangeable K, Ca and Mg were positively correlated with water content, silt fraction, soil organic matter and CEC. Soil pH showed negative correlation with clay fraction and positive with degree of base saturation.

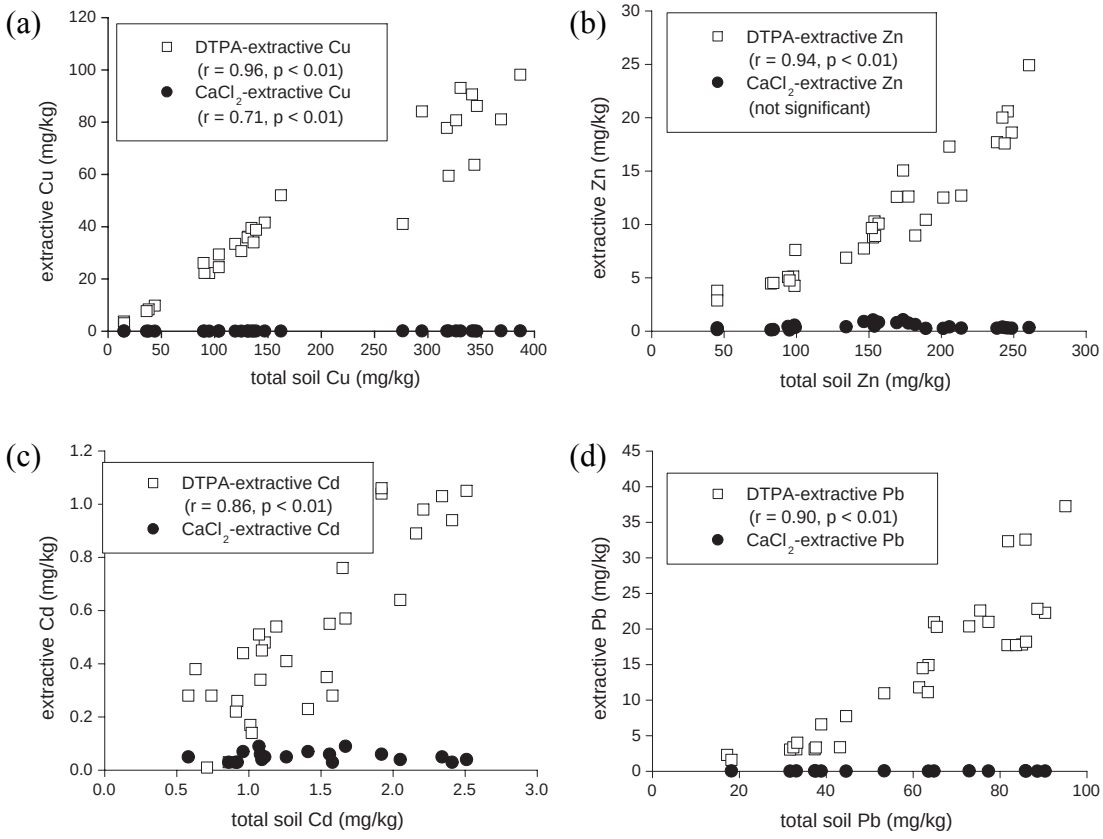

Fig. 1. Soil heavy metal concentrations extractable with 0.01 $CaCl_2$ and DTPA at thirty sampling plots [Cu (a), Zn (b), Cd (c) and Pb (d)]. Pearson correlation coefficients between total and extractive heavy metal are shown in each graph. The $CaCl_2$-extractive Cd and Pb were too low to be detected at every sampling plot.

Total metal concentrations were positively correlated to water content, and also positively correlated to pH except Pb. Total Zn, Cd and Pb concentrations were also positively correlated with organic matter, silt fraction, CEC and exchangeable cations, while Cu hardly showed a correlation with soil properties (Table 2). DTPA-extractive heavy metals showed similar pattern to total metals. $CaCl_2$-extractive Cu showed positive correlation with pH, while that of Zn was negatively correlated.

Table 2. Pearson correlation coefficients between soil heavy metal concentrations and soil properties

Variable[a]	pH(H_2O)	WC	LOI	C	N	CN ratio	sand	silt	clay	CEC	Na	K	Ca	Mg	BS
Cu_{total}	0.729**	0.362*	-	-	-	-	-	-	-	-	-	-	0.612**	0.450*	0.383*
Zn_{total}	0.515**	0.588**	0.563**	0.577**	0.599**	-	-0.534**	0.582**	-	0.501**	-	0.386*	0.731**	0.570**	-
Cd_{total}	0.532**	0.482**	0.524**	0.424*	0.489**	-	-0.526**	0.589**	-	0.426*	-	0.505**	0.709**	0.570**	-
Pb_{total}	-	0.793**	0.790**	0.749**	0.816**	-	-0.843**	0.849**	0.710**	0.752**	-	0.381*	0.615**	0.499**	-
Cu_{DTPA}	0.639**	0.515**	0.452*	0.465**	0.469**	-	-0.417*	0.473**	-	-	-	-	0.711**	0.569**	-
Zn_{DTPA}	0.497**	0.588**	0.626**	0.649**	0.650**	-	-0.490**	0.538**	-	0.536**	-	0.427*	0.792**	0.649**	-
Cd_{DTPA}	0.624**	0.585**	0.524**	0.524**	0.528**	-	-0.442*	0.501**	-	0.389*	-	0.365*	0.747**	0.607**	-
Pb_{DTPA}	-	0.684**	0.692**	0.716**	0.758**	-	-0.741**	0.709**	0.756**	0.691**	-	-	-	-	-0.510**
Cu_{CaCl2}	0.603**	-	-	-	-	-	-	-	-	-	-	-	-	-	0.414*
Zn_{CaCl2}	-0.701**	-	-	-	-	-	-0.394*	-	0.667**	0.386*	0.385*	-0.438*	-	-	-0.813**

a Me_{total}: concentration of total metal; Me_{DTPA}: concentration of DTPA-extractive soil metal; Me_{CaCl2}: concentration of $CaCl_2$-extractive soil metal; WC: water content (water/wet soil); LOI: loss on ignition; CEC: cation exchangeable capacity; Na, K, Ca, Mg: exchangeable cations; BS: degree of base saturation

*: significant at the level of $P<0.05$, **: significant at the level of $P<0.01$, -: not significant at the level of $P>0.05$

3.2. Earthworm community structure

All earthworms were sampled from soil, because the litter layer was reduced by burning grasses in early spring. Large differences in the number and biomass of earthworms were observed among sampling plots (Fig. 2). Three megascolecid

earthworms [*Amynthas corticis* (Kinberg, 1867), *A. hupeiensis* (Michaelsen, 1895) and *A. micronarius* (Goto & Hatai, 1898)] were found at all levels of soil metals. *Drawida japonica* (Michaelsen, 1892) and another species, immature *Drawida* sp. (family Moniligastridae) showed s contrasting distribution in the study area; *D. japonica* was observed at low or especially high level contaminated plots, while *Drawida* sp., which was in large number and small individual size, was found only at intermediate level contamination. *D. japonica* and *Drawida* sp. were easily distinguished by the number of gizzards. A large number of *Eisenia japonica* (Michaelsen, 1891) (family Lumbricidae) was also observed at intermediately contaminated plots. In the whole biomass of sampled earthworms, Megascolecidae was most abundant (46%), followed by Lumbricidae (31%), and Moniligastridae (23%).

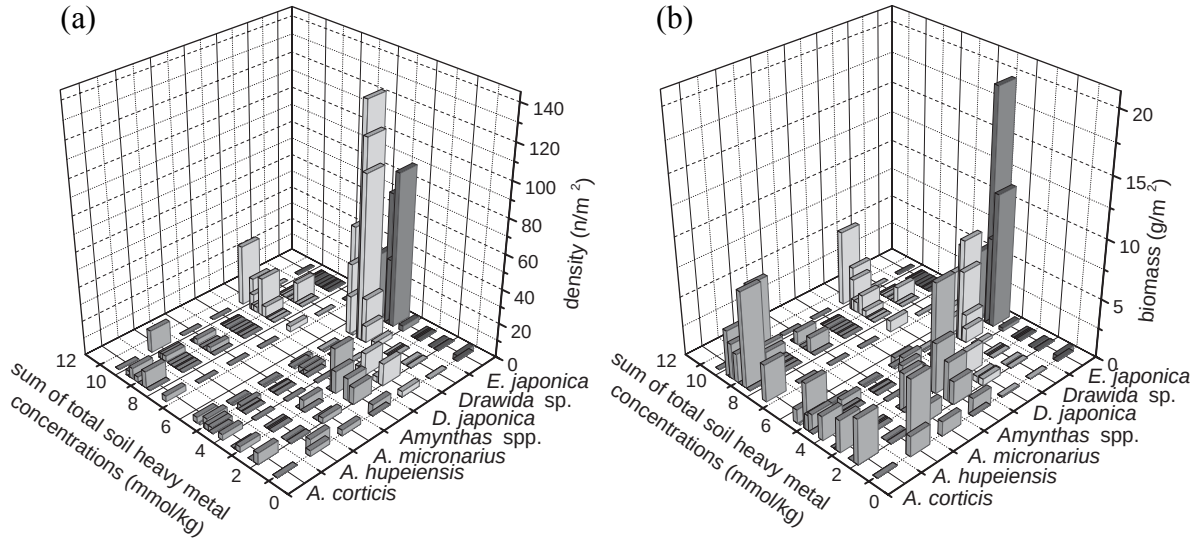

Fig. 2. Density (a) and fresh biomass (b) of earthworm species in the study area. Density and biomass are mean value (n = 5) in each plot. *Amynthas* spp. indicates immature and unidentifiable specimens of Megascolecidae.

CCA ordination for the earthworm communities showed that among selected environmental variables (total soil heavy metal concentrations, pH, water content, LOI, silt and clay fractions and CEC), pH and clay fraction were significant in the forward selection ($P < 0.05$) (Fig.3). *Drawida* sp. and *E. japonica* were situated on the left side of axis 1, indicating a positive correlation with the clay fraction. *A. hupeiensis*, *A micronarius* and *D. japonica* were situated on the right side of axis 1, indicating positive correlation with pH. *A. corticis*, situated on the center of coordinate, had no correlation with various soil properties.

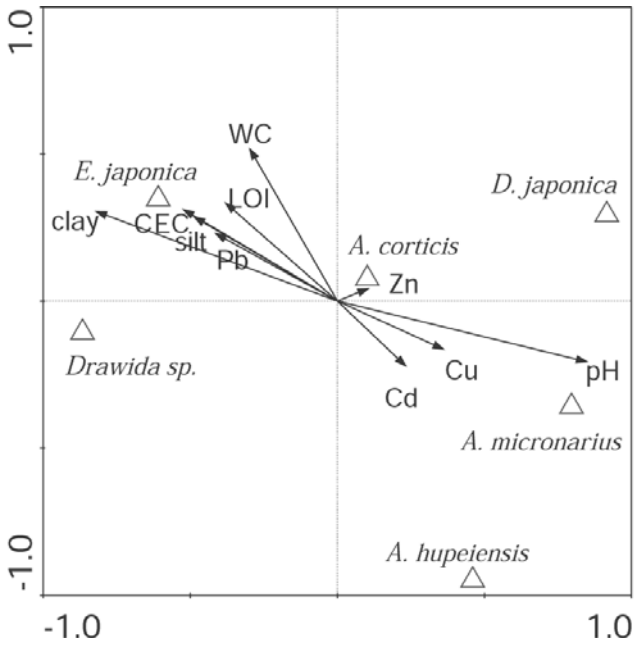

Fig. 3. CCA ordination biplot showing species density and environmental variables for earthworm community in the study area. Environmental variables are shown with arrows, and earthworm species distribution with triangular dots. Cu, Zn, Cd and Pb are total soil heavy metal. WC: water content (water/wet soil); LOI: loss on ignition; CEC: cation exchangeable capacity.

3.3. Heavy metals in earthworms

Concentrations of Cu, Zn, Cd and Pb in earthworms showed species-specific patterns (Fig. 4). All megascolecid (family Megascolecidae) worms showed similar heavy metal accumulation patterns in their tissue; the Cu and Cd in tissue slightly increased with total soil heavy metals, while Zn and Pb did not show a positive increase. *E. japonica* accumulated Cu and Zn to the same levels as megascolecid worms, while relatively higher concentrations of Cd and Pb in tissues were observed. Moniligastrid species showed relatively high heavy metal accumulation patterns; *D. japonica* accumulated higher concentrations of Zn and Cd than megascolecids, while *Drawida* sp. accumulated all metals to notably high levels. Bioaccumulation factor (BAF): the ratio of heavy metals in tissue to those in soil, was ranked as Cd > Zn > Cu = Pb in megascolecids and *D. japonica*, on the contrary, Cd > Zn = Pb > Cu in *E. japonica* and *Drawida* sp. The BAF of Cu and Pb in the range of high concentration were less than 1 except for Pb in *Drawida* sp.. The BAF of Zn was also less than 1 in megascolecids and *E. japonica*.

A stepwise multiple linear regression was used to explore the potential for improving predictions of the heavy metal concentration in tissue of *A. corticis* and *D. japonica*, which were found along a wide range of soil heavy metal concentration in this study area. Total, DTPA- or $CaCl_2$-extractive heavy metals and selected soil properties: pH, organic matter (LOI), clay contents and CEC, were considered. Only six models included heavy metal parameters (Table 3). Multiple linear regression demonstrated that the most successful models were relatively simple, including only total or DTPA-extractive metals.

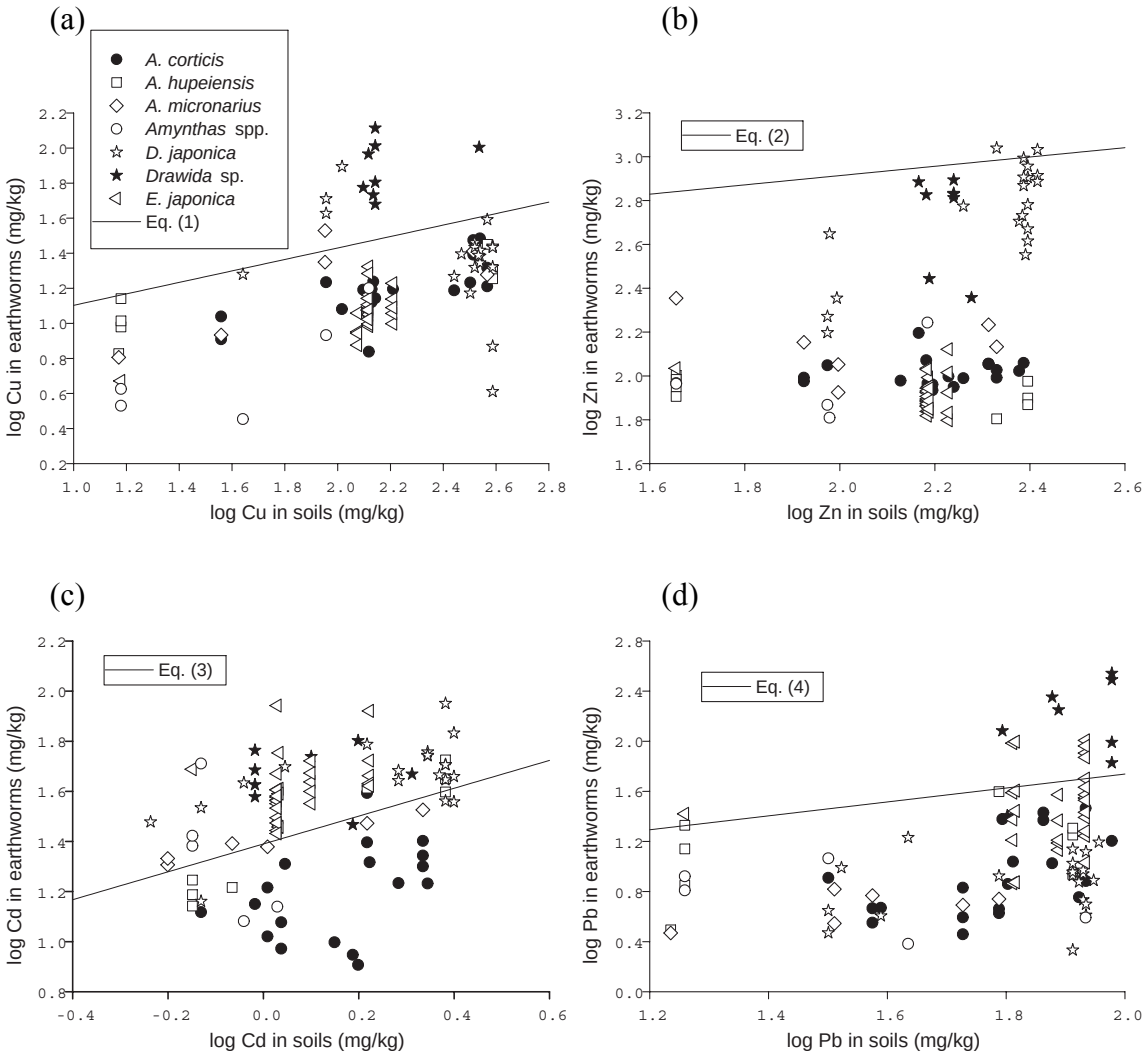

Fig. 4. Relationship between Concentration of heavy metals [Cu (a), Zn (b), Cd (c) and Pb (d)] in soil (HNO_3 extract) and in earthworms. The calculated values by multiple regression models (Ma, 2004) was shown in parallel, relating heavy metal concentration in endogeic groups of lumbricid earthworms (Me_w) to total soil metal concentrations (Me_s); Eq. 1: $\log[Cu_w] = 0.327 \log[Cu_s] + 0.776$; Eq. 2: $\log[Zn_w] = 0.212 \log[Zn_s] + 2.49$; Eq. 3: $\log[Cd_w] = 0.556 \log[Cd_s] + 1.39$; Eq. 4: $\log[Pb_w] = 0.556 \log[Pb_s] + 0.626$.

Table 3. Regression models and coefficients of determination (R^2) relating metal concentration in earthworms (mg/kg) to acid or DTPA extractable soil metal concentration (mg/kg), pH, LOI, clay fraction and CEC. The number of individuals was 19 in *A. corticis* and 20 in *D. japonica*.

Model	R^2	Adj. R^2	s.e.
$\log[Cu_{A.\ corticis}] = 0.395 \log [Cu_{total}] + 0.327$	0.534	0.507	0.1190
$\log[Cu_{A.\ corticis}] = 0.384 \log [Cu_{DTPA}] + 0.588$	0.521	0.493	0.1207
$\log[Cu_{A.\ corticis}] = 0.254 \log [Cu_{DTPA}] + 0.276\ pH - 0.793$	0.648	0.604	0.1067
$\log[Zn_{D.\ japonica}] = 1.12 \log [Zn_{total}] + 0.170$	0.592	0.570	0.1607
$\log[Zn_{D.\ japonica}] = 0.683 \log [Zn_{DTPA}] + 1.96$	0.520	0.493	0.1743
$\log[Cd_{A.\ corticis}] = 0.426 \log [Cd_{DTPA}] + 1.35$	0.361	0.323	0.1581

$Me_{A.\ corticis}$: metals in mg/kg *A. corticis*; $Me_{D.\ japonica}$: metal in mg/kg *D. japonica*; Me_{total}: concentration of total metal (mg/kg); Me_{DTPA}: concentration of DTPA-extractive soil metal (mg/kg)

4. Discussion

4.1 Abundance and biomass of earthworms in heavy metal contaminated soil

The present study site was complexly contaminated with heavy metals (Table 1). Three megascolecid, two moniligastrid, and one lumbricid earthworms were found, and the abundance and biomass of earthworm varied among sampling plots (Fig. 2). Even at the most contaminated end of our sampling plot, 12.2 g/m^2 of worms was found, which has twice the biomass of average weight in the study site (6.5 g/m^2). Thus, the total

abundance and biomass of earthworms did not seem to be mostly influenced by the soil contamination level.

Spurgeon and Hopkin (1999a) have shown that total abundance and biomass of earthworms decreased with proximity to a primary Pb, Zn and Cd smelting works, and sensitive species were absent from the intensively contaminated soils, leading to reduced species richness. However, in their study, the detrimental effect of metals on earthworm community had emerged at notably high concentration (Pb: 411 - 20,700 mg/kg; Zn: 1,530 - 37,300 mg/kg; Cd: 13.8 - 275 mg/kg). At the relatively low concentration just as this study site, the contamination was found to be of less important for species composition, and we should take natural factors into account for estimating the impact of soil contamination on earthworms, e.g. soil acidity (Vorobeichik, 1998), and grain size distribution (Vandecasteele et al., 2004). In the present study, heavy metal concentrations were significantly correlated with various soil properties (Table 2). Although the number of *D. japonica* seemed to be correlated positively to the soil heavy metal concentration (Fig. 2), CCA suggested that only pH and clay fraction, which were significant among soil properties analyzed, might have determined earthworm community (Fig. 3). *A. hupeiensis*, *A. micronarius* and *D. japonica* seemed to preferentially dwell at relatively high pH sites, while *Drawida* sp. and *E. japonica* distributed at clayey soil. *A. corticis* would not be affected by any soil properties and heavy metal contaminations.

4.2. Heavy metal accumulation in earthworms relating to bioavailability and uptake routes

There was no litter layer on the floodplain, and humus layer was not developed, because the grasses are burned in spring and most of soil organic matter (LOI) consists of crumble charcoal. Since the collected earthworms inhabit mineral soil horizon and mainly feed on soil, we can regard them as endogeic species. *A. micronarius* and *E. japonica* have been also classified as endogeic species by Uchida and Kaneko (2004) and Uchida et al. (2004), respectively. In spite of the same endogeic categories, the degrees of heavy metal accumulation in each earthworm showed different, species-specific patterns (Fig. 4). Ma (2004) has summarized and generated multiple regression models relating heavy metal concentration in ecological groups of lumbricid earthworms to soil metal concentration (Fig. 4). Compared to his model of lumbricid endogeic earthworms including *Apporectodea* sp. and *Allolobophora* sp., the species of Megascolecidae in the present study accumulated heavy metals relatively less. One moniligastrid species, *Drawida* sp., showed relatively higher accumulation in Cu, Cd and Pb. Another species, *D. japonica*, showed relatively lower concentrations in Pb, higher in Cd. A lumbricid earthworm, *E. japonica*, accumulated lower in Cu and Zn, higher in Cd. We attempted to generate multiple regression models on the line of Ma (2004) for two species; *A. corticis* and *D. japonica*, using total, DTPA- or $CaCl_2$-extractive heavy metals and selected soil properties. As shown in Table 3, a few, simple models were successful, indicating that soil properties did not relate to accumulation of heavy metals in earthworms and that Cu and Cd in *A. corticis* and Zn in *D. japonica* could be predicted with total or DTPA-extractive soil heavy metals. Positive relation of metals in soil and most of the worm species were not observed (Fig. 4).

Two different pathways: dermal and intestinal uptake, have been considered for their

potential impacts on the heavy metal accumulation patterns among earthworm species (Oste et al., 2001). We assumed two available fractions, extracted by $CaCl_2$ and DTPA adjusted to pH 7.2, to represent the conditions of pore water and gut, respectively. The two extraction methods showed different extraction patterns in the present study. The $CaCl_2$-extractive fractions were remarkably low at all contamination levels (Fig. 1). This could result from an aging period, more than 30 years in this study area, which leached the soluble fractions and increased the binding of metals to soil (Van Gestel, 1997). Thus, exposure of heavy metals to a certain earthworm by dermal uptake route would not have been different at all sampling plots. Although uniform low availability at various contamination levels could explain the absence of positive relationship between metals in some earthworms and total amount in soil, the increase in heavy metals of particular earthworms, e.g. Cu for *A.corticis* and Zn for *D. japonica* were not proportional to the $CaCl_2$-extractive fractions. The DTPA-extractive heavy metal showed positive relation with total amounts (Fig. 1), indicating that mobilizable fraction of heavy metals in the gut would potentially increase with total soil concentration. If intestinal uptake is an important pathway, the free heavy metal concentrations in the gut will determine heavy metal uptake (Oste et al., 2001; Hobbelen et al., 2004). Thus, a positive increase in tissue with soil heavy metal would be caused by uptake from the gut.

In the present study, earthworms accumulated heavy metals in the following orders of increasing efficiency: Cd > Zn > Cu = Pb in megascolecids and *D. japonica*, and Cd > Zn = Pb > Cu in *E. japonica* and *Drawida* sp. The former order of BAF was consistent with the data of previous studies of lumbricid worms (Ma, 1982; Dai et al., 2004). They reflected the affinity order of the specific adsorption of metal cations in

soil: Pb > Cu > Zn > Cd. However, the BAF of Zn in megascolecids was less than 1, which was lower than the lumbricid data of Dai et al. (2004); more than 5 in *Aporrectodea caliginosa* at the same concentration level. In the latter order, the BAF of Zn in *E. japonica* was also less than 1. The low accumulation of Zn might be explained partly by the low extractive efficiency with DTPA, which was ranked as Cd > Cu = Pb >Zn in the present study. The proportion of Zn contained in the DTPA-extract was only 5 % of total concentration, while that of Dai et al. (2004) was about 34 %. Thus, the accumulation rank of heavy metals depended on availability of heavy metals in gut, as well as species specific physiology for assimilation and depuration.

Difference in structure of alimentary tract might also affect the change of heavy metal accumulation among species. The family Moniligastridae has multiple gizzards: 2-3 in *D. japonica* and ≥3 in *Drawida* sp., while the megascolecid and lumbricid earthworms found in this study had only one gizzard. The gizzard is a very firm and thick-walled muscular region of the alimentary tube functioning as trituration of food (Stephenson, 1930). Organic matters strongly binding heavy metals would be ground intensively in the multiple gizzards and mixed with intestinal digestives. The bioavailability of heavy metals through the digestive tract in moniligastrid species might be enhanced by such processes, probably resulting high accumulation rates of metals in the present study. Thus, putting the emphasis on uptake process, the species specific accumulation patterns would attribute partly to the bioavailable fraction in gut and assimilation efficiency of each species.

4.3. Factors affecting patterns of heavy metal accumulation in earthworms

The complicated kinetics of heavy metals in each earthworm would also affect the mechanisms of accumulation in tissue. Essential trace metals such as Cu and Zn are physiologically regulated in some parts of body (Panda et al., 1999; Peijnenburg et al., 1999a; Lukkari et al., 2004), in contrast Cd is a nonessential element that is hardly eliminated and therefore accumulates for long periods (Spurgeon and Hopkin, 1999b). In the present study, essential trace metals were not necessarily regulated especially by *D. japonica* in Zn concentration. The body concentration in this species would not reach the regulation level for this species. Life history of earthworms was another important requirement, because metal concentrations may vary considerably according to the stage of development (Ma, 2004). We can neglect the effect of body size difference in the present study, because earthworms used for the body metal analysis were selected with approximately same size. Contrary to our expectation, *Drawida* sp. accumulated heavy metals to a relatively high level, although they were all immature, indicating that this species might have tolerance to heavy metals by accumulating in tissue, but not by egesting. Our present study did not support characteristics of this species on adult stage. Further works for physiology and life history of such unknown species is necessary to recognize its strategy for heavy metal contamination.

The difference of feeding preference and vertical distribution among species might also explain the difference of heavy metal concentration in tissue, although all earthworms in the present study were recognized as geophagous species. A number of plant roots and several stages of decomposing litter exist in soil. The stock and root of *M. sacchariflorus* were distributed mainly within 10 cm from the soil surface, while those of *P. australis* were much deeper. Frequent flooding events often form the vertical distribution of heavy metals in soil: clean sediments cover heavy metal contaminated

stratifications (Zorn et al., 2005). In our study site, distinct clean sediment on top layer was not observed, and the differences of vertical distribution among earthworm species were not found at sampling time. However, they probably exploit different habitat in soil, e.g. transit soil mineral layers or stay in the rhizosphere, and specifically select palatable food, or consume communities of the lower levels in detrital food webs as a whole (Pokarzhevskii et al. 2003). The heterogeneity of contamination in such a small scale level may result in variability in the exposure of earthworms. More detail analyses for the specie-specific feeding habit were needed to ensure the materials of feeding and assimilating in the gut of earthworms. Isotopic analysis will be one of the effective methods to clarify their feeding habit (Uchida et al., 2004).

5. Conclusions

Three species of the family Megascolecidae, two species of Moniligastridae, and one species of Lumbricidae were observed on a floodplain contaminated moderately with heavy metals. The earthworm community structure was affected by soil properties, especially pH and clay fractions, rather than heavy metals. In moderately contaminated areas, the heavy metal concentration in earthworms are very important for ecological risk assessment, since the contaminants in earthworms itself poses a serious risk of secondary poisoning of vertebrate predators due to biomagnification, even if the body concentration does not achieve toxic level for earthworms. The degree of heavy metal accumulation in earthworms showed different, species-specific patterns. Compared to previous studies of lumbricid worms, Megascolecidae accumulated heavy metals relatively less, while Moniligastridae, especially *Drawida* sp., showed higher

accumulation. A lumbricid earthworm, *E. japonica*, accumulated with the same levels of Cu and Zn as megascolecid worms, but higher Cd. In the case of risk assessment of heavy metal biomagnification, a place dominated by moniligastrid worm would possess higher potential of risk for secondary predators than a place in which megascolecid worm is dominant, even if the soil heavy metal concentrations were similar.

The aged contamination caused remarkably low $CaCl_2$-extractive heavy metal fractions, indicating low availability by dermal uptake route. The positive increase of heavy metals in some earthworm species and the difference in accumulation rank of heavy metals among species would partly reflect the bioavailable fraction in gut, which was represented by DTPA-extractive fractions. Further works for physiology concerning to assimilation efficiencies, life history and feeding habitat of such domestic species is needed to recognize their strategy on heavy metal contamination.

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Chapter 5

The Earthtron facility for below-ground manipulation study

Kamitani T and N Kaneko

Abstract: A controlled environmental facility is necessary for investigation of the interaction between above-ground and below-ground components in microcosm experiments. The Earthtron is a simple computer-controlled chamber simulating natural environments: diurnal light/dark cycles, and separately controlled soil and air temperature, humidity and CO_2 concentration. In soil core incubation experiments, the Earthtron was able to simulate the dynamics of soil temperature field conditions. Environmental control also affects the dynamics of soil water and distribution patterns of nutrients in the microcosm, linking to the distribution of plant roots and soil biota. The Earthtron can not only reproduce field conditions but also predict the effects of global climatic change on terrestrial ecosystems.

Keywords: Earthtron; environmental control; microcosm; soil temperature; N mineralization

1. Introduction

There is increasing recognition of the influence of above-ground and below-ground components on one another. A combined above-ground/below-ground approach to community and ecosystem ecology has enhanced our understanding of the regulation and functional significance of biodiversity and of the environmental impact of human-induced global change phenomena (Wardle et al. 2004). Since field manipulation experiments cannot control environmental conditions, e.g. temperature and moisture variations, resulting in a low repeatability and leading to difficulties with interpretation of the results, simplified laboratory 'microcosms' have been constructed as surrogates for the complicated field situation (Beyer and Odum 1993; Verhoef 1996). Although several experiments have been conducted to investigate the effects of a decomposer on above-ground plant productivity with microcosms in climate chambers, not so much attention has been paid to controlling soil temperature (e.g. Laasko and Setälä 1999; Liiri et al. 2002). Recently, a type of soil microcosm called the terrestrial model ecosystem (TME) has been reported to be a valid method for use by soil ecologists and especially soil ecotoxicologists (Van Straalen 2002; Knacker et al. 2004). The TME approach uses undisturbed soil columns taken from the field and includes living vegetation growing on the soil to allow interactions between living soil organisms and plant roots. Soil organisms are so small that the soil column can contain multiple soil biota within a small volume. Such a miniature ecosystem can be manipulated under environmentally controlled conditions to take the changes in above-ground and below-ground conditions in the field into consideration.

An environmental chamber, the 'Earthtron', was built at Yokohama National

University in 2004. It allows us to construct replicate terrestrial compartments at ecosystem level under controlled environmental conditions. We describe here the facility emphasizing its unique features and report the effects of environmental control on the dynamics of temperature, water and nutrients in the soil core.

2. Materials and Methods

The Earthtron (Dalton Company, Tokyo, Japan) is a computer-controlled chamber simulating natural environments. It consists of a large environmental chamber (12.4 m^2) with two cabinets in it (Fig. 1). The chamber can provide diurnal light/dark cycles, and control of air temperature (10–25°C), humidity (40–95%) and CO_2 concentration. Controlled air is blown from the side faces into the chamber with low wind speed to minimize the effects on plant growth. Each cabinet can accommodate 30 soil cores (maximum 80 cm in height and 20 cm in diameter), exposing the soil surface to the controlled air. The cabinets can also be connected to another temperature control unit which allows the "soil temperature" around the soil cores to be controlled (10–25°C). The environmental control is a feed-back system: the chambers are supplied with air to adjust to a set temperature, humidity and CO_2 concentration near the air conditioning system in the chamber. The cabinets also have the same system. In contrast, the Ecotron facility, which contains 16 physically and electronically integrated environmental chambers (1 m^2 terrestrial ecosystem in each) in the Centre for Population Biology at Silwood Park (Ascot, UK), is a feed-forward system: chambers are supplied with air at a known temperature and humidity (Lawton 1996). The Ecotron is a more macroscale facility than the Earthtron, but the way the air is controlled in the Earthtron is closer to

natural macroclimatic conditions. Then, what is the advantage of such environmental control for microcosm experiments? We conducted an experiment to investigate the dynamics of temperature, moisture and nutrients in soil cores set in the Earthtron.

Fig. 1 The Earthtron facility. Two cabinets are fixed to opposite sides of the chamber. The cabinets can be assigned with experimental pots (the experiment shown in this photograph is not the present study)

We used white high-impact styrol pots (14 cm height and 11.5 cm diameter). Each pot was filled with fresh soil to 11 cm height (from Watarase retarding basin, central Japan, sieved through a 2-mm mesh; pH 5.89, organic matter 11%, clay content 23%, dry bulk density 0.52 g cm^{-3}, 47% total water holding capacity). Soil cores were divided into five treatments: EA1, EA2, ES, IS1 and IS2. Treatment pots EA1 and EA2 were placed on the top of the cabinet in the Earthtron. The temperature over the cabinet (air temperature) was controlled at 25°C during the day (14 h) and at 16°C during the night (10 h). Relative humidity and CO_2 concentration were controlled at 65% and 380 ppm during the day, and 80% and 400 ppm during the night, respectively. Treatment pots

EA2 were wrapped with aluminum foil to prevent radiant heating from the fluorescent lighting. Treatment pots ES were set in the cabinet. The temperature in the cabinet (soil temperature) was set at 22°C during the day and at 19°C during the night with the soil surface exposed to controlled air over the cabinet (air temperature over the cabinet was controlled at 25°C during the day and 16°C during the night). Treatment pots IS1 and IS2 were placed in an incubator (Eylatron FLI-161, Tokyo Rikakikai Company, Tokyo) which was controlled at 22°C during the day and 19°C during the night, the same as the Earthtron cabinet temperature (soil temperature). Treatment pots IS2 were also wrapped with aluminum foil. The incubator used in this study had no capacity to control humidity or CO_2 level. The illumination was 4500 lux during the day in both the Earthtron and incubator. There were six replicates of each treatment. The environmental conditions and management for each treatment are summarized in Table 1. The pots were incubated for 28 days. The soil was wetted manually with distilled water every other day to maintain the initial water content during the experiment, and loss of water per day was calculated for each pot.

Table 1 Summary of the conditions of each treatment in the pot experiment

Instrument with controlled factors (day/night)	Temperature (day/night)	Treatment	Management	Replicate
Earthtron illumination 4500 lux; CO_2 380/400 ppm; relative humidity 65/80 %	Air 25/16 ^oC	EA1	On top of the cabinet	6
		EA2	On top of the cabinet wrapped with aluminum foil	6
	Soil 22/19 ^oC	ES	In the cabinet with soil surface exposed to air temperature	6
Incubator Illumination 4500 lux; no control of CO_2 or humidity	Soil 22/19 ^oC	IS1	In the incubator	6
		IS2	In the incubator wrapped with aluminum foil	6

The soil temperatures at depths of 5 and 10 cm in the center of the soil cores were measured during the experiment with thermal probes in one pot per treatment. Relative humidity was also measured near the pots in each facility. The soil solution was sampled from every pot on days 7 and 21 of the incubation with a Rhizon soil moisture sampler (Rhizon MOM, Rhizosphere Research Products, Wageningen, The Netherlands) from the top of the soil. About 10 ml of soil solution was extracted by vacuum applied using a syringe for analysis of cations (Na, K, Mg and Ca) and anions (Cl, SO_4 and NO_3) with an ion chromatograph (IC-20, DIONEX, Calif.). After 28 days of incubation, extractable mineralized N ($N–NO_3$ and $N–NH_4$) of the soil at depths of 5 and 10 cm was determined in 1 *M* KCl extracts using an autoanalyzer (Integral Futura, Alliance Instruments, Frépillon, France). Soil pH (soil water ratio 1:2.5) was also determined at each depth.

3. Results and Discussion

Dynamics of soil temperature and water

Relative humidity just near the pots was controlled at about 45% during the day and 70% during the night in the Earthtron. In contrast, relative humidity in the incubator oscillated in a short cycle from 25% to 65% every day, probably because of periodical activity of the conditioning fan. The low wind speed in the Earthtron would have resulted in a steady humidity. The temperatures in both facilities were well controlled to the set values (Fig. 2). The EA1 soil cores showed a higher temperature than the controlled air temperature during the day, while the temperature of the EA2 cores

coincided with the controlled values (Fig. 2a, b). The aluminum foil wrapped around the EA2 pot efficiently cut radiant heating from the fluorescent lamps. No difference in temperature was observed between the 5 cm and 10 cm depths in each treatment. The IS1 soil core adjusted well to the controlled soil temperature at 10 cm depth, but the temperature was about 1°C lower at 5 cm than at 10 cm (Fig. 2d). The IS2 soil core showed similar thermal fluctuation to the IS1 core, but was about 2°C lower than the IS1 soil core during the day (Fig. 2e). The lower soil core temperature at 5 cm depth in the incubator was the result of evaporation resulting from the saturation deficit, which took the heat of vaporization from the soil surface (Nakano 1991). The fluctuation in temperature at the 5 cm depth in the ES soil core was smaller than that of the controlled air temperature and larger than that of the controlled soil temperature (Fig. 2c). The amplitude was damped at the 10 cm depth. Under field conditions, both the highest and lowest soil temperatures in a day appear at the surface and the soil temperature becomes constant toward the deeper layers (Miyazaki 1993). Soil cores controlled by the Earthtron showed similar temperature dynamics to those under field conditions. At both soil depths, the cumulative temperatures were higher in the EA1 and EA2 soil cores than in cores of the other treatments (Table 2). The cumulative temperature at 5 cm depth was higher than at 10 cm depth in the ES soil cores, while the cumulative temperature at the 10 cm depth was higher than at 5 cm depth in the IS1 and IS2 cores. The loss of water from the soil core was also different among treatments (Table 2). Treatment ES showed the lowest water loss. The soil water in the EA1 and EA2 soil cores was considered to evaporate more as a result of the higher soil temperature, while the higher saturation deficit between soil and air would result in a higher water loss in the IS1 and IS2 soil cores. Thus, the dynamics of soil temperature and water changed typically according to the environmentally controlled conditions.

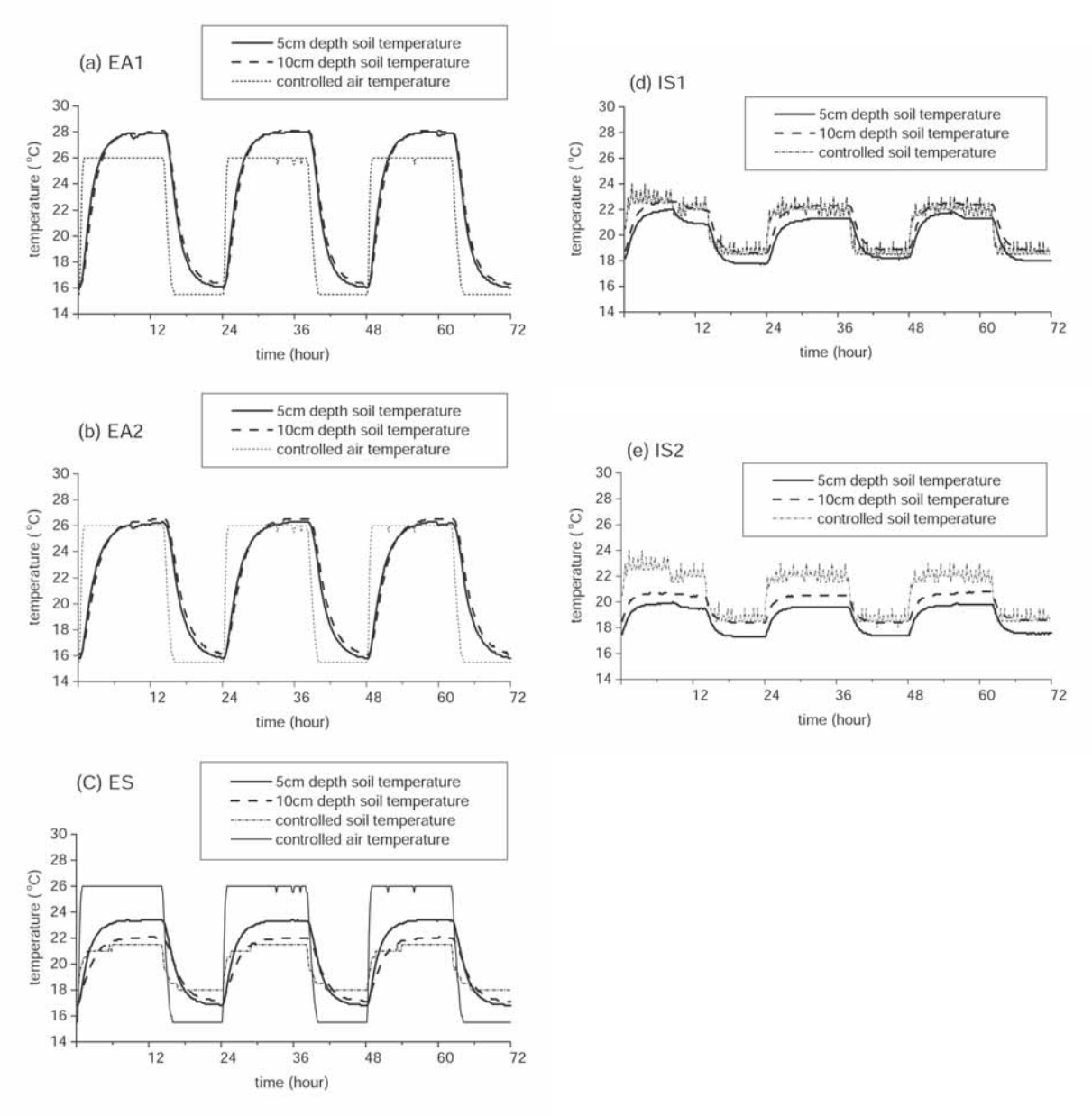

Fig. 2 Time-course of changes in soil core temperature at 5 and 10 cm depth over 72 h during incubation with the surrounding temperature controlled in each treatment

Table 2 Mean rate of water loss per pot, cumulative temperature during incubation, soil pH and mineralized N after incubation (mean ± S.D., n = 6) for each treatment

Treatment	Water loss (g/day per pot)	Cumulative temperature (°C)		Soil pH		Mineralized N (μg/g dry soil)	
		5 cm	10 cm	5 cm	10 cm	5 cm	10 cm
EA1	23.7	648	651	5.98 ± 0.02[a]	5.92 ± 0.02[a]	14.3 ± 2.2[a]	20.5 ± 1.4[a]
EA2	19.5	616	623	6.00 ± 0.02[a]	5.96 ± 0.01[ab]	12.9 ± 1.5[a]	17.4 ± 1.3[b]
ES	15.9	581	561	6.01 ± 0.01[b]	5.99 ± 0.01[b]	12.4 ± 0.7[a]	13.1 ± 0.5[c]
IS1	38.7	559	584	5.99 ± 0.02[ab]	5.93 ± 0.03[a]	8.0 ± 1.2[b]	15.1 ± 2.6[bc]
IS2	34.7	525	551	5.98 ± 0.01[a]	5.94 ± 0.02[a]	9.1 ± 1.0[b]	14.9 ± 1.5[bc]

Different letters in each column indicate that the values are statistically significantly different in the Sheffé test ($P < 0.05$).

Dynamics of soil nutrients

The soil pH with every treatment increased slightly after incubation from 5.89 on day 0 (Table 2). There were few differences between the 5 and 10 cm depths and the soil pH with treatment ES seemed to be slightly higher than with the other treatments. The cumulative amounts of mineralized N at the end of the incubation differed significantly among treatments (Table 2). The amounts of mineralized N in treatments EA1, EA2 and ES were larger than in treatments IS1 and IS2 at the 5 cm depth. There was no difference in concentrations of mineralized N between the 5 and 10 cm depths with treatment ES, while the other treatments showed higher concentrations at the 10 cm depth. The distribution pattern of mineralized N in the profile may be related to the dynamics of temperature and water in the soil. High cumulative temperature seemed to enhance N mineralization at each depth, but water flow in the soil complicates the distribution in the profile. The soil solution was investigated on days 7 and 21. An increase in cations and NO_3 concentrations in soil solutions was observed on day 21 compared to day 7 for every treatment (Table 3). The concentrations with treatment ES were smaller than with treatments EA1 and EA2, but larger than with treatments IS1

and IS2. The concentrations of NO_3 showed similar patterns to the amount of mineralized N in the whole core. Variations in environmental conditions affect the solubility of cations and anions as well as the distribution patterns of mineralized N in the profile. It was notable that dispersion of all data was suppressed with treatment ES, indicating accurate control by the Earthtron system.

Table 3 Cations and anions in soil solution extracted on day 7 and 21 of incubation (mean ± SD, n = 6) for each treatment

Treatment	Cations (ppm)				Anions (ppm)		
	Na	K	Mg	Ca	Cl	SO_4	NO_3
7 days incubation							
EA1	1.15 ± 0.07^{a}	11.4 ± 0.5^{a}	2.66 ± 0.16^{ab}	8.9 ± 1.2^{ab}	3.10 ± 0.16	2.25 ± 0.28	58.8 ± 3.8^{a}
EA2	1.16 ± 0.07^{a}	10.6 ± 0.4^{ab}	2.75 ± 0.30^{a}	12.1 ± 3.7^{a}	3.16 ± 0.13	2.73 ± 0.35	53.8 ± 3.8^{ab}
ES	1.02 ± 0.03^{b}	9.7 ± 0.3^{bc}	2.36 ± 0.16^{abc}	7.6 ± 0.5^{b}	3.19 ± 0.22	2.32 ± 0.27	50.3 ± 3.0^{bc}
IS1	0.99 ± 0.08^{b}	9.1 ± 0.6^{c}	2.18 ± 0.27^{c}	8.3 ± 2.5^{ab}	3.38 ± 0.29	2.58 ± 0.21	45.1 ± 5.5^{c}
IS2	0.99 ± 0.07^{b}	8.9 ± 0.6^{c}	2.26 ± 0.21^{bc}	8.8 ± 2.5^{ab}	3.21 ± 0.16	2.54 ± 0.46	44.7 ± 5.5^{c}
21 days incubation							
EA1	2.01 ± 0.14^{a}	18.2 ± 1.2^{a}	6.56 ± 0.69^{a}	19.7 ± 2.1^{ab}	3.30 ± 0.05	2.05 ± 0.11	120.2 ± 11.9^{a}
EA2	2.01 ± 0.10^{a}	17.2 ± 1.0^{a}	6.21 ± 0.54^{ab}	20.7 ± 2.9^{a}	3.36 ± 0.18	2.14 ± 0.30	112.5 ± 10.3^{ab}
ES	1.76 ± 0.02^{b}	15.0 ± 0.4^{b}	5.41 ± 0.15^{b}	16.0 ± 0.5^{bc}	2.86 ± 0.18	1.81 ± 0.07	97.3 ± 2.8^{b}
IS1	1.61 ± 0.10^{b}	13.1 ± 0.8^{c}	4.18 ± 0.44^{c}	12.6 ± 1.3^{c}	3.23 ± 0.71	2.35 ± 0.18	73.5 ± 8.2^{c}
IS2	1.60 ± 0.13^{b}	12.9 ± 1.0^{c}	4.22 ± 0.56^{c}	12.9 ± 1.7^{c}	3.17 ± 0.30	2.30 ± 0.36	73.5 ± 11.5^{c}

Different letters in each day and column indicate that the values are statistically significantly different in the Sheffé test ($P < 0.05$).

Advantages of using Earthtron

In the soil incubation experiment, the dynamics of temperature and moisture in the soil cores were changed by the surrounding temperature and humidity. The Earthtron was able to construct the characteristics of soil temperature under field conditions by means of separate temperature control systems for above-ground and below-ground conditions. Furthermore, the distribution patterns of soil nutrients in the profile reflected

the dynamics of soil temperature and moisture. Nutrient mineralization in the soil profile will dictate root response (Fujimaki et al. 2004), linking to the microbial activity in relation to root surfaces and rhizospheres (Coleman et al. 2004), and microbivorous nematodes (Papatheodorou et al. 2004). The distribution of soil temperature and moisture also affects the transition of soil invertebrates (Coleman et al. 2004). Such effects on microbe and soil invertebrates lead to changes in the decay rate of litter (Salamanca et al. 1998). Thus, accurate environmental control is necessary to measure and understand the interaction between above-ground and below-ground components in microcosm experiments.

In addition, the CO_2 concentration can be controlled in the Earthtron, providing an accurate estimation of plant growth in microcosm experiments. The TME (Knacker et al. 2004) described in the introduction did not take CO_2 concentration into consideration. Moreover, the Earthtron can be used to explore the effects of enhanced CO_2 and temperature on plant, soil biota and ecosystem dynamics, simulating global environmental changes. Microcosm experiments in the Earthtron provide not only a biologically realistic bridge between simple laboratory experiments and the complicated real world, but also allow prediction of the effects of global climatic changes on terrestrial ecosystems.

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Chapter 6

Soil ecological risk assessment of heavy metal pollution by using Terrestrial Model Ecosystems

Kamitani T, N Kaneko and H Oba

Abstract: Using intact soil system, called Terrestrial Model Ecosystem (TME), has recently been regarded as a valid method by soil ecotoxicologists. Our objective was to investigate the effect of applied Cu and aged heavy metal pollution on soil ecosystems including soil biota (microbial community, microarthropods and macrofauna) and ecological functions (N mineralization, elements in leachate, organic matter decomposition and plant growth) using TME approach, and to demonstrate the utility of the TME approach for soil ecotoxicological studies by comparison with a homogenized soil system. Soils for experiments were taken from heavy metal polluted and reference site on a floodplain. The community structure of microbes and oribatid mites, and earthworm biomass were soil biological parameters affected significantly by spiked Cu. The Cu application also had significant effects on several functions including soil ammonium-N concentration and plant growth. However, these results did not necessarily predict the field situations affected by aged heavy metal pollution. The intact soil system was able to mimic the functional parameters *in situ* compared to homogenized systems. In conclusion, despite several limitations and requirements for further research, TMEs contribute valuable systems for ecological risk assessment.

Keywords: earthworm, ecological function, heavy metal pollution, soil biota, Terrestrial Model Ecosystem

1. Introduction

Ecotoxicological effects of soil pollution have been assessed by use of single species tests, such as acute and chronic tests in homogenized systems in the laboratory. A homogenized system may be set up easily and ensure uniform exposure of the organisms to the test chemical (Burrows and Edwards, 2002). Such a testing based approach of ecotoxicology greatly benefited environmental regulation for deriving maximum acceptable chemical concentrations (Van Straalen, 2003). However, further problems arise because many of the species used in such tests are not found commonly in soil (Bogomolov et al. 1996). For example, the earthworm *Eisenia fetida*, used widely as a test earthworm, is a coprophagous species living in manure, not in soil (Paoletti, 1999). Moreover, since studies have focused on only a few and single species, it is also recognized that these tests cannot predict effects that critically depend on the interaction among many species in field conditions (Cortet et al., 1999; Van Straalen, 2002). Therefore, simplified laboratory 'microcosm' tests, constructed to surrogate for the complicated field situation (Beyer and Odum, 1993; Verhoef, 1996), were begun to be used in soil ecotoxicology. Studies in microcosms could provide the effect of pollutants on soil biota under predator-prey relationships [e.g. Gamasida and Collembola (Hamers and Krogh, 1997); centipede and housefly larvae (Kramarz and Laskowski, 1999)], soil faunal community and trophic structure (Parmelee et al., 1997), and ecological function [litter decomposition by isopods (Vink and van Straalen, 1999)]. More complicate microcosms in homogenized system have been also used for an ecosystem approach to soil toxicity testing, allowing for integration of the effects of chemicals on community structure and ecological function in soil (Bogomolov et al,

1996; Martikainen et al., 1998; Burrows and Edwards, 2002).

Recently, using a type of soil microcosm, called Terrestrial Model Ecosystem (TME), has been regarded as a valid method by soil ecologists and especially, soil ecotoxicologists (Van Straalen 2002). TME approach uses undisturbed soil columns taken from field and includes living vegetation growing on the soil allowing for interactions between soil-living organisms and plant roots. Thus TME includes the structure of soil system, which was defined as "the composition and distribution of the soil biological community, and the quality and distribution of abiotic materials such as nutrient and contaminants" (Sheppard, 1997). In international programs sponsored by the EU, significant progress has been made in the standardization and field validation of TME, which was published as a series of papers in Ecotoxicology vol. 13 (Knacker et al. 2004).

Our objective was to investigate the effect of applied Cu and aged heavy metal pollution on soil ecosystems including both soil organisms and functions using TME approach, and to demonstrate the utility of a TME approach for soil ecotoxicological studies by comparison with homogenized soil system. In Japan, there are few basic toxicological studies concerning domestic soil animals, which have probably different toxicological characteristics from European species dealt in most ecotoxicological studies.

2. Material and methods

2.1. Microcosm design and soil preparation

Microcosms consisted of cylinders made from polyvinyl chloride (PVC) pipe, 15.5 cm i.d. and 30 cm high. The bottom of each cylinder was sealed with a PVC lid, and small hole (6 mm i.d.) was opened on side face at 15 mm high from bottom to allow the installation of Rhizon soil moisture sampler (Rhizon MOM, Rhizosphere Research Products, Wageningen, the Netherlands).

We conducted two types of examination: tier I (homogenized soil system) and tier II (intact soil system). In tier I, freshly collected field soils, both heavy metal polluted and reference soil, from Watarase Retarding basin (Kamitani et al., in press a, chapter 2), were sieved through a 5 mm screen, packed gently into cylinders with dry bulk density at 0.47 g cm^{-3}, and moistened with deionized water to 60 % of the total water holding capacity (25 cm depth). Twenty replicates of polluted and reference soils were prepared (named HP and HR, respectively), and each microcosm was implanted with two seedlings of *Miscanthus sacchariflorus*, which were germinated from seed sampled from the reference site of Watarase Retarding basin.

In tier II, intact system called Terrestrial Model Ecosystems (TMEs) were used to study the effects of spiked Cu or aged heavy metal pollution on soil biota and ecological finction. TME soil core was taken at the study site by hand shovel in April 2004. The cylinder mentioned above was set on the ground to let the sprout of *M. sacchariflorus* centered in the core, inserted carefully by digging around the sidewall to the depth at 25 cm, and then taken up. The dry weight of soil in each cylinder was about 3.2 kg. The sprouts in TMEs were cut, and after an acclimatization period of 2 weeks, the TMEs found new sprout from the *M. sacchariflorus* stock were used for analysis. A Rhizon soil moisture sampler inserted vertically from soil surface to 10 cm depth was used for application of $CuSO_4$ to TMEs in order to prevent the chemical to be adsorbed at the

surface of soil core. We started the experiment just after applying Cu solution. Twelve replicates in each two treatment levels (300 and 600 mg Cu kg^{-1} dry soil, inoculation of 100 ml of 10,000 ppm Cu and 20,000 ppm Cu, respectively), twelve controls and six field polluted cores were used (IT1, IT2,IR and IP, respectively).

2.2 Environmental design

Microcosms and TMEs were placed in Earthtron (DALTON Company, Tokyo, Japan), which is a computer-controlled chamber with cabinets for incubation of soil core, simulating natural environments: diurnal light/dark cycles; separately controlled soil and air temperature; humidity; CO_2 concentration (Kamitani and Kaneko, in press b, chapter 5). Temperature over the cabinet (air temperature) was controlled at 25^{o}C during days (14 h) and 18^{o}C in nights (10 h) during experiment period. Relative humidity and CO_2 concentrations were controlled 65 % and 380 ppm in days, 80 % and 400 ppm in nights, respectively. Tier I started from February 2004 for 7 week, and tier II started May 2004 for 10 weeks. Soil cores were irrigated with automatic water supply system (EY4200, National, Japan). Based on the average precipitation of the field site, the amount of supplied water had been determined to 50 mL every third day. However, we adjusted the amount of water manually dependent on the amount of leachate from column.

2.3. Measurement endpoints

Heavy metals in soil were analyzed in both tier experiments. The measurement

endpoints were chosen based on the soil biota (tier II) and ecological function (tier I and II) (Table 1).

Table 1. Measurement endpoints analyzed, and the depth of the soil profile investigated.

Endpoint	Tier 1 (microcosms)	Tier 2 (TMEs)
Soil properties		
Total heavy metals	Soil layer: 0-5 cm	Soil layer: 0-5 cm
		Soil layer: 5-25 cm
Exchangeable heavy metals	Soil layer: 0-5 cm	Soil layer: 0-5 cm
Soil pH	(see chapter 2)	Soil layer: 0-5 cm
Soil biota		
Microbial community (BIOLOG test)	-	Soil layer: 0-5 cm
Microarthropods	-	Soil layer: 0-5 cm
Macrofauna	-	Soil layer: 0-5 cm
		Soil layer: 5-25 cm
Ecological function		
Soil inorganic N (NH_4-N and NO_3-N)	Soil layer: 0-5 cm	Soil layer: 0-5 cm
Leaching ions	After passage through soil core (each 2 weeks)	After passage through soil core (each 2 weeks)
Organic matter decomposition (cellulose paper)	Soil layer: 0-5 cm	Soil layer: 0-5 cm

Phospholipid fatty acid (PLFA), mycorrhiza, and plant growth were also analyzed by a partner. We do not discuss these results in this thesis.

Heavy metal and pH analysis

The soil after experiment was sampled, sieved through a 2 mm mesh and dried at 60 ^{o}C for 72 h. To estimate total amount of heavy metals (Cu, Zn, and Pb), 1 g dry soil was heated with 10 mL concentrated HNO_3, starting at 80 ^{o}C (Kamitani et al., in press,

chapter 2). The samples were then dried at 135 ^{o}C, redissolved in 40 mL 0.1 M HNO_3, centrifuged and filtered. The exchangeable (available) fraction of heavy metals was measured by extraction with 0.01 M $CaCl_2$ (Novozamsky et al., 1993). Four grams of dry soil was shaken with 20 mL $CaCl_2$ solution for 20 h, and then centrifuged and filtered. Heavy metals were analyzed with ICPAES (ICPS-8000E, SHIMADZU Co., Kyoto, Japan). In tier II, the soil was separated into 0-5 cm and 5-25 cm deep, analyzed for total concentration at each layers, and for available fraction at only 0-5 cm deep.

We measured soil pH with 0.01 M $CaCl_2$ extract of 0-5 cm soil in tier II, which was also used for the exchangeable fraction of heavy metals mentioned above.

Soil biota analysis

The measurements on soil biota were conducted only at TME experiment (tier II). Microbial community after measurement was determined by using BIOLOG® Ecoplates (Biolog Inc., Hayward, CA, USA) after Kamitani et al. (in press, chapter 2). Ten grams of soil, sampled at 5 cm depth after 10 weeks incubation, was shaken in 90 g sterile water with a reciprocal shaker set at 180 oscillations min^{-1} for 10 min, and 1 mL supernatant was diluted with 99 mL sterile water. Ecoplates were inoculated with 150 μL of the 10^{-3} diluted soil suspension to each well. The plates were incubated at 25^{o}C for 7 days and absorbance of 590 nm (A_{590}) was measured every 24 h for 7 days with a microplate reader (Multiskan JX, Thermo Labsystems, Helsinki, Finland). Wells were considered to be positive in terms of substrate utilization if the value of A_{590} in wells was 0.25 units greater than that in control wells (Toyota et al., 2000). The values of average well color development (AWCD) were calculated for each plate. The data was

standardized by subtracting the mean value of the control wells from all well color values and then dividing by AWCD (Garland and Mills, 1991).

Microarthropod in 5 cm deep soil was extracted after the experiment using a Tullgren apparatus for three days. Specimens of mites and collembola were preserved in 80 % ethyl alcohol, and put on glass slide. Oribaited mites were identified at the species level, and the individual number of mites and collembola were counted using an optical microscope. Biomass of Collembola was calculated by multiplying estimated individual number by typical average individual weight 2.7 μg (Petersen and Luxton, 1982). The length and width of mite was measured and the corresponding dry weight was calculated based on regression equations of length and width on dry weight (Engelmann, 1961, see chapter 3).

Macrofauna was collected from soil cores after the experiment. The soil was separated into 0-5 cm and 5-25 cm deep. Macrofauna in 0-5 cm soil was hand-sorted, followed by extraction from soil using Tullgren apparatus for three days. Macrofauna in 5-25 cm was only hand-sorted. All specimens were preserved in 80 % ethyl alcohol, identified at order or class level using a stereomicroscope. Earthworms were identified at family level and weighted.

Soil function analyses

Soil inorganic N (NH_4-N and NO_3-N) was measured after both experiments. Fresh soil (5 g) from 5 cm depth was extracted with 30 mL of 1 M KCl. Extracted NH_4-N and NO_3-N were analyzed with an auto-analyzer (INTEGRAL FUTURA, ALLIANCE Instruments, Frépillon, France). In tier I, the soil samples were randomly selected from

10 microcosms in each treatment, and in tier II, five TMEs were selected from each treatment for inorganic N analysis.

Soil leachates were collected from the bottoms of soil core by using Rhizon soil moisture samplers and 10 ml syringes. Soil leachates were collected five times for two weeks, each sample was approximately 10 ml. Leachates collected for 2 weeks from a certain soil core were accumulated in the same 50 ml polypropylene bottle at -30 ^{o}C. After each two week, leachate samples were defrosted and filtered with 0.45 μm mesh for cations (NH_4, Na, K, Ca, Mg) and anions (Cl, NO_3, SO_4) analyses by an ion chromatograph (IC-20, Dionex Co., CA, USA).

The potential of organic matter decomposition in the study soils was determined with Whatman Benchcote sheet (Whatman Ltd., Brentford, Middlesex, UK), which is a polyethylene backed absorbent cellulose paper (Kamitani et al., in press, chapter 2). Before burying, the papers were dried in a desiccator and weighted. The dimension of cellulose paper was 3 cm in width by 5 cm in length. We inserted one paper per microcosm (tier I) and three papers per TME (tier II) vertically into upper soil layer (0-5 cm). In tier I, the paper was removed from each microcosm at 32 days. In tier II, the papers were removed from each TME at 20, 40 and 70 days. The papers were dried (18h at 60^{o}C) after loose soil particles and debris had been eliminated. The final weight of paper was determined as a weight loss after ignition (1.5 h at 750 ^{o}C) of the dried paper.

The cellulose decomposition rate was expressed by the percentage of dry weight of cellulose after an incubation period prior to burial, as follows:

weight = $(w_0 - w_t)/(w_0 - w_p)$

where, w_0 and w_t were the weight of the benchcote sheet at time 0 and t, respectively. w_p is the weight of polyethylene, which is 21 % of the benchcote sheet weight.

2.4. Statistical analyses

T-tests were conducted for the comparison of heavy metal concentrations, leaching cations and anions concentrations, and remaining weights of cellulose papers between HR and HP in tier I. Mann-Whitney U-test was carried out to compare soil inorganic N concentrations between HR and HP in tier I and to compare the abundance of macrofauna and biomass of earthworm among treatments in tier II. One-way ANOVA was conducted for soil pH, the number of positive wells in BIOLOG test, the (*log* x + 1) transformed number and biomass of microarthropods, soil inorganic N concentrations, leaching canions and anions concentrations, and remaining weights of cellulose papers. Post hoc tests (Tukey-Kramer or Sheffe) were performed to compare means if the anova indicated significance.

The data from the BIOLOG tests based on equivalent AWCD values of about 1.0 for each plate, and the species composition of Oribatida were subjected to principal component analyses (PCA) using Canoco for Windows, version 4.5. In the analysis of Oribatida, the species data were (*log* x + 1) transformed. Rare species, i.e. those having fewer than 10 individuals in the total sample, were excluded from this analysis.

3. Results

3.1. Tier I (homogenized soil system)

The total heavy metal concentrations (Cu, Zn and Pb) were significantly higher in

HP than HR (Fig. 1(a)). Cu concentration was highest, followed by Zn and Pb, which reflected the contaminated condition *in situ* (Kamitani et al., in press, chapter 2). The exchangeable fractions of heavy metals were very low and only Zn was significantly higher in HP than HR (Fig. 1(b)). Soil inorganic N did not change significantly between HR and HP (Fig.2). High variability was found in values of both NH_4-N and NO_3-N in HR. The leaching of cations and anions were significantly different between treatments until about 6 weeks after incubation (Fig. 3). Ammonium ion was not detected at all sampling time in both treatments. Microcosms of HR had significantly higher K, Mg, Ca and NO_3 concentrations in the leachates collected 2, 4 and 6 weeks after incubation. Conversely, Microcosms of HP had significantly higher SO_4 concentrations in the leachates collected 2 and 4 weeks after incubation. Cations and anions in leachates except for Cl did not differ significantly between treatments 8 weeks after incubation. Cellulose filter paper inserted in the top soil remained on average 34.1 % of the initial weight in HR and 68.6 % in HP until 32 days after incubation (Fig. 4). The decomposition rate of cellulose was significantly retarded in HP compared to HR.

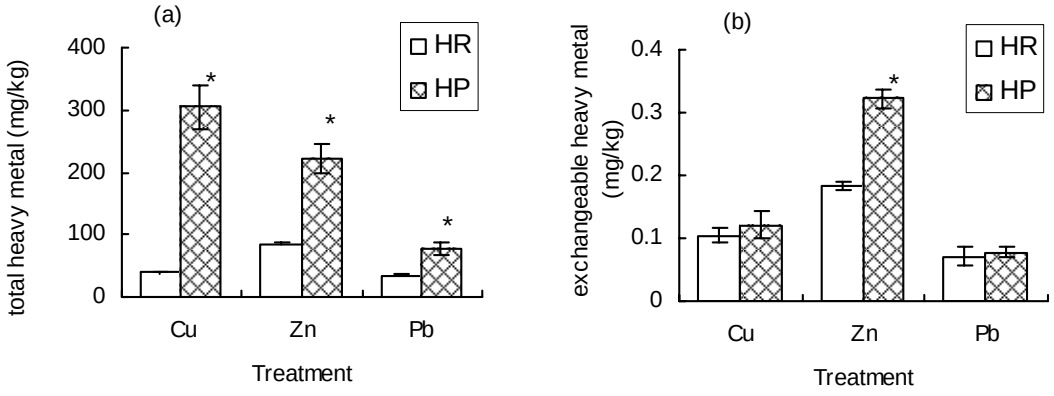

Fig. 1 Heavy metal concentrations in soil [total concentration (a) and exchangeable fraction with $CaCl_2$ (b), mean ± SD, n = 3] of tier I. Stars indicate statistically significant different values in the t-test ($P < 0.05$).

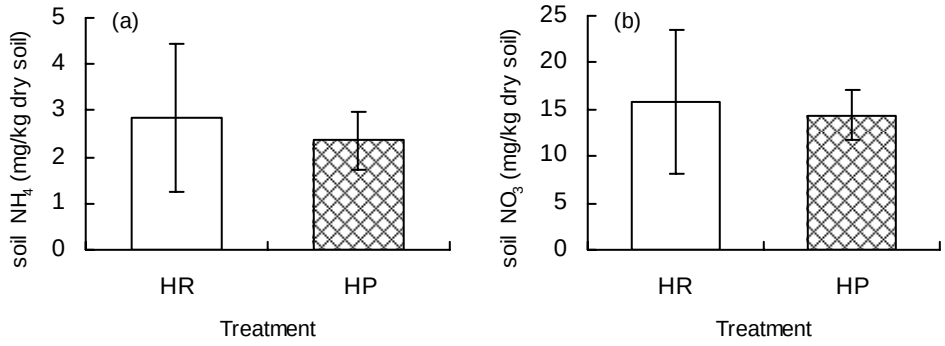

Fig. 2 Soil inorganic N concentrations [NH_4 (a) and NO_3 (b), mean ± SD, n = 10] of tier I. No significant difference was observed between treatments.

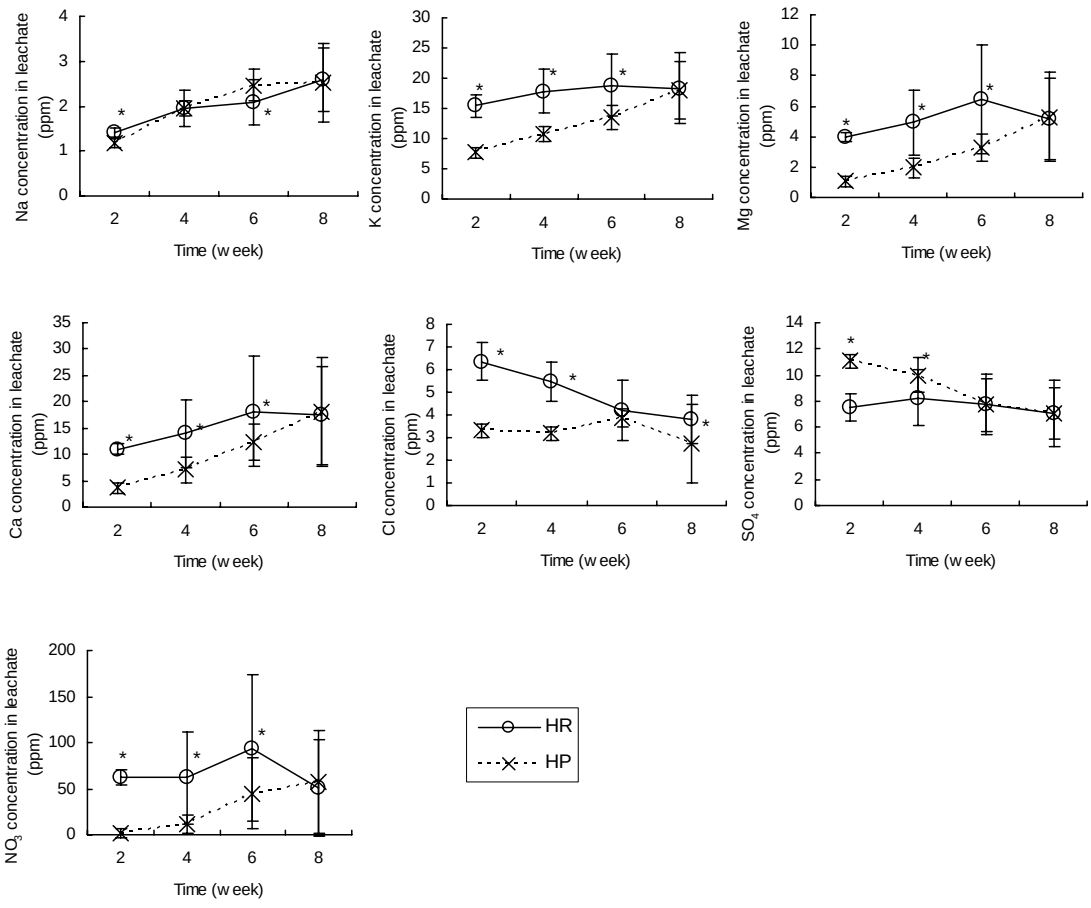

Fig. 3 leaching cations and anions concentrations (mean ± SD, n = 20) of tier I at several times after incubation. Stars indicate statistically significant different values in the t-test ($P < 0.05$).

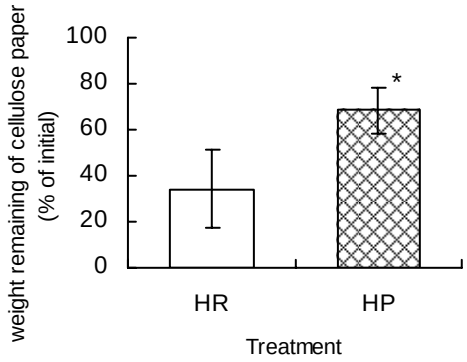

Fig. 4 The remaining weight of cellulose paper inserted in the top soil (mean ± SD, n = 20) of tier I. Stars indicate statistically significant different values in the t-test ($P < 0.05$).

3.2. Tier II (intact soil system)

Heavy metal concentrations and soil pH

The total heavy metal concentrations of IR and IP were similar to those of HR and HP in tier I, respectively (Fig. 5(a)). The Cu concentrations at 5 cm depth were higher in IT1 (average 1220 mg/kg) and IT2 (average 1740 mg/kg) than expected (300 and 600 mg/kg, respectively). We attempted to prevent Cu to be adsorbed at the surface of soil. However, Cu remained in the top layer of soil, resulting in low concentrations in deeper layers (217 and 291 mg/kg, respectively). The Zn and Pb concentrations in IT1 and IT2 decreased significantly compared to IR. The exchangeable Cu concentration showed typical increase in IT1 and IT2 (Fig. 5(b)) in comparison with IR and IP. The exchangeable Cu concentration Zn and Pb also increased in IT1 and IT2 in spite of no application of these metals. Soil pH in IT1 and IT2 were significantly lower than IR and IP, but the values in all treatments converged on 5.1-5.7 (Fig. 6).

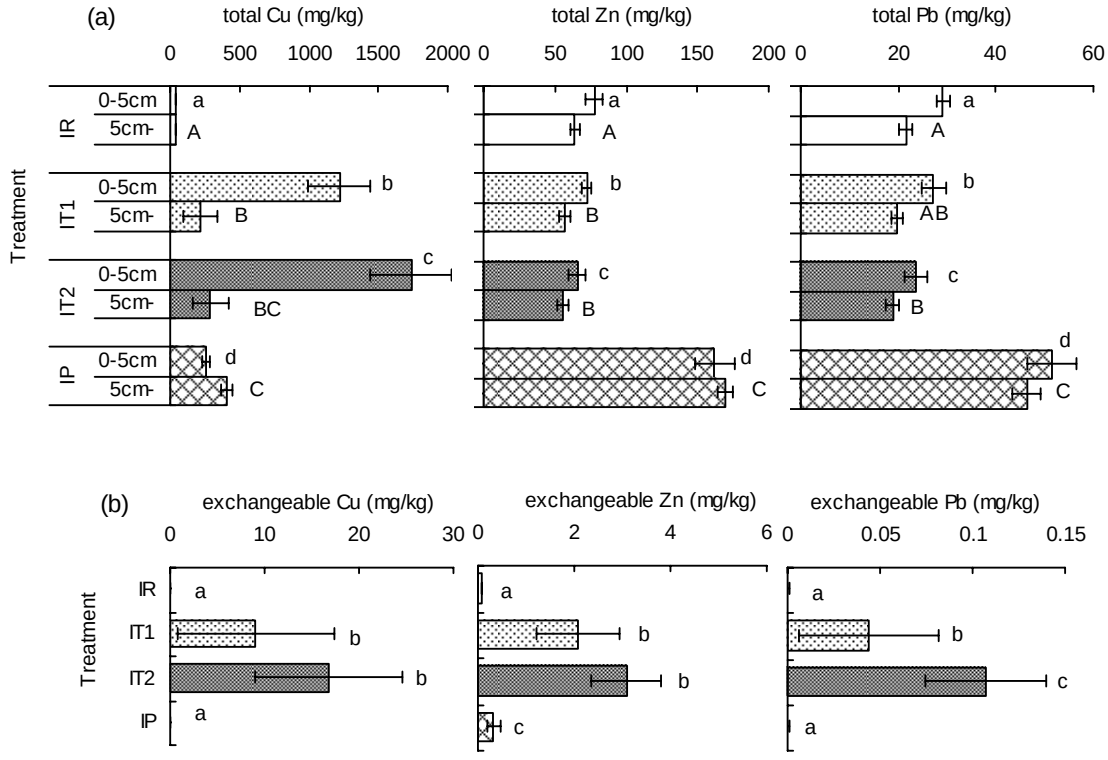

Fig. 5 Heavy metal concentrations in soil [total concentration (a) and exchangeable fraction with $CaCl_2$ (b), mean ± SD] of tier II. The replicates in total concentrations are 12 (0-5 cm of IR, IT1 and IT2), 6 (0-5 cm of IP), and 5 (5-25 cm of all treatments). The replicates in exchangeable concentrations are 5. Bars for the same soil layer having the same letter do not differ significantly from each other ($P<0.05$).

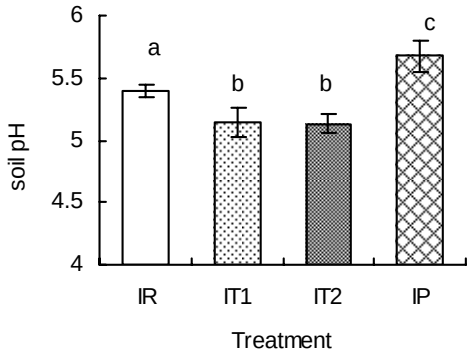

Fig. 6 Effect of Cu addition on soil pH (mean ± SD, n = 5) of tier II. Bars having the same letter do not differ significantly from each other.

Soil biota

Ability of microbial C substrate utilization expressed by the number of positive wells in BIOLOG test was significantly higher in IR from 2 to 7 days after incubation compared to IT1, and from 3 to 5 after incubation compared to IT2 (Fig. 7). The number of positive wells in IP did not differ significantly from those in IR. The result of statistical analysis by PCA enabled us to separate roughly IT1, IT2 and IP from IR (Fig. 8). IR plots were situated on the center of axis 1, and center and positive side of axis 2. IP plots were situated on the positive side of axis 1, while IT1 was on the negative side of axis 1. IT2 plots were situated widely on axis 1, and mainly on the negative side of axis 2.

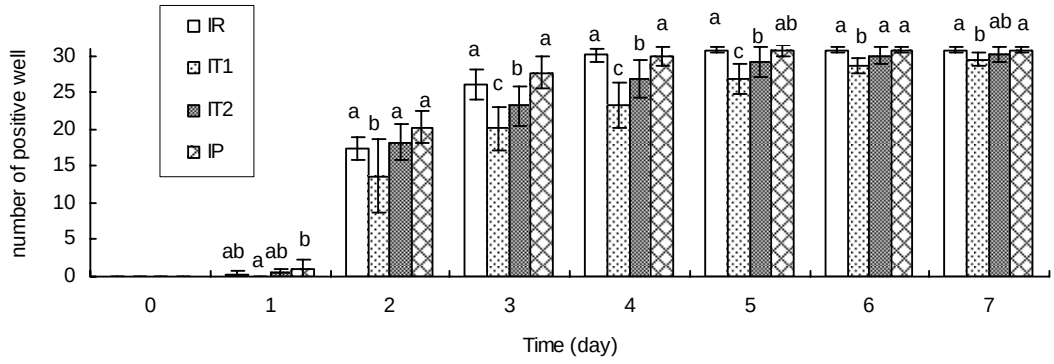

Fig. 7 Effect of Cu addition on the ability of C substrate utilization (BIOLOG Ecoplate) by soil microbial community (mean ± SD, n = 15) of tier II. Bars for a certain time having the same or no letter do not differ significantly from each other.

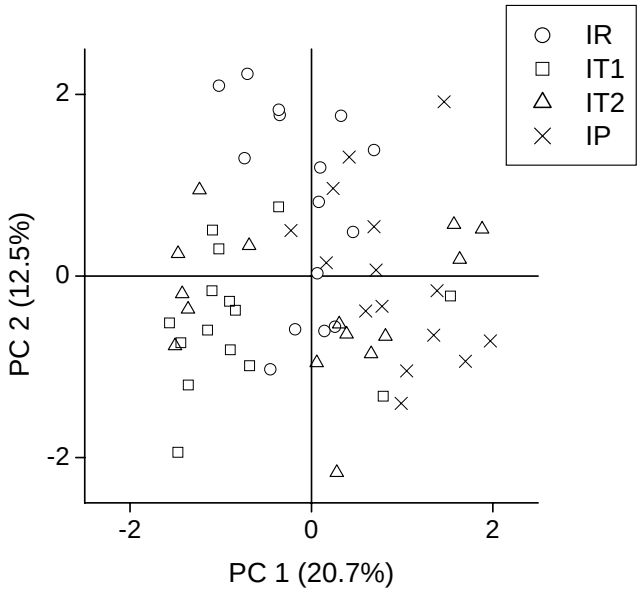

Fig. 8 Two-dimensional principal component diagram of C substrate utilization profiles. Data based on AWCD values of ca. 1.0.

The numbers of soil microarthropods were not significantly different among treatments except for Gamasida, which showed large abundance in IP (Fig. 9a). Oribatida showed the largest numbers among other arthropods in each treatment. The numbers of oribatid mites at the species level in each treatment were listed in Appendix 1. The number of species was higher in IR (33 species), followed by IT1 (31 species), IT2 (27 species) and IP (24 species), while Shannon-Wiener index was higher in IP (2.45), followed by IR (2.25), IT1 (1.98) and IT2 (1.65). *Oppiella nova* was the most dominant species in all treatments, but the percentage in oribatid mite community was quite variable: 44 % in IR; 53 % in IT1; 62 % in IT2; and 26 % in IP. The difference in species composition of oribatid mites was investigated by PCA. The results of PCA showed no difference of oribatid mite species composition among IR, IT1 and IT2: these plots aggregated on the center and negative side of axis 1 (Fig. 10). On the other hand, the plots of IP were situated on the positive side of axis 1. The species

Cosmohermannia frondosa, *Masthermannia hirsute* and *Nippohermannia* sp., which are the same family Nanhermanniidae, were situated on the positive side of axis 1, while Oppiidae sp. 2, *Oppiella nova*, which was dominant in all treatments, and *Eohypochthonius magnus* were situated on the negative side of axis 1. Among the biomass of arthropods, only Oribatida showed significant difference among treatments: biomass in IP was about two times larger than other treatments, although no significant change was observed in the number of individuals (Fig. 9b).

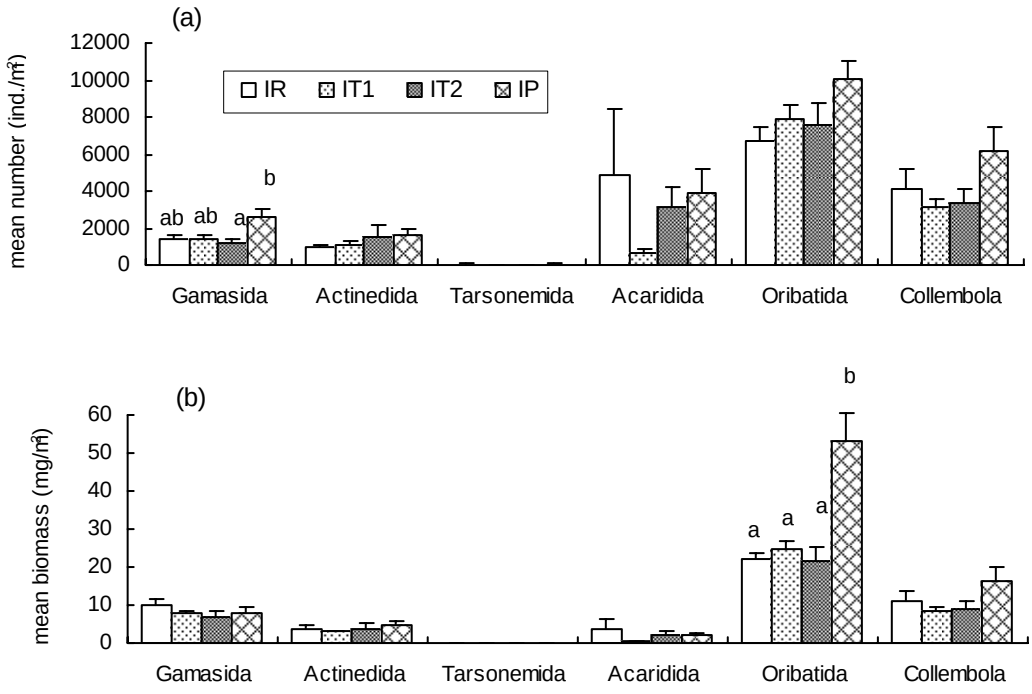

Fig. 9 Effect of Cu addition on the number (a) and biomass (b) of soil microarthropods [mean ± SE, n = 12 (IR, IT1 and IT2), 6 (IP)] of tier II. Bars for a certain group having the same or no letter do not differ significantly from each other.

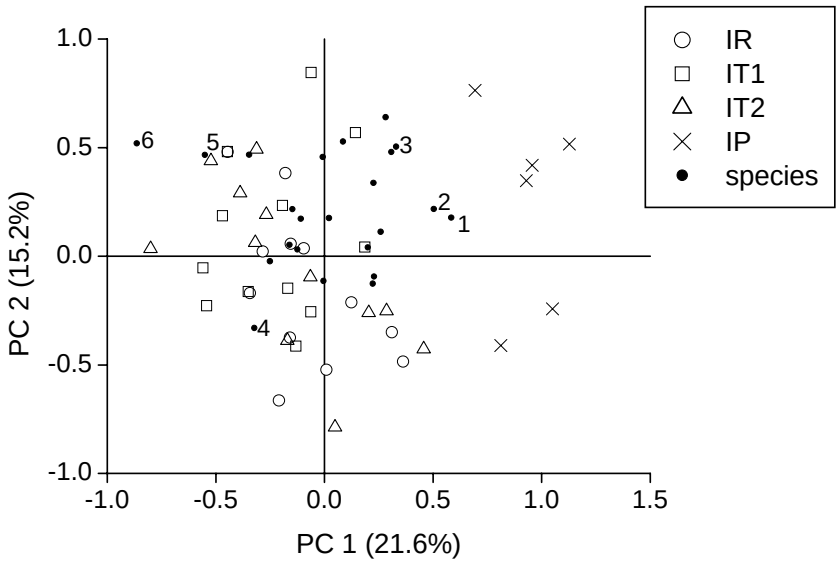

Fig. 10 Two-dimensional principal component diagram of oribatid mite community. The letters on the right of the dots mean species below, 1 = *Cosmohermannia frondosa*, 2 = *Masthermannia hirsute*, 3 = *Nippohermannia* sp., 4 = *Eohypochthonius magnus*, 5 = *Oppiella nova*, and 6 = Oppiidae sp. 2

The abundance of macrofauna except for earthworms is shown in Fig. 11. A high variability in the same treatment was observed, and no effect of Cu addition was detected for any group. Geophilomorpha in IP was significantly large in number, while Lithobiomorpha in IP tended to decrease compared to other treatments. Earthworm abundance appeared to decrease with Cu addition, and was significantly reduced in IT2 (Fig. 12a). In the family level, all three groups (Megascolecidae, Moniligastridae and Lumbricidae) tended to decrease in number, but no statistically significant difference was observed among IR, IT1 and IT2. The abundance of Moniligastridae in IP was significantly higher than in other treatments. Earthworm biomass decreased in response to Cu addition (Fig. 12b). The biomass of megascolecid worms in IT2 and IP were significantly lower than IR, while the biomass of moniligastrid worms in IP was higher than other treatments.

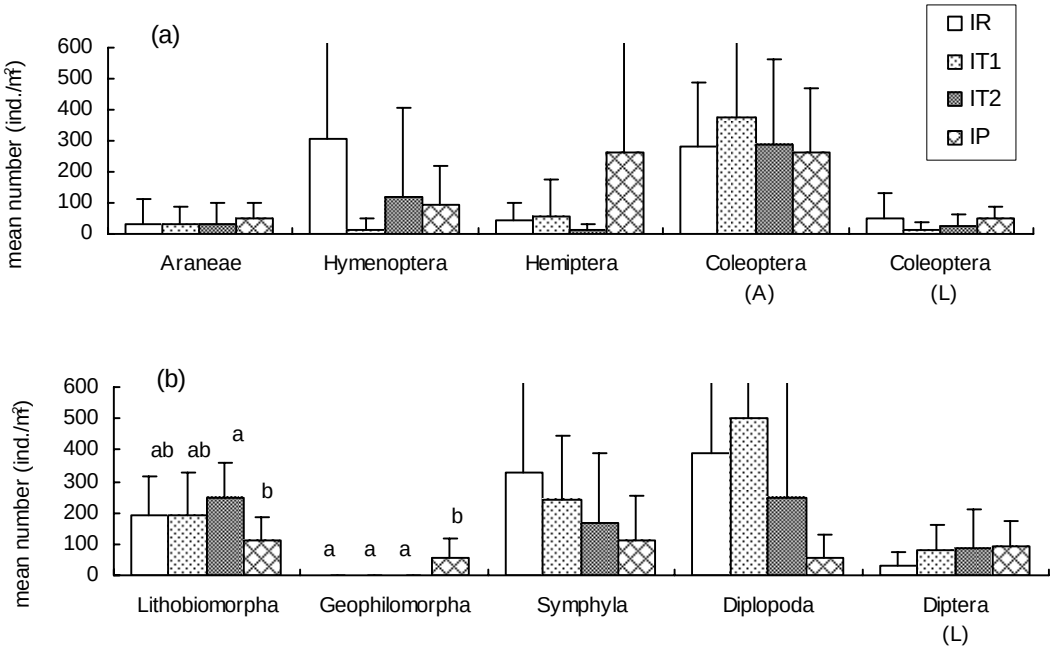

Fig. 11 Effect of Cu addition on the number of soil macrofauna [mean ± SD, n = 12 (IR, IT1 and IT2), and 6 (IP)] of tier II. Bars for a certain group having the same or no letter do not differ significantly from each other.

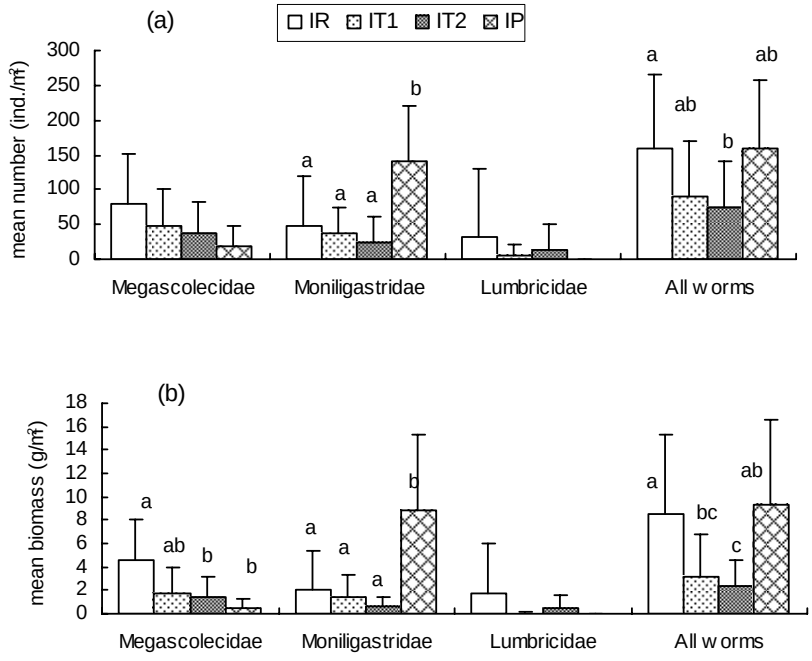

Fig. 12 Effect of Cu addition on the number (a) and biomass (b) of earthworm [mean ± SD, n = 12 (IR, IT1 and IT2), and 6 (IP)] of tier II. Bars for a certain group having the same or no letter do not differ significantly from each other.

Ecological functions

A significant increase of soil NH_4-N was observed for IT2 (Fig. 13) compared to IR and IP, while there was no significant change in NO_3-N among treatments, although the values tended to increase in response to Cu additions. The leaching of other cations and anions were not affected significantly by Cu additions (Fig. 14). Ammonium ion was not detected at all sampling time in both treatments. Leachate in IP tended to decrease in K, Mg, Ca and NO_3, and increase in Na, Cl and SO_4. Leaching NO_3 was significantly higher from the beginning up to 8 weeks in IR, IT1 and IT2 compared to IP. With respect to SO_4, IT2 increased and dispersed at 6, 8 and 10 weeks after incubation. The mean weight of remaining cellulose decreased gradually at all treatments (Fig. 15), and the decomposition rate of cellulose did not significantly differ among treatments at any time.

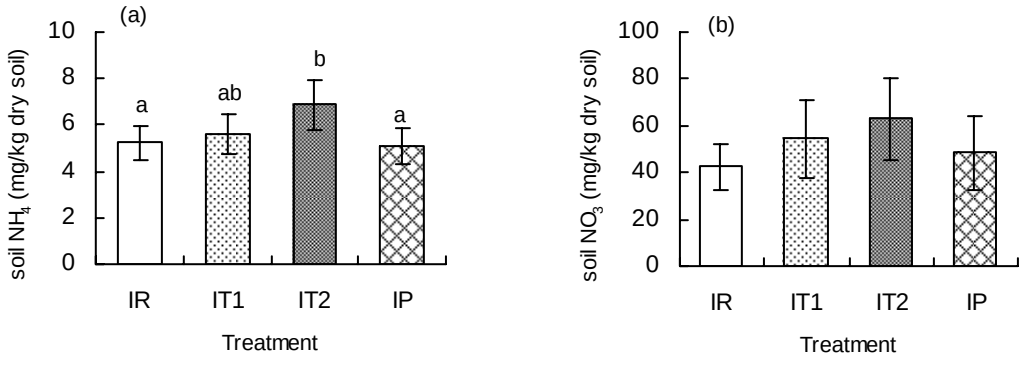

Fig. 13 Effect of Cu addition on soil inorganic N concentrations: NH_4 (a) and NO_3 (b) (mean ± SD, n = 5) of tier II. Bars for a certain treatment having the same or no letter do not differ significantly from each other.

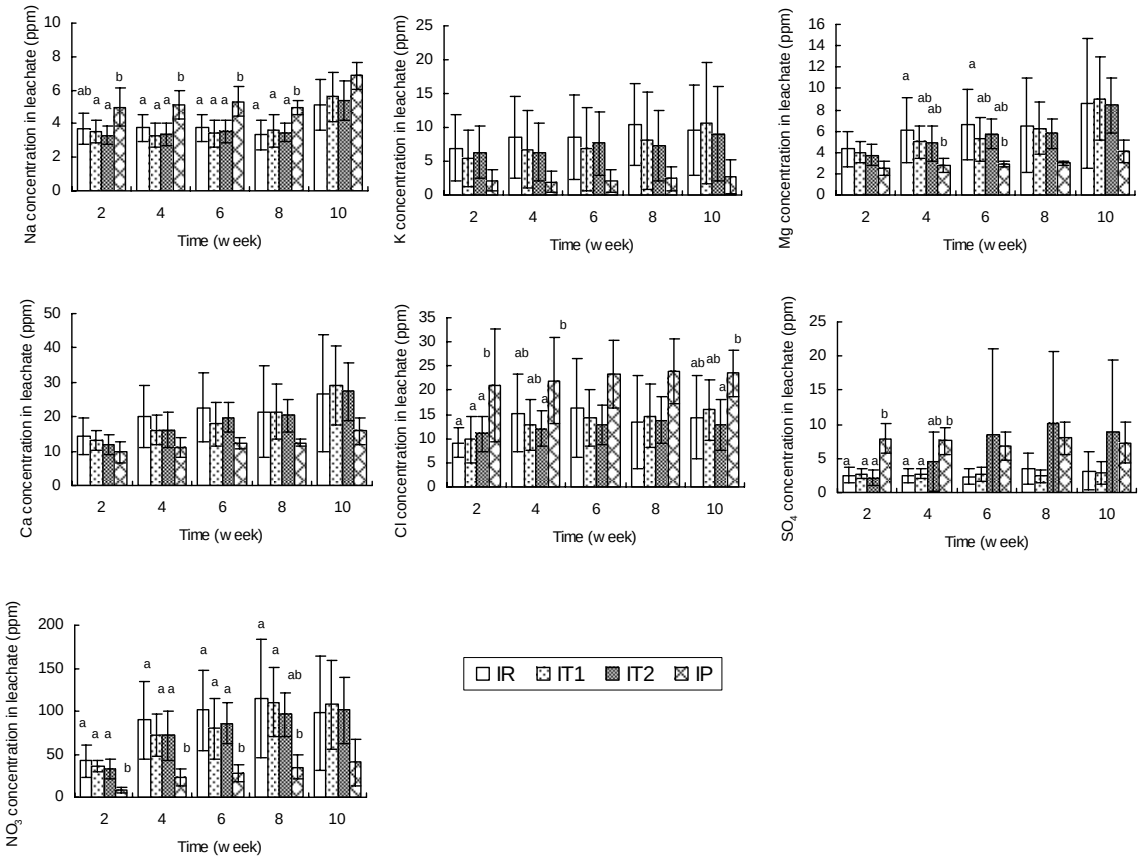

Fig. 14 Effect of Cu addition on leaching of various cations and anions [mean ± SD, n = 12 (IR, IT1 and IT2), and 6 (IP)] of tier II. Bars for a certain time having the same or no letter do not differ significantly from each other.

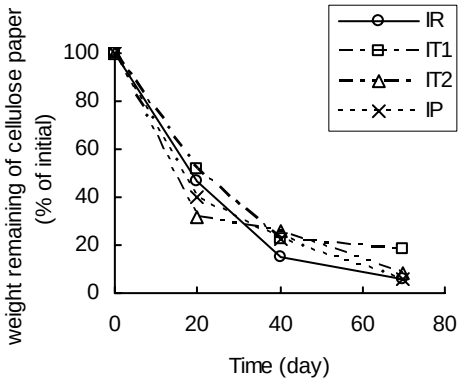

Fig. 15 Effect of Cu addition on the remaining weight of cellulose paper inserted in the top soil [mean ± SD, n = 12 (IR, IT1 and IT2), and 6 (IP)] of tier II. No significant differences were observed.

4. Discussion

First, we discuss the effects of spiked Cu and aged heavy metal pollution on soil biota and ecological function in intact system (tier II), and compared the functional aspects in intact system with the homogenized system (tier I). Then, we mentioned the validation of using intact soil systems for ecological risk assessment.

4.1. Heavy metal distribution in intact soil core

Although we attempted to inject Cu solution to deeper layer of intact soil core, high Cu concentrations were observed within 5 cm deep (Fig. 5a). It was very difficult to reproduce the heavy metal soil profile *in situ* in intact soil system because of high adsorption efficiency of Cu to soil (Sparks, 1986). Using chemicals sprinkled on the soil surface, e.g. fungicide, would be appropriate to intact soil systems for ecotoxicological studies, but another method is necessary when we need to spike toxicants to deeper soil layer. The exchangeable fraction of heavy metals, extracted with $CaCl_2$ solutions, was at low levels in IP (Fig. 5b), which was also observed in the study site *in situ*, resulting partly from a long aging period, which increased the binding of metals to soil (Kamitani et al., in press, chapter 2). The effect of ageing on extractability was also revealed in IT1 and IT2 for 10 weeks incubation: $CaCl_2$-extractable Cu fractions never exceeded 1% of the total soil Cu (9.0 and 16.7 mg/kg, respectively). However, $CaCl_2$-extractable Cu in IT1 and IT2 were much higher than IR and IP. Because the bioavailable fraction of the pollutant is usually more strongly related to free Cu concentrations in the pore water than the total concentration in soils (Van Straalen, 2002), the effects on soil biota and

ecological functions would be different between spiked Cu and aged Cu in soil (mentioned below). Zn and Pb also were dissolved in $CaCl_2$ extracts at low levels, but statistically larger in IT1 and IT2, probably due to depression of specific sorption of these metals by competition with high concentrations of applied Cu (Atanassova, 1999).

4.2. Effect of spiked Cu and aged heavy metals on soil biota

We compare soil biota in unpolluted field soil (IR) to Cu spiked field soils (IT1 and IT2) for estimation of applied Cu effects on soil organisms in the condition of semi-field intact system. The differences between Cu spiked field soils and heavy metal polluted soil (IP) are also surveyed.

The ability of microbial substrate utilization was reduced in IT1 and IT2 compared to IR (Fig. 7). The statistical analysis by PCA also supported the difference in microbial functional diversity of IR from IT1 and IT2 (Fig. 8). The C substrate utilization of microbes seemed to be suppressed by the adverse effects of spiked Cu. Conversely, C utilization ability in IP was similar to IR, although the C substrate utilization patterns of IP could be separated from IR. The difference of microbial response to Cu between spiked soils and aged polluted soil may result from the difference of the free Cu concentrations in the pore water. The $CaCl_2$-extractive heavy metals were much higher in Cu spiked treatments, therefore the available fractions of IT1 and IT2 would be higher than IP. Differential microbial community structure could also be a reason for the different response to Cu between spiked soils and aged polluted soil. In the present study area, the microbial community of the polluted site has shown enhanced tolerance to Cu stress (Kamitani et al. in press, chapter 2). The microbial community in IT1 and

IT2 would be vulnerable to Cu compared to those in IP. Fungi are known to be sensitive to Cu (cf. the use of copper oxychloride as a fungicide in viniculture). Further investigation into the microbial community structure including fungi will be needed to determine the effects of Cu.

In the present study, the number and biomass of microarthropods showed rather large variations and it was difficult to find significant effects of Cu treatments (Fig. 9). Throughout the experiment, concentrations of exchangeable Cu became lower compared to the initial Cu concentrations in Cu spiked treatments, becoming less available to microarthropods. For example, the values of $CaCl_2$-extractable Cu in Cu-spiked treatments were much lower than the EC_{50} values for reproduction of *Folsomia fimetaria* (more than 55 mg/kg) reported by Bruus Pedersen and van Gestel (2001). However, the total Cu concentrations in 5 cm deep in IT1 and IT2 were much higher than those reported as significantly toxic for oribatid mites. For example, Streit (1984) have found that the oribatid mite *Platynothrus peltifer*, which is "often" used as a test organism in ecotoxicology (Van Gestel and Doornekamp, 1998), showed a significant reduction at 200 mg Cu/kg, whereas Parmelee et al. (1993) found a 47% reduction of oribatid mites at 100 mg Cu/kg. Actually, the species number and Shannon-Wiener index of oribatid mites were smaller in IT1 and IT2 compared to IR in the present study. The superdominant species was *Oppiella nova* in each site, and the percentage of this species became larger in response to applied Cu concentrations. *Oppiella nova* has been categorized as a microphytophagous species (Kaneko, 1988), and in general, concentrations of Cu were consistently higher in this species compared to the panphytophagous or macrophytophagous feeding groups. A high accumulation capacity for Cu has been discovered in *Oppiella nova* (Skubała and Kafel, 2004). We

need further information on the resistance strategy of this species for heavy metal pollution. In spite of the change in species number, the result of PCA for oribatid mite community, which excluded rare species from analysis, showed no difference of species structure between IT1, IT2 and IR (Fig. 10). Therefore, the effects of spiked Cu would also appear in the decrease of rare species in IR. Thus, the differences in community structure between reference and Cu applied treatments were mainly due to the change in the densities of dominant taxa and rare species, not to the decrease in the total abundance of this group. The high biomass of oribatid mites in IP showed the same tendency to field survey (chapter 3): large abundance and dominance of large sized species (the family Nanhermanniidae).

Adverse effects of spiked Cu were not revealed on the density of macrofauna except for earthworms (Fig. 11 and 12). The Cu concentrations in IT1 and IT2 were much higher than previously reported LC_{50}s for lumbricid worms (e.g. Khalil et al. (1996a); Maboeta et al. (2004); Spurgeon et al. (2004a), see Table 1 in chapter 1). The significant decrease in density and biomass of earthworms by spiked Cu was attributed mainly to the decrease in megascolecid worms, which also showed low density and biomass in IP (Fig. 12). In the field survey, low biomass and dominance of megascolecid worms have been also observed in the polluted sites (chapter 3). Thus, this family appears to be sensitive to heavy metal pollution. In the present study, megascolecid worms tended to decrease their number at the top 5 cm deep in IT1 and IT2 (Fig. 16), indicating not only high mortality by high Cu concentrations, but also avoidance behavior to the deeper layer which was less contaminated. In recent years, such an avoidance behavior of earthworm from toxic materials was noticed as a sensitive parameter for soil quality and risk assessment (Van Zwieten et al., 2004; Eijsackers et al., 2005; Loureiro et al., 2005;

Lukkari and Haimi, 2005). In TME analysis, to assess the avoidance behavior would be one of the key parameters, because spiked toxicants may show a heterogeneous distribution in soil cores. The dominance of moniligastrids, low density of megascolecids and absence of lumbricids in IP reflected the earthworm community *in situ* in the polluted site (chapter 3). The density of other macrofauna in IR and IP, including the large numbers of Lithobiomorpha in IR and Geophilomorpha in IP, also reflected the data in field survey (chapter 3).

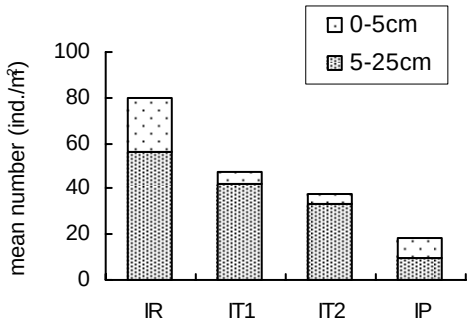

Fig. 16 Distribution of megascolecid worms in intact soil core [mean value, n = 12 (IR, IT1 and IT2), and 6 (IP)] of tier II.

Thus, the effects of spiked Cu were observed mainly in the microbial functional diversity, oribatid mite community and earthworm number and biomass. It is the intact soil systems using TMEs that such community level effects were able to be assessed by. On the other hand, the intact cores of aged heavy metal polluted soil reflected the community structure *in situ*, which was different from those in Cu applied soils. Within the test duration of TMEs, we could not observe build-up of increased resistance to toxicants in decomposer community structure, which may partly result in the large structural difference between Cu applied and aged polluted treatments. The key test species should be present in a wide range of soil ecosystems, should exist in a large,

dominant populations, play a key role in the soil ecosystem, and be testable under natural or simulated laboratory conditions. The result presented here indicated that megascolecid worms, which were adversely affected by spiked heavy metal, and native lumbricid worm, which were not observed in aged heavy metal polluted soil, should be given priority for the environmental risk assessment and more toxicological data are necessary for such native earthworm species.

4.3. Effect of spiked Cu and aged heavy metals on ecological functions

We compared estimated ecological functions in IR to IT1 and IT2 for estimation of Cu effects on these functions in the condition of semi-field intact system. The differences between Cu spiked field soils and heavy metal polluted soil (IP) are also surveyed.

Soil NH_4-N at 10 weeks after spiking tended to increase in response to Cu concentrations and was significantly higher in IT2 compared to IR (Fig. 13). Conversely, there was no difference in NH_4-N between IR and IP. There was no significant difference in soil NO_3-N among the treatments. Kostov and van Cleemput (2001) have shown that nitrification was more sensitive than ammonification to Cu toxicity (0-3000 mg Cu/kg) and they have concluded that applied Cu inhibited nitrification, resulting in accumulation of NH_4-N. Khan and Scullion (2002) have reported that additions of Cu as amended sludge increased the accumulation of mineral N, explained by a combination of decrease in microbial biomass N and increases in mineralization with higher input of Cu. In the present study, microbial biomass was not studied, but a high concentration of applied Cu would enhance the release of nitrogen from dead microbial cells, resulting in

large increase in NH_4-N compared with the reference soil, as suggested by Bogomolov et al. (1996).

Cations and anions in the leachate did not change significantly between reference and Cu spiked soils (Fig. 14). The typical increase and high variability of SO_4 in IT2 in 6-10 weeks after incubation could result from leaching of high concentration of SO_4, amended as $CuSO_4$ conformation. The large variance within treatments in both systems would be partly caused by the uptake to plant roots, which obscured the difference between treatments. In the intact system, drainage channels or different soil pore size in individual soil cores would also cause high variations (Burrows and Edwards, 2004). The cations and anions in IP showed quite different patterns from the other treatments: decrease in K, Mg, Ca and NO_3, and increase in Na, and SO_4 in the polluted soil at the initial stage of incubation. The change in the amounts of ions in IP may result from the initial difference of the amount of inherent ions in soil. The exchangeable Na tended to be higher concentration in the polluted site compared to the reference site in the present study area (Kamitani et al. in press, chapter 2). The larger amount of dissolved SO_4 in IP might inhibit the desorption of NO_3 from surface area of soil particles. The decrease in NO_3 in the aged polluted soil could also result from the depression of bacteria, fungi and their predators. Hunt et al. (1987) estimated that bacteria mineralized most of the N in their food web model analysis. Among soil fauna, amoebae and bacterivorous nematodes have been recognized as the most important contributors to N mineralization (Hunt et al. 1987; de Ruiter et al., 1993a). Although we did not estimate bacteria and bacterivorous microfauna, the bacterial channel could be depressed seriously because bacteria are mainly associated with silt and clay, substrate where heavy metals bind (Kandeler et al., 2000), and bacterivorous predators (flagellates, amebae, nematodes)

live mainly in water films on soil surfaces (Coleman et al., 2004), where they are chronically exposed by desorbed heavy metals. The turnover of N might be delayed in the aged polluted soil.

The decomposition process has implications for cycling of other nutrients, and hence is of central importance to soil fertility (Obbard and Jones, 1993). Van Gestel et al. (2003) have suggested that cellulose degradation was more dependent on microbial activity rather than on the abundance and activity of soil invertebrates. Conversely, Förster et al. (2004) have reported that earthworm density affected decomposition rate of cellulose paper. In the present study, the decomposition rate of cellulose did not differ among treatments at any time (Fig. 15). The ability of microbial C substrate utilization reduced in IT1 and IT2 compared to IR (Fig. 7). Earthworm density also decreased in IT1 and IT2 (Fig. 12). Therefore the maintenance of cellulose decomposition rate at increasing Cu concentrations could neither explained by soil fauna or microbial activity. In the study site, no significant differences have been observed in the decomposition of cellulose between heavy metal polluted and reference sites (chapter 3), but the decomposition was slower compared to laboratory experiments. The steady state of environmental conditions in Earthtron facility rather than the conditions *in situ* would enhance microbial and faunal activity for organic matter decomposition.

The shoot biomass of *M. sacchariflorus* was suppressed by Cu applications (IT1 and IT2) probably because of the diminution of roots (Oba et al., unpublished data). In the field condition, the individual shoot biomass and shoot/root ratio of *M. sacchariflorus* were lower in the polluted site (Oba et al., unpublished data). Contrary to the field survey, a high shoot/root ratio was detected in IP, similar to the ratio in IR. The competition-free condition in TME might result in high shoot/root ratio in IP. In the

field condition, high density of *M. sacchariflorus* was observed in the polluted site compared to reference site, which can cause low shoot/root ratio to gain for soil nutrients.

Thus, the effects of spiked Cu were observed in mineralized N, especially the increase in NH_4-N, and plant growth. Such reactions were different from those in aged polluted soil. The cellulose decomposition showed the same reaction, indicating high redundancy, which would maintained by whole soil community.

4.4 Comparison between TMEs and homogenized systems

The intact system contains a soil biological community, and a soil structure with the quality of abiotic materials. We discuss here the differences between intact soil systems (tier II) and homogenized soil systems (tier I) for functional analysis.

Soil inorganic N (NH_4-N and NO_3-N) was higher in intact systems than homogenized systems, but there was no difference between heavy metal polluted soil and reference soil in each system (Fig. 2 and Fig. 13). Cations and anions in the leachate showed the same patterns between intact and homogeneous systems (Fig. 3 and Fig. 14). The weight loss of cellulose paper as a measurement endpoint for decomposition of organic material showed different results between intact and homogenized systems. The decomposition rate of cellulose was significantly retarded in the polluted soil compared to the reference soil in homogenized system (Fig. 4), while no significant change was observed between the polluted and reference soil in intact system (Fig. 15), which was consistent with the reaction *in situ* (chapter 3). The retarded decomposition rate in homogenized system would be caused by the vulnerability of the hyphal network to

disturbance in the polluted soil. In this study area, fungi/bacteria ratio, which was estimated by phospholipids fatty acid (PLFA) analysis (Oba et al., unpublished data), was larger in the polluted site compared to reference site. Hedlund et al. (2004) have claimed that decomposition is conducted through bacterial- and fungal-based channels, and organisms in the fungal pathway were less resistant to disturbance because of low abilities to disperse. The lack of macrofauna in the homogenized system would be another reason for low decomposition rate in the polluted soil. The cellulose papers in this study were allowed to be attacked directly by all soil organisms. Therefore the feeding activity of macrofauna like earthworms would accelerate the diminution of cellulose paper in the polluted soil *in situ* and TMEs. Thus, disturbance of fungal hyphal network and elimination of soil macrofauna would reduce cellulose decomposition rate in the polluted soil in homogenized system. In a homogenized microcosm system, the shoot biomass and shoot/root ratio of *M. sacchariflorus* were lower in the polluted soil. The decrease in shoot/root ratio would be a mechanism by which the plants can tolerate high heavy metal concentrations (Weng et al., 2005). Contrary to homogenized systems and the field survey, mentioned above, high shoot/root ratio was detected in the polluted soil in intact system. It will be difficult to account the difference of shoot/root ratio between homogenized and intact system, because one was seedling without stock, while another was sprout from stock.

Thus, the intact soil system presented by using TME mimics the soil functional reaction to heavy metal pollution in the field condition compared to homogenized system, especially in the decomposition of organic matter which is the most integrating process within the soil ecosystem because of containing inherent soil biota in it.

4.5 Validation of using intact soil systems for ecological risk assessment

The result presented here indicated that using intact TME would be adequate to investigate the toxic effects on the soil ecosystems including soil biota and ecological functions. In ecological risk assessment, there are two approaches: argued from concentration to risk, and from risk to concentration. A site where certain concentrations of pollutants are present, which applies to the former type, we can use TME approach for estimation of the effects of the pollutants on ecological function. In the present study, we could indicate that the intact cores of aged heavy metal polluted soil reflected the community structure *in situ*, and that inherent soil biota produced soil functional reaction to heavy metal pollution in the field condition, which could not be reproduced by homogenized soil system. Moreover, the TME approach conducted under controlled environmental conditions can exclude the effects of uncontrolled field conditions on soil biota and ecological functions. To derive a concentration level such that accepted risk is not exceed, which applies to the latter type, an assessment system taking a local area into account would be appropriate to Japanese soil because of a large diversity of soil types due to a variety of base materials, climates and landform. TME would be useful for a site-specific approach, in spite of several limitations. In the present study, the method of spiked Cu could not necessarily predict the effects of aged heavy metal pollution. The way to application of chemicals, the number of replicates for intact core and the number of treatments would be perplex problem to use TME for ecological risk assessment. The homogenized systems are more suitable for the “risk to concentration” approach when generic environmental regulation is aimed. In the test system, native earthworms should serve as key test organisms because of their sensitivity and

important role in soil ecosystem.

Appendix 1. Density (individuals m^{-2}) of oribatid mite species found in each treatment. Each value in the table is the average numbers, number of species and diversity index.

	No. per m^2				
	IR	IT1	IT2	IP	total average
Collembola	4129	3121	3329	6130	4,177
Gamasida	1384	1427	1139	2542	1,623
Actinedida	956	1083	1483	1667	1,297
Tarsonemida	52	14	0	38	26
Acaridida	4915	692	3136	3879	3,156
Total Oribatida	6737	7943	7561	10028	8,067
Oribatida (adult)					
Oppiella nova	2048	3253	3512	1478	2,573
Scheloribates latipes	391	579	438	744	538
Oppiidae sp.2	461	391	367	0	305
Suctobelbidae sp.1	212	207	141	537	274
Nippohermannia sp.	108	193	80	377	190
Masthermannia hirsuta	80	151	71	414	179
Ischeloribates lanceolatus	179	146	104	273	175
Hypochthonius rufulus	89	80	71	424	166
Quadroppia quadricarinata	75	188	160	198	155
Cosmohermannia frondosa	33	19	19	499	142
Eohypochthonius magnus	188	221	137	0	137
Eremobelba japonica	47	137	155	9	87
Tectocepheus velatus	113	47	56	132	87
Eremulus sp.	94	61	28	122	77
Brachychthoniidae sp.	61	33	33	151	69
Galumnidae sp.2	127	71	33	9	60
Dolicheremaeus elongatus	0	108	75	0	46
Rhysotritia ardua	28	14	33	104	45
Fosseremus quadripertitus	14	9	14	104	35
Oppiidae sp.3	14	33	9	66	31
Galumnidae sp.3	19	52	19	9	25
Galumnidae sp.1	42	19	33	0	24
Arcoppia viperea	19	24	14	0	14
Papillacarus hirsutus	56	0	0	0	14
Nothrus silvestris	19	5	0	28	13
Xylobates sp.	5	33	0	0	9
Punctoribates sp.	24	5	0	9	9
Ceratozetes sp.	5	5	5	19	8
Scheloribates sp.2	0	33	0	0	8
Palaeacarus hystricinus	24	0	5	0	7
Nothrus palustris	14	0	0	0	4
Oppiidae sp.8	5	0	5	0	2
Suctobelbila tuberculata	5	5	0	0	2
Peloribates sp.	0	0	0	9	2
Oribatella sp.	0	0	0	9	2
Oppiidae sp.10	0	0	0	9	2
Oppiidae sp.1	5	0	0	0	1
Oppiidae sp.6	0	5	0	0	1
Suctobelbidae sp.2	0	5	0	0	1
Suctobelbidae sp.3	5	0	0	0	1
Oppiidae sp.9	0	0	5	0	1
Total no. individuals (adult)	4609	6130	5621	5734	5,524
No. of species	33	31	27	24	41
Shannon-Wiener index	2.25	1.98	1.65	2.45	

Chapter 7

General discussion

General discussion

In Japan, high concentrations of heavy metals are attributed to the mineral deposits (mines) (Imai et al., 2004). Heavy metal pollution from the mine has caused several kinds of damage and has troubled fishermen and farmers in the basin. The study area of this thesis, Watarase retarding basin on the Watarase River, is located downstream of thee Ashio copper mine. Heavy metal pollution in the Watarase River basin had ever attracted a great deal of public attention as a primary point of environmental disruption in Japan, since mining activity rapidly enlarged in 1877 (Morishita, 1981). The mine was closed in 1973, and water quality of the Watarase River is better now. However, complex heavy metals still remain in the sediment of Watarase retarding basin.

Environmental Quality Standards (EQS) for soil pollution in Japan were issued in 1991, and now regulate 25 substances including heavy metals and organic compounds (http://www.env.go.jp/en/lar/regulation/sp.html). The values of substances, except for Cu, were examined through leaching test: contents in sample solution (solvent is pH 5.8-6.3 regulated with HCl). These target values accord to EQS for water and ground water pollution, used as the standard for public water resources to protect human health. In paddy fields, target level of Cd is regulated less than 1 mg/kg in rice, and As and Cu are regulated less than 15 and 125 mg/kg in soil (extracted with 0.1M HCl), respectively. Thus, the main purpose for Japanese EQS is to protect human health or food production, not wildlife and soil ecosystems. Conversely, not only soil scientists and ecologist, but also policy makers and the public have noticed recently that soil biological community and ecological functions of the soil are important to humankind and should be protected,

especially in the European Union (Beck et al. 2005). Soil ecologists and ecotoxicologists in EU now try to develop biological concepts for the classification and assessment of soils (Breure et al., 2005). In Japan little data are available to predict the effects of soil pollution on a domestic soil ecosystem. Therefore, the general objectives of this thesis were set to investigate the effects of heavy metal pollution on a soil ecosystem in Japan, including soil biota, ecological function and bioaccumulation and to evaluate the validity of semi-field test using Terrestrial Model Ecosystems (TME) for soil ecological assessment in Japan. TME approach has recently been regarded as a valid method in the EU (Van Straalen, 2002). This chapter summarizes the results of this thesis to highlight the present degree of heavy metal pollution in Watarase retarding basin from the viewpoint of soil ecological risk, and to suggest a perspective of the application of soil ecosystem analysis to ecological risk assessment in Japan.

1. The effects of heavy metal pollution in Watarase retarding basin on the soil ecosystem

Based on the results of the field survey, the effects of heavy metal pollution on soil ecosystem in Watarase retarding basin were summarized in Fig. 1. The low availability of heavy metals in field condition due to the ageing effect did not exert notable toxic effects on abundance and biomass of soil biota. However, species composition of some trophic groups changed at a community level in the polluted area, e.g. microbial functional community, oribatid mite community, predator group and earthworm community structure (chapter 2 and 3). A long period of low but chronically stressed ecosystem could change community structure, probably because of differences in

sensitivity of species, life cycle strategies (Van Straalen, 2004) and genetic adaptation to stress (Posthuma, 1990). Soil biota was complementally replaced in part by other sets of species in the same functional groups. The ecological functions did not decrease and had redundancy at the pollution levels in Watarase retarding basin, which could be maintained by alternative species. Ramsey et al. (2005) have shown an opposite case that functional redundancy in a microbial community did not prevent depression of ecosystem functions in a 93-year-old mine waste contamination. Our results were not able to compare simply with theirs due to the difference of heavy metal concentration range (quite wide in Ramsey et al. (2005), e.g. 110-4600 mg Cu/kg). They have investigated mainly microbial parameters (e.g. *in situ* soil respiration, microbial biomass, and phospholipids fatty acid (PLFA) abundance and richness). In the present study, we calculated N mineralization rates by fungal based food web model, but this was incomplete because of the lack of bacterial channel including bacterivorous microfauna in food web diagram. Because bacteria are mainly associated with silt and clay, substrate where heavy metals bind (Kandeler et al., 2000), and bacterivorous predator (flagellates, amebae, nematodes) live mainly in water films on soil surfaces (Coleman et al., 2004), the bacterial channel in Watarase retarding basin may be depressed seriously rather than fungal channel. Actually, we consider the exposure of bacteria to heavy metal at the microscopic level has induced the shift to a tolerant community in the aged polluted site. In laboratory experiments presented in chapter 6 have shown significantly decreased NO_3 in leachate in polluted soil. The process parameter concerning mainly to bacteria and bacterivores might be affected even by aged heavy metal pollution.

In the present study, the degrees of heavy metal accumulation in earthworms showed different, species-specific patterns (chapter 4). Despite the same endogeic

categories, species in Megascolecidae and Lumbricidae had relatively lower concentrations compared to those of Moniligastridae. Although earthworm community structure seemed to be mostly influenced by soil properties, especially pH and clay fraction (chapter 4), high abundance of moniligastrid worms in the polluted sites and megascolecid worms in the reference site was observed when the soil properties were similar (chapter 3). In a moderately contaminated area such as Watarase retarding basin, the heavy metal concentration in earthworms are very important for ecological risk assessment, because the contaminants in earthworms itself poses a serious risk of secondary poisoning of vertebrate predators due to biomagnification, even if the body concentration does not achieve toxic level for earthworms. In the present study, we did not investigate the heavy metal concentrations in vertebrate predators. Further work for estimating the heavy metal concentration in vertebrate predators would confirm the degree of present ecological risk in Watarase retarding basin.

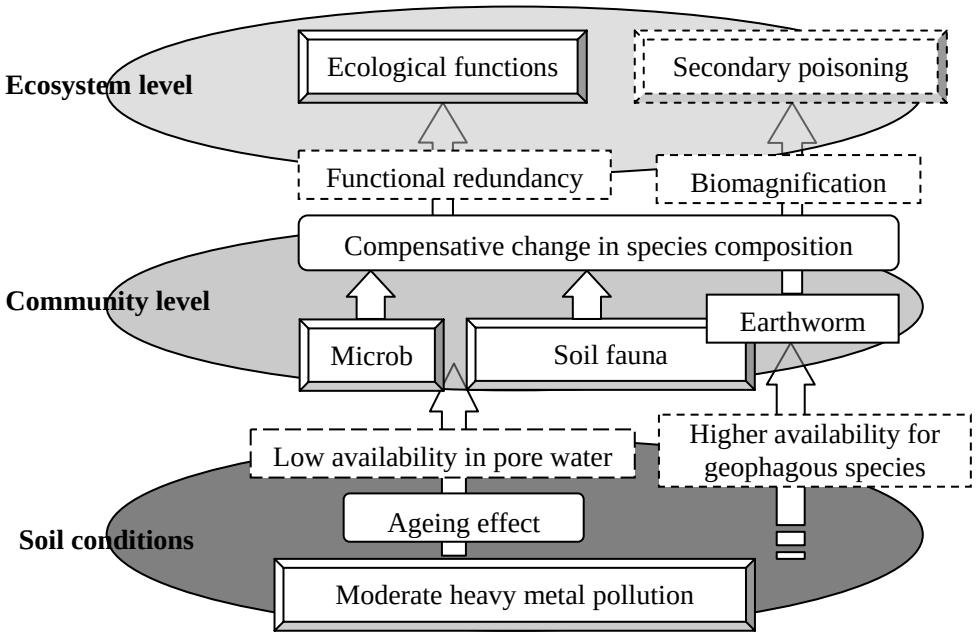

Fig. 1 Schematic model of the effects of heavy metal pollution on soil ecosystems in Watarase retarding basin.

2. Soil ecological risk assessment in Japan –a perspective-

I discuss here about an perspective of soil ecological risk assessment in Japan based on the results of the present study. In ecological risk assessment, there are two approaches as mentioned in chapter 6: argued from concentration to risk, which should be considered where certain concentrations of pollutants are present, and from risk to concentration, which should be considered when we derive a concentration level such that accepted risk is not exceed.

From concentration to risk

In former type of ecological risk assessment, the triad approach, the combination of chemical measurement, ecological surveys and bioassays, would be recommendable method, as suggested by many soil ecotoxicologists (e.g. Van Straalen, 2004; Römbke et al. 2005a). In such a site-specific assessment, an adequate reference location is needed to decide on the degree of the toxic effects at a target location, because the value of an indicator is dependent not only on stress factors, but also on soil type, land use, and vegetation (Beck et al. 2005). In the present study, one of the no polluted site, where the soil properties were different from the polluted sites, did not work as a reference site (chapter 2 and 3). In chemical analysis, the estimation of an available fraction of toxicants is important rather than total concentration. In the present study, we used $CaCl_2$ and DTPA extracts for estimation of available fraction. $CaCl_2$ would be useful for microbes and many soil organisms exposed through the skin, while DTPA would be useful for geophagous species like some earthworms (chapter 2, 3 and 4).

Van Straalen (2004) has provided an overview of the various bio-indicator systems

using soil invertebrates and recommended three systems: the maturity index of nematodes, species richness of microarthropods and ecological classifications of earthworms as the best candidates to be taken up in a general system of ecological survey. I mostly agree this suggestion. Although we did not investigate the nematode community in Watarase retarding basin, nematode maturity index can be also applied to heavy metal polluted soil (Bongers, 1990; Korthals et al., 1996; Korthals et al., 2000). Integration of nematode analysis with microbial indicators (e.g. microbial biomass, PLFA, or BIOLOG test) would provide more information of functional aspect: nutrient cycling. The microarthropods survey in this study showed higher sensitivity in Oribatida community than Collembolan community (chapter 3). The community level analysis could perform as good indicators for moderately polluted soil.

In Japan, I suggest that earthworm community should be divided not only to ecological group: epigeic, endogeic and anecic, but also to species, at least family level, because several families are distributed in Japan: Megascolecidae is mainly observed, followed by Lumbricidae and Moniligastridae (Blakemore, 2003), which is different from Europe: domination of Lumbricidae (Römbke et al., 2005b). In addition, the concentration in earthworm should be included as an indicator for secondary poisoning, even if the body concentration does not achieve toxic level for earthworms. Spurgeon and Hopkin (1996) have suggested the risk to predators of toxicants in the food chain soil-earthworm-vertebrate, using bioconcentration factors (from soil to earthworm). However, our studies suggested that bioconcentration factors were different among species. In the case of risk assessment of heavy metal biomagnification, a place dominated by moniligastrid worm would possess higher potential of risk for secondary predators than a place in which megascolecid worm is dominant, even if the soil heavy

metal concentrations were similar. We presented some regression models relating metal concentration in earthworms to acid or DTPA extractable soil metal concentration (Table 3 in chapter 4). More data should be needed to establish the reliability of regression model for native worms and to calculate biomagnification to birds and mammals by terrestrial food web models.

Ecological functions are also important factors to investigate in soil ecological assessment, because they are related to soil biodiversity and species composition and one of vulnerable functions to a pollution (Van Straalen, 2002). Organic matter decomposition, using cellulose paper in this study, seems to be a good indicator, because this indicator can include both soil microbial and faunal activities. Plant biomass might be a good indicator, but it is difficult to decide on direct or indirect effects of toxicants on plants. Moreover, the competition for photosynthesis and soil nutrients may obscure the direct and indirect toxic effects on plants in the field conditions.

For bioassays using polluted soil, earthworms would be the most important soil invertebrates, because they are known to influence soil structure, soil chemistry and processes like organic matter decomposition (Römbke et al., 2005b). The results presented in this study (chapter 3 and 6) indicate that megascolecid worms, which were adversely affected by spiked and aged heavy metal soil, and native lumbricid worm (*Eisenia fetida*), which was not observed in aged heavy metal polluted soil, should be used as test organisms. Intact soil system would provide much profitable information for functional analysis in bioassays. Moreover, bioassays conducted under controlled environmental conditions can exclude the effects of uncontrolled field conditions on soil biota and ecological functions. In the present study, we could indicate that the intact

cores of aged heavy metal polluted soil reflected the community structure *in situ*, and that inherent soil biota produced soil functional reaction (organic matter decomposition) to heavy metal pollution in the field condition, which could not be reproduced by homogenized soil system (chapter 6).

In conclusion, the triad approach, including chemical analysis of available fractions, ecological research for community level, bioaccumulation in earthworm and ecological functions *in situ*, and laboratory tests for native earthworm and TME approach, can assess the effects of pollutants on whole soil ecosystems.

From risk to concentration

There are few basic toxicological studies concerning domestic soil animals in Japan. The results of this study indicated that native earthworm was one of the most sensitive organisms to heavy metal. Native earthworms should serve as key test organisms because of not only their sensitivity, but also important role in soil ecosystem. Therefore, they should be given priority for ecotoxicological analysis by established test methods (one species in homogenized soil system) to obtain basic toxicological data, which would be probably different from toxicological characteristics of European lumbricid species dealt in most ecotoxicological studies.

At the next step, an assessment system taking a local area into account would be appropriate to Japanese soil because of a large diversity of soil types due to a variety of base materials, climates and landscapes. The results of this thesis (chapter 6) indicated that intact soil system using TME would be adequate to investigate the toxic effects on local soil ecosystems, because TME could mimic not only soil biota, but also functional

parameters *in situ*. We investigated the effect of applied Cu and aged heavy metal pollution on soil ecosystems including soil biota (microbial community, microarthropods and macrofauna) and ecological functions (N mineralization, elements in leachate, organic matter decomposition and plant growth). The community structure of microbes and oribatid mites, and earthworm biomass were soil structural parameters affected significantly by spiked Cu. The Cu application also had significant effects on several functions including soil ammonium-N concentration and plant growth. Organic matter decomposition showed resilience to the stress of Cu. These biological and functional parameters could be used as endpoints in ecotoxicological studies with TMEs.

In the present study, the method of Cu spike could not necessarily predict the effects of aged heavy metal pollution. We used two Cu treatments, but we need more treatments with enough replicates to derive EC_x or NOEC for each endpoint parameter. Moreover, the data of TMEs are subject to show high variability due to heterogeneous conditions in soil core, which may keep from finding consistent effects of toxicant on target endpoints. Thus, to decide the way to application of chemicals, the number of replicates for intact core and the number of treatments would be perplex problem to use TME approach for ecological risk assessment.

In conclusion, despite several limitation and requirements for further research, TME is valuable approach for ecological risk assessment.

Future work using TME approach

Currently, the most widely used method in ecological risk assessment is the hazard

quotient (HQ) method, which is normally calculated from the effect concentration of the most sensitive organism or group of organisms and comparing this to the greatest exposure concentration (Solomon and Sibley, 2002). Because TME approach can estimate effect concentrations of some soil organisms and ecological functions simultaneously, I think all estimated toxicity data should be used for ecological risk assessment. Probabilistic approaches would be better methods, which use distributions of species sensitivity combined with distributions of exposure concentrations to better describe the likelihood of exceedences of effect thresholds and thus the risk of adverse effects (Solomon and Sibley, 2002). The toxicological data set of each soil and land use type by using TMEs can provide more realistic quantitative estimations of risk in each local site with probabilistic approaches. It is a long way and very laborious to establish the TME methods and collect soil ecotoxicological data set with the approach. But soil ecologists in Japan are hopefully expected to take steps for ecotoxicological work in anticipation of legal requirement into the future.

References

References

Adamo, P., Denaix, L., Terribile, F., Zampella, M., 2003. Characterization of heavy metals in contaminated volcanic soils of the Solofrana river valley (southern Italy). Geoderma 117, 347-366.

Aoyama, M. and Nagumo, T., 1997. Comparison of the effects of Cu, Pb, and As on plant residue decomposition, microbial biomass, and soil respiration. Soil Sci. Plant Nutr. 43, 613-622.

Atanassova, I., 1999. Competitive effect of copper, zinc, cadmium and nickel on ion adsorption and desorption by soil clays. Water, Air, and Soil Pollution 113, 115-125.

Bardgett, R.D., Lovell, R.D., Hobbs, P.J. and Jarvis, S.C., 1999. Seasonal changes in soil microbial communities along a fertility gradient of temperate grasslands. Soil Biology and Biochemistry 31, 1021-1030.

Beck, L., Römbke, J., Breure, A.M., Mulder, C., 2005. Considerations for the use of soil ecological classification and assessment concepts in soil protection. Ecotoxicology and Environmental Safety 62, 189-200.

Belfroid, A.C., Sijm, D.T.H.M., van Gestel, C.A.M., 1996. Bioavailability and toxicokinetics of hydrophobic aromatic compounds in benthic and terrestrial invertebrates. Environmental Reviews 4, 276-299.

Belotti, E., 1998. Assessment of a soil quality criterion by means of a field survey. Applied Soil Ecology 10, 51-63.

Berg, M., de Ruiter, P., Didden, W., Janssen, M., Schouten, T., Verhoef, H., 2001. Community food web, decomposition and nitrogen mineralization in a stratified Scots pine forest soil. Oikos 94, 130-142.

Beyers, R.J., Odum, H.T., 1993. Ecological microcosms. Springer-Verlag, New York.

Blackburn, J., Farrow, M., Arthur, W., 2002. Factors influencing the distribution, abundance and diversity of geophilomorph and lithobiomorph centipedes. Journal of Zoology 256, 221-232.

Blakemore, R.J., 2003. Japanese earthworms (Annelida: Oligochaeta): a review and checklist of species. Organisms Diversity and Evolution 11, 1-43.

Blume, E., Bischoff, M., Reichert, J.M., Moorman, T., Konopka, A. and Turco, R.F., 2002. Surface and subsurface microbial biomass, community structure and metabolic activity as a function of soil depth and season. Applied Soil Ecology 20, 171-181.

Bogomolov, D.M., Chen, S.K., Parmelee, R.W., Subler, S., Edwards, C.A., 1996. An ecosystem approach to soil toxicity testing: a study of copper contamination in

laboratory soil microcosms. Applied Soil Ecology 4, 95-105.

Boivin, M.E.Y., Breure, A.M., Posthuma, L., Rutgers M., 2002. Determination of field effects of contaminants – significance of pollution-induced community tolerance. Human and Ecological Risk Assessment 8, 1035-1055.

Boivin, M.E.Y., Massieux, B., Breure, A.M., van den Ende, F.P., Greve, G.D., Rutgers M., Admiraal, W., 2005. Effects of copper and temperature on aquatic bacterial communities. Aquatic Toxicology 71, 345-356.

Bongers, T., 1990. The maturity index: an ecological measure of environmental dsiturbance based on nematode species composition. Oecologia 83, 14-19.

Bossuyt, H., Six, J., Hendrix, P.F., 2005. Protection of soil carbon by microaggregates within earthworm casts. Soil Biology and Biochemistry 37, 251-258.

Bouché, M.B., 1977. Strategies Lombriciennes. In: Lohm, U., Persson, T. (Eds.), Soil Organisms as Components of Ecosystems. Ecology Bulletin 25, Stockholm, pp. 122-132.

Bradford, M.A., Jones, T.H., Bardgett, R.D., Black, H.I.J., Boag, B., Bonkowski, M., Cook, R., Eggers, T., Gange, A.C., Grayston, S.J., Kandeler, E., McCaig, A.E., Newington, J.E., Prosser, J.I., Setälä, H., Staddon, P.L., Tordoff, G.M., Tscherko, D., Lawton, J.H., 2002. Impacts of soil faunal community composition on model grassland ecosystems. Science 298, 615-618.

Breure, A.M., Mulder, C., Römbke, J., Ruf, A., 2005. Ecological classification and assessment concepts in soil protection. Ecotoxicology and Environmental Safety 62, 211-229.

Bruus Pedersen, M., Axelsen, J.A., Strandberg, B., Jensen, J., Attrill, M.J., 1999. The impact of a copper gradient on a microarthropod field community. Ecotoxicology 8, 467-483.

Bruus Pedersen, M., van Gestel, C.A.M., 2001. Toxicity of copper to Collembolan *Folsomia fimetaria* in relation to the age of soil contamination. Ecotoxicology and Environmental Safety 49, 54-59.

Burrows, L.A., Edwards, C.A., 2002. The use of integrated soil microcosms to predict effects of pesticides on soil ecosystems. European Journal of Soil Biology 38, 245-249.

Burrows, L.A., Edwards, C.A., 2004. The use of integrated soil microcosms to assess the impact of carbendazim on soil ecosystems. Ecotoxicology 13, 143-161.

Chander, K., Dyckmans, J., Joergensen, R.G., Meyer, B., Raubuch, M., 2001. Different sources of heavy metals and their long-term effects on soil microbial properties. Biology and Fertility of Soils 34, 241-247.

Chapman, P.M., 1986. Sediment quality criteria from the sediment quality triad: an

example. Environmental Toxicology and Chemistry 5, 957-964.

Chen, C.R., Condron, L.M., Davis, M.R., Sherlock, R.R., 2003a. Seasonal changes in soil phosphorus and associated microbial properties under adjacent grassland and forest in New Zealand. Forest Ecology and Management 177, 539-557.

Chen, C.R., Xu, Z.H., Blumfield, T.J., Hughes, J.M., 2003b. Soil microbial biomass during the early establishment of hoop pine plantation: seasonal variation and impacts of site preparation. Forest Ecology and Management 186, 213-225.

Chew, I., Obbard, J.P., Stanforth, R.R., 2001. Microbial cellulose decomposition in soils from a rifle range contaminated with heavy metals. Environmental Pollution 111, 367-375.

Coleman, D.C., Crossley, D.A., Hendrix, P.F., 2004. Fundamentals of soil ecology 2nd Edition. Elsevier Academic Press, Burlington.

Cortet, J., Gomot, A., Poinsot, N., Gomot, L., Texier, C., Cluzeau, D., 1999. The use of invertebrate soil fauna in monitoring pollutant effects. European Journal of Soil Biology 35, 115-134.

Crommentuijn, T., Doodeman, C.J.A.M., van der Pol, J.J.C., Doornekamp, A., Rademaker, M.C.J., van Gestel, C.A.M., 1995. Sublethal sensitivity index as an ecotoxicity parameter measuring energy allocation under toxicant stress: application to cadmium in soil arthropods. Ecotoxicology and Environmental Safety 31, 192-200.

Dai, J., Becquer, T., Rouiller, J.H., Reversat, G., Bernhard-Reversat, F., Nahmani, J., Lavelle, P., 2004. Heavy metal accumulation by two earthworm species and its relationship to total and DTPA-extractable metals in soils. Soil Biology and Biochemistry 36, 91-98.

De Ruiter, P.C., Moore, J.C., Zwart, K.B., Bouwman, L.A., Hassink, J., Bloem, J., de Vos, J.A., Marinissen, J.C.Y., Didden, W.A.M., Lebbink, G., Brussaard, L., 1993a. Simulation of nitrogen mineralization in the belowground food webs of two winter wheat fields. Journal of Applied Ecology 30, 95-106.

De Ruiter, P.C., van Veen, J.A., Moore, J.C., Brussaard, L., Hunt, H.W., 1993b. Calculation of nitrogen mineralization in soil food webs. Plant and Soil 157, 263-273.

De Ruiter, P.C., Griffiths, B., Moore, J.C., 2002. Biodiversity and stability in soil ecosystems: patterns, processes and the effects of disturbance. In: Loreau, M., Naeem, S., Inchausti, P. (Eds.), Biodiversity and Ecosystem Functioning. Oxford University Press, New York, pp. 102-113.

Dobler, R., Saner, M., Bachofen, R., 2000. Population changes of soil microbial communities induced by hydrocarbon and heavy metal contamination. Bioremediation Journal. 4, 41-56.

Dumestre, A., Sauvé, S., McBride, M., Baveye, P., Berthelin, J., 1999. Copper speciation and microbial activity in long-term contaminated soils. Archives of Environmental Contamination and Toxicology 36, 124-131.

Easton, E.G., 1981. Japanese earthworms: a synopsis of the Megadrile species (Oligochaeta). Bull. Br. Mus. Nat. Hist. (Zool.) 40, 33-65.

Edwards, C.A., 2002. Assessing the effects of environmental pollutants on soil organisms, communities, processes and ecosystems. European Journal of Soil Biology 38, 225-231.

Edwards, C.A., Bohlen, P.J., 1996. Biology and Ecology of Earthworms 3rd Edition. Chapman & Hall, London.

Eijsackers, H., Beneke, P., Maboeta, M., Louw, J.P.E., Reinecke, A.J., 2005. The implications of copper fungicide usage in vineyards for earthworm activity and resulting sustainable soil quality. Ecotoxicology and Environmental Safety 62, 99-111.

Engelmann, M.D., 1961. The role of soil arthropods in the energetics of an old field community. Ecological Monographs 31, 221-238.

Fang, P., Wu, W., Xu, Q., Jiahai, H., Han, C., Paoletti, M.G., 1999. Assessing bioindication with earthworms in an intensively farmed rural landscape (Yuanqiao and Daqiao villages in Qianjiang Municipality, located in Hubei Province, subtropical China). Critical Reviews in Plant Sciences 18, 429-455.

Fountain, M.T., Hopkin, S.P., 2001. Continuous monitoring of *Folsomia candida* (Insecta: Collembola) in a metal exposure test. Ecotoxicology and Environmental Safety 48, 275-286.

François, M., Dubourguiter, H.C., Li, D., Douay, F., 2004. Prediction of heavy metal solubility in agricultural topsoils around two smelters by the physico-chemical parameters of the soils. Aquatic Sciences 66, 78-85.

Fujimaki, R., McGonigle, T.P., Takeda, H., 2004. Soil micro-habitat effects on fine roots of *Chamaecyparis obtusa* Endl.: A field experiment using root ingrowth cores. Plant and Soil 266, 325-332.

Förster, B., van Gestel, C.A.M., Koolhaas, J.E., Nentwig, G., Rodrigues, J.M.L., Sousa, J.P., Jones, S.E., Knacker, T., 2004. Ring-testing and field-validation of a terrestrial model ecosystem (TME) - An instrument for testing potentially harmful substances: effects of carbendazim on organic matter breakdown and soil fauna feeding activity. Ecotoxicology 13, 129-141.

Gardi, C., Tomaselli, M., Parisi, V., Petraglia, A., Santini, C., 2002. Soil quality indicators and biodiversity in northern Italian permanent grasslands. European Journal of Soil Biology 38 103-110.

Garland, J.L., Mills, A.L., 1991. Classification and characterization of heterotrophic microbial communities on the basis of patterns of community-level sole-carbon-source utilization. Applied and Environmental Microbiology 57, 2351-2359.

Gee, G.W., Or, D., 2002. Particle-size analysis. In: Dane, J.H., Topp, G.C. (Eds.), Methods of Soil Analysis, Part 4. Physical Methods. Soil Science Society of America Inc, Wisconsin, pp. 255-294.

Grelle, C., Fabre, M.C., Leprêtre, A., Descamps, M., 2000. Myriapod and isopod communities in soils contaminated by heavy metals in northern France. European Journal of Soil Science 51, 425-433.

Guo, Y., Wang, Z., Zhang, Y., Mo, X., 1998. Bioconcentration effects of heavy metal pollution in soil on the mucosa epithelia cell ultrastructure injuring of the earthworm's gastrointestinal tract. Bulletin of Environmental Contamination and Toxicology 60, 280-284.

Haimi, J., 2000, Decomposer animals and bioremediation of soils. Environmental Pollution 107, 233-238.

Haimi, J., Siira-Pietikäinen, A., 1996. Decomposer animal communities in forest soil along heavy metal pollution gradient. Fresenius Journal of Analytical Chemistry 354, 672-675.

Hamers, T., Krogh, P.H., 1997. Predator-prey relationships in a two-species toxicity test system. Ecotoxicology and Environmental Safety 37, 203-212.

Hedlund, K., Griffiths, B., Christensen, S., Scheu, S., Setälä, H., Tscharntke, T., Verhoef, H., 2004. Trophic interactions in changing landscapes: responses of soil food webs. Basic and Applied Ecology 5, 495-503.

Heemsbergen, D.A., Berg, M.P., Loreau, M., van Hal, J.R., Faber, J.H., Verhoef, H.A., 2004. Biodiversity effects on soil processes explained by interspecific functional dissimilarity. Science 306, 1019-1020.

Hobbelen, P.H.F., Koolhaas, J.E., van Gestel, C.A.M., 2004. Risk assessment of heavy metal pollution for detritivores in floodplain soils in the Biesbosch, the Netherlands, taking bioavailability into account. Environmental Pollution 129, 409-419.

Hopkin, S.P., 1989. Ecophysiology of metals in terrestrial invertebrates. Elsevier Applied Science, London.

Hopkin, S.P., 1990. Species-specific differences in the net assimilation of zinc, cadmium, lead, copper and iron by the terrestrial isopods *Oniscus asellus* and

Porcellio scaber. Journal of Applied Ecology 27, 460-474.

Hund-Rinke, K., Kördel, W., 2003. Underlying issues in bioaccessibility and bioavailability: experimental methods. Ecotoxicology and Environmental Safety 56, 52-62.

Hunt, H.W., Coleman, D.C., Ingham, E.R., Ingham, R.E., Elliott, E.T., Moore, J.C., Rose, S.L., Reid, C.P.P., Morley, C.R., 1987. The detrital food web in a shortgrass prairie. Biology and Fertility of Soils 3, 57-68.

Hågvar, S., 1998. The relevance of the Rio-Convention on biodiversity to conserving the biodiversity of soils. Applied Soil Ecology 9, 1-7.

Imai, N., Terashima, S., Ohta, A., Mikoshiba, M., Okai, T., Tachibana, Y., Togashi, S., Matsuhisa, Y., Kanai, Y., Kamioka, H., Taniguchi, M., 2004. Geochemical Map of Japan. Geological Survey of Japan, AIST.

Inubushi, K., 1997. Method of microbial biomass C. In: Society for the Study of Soil Microbes (ed.), Methods of Experiment with Soil Microbes, Yokendo Press, Tokyo, pp. 173-183 (in Japanese).

Jongmans, A.G., Pulleman, M.M., Balabane, M., van Oort, F., Marinissen, J.C.Y., 2003. Soil structure and characteristics of organic matter in two orchards differing in earthworm activity. Applied Soil Ecology 24, 219-232.

Kamitani, T., Kaneko, N., Niwa, N., Maeda, H., 2004. A case study of assessment of oil spill soil and remediation process from ecological viewpoint. Edaphologia 75, 1-9. (in Japanese)

Kamitani, T., Kaneko, N., (in press a). Species-specific heavy metal accumulation patterns of earthworms on a floodplain in Japan. Ecotoxicology and Environmental Safety

Kamitani, T., Kaneko, N., (in press b). The Earthtron facility for below-ground manipulation study. Ecological Research

Kamitani, T., Oba, H., Kaneko, N., (in press). Microbial biomass and tolerance of microbial community on an aged heavy metal polluted floodplain in Japan. Water, Air, and Soil Pollution

Kandeler, E., Tscherko, D., Bruce, K.D., Stemmer, M., Hobbs, P.J., Bardgett, R.D., Amelung, W., 2000. Structure and function of the soil microbial community in microhabitats of a heavy metal polluted soil. Biology and Fertility of Soils 32, 390-400.

Kaneko, N., 1988. Feeding habits and cheliceral size of oribatid mites in cool temperate forest soils in Japan. Revue d'Ecologie et de Biologie du Sol 25, 353-363.

Kelly, J.J., Tate, R.L., 1998. Use of BIOLOG for the analysis of microbial communities from zinc-contaminated soils. Journal of Environmental Quality 27, 600-608.

Kelly, J.J., Häggblom, M., Tate, R.L., 1999. Changes in soil microbial communities over time resulting from one time application of zinc: a laboratory microcosm study. Soil Biology and Biochemistry 31, 1455-1465.

Khalil, M.A., Abdel-Lateif, H.M., Bayoumi, B.M., van Straalen, N.M., 1996a. Analysis of separate and combined effects of heavy metals on the growth of *Aporrectodea caliginosa* (Oligochaeta; Annelida), using the toxic unit approach. Applied Soil Ecology 4, 213-219.

Khalil, M.A., Abdel-Lateif, H.M., Bayoumi, B.M., van Straalen, N.M., van Gestel, C.A.M., 1996b. Effects of metals and metal mixtures on survival and cocoon production of the earthworm *Aporrectodea caliginosa.* Pedobiologia 40, 548-556.

Khan, M., Scullion, J., 2002. Effects of metal (Cd, Cu, Ni, Pb or Zn) enrichment of sewage-sludge on soil micro-organisms and their activities. Applied Soil Ecology 20, 145-155.

Khodadoust, A.P., Reddy, K.R., Maturi, K., 2005. Effect of different extraction agents on metal and organic contaminant removal from a field soil. Journal of Hazardous Materials B117, 15-24.

Klumpp, A., Hintemann, T., Lima, J.S., Kandeler, E., 2003. Bioindication of air pollution effects near a copper smelter in Brazil using mango trees and soil microbiological properties. Environmental Pollution 126, 313-321.

Knacker, T, van Gestel, C.A.M., Jones, S.E., Soares, A.M.V.M., Schallnaß, H.J., Förster, B., Edwards, C.A., 2004. Ring-testing and field-validation of a terrestrial model ecosystem (TME) - An instrument for testing potentially harmful substances: conceptual approach and study design. Ecotoxicology 13, 9-27

Knight, B.P., McGrath, S.P., Chaudri, A.M., 1997. Biomass carbon measurements and substrate utilization patterns of microbial populations from soils amended with cadmium, copper, or zinc. Applied and Environmental Microbiology 63, 39-43.

Korthals, G.W., de Goede, R.G.M., Kammenga, J.E., Bongers, T., 1996. The maturity index as an instrument for risk assessment of soil pollution, In: Van Straalen, N.M., Krivolutsky, D.A. (Eds.), Bioindicator Systems for Soil Pollution. Kluwer Academic Publishers, Dordrecht, pp. 85-93.

Korthals, G.W., Bongers, M., Fokkema, A., Dueck, T.A., Lexmond, T.M., 2000. Joint toxicity of copper and zinc to a terrestrial nematode community in an acid sandy soil. Ecotoxicology 9, 219-228.

Kostov, O., van Cleemput, O., 2001. Nitrogen transformations in copper-contaminated soils and effects of lime and compost application on soil resiliency. Biology and Fertility of Soils 33, 10-16.

Kramarz, P., Laskowski, R., 1999. Toxicity and possible food-chain effects of copper,

dimethoate and a detergent (LAS) on a centipede (*Lithobius mutabilis*) and its prey (*Musca domestica*). Applied Soil Ecology 13, 177-185.

Kunito, T., Saeki, K., Oyaizu, H., Matsumoto, S., 1999. Influences of copper forms on the toxicity to microorganisms in soils. Ecotoxicology and Environmental Safety 44, 174-181.

Laakso, J., Setälä, H., 1999. Sensitivity of primary production to changes in the architecture of belowground food webs. Oikos 87, 57-64.

Lanno, R., Wells, J., Conder, J., Bradham, K., Basta, N., 2004. The bioavailability of chemicals in soil for earthworms. Ecotoxicology and Environmental Safety 57, 39-47.

Lavelle, P., Bignell, D., Lepage, M., Wolters, V., Roger, P., Ineson, P., Heal, O.W., Dhillion, S., 1997. Soil function in a changing world: the role of invertebrate ecosystem engineers. European Journal of Soil Biology 33, 159-193.

Laverack, M.S., 1963. The Physiology of Earthworms. Pergamon. Oxford.

Lawton, J.H., 1994. What do species do in ecosystems? Oikos 71, 367-374.

Lawton, J.H., 1996. The Ecotron facility at Silwood Park: The value of "big bottle" experiments. Ecology 77, 665-669.

Lee, K.E., 1985. Earthworms: Their Ecology and Relationships with Soils and Land Use. Academic Press, Sydney.

Liang, J., Zhou, Q., 2003. Single and binary-combined toxicity of methamidophos, acetochlor and copper acting on earthworms *Eisenia Foelide*. Bulletin of Environmental Contamination and Toxicology 71, 1158-1166.

Liiri, M., Setälä, H., Haimi, J., Pennanen, T., Fritze, H., 2002. Relationship between soil microarthropod species diversity and plant growth does not change when the system is disturbed. Oikos 96, 137-149.

Lock, K., Janssens, F., Janssen, C.R., 2003. Effect of metal contamination on the activity and diversity of springtails in an ancient Pb-Zn mining area at Plombières, Belgium. European Journal of Soil Biology 39, 25-29.

Loreau, M., Naeem, S., Inchausti, P., Bengtsson, J., Grime, J.P., Hector, A., Hooper, D.U., Huston, M.A., Raffaelli, D., Schmid, B., Tilman, D., Wardle, D.A., 2001. Biodiversity and ecosystem functioning: current knowledge and future challenges. Science 294, 804-808.

Loureiro, S., Soares, A.M.V.M., Nogueira, A.J.A., 2005. Terrestrial avoidance behaviour tests as screening tool to assess soil contamination. Environmental Pollution 138, 121-131.

Lukkari, T., Taavitsainen, M., Väisänen, A., Haimi, J., 2004. Effects of heavy metals on earthworms along contamination gradients in organic rich soils. Ecotoxicology and

Environmental Safety 59, 340-348.

Lukkari, T., Aatsinki, M., Väisänen, A., Haimi, J., 2005. Toxicity of copper and zinc assessed with three different earthworm tests. Applied Soil Ecology 30, 133-146.

Lukkari, T., Haimi, J., 2005. Avoidance of Cu- and Zn-contaminated soil by three ecologically different earthworm species. Ecotoxicology and Environmental Safety 62, 35-41.

Ma, W.C., 1982. The influence of soil properties and worm-related factors on the concentration of heavy metals in earthworms. Pedobiologia 24, 109-119.

Ma, W.C., 2004. Estimating heavy metal accumulation in Oligochaete earthworms: a meta-analysis of field data. Bulletin of Environmental Contamination and Toxicology 72, 663-670.

Ma, W.C., 2005. Critical body residues (CBRs) for ecotoxicological soil quality assessment: copper in earthworms. Soil Biology and Biochemistry 37, 561-568.

Ma, W.C., Talmage, S., 2001. Insectivora. In: Shoreand, R.F., Rattner, B.A. (Eds.) Ecotoxicology of wild mammals. John Wiley & Sons Ltd., New York, pp. 123-158.

Maboeta, M.S., Reinecke, S.A., Reinecke, A.J., 2004. The relationship between lysosomal biomarker and organismal responses in an acute toxicity test with *Eisenia Fetida* (Oligochaeta) exposed to the fungicide copper oxychloride. Environmental Research 96, 95-101.

Maiz, I., Arambarri, I., Garcia, R., Millán, E., 2000. Evaluation of heavy metal availability in polluted soils by two sequential extraction procedures using factor analysis. Environmental Pollution 110, 3-9.

Martikainen, E., Haimi, J., Ahtiainen, J., 1998. Effects of dimethoate and benomyl on soil organism and soil processes - a microcosm study. Applied Soil Ecology 9, 381-387.

Migliorini, M., Pigino, G., Bianchi, N., Bernini, F., Leonzio, C., 2004. The effects of heavy metal concentration on the soil arthropod community of a shooting range. Environmental Pollution 129, 331-340.

Mikola, J., Bardgett, R.D., Hedlund, K., 2002. Biodiversity, ecosystem functioning and soil decomposer food webs. In: M. Loreau, S. Naeem, P. Inchausti (Eds.), Biodiversity and Ecosystem Functioning. Oxford University Press, New York, USA, pp. 169-180.

Millennium Ecosystem Assessment, 2005. Ecosystems and human well-being: Biodiversity synthesis. World Resources Institute, Washington DC.

Ministry of Land, Infrastructure and Transport Government of Japan, 1976. Report of soil research in investigation of development in Watarase Retarding Basin (part 2). (in Japanese)

Ministry of Land, Infrastructure and Transport Government of Japan, 1991. Report of schematic design of excavation in the second regulation pond. (in Japanese)

Miyazaki, T., 1993. Water flow in soils. Marcel Dekker, New York

Morgan, J.E., Morgan, A.J., 1999. The accumulation of metals (Cd, Cu, Pb, Zn and Ca) by two ecologically contrasting earthworm species (Lumbricus rubellus and Aporrectodea caliginosa): implications for ecotoxicological testing. Applied Soil Ecology 13, 9-20.

Morgan, A.J., Turner, M.P., Morgan, J.E., 2002. Morphological plasticity in metal-sequestering earthworm chloragocytes: morphometric electron microscopy provides a biomarker of exposure in field populations. Environmental Toxicology and Chemistry 21, 610-618.

Morishita, T., 1981. The Watarase River basin: contamination of the environment with copper discharged from Asio mine. In: Kitagishi, K., Yamane, I. (Eds.) Heavy Metal Pollution in Soils of Japan. Japan Scientific Societies Press, Tokyo, pp. 165-179.

Nahmani, J., Lavelle, P., 2002. Effects of heavy metal pollution on soil macrofauna in a grassland of northern France. European Journal of Soil Biology 38, 297-300.

Nakano, M., 1991. Transport phenomena in soils (in Japanese). University of Tokyo Press, Tokyo.

Nakano, M., Miyazaki, T., Shiozawa, S. Nishimura, T., 1995. Physical and Environmental Analysis of Soils. University of Tokyo Press, Tokyo, (in Japanese).

Niklińska, M., Chodak, M., Laskowski, R., (in press). Pollution-induced community tolerance of microorganisms from forest soil organic layers polluted with Zn or Cu. Applied Soil Ecology

Nimmo, J.R., Perkins, K.S., 2002. Aggregate stability and size distribution. In: Dane, J.H., Topp, G.C. (Eds.), Methods of Soil Analysis, Part 4. Physical Methods. Soil Science Society of America Inc., Wisconsin, pp. 317-328.

Novozamsky, I., Lexmond, T.M., Houba, V.J.G., 1993. A single extraction procedure of soil for evaluation of uptake of some heavy metals by plants. International Journal of Environmental Analytical Chemistry 51, 47-58.

Obbard, J.P., Jones, K.C., 1993. The use of the cotton-strip assay to assess cellulose decomposition in heavy metal-contaminated sewage sludge-amended soils. Environmental Pollution 81, 173-178.

Oste, L.A., Dolfing, J., Ma, W.C., Lexmond, T.M., 2001. Cadmium uptake by earthworms as related to the availability in the soil and the intestine. Environmental Toxicology and Chemistry 20, 1785-1791.

Panda, R., Pati, S.S., Sahu, S.K., 1999. Accumulation of zinc and its effects on the growth, reproduction and life cycle of *Drawida willsi* (Oligochaeta), a dominant

earthworm in Indian crop fields. Biology and Fertility of Soils 29, 419-423.

Paoletti, M.G., 1999. The role of earthworms for assessment of sustainability and as bioindicators. Agriculture, Ecosystems and Environment 74, 137-155.

Paoletti, M.G., Iovane, E., Cortese, M., 1988. Pedofauna bioindicators and heeavy metals in five agroecosystems in north-east Italy. Revue d'Écologie et de Biologie du Sol 25, 33-58.

Paoletti, M.G., Hassall, M., 1999. Woodlice (Isopoda: Oniscidea): their potential for assessing sustainability and use as bioindicators. Agriculture, Ecosystems and Environment 74, 157-165.

Papatheodorou, E.M., Argyropoulou, M.D., Stamou, G.P., 2004. The effects of large- and small-scale differences in soil temperature and moisture on bacterial functional diversity and the community of bacterivorous nematodes. Applied Soil Ecology 25, 37-49.

Parmelee, R.W., Wentsel, R.S., Phillips, C.T., Simini, M., Checkai, R.T., 1993. Soil microcosm for testing the effects of chemical pollutions on soil fauna communities and trophic structure. Environmental Toxicology and Chemistry 12, 1477-1486.

Parmelee, R.W., Phillips, C.T., Checkai, R.T., Bohlen, P.J., 1997. Determining the effects of pollutants on soil faunal communities and trophic structure using a refined microcosm system. Environmental Toxicology and Chemistry 16, 1212-1217.

Peijnenburg, W.J.G.M., Baerselman, R., de Groot, A.C., Jager, T., Posthuma, L., van Veen, R.P.M., 1999a. Relating environmental availability to bioavailability: soil-type-dependent metal accumulation in the Oligochaete *Eisenia andrei*. Ecotoxicology and Environmental Safety 44, 294-310.

Peijnenburg, W.J.G.M., Posthuma, L., Zweers, P.G.P.C., Baerselman, R., de Groot, A.C., van Veen, R.P.M., Jager, T., 1999b. Prediction of metal bioavailability in Dutch field soils for the Oligochaete *Enchytraeus crypticus*. Ecotoxicology and Environmental Safety 43, 170-186.

Peijnenburg, W.J.G.M., Jager, T., 2003. Monitoring approaches to assess bioaccessibility and bioavailability of metals: Matrix issues. Ecotoxicology and Environmental Safety 56, 63-77.

Pennanen, T., 2001. Microbial communities in boreal coniferous forest humus exposed to heavy metals and changes in soil pH -a summary of the use of phospholipid fatty acids, Biolog® and ^{3}H-thymidine incorporation methods in field studies. Geoderma 100, 91-126.

Pennanen, T., Frostegård, Å., Fritze, H., Bååth, E., 1996. Phospholipid fatty acid composition and heavy metal tolerance of soil microbial communities along two

heavy metal-polluted gradients in coniferous forests. Applied and Environmental Microbiology 62, 420-428.

Petersen, H., Luxton, M., 1982. A comparative analysis of soil fauna populations and their role in decomposition processes. Oikos 39, 1982.

Pietrzak, U., McPhail, D.C., 2004. Copper accumulation, distribution and fractionation in vineyard soils of Victoria, Australia. Geoderma 122, 151-166.

Pokarzhevskii, A.D., van Straalen, N.M., Zaboev, D.P., Zaitsev, A.S., 2003. Microbial links and element flows in nested detrital food-webs. Pedobiologia 47, 213-224.

Posthuma, L., 1990. Genetic differentiation between populations *Orchesella cincta* (Collembola) from heavy metal contaminated sites. Journal of Applied Ecology 27, 609-622.

Prinzing, A., Kretzler, S., Badejo, A., Beck, L., 2002. Traits of oribatid mite species that tolerate habitat disturbance due to pesticide application. Soil Biology and Biochemistry 34, 1655-1661.

Ramsey, P.W., Rillig, M.C., Feris, K.P., Gordon, N.S., Moore, J.N., Holben, W.E., Gannon, J.E., 2005. Relationship between communities and processes; new insights from a field study of a contaminated ecosystem. Ecology letters 8, 1201-1210.

Rasmussen, L.D., Sørensen, S.J., 2001. Effects of mercury contamination on the culturable heterotrophic, functional and genetic diversity of the bacterial community in soil. FEMS Microbiology Ecology 36, 1-9.

Reinecke, A.J., Reinecke, S.A., 2004. Earthworms as test organisms in ecotoxicological assessment of toxicant impacts on ecosystems. In: Edwards, C.A. (Ed.) Earthworm Ecology, 2nd edition. CRC Press, New York, pp. 299-320.

Rensing, C., Maier, R.M., 2003. Issues underlying use of biosensors to measure metal bioavailability. Ecotoxicology and Environmental Safety 56, 140-147.

Rogers, B.F., Tate, R.L., 2001. Temporal analysis of the soil microbial community along a toposequence in Pineland soils. Soil Biology and Biochemistry 33, 1389-1401.

Römbke, J., Breure, A.M., Mulder, C., Rutgers, M., 2005a. Legislation and ecological quality assessment of soil: implementation of ecological indication systems in Europe. Ecotoxicology and Environmental Safety 62, 201-210.

Römbke, J., Jänsch, S., Didden, W., 2005b. The use of earthworms in ecological soil classification and assessment concepts. Ecotoxicology and Environmental Safety 62, 249-265.

Salamanca, E.F., Kaneko, N., Katagiri, S., 1998. Effects of leaf litter mixtures on the decomposition of *Quercus serrata* and *Pinus densiflora* using field and laboratory microcosm methods. Ecological Engineering 10, 53-73.

Sheppard, S.C., 1997. Toxicity testing using microcosms. In: Tarradellas, J., Bitton, G., Rossel, D. (Eds.), Soil Ecotoxicology. CRC Press Inc., Boca Raton, pp. 345-373.

Siepel, H., 1995. Applications of microarthropod life-history tactics in nature management and ecotoxicology. Biology and Fertility of Soils 19, 75-83.

Sijm, D., Kraaij, R., Belfroid, A., 2000. Bioavailability in soil or sediment: exposure of different organisms and approaches to study it. Environmental Pollution 108, 113-119.

Skubała, P., Kafel, A., 2004. Oribatid mite communities and metal bioaccumulation in oribatid species (Acari, Oribatida) along the heavy metal gradient in forest ecosystems. Environmental Pollution 132, 51-60.

Solomon, K.R., Sibley, P., 2002. New concepts in ecological risk assessment: where do we go from here? Marine Pollution Bulletin 44, 279-285.

Sparks, D.L., 1986. Kinetics of reactions in pure and mixed systems, In: Sparks, D.L. (Ed.), Soil Physical Chemistry. CRC Press, Inc., Boca Raton, pp. 83-145.

Spurgeon, D.J., Hopkin, S.P., 1996. Risk assessment of the threat of secondary poisoning by metals to predators of earthworms in the vicinity of a primary smelting works. The Science of Total Environment 187, 167-183.

Spurgeon, D.J., Sandifer, R.D., Hopkin, S.P., 1996. The use of macro-invertebrates for population and community monitoring of metal contamination -indicator taxa, effect parameters and the need for a soil invertebrate prediction and classification scheme (SIVPACS). In: Van Straalen, N.M., Krivolutsky, D.A. (Eds.), Bioindicator Systems for Soil Pollution. Kluwer Academic Publishers, Dordrecht, pp. 95-110.

Spurgeon, D.J., Hopkin, S.P., 1999a. Seasonal variation in the abundance, biomass and biodiversity of earthworms in soils contaminated with metal emissions from a primary smelting works. Journal of Applied Ecology 36, 173-183.

Spurgeon, D.J., Hopkin, S.P., 1999b. Comparisons of metal accumulation and excretion kinetics in earthworms (*Eisenia fetida*) exposed to contaminated field and laboratory soils. Applied Soil Ecology 11, 227-243.

Spurgeon, D.J., Weeks, J.M., van Gestel, C.A.M., 2003. A summary of eleven years progress in earthworm ecotoxicology. Pedobiologia 47, 588-606.

Spurgeon, D.J., Stürzenbaum, S.R., Svendsen, C., Hankard, P.K., Morgan, A.J., Weeks, J.M., Kille, P., 2004a. Toxicological, cellular and gene expression responses in earthworms exposed to copper and cadmium. Comparative Biochemistry and Physiology, Part C 138, 11-21.

Spurgeon, D.J., Svendsen, C., Kille, P., Morgan, A.J., Weeks, J.M., 2004b. Responses of earthworms (*Lumbricus rubellus*) to copper and cadmium as determined by measurement of juvenile traits in a specifically designed test system. Ecotoxicology

and Environmental Safety 57, 54-64.

Stephenson, J., 1930. The Oligochaeta. Oxford University Press, London.

Streit, B., 1984. Effects of high copper concentrations on soil invertebrates (earthworms and oribatid mites): Experimental results and a model. Oecologia 64, 381-388.

Ter Braak, C.F.J., 1986. Canonical correspondence analysis: a new eigenvector technique for multivariate direct gradient analysis. Ecology 67, 1167-1179.

Tipping, E., Rieuwerts, J., Pan, G., Ashmore, M.R., Lofts, S., Hill, M.T.R., Farago, M.E., Thornton, I., 2003. The solid-solution partitioning of heavy metals (Cu, Zn, Cd, Pb) in upland soils of England and Wales. Environmental Pollution 125, 213-225.

Toyota, K., Kimura, M., Takechi, S., Kuninaga, S., 2000. Comparison of microbial community structure between soils suppressive and conducive to Fusarium-wilt of radish. Soil Microorganisms 54, 69-79.

Turpeinen, R., Kairesalo, T., Häggblom, M.M., 2004. Microbial community structure and activity in arsenic-, chromium- and copper-contaminated soils. FEMS Microbiology Ecology 47, 39-50.

Uchida, T., Kaneko, N., 2004. Life history of Megascolecidae earthworms in forest soils at Kanagawa, Japan. Edaphologia. 74, 35-45. (in Japanese)

Uchida, T., Kaneko, N., Ito, M.T., Futagami, K., Sasaki, T., Sugimoto, A., 2004. Analysis of the feeding ecology of earthworms (Megascolecidae) in Japanese forests using gut content fractionation and $\delta^{15}N$ and $\delta^{13}C$ stable isotope natural abundances. Applied Soil Ecology 27, 153-163.

Van Gestel, C.A.M., 1992. The influence of soil characteristics on the toxicity of chemicals for earthworms: a review. In: Greig-Smith, P.W., Becker, H., Edwards, P.J., Heimbach, F., (Eds.) Ecotoxicology of Earthworms. Intercept Ltd., Andover, pp. 44-54.

Van Gestel, C.A.M., 1997. Scientific basis for extrapolating results from soil ecotoxicity tests to field conditions and the use of bioassays. In: Van Straalen, N.M., Løkke, H. (Eds.), Ecological Risk Assessment of Contaminants in Soil. Chapman & Hall, London, pp. 25-50.

Van Gestel, C.A.M., Hensbergen, P.J., 1997. Interaction of Cd and Zn toxicity for *Folsomia candida* Willem (Collembola: Isotomidae) in relation to bioavailability in soil. Environmental Toxicology and Chemistry 16, 1177-1186.

Van Gestel, C.A.M., Doornekamp, A., 1998. Tests on the oribatid mite *Platynothrus peltifer*. In: Løkke, H., van Gestel, C.A.M. (Eds.), Handbook of Soil Invertebrate Toxicity Tests. John Wiley & Sons Ltd., Chichester, pp. 113-130.

Van Gestel, C.A.M., Kruidenier, M., Berg, M.P., 2003. Suitability of wheat straw

decomposition, cotton strip degradation and bait-lamina feeding tests to determine soil invertebrate activity. Biology and Fertility of Soils 37, 115-123.

Van Straalen, N.M., 1998. Evaluation of bioindicator systems derived from soil arthropod communities. Applied Soil Ecology 9, 429-437.

Van Straalen, N.M., 2002. Assessment of soil contamination - a functional perspective. Biodegradation 13, 41-52.

Van Straalen, N.M., 2003. Ecotoxicology becomes stress ecology. Environmental Science and Technology 37, 324A-330A.

Van Straalen, N.M., 2004. The use of soil invertebrates in ecological surveys of contaminated soils. In: Doelman, P., Eijsackers, H.J.P. (Eds.), Vital Soil -Function, Value and Properties-. Elsevier, Amsterdam, pp. 159-195.

Van Straalen, N.M., Verhoef, H.A., 1997. The development of a bioindicator system for soil acidity based on arthropod pH preferences. Journal of Applied Ecology 34, 217-232.

Van Straalen, N.M., Butovsky, R.O., Pokarzhevskii, A.D., Zaitsev, A.S., Verhoef, S.C., 2001. Metal concentrations in soil and invertebrates in the vicinity of a metallurgical factory near Tula (Russia). Pedobiologia 45, 451-466.

Van Zwieten, L., Rust, J., Kingston, T., Merrington, G., Morris, S., 2004. Influence of copper fungicide residues on occurrence of earthworms in avocado orchard soils. Science of Total Environment 329, 29-41.

Vance, E.D., Brookes, P.C., Jenkinson, D.S., 1987. An extraction method for measuring soil microbial biomass C. Soil Biology and Biochemistry 19, 703-707.

Vandecasteele, B., Samyn, J., Quataert, P., Muys, B., Tack, F.M.G., 2004. Earthworm biomass as additional information for risk assessment of heavy meyal biomagnification: a case study for dredged sediment-derived soils and polluted floodplain soils. Environmental Pollution 129, 363-375.

Verhoef, H.A., 1996. The role of soil microcosms in the study of ecosystem processes. Ecology 77, 685-690.

Vijver, M.G., Vink, J.P.M., Miermans, C.J.H., van Gestel, C.A.M., 2003. Oral sealing using glue: a new method to distinguish between intestinal and dermal uptake of metals in earthworms. Soil Biology and Biochemistry 35, 125-132.

Vijver, M.G., van Gestel, C.A.M., Lanno, R.P., van Straalen, N.M., Peijnenburg, W.J.G.M., 2004. Internal metal sequestration and its ecotoxicological relevance - a review. Environmental Science and Technology 38, 4705-4712.

Vink, K., van Straalen, N.M., 1999. Effects of benomyl and diazinon on isopod-mediated leaf litter decomposition in microcosms. Pedobiologia 43, 345-359.

Vorobeichik, E.L., 1998. Populations of earthworms (Lumbricidae) in forests of the

middle Urals in conditions of pollution by discharge from copper works. Russian Journal of Ecology 29, 85-91.

Wardle, D.A., 2002. Communities and Ecosystems. Linking the aboveground and belowground components. Princeton University Press, New Jersey.

Wardle, D.A., Bardgett, R.D., Klironomos, J.N., Setälä, H., van der Putten, W.H., Wall, D.H., 2004. Ecological linkages between aboveground and belowground biota. Science 304, 1629-1633.

Welp, G., Brümmer, G.W., 1997. Microbial toxicity of Cd and Hg in different soils related to total and water-soluble contents. Ecotoxicology and Environmental Safety 38, 200-204.

Weng, G., Wu, L., Wang, Z., Luo, Y., Christie, P., 2005. Copper uptake by four *Elsholtzia* ecotypes supplied with varying levels of copper in solution culture. Environment International 31, 880-884.

Whalen, J.K., Parmelee, R.W., 1999. Quantification of nitrogen assimilation efficiencies and their use to estimate organic matter consumption by the earthworms *Aporrecetodea tuberculata* (Eisen) and *Lumbricus terrestris* L.. Applied Soil Ecology 13, 199-208.

Yeates, G.W., Bongers, T., 1999. Nematode diversity in agroecosystems. Agriculture, Ecosystems and Environment 74, 113-135.

Yonebayashi, K., Okazaki, M., Pechyapisit, J., Vijarnsorn, P., Zahari, A.B., Kyuma, K., 1994. Distribution of heavy metals among different bonding forms in tropical peat soils. Soil Science and Plant Nutrition 40, 425-434.

Zaitsev, A.S., van Straalen, N.M., 2001. Species diversity and metal accumulation in oribatid mites (Acari, Oribatida) of forests affected by a metallurgical plant. Pedobiologia 45, 467-479.

Zhang, M.K., Xu, J.M., 2003. Difference of lead, copper and zinc concentrations between interiors and exteriors of peds in some contaminated soils. Chemosphere 50, 733-738.

Zorn, M.I., van Gestel, C.A.M., Eijsackers, H., 2005. The effect of two endogeic earthworm species on zinc distribution and availability in artificial soil columns. Soil Biology and Biochemistry 37, 917-925.

Summary

Summary

General objectives

Soil pollution by hazardous chemicals has become a serious problem in many countries, impeding the use of land. The main purposes of soil quality Environmental Quality Standards (EQS) for soil pollution in Japan are to protect human health, and the effects on terrestrial ecosystems are hardly noticed. The reasons for few attentions to ecological aspects would be partly the lack of basic knowledge about native soil organisms and their functions, and toxicological data on them. Soil ecological functions: e.g. cycling of nutrients, litter decomposition and primary production, are vulnerable to a pollution, therefore the impacts of soil pollution can be evaluated as decrease in such a functional performance. In addition, macrofauna like earthworms serve as food for a variety of vertebrates, therefore the presence of contaminants in them poses a serious risk of secondary poisoning of predators due to biomagnification. The general objectives of this thesis were to determine the effects of heavy metal pollution on a soil ecosystem, including soil biota, ecological function and bioaccumulation, and to evaluate the validity of semi-field test using Terrestrial Model Ecosystems (TMEs) for soil ecological assessment in Japan. The research was proceeded on the line of the triad approach: chemical analysis, ecological research *in situ*, and laboratory tests with field soil.

Field survey

The study area of this thesis, Watarase retarding basin on Watarase River, locates downstream of Ashio Copper Mine. Although the mine was closed in 1973, complex heavy metal pollution remains in the Watarase retarding basin until today. The study area is dominated by tall grasses (reed; *Phragmites australis* and *Miscanthus sacchariflorus*). The amounts of heavy metals in soil were estimated at two polluted and two reference sites (chapter 2), and thirty plots of gradual concentrations (chapter 4). A complex heavy metal pollution, with moderate but wide range concentrations, was observed in Watarase retarding basin; total Cu concentration (386.7-24.8 mg/kg) was highest followed by Zn (260.6-45.3 mg/kg) and Pb (95.1-17.2 mg/kg). Total Cd concentration was much lower (2.51-0.58 mg/kg). A positive relationship was also shown between total and DTPA-extractive heavy metal concentrations, while the available concentrations in $CaCl_2$ extraction were very low and similar irrespective of total concentrations. The aging period would increase the binding of heavy metals to soil, and decrease the available fractions.

The effects of heavy metals on microbial biomass and microbial functional community were focused on chapter 2. The soil microbial biomass was affected by ambient soil properties (e.g. total C, N, pH, moisture content, and CEC) rather than by the heavy metal pollution. However, the aged pollution tended to impact on the composition of the microbial community. PICT (pollution-induced community tolerance) test using BIOLOG Ecoplates showed enhanced tolerance of the microbial community to Cu stress in the polluted site. Chapter 3 focused on the effects of heavy metals on soil macrofauna, microarthropods and ecological functions in the study sites. In the predator group of macrofauna, Geophilomorpha was found mainly in the polluted sites, while Lithobiomorpha was found in the reference site. Geophagous earthworms

notably changed in biomass and community structure: low biomass and dominance of moniligastrid worms in the polluted sites, while high biomass and dominance of megascolecid worms were observed in the reference site. In microarthropods, Collembolan community structure did not change conspicuously, probably due to low available heavy metal fraction. However, Oribatida changed the species composition. The total abundance of microarthropods was larger in the polluted sites, which partly results from the low earthworm biomass and small adverse effects of ecosystem engineer. Analysis of fungal based food web model showed in similar or lower N mineralization rates in the polluted sites compared to reference sites, indicating low N turnover rate in spite of high biomass of microarthropods at the polluted sites. Ecological functions, such as organic matter decomposition, soil aggregates and primary production, were not significantly influenced by heavy metal pollution. Thus, soil biota was not affected seriously, but complementally replaced in part by other sets of species in the same functional groups in such a moderately polluted site. The ecological functions are characterized by redundancy at the pollution levels in Watarase retarding basin, which would be maintained by replaced species in the same functional group.

Chapter 4 presented the heavy metal concentrations in earthworms at the species level to explore a risk of secondary poisoning of vertebrate predators due to biomagnification. There were six species in the basin, belong to three families: Megascolecidae; Moniligastridae and Lumbricidae. Despite the same endogeic categories, species-specific patterns of heavy metal accumulation were observed: species of Megascolecidae and Lumbricidae had relatively lower concentrations compared to those of Moniligastridae. Even in the same family of Moniligastridae, *Drawida* sp. accumulated Cu and Pb markedly higher than *Drawida japonica*. Soil

heavy metal concentrations in extracts of $CaCl_2$ and DTPA indicated that the different patterns of heavy metal accumulation among species must partly result from species specific gut process. In moderately contaminated area just as Watarase retarding basin, the earthworm community structure and heavy metal concentrations in earthworms indicate very important indicators for ecological risk assessment.

TME approaches

We used the Earthtron facility for laboratory experiments. The Earthtron is a simple computer-controlled chamber simulating natural environments: diurnal light/dark cycles, and separately controlled soil and air temperature, humidity and CO_2 concentration. In soil core incubation experiments conducted in chapter 5, the Earthtron was able to simulate the dynamics of soil temperature in field conditions. Environmental control also affects the dynamics of soil water and distribution patterns of nutrients in the microcosm, linking to the distribution of plant roots and soil biota. Thus, accurate environmental control is necessary to measure and understand the interaction between above-ground and below-ground components in microcosm experiments.

Chapter 6 focused on the effect of applied Cu and aged heavy metal pollution on soil ecosystems including soil biota (microbial community, microarthropods and macrofauna) and ecological functions (N mineralization, elements in leachate, organic matter decomposition and plant growth) using TME approach, and demonstrated the utility of TME approach for soil ecotoxicological studies by comparison with a homogenized soil system. The community structure of microbes and oribatid mites, and

earthworm biomass were soil biological parameters affected significantly by spiked Cu. The Cu application also had significant effects on several functions including soil ammonium-N concentration and plant growth. However, these results did not necessarily predict the field situations affected by aged heavy metal pollution. The intact soil system was able to mimic the functional parameters *in situ* compared to homogenized systems. Thus, despite several limitation and requirements for further research, TME is valuable approach for ecological risk assessment.

A perspective for soil ecological risk assessment in Japan

In field survey for heavy metal polluted soil, the triad approach would be recommendable method. An adequate reference location is needed to decide on the degree of toxic effects at a target location. In chemical analysis, the estimation of an available fraction of toxicants is important rather than total concentration: $CaCl_2$ would be useful for microbes and many soil organisms exposed through the skin, while DTPA would be useful for geophagous species like some earthworms. In ecological surveys, community level analysis for microbe, microarthropods and earthworm perform as good indicators for moderately polluted soil. In addition, the concentration in earthworm at the species level should be included as an indicator for secondary poisoning. Organic matter decomposition was also a good indicator for assessment of the effects on ecological functions, because this indicator can include both soil microbial and faunal activities. In bioassay, native earthworms should be used as test organisms. Intact soil system would also provide much profitable information for functional analysis in bioassays. Moreover, bioassays conducted under controlled environmental conditions

can exclude the effects of uncontrolled field conditions.

When we derive a concentration level such that accepted risk is not exceed, few basic toxicological data are available for native soil animals in Japan. Native earthworms should serve as key test organisms because of not only their sensitivity, but also important role in soil ecosystem and should be given priority for ecotoxicological analysis by established test methods. At the next step, an assessment system taking a local area into account would be appropriate to Japanese soil because of a large diversity of soil types. Intact soil system using TME would be adequate to investigate the toxic effects on local soil ecosystems, because TME could mimic not only soil biota, but also functional parameters *in situ*. Biological and functional parameters estimated in this study could be used as endpoints in ecotoxicological studies with TMEs. The toxicological data sets of each soil type by using TMEs can provide more realistic quantitative estimations of risk in each local site with probabilistic approaches, rather than hazard quotient method. It is a long way and very laborious to establish the TME methods and collect ecotoxicological data set with the approach. But soil ecologists in Japan are hopefully expected to take steps for ecotoxicological work in anticipation of legal requirement into the future.

要　約

要　約

１．研究の背景（Chapter 1）

人為的な汚染による土壌の劣化は世界各国で重大な問題となっている．我が国においても近年土壌汚染に関する法整備が進んでいるが，現行の制度は人の健康や生活影響に注目しており，土壌生態系については言及されていない．一方欧米では，土壌汚染の生態系影響について土壌生物も取り入れた評価手法が検討されている．我が国で土壌生態系への影響が考慮されていない要因として，土壌動物やそれらの有する機能，およびそれらへの毒性影響に関する基礎的な知見が不足していることが挙げられる．そのため，土壌生態リスク評価を我が国で導入するにあたり，国内の土壌生態系への土壌汚染影響を把握する必要がある．本研究では，重金属汚染が土壌生態系に及ぼす影響について，汚染地における生態学的機能や生物蓄積も考慮して体系的に把握すること，また Terrestrial Model Ecosystem (TME)とよばれる現地の生物をそのまま含んだ未撹乱土壌を用いた室内ポット実験による生態学的評価手法の検討を行うことを目的とした．本研究は，汚染物質の定量，生態学的調査および生態毒性試験といった情報を統合して解析した．

２．結果および考察

１）野外調査

重金属濃度

本研究では，調査地を栃木県にある渡良瀬遊水地に設定した．この地域は渡良瀬川下流部にあたり，過去に上流の足尾銅山の採掘作業の影響で重金属の複合汚染に曝された．現在は，ヨシ・オギの高茎草本が優占する草原が広がっている．土壌重金属濃度は，汚染地の２区，非汚染地（対照地）の２区（Chapter 2），および遊水地内で濃度勾配が得られるような 30 地点（Chapter 4）で測定した．その結果，渡良瀬遊水地では中程度ながら複合汚染がみられ，全濃度（硝酸抽出）ではCuが高く (386.7-24.8 mg/kg)，次いでZn (260.6-45.3 mg/kg)，Pb (95.1-17.2 mg/kg)となり，Cdは最も低濃度であった(2.51-0.58 mg/kg)．生物に取り込まれる可能性（bioavailability）を考慮して測定したDTPA抽出による重金属濃度は全濃度と正の相関を示したが，$CaCl_2$抽出による濃度は全濃度によらず著しく低くなった．長期間の汚染により重金属の土壌への吸着力が増し，土壌水に溶け出す画分は汚染区でもごく微量であることが示された．

土壌生物相と生態学的機能

Chapter 2 では，汚染区と非汚染区における微生物バイオマスや群集構造の違いを，土壌特性および重金属濃度に注目して評価した．微生物バイオマスは同じ土壌特性を持つ調

査区間で差がなく，重金属濃度よりも全炭素，全窒素，pH，含水量，CEC といった土壌特性が大きく影響していた．しかし，BIOLOG エコプレートを用いた PICT テスト（Pollution Induced Community Tolerance: 汚染誘導群集耐性）により，微生物群集が汚染地では耐性のある群集構造になることが示唆された. Chapter 3 では, 大型土壌動物, 小型節足動物および生態学的機能への汚染影響を把握した．捕食者として汚染区ではジムカデが，非汚染区ではイシムカデが優占する傾向があった．また，ミミズは調査区間で群集構造に違いがみられ，汚染区ではジュズイミミズ類が，非汚染区でフトミミズ類が優占していた．トビムシ群集は大きな違いがなかったものの，ササラダニ群集は種構成が異なる傾向がみられた．これらの小型節足動物の個体数は汚染区で多く，これはミミズのバイオマスが小さく，撹乱等による負の影響が小さいことに起因すると考えられる．小型節足動物のバイオマスが大きいものの，糸状菌をベースとした食物連鎖による窒素の無機化量は汚染区で同程度か若干低くなった．生態学的機能として測定した有機物の分解率や耐水性団粒量，オギの一次生産量には汚染影響による有意な差が認められなかった.

ミミズによる生物蓄積

Chapter 4 では，生物濃縮による脊椎動物の捕食者への二次汚染によるリスクを想定して，現地のミミズの体内重金属濃度を種レベルで測定した．計 30 調査地点において採取したミミズは３科６種が同定され，群集組成には重金属濃度よりもｐHや粘土量といった土壌特性が影響していた．すべて土壌を摂食する地中性ミミズであったが，フトミミズ科やツリミミズ科の種よりもジュズイミミズ科の種の体内重金属濃度が高く，中でも*Drawida* sp.は*Drawida japonica*よりも銅や鉛を多く蓄積するなど，金属種，ミミズ種間によって蓄積の程度が大きく異なることが本研究で明らかになった．このような種間の差は，$CaCl_2$溶液およびDTPA溶液による土壌抽出実験により，体表面からの浸透による曝露よりも，腸内における吸収量が関係すると推察された.

野外調査のまとめ

調査対象とした渡良瀬遊水地の重金属汚染は長期間に及ぶため，土壌粒子や有機物に強く吸着されるなど，土壌水に溶け出す画分はきわめて少なく，土壌生物相の激減といった過度の影響は観察されなかった．ただし，微生物群集は汚染に耐性を持つような構成に移行し，大型土壌動物や小型節足動物も機能グループの種が入れ替わるなど，汚染影響による種構成の変化が示唆された．これらの構成の変化はそれぞれのグループが有する機能に補完的作用をもたらすと考えられ，その結果，現地では生態学的機能の目立った低下はみられず，冗長性が示された．一方，土壌食のミミズに関しては，種によっては体内重金属量が土壌中の濃度と比例して増加する傾向がみられ, 本調査地のような中程度の汚染では, 生物蓄積による食物網を通じた生物濃縮が最も重要な生態リスク指標となることが示された.

2）モデル生態系（TMEs）による生態リスク評価

横浜国立大学に導入された環境制御室（Earthtron）は，土壌を 60 ポット設置でき，地上部と地下部の温度の個別制御や，地上部の湿度・CO_2濃度調節が可能である．Chapter 5 では，Earthtronによる環境制御が野外に類似した土壌温度動態を再現できることを比較実験によって確認し，それが土壌水分や栄養塩の挙動を決定する要因となることを明らかにした．このような室内実験における適正な環境制御は，地上部－地下部の相互作用を理解し，野外での反応を推定する上で必要となることが示された．

Chapter 6 では，新たに Cu を添加した土壌および長期間汚染された野外土壌を用いて，土壌生物（微生物群集，小型節足動物，大型土壌動物）および生態学的機能（窒素無機化，土壌水中の栄養塩，有機物分解率，一次生産量）への影響を TME 手法により評価した．また，従来のような撹乱土壌を用いたポット試験結果と比較することで，土壌生態毒性研究における TME 手法の妥当性を探った．Cu 添加試験では，微生物群集構造，ササラダニの種組成，ミミズバイオマスの感受性が高く，土壌アンモニア態窒素の増加や植物成長の低下がみられたが，これらの結果は必ずしも長期間重金属汚染が存在する野外の状態を反映するものではなかった．一方，未撹乱土壌は撹乱土壌よりも野外における生態学的機能の状態をよく反映することが明らかになった．以上のように，いくつかの制限条件や改良の必要性があるものの，土壌生態リスク評価における TME 手法の有効性が確認された．

3．我が国における土壌生態リスク評価－今後の展望－

本研究結果から，土壌汚染が発覚した地域において，化学・生物情報の統合により正確な土壌生態系の影響評価を行うことができ，それには適正な対照地が必要であることが明らかになった．化学性の測定では，汚染物質の全量よりも生物に取り込まれる可能性のある画分の推定が重要であり，経皮曝露においては$CaCl_2$抽出が，ミミズのような土壌を摂取する動物にはDTPA抽出が有効であることが示唆された．生態調査では，微生物，小型節足動物，ミミズの群集レベルの解析が中程度の汚染地において有効な指標性を持つことが確認された．加えて，ミミズの体内濃度は二次汚染リスクの指標として重要であり，生態学的機能では有機物分解率が微生物や土壌動物の活性を統合する指標として有効であると考えた．毒性試験では，在来ミミズの試験生物としての利用が必要であり，未撹乱土壌を用いた試験が機能面への影響評価に有効であることが示された．

土壌生態系への影響から汚染物質の規制値を設定するには，我が国では毒性データが著しく不足している．在来ミミズは感受性の高さだけでなく生態系における役割からも重要であり，その生態毒性情報の把握が急務である．次の段階として，我が国は土壌タイプが多様なことから，評価システムは地域ごとに考慮する必要があり，現地の生物情報・機能情報をともに包含する TME 手法による未撹乱土壌を用いた毒性影響評価が有効であると考えた．本研究で評価対象としたような複数の生物および機能指標を用いて定量的なリス

ク評価を行うには，従来のようなハザード比を用いた評価よりも個々の感受性を含んだ確率論的手法の導入が必要である．

以上のような手法の確立やデータ収集には大きな労力と時間が必要となるが，将来の法制度化を見据えた，我が国のより多くの土壌生態研究者による生態毒性研究への取り組みが期待される．

Publications

Publications

Kamitani, T., Oba, H., Kaneko, N. (in press) Microbial biomass and tolerance of microbial community on an aged heavy metal polluted floodplain in Japan. *Water, Air, and Soil Pollution*. ..…….<Chapter 2>

Kamitani, T., Kaneko, N. (in press) The Earthtron facility for below-ground manipulation study. *Ecological Research* (DOI: 10.1007/s11284-005-0139-5). ..…….<Chapter 4>

Kamitani, T., Kaneko, N. (in press) Species-specific heavy metal accumulation patterns of earthworms on a floodplain in Japan. *Ecotoxicology and Environmental Safety* (DOI: 10.1016/j.ecoenv.2005.10.009). ..…….<Chapter 5>

金子信博・金田哲・橋本みのり・豊田鮎・古川祐美・神谷貴文 (2005) 土壌生態系研究におけるマイクロコズム手法. *Edaphologia*, 78: 19-30.

神谷貴文・金子信博・丹羽尚志・前田浩之助 (2004) 生態学的視点による重油汚染土壌の回復状況調査, *Edaphologia*, 75: 1-9.

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