ENVIRONMENTAL SPECIMEN BANK AT THE NATIONAL INSTITUTE FOR ENVIRONMENTAL STUDIES, JAPAN; ESB AS AN EFFECTIVE TOOL TO SUPPLMENT ENVIRONMENTAL MONITORING AND ASSESSMENT

Shibata Y[1], Tanaka A[1], Horiguchi T[1], Hashimoto S[1], Takazawa Y[1], Mukai H[1], Yoshikane M[1], Hirota M[2], Kanda Y[1], Komori S[1], Kobayashi M[1], Uehiro T[1]
[1]National Institute for Environmental Studies, Tsukuba, Ibaraki 305-8506 Japan; [2]Present address; Paleo Labo Co Ltd, Tokai Branch, Hashima, Gifu 501-6264, Japan

Abstract
After two decades of operation, the environmental specimen bank (ESB)[1] at the National Institute for Environmental Studies (NIES), Japan, up-dated and reorganized recently to be a part of "Environmental Time Capsule Program" which covers collection and long-term preservation of both environmental samples and cells/tissues of endangered species. Newly constructed banking facility includes 19 liquid nitrogen-cooled storage tanks (550 little each), 10 freezers operating at -80 C, and two 68 m^2 walk-in freezers operating at -60 C, and the collected environmental samples include bivalves (mussels and oysters) along coastline of Japan, fish (sting ray) livers and sediments collected in Tokyo Bay, filters and adsorbents for atmospheric pollutants collected at a remote station, and human breast milk. Part of other environmental samples, including fishes, birds, bivalves, sediments and foods, collected at various places in Japan by the Ministry of the Environment, Japan, for their regular environmental POPs monitoring[2] (reported in "Chemicals in the Environment" every year) have been stored, too, for realizing future retrospective analysis. Aim and design of the current ESB as well as some of the monitoring data are presented.

Introduction
The idea of Environmental Specimen Bank (ESB) as an effective supplemental activity for regular environmental monitoring by storing part of the collected samples for future retrospective analysis had attracted attention in 1960's to early 1970's, and was realized in several countries, including Sweden, Canada, USA, Germany and Japan.[3] In Japan, a pilot ESB, including -20 C cold room as the main storing facility, constructed in FY 1979 at the National Institute for Environmental Studies (NIES), and the collection of environmental samples as well as researches on preservation techniques etc started in 1980 as part of Special Research Program on the method of long-term environmental monitoring for chemical pollution.[4] Since then collection of a variety of environmental samples has been conducted under collaboration with several environmental monitoring programs conducted within the institute. Part of the samples stored in this ESB were collected by other agencies / institutes, such as fishes, birds, bivalves, sediments and foods collected by the regular environmental monitoring conducted by the Environment Agency (now Ministry of the Environment), Japan, and bivalves / birds in Tokyo Bay collected by Tokyo Metropolitan Institute of Health. Retrospective analysis of these samples have been conducted occasionally; for example, the effects of regulation of organotin usage for antifouling agents could be assessed by the analysis of their levels in bivalves and birds in Tokyo Bay using highly sensitive GC/AED technique,[5] and residual pollution levels after oil pollution by the tanker accident could be assessed properly by the comparison of PAHs levels in bivalves collected in the affected area with those collected and stored in the ESB before the accident.[5]
After two decade of operation, the ESB program at NIES was up-dated and reorganized with the construction of a new facility, Environmental Time Capsule building, whose long-term sample storage capacity was strengthened by liquid nitrogen-cooled storage tanks and -60 C walk-in freezers. Sample collection and processing protocol was also up-dated in order to utilize potential of these storage capacities at maximum level. Here we report the outline and characteristics of the protocol, and some analytical results of stored samples.

Materials and Methods
Monitoring of coastal environment along coastline of Japan, which was supposed to be affected by the human activity most strongly, is the primary target of the up-dated ESB activity at NIES. For this we collected bivalves, such as mussels *Mytilus galloprovincialis*, *Septifer virgatus* and *Perna viridis*, and oysters Sacco*strea mordax* in several tens of sampling locations set along the coastline of Japan (Figure 1), and fishes and sediments

at twenty sampling locations set in Tokyo Bay (Figure 2), a semi-enclosed bay along the most densely populated Metropolitan area in Japan. In many places, bivalve samples were de-shelled and frozen by liquid nitrogen on site immediately after the sampling, and were kept frozen through transport, temporal storage, homogenization and sub-sampling procedures for long-term storage in order to avoid decomposition / reaction of even unstable pollutants as well as to preserve biomarkers, such as induced genes / proteins by the exposure to particular types of pollutants and DNA / protein adducts and metabolites. Part of bivalve samples were frozen and stored per se for future morphological as well as localization studies. As for fish in Tokyo Bay, we counted and weighed all fishes, crustaceans, mollusks species collected in each location in each season, by the trawling net under fixed condition, and selected stingray liver as target stored samples because of their higher trophic level as well as dominance in the bay. The collected fishes were sent to the institute and dissected within a few hours after the sampling, and liver was frozen by liquid nitrogen, stored and processed in the same manner as bivalves.

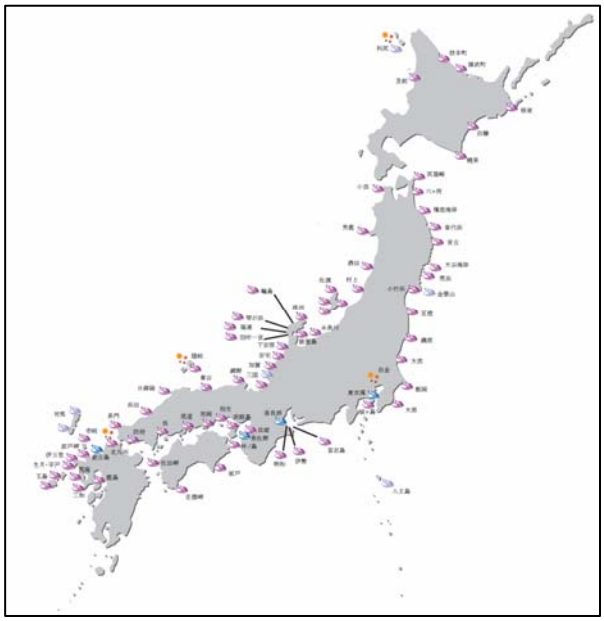

Figure 1 Sampling locations of bivalve along coastline of Japan

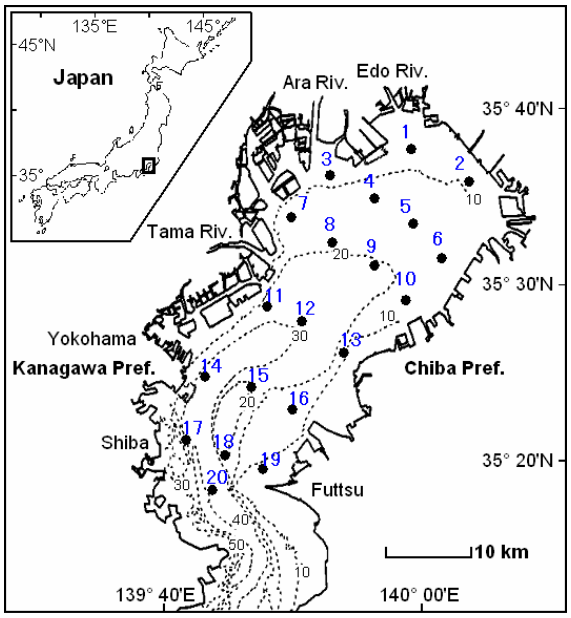

Figure 2 Sampling sites of fishes and sediments in Tokyo Bay

Purified, deionized water was exposed to the ambient air during sample processing at sampling site / in the institute. In addition, purified water was cryo-homogenized in the same manner as the biological samples. These water samples were kept frozen for future in order to record contamination status during the sample processing. Some pollutants, such as plastic additives, fluorinated surfactants and heavy metals, in the water samples were analyzed regularly in order to check and minimize the contamination levels.

Cryo-homogenization of biological samples were conducted in stepwise manner by coarse fracturing with air-driven (or manual) hummer mill made of titanium followed by fine fracturing with a planetary ball mill made of either zirconium oxide or titanium. Powder sizes were checked by a laser scattering particle size analyzer (LA-300, Horiba Co. Ltd, Japan) after cryo-homogenization. Acceptable criteria of the median size is less than 100 micrometers in diameter with unimodal normal distribution; in fact, typical median sizes were less than 50 micrometers. The homogeneity of the powder was finally checked by the elemental analysis.

Results and Discussion

Regular sampling, homogenization and analysis

Cryo-homogenization has been developed as a most appropriate homogenization method of biological samples for long-term storage for future chemical analysis. While ESB activity in US adopted a Teflon-covered mill for minimizing metal contamination during sample processing[6], German ESB adopted a different approach by using

a rod-mill made of titanium for cryo-powdering the samples to minimize contamination by organic pollutants[7]. We also adopted the latter approach to minimize contamination by organic pollutants. In our program, bivalve samples were de-shelled on site and the soft tissues were frozen as soon as possible in order to preserve induction status of genes / proteins so that biomarker analysis will be conducted successfully in future. This approach, on the other hand, is not idealistic for elemental analysis, for sediment particles, which will mask the true elemental composition of soft tissues, will remain in the gastrointestinal tracts. Thus we concentrated on the elimination of organic pollutants rather than elemental compositions.

By the careful examination of several different cryo-homogenization systems, we have developed an independent approach by the combination of a stump mill for coarse fracturing and a planetary ball mill for fine fracturing, both of which are made of either metal or ceramics in order to minimize contamination by the organic pollutants. A typical particle size distribution of bivalve tissues after cryo-homogenization by the above system is shown in Figure 3. By using this system, we checked the pollution status during sample homogenization by cryo-homogenizing frozen pure water (Milli-Q, Millipore Co. Ltd.). As a result, we have experienced difficulties to control contamination of plastic additives / perfluorosurfactants during sample processing, and thus extensive efforts have been paid to eliminate / decrease addition of contamination during cryo-homogenization of the samples. We have found and eliminated their use of, for example, a highly contaminated polyethylene balance tray by nonylphenol, clean shoes contaminated by PFOA, a water-proof apron highly contaminated with bisphenol A, and several experimental items contaminated with nonylphenol, all of which were thought to cause uncontrollable contamination of samples by these chemicals during pretreatment for the long-term preservation.

As for environmental monitoring, we put priorities to monitor coastal environment along coastline of Japan, and collected bivalves, i.e., blue mussel, green mussel, and oysters (Figure 2). Every year we collected bivalve samples at 10 fixed sampling sites, i.e., four densely populated areas (Tokyo Bay, Ise Bay, Osaka Bay and Hakata Bay), six background areas (3 at Japan sea side and 3 at Pacific side), while 10 to 15 additional sampling sites will be selected in other places; which will be re-visited several years later in order to cover the Japanese coastline as widely as possible.

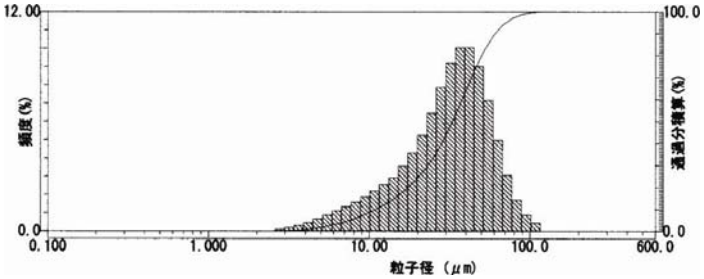

Figure 3 Size distribution of cryo-homogenized bivalve tissues analyzed by a laser particle size analyzer Distribution maximum is around 30 micron meters in diameter.

For the monitoring purposes, we have been analyzing POPs pesticides, perfluorosurfactants, and elemental compositions. The development of the analytical methods, and detailed description of the data have been or will be presented separately; in brief, we have found several “hot-spots” of perfluorosurfactants levels along the coastline of Japan where either a factory producing perfluorochemicals or textile industries utilizing these chemicals may contribute to the levels.[8,9]

Analysis of samples killed by the accidents

Occasionally samples of wildlife killed by some accidents were sent to us in order to identify the causes. In an endangered bird species, Japanese crane, mortality in Dec 2002, it was found to be an organophosphorus pesticide, fenthion, pollution.[10] Although the reason of mass mortality of Rhinoceros Auklet occurred in 1999 could not be clarified, their dead bodies gave us valuable opportunities to reveal pollution status of wildlife species living along the Japanese coastline. As an example, the results of dioxin analysis in the Rhinoceros Auklet are summarized in Table 1 together with the results of dioxin analysis by the MOE (Table 2).

Table 1 Dioxins concentrations in liver (pg/g wet tissue TEQ : WHO TEF(1997))

Sample	PCDDs	PCDFs	co-PCBs	total
Teuri mix	12	29	61	102
Nemuro mix	23	14	31	68

Table 2 Dioxins concentrations in birds (reported by the Ministry of the Environment)

Bird	Place	Year	Tissue	Lipid content	total Dxns min～max（Average）
Pigeon	Tokyo	1998	Muscle+liver	5.3%	0.4～1.3（0.79）
Kite	Kanagawa	1998	Muscle	6.9%	9.4～390（120）
Kite	Hokkaido	1999	Muscle	5.0%	3.8～12（8.3）
Kite	Tokyo	1999	Muscle	7.0%	8.2～110（55）
Raptors	Japan	1998	Liver	10.4%	14～530（158）
Cormorant	Aichi	1999	Muscle	4.3%	24～300（110）
Cormorant	Shiga	1999	Muscle	3.7%	7.3～210（87）
Cormarant	Tokyo	1999	Muscle	4.4%	54～370（190）

As shown in the Tables, dioxin levels in the Rhinoceros Auklet, which live in remote, off-shore region, were fairly high, comparable to the fish-eating birds, such as cormorant and kite, which live in coastal area and thus are expected to be affected by the human activity strongly. The levels do not seem to cause mass mortality of the birds directly, but such a level of dioxins might affect the metabolism of the birds through the interference / disruption of thyroid hormone action and thus might cause decreases of their physical strength and eventually cause their death due to hard work during breeding season.

In conclusion, ESB has been conducted at NIES for more than a quarter of the century as an efficient supplemental activity for the regular environmental monitoring. After construction of the new facility, the program has been up-dated to the second generation with capability to preserve not only pollutants but also the exposure biomarkers for future. It should be kept in mind, however, that the priority should be put on monitoring in order to find danger and present warning sign as soon as possible, and that development of more comprehensive and systematic monitoring approach will be needed.

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