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Unique amnesic shellfish toxin composition found in the South East Asian diatom *Nitzschia navis-varingica*

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ABSTRACT

Nitzschia navis-varingica is a diatom that is known to produce significant levels of amnesic shellfish poisoning (ASP) toxins. A total of 33 *N. navis-varingica* strains were isolated from four brackish water localities in the Philippines and Indonesia, and cultured to characterize the toxins produced. The isolates were analyzed for domoic acid (DA) and isodomoic acids A (IA) and B (IB) by HPLC with fluorescence detection. Two toxin composition types were detected that have not been previously described: strains producing only IB and strains producing DA–IA–IB. These two types were isolated from two different localities. Eighteen strains were isolated from the Philippines (northern Luzon Island). Among them, 10 isolates from Alaminos produced only IB with an average toxin content of 3.05 pg cell^{-1}, seven isolates from Bulacan produced DA and IB with average toxin contents of 0.68 pg cell^{-1} and 1.18 pg cell^{-1}, respectively. One isolate from Cavite produced DA, IA, and IB with a toxin content of 0.58, 0.20, and 0.92 pg cell^{-1}, respectively. Fifteen isolates from Indonesia (Bone, South Sulawesi) produced only DA (four isolates) or DA with trace amounts of IB (eleven isolates), with an average toxin content of 2.38 pg cell^{-1} and 0.06 pg cell^{-1}, respectively. Sub-strains were established from strains producing either of the three toxin types: IB, DA–IA–IB, and DA-trace IB. Results showed that the toxin composition type was the same for parent and sub-strains, indicating that the toxin composition is a stable character for a strain.

1. Introduction

Domoic acid (DA) and its derivatives (e.g., isodomoic acids A, B, C, G, and H) (Maeda et al., 1986; Zaman et al., 1997) are insecticidal agents first isolated from the macro-alga *Chondria armata* (Takemoto & Daigo, 1958). Later, DA was identified as the toxin responsible for amnesic shellfish poisoning (ASP) (Wright et al., 1989) followed by identification of the isomers isodomoic acids D, E, and F as minor components (Wright et al., 1990), and the causative organism was traced and identified as the diatom *Pseudo-nitzschia multiseries* (Bates et al., 1989). A number of DA-producing *Pseudo-nitzschia* spp. have been found in subsequent searches primarily in temperate areas, although the toxin level varied widely (Garrison et al., 1992; Martin et al., 1990; Lundholm et al., 1994; Rhodes et al., 1996; Rhodes, 1998; Orsini et al., 2002). Among them, three *Pseudo-nitzschia* species (*P. multiseries*, *P. australis*, and *P. seriata*) are known to produce high levels of DA (Bates et al., 1989; Garrison et al., 1992; Lundholm et al., 1994; Rhodes et al., 1996).

During a more recent search for DA-producing diatoms in tropical waters, a major DA-producing benthic diatom was isolated from Vietnam (Kotaki et al., 2000). This organism was identified as a new species, *Nitzschia navis-varingica* (Lundholm and Moestrup, 2000; Bates, 2000). This species has also been isolated from brackish water areas in the Philippines and Japan (Kotaki et al., 2004). Recently, some of the Philippine strains of *N. navis-varingica*

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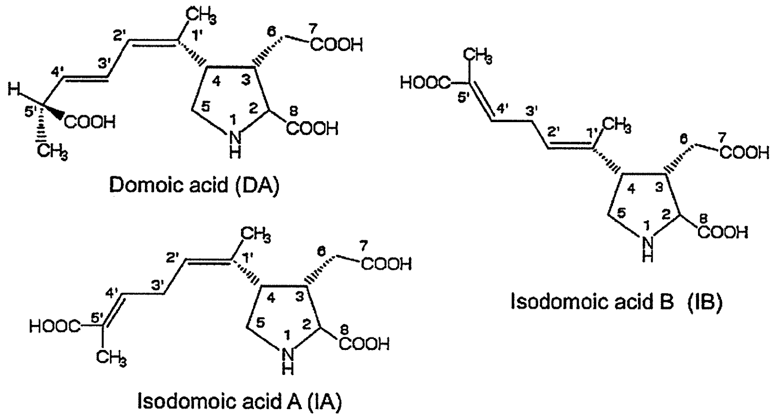
Domoic acid (DA)

Isodomoic acid A (IA)

Isodomoic acid B (IB)

Fig. 1. Structure of domoic acid and isodomoic acids A and B.

were found to produce not only DA but also isodomoic acids A (IA) and B (IB) as major toxin components (Kotaki et al., 2005) (Fig. 1). ASP toxin production was then re-investigated using the strains of *N. navis-varingica* from the Philippines and a few other Asian countries (Japan, Vietnam, and Thailand) and three types of ASP toxin composition (DA, DA–IB, IA–IB) were detected. The most abundant toxin composition type was a combination of DA and IB (DA–IB), with the ratio of IB ranging from trace to ca. 80%. This type was found in strains from Japan, Thailand, Vietnam, and the Philippines (Kotaki, 2008; Kotaki et al., 2008; Romero et al., 2008). Isolates from restricted areas in the northern part of the Philippines had another toxin composition (IA and IB) (Bajarias et al., 2006; Kotaki, 2008; Kotaki et al., 2008). However, the composition and geographical distribution of toxins from southernmost Asia, such as Indonesia and Malaysia, and most of the northern part of the Philippines have not been studied.

We report here the results of a toxin screening of 33 *N. navis-varingica* strains from South Sulawesi in Indonesia and three localities in the Philippines. We document two new toxin composition types (IB and DA–IA–IB) from two different localities in the Philippines, although IA and IB might not pose a major health risk because of their lower toxicity than DA (Munday et al., 2008).

2. Materials and methods

2.1. Isolation and identification of N. navis-varingica

Samples were collected from the surface of mangrove roots or palm tree leaves hanging down into the water in estuarine areas using a handy scoop net (20 μm). Sampling was conducted in three areas of Luzon Island, Philippines, including several brackish sites, in December 2007 and December 2008: Alaminos in Pangasinan, and Bulacan and Cavite in Manila Bay (Fig. 2). Sampling was also conducted in several other brackish sites: Panyula, Bone in South Sulawesi (July 2008), Jakarta Bay (March 2009), Lampung Bay (March 2009), and Sangihe Island (May 2009), all in Indonesia, and finally in Kota Kinabalu, Malaysia (May 2008) (Fig. 2).

Crude cultures were prepared by mixing aliquots (0.2 mL or 1.0 mL) of net sample with 30 mL of f/2 medium in 50 mL tissue culture flasks (Greiner bio-one, Tokyo, Japan), and incubating them at 25 °C under an irradiance level of 60 μmol photons m^{-2} s^{-1} with a 16:8 h light:dark cycle. f/2 medium was prepared (Guillard, 1983) using sea water diluted with distilled water to a salinity of ca. 27 psu. Uni-algal cultures of *N. navis-varingica* were established from crude cultures by capillary washing of single cells under a light microscope (LM) according to the morphological characteristics of *N. navis-varingica* (Lundholm and Moestrup, 2000; Kotaki et al., 2005). The cells possess two yellow-brown chloroplasts at each end of the cell and are lanceolate in valve view. Cells are 38–110 μm long and 9–11 μm wide. In girdle view, the cells are rectangular and slightly indented at the middle. Most cells form ribbon-shaped colonies while growing (Kotaki et al., 2004). Experience have shown, that using these light microscopical characters allow us to isolate strains that have a high likelihood of being *N. navis-varingica*. One representative strain from each collection site was further investigated morphologically to confirm the identification using transmission electron microscopy (TEM) according to the method of Lundholm and Moestrup (2000). Subsamples were fixed in formalin (final concentration 3.7%). Representative strains identified using TEM were PBULD 07-4 (Bulacan, Manila Bay), PCAVA 07-2 (Cavite, Manila Bay), PALMC 07-1 (Alaminos, Pangasinan) (all from the Philippines), and IBNA 08-3, IBNB 08-4, and IBNC 08-3 (Panyula, Bone in South Sulawesi, all from Indonesia).

2.2. Analysis of ASP toxins in N. navis-varingica cultures

Isolates were cultured in 30 mL f/2 medium in 50 mL tissue culture tubes under the same culture conditions described in Section 2.1. The cultures were harvested after a 3-week culture period (mid-stationary growth phase). A 3 mL aliquot of whole culture was retrieved, boiled for 5 min and then centrifuged after being cooled to room temperature (Bajarias et al., 2006). A 2 mL aliquot was also retrieved for determining the cell density (Kotaki et al., 2000). Toxin profile analysis was done for DA, IA, and IB using HPLC-fluorescence analysis with pre-column derivatization using

Fig. 2. Sampling areas in the Philippines, Indonesia, and Malaysia.

9-fluorenylmethylchloroformate (FMOC-Cl) according to the slightly modified method of Pocklington et al. (1990) in which a Develosil ODS-5 column (4.6 × 250 mm, Nomura, Seto, Aichi, Japan) and a mobile phase of 40% acetonitrile in 20 mM phosphate buffer (pH 2.5) was used. The cellular toxin content was expressed on a per cell basis (pg cell^{-1}), which is calculated by dividing the total amount of each toxin (ng mL^{-1}) in the entire culture by the corresponding density of cells in the culture. The number of cells was determined using 5 μL of culture under LM in a Sedgewick-Rafter counting chamber. Samples were counted in triplicate and the average cell number was used for the cellular toxin content calculation. Standard toxins were purified from a mass culture of *N. navis-varingica* that produces both IA and IB (for IA and IB standards) and an extract of the red alga *C. armata* that produces high amount of DA and small/trace amount of IA, IB, 5′-epi-DA, etc. (for DA standard) collected in Kagoshima Prefecture, Japan. These were calibrated using commercial Canadian standard toxin (DACS-1D, DA) or purified toxins (IA and IB) (Kotaki et al., 2005).

Confirmation of toxin production of the representative strains was performed by LC–MS/MS using multiple reaction monitoring (MRM) (Takata et al., 2009). A 1 L mass culture of each strain was filtered (mixed cellulose ester 3 μm pore size filters, Advantec, Tokyo, Japan) at the end of the three-week culture period and the cells were extracted by boiling followed by ultrafiltration (10,000 Da cut-off). LC–MS was performed on an HP1100 LC system (Agilent Technologies, Santa Clara, CA) coupled to an Applied Biosystems/MDS Sciex API 2000 triple quadruple mass spectrometer (Applied Biosystems, Foster City, CA). LC separation was performed using a slight modification of the method described in the Canadian standard DACS-1D manual. A Wakosil Navi C18-5 column (2 mm × 250 mm; Wako, Tokyo, Japan) was employed, and analytes were eluted over 20 min using a linear gradient from 10% acetonitrile in 0.1% formic acid to 20% acetonitrile in 0.1% formic acid with a flow rate of 0.2 mL min^{-1}. The MS analysis was achieved by MRM with a turboion-spray interface in positive ion mode. Three MRM transitions from the protonated DA ion were monitored (m/z 312–266, m/z 312–248, and m/z 312–161). Standard toxin was prepared from an extract of *C. armata* and calibrated using commercial Canadian standard toxin (DACS-1D) or purified toxins (IA and IB) (Kotaki et al., 2005).

2.3. Toxin composition of *N. navis-varingica* sub-strains

Sub-strains were prepared by capillary washing of single cells of two representative parental Philippine strains: PALMC 07-1 (Alaminos, IB type), PCAVA 07-2 (Cavite, DA–IA–IB type), and one Indonesian parental strain: IBNB 08-2 (Panyula, Bone, DA-trace IB type), after having been pre-cultured for one week under the same conditions as the batch cultures in Section 2.1. Sub-strains were cultured for three weeks as described in Section 2.1, harvested and analyzed for toxin production as described in Section 2.2. The toxin composition was then compared with that of the parental strain. Parental strains were maintained for more than half year by inoculating aliquots of old culture into new f/2 medium every month followed by culturing for one week under the same conditions in Section 2.1.

3. Results

3.1. Isolation and identification of *N. navis-varingica*

Ten isolates of *N. navis-varingica* were obtained from Alaminos (PALM 07/08 strains, one in 2007 and nine in 2008), seven were

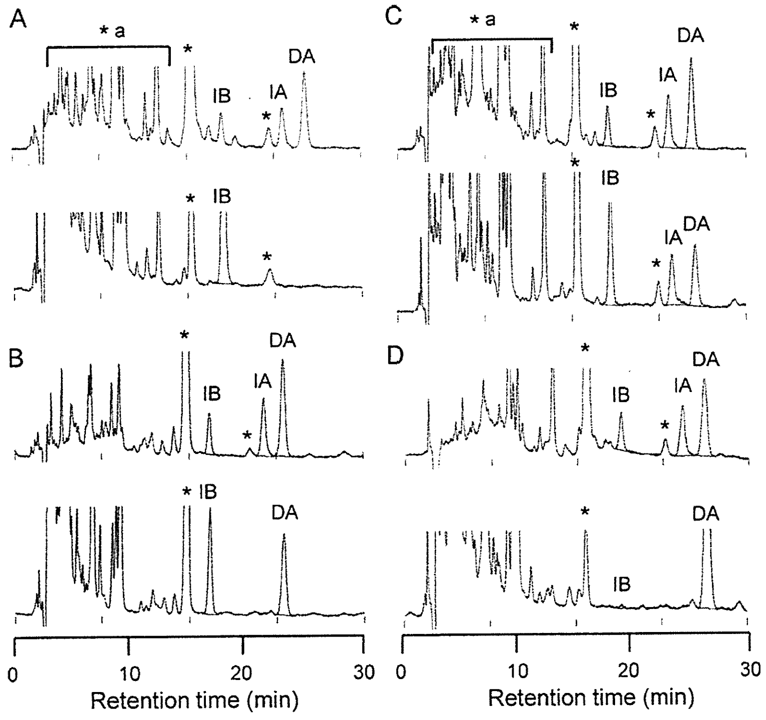

Fig. 3. HPLC-fluorescence analyses of ASP toxins from representative *Nitzschia navis-varingica* strains. Upper chromatogram of (A), (B), (C) and (D); standard toxins DAKS 04-1 containing IA, IB and DA (8.7, 9.2, and 17.5 ng/mL) analyzed at the same time as the respective sample. Lower chromatogram of (A), (B), (C) and (D); sample extracts of (A) PALMC 08-1 strain isolated from Alaminos, Pangasinan, (B) PBULD 07-5 isolated from Bulacan, Manila Bay, (C) PCAVA 07-2 isolated from Cavite, Manila Bay, all in the Luzon Island, the Philippines and (D) IBNB 08-3 isolated from Panyula, Bone, South Sulawesi, Indonesia. DA, domoic acid; IA, isodomoic acid A; IB, isodomoic acid B. *Side-products derived from the FMOC-Cl reagent. The amount of each derivative often varies due to contamination with compounds (e.g., other amino acids) that compete with ASP toxins and/or due to reaction conditions (Pocklington et al., 1990), *a: Side-products and/or reaction products derived from contaminants in the medium and other compounds in the culture (e.g., neutral amino acids) that react with the FMOC-Cl reagent. Analytical conditions: Develosil ODS-5 column (4.6 mm × 250 mm, Nomura); mobile phase, 40% acetonitrile in 20 mM phosphate buffer (pH 2.5); column temperature, 55 °C; flow rate, 1 mL min^{-1}; fluorescence detection, Ex. = 264 nm, Em. = 313 nm.

obtained from Bulacan (PBULD 08 strains), and one from Cavite (PCAVA 07 strain), all from the Philippines (Fig. 2). Fifteen strains of *N. navis-varingica* were obtained from Panyula, Bone, South Sulawesi, Indonesia (IBN 08 strain). No strains of *N. navis-varingica* were isolated from Jakarta Bay, Lampung Bay and Sangihe Island, all in Indonesia, or from Kota Kinabalu, in Malaysia (Fig. 2).

Morphological TEM studies of the strains PALMC 07-1 (Alaminos, Pangasinan), PBULD 07-4 (Bulacan, Manila Bay), PCAVA 07-2 (Cavite, Manila Bay) (all from the Philippines), and IBNA 08-3, IBNB 08-4, IBNC 08-3 (Panyula, Bone, all from Indonesia) that were positive for ASP toxin production agreed with Lundholm and Moestrup's (2000) description of *N. navis-varingica*. Characteristic silica ridges were observed in the wall of the raphe canal and on the mantle. The girdle bands were ornamented by silica warts and the valvocopula had 1–3 rows of poroids. On the valve, the density of interstriae was 25–28 in 10 μm, and the poroids in the uniseriate striae had a density of 3–5 poroids in 1 μm. The raphe was interrupted in the middle by a central interspace and the fibulae that did not show a central larger interspace had a density of 8–11 in 10 μm. The density of the poroids on the girdle bands was 32–42 in 10 μm.

3.2. ASP toxin production in N. navis-varingica cultures

Representative HPLC chromatograms and the average toxin composition of each strain positive for ASP production are shown in Figs. 3 and 4. Ten isolates from Alaminos, Pangasinan, the Philippines (PALM 07/08 strains) showed a new toxin composition type consisting only of IB. The isolates had an average toxin content of 3.05 pg cell^{-1} ($\sigma = 3.65$) (Fig. 3A, lower chromatogram). All seven isolates from Bulacan, Manila Bay, the Philippines (PBUL 07 strains) had the typical DA–IB toxin composition similar to that of strains isolated from the southern Philippines (Kotaki et al., 2005, 2008; Kotaki, 2008). The average content of DA and IB in the toxin across all strains was 0.68 pg cell^{-1} ($\sigma = 0.35$) and 1.18 pg cell^{-1} ($\sigma = 0.46$), respectively (1.86 pg cell^{-1} in total), and the DA:IB ratio was 37:63 (Fig. 3B, lower chromatogram). The strain isolated from Cavite, Manila Bay, the Philippines (PCAVA 07) had a new toxin composition type containing all three toxins (DA–IA–IB). The total toxin content for this strain was 1.70 pg cell^{-1} (DA, IA, and IB toxin content: 0.58, 0.20, and 0.92 pg cell^{-1}, with a DA:IA:IB ratio of 33:14:53) (Fig. 3C, lower chromatogram). The toxins produced by the 15 strains from Panyula, Bone, South Sulawesi, Indonesia consisted either of DA (4 strains) or DA-trace IB (11 strains). The average DA and IB toxin content across all the strains was 2.38 pg cell^{-1} ($\sigma = 1.13$) and 0.06 pg cell^{-1} ($\sigma = 0.06$), respectively (2.44 pg cell^{-1} in total), and the DA:IB ratio was 98:2 (Fig. 3D, lower chromatogram).

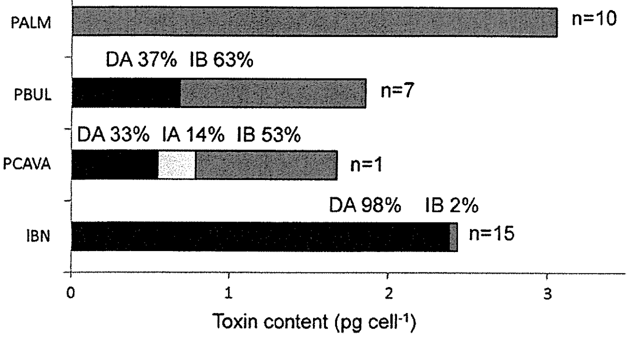

Fig. 4. Toxin composition of *Nitzschia navis-varingica* isolated from Luzon Island, Philippines and South Sulawesi, Indonesia. Black, domoic acid (DA); white, isodomoic aid A (IA); grey, isodomoic acid B (IB). Strains tested: PALM (Alaminos, Pangasinan, Philippines, One isolate in 2007 and 9 isolates in 2008), PBUL (Bulacan, Manila, Philippines, 2007), PCAVA (Cavite, Manila, Philippines, 2007), IBN (Panyula, Bone, South Sulawesi, Indonesia, 2008).

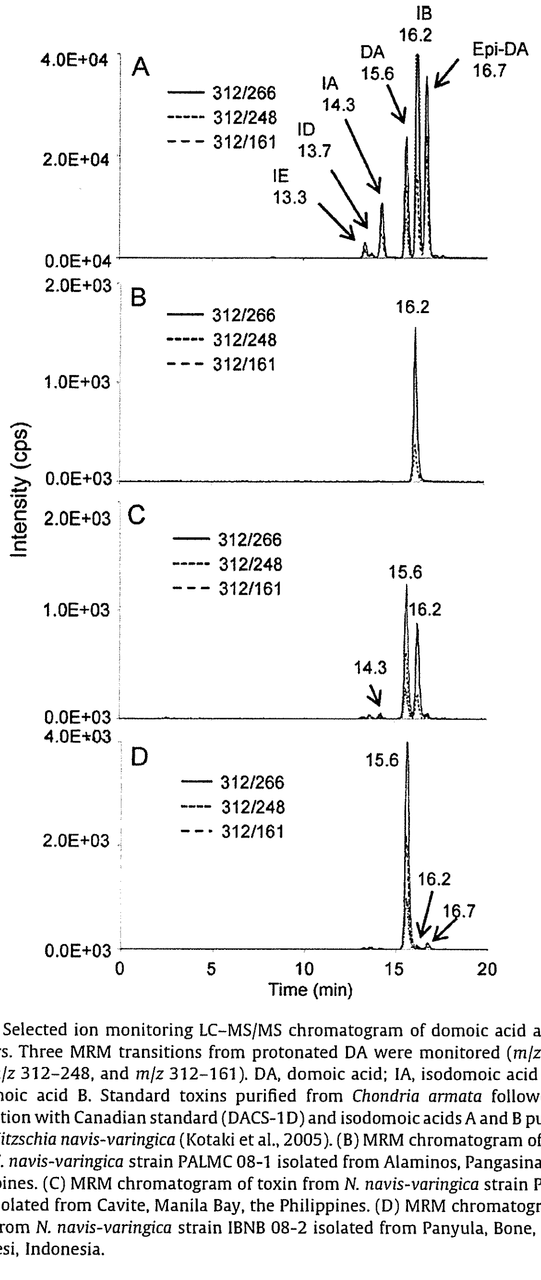

Fig. 5. Selected ion monitoring LC–MS/MS chromatogram of domoic acid and its isomers. Three MRM transitions from protonated DA were monitored (*m/z* 312–266, *m/z* 312–248, and *m/z* 312–161). DA, domoic acid; IA, isodomoic acid A; IB, isodomoic acid B. Standard toxins purified from *Chondria armata* followed by calibration with Canadian standard (DACS-1D) and isodomoic acids A and B purified from *Nitzschia navis-varingica* (Kotaki et al., 2005). (B) MRM chromatogram of toxin from *N. navis-varingica* strain PALMC 08-1 isolated from Alaminos, Pangasinan, the Philippines. (C) MRM chromatogram of toxin from *N. navis-varingica* strain PCAVA 07-2 isolated from Cavite, Manila Bay, the Philippines. (D) MRM chromatogram of toxin from *N. navis-varingica* strain IBNB 08-2 isolated from Panyula, Bone, South Sulawesi, Indonesia.

As shown in Fig. 5, the major toxin types produced by representative strains were confirmed by LC–MS/MS using multiple reaction monitoring (MRM) and comparing the results with LC chromatograms of standard toxins (Fig. 5A). Production of the following toxins was confirmed: (1) only IB was produced by a strain from Alaminos, the Philippines (PALMC 08-1 strain, Fig. 5B), (2) DA, IA, IB were produced by a strain isolated from Cavite, the Philippines (PCAVA 07-2 strain, Fig. 5C), and (3) DA and IB were produced by the strain isolated from Panyula, Bone, South Sulawesi Island, Indonesia (IBNB 08-2 strain, Fig. 5D).

3.3. Toxin composition of the N. navis-varingica sub-strains

Results of toxin composition analyses of the sub-strains are shown in Fig. 6 together with those of the corresponding parent. Thirty-three sub-strains were established from the IB type Philippine parental strain (PALMC 07-1 strain) with the IB toxin content of 4.59 pg cell^{-1}. All sub-strains had the same IB type toxin

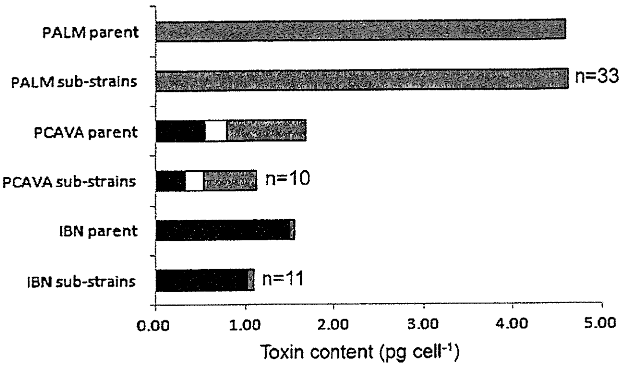

Fig. 6. Toxin composition of the sub-strains of *Nitzschia navis-varingica* compared with the respective parental strain. Black, domoic acid (DA); white, isodomoic aid (IA); grey, isodomoic acid B (IB). Parental strains used: PALM 07-1 (Alaminos, Pangasinan, Philippines, 2007), PCAV 07-2 (Cavite, Manila, Philippines, 2007), IBN 08-2 (Bone, South Sulawesi, Indonesia, 2008).

composition. The toxin composition was 100% IB with an average toxin content of 4.63 pg cell^{-1} ($\sigma = 1.28$), entirely lacking IA and DA (Fig. 6). Ten sub-strains were established from the DA–IA–IB type Philippine parental strain (PCAVA 07-2 strain) which had a total toxin content of 1.70 pg cell^{-1} with a DA:IA:IB toxin ratio of 33:14:53. The toxin in all the sub-strains was composed of DA–IA–IB, similar to the parental strain, with an average toxin content of 0.33 pg cell^{-1} ($\sigma = 0.30$), 0.19 pg cell^{-1} ($\sigma = 0.15$), and 0.60 pg cell^{-1} ($\sigma = 0.45$), respectively, and with a DA:IA:IB toxin ratio of 29:17:54 (Fig. 6). Eleven sub-strains were established from the Indonesian parental strain having a DA–IB type toxin composition (IBNB 08-2 strain) showing the total toxin content of 1.55 pg cell^{-1} with a DA:IB toxin ratio of 96:4. All sub-strains had the same toxin composition as the parental strain (DA–IB). The average DA and IB toxin content was 1.09 pg cell^{-1} ($\sigma = 0.64$) and 0.07 pg cell^{-1} ($\sigma = 0.02$), respectively, with an average DA:IB ratio of 94:6 (Fig. 6).

4. Discussion

We confirmed that *N. navis-varingica* can be found in estuarine areas of the northern part of Luzon Island, the Philippines and in South Sulawesi, Indonesia (Fig. 2). Interestingly, all 10 strains isolated from Alaminos, Luzon Island had a toxin composition only of IB without even trace amounts of any other isomers. The single isolate from Cavite, Luzon Island had the toxin content composed of DA–IA–IB. Neither of these two toxin composition types has been detected before in any diatom as major toxin components, and is described for the first time in the present study. Other isolates from Bulacan, Luzon Island, the Philippines and Bone, South Sulawesi, Indonesia showed the major toxin composition of DA–IB (including only DA) similar to *N. navis-varingica* from areas other than Luzon Island (Kotaki et al., 2005, 2008; Bajarias et al., 2006; Kotaki, 2008). Though total toxin content of each isolate had rather high variability, toxin composition was the same within each toxin composition type. The variability might come from a difference of culture conditions (e.g. difference of co-existing bacteria, difference of light intensity due to the location of tissue culture tube, etc.).

In this study, *N. navis-varingica* samples were collected mainly in dry season. It is thus unknown whether there is a seasonal difference in the distribution and toxin compositions of *N. navis-varingica*. Furthermore, we have not explored the possibility of other benthic diatoms producing ASP toxins. However, in Sakari Estuary, near our college, in Japan, *N. navis-varingica* could be isolated throughout the year (even in winter season, data not shown), all strains showing the same toxin composition (DA–IB). Our data indicate that *N. navis-varingica* might show the same toxin composition during all seasons in tropical areas. Detailed studies in different seasons at the same locality will be needed to further explore the seasonality of the distribution and toxin composition of *N. navis-varingica* in tropical areas.

Toxin compositions of *N. navis-varingica* isolates are summarized in Fig. 7 together with previously published results (Kotaki et al., 2005, 2008; Bajarias et al., 2006; Romero et al., 2008; Kotaki, 2008). The strains isolated from Panyula, Bone, South Sulawesi, Indonesia showed a DA-trace IB toxin composition type that was similar to those of strains isolated from Northern Japan (Kotaki et al., 2008; Kotaki, 2008) and Thailand (Romero et al., 2008), in contrast to the typical DA–IB type strains from Okinawa, southernmost Japan, strains from Vietnam, and some strains from the southern Philippines which had a higher proportion of IB (more than ca. 20%) (Kotaki et al., 2005, 2008; Kotaki, 2008). Our results show that toxin composition is not related to latitude, in contrary to what has previously been suggested, namely that the proportion

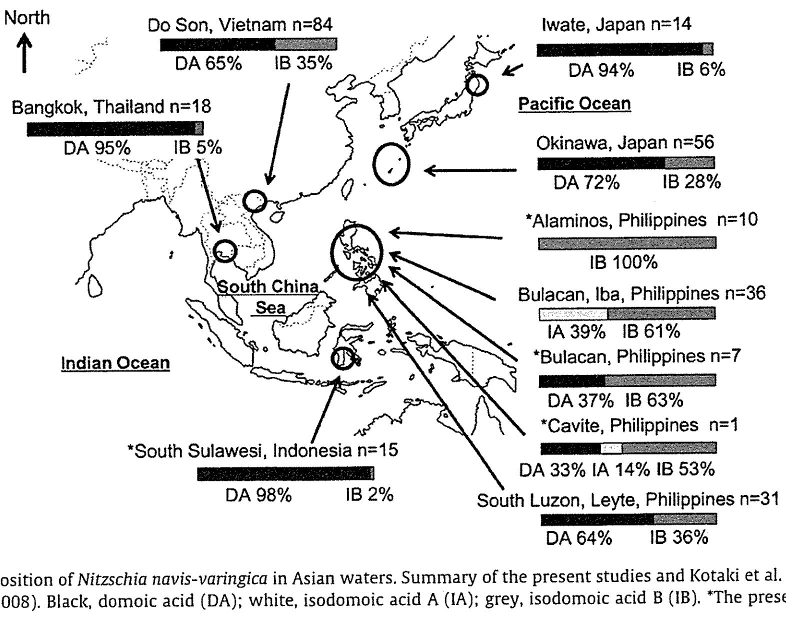

Fig. 7. Distribution and toxin composition of *Nitzschia navis-varingica* in Asian waters. Summary of the present studies and Kotaki et al. (2005, 2008), Bajarias et al. (2006), Romero et al. (2008) and Kotaki (2008). Black, domoic acid (DA); white, isodomoic acid A (IA); grey, isodomoic acid B (IB). *The present study.

of IB in the DA–IB type toxin should be higher in lower latitudes (Kotaki et al., 2005, 2008; Kotaki, 2008).

Sub-strains were established from strains representative of three different toxin composition types (IB, DA–IA–IB, and DA-trace IB), followed by toxin analysis. All of the sub-strains had the same toxin composition as the parental strains. As the parental strains were maintained for more than half year until use, above results demonstrate that toxin composition is most likely a stable character, although the exact ratio of each toxin component varies within each toxin composition type (Fig. 6). The stability of the character is also supported by supplemental batch culture experiments that were done using PALMC 07-1 (IB type) and PCAVA 07-2 (DA–IA–IB type) strains for up to 36 days resulting in confirmation that they maintained the same toxin profile type during the entire experimental period (PALMC strain; toxin content of IB increased up to 4.3 pg cell^{-1} at 36 day and PCAVA strain; total toxin content of DA–IA–IB showed maximum of 5.0 pg cell^{-1} at 28 day with the DA:IA:IB ratio of 37:14:49 and decreased to 4.2 pg cell^{-1} at 36 day with the DA:IA:IB ratio of 34:13:53).

As shown in Fig. 6, toxin content per cell between parent and sub-strain of PALMC 07-1 was almost the same (ca. 4.6 pg cell^{-1}) but in case of PCAVA 07-2 (DA–IA–IB type) and IBNB 08-3 (DA–IB type), average toxin content per cell of the sub-strains was a little lower than those of the parents. However, these differences are included in the variability of sub-strains (PCAVA; $\sigma = 0.87$, IBN; $\sigma = 0.66$).

Trace amounts of other isomers (e.g., isodomoic acids D and E together with DA, IA, and IB in the PCAVA 07 strain (Fig. 5C) and 5′-epi-DA together with DA and IB in the IBNB 08-2 strain (Fig. 5D) were detected in LC–MS/MS chromatograms of toxins from representative strains. However, the presence of these additional minor isomers was not confirmed in HPLC chromatograms with fluorescence detection because of low concentrations using an analysis method to extract samples without concentration (Pocklington et al., 1990; Kotaki et al., 2004). It is uncertain whether these minor isomers were artifacts produced by UV or heating (Wright et al., 1990; Quilliam, 2003) or whether they were bio-synthesized. Nevertheless, we did confirm that DA, IA, and IB are the major components of *N. navis-varingica* toxin, and that the toxin composition is more complex than previously reported (Kotaki et al., 2005, 2006; Bajarias et al., 2006; Kotaki, 2008). The presence of isodomoic acid C (IC) simultaneously with DA was recently reported in toxin of *P. australis* and shellfish that have fed on *P. australis* (Holland et al., 2003, 2005; Rhodes et al., 2003). We did not determine whether IC was present in this study due to a lack of standard toxin for comparison. However, it is unlikely that IC constitutes a major component of the toxin profile because no other apparent peak was observed in any of the HPLC and LC–MS/MS chromatograms.

Interestingly, only *N. navis-varingica* isolates from northern Luzon Island had a toxin composition different from the major DA–IB type. In total, three different toxin profiles have been confirmed in isolates from Luzon Island: IA–IB (Kotaki et al., 2005, 2008; Bajarias et al., 2006), IB, and DA–IA–IB (present study) (Fig. 7). The strains are morphologically similar to those found in other/close localities (Haiphong in Vietnam, Okinawa in Japan, the southern Philippines and Bulacan Estuary, Manila Bay in the Philippines).

One of the strains showing DA–IB toxin composition has been shown to change its toxin composition to the IA–IB type when grown in axenic culture and it returned to the original DA–IB type when the culture medium was replaced with the cell-free but non-axenic medium of the parental strain (Kotaki et al., 2008). This suggests that bacteria might play a role in controlling the toxin composition, but the mechanism is presently unknown. One possibility is that some kinds of bacteria might enhance DA production and/or block IA production and/or consume IA. Such bacteria might be absent at sites from which strains having a toxin composition that includes IA were isolated. Five types of ASP toxin compositions have been identified to date, namely DA, DA–IB, IA–IB, IB, and DA–IA–IB, although the difference between DA and DA–IB is uncertain because the ratio of IB varies (Fig. 7).

For the primary screening of ASP toxin-producing *N. navis-varingica*, the culture conditions described in Section 2.1 were used. The conditions are considered to be sufficiently similar to those of the seawater from which these *N. navis-varingica* strains were isolated. Temperature and salinity appear to not affect the toxin composition based on preliminary experiments (data not shown), but systematic examination of the factors affecting the toxin composition of this diatom, including co-existing bacteria, is needed. The detailed processes of the production of each toxin component are being investigated for determining the toxin production mechanism.

The toxicity of IA, IB, and IC is reported to be significantly lower than DA alone (Munday et al., 2008), suggesting that these toxins pose a lower risk to humans. However, the structural difference of DA and IB is based on the position of only one double bond: 3′–4′ (DA) and 4′–5′ (IA), and in IA and IB, the difference is due to only the configuration of Z (IA) and E (IB) at the 1′–2′ double bond (Fig. 1). Because of the structural similarities, there is a possibility that marine animals such as shellfish, fish and mammals may act as vectors of the toxin isomers, which might then be converted from IB to IA and finally to DA in the animal tissue, although there is no report showing these bioconversions in the animal tissue. Studies of such conversions are currently ongoing.

In conclusion, we isolated ASP toxin-producing diatom *N. navis-varingica* from selected areas of Luzon Island, the Philippines and South Sulawesi, Indonesia. Two new toxin composition types: (1) only IB and (2) DA, IA and IB were confirmed in the *N. navis-varingica* isolates from Luzon Island, the Philippines. The stability of these toxin compositions was confirmed by comparing the toxin composition of a parental strain and respective sub-strains. We summarized, in total, five ASP toxin composition types in *N. navis-varingica*. Investigation of factors affecting the toxin composition of *N. navis-varingica* might help in determining the toxin production mechanism of diatoms.

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連載

社会と健康を科学するパブリックヘルス(4)
「コンピューターシミュレーションによる環境中化学物質のヒト曝露評価法」

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1.　はじめに

現代社会において，おびただしい数の化学物質が活用され，常に新種の物質が生み出されている。特に近年の開発速度には目を見張るものがあるが，それは同時に環境中に排出される化学物質が増え続けているということでもある。現在，工業目的で使用されている化学物質はおよそ70000種あるとされており，ヒトの健康への影響が懸念されているものも少なくないが，その環境中での動態が明らかになっているものはわずかでしかない。

職業曝露を受けていない一般的なヒト集団の，環境中化学物質への曝露状況の実態を把握するには，実際のヒト集団から生体試料を採取することが最も有効であろう。京都大学大学院医学研究科では，ヒトの血液と陰膳法による食事試料を中心とする生体試料の収集を行ってきた。調査は1970年代に開始され，対象は日本国内のみならず，韓国，中国，ベトナムなど広く東アジアに及ぶ[1]。現在まで継続的に収集されてきた試料は京都大学生体試料バンクとして冷凍保存され，貴重なデータを提供している。しかしながら，このような調査で得られる情報は，調査が行われた地域，時代における情報でしかない。言うなれば，時間，空間について限定的かつ離散的な情報である。曝露状況を包括的に理解するにはこれらの範囲を広げるとともに，密度を向上させるべきであるが，財政的および人的資源が限られている中で，それは非常に困難であり，現実的でないとも言える。ここでは，コンピューターシミュレーションを活用してヒト曝露評価を行う手法である，Environmental ecological modeling（EEM）について紹介する。

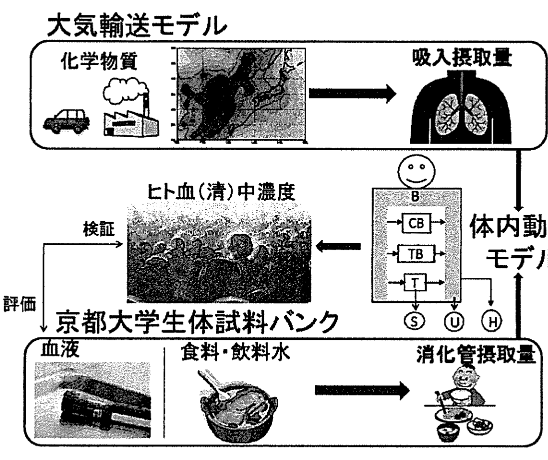
2.　EEM の概念と手法

EEM は3つのパートから構成される（図1）。大気輸送モデル，体内動態モデル，生体試料である。環境中化学物質に対するヒトの曝露経路には大きく分けて2つある。呼吸による吸入摂取と，食事による消化管摂取である。吸入摂取は化学物質の大気中濃度に依存するが，これは大気の流れによって輸送されるため排出源の影響が広範囲に及び，国境を越えることも少なくない。一方，消化管摂取は化学物質の食料，飲料水中の含有量に依存するが，これはそれぞれの国における供給体制を反映するものと考えられる。

そこで，吸入摂取量は大気輸送モデルにより計算した大気中濃度に基づいて算出する。その際，大気への排出の強度，分布，時間による推移は，その物質の排出に関する知見と経済統計などから推定し，大気モデルに入力する。従って，吸入摂取量は位置と時間の関数として与えられる。消化管摂取量は生体試料バンクの陰膳食事試料における含有量の実測値に基づいて算出し，国ごとに時間の関数として与える。算出した吸入および消化管摂取量をヒトの体内動態モデルに入力し，血（清）中濃度を計算する。

この計算値を，生体試料バンクの血液試料における濃度と比較して検証を行う。計算値と実測値がうまく一致していれば，大気モデル，体内動態モデルともに正しく機能しているということになる。そして検証されたモデルの結果を解析すれば各地域の任意の時間におけるヒト曝露状況を評価することができる。

図1　EEM の概念図。



3. EEMの適用例-血中鉛のシミュレーションと曝露評価

EEMの適用例として，血中鉛のシミュレーションを紹介する。鉛は古くから工業目的で広く活用され，有毒な重金属としては環境中に最も多量に存在する。大気中では微小粒子中に含まれ，広範囲に輸送される。大気への排出は有鉛ガソリンの使用によるものが最も多く，次いで非鉄金属の精錬工程からのものが多い。さらに近年は石炭などの化石燃料燃焼の影響も指摘されている。多くの先進国では有鉛ガソリンの禁止など環境中鉛の排出削減努力により既に大きな成果が得られている。一方，東アジアでは削減がいまだ不十分な国が少なくなく，そういった中で急速な経済成長が進行している。我が国における大気を通じた越境汚染も懸念されており，ヒト曝露の実態把握が急がれる。そこで筆者らはEEMを用いて，日本，韓国，中国，ベトナムにおける成人女性の過去の血中鉛濃度の再現を行い，環境中鉛に対する曝露評価を行った2)。対象期間は1979年から2009年である。

3-1. シミュレーション

大気モデルには水平解像度1.25度の全球輸送モデルを用いた。既存のデータや経済統計などを基に4カ国からの鉛排出量を推定して大気モデルに入力し，大気中濃度分布を計算して過去30年間の観測データと比較したところ，概ね良い一致が見られた2,3)。各地の鉛の吸入摂取量はこのモデルによる地表面大気中濃度から算出した。消化管摂取量は，生体試料バンクの食事試料における鉛含有量データに対して，各国ごとに指数回帰を適用し，時間の関数として算出した。

算出した鉛摂取量を，4つの体内区画から成る動態モデル4)に入力して血中鉛濃度を計算した。4カ国の成人女性の血中鉛濃度の計算値を実測値と比較した（図2）。実測データにおける血中濃度の幾何平均（GM）値は，日本では1980年頃に32.8 μg L^{-1}であったのが1990年代には24.0 μg L^{-1}に減少し，その後2000年代には15.7 μg L^{-1}まで減少している。韓国でのGM値は1994年の44.3 μg L^{-1}から2000年代には17.7 μg L^{-1}まで減少した。中国のGM値は1980年代は60.5 μg L^{-1}，1990年代は53.6 μg L^{-1}，2000年代は53.9 μg L^{-1}である。ベトナムでは2009年のGM値は28.0 μg L^{-1}であり，これは1980年頃の日本，あるいは2000年代初頭の韓国の水準に近い。EEMは過去の実測値のほとんどすべてを2倍の誤差の範囲で再現できていることが分かる。

3-2. 曝露評価

図3は東京，ソウル，北京，ハノイにおける成人女性の血中鉛濃度の計算値(μg L^{-1})の推移である。計算値に見られる振動は地表面大気中鉛濃度の季節変化によるもので，冬季に極大となることが多い。呼吸による吸入摂取量を含まない場合（no-air run）の結果も図中に示す。control runとno-air runの差が，血中鉛全体に対する大気由来成分の寄与を表すことになる。

東京での計算値は1980年の44 μg L^{-1}から2009年の18 μg L^{-1}まで58％減少している。ソウルでは1990年代初頭は50 mg L^{-1}を超えていたのが，2009

図2　成人女性の血中鉛濃度の計算値と実測値との比較(μg L^{-1})。中央の実線は計算値と実測値が一致すること，2本の破線は誤差が2倍であることを表す。（＋）日本1980年頃，（●）日本1990年代，（×）日本2000年以降，（□）韓国2000年以前，（@）韓国2000年以降，（△）中国1980年代，（＊）中国1990年代，（○）中国2005年以降，（V）ベトナム2005年以降。

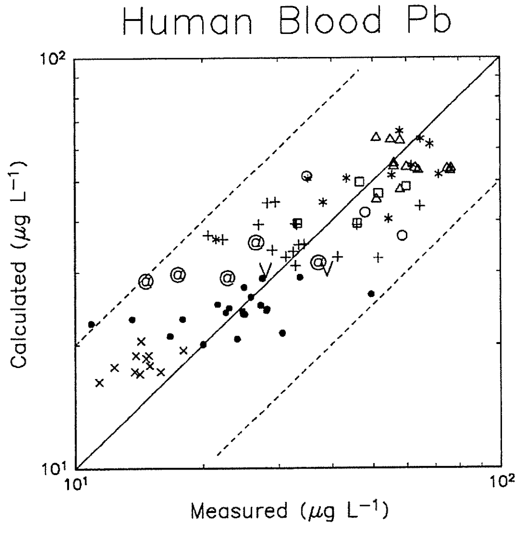
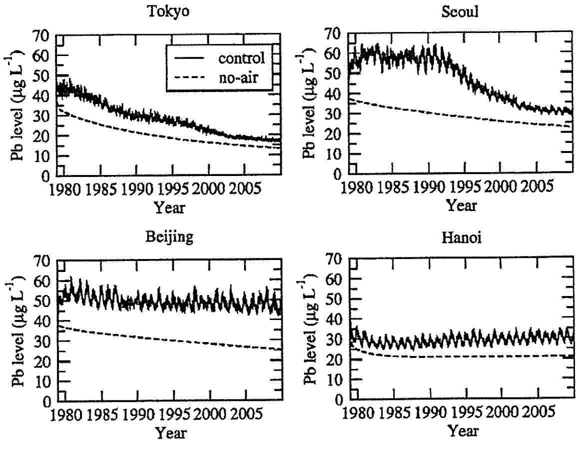


図3　モデルによる，東京，ソウル，北京，ハノイにおける成人女性の血中鉛濃度の1979年から2009年までの推移。破線は呼吸器からの曝露を含まない場合（no-air）の計算値。



年には29 μg L^{-1}となり45%の減少である。東京での大気由来成分の寄与は小さく，1983年の30%から2003年の19%の範囲である。ソウルでは1990年に48%であったのが2005年の24%まで減少している。北京における血中鉛濃度の計算値はおよそ50 μg L^{-1}で，現在の東京の計算値よりずっと大きい。計算値の減少の度合いが東京やソウルより緩やかであることは，実測値の経年変化が中国では日本や韓国ほどはっきりしない（図2）ことと整合性がとれている。北京では大気由来成分の寄与は1980年の32%から2009年の43%へと増加している。ハノイでは鉛の大気への排出量と食料，飲料水中含有量が一定であると仮定したときの血中濃度の計算値はおよそ30 μg L^{-1}で，大気由来成分の寄与は30%である。血中濃度も大気由来成分の寄与も現在のソウルでの計算値と同程度である。しかしながら，トレンドに関しては，継続的な測定データがなければ評価することはできない。

東京，ソウルにおける成人女性の血中鉛濃度のモデル値に減少トレンドが見られたのは日本，韓国における環境中鉛の削減を反映するものである。有鉛ガソリンが禁止された時期を含む10年間の血中鉛濃度の減少の大きさは，東京で1980年から1990年にかけて30.6%，ソウルで1990年から2000年にかけて37.8%である。これは先進国で実際に見られた減少率に近い値である。例えば米国では1991年から1994年の調査と1999年から2002年の調査の結果では30.2%減少しており5)，ドイツでは1990年から1992年の調査と1998年の調査の結果では30.5%減少している6)。

モデルによる北京の血中鉛濃度は他の都市の現在の血中濃度よりずっと高い。また1999年から2009年にかけて，モデル値はわずか4.1%減少したに過ぎない。また，大気由来成分の寄与もはっきりと増加している。中国の有鉛ガソリンは2001年に禁止されているが，北京の大気中鉛濃度は近年漸増していることが観測されており，石炭消費量の増加によるものと考えられている7)。今後，中国の急激な経済成長により，中国だけでなく周辺国でも大気中濃度が大きく増加する可能性が否定できない。従って，東アジア全体で環境中鉛を継続的にモニターしていく必要がある。

4. EEMの展望

EEMは関西地方におけるパーフルオロオクタン酸（PFOA）の血清中濃度のシミュレーションに初めて使用された8)。ある化学工場からのPFOAの排出量，大気による輸送量および周辺住民の曝露量を評価した。これにより周辺住民の血清中に見られた高いPFOA濃度の原因が明らかになった。鉛に関しては，ハノイ市の児童におけるリスク評価にも応用している2)。血液と食における汚染レベルについての情報があれば，EEMは他の物質や地域にも適用できる。さらに，過去のトレンドや現在のリスクを評価するだけでなく，未知の汚染源の推定や，曝露シナリオを想定しての将来のトレンドやリスクの予測を行うこともできる。また実行に際して，財政的負担が小さいことも指摘しておくべきであろう。生体試料により得られる情報は極めて重要ではあるが，時間，空間について限定的，離散的であると先に述べた。以上見てきたように，EEMはコンピューターを活用することにより，限定的，離散的情報を包括的，連続的情報に拡張して活用する手法であるとも言え，環境評価や政策決定にも大いに貢献できるものと確信している。

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2,2',3,4,4',5'-六塩素化ビフェニル（CB138）のモルモットにおける *in vivo* 代謝

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In Vivo Metabolism of 2,2',3,4,4',5'-Hexachlorobiphenyl (CB138) in Guinea Pigs

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Abstract Our preceding studies reported using animal liver microsomes that 2, 2', 3, 4, 4', 5'-hexachlorobiphenyl (hexaCB) (CB138), a worldwide and persistent organohalogen pollutant, was metabolized to two major hydroxy (OH)-metabolites, 3'-OH-CB138 (M-3) and 2'-OH-2, 3, 3', 4, 4', 5'-hexaCB (M-4), and two dechlorinated OH-metabolites (M-1 and M-2) in guinea pigs at much faster rate than in rats and hamsters. In this study, the distribution of four CB138 metabolites to the serum and liver 4 days after exposure and their fecal excretion were studied in guinea pigs administered with CB138 intraperitoneally. 3'-OH-CB138 (M-3) was a major metabolite in the liver, serum and feces. M-1 was observed as a minor metabolite in guinea pig feces. In contrast, trace amount of M-2 was present in guinea pig serum. However, 2'-OH-2, 3, 4, 3', 4', 5'-hexaCB (M-4) which was a major metabolite in the *in vitro* system using guinea pig liver microsomes was not found in all tissues and feces tested in this study. On the other hand, the exact chemical structures of M-1 and M-2 were determined to be 6'-OH-2, 3, 3', 4, 4'-pentaCB and 4'-OH-2, 2', 3, 4, 5'-pentaCB, respectively, by comparison of the retention time and mass fragmentation of the synthetic authentic samples in GC-MS. From these results, it is suggested that the metabolism of CB138 in guinea pigs may proceed by three pathways, a direct hydroxylation at 3'-position, and also the formation of 2', 3'- or 3', 4'-epoxide and subsequent dechlorination and that three metabolites show the different mode of distribution and excretion.

Key words：CB138, Metabolism, Guinea pig, PCB

はじめに

ポリ塩素化ビフェニル（PCB）は，周知の通り，カネミ油症の原因物質1)であるとともに世界的な環境汚染物質2)としても有名である．PCB 異性体のうち，2, 2', 3, 4, 4', 5'-hexachlorobiphenyl （hexaCB）（CB138）は，2,2',4,4',5,5'-hexaCB（CB153）や 2,2',3,4,4',5,5'-heptachlorobiphenyl（heptaCB）（CB180）とともに，生体への残留性が非常に高いPCB異性体として知られている．例えば，海棲哺乳動物の血液や脂肪組織3)4)，ヒト母乳5)6)，ヒト血液およびヒト脂肪組織5)7)~9)では，CB153に次いで高濃度で検出される．一方，ヒト血液中では，これらのPCB異性体とともに，4-hydroxy（OH）-2,2',3,4',5,5',6-heptaCB（CB187），4-OH-2,2',3,4',5,5'-hexaCB（CB146），4-OH-2,3,3',4',5-pentachlorobiphenyl（pentaCB）（CB107）などのPCB水酸化体が比較的高濃度で検出されている10)~14)．

CB138の毒性は，3,3',4,4'-tetrachlorobiphenyl（CB77），3,3',4,4',5-pentaCB（CB126）および3,3',4,4',5,5'-hexaCB（CB168）などのダイオキシン類（コプラナーPCB）に比べはるかに弱いため，世界保健機関（WHO）がダイオキシン類の毒性の強さを表わすために提唱している毒性等価係数は設定されていない15)．しかしながら，CB138は，CB153と同様にチトクロムP450を含む肝薬物代謝酵素の強いphenobarbital（PB）型誘導能を有することから16)，何らかの生体影響が危惧される．

これまでにCB138代謝に関する報告はほとんどなかったが，CB138の代謝物と思われる3'-OH-CB138がヒト血中や肝中から検出されるに至り11)~14)17)，少なくとも代謝を受けることが判明した．また，ヒト肝では3'-OH-CB138が特異的に高濃度で分布していることが報告された18)．一方，当研究室では，CB138の動物肝ミクロゾームによる*in vitro*代謝を調べ，1）ラットでは1種類，ハムスターでは3種類およびモルモットでは4種類の代謝物が生成されること，2）そのうち主代謝物は3'-OH-CB138および2'-OH-2,3,3',4,4',5'-hexaCB（CB157）であること，3）モルモットが最も高い代謝活性を有すること，4）2種類の一脱塩素化OH体（OH-pentaCB）も生成されること，さらに，5）これらの生成はPB前処理により著しく増加することを明らかにした19)．そこで，本研究では，これまで不明であった2種類のOH-pentaCBの化学構造を明らかにするとともに，CB138代謝物の生体内運命を明らかにするため，代謝活性が最も強いモルモットにCB138を投与し，投与後4日目の代謝物の血液および肝への分布と4日間の糞中への排泄を調べた．

実験方法

1．実験材料

(1)CB138および代謝物

CB138はCadoganの方法20)で合成した．まず，1,2,3-trichlorobenzeneおよび2,4,5-trichloroanilineをtetrachloroethyleneで溶解し，さらにisoamyl nitriteを加えて，110℃で24時間反応させた．反応物はアルミナカラム（100 g，Merck）およびシリカゲルカラム（65 g，Merck）で部分精製した後，高速液体クロマトグラフィー（HPLC）に付した．HPLC条件は次の通りである．カラム，ODSカラム（250 × 20 mm i.d.，5 μm，YMC製）；プレカラム，ODSプレカラム（20 mm i.d.× 50 mm，YMC製）；移動相，acetonitrile；流速，4 ml/min；検出波長，254 nm．なお，CB138の純度は電子捕獲型検出器付ガスクロマトグラフィー（GC-ECD）による検討結果，最終的に94.0%以上であった．

<u>CB138</u>：MS（EI）*m/z*（relative intensity）358（100）$[M^+]$，360（193）$[M^++2]$，362（157）$[M^++4]$，364（63）$[M^++6]$，366（16）$[M^++8]$，323（32）$[M^+-Cl]$，288（89）$[M^+-Cl_2]$．

(2)代謝物の合成

M-1（メチル化体）の予想代謝物として，6'-Methoxy（MeO）-2,3,3',4,4'-pentaCB（CB105）を合成する場合，2,3,4-trichloroanilineと3,4-dichloroanisoleを合成原料として用い，Caduganの方法20)で行った．なお，3,4-dichloroanisoleは，3,4-dichlorophenolをアルカリ性条件下，dimethyl sulfateの添加によりメチル化したものを用いた．一方，M-2（メチル化体）の予想代謝物として，5'-MeO-CB105を合成する場合，2,3,4-trichloroanilineと2,3-dichloroanisoleを，また5'-MeO-2,2',3,4,4'-pentaCB（CB85）を合成する場合，2,3,4-trichloroanilineと2,4-dichloroanisoleを，さらに4'-MeO-2,2',3,4,5'-pentaCB（CB87）を合成する場合，2,3,4-trichloroanilineと2,5-dichloroanisoleを，合成原料として用いた．得られたMeO体はCB138と同様に，アルミナカラムとシリカゲルカラムを用いて部分

精製した後，HPLC にて精製した．

6'-MeO-CB105：MS(EI) m/z (relative intensity) 354 (100) [M^+]，356 (152) [$M^+ + 2$]，358 (100) [$M^+ + 4$]，360(34) [$M^+ + 6$]，362(6) [$M^+ + 8$]，304(97) [M^+-CH_3Cl]，241(39) [M^+-$COCH_3Cl_2$].

5'-MeO-CB105：MS(EI) m/z (relative intensity) 354(100) [M^+]，356(148) [$M^+ + 2$]，358(96) [$M^+ + 4$]，360(33) [$M^+ + 6$]，362(4) [$M^+ + 8$]，304(5) [M^+-CH_3Cl]，311(31) [M^+-$COCH_3$]，241(41) [M^+-$COCH_3Cl_2$].

5'-MeO-CB85：MS(EI) m/z (relative intensity) 354(100) [M^+]，356(154) [$M^+ + 2$]，358 (107) [$M^+ + 4$]，360(32) [$M^+ + 6$]，362(6) [$M^+ + 8$]，339(16) [M^+-CH_3]，311(29) [M^+-$COCH_3$]，304(7) [M^+-CH_3Cl]，241(37) [M^+-$COCH_3Cl_2$].

4'-MeO-CB87：MS(EI) m/z (relative intensity) 354(100) [M^+]，356(138) [$M^+ + 2$]，358 (118) [$M^+ + 4$]，360(34) [$M^+ + 6$]，362(4) [$M^+ + 8$]，339(17) [M^+-CH_3]，311(35) [M^+-$COCH_3$]，241(46) [M^+-$COCH_3Cl_2$].

2．動物の薬物処理

代謝実験は，Hartley 系雄性モルモット（体重約 300〜350 g）を 5 匹用いて行った．実験期間中は，床敷き用ケージにて個別に飼育し，飼料 RC4（オリエンタル酵母製）を，水とともに自由に摂取させた．実験室は，温度 23.0 ± 0.5℃，湿度 60 ± 5% に保持し，照明は 12 時間の暗期／明期サイクル（明期：7:00〜19:00）とした．CB138 はコーン油に溶解し，モルモット 1 匹あたり 10 mg を腹腔内に投与した．投与後，糞を 2 日間ごとに採取するとともに，4 日目には頸動脈より全血液を採取することにより屠殺した．血液は血清分離剤（栄研製）で処理し，血清として得た．また，肝は屠殺後直ちに摘出し，生理食塩水で灌流した後，分析まで-80℃に保管した．なお，これらの動物実験は動物実験研究倫理審査委員会の承認を得た上で，「中村学園大学（含む短期大学部）における実験動物のための指針」を遵守し実施した．

3．代謝物の抽出

(1) 糞中代謝物

糞は 60℃で 48 時間乾燥後，コーヒーミルで粉砕した．乾燥糞の粉末 10 g に内部標準物質として 2, 2', 3, 3', 4, 5, 5', 6, 6'-nonachlorobiphenyl (CB208) を添加後，acetone-n-hexane（2:1, v/v）で 16 時間，ソックスレー抽出器で連続抽出した．次に代謝物をメチル化するため，得られた抽出物を chloroform に溶解した．次に，この 20 分の 1 を採り，2M 水酸化カリウム水溶液 2.5 ml で懸濁した後，dimethyl sulfate を 0.5 ml 添加し，100℃で 60 分間還流した．その後，chloroform で抽出し，濃縮した後，n-hexane に溶解したものを GC サンプルとした．

(2) 血中代謝物

血清 0.5 ml に CB208 を添加し，0.5M 硫酸 0.25 ml 添加して酸性にした後，chloroform-methanol（2:1, v/v）1 ml および n-hexane 3 ml の混合溶媒で 3 回抽出した．また，抽出物は diazomethane でメチル化した．

(3) 肝中代謝物

肝臓 1 g に CB208 を添加して，Potter-Elvehjem ホモジナイザーを用いて acetone-n-hexane (2:1, v/v) 15 ml でホモジナイズした．次に，脱水するため，上清は硫酸ナトリウムカラム（12 g）にかけた．また残渣は n-hexane 10 ml で 2 回ホモジナイズを繰り返した後，前述の硫酸ナトリウムカラムにかけた．得られた抽出物は diazomethane でメチル化した．

4．分析機器

CB138 とその代謝物の分析は，GC-ECD および質量分析計付 GC（GC-MS）により行った．なお，これらの定量は，CB138 の検量線を用いて GC-ECD により行った．GC-ECD の条件は次の通りである．分析機器，ECD 付 HP5890 Series II ガスクロマトグラフ（Hewlett-Packard 製）；カラム，DB-1 フューズドシリカキャピラリーカラム（30 m × 0.25 mm i.d., 0.25 μm 膜厚，J&W Scientific 製）；オーブン温度，230℃；注入口温度，250℃；検出器温度，250℃；キャリアーガス，N_2 (1 ml/min)．一方，代謝物の分子量は，GC-MS 2010（島津製作所製）を用いて，EI モードで測定した．GC-MS 分析条件は次の通りである．カラム，DB-1 フューズドシリカキャピラリーカラム（30 m × 0.25 mm i.d., 0.25 μm 膜厚，J&W Scientific 製）；オーブン温度，70℃（1.5 min）-

20℃/min- 230℃（0.5 min）- 4℃/min- 280℃（5 min）；注入口温度，250℃；検出器温度，280℃；キャリアーガス，He（1 ml/min）.

実験結果

1．血中代謝物の検索

CB138投与後4日目のモルモット血中のCB138およびその代謝物（メチル化体）のGC-ECDクロマトグラムをFig. 1Aに示す．未変化体のCB138以外に，2本の代謝物ピークが，それぞれ保持時間15.1分および18.9分に検出された．これらのピークは，既報[19]の代謝物の保持時間との比較から，それぞれOH-pentaCB（M-2）および3'-OH-CB138（M-3）のメチル化体であることが確認された．

次に，これらの血中濃度を定量した（Table 1）．定量にはCB138の検量線を用いた．その結果，未変化体の血中濃度は，0.25 ± 0.05 nmol/ml serumであった．これに対し，主代謝物のM-3の血中濃度は，0.18 ± 0.07 nmol/ml serumであり，未変化体と同程度が検出された．一方，M-2の血中濃度は，0.04 ± 0.01 nmol/ml serumとかなり低かった．

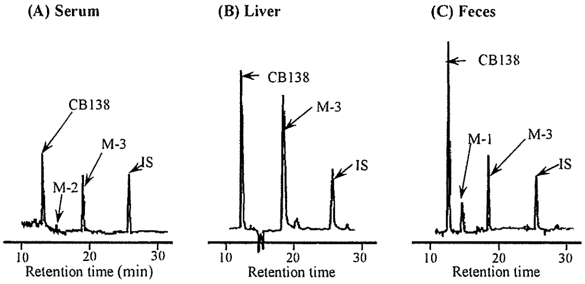

Fig. 1 Gas chromatograms of CB138 and the methylated derivatives of CB138 metabolites detected in the serum (A), liver (B) and feces (C) of guinea pigs injected CB138 intraperitoneally.
IS, internal standard (CB208).

Table 1 Distribution of CB138 and its metabolites to the serum and liver 4 days after CB138 injection to guinea pigs

Compound	Serum (nmol/ml serum)	Liver (nmol/g wet wt.)
CB138	0.25 ± 0.05	1.87 ± 0.05
M-1	N.D.	N.D.
M-2	0.04 ± 0.01	N.D.
M-3	0.18 ± 0.07	1.26 ± 0.09
M-4	N.D.	N.D.

N.D., not detected.
Each value represents the mean ± S.D. of five guinea pigs.

2．肝中代謝物の検索

CB138投与後4日目のモルモット肝中のCB138およびその代謝物（メチル化体）のGC-ECDクロマトグラムをFig. 1Bに示す．CB138以外に，代謝物として3'-OH-CB138（M-3）のメチル化体のみが検出された．そこで，肝中の未変化体およびM-3の定量を試みたところ，肝中濃度はそれぞれ1.87 ± 0.35および1.26 ± 0.09 nmol/g wet wt.であり，肝においてもM-3は未変化体に匹敵するほどの高濃度で分布していた（Table 1）．なお，モルモット5匹の平均肝湿重量を約17 gとして換算すると，4日目の肝に分布する未変化体とM-3の総量はそれぞれ投与量の0.12%と0.08%に相当していた．

3．糞中代謝物の検索

Fig. 1Cに，CB138投与後2日間の糞中の未変化体および代謝物（メチル化体）のGC-ECDクロマトグラムを示す．未変化体とともに2種類の代謝物ピークが，保持時間14.9分および18.9分に検出された．なお，これらはGC保持時間から，それぞれ，既報[19]のOH-pentaCB（M-1）および3'-OH-CB138（M-3）のメチル化体と推定された．次に，4日間で糞中へと排泄された未変化体および代謝物（M-1とM-3）を定量し，投与後2日間および3～4日間で比較した（Fig. 2）．

まず，未変化体の糞中への総排泄量は，投与後2日間で76.8 nmol，3～4日間で31.6 nmolであった．これに対し，M-3の総排泄量は，投与後2日間で20.8 nmol，3～4日間で10.0 nmolであった．また，M-1の総排泄量は，投与後2日間

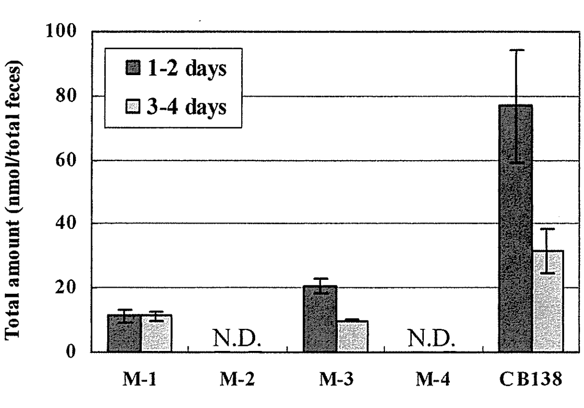

Fig. 2 Fecal excretion of CB138 and its metabolites in guinea pigs injected CB138 intraperitoneally.
N.D., not detected.

で11.3 nmol，3～4日間でも11.3 nmolと変わらなかった．このように，代謝物（M-3およびM-1）の排泄量は，未変化体の半分以下であった．なお，両代謝物の4日間の糞中排泄量は投与量のほんの0.19％であった（データ未掲載）．

4．代謝物M-1とM-2の同定

今回CB138代謝物として，M-1およびM-2が微量ながら，それぞれ糞中および血中から検出された．そこで，これらの分子量を確かめるために，各試料の抽出物をGC-MSに付した．その結果，Table 2に示すように，M-1およびM-2のメチル化体はいずれも分子量354を有することから，既報19)と同様に，塩素が1個脱離したMeO-pentaCBであることが確認された．次に，予想代謝物を合成し，両代謝物の同定を試みた．

まず，M-1（メチル化体）のマススペクトルをみると，フラグメントイオン［M^{+}-50］が強く検出されたことから，M-1（メチル化体）は2(2')位に，あるいは6(6')位にMeO基を有するpentaCB21)と推定された．そこで，予想代謝物として6'-MeO-2,3,4,4',5'-hexaCB（CB105）を合成し，GC-MSでの保持時間およびマスフラグメンテーションをM-1（メチル化体）と比較した結果，いずれも完全に一致した．以上の結果から，M-1は6'-OH-CB105であると決定された．

一方，M-2（メチル化体）のマススペクトルでは，弱いフラグメントイオン［M^{+}-15］とともにフラグメントイオン［M^{+}-43］が比較的強く検出されたことから，3(3')位あるいは5(5')位にMeO基を有するpentaCB21)と推定された．そこで，予想代謝物として別途，5'-MeO-CB105および5'-MeO-CB85を合成し，GC保持時間を比較した．しかしながら，いずれもM-2（メチル化体）と保持時間が一致しなかった．次に，4'-MeO-CB87を合成し比較したところ，GC-MSでの保持時間およびマススペクトルがM-2（メチル化体）とほぼ完全に一致した．以上の結果から，M-2は4'-OH-CB87であることが明らかになった．

考　察

モルモットによるCB138の代謝を調べたところ，肝ミクロゾームを用いた*in vitro*代謝系とかなり異なる代謝パターンを示すことが明らかとなった．すなわち，*in vitro*代謝系では4種類の代謝物が生成されたが19)，今回，*in vivo*代謝系ではそのうちの3種類が検出された．主代謝物は3'-OH-CB138（M-3）であり，血液，肝および糞のすべてから検出された．他に，微量ではあるが，2種類のOH-pentaCB（M-1，M-2）がそれぞれ糞中と血中から検出された．

本研究では，上記2種類のOH-pentaCBの化学構造が明らかとなった．予想代謝物を合成し，GC-MSで比較したところ，M-1とM-2はそれ

Table 2　GC-MS data of CB138 metabolites and synthetic authentic samples

Compound	Molecular weight	Mass spectral data [M^{+}]	[M^{+}-15]	[M^{+}-43]	[M^{+}-50]	Retention time (min)
Serum						
M-2	354	100	18	39	-	13.81
M-3	388	100	7	38	10	15.08
Liver						
M-3	388	100	5	34	8	15.08
Feces						
M-1	354	100	-	-	84	13.75
M-3	388	100	5	33	7	15.08
Standards						
6'-MeO-CB105	354	100	-	-	97	13.75
5'-MeO-CB105	354	100	-	31	5	15.17
5'-MeO-CB85	354	100	16	29	7	13.58
4'-MeO-CB87	354	100	17	35	-	13.81
3'-MeO-CB138 (M-3)*	388	100	8	40	-	15.08
2'-MeO-CB157 (M-4)*	388	100	-	-	155	15.23

-, not detected. *cited from the reference19).
CB85 (2,2',3,4,4'-pentaCB) ; CB87 (2,2',3,4,5'-pentaCB) ; CB105 (2,3,3',4,4'-pentaCB) ; CB138 (2,2',3,4,4',5'-hexaCB) ; CB157 (2,3,3',4,4',5'-hexaCB).

ぞれ6'-OH-CB105と4'-OH-CB87であることが示唆された．この事実から，モルモットでは3つの水酸化経路が同時に進行していることが示唆された．Fig. 3にモルモットにおけるCB138の推定代謝経路を示した．すなわち3'-OH-CB138は主として3'位の直接水酸化により，また，6'-OH-CB105（M-1）と4'-OH-CB87（M-2）は2',3'-epxideと3',4'-epoxideを中間体とし[22)~24)]，さらに脱塩素化が起こって生成されたものと考えられる．

生成された3種類の代謝物はそれぞれ異なる分布を示した．CB138投与後4日目では，主代謝物の3'-OH-CB138（M-3）は，血中でも検出されたが，肝で特に高濃度分布していた．この結果は，Guveniusら[18)]のヒト肝の報告とよく一致したが，本代謝物がなぜ肝に蓄積しやすいかは不明である．さらに，2種類のOH-pentaCBのうち，4'-OH-CB87（M-2）は微量ながら血中へと分布し，一方，6'-OH-CB105（M-1）は容易に糞中へと排泄されることが明らかとなった．これまで血中に残留しているPCB代謝物は，共通して4-OH-3,5-dichlorobenzeneの構造を有しており，血中の甲状腺ホルモン結合タンパクtransthyretinと高い親和性を示すことが知られている[25)]．4'-OH-CB87（M-2）は，この条件を満たしていることから，transthyretinに結合し血中へ残留しているものと推定される．なお，モルモット肝ミクロゾームを用いた*in vitro*代謝系において主代謝物の1つであった2'-OH-CB157（M-4）は，今回の*in vivo*代謝系では，全く検出されなかった．この理由は不明であるが，今回調べた血液，肝および糞以外に特異的に分布しているのかもしれない．あるいは，代謝物が生体高分子と結合しており，抽出されなかったためかもしれない．この点は今後の研究課題である．

今回，腹腔内投与されたCB138は血液，肝だけではなく，糞中にも高濃度で検出された．腹腔から糞への排泄経路は，2つ考えられる．1つは，腹腔内から，血液，肝，さらに胆汁を介して糞中へ排泄される経路である．この場合，CB138は脂溶性が高いことから小腸でCB138のほとんどが再吸収されると思われる．もう1つは，小腸上皮細胞からの排出である．吉村と神村[26)]は，カネミ油症の主たる原因物質の1つである2,3,4,7,8-pentachlorodibenzofuran（pentaCDF）が，ほとんど代謝されることなく，毎日微量ではあるが，胆汁を介さずに，ラット小腸管腔内に排泄されることを報告している．現在，小腸上皮細胞膜に存在するトランスポーターのP糖タンパク質（MDR1）が種々の薬物の細胞外への排出を担っていることが知られており[27)]，前述のpentaCDFや本研究のCB138もMDR1の基質になっているのかもしれない．

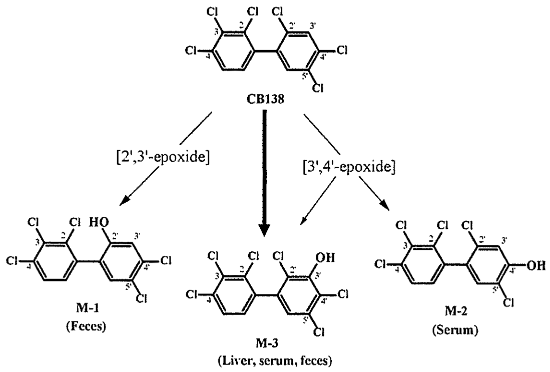

Fig. 3 Postulated metabolic pathways of CB138 in guinea pigs.

前述のように，ヒト血中ではPCB異性体とともに，代謝物の4-OH-CB187や4-OH-CB146が検出されている．4-OH-CB146はヒト血中で2番目に多いPCB代謝物であるが，もしCB138が酸化されて，4,5-epoxide中間体を生成し，さらに4位の塩素が5位にNIH転位すると4-OH-CB146が生成されることになる[28)]．しかしながら今回，CB138投与モルモットの血液，肝および糞のいずれからも4-OH-CB146は検出されなかったことから，4-OH-CB146はCB138からは生成されないことが示唆された．

総　　括

1．CB138をモルモットに腹腔内投与し4日目のCB138代謝物の血液と肝への分布および糞中排泄を調べた．3種類の代謝物（M-1，M-2，M-3）が検出され，このうち，M-3（3'-OH-CB138）は血液，肝および糞中のいずれでも最も多く検出された．

2．CB138投与モルモット血中から，未変化体および3'-OH-CB138とともに微量のM-2が検出された．肝中では未変化体と3'-OH-CB138が

高濃度で検出された.

3. CB138投与後4日間のモルモット糞中から，未変化体および3'-OH-CB138とともに少量のM-1が検出された.
4. M-1およびM-2の予想代謝物を合成し，GC-MSにより比較したところ，M-1とM-2は，それぞれ6'-OH-CB105と4'-OH-CB87であることが明らかになった.

以上の結果からモルモットでのCB138の代謝は3'位への直接水酸化が主であること，また，一部は中間体の2',3'-epxideと3',4'-epoxideを経由して脱塩素化が起こること，さらに，これらの代謝物は肝，血液への分布および糞への排泄がそれぞれ異なることが示唆された.

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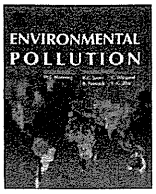

Regional variation and possible sources of brominated contaminants in breast milk from Japan

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ABSTRACT

This study focuses on the regional trends and possible sources of brominated organic contaminants accumulated in breast milk from mothers in southeastern (Okinawa) and northwestern (Hokkaido) areas of Japan. For persistent brominated flame retardants, polybrominated diphenyl ethers (PBDEs; major components, BDE-47 and BDE-153) were distributed at higher levels in mothers from Okinawa (mean, 2.1 ng/g lipid), while hexabromobenzene (HeBB) and its metabolite 1,2,4,5-tetrabromobenzene were more abundantly detected in mothers from Hokkaido (0.86 and 2.6 ng/g lipid), suggesting that there are regional differences in their exposure in Japan. We also detected naturally produced brominated compounds, one of which was identified as 2′-methoxy-2,3′,4,5′-tetrabromodiphenyl ether (2′-MeO-BDE68) at higher levels in mothers from Okinawa (0.39 ng/g lipid), while the other was identified as 3,3′,4,4′-tetrabromo-5,5′-dichloro-2,2′-dimethyl-1,1′-bipyrrole in mothers from Hokkaido (0.45 ng/g lipid). The regional variation may be caused by source differences, i.e. southern seafood for MeO-PBDEs and northern biota for halogenated bipyrroles in the Japanese coastal water.

1. Introduction

Persistent organic pollutants (POPs) are biomagnified in the food chain (Borgå et al., 2001). Irrespective of the nature of their source, they are widespread and probably undergo extensive transport and fates that are governed by their physicochemical properties such as vapor pressure, aqueous solubility, Henry's Law constant and octanol/water partition coefficient (K_{OW}) (Hackenberg et al., 2003; Tittlemier et al., 2004; Vetter et al., 2004). As a result, their residues accumulate in the human body by way of dietary intake or inhalation throughout a person's lifetime. Therefore, regular monitoring of POP contamination in human milk can help to identify specific sources of pollutants, exposure trends and potential risks of exposure to mothers and infants.

It seems likely that bioaccumulative brominated flame retardants (BFRs), such as polybrominated diphenyl ethers (PBDEs), hexabromocyclododecane and hexabromobenzene (HeBB) are globally spreading throughout the marine biosphere. Some of these compounds have been reported to transfer via the placenta and breast milk from mothers to offspring in humans and exhibit endocrine-disrupting effects (Kawashiro et al., 2008) or developmental neurotoxic effects (Costa and Giordano, 2007). In Japan, PBDEs have been used to prevent combustion in consumer products, such as electronics, construction materials and textiles (Ueno et al., 2004), but have leveled off in recent years after voluntary phasing out of penta- and octa-PBDE formulations in the 1990s (Ueno et al., 2010). The residue levels of PBDEs have recently been reported in human milk (Eslami et al., 2006; Haraguchi et al., 2009c) and blood (Kawashiro et al., 2008) as well as in seafood from Japanese coastal water (Ueno et al., 2004). The sources are probably house dust and/or electric waste (Fromme et al., 2009; Thomsen et al., 2010) as well as seafood (Ueno et al., 2004). Although the temporal trends in human exposure to PBDEs are steadily decreasing in Japan, the current status of BFR use seems to differ from region to region and from country to country (Watanabe and Sakai, 2003). Similar to PBDEs, HeBB has been used as an additive flame retardant for paper, plastic and electronic goods and is still used at low volumes in Japan (350 tons per year between 1994 and 2001) (Watanabe and Sakai, 2003). Thus far, the levels of HeBB in adipose tissues of Japanese people have been reported (Yamaguchi et al., 1988), but no recent trends for HeBB levels in breast milk are available.

Regarding related organobromine residues, methoxylated PBDEs (MeO-PBDEs) and halogenated bipyrroles of natural origin have been found in biota from Japanese coastal water (Haraguchi et al., 2009b; Marsh et al., 2005). MeO-PBDEs can biomagnify in higher-trophic

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organisms via the food chain from the Pacific Ocean (Haraguchi et al., 2010; Vetter et al., 2009). A series of mixed halogenated bipyrroles, i.e. 1,1′-dimethyl-3,3′,4,4′-tetrabromo-5,5′-dichloro-2,2′-bipyrrole (DBP-Br_4Cl_2) and 2,3,3′,4,4′,5,5′-heptachloro-1′-methyl-1,2′-bipyrrole (MBP-Cl_7), have also been found to biomagnify at higher-trophic levels via the food chain to similar extents to recalcitrant POPs. In fact, these two bipyrroles have been found in fish, seabirds and marine mammals from the North Pacific (Gribble et al., 1999; Tittlemier et al., 2002; Tittlemier, 2004) and Oceania (Vetter et al., 2001, 2009), owing to their similar physical properties to PBDEs (Hackenberg et al., 2003; Tittlemier et al. 2004; Vetter et al., 2004). Therefore, human exposure to these brominated compounds is of concern for the health of mothers and infants, because DBP-Br_4Cl_2, for example, has displayed some *in vitro* dioxin-like ability (Tittlemier et al., 2003). However, the regional trends in the contamination status of MeO-PBDEs and halogenated bipyrroles in human breast milk are poorly understood.

The aim of this study was to investigate the trends and sources of anthropogenic PBDEs and HeBB, as well as naturally occurring MeO-PBDEs and halogenated bipyrroles, in human breast milk from Japan. To investigate the regional trends in these brominated contaminants, we selected human milk samples from the most northeast area (Hokkaido) and the most southwest area (Okinawa) of Japan (Fig. 1).

2. Materials and methods

2.1. Sample collection

Human milk samples were obtained from the Kyoto University Human Specimen Bank using a standardized protocol (Koizumi et al., 2005, 2009). A total of 40 samples were collected during 2005–2006 from volunteers living in Hokkaido ($n = 20$) and Okinawa ($n = 20$) as shown in Table 1. Milk samples (30–50 mL) were collected manually during breastfeeding at 4–8 weeks after childbirth, either by the subjects themselves or with the assistance of midwifes. The breast milk was kept frozen (−20 °C) prior to analysis. The Ethics Committee of Kyoto University approved the protocol of the present study (E25) and appropriate written informed consent was obtained from all the participants.

Table 1
Information regarding the participants and lipid contents of milk samples from Hokkaido and Okinawa.

Region	Location		Year	n	Mean age	Lipid (%)
	Latitude	Longitude				
Hokkaido	42–90′N	140–99′E	2005	20	30.5	2.30
Okinawa	26–20′N	127–69′E	2005–2006	20	30.3	2.63
All				40	30.4	2.45

2.2. Chemicals

Two standards, 4′-methoxy-2,3′,4,5′,6-pentachlorodiphenyl ether (4′-MeO-BDE121), as an internal standard for the determination of all brominated contaminants, and 2,2′-dimethoxy-3,3′,4,4′-tetrabromobiphenyl (2,2′-diMeO-BB80) were donated by Dr. G. Marsh (Stockholm University). Native BDE-28, BDE-47, BDE-99, BDE-100, BDE-153, BDE-154, hexabromobenzene (HeBB), 1,2,4,5-tetrabromobenzene (TeBB), 2′-hydroxy-2,3′,4,5′-tetrabromodiphenyl ether (2′-MeO-BDE68) and 6-methoxy-2,2′,4,4′-tetrabromodiphenyl ether (6-MeO-BDE-47) were purchased from Cambridge Isotope Laboratories (Andover, MA, USA). Two bipyrrole standards, 1,1′-dimethyl-3,3′,4,4′-tetrabromo-5,5′-dichloro-2,2′-bipyrrole (DBP-Br_4Cl_2) and 2,3,3′,4,4′,5,5′-heptachloro-1′-methyl-1,2′-bipyrrole (MBP-Cl_7), were synthesized according to the methods outlined in Gribble et al. (1999) and Wu et al. (2002), respectively. The purities of the compounds were >99% by gas chromatography. The standards were used for the calibration, recovery and quantification of target compounds. All solvents of pesticide grade quality were purchased

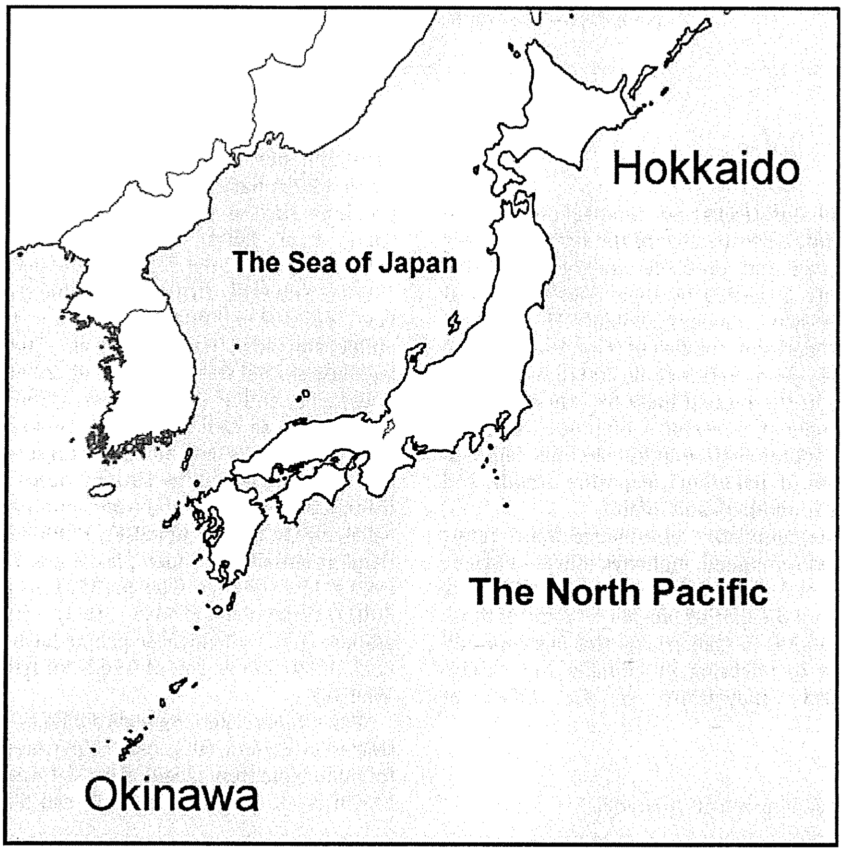

Fig. 1. Sampling sites of breast milk in Japan (Hokkaido and Okinawa Prefecture).