in a decrease in pesticide concentrations in river water compared to artificial drainage. According to studies on pesticide runoff from paddy plots, a prolonged water-holding period by paddy closure after pesticide application effectively reduces pesticide discharge (Karpouzas *et al.* 2006; Vu *et al.* 2006). However, this agricultural practice may not always be effective because rainfall, and in particular the heavy rainfalls that occur during the Asian monsoons, including in Japan, may cause spillover of paddy-ponding water to the rivers.

For the fungicide isoprothiolane and the herbicide pretilachlor, model simulations were conducted where percolation instead of artificial drainage were practiced during the intermittent irrigation period. The other model simulation conditions were the same as those of Figure 4, and therefore the effect of percolation/artificial drainage could be compared. As comparing Figure 8A with Figure 4A, the model simulation revealed that percolation instead of artificial drainage resulted in a decrease in the total amount of fungicide runoff, from 65 kg to 30 kg. However, no substantial change in herbicide runoff (Figures 8B and 4B). The result of fungicide suggests that 35 kg of the 153 kg applied to rice paddy fields flows to the river during artificial drainage of the fields and about 30 kg can potentially flow to the river from spillover caused by precipitation. Precipitation of 47 mm on July 19 and 30 mm on July 26 could have resulted in pesticide spillover as almost all of the runoff of the pesticides occurred during this short period of time. As reported elsewhere (Sudo *et al.* 2002; Phong *et al.* 2008), pesticide runoff from spillover is dependent on the frequency and intensity of precipitation and the height of the paddy water outlet to accommodate the excess precipitation without spilling over. Therefore, the quantity of runoff and the concentration of a fungicide in river water should vary depending on the structure of the paddy and the amount of precipitation, although in our sensitivity analyses using model simulations the concentration of the fungicide in the river water decreased by about one-third when intermittent irrigation was combined with percolation.

The results of the sensitivity analyses shown in Figure 3 were based on simulations of artificial drainage in intermittent irrigation periods. We then conducted similar sensitivity analyses for percolation in intermittent irrigation periods. The effect of the degradability in soil on the fungicide concentration did not change; the overall concentrations were merely shifted downward (data not shown). Changing the drainage practice did not alter the effect of the adsorbability on the fungicide concentration. Even with percolation, the majority of the fungicide runoff occurred through spillover of paddy water, with soil permeation only slightly affecting total runoff. The effect of the degradability in soil, therefore, on fungicide concentrations was not particularly large.

Synergetic effects of pesticide degradability and adsorbability

We carried out a sensitivity analysis of the effects of the degradability-in-soil on the concentrations of pretilachlor and isoprothiolane in the river water. The value of the degradation rate constant in soil was changed under the three conditions of adsorption coefficients and the two

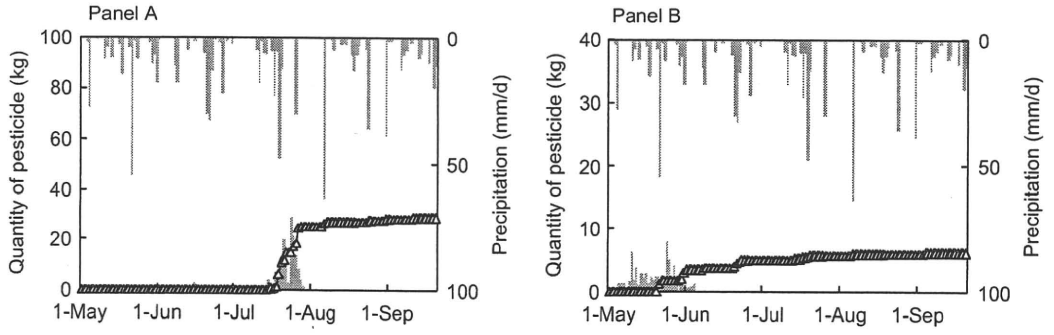

Figure 8 | Time-series runoff behavior of the fungicide isoprothiolane (Panel A) and the herbicide pretilachlor (Panel B) with percolation instead of artificial drainage during the intermittent irrigation period. Bars along the *x*-axis indicate the daily quantity of applied pesticide (in kg), and bars along the top axis indicate the amount of precipitation. Open triangles indicate the cumulative amount of pesticide runoff into the river.

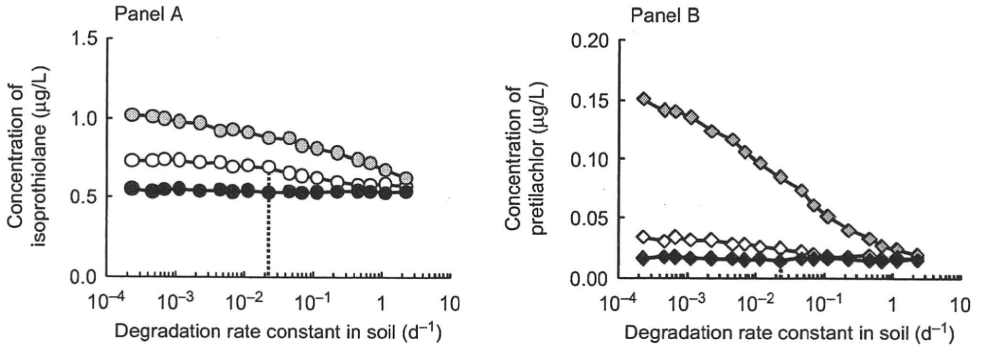

Figure 9 | Effect of the pesticide degradability-in-soil on the average concentrations of isoprothiolane (Panel A) and pretilachlor (Panel B) under the conditions of three adsorption coefficient values. Gray circles, white circles, and black circles indicate the results with the low, actual, and high adsorption coefficient values: 12.3 mL/g, 1230 mL/g, and 123 L/g, respectively, for isoprothiolane; 11.6 mL/g, 1160 mL/g, and 116 L/g, respectively, for pretilachlor.

drainage practices. As shown in Figure 9, with a low pesticide adsorbability to soil (adsorption coefficients of 12.3 mL/g for the fungicide and 11.6 mL/g for the herbicide, which are 0.01 times the default values; see Table 1), the concentrations of the pesticides increased as the degradation rate constant in soil decreased. With a high pesticide adsorbability (123 L/g for the fungicide and 116 L/g for the herbicide), concentrations were not affected by degradability. We interpret this as pesticides with high adsorbability existing mostly in soil, which minimizes the potential pesticide loads to river water regardless of their degree of degradability. When the pesticide degradation rate constant in soil was high (degradation rate constants were increased to 2.3 d^{-1} for both pesticides, 100 times the default value), the effect of adsorption decreased, suggesting that pesticide loads are minimal when either adsorbability or degradability are high.

When the pesticide adsorbability in soil was low, pesticide concentrations were decreased with the increase in the degradation rate constant in soil. The concentration of the herbicide was more affected by the degradation rate constant compared to the fungicide because fungicides are removed with drainage water and spillover by rain within a short time after application. The effect of the degradability on the fungicide concentration was more prominent with percolation compared to artificial drainage. Fungicides with low soil adsorbability may permeate underground or flow by lateral water seepage/leakage through the ridge of paddy fields to adjacent drainage canals, which suggests that percolation may not be effective in preventing fungicide runoff when soil adsorbability is low.

Overall, concentrations of pesticides were highest with the simultaneous condition of low adsorption coefficients and low degradation constants. The pesticide degradability in soil affected the pesticide concentration only when the pesticide adsorbability was low.

CONCLUSIONS

(1) The rice pesticide concentrations in downstream river water differed according to the application period of the pesticide and the irrigation schedule. When the pesticide is applied more than a month before the paddy fields are drained (as in the case of herbicide application), the pesticide runs off slowly, and the pesticide concentration in river water is affected by the degradability and adsorbability in soil. In contrast, when pesticides are applied just prior to intermittent irrigation and artificial drainage (as in the case of fungicide application), the pesticide runs off rapidly, and neither the degradability nor the adsorbability has a notable effect on the pesticide concentration in the river water. Therefore, even if pesticides have similar characteristics, pesticide concentrations vary greatly in river water depending upon the application timing.

(2) Only degradation rate constant in-water larger than approximately $10^{-2}\,d^{-1}$ and water solubility values less than 1 mg/L influenced pesticide concentrations.

(3) The total runoff quantity of fungicides, which were applied shortly before the start of artificial drainage of

the paddy fields, was partly depressed by using percolation rather than artificial drainage; fungicide still entered the river via spillover of paddy field water during rain events. Therefore, the effect of soil adsorbability on fungicide concentrations was not large, regardless of whether artificial drainage or percolation was used during intermittent irrigation.

(4) The concentrations of pesticides decreased when either the degradation rate constant in soil or the adsorption coefficient was large. The concentrations of pesticides greatly increased when both the degradation rate constant in soil and adsorption coefficients were small.

We obtained these findings from the sensitivity analysis, but further observations are needed for their confirmation.

ACKNOWLEDGEMENTS

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Risk Assessment of Fenthion Oxide Derivatives in Aqueous Environment

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ABSTRACT

Fenthion (MPP), an organophosphorus pesticide, is widely used as an agricultural and household insecticide. The oxons are known to be the actual toxic forms of organophosphorus pesticides. Using an *in vitro* cytochrome P450 (CYP) metabolism system, MPP was metabolized to produce five metabolites: MPP sulfoxide, MPP sulfone, MPP oxon, MPP oxon sulfoxide and MPP oxon sulfone. MPP sulfoxide was the main product, while MPP oxon sulfone and the other metabolites were produced in small amounts. On the other hand, MPP was converted to MPP oxon sulfone by chlorination in a water purification system, raising the possibility of human exposure to MPP oxon sulfone through drinking water. MPP oxon sulfone showed the highest acute toxicity among MPP and its metabolites. In addition, MPP oxon sulfone was not metabolized by CYP3A4, the major CYP isomer in humans. It is important that MPP and its oxides are monitored and their health risk assessed to control drinking water safety because MPP was detected in river water.

Keywords: MPP, oxide derivative, risk assessment.

INTRODUCTION

Many chemicals from domestic wastewater, industrial effluent, agricultural run-off, and other sources flow into natural waters such as rivers. For this reason, it is relevant to consider that those pollutants could affect human health. In particular, the general public has a great concern for pesticides because of their adverse effects on human health. Therefore, numerous monitoring surveys of pesticides in natural water have been performed, and the detection data have been reported (Sancho *et al.,* 2004; Quintana *et al.,* 2001; Frenich *et al.,* 2001; Sabik *et al.,* 2000). In this study, we focused on fenthion (MPP), an insecticide from a class of organophosphorus pesticides, which is used in large amounts and has a high detection frequency. Organophosphorus pesticides may be converted to oxons, which are known to be their actual toxic derivatives (Eaton *et al.,* 2008; Casida and Quistad, 2004; Cox, 1994). Five oxide derivatives of MPP were determined including the side-chain oxidations and their oxons.

Concerns of the potential risk to human health of MPP have recently increased. We therefore sought to determine whether MPP could be biotransformed to oxon derivatives. Ingested chemicals are typically modified by the phase I oxidative reaction, of which the cytochrome P450 (CYP) proteins are the principal oxidative enzymes. CYP3A4 is found to be the major isomer in many organs (Denisov *et al.,* 2007; Guengerich, 1999). We examined and identified the *in vitro* metabolites of MPP mediated by human CYP3A4 and we also quantified the oxons.

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In this study, we measured MPP and its oxides in river water. On the basis of the metabolic profile and from the knowledge of environmental exposure routes, we further discussed the possible acute toxicity from exposure to MPP oxide derivatives.

MATERIALS AND METHODS

Chemicals

Fenthion (*O,O*-dimethyl *O*-4-methylthio-*m*-tolyl phosphorothioate, MPP) and MPP sulfoxide were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). MPP sulfone, MPP oxon, MPP oxon sulfoxide, and MPP oxon sulfone were purchased as 10 mg/L solutions in acetonitrile from Kanto Chemical Co., Inc. (Tokyo, Japan). Dichloromethane and acetone (pesticide residue analysis grade), acetonitrile and methanol (high performance liquid chromatography grade), and acetic acid were purchased from Wako Pure Chemical Industries, Ltd. The water used in the experiment was purified using a Milli-Q gradient A10 and Elix with EDS polisher water purification system (Millipore, Bedford, MA, USA). Microsomal enzyme solution and an NADPH regenerating system for the metabolic reaction were purchased from BD Biosciences (Woburn, MA, USA). The microsomal enzyme solution contained recombinant human CYP, human CYP reductase, and human cytochrome b_5, which were expressed from cDNA using baculovirus-infected insect cells.

Sampling procedure

River water samples were taken from the Naka River that flows through urban areas, Saitama and Tokyo, Japan. Sampling was performed twice a month from December 2007 to December 2008 in the riverside of Taniguchi Town, Misato City, Saitama Prefecture. A 2-liter glass bottle was washed twice with river water before the sample was placed and then the bottle was tightly covered so that air would not enter. The glass bottle containing the sample was kept at 4°C until the time of concentration and analysis which were done on the same day.

Pre-treatment of river water and GC/MS analysis

Analytes were extracted by solid-phase extraction (SPE) and were analyzed as previously reported (Tahara *et al.*, 2006). Two liters of river water samples were concentrated using a Sep-Pak Plus C18 SPE cartridge (Waters, Milford, MA, USA) with an automatic concentrator, Sep-Pak Concentrator Plus (Waters). The final volume used was 1 mL with dichloromethane for gas chromatography/mass spectrometry (GC/MS) analysis. The instrument was operated in selected-ion monitoring (SIM) mode. Two selected ions for each target compound were monitored for quantification and identification, and the retention times are summarized in Table 1.

Metabolic reaction

A 1 mM standard solution of pesticide was prepared in acetone and stored at –20°C. Working solutions were freshly prepared before use by diluting with acetonitrile. The reduction of MPP sulfoxide back to MPP by the cytosolic aldehyde oxidase enzyme was ruled out in this study. Each 0.5 mL reaction mixture contained 1.6 mM $NADP^+$, 3.3 mM glucose-6-phosphate, 3.3 mM magnesium chloride, 0.01 mM pesticide, and 0.4 U/mL glucose-6-phosphate dehydrogenase in 100 mM potassium phosphate buffer (pH 7.4). After incubation at 37°C for 10 min, the microsome solution was added to make up

a 10 pmol CYP isomer. Reactions were stopped after 5, 10, 15, 20, 30, 40, 50 and 60 min by the addition of 250 μL acetonitrile. And then they were centrifuged at 10,000×*g* for 3 min. The supernatant was analyzed by liquid chromatography/mass spectrometry (LC/MS).

Quantification using LC/MS analysis

Analysis by LC/MS was performed as previously reported (Tahara *et al.*, 2008a). The retention time and selected ions for each target compound were monitored for quantification and are summarized in Table 1.

Table 1 - Analysis of monitored ions and retention times of MPP and its oxides using GC/MS and LC/MS.

Compound	GC/MS			LC/MS	
	Retention time (min)	Quantitation ion (*m/z*)	Identification ion (*m/z*)	Retention time (min)	Quantitation ion (*m/z*)
MPP	16.0	278	125	18.3	279
MPP sulfoxide	23.0	125	278	13.3	295
MPP sulfone	23.3	310	125	16.9	311
MPP oxon	14.7	262	109	16.2	263
MPP oxon sulfoxide	21.3	263	109	4.0	279
MPP oxon sulfone	21.1	294	109	6.6	295

RESULTS AND DISCUSSION

MPP and its oxides in river water

MPP is widely used as a pest control agent for rice, leguminous plants, and fruit trees. As mentioned previously, the existence of MPP and its oxides in river water was studied. From the results of the GC/MS analysis for the period April to August, a peak was observed on the chromatogram which was recognized to be MPP with *m/z* = 278 at a retention time of 16 min (Fig. 1). The peaks of oxides were not detected in river water. The maximum concentration of MPP was 0.12 μg/L on April (Fig. 2). As previously reported, insecticides and herbicides used in paddy fields become detectable in river water immediately after spraying (Tsuda *et al.*, 1998). Their concentrations show seasonal changes depending upon their usage.

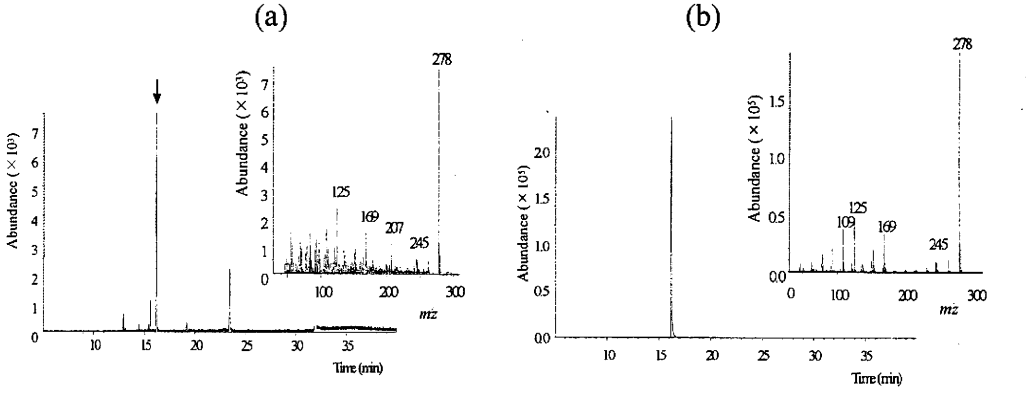

Fig. 1 - Ion chromatograms and mass spectra of MPP; (a) detected in river water, (b) 10 mg/L standard solution

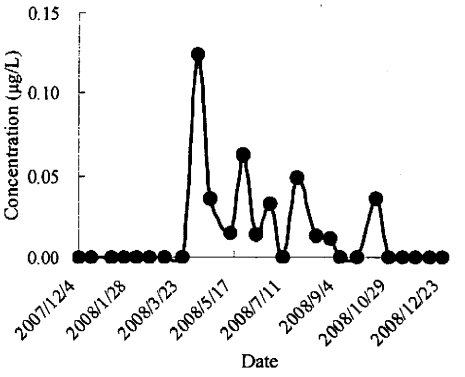

Fig. 2 - Detected concentration of MPP in river water sampled from Naka River in 2008

Metabolism of MPP by human CYP3A4

Among CYP isomers, CYP3A4 is found to have the largest quantity *in vivo*. In order to detect the metabolites of MPP and to help determine its potential risks to human health, MPP was allowed to react with human CYP 3A4. MPP sulfoxide was detected by LC/MS as the main metabolite, and four other metabolites (MPP sulfone, MPP oxon, MPP oxon sulfoxide and MPP oxon sulfone) were also detected at trace levels (Fig. 3). This metabolic reaction continued linearly for 10 min. Among the metabolites of MPP, we focused on the formation of oxon metabolites, which are known inhibitors of cholinesterase activity. The conversion rates of MPP to MPP oxon, MPP oxon sulfoxide and MPP oxon sulfone by CYP3A4 were 1.5, 0.52, and 0.02 nmol/min/nmol P450, respectively, calculated from the molarities at 10 min.

In addition, MPP was similarly metabolized by seven other CYP isomers: CYP1A2, CYP1B1, CYP2A6, CYP2C9, CYP2C19, CYP2D6 and CYP2E1. The major products of metabolism by these seven CYP isomers were MPP sulfoxide and MPP oxon. Trace amounts of MPP sulfone, MPP oxon sulfoxide, and MPP oxon sulfone were detected from CYP1A2 and CYP2C19.

Only small quantities of the bioactive derivatives, MPP oxon, MPP oxon sulfoxide and MPP oxon sulfone, were formed from the metabolism by any of the eight CYP isomers.

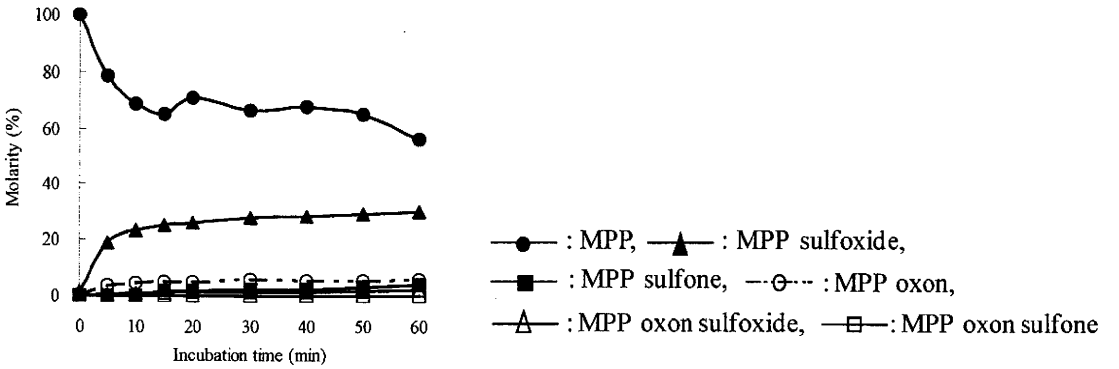

Fig. 3 - Formation of MPP metabolites by CYP3A4 up to an incubation time of 60 min. The molarity of MPP (10 μM) at the beginning of the reaction was defined as 100%.

Two major metabolites, MPP sulfoxide and MPP oxon were formed from MPP by some CYP and flavin-containig monooxygenase in the previously reported papers (Leoni *et al.*, 2008; Kitamura *et al.*, 2003). In their results, MPP sulfone and MPP oxon sulfone were not detected on chromatograms by HPLC/UV and GC/MS. Thus, we compared sensitivity on metabolites using GC/MS and LC/MS, and set the limit of detection of MPP sulfoxide, MPP sulfone, MPP oxon, MPP oxon sulfoxide and MPP oxon sulfone to 10, 2, 5, 50 and 20 μg/L for GC/MS and 0.02, 0.2, 0.05, 0.2 and 0.1 μg/L for LC/MS, respectively. Five compounds except MPP were able to be analyzed more effectively by LC/MS as compared with GC/MS. Since LC/MS had 10-500 times higher sensitivity in measurement of metabolites than GC/MS, it yielded satisfactory separation and it was used to analyze the metabolites. Therefore, traces of MPP sulfone, MPP oxon sulfoxide and MPP oxon sulfone were able to be detected.

Toxicity evaluation of MPP oxide derivatives

In water, MPP is gradually converted to MPP sulfoxide and then to traces of MPP oxon sulfoxide and MPP oxon sulfone (Tahara *et al.*, 2008a). Moreover, MPP has been thought to react with chlorine in the water purification process. The formation of four oxides, MPP sulfoxide, MPP sulfone, MPP oxon sulfoxide and MPP oxon sulfone, through chlorination process was observed, and after 24 hours, MPP was almost completely converted to MPP oxon sulfone (Tahara *et al.*, 2008a). The four MPP oxide derivatives have not received careful evaluation as toxic agents since they are not used as pesticides. Thus, the three MPP oxons were evaluated for toxicity by *in vitro* system using cholinesterase activity as an indicator (Tahara *et al.*, 2005). It was found out that MPP oxon sulfone has the most pronounced adverse effect followed by MPP oxon sulfoxide, and then MPP oxon. Consequently, human risk assessment for MPP must be done on the basis of the contributions of its oxides.

The stability of MPP oxon sulfone with CYP3A4 was examined next. The concentration of MPP oxon sulfone did not change for 60 min, which suggests that MPP oxon sulfone might accumulate and show toxicity without further *in vivo* modification. It was also found out that the oxidation of MPP to MPP oxon sulfone occurs more readily in the environment and in the water purification process than by *in vivo* metabolism.

The inhibition of cholinesterase activity was reported to be 280 times stronger with MPP oxon sulfone as compared with MPP (Tahara *et al.*, 2008b). Therefore, careful monitoring of MPP oxides in drinking water is required to evaluate the acute toxicity of MPP because human population could be exposed to MPP oxon sulfone through the drinking water and other aqueous environment.

CONCLUSIONS

In this study, the possible genesis of MPP oxide derivatives in the environment was investigated as well as their potential toxicity to humans in a cholinesterase assay. The *in vitro* metabolic profile of MPP from aqueous environment was observed to mimic *in vivo* metabolism. It was clarified that CYP3A4, one of the most common human oxidative enzymes, catalyzed the conversion of MPP to five oxide derivatives. In public health, characterizing metabolic pathways for widely used pesticides is of high significance, especially with organophosphorus oxons, which have shown acute toxicity.

Moreover, the behavior of MPP oxon sulfone using CYP3A4 was examined because it is the product of the chlorination of MPP in the water purification process and it displays the strongest acute toxicity. As a result, the concentration of MPP oxon sulfone did not change and no product peak was observed. In addition, MPP was detected from river water in the summer months and lower amounts found in the winter months. MPP has a potential risk to human health by its conversion to MPP oxon sulfone through the water purification process; the environmental conversion end point for organophosphorus pesticides is thought to be the same. As shown in this study, the careful monitoring of organophosphorus pesticides and their oxides in aqueous environment and in tap water is an important matter for health assessment from the viewpoint of controlling water purity and risk management.

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Exposure assessment of metal intakes from drinking water relative to those from total diet in Japan

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ABSTRACT

Daily intakes of 17 metals (boron, aluminium, chromium, manganese, nickel, copper, zinc, arsenic, selenium, molybdenum, cadmium, antimony, lead, uranium, magnesium, calcium, and iron) via drinking water and total diet were investigated in six cities in Japan. The daily metal intakes were estimated and compared with tolerable daily intake (TDI) values proposed by the WHO or Joint FAO/WHO Expert Committee on Food Additives for toxic metals and with recommended dietary allowances (RDAs) or adequate intake (AI) values proposed for essential metals by the Japanese Ministry of Health, Labour and Welfare. Among the 13 toxic metals, mean dietary intakes of 10 (except arsenic, selenium, and molybdenum) were less than 50% of TDI, suggesting that for these 10 metals the allocation of intake to drinking water in establishing guidelines or standards could possibly be increased from the normal allocation of 10–20% of TDI. For the 13 toxic metals, the contribution of drinking water to TDI was 2% or less in all six cities. Mean dietary intakes of the essential elements magnesium, calcium, and iron were less than the RDA or AI values. Drinking water did not contribute much to essential metal intake, accounting for less than 10% of RDA or AI.

Key words | dietary metal intake, drinking water quality, health risk assessment

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INTRODUCTION

In the *Guidelines for Drinking-water Quality* of the World Health Organization (WHO), the guideline values for non-carcinogenic chemicals are derived from a certain percentage of tolerable daily intake (TDI). This percentage should be allocated on the basis of actual data on daily intakes from other sources such as food, air, and soil, but such data are not always available. As noted in the third edition of the guidelines (WHO 2004), when data on daily intake of the chemical are insufficient, the default allocation percentage to drinking water is 10% of TDI. In recent addenda to the guidelines, however, the allocation approach has changed twice. In the first addendum, the description of a specific percentage as the default allocation was changed to: "Where appropriate information is not available, values are applied that reflect the likely contribution from water for various chemicals" (WHO 2006). The values generally vary from 10 to 80%, considering the exposure from all sources (WHO 2006). In the second addendum, the use of a default allocation value was re-established and the value was changed from 10 to 20% of TDI (WHO 2008). The second addendum stated that an allocation of 20% is still protective and that 10% was found to be excessively conservative (WHO 2008). The collection of chemical intake data from other sources such as food is becoming more crucial for the review and establishment of local drinking water quality standards that reflect actual exposures to chemicals.

Although numerous studies have measured daily metal intake from total diet (Biego *et al.* 1998; Bordajandi *et al.* 2004; Maitani 2004; Santos *et al.* 2004; Turconi *et al.* 2009),

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dietary patterns as well as dietary metal intake vary among countries and regions. For example, like many Asians, Japanese eat rice as a staple food and consume a lot of seafood, and this diet differs from that in other parts of the world. When reviewing or establishing local drinking water quality standards, it is important to take into account these local dietary patterns. Recently, an issue about risk assessment of essential yet toxic metals has been posed (Aggett 2008; WHO 2008). For example, copper plays an essential role as a central component of many redox active enzymes (Stern 2008), and manganese is essential for skeletal development, immune system function, and energy metabolism (Santamaria 2008). Deficiencies in such essential metals linked to enzyme activities contribute to the progression of disease (Gambling 2008). Although these elements are essential to biological processes in humans, they can also be toxic when consumed in excessive amounts (Fraga 2005). Therefore, both deficient and excessive intake of certain essential elements poses health risks.

In the present study, we investigated daily metal intake (DMI) of 17 metals (toxic, essential yet toxic, and essential metals) from total diet and drinking water in six cities in Japan. We estimated the contribution to TDI of toxic elements as proposed by the WHO or Joint FAO/WHO Expert Committee on Food Additives (JECFA). We also estimated the contribution to recommended dietary allowances (RDAs) proposed by the Japanese Ministry of Health, Labour and Welfare (JMHLW). The RDA represents the dietary intake level that is sufficient to meet the nutrient requirements for 97–98% of a Japanese population of a certain age and gender (JMHLW 2005). We focused on the contribution of metal intake via drinking water to TDI and/or RDA. For toxic metals, the margins of TDI that could be allocated to drinking water for establishment or review of drinking water quality standards are discussed. The contribution of drinking water to daily essential metal intake is also discussed.

METHODS

Sample collection

Samples were collected by the market-basket method from six cities across Japan. At each sampling location, about 150 kinds of food were purchased from grocery stores according to the methods used by the National Nutrition Survey (JMHLW 2004). Food items that are generally cooked before consumption were cooked by the usual methods such as boiling or baking. The items were cooked without addition of any oil or seasoning, because these additives belonged to different categories. Then, all food items were categorized into 13 groups in accordance with the classification of the National Nutrition Survey (Figure 1). Finally, food samples of each group were homogenized to make a composite sample. Drinking water samples were collected from the tap where the preparation of the food was performed. In all six cities, drinking water was processed in municipal drinking water treatment plants that use surface water as the source. The drinking water samples were assigned to the 14th group. The food composite samples were stored at −30°C in a deep freezer and were defrosted just before analysis. Drinking water samples were stored at 4°C in a refrigerator.

Analytical methods

The composite food samples were digested in a microwave digestion system (ETHOS TC, Milestone S.r.l., Bergamo, Italy) by the following procedure. A portion of 0.5–1.0 g (wet weight) was weighed into a PTFE vessel, and 4 mL of nitric acid and 1 mL of hydrogen peroxide (Ultra Pure Grade; Kanto Chemical Co., Inc., Tokyo, Japan) were added. The basic program of the microwave digester was as follows: increase the temperature from room temperature

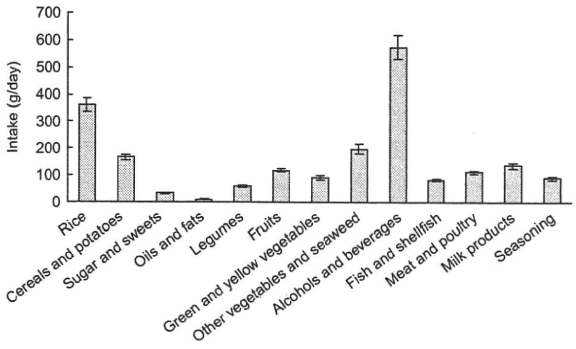

Figure 1 | Thirteen food sample groups and intake of every group, as determined by methods used by the National Nutrition Survey (JMHLW 2004). Columns represent the average of six cities, and error bars are one standard deviation.

to 210°C over 30 min, maintain that temperature for 15 min, and then cool to room temperature over 10 min; the maximum power was 1,000 W (Dolan & Caper 2002). Digested solution was brought up to 50 mL with Milli-Q water (Milli-Q Advantage, Millipore, Billerica, MA, USA).

The concentrations of 14 metals (boron, aluminium, chromium, manganese, nickel, copper, zinc, arsenic, selenium, molybdenum, cadmium, antimony, lead, and uranium) in drinking water and in the digested solutions of the food composite samples were determined using an inductively coupled plasma-mass spectrometer (ICP-MS; HP-4500, Agilent Technologies, Inc., Palo Alto, CA, USA). The instrumental parameters were as follows: RF power, 1,200 W; RF matching, 1.8 V; sample skimmer cone in Ni; plasma flow rate, 16 L/min; auxiliary flow rate, 1.1 L/min; nebulizer flow rate, 1.2 L/min. Gallium (m/z = 69) and yttrium (m/z = 89) were used as the internal standards. The concentrations of magnesium, calcium, and iron in drinking water and in the digested solutions were determined by ICP-atomic emission spectrometry (ICPS-7510, Shimadzu Corp., Kyoto, Japan).

Validation of measurement method

To check the validity of the analysis, seven standard reference material (rice flour [SRM1568a], spinach leaves [SRM1570a], bovine muscle powder [SRM8414], oyster tissue [SRM1566b], nonfat milk powder [SRM1549], wheat flour [SRM1549a], and "typical diet" [SRM1548a]) were purchased from the National Institute of Standards and Technology (Gaithersburg, MD, USA). These standard reference materials were analyzed in the same manner as the food composite samples in this study and compared with the certified values of the standard reference materials.

The standard reference material–certified or reference values and our analyzed values were compared by the recovery ratio and z-score (Dolan & Caper 2002). For almost all metals, the recovery ratio was between 75 and 125% and/or the absolute value of the z-score was less than 2.5, which showed good agreement between the certified or reference values and our analyzed values. However, our analyzed values were outside of these ranges for selenium in the spinach leaves and the nonfat milk powder and for arsenic and molybdenum in the bovine muscle powder. The main cause of this disagreement may be that the certified or reference values are very low and near the detection limits of the present study. Because these values are very small, the effect of the disagreement was estimated to be negligible in the calculation of DMI in this study.

Detection limits were estimated to three times the standard deviation (SD) of the metal concentration derived from 10 measurements of the method blank. The method blank was a blank sample pretreated by the same procedure as for food composite samples.

Estimation of DMI

The National Nutrition Survey provides daily intake data for each food sample group (Figure 1). In the case of drinking water (14th group), the daily drinking water intake was assumed to be 2 L/day. To calculate DMI, the metal concentration in a food composite sample or drinking water was multiplied by the daily intake of each group, and the values were summed, as follows:

$$\sum_{i=1}^{14} a_i b_i$$

a_i = metal concentration in the food composite sample or drinking water of ith group

b_i = daily intake (consumption rate) of ith group

Calculation of TDI and RDA values and their assumptions

In this study, the values of TDI and RDA were calculated as for an average Japanese adult. Basically, the values of TDI (mg/kg/day) were derived from either the TDI reported in the *Guidelines for Drinking-water Quality* (WHO 2008) or from that proposed by JECFA (1982, 1983, 1989, 1993). TDI is not necessarily expressed as TDI but other similar terms, such as PTWI (provisional tolerable weekly intake) and PMTDI (provisional maximum tolerable daily intake). In this study, for simplification, the term "TDI" is used for all of these similar terms after weekly intakes were converted to daily intakes. Exceptions were the TDI values of manganese, molybdenum, and selenium. The original

TDI for manganese in the WHO guidelines is considered to apply only to drinking water, because it includes an uncertainty factor (UF) of 3 to take into consideration the possible increased bioavailability of manganese from water (WHO 2008). Therefore, the TDI of manganese was set to be 11 mg as daily intake per capita by excluding the UF of 3. This value was also applied as the upper limit for dietary reference intakes for Japanese (JMHLW 2005). For molybdenum, we applied the reference dose set for chronic oral exposure (0.005 mg/kg/day) by the U.S. Environmental Protection Agency (USEPA 1993). This value was also applied as the upper limit for Japanese (JMHLW 2005). For selenium, the no observed adverse effect level (NOAEL) of 4 μg/kg/day in humans was used directly for the derivation of the WHO guideline value (WHO 2003, 2008); this approach is the same as the TDI approach with a UF of 1. Therefore, we assume the NOAEL should be equivalent to TDI in the case of selenium. This value is more conservative than the upper limit for Japanese of 6.7 μg/kg/day (JMHLW 2005), which is based on a NOAEL of 13.3 μg/kg/day as determined by the study of Yang & Zhou (1994) and a UF of 2. To convert these TDI values to the unit of weight per capita, the average weight per capita was assumed to be 50 kg, which is the assumption used in establishing the drinking water quality standards in Japan.

The JMHLW (2005) provides separate RDA values for each gender and age group. In this study, the average of RDA values of both males and females from 18 to 69 years old was used as the RDA for an average Japanese person, except in the case of calcium and manganese. For these elements, JMHLW does not set a RDA but an adequate intake (AI) value. AI is defined as the amount of daily intake sufficient to maintain a stable nutritional state (JMHLW 2005). AI is set only when an RDA cannot be set because of the lack of experimental and epidemiological data. The values of TDI and RDA calculated in this study for an average Japanese person are shown in Table 1 with the results of DMI.

RESULTS AND DISCUSSION

Margin to TDI values for toxic metals

The mean and SD of DMI for the 17 elements, as well as the DMI of each food group, are presented in Table 1. The percentage of DMI to TDI was calculated for each of the 13 toxic metals (Figure 2). The mean DMIs of 10 toxic metals (excluding selenium, molybdenum, and arsenic) were less than 50% of TDI. A simple interpretation of this result indicates that there may be a substantial margin between TDI and mean DMI. Accordingly, for these 10 metals the total daily intake percentages allocated to drinking water could possibly be increased from the normal allocation percentage (i.e. 10–20% of TDI) in reviewing or establishing drinking water quality standards. In this case, however, only the mean intakes were considered. Variation of the intakes among individuals and areas should be further investigated and considered. Exposure from other possible sources such as air and soil should be considered as well. Furthermore, it may not be necessary to increase the allocation ratio if drinking water can meet the current standards with commonly-used drinking water treatment processes.

The six-city average of daily arsenic intake exceeded TDI by a considerable degree (280% of TDI); the highest intake was 359% of the TDI. The toxicity of arsenic varies greatly according to its chemical form (i.e. inorganic or organic). The toxicity of organic arsenic is much lower than that of inorganic arsenic. In this study, the chemical form was not considered, and the DMI of arsenic was calculated on the basis of the total arsenic. According to the analysis of daily arsenic intake (Table 1), among the 13 food groups two groups ("Other vegetables and seaweeds" and "Fish and shellfish") accounted for high ratios. The sum of arsenic intake from the other 11 food groups was lower than the TDI; therefore, the chemical forms of arsenic in these two groups should be further investigated. The mean molybdenum intake was 99% of the TDI, and maximum ratio was 134%. The excess intake of molybdenum does not pose an immediate health hazard to humans, because the TDI represents a tolerable intake for a lifetime, and short-term exposure to levels exceeding the TDI is not a cause for concern (WHO 2008). Uncertainties exist in risk assessment of long-term exposure, however, including large UFs generally involved in establishing TDIs (WHO 2008) and the lack of quantitative risk information on intakes exceeding the TDI. These uncertainties are limitations in the current risk assessment approaches and should be improved in the future.

Table 1 | Daily metal intake of each food group, average of six cities

	B mg/day	Mg mg/day	Al mg/day	Ca mg/day	Cr μg/day	Mn mg/day	Fe mg/day	Ni μg/day	Cu mg/day
TDI × 50-kg body weight	8*	–	–	–	–	11†,*	40‡	600*	25§
RAD†	–	318	–	694 (Al)	34	3.75 (Al)	9	–	0.75
Food group	Intake SD	Intake SD	Intake SD	Intake SD	Intake SD	Intake SD	Intake SD	Intake SD	Intake SD
Rice	0.1 ± 0.04	14 ± 4.1	0.07 ± 0.01	8.8 ± 2.5	197 ± 148	1.0 ± 0.2	0.04 ± 0.03	24 ± 8.7	0.3 ± 0.1
Cereals and potatoes	0.09 ± 0.02	30 ± 7.0	0.4 ± 0.1	41 ± 10	22 ± 11	0.5 ± 0.2	0.9 ± 0.2	13 ± 8.9	0.2 ± 0.03
Sugar and sweets	0.04 ± 0.01	7.2 ± 1.6	0.4 ± 0.3	17 ± 4.9	21 ± 24	0.1 ± 0.04	0.2 ± 0.1	4.0 ± 2.2	0.04 ± 0.01
Oils and fats	0.003 ± 0.0001	0.02 ± 0.02	0.004 ± 0.01	0.3 ± 0.3	29 ± 7.9	0.0001 ± 0.0001	0.004 ± 0.002	0.1 ± 0.1	0.001 ± 0.0004
Legumes	0.2 ± 0.05	47 ± 12	0.1 ± 0.04	52 ± 15	21 ± 7.3	0.4 ± 0.06	0.7 ± 0.2	38 ± 9.3	0.1 ± 0.01
Fruits	0.3 ± 0.03	13 ± 0.8	0.03 ± 0.02	14 ± 1.7	10 ± 7.9	0.2 ± 0.09	0.1 ± 0.03	6.2 ± 2.8	0.05 ± 0.01
Green and yellow vegetables	0.2 ± 0.02	23 ± 7.6	0.2 ± 0.2	34 ± 14	11 ± 10	0.2 ± 0.06	0.3 ± 0.04	5.8 ± 5.1	0.04 ± 0.01
Other vegetables and seaweed	0.4 ± 0.2	30 ± 10	0.6 ± 0.5	65 ± 21	23 ± 12	0.4 ± 0.1	0.6 ± 0.3	10 ± 1.2	0.09 ± 0.03
Alcohols and beverages	0.2 ± 0.1	20 ± 13	1.1 ± 1.1	18 ± 23	34 ± 19	0.8 ± 0.6	0.2 ± 0.1	21 ± 18	0.08 ± 0.06
Fish and shellfish	0.06 ± 0.02	36 ± 13	0.2 ± 0.1	109 ± 48	44 ± 19	0.05 ± 0.02	0.6 ± 0.2	2.9 ± 1.2	0.1 ± 0.06
Meat and poultry	0.05 ± 0.04	26 ± 8.2	0.1 ± 0.1	33 ± 20	64 ± 25	0.03 ± 0.005	1.7 ± 0.6	3.0 ± 4.7	0.08 ± 0.02
Milk products	0.06 ± 0.02	17 ± 5.2	0.02 ± 0.02	188 ± 90	39 ± 11	0.01 ± 0.01	0.1 ± 0.07	2.0 ± 1.5	0.01 ± 0.01
Seasoning	0.2 ± 0.05	27 ± 3.2	0.2 ± 0.1	22 ± 7.7	51 ± 10	0.4 ± 0.1	0.5 ± 0.2	25 ± 2.8	0.05 ± 0.02
Drinking water	0.07 ± 0.03	6.1 ± 4.1	0.08 ± 0.07	31 ± 17	0.2 ± 0.1	0.004 ± 0.005	0.14 ± 0.30	0.7 ± 0.6	0.005 ± 0.003
DMI	1.93 ± 0.22	295 ± 38.1	3.60 ± 1.37	631 ± 126	568 ± 194	4.15 ± 0.66	6.13 ± 1.11	156 ± 0.03	1.20 ± 0.17

	Zn mg/day	As μg/day	Se μg/day	Mo μg/day	Cd μg/day	Sb μg/day	Pb μg/day	U μg/day
TDI × 50-kg body weight	50§	107‖	200*	250†,¶	50*	300*	179*,**,***	30*
RAD†	8	–	28	23	–	–	–	–
Food group	Intake SD	Intake SD	Intake SD	Intake SD	Intake SD	Intake SD	Intake SD	Intake SD
Rice	2.4 ± 0.4	15 ± 4.2	6.7 ± 2.2	122 ± 38	4.3 ± 2.6	0.4 ± 0.3	9.1 ± 5.9	0.09 ± 0.08
Cereals and potatoes	0.7 ± 0.1	1.5 ± 0.4	23 ± 5.5	12 ± 4.9	2.6 ± 0.5	0.2 ± 0.1	4.1 ± 2.9	0.0003 ± 0.001
Sugar and sweets	0.1 ± 0.02	0.8 ± 0.4	1.1 ± 0.4	3.6 ± 0.3	0.5 ± 0.3	0.04 ± 0.02	0.7 ± 0.5	0.05 ± 0.02
Oils and fats	0.003 ± 0.005	0.1 ± 0.06	0.4 ± 0.02	0.2 ± 0.2	0.08 ± 0.004	0.01 ± 0.001	0.5 ± 0.3	0.004 ± 0.01
Legumes	0.6 ± 0.07	0.5 ± 0.3	3.4 ± 1.7	40 ± 15	1.0 ± 0.3	0.07 ± 0.06	2.1 ± 1.2	0.03 ± 0.03
Fruits	0.07 ± 0.01	0.6 ± 0.3	2.5 ± 0.1	2.5 ± 0.4	0.4 ± 0.0	0.2 ± 0.09	2.3 ± 2.0	0.07 ± 0.08
Green and yellow vegetables	0.3 ± 0.04	0.4 ± 0.2	1.9 ± 0.1	4.0 ± 2.0	1.8 ± 0.2	0.08 ± 0.04	2.5 ± 2.1	0.06 ± 0.07
Other vegetables and seaweed	0.4 ± 0.04	103 ± 64	7.3 ± 4.6	11 ± 5.3	3.7 ± 1.2	0.2 ± 0.1	4.3 ± 2.9	0.7 ± 0.3
Alcohols and beverages	0.2 ± 0.2	2.1 ± 1.4	10 ± 4.0	13 ± 8.5	2.0 ± 0.8	0.7 ± 0.4	8.7 ± 1.1	0.4 ± 0.4

Table 1 | *(continued)*

	Zn mg/day	As μg/day	Se μg/day	Mo μg/day	Cd μg/day	Sb μg/day	Pb μg/day	U μg/day
TDI × 50-kg body weight	50[§]	107[‖]	200[*]	250[†,¶]	50[*]	300[*]	179[*,**]	30[*]
RAD[†]	8	-	28	23	-	-	-	-
Food group	Intake SD	Intake SD	Intake SD	Intake SD	Intake SD	Intake SD	Intake SD	Intake SD
Fish and shellfish	0.9 ± 0.2	159 ± 44	42 ± 5.4	2.3 ± 1.6	3.8 ± 3.2	0.2 ± 0.2	2.6 ± 3.0	0.4 ± 0.1
Meat and poultry	2.2 ± 0.5	1.9 ± 0.3	36 ± 6.9	7.5 ± 2.0	0.6 ± 0.2	0.3 ± 0.2	2.5 ± 1.1	0.2 ± 0.06
Milk products	0.5 ± 0.06	0.8 ± 0.2	8.0 ± 2.2	6.9 ± 0.9	0.6 ± 0.1	0.1 ± 0.08	2.0 ± 0.5	0.1 ± 0.04
Seasoning	0.3 ± 0.04	13 ± 3.2	12 ± 2.8	21 ± 5.1	1.4 ± 0.8	0.2 ± 0.2	2.4 ± 1.2	0.1 ± 0.03
Drinking water	0.06 ± 0.1	0.7 ± 0.3	1.3 ± 0.0	1.3 ± 0.7	0.07 ± 0.06	0.2 ± 0.1	0.7 ± 0.3	0.04 ± 0.01
DMI	8.65 ± 0.74	300 ± 58.2	156 ± 16.1	247 ± 46.4	22.8 ± 3.08	2.90 ± 1.25	44.4 ± 18.1	2.38 ± 0.64

*WHO (2008).
†JMHLW (2005).
‡JECFA (1983).
§JECFA (1982).
‖JECFA (1989).
¶USEPA (1993).
** JECFA (1993).
TDI: tolerable daily ntake; RDA: recommended dietary allowances; DMI: daily metal intake; SD: standard deviation; AI: adequate intake.

Daily essential metal intakes relative to RDA

The percentages of DMI to RDA or AI were calculated for nine essential metals (Figure 3). The mean dietary intakes of magnesium, calcium, and iron were less than the RDA or AI. In terms of minimum daily intake among the six cities, the intakes of five metals (magnesium, calcium, manganese, iron, and zinc) were lower than the RDA or AI. Magnesium and calcium intakes were lower than the RDA or AI in five of the six cities. The differences between the RDA or AI and the intakes, however, were not very large, and the variation of the intake among cities and among years should be further investigated. As discussed for TDI, RDA and AI values also include many uncertainties that should be improved in the future, which limit our ability to discuss risks associated with deficiency of essential metals.

Iron intake was lower than the RDA in all six cities, and manganese and zinc intakes were lower in one city. Iron, manganese, and zinc are considered to be both essential and toxic metals. In terms of toxicity, the DMI values of these three metals were less than 50% of the TDI. Therefore, Japanese may consume more of these metals to satisfy the RDA or AI value.

Daily intakes of chromium, selenium, and molybdenum were far greater than the RDA values. In terms of toxicological aspects, the DMI values of selenium and molybdenum were near, or a slightly higher than, the TDI (Figure 2). Thus, it is not recommended that Japanese consume more selenium or molybdenum. For chromium, JMHLW (2005) does not provide a tolerable upper intake level, and the WHO does not describe the quantitative risk of chromium intake, although it has established a provisional drinking-water quality guideline value 0.05 mg/L for total chromium (WHO 2006).

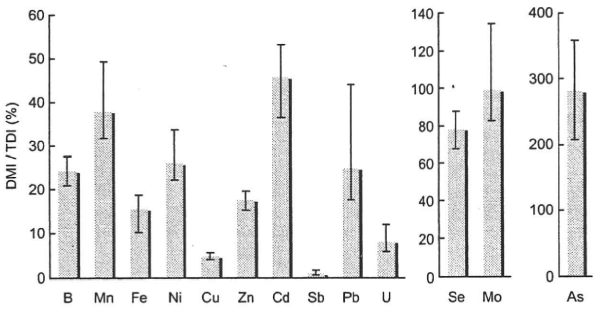

Figure 2 | Daily toxic metal intake as a percentage of TDI. Columns represent the average of six cities; bars represent minimum and maximum values.

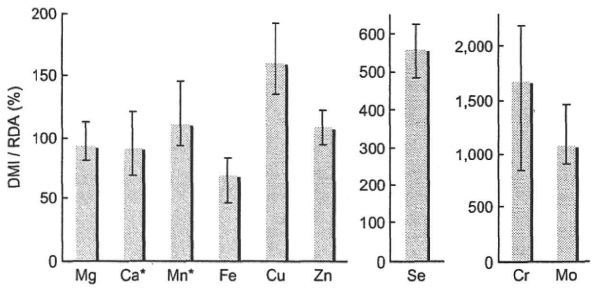

Figure 3 | Daily essential metal intake as a percentage of RDA. Columns represent the averages of six cities; bars represent minimum and maximum values. Asterisk indicates AI instead of RDA.

Contribution of drinking water to TDI and/or RDA

For the 13 toxic metals analyzed, the contribution of drinking water to TDI was 2% or less in all six cities (Figure 4). Basically, in establishing the Japanese water quality standards, 10 of the TDI is allocated to intake via drinking water for most metals. The actual contribution is much less than 10% of the TDI, because the metal contents are controlled to be about 20–30% of the Japanese drinking water quality standard values in most water purification plants in Japan (Japan Water Works Association 2009).

The drinking water contribution to the RDA or AI of essential metals is less than 10% in all cities. On average, drinking water contributed only about 5% of the AI of calcium. Hardness in water is derived from calcium and magnesium. According to the WHO *Guidelines for Drinking-water Quality*, very soft waters may have an adverse effect on mineral balance and cardiovascular health (WHO 2008). The calcium concentration in drinking water is low in many cities in Japan (Japan Water Works Association 2009), and calcium compounds are added at some drinking water treatment plants where the hardness of the water is very low. This calcium addition was originally designed to prevent the corrosion of water pipes, but it may also help to reduce the health risks posed by very soft water. In general, the process increases the calcium concentration by ~8 mg/L (Japan Water Works Association 2009), which is equivalent to a daily intake of 16 mg/day or 2.3% of the AI of calcium. Therefore, this addition of calcium to drinking water does not help with calcium intake deficiency; it is better to consume calcium in foods such as milk.

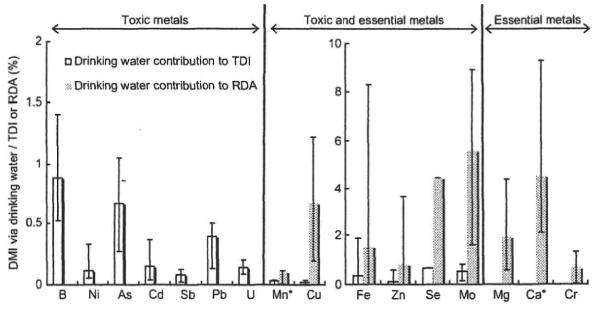

Figure 4 | Drinking water contributions to TDI or RDA. Columns represent the averages of six cities; bars represent minimum and maximum values. Asterisk indicates AI instead of RDA.

CONCLUSION

The daily intakes of 17 metals in six cities in Japan were estimated by analyzing the concentrations of metals in locally obtained food composite samples and drinking water samples. The mean daily intake of 10 of the 13 toxic metals was less than 50% of TDI, except in the cases of arsenic, selenium, and molybdenum. The allocation ratio of intake to drinking water in establishing drinking water quality standards could possibly be increased from the normal allocation of 10–20% of TDI for these 10 metals. However, not only the mean intakes but also variation of the intakes among individuals and areas should be further investigated and considered. For the 13 toxic metals analyzed in this study, the contribution of drinking water to TDI was 2% or less in all six cities. For essential metals, the DMI values of magnesium, calcium, iron, manganese, and zinc were lower than RDA or AI in at least one city. The drinking water contribution to RDA or AI was less than 10% for all essential metals in all the six cities, indicating that drinking water did not contribute very much to essential metal intake.

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Fetal malformations and early embryonic gene expression response in cynomolgus monkeys maternally exposed to thalidomide☆

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ABSTRACT

The present study was performed to determine experimental conditions for thalidomide induction of fetal malformations and to understand the molecular mechanisms underlying thalidomide teratogenicity in cynomolgus monkeys. Cynomolgus monkeys were orally administered thalidomide at 15 or 20 mg/kg-d on days 26–28 of gestation, and fetuses were examined on day 100–102 of gestation. Limb defects such as micromelia/amelia, paw/foot hyperflexion, polydactyly, syndactyly, and brachydactyly were observed in seven of eight fetuses. Cynomolgus monkeys were orally administered thalidomide at 20 mg/kg on day 26 of gestation, and whole embryos were removed from the dams 6 h after administration. Three embryos each were obtained from the thalidomide-treated and control groups. Total RNA was isolated from individual embryos, amplified to biotinylated cRNA and hybridized to a custom Non-Human Primate (NHP) GeneChip® Array. Altered genes were clustered into genes that were up-regulated (1281 genes) and down-regulated (1081 genes) in thalidomide-exposed embryos. Functional annotation by Gene Ontology (GO) categories revealed up-regulation of actin cytoskeletal remodeling and insulin signaling, and down-regulation of pathways for vasculature development and the inflammatory response. These findings show that thalidomide exposure perturbs a general program of morphoregulatory processes in the monkey embryo. Bioinformatics analysis of the embryonic transcriptome following maternal thalidomide exposure has now identified many key pathways implicated in thalidomide embryopathy, and has also revealed some novel processes that can help unravel the mechanism of this important developmental phenotype.

1. Introduction

Thalidomide (α-phthalimidoglutarimide) was synthesized in West Germany in 1953 by the Chemie Grünenthal pharmaceutical firm, and was marketed from October 1957 into the early 1960s. It was used for treating nausea and vomiting late during pregnancy and was also said to be effective against influenza. The first case of the phocomelia defect, although not recognized at the time as drug-related, was presented by a German scientist in 1959; subsequently, malformed children were reported in 31 countries [1]. A pattern of defects of limbs as well as the ocular, respiratory, gastrointestinal, urogenital, cardiovascular and nervous systems caused by maternal thalidomide exposure during early pregnancy was observed. Limb defects such as phocomelia, amelia, micromelia, oligodactyly, and syndactyly were the most common malformations [2]. After removal from the global market in 1962, thalidomide was reintroduced in 1998 by the biotechnology firm Celgene as an immunomodulator for the treatment of erythema nodosum leprosum, a serious inflammatory condition of Hansen's disease, and in orphan status for treating Crohn's disease and several other diseases [1].

Animal species are not equally susceptible or sensitive to the teratogenicity of chemical agents, and some species respond more readily than others [3]. For thalidomide, a variety of developmental toxic effects were reported in 18 animal species, but the responses have been highly variable across species. Limb defects that mimic

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