NIMD Annual Report 2018

（April 2018 to March 2019）

National Institute for Minamata Disease

Ministry of the Environment

Japan

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Report on Research and Other Activities in the Fiscal Year 2018

1. Pathomechanism Group

The aim of the study group is to understand the molecular mechanisms underlying methylmercury (MeHg) toxicity in humans. Our goals are as follows to: 1. understand the initial signs of MeHg poisoning; 2. evaluate MeHg toxicity; 3. develop methods protecting against MeHg-induced disorders; and 4. develop new treatment options for MeHg poisoning. In this study, we used techniques from the field of biochemistry, molecular biology, and pathology in cell cultures and in animal models to determine the differences in MeHg sensitivity of various organs and individuals.

This group conducted the following researches during the fiscal year 2018.

[Research theme and summary]

(1) Fundamental research on prevention and treatment of methylmercury toxicity (Project research)

Masatake Fujimura

(Department of Basic Medical Sciences)

We found that Fasudil, a ROCK inhibitor, reversed MeHg-induced neurological symptoms and axonal degeneration in dorsal spinal root nerves in rats by directing microglia phenotype change from the pro-inflammation to the anti-inflammation type. Furthermore, vibration stimuli on the plantar surface of a rat hind limb were effective in an immobilization-induced muscle atrophy model by activating the mechano growth factor (MGF) and insulin-like growth factor-1 (IGF-1) pathways. We also determined that anti-oxidative drugs prevented MeHg-induced neuronal cell death by suppression of neuronal hyperactivation in differentiated SH-SY5Y cells.

This year, we presented our findings at one conference, and published two peer-reviewed articles.

(2) Research on selective cytotoxicity and sensitivity of individuals toward methylmercury (Fundamental research)

Masatake Fujimura

(Department of Basic Medical Sciences)

We determined that MeHg exposure during pregnancy decreased synaptic vesicle proteins by synaptic pruning in rat cerebellum. Next, we analyzed the relationship between blood oxidation indexes and neurobehavior (hind-limb crossing) in MeHg-intoxicated rats. Thiol antioxidative ability in blood was identified as a potential MeHg toxicity biomarker. This result suggests that thiol antioxidative ability is an important factor underlying individual sensitivity differences to MeHg.

This year, we presented our findings at four conferences, and published two peer-reviewed articles.

(3) Study on changes in gene expression induced by methylmercury exposure, their effects on pathological conditions, and protection against toxicity (Fundamental research)

Fusako Usuki

(Research Adviser)

We investigated the behavior of nonsense -mediated mRNA decay (NMD), a cellular mechanism that eliminates mRNAs harboring premature translation termination codons (PTCs) under environmental stresses (MeHg-induced oxidative stress and

endoplasmic reticulum (ER) stress). NMD suppression, evidenced by upregulation of NMD-sensitive mRNAs and decrease in UPF1 phosphorylation, was observed in MeHg-treated cultured cells. To confirm that the upregulation of NMD-sensitive mRNA was not caused by transcriptional change, but rather by NMD suppression, we investigated the effects of NMD effectors depletion on NMD-sensitive *heterogenous nuclear ribonucleoprotein L (Hnrnpl_NMD)* and NMD-non-sensitive *Hnrnpl* isoform mRNA expression during stress. The results indicated that the observed NMD-sensitive isoform mRNA upregulation is not a transcriptional effect, but rather represents true NMD suppression. Our data indicate that stress-induced NMD suppression has the potential to affect cellular conditions under environmental stresses by stabilizing NMD-targeted gene expression. Thus, NMD activity is an important factor determining individual susceptibility to environmental stresses. The findings were published as a research article in a peer-reviewed English language journal (Usuki et al., *Scientific Reports*, 9: 1279).

To study potential epigenetic implications of MeHg toxicity, we investigated the effect of sex-determining region Y (Sry) gene abnormal expression on birth rate decrease of male in 8 ppm MeHg-exposed rats. However, no female rats had Sry gene, indicating that MeHg-induced decrease in birth rate of male was not caused by abnormal epigenetic regulation of the Sry gene during the fetal period.

(4) Study on the modifying factors in the toxicity of methylmercury (Fundamental research)

Masaaki Nagano
(Department of Basic Medical Science)

In this study, we investigated the effect of three modifying factors (wheat bran (Bran), fructooligosaccharide (FOS), and glucomannan) in the toxicity of MeHg. We had previously demonstrated that daily intake of bran or FOS significantly reduces tissue Hg levels after single administration of MeHg in mice.

In this past year, we examined the effect of bran and FOS on tissue Hg concentration after continuous administration of MeHg in mice. At the end of the experiment, the bran-fed group significantly reduced total Hg levels in blood, brain, liver and kidney compared to the control group. On the other hand, the FOS-fed group showed lower total Hg levels in blood and liver than the control group. These results suggest that daily intake of bran or FOS may reduce tissue Hg levels after MeHg exposure in animals and human.

(5) Research on selective cytotoxicity of methylmercury focusing on sulfur nucleophiles (Fundamental research)

Takamitsu Unoki
(Department of Basic Medical Sciences)

MeHg can covalently modify cellular proteins, causing their dysfunction and thereby exerting deleterious effects. Recent findings indicate that reactive sulfur species (RSS), which are highly nucleophilic molecules, easily react with MeHg to form bismethylmercury sulfide, a less toxic sulfur adduct than MeHg. Metabolomic analyses revealed that CysSSH and H_2S, two types of RSS, increase in an age-dependent manner in rat brain. These results support the contention that embryo and juvenile rats are more sensitive to MeHg toxicity than adult rats, due to low RSS levels during the early stages of development.

(6) Research on the mechanism of the late stage of methylmercury neurotoxicity (Fundamental research)

Akio Sumioka
(Department of Basic Medical Sciences)

Methylmercury (MeHg) exposure induces oxidative stress in the brain and consequently causes neurodegeneration. Elucidation of the neurodegeneration process will help understand the molecular mechanism of brain region-specific MeHg sensitivity and improve therapeutic options. Therefore, we investigate the mechanism of late stage MeHg neurotoxicity.

To monitor the process of MeHg toxicity, we develop a sensor vector, which quickly responds to MeHg. Deficient selenocysteine (SeC) insertion during selenoprotein translation mediated by MeHg exposure is utilized for sensor vector development by using genetic engineering. During this year, an expression vector for Thioredoxin reductase (TrxR), which is reportedly impaired by MeHg, was constructed. By transfecting TrxR in cultured cells, it was confirmed that MeHg exposure regulates the expression of full-length and partial-length TrxR.

Oxidative stress is a significant risk factor for excitotoxicity. We investigate excitatory synapses dysfunction caused by MeHg exposure to understand the molecular mechanism of MeHg toxicity. During this year, we introduced a mouse experiment and initiated the administration of MeHg to mice for a preliminary study. Additionally, a method was established to biologically fractionate synaptosomes from mouse brain to monitor receptors at the excitatory synapse.

Furthermore, we focus on Tau pathology, for which oxidative stress is a major risk factor. Phosphorylation and aggregation of Tau by MeHg is examined to understand the molecular mechanism of MeHg toxicity. During this year, we introduced and bred transgenic mice, which show Tau pathology, and started administration of MeHg to the mice for a preliminary experiment. Further, we established a platform for validation of Tau phosphorylation and aggregation and reported the results at MBSJ 2018.

2. Clinical Group

Research

Minamata disease (MD) is caused by severe Hg poisoning. The incidence of MD comorbidities, such as cervical spondylosis or metabolic syndrome, tends to increase with age, rendering the MD diagnosis difficult, as it relies exclusively on clinical neurological symptoms. Therefore, the development of objective methods to accurately identify MeHg poisoning is necessary. Furthermore, effective therapies are needed for spasticity, dystonia (involuntary movements), and intractable chronic pain, as they affect the quality of life (QOL) of patients with MD.

Our research group conducted experiments to evaluate the neurological function of patients with MD, using magnetoencephalography (MEG) and magnetic resonance imaging (MRI). To develop an effective therapy for MD, we performed repetitive transcranial magnetic stimulation (rTMS) therapy for ataxia, pain, numbness, and tremor. Furthermore, we have continued botulinum toxin therapy for treating spasticity in patients with MD.

The research conducted by our group during FY 2018 is outlined below:

[Research theme and summary]

(1) Research on methylmercury exposure health effects in humans and therapeutic development

(Project research)

Masaaki Nakamura

(Department of Clinical Medicine)

To develop an objective evaluation protocol using MEG and MRI, we compared somatosensory evoked fields (SEF) and morphological features in patients with MD and control subjects from the Kumamoto district (not polluted with MeHg). We quantified SEF from the points of view of N20m amplitude, N20m latency stability, N20m and P35m direction, and somatic sensory area frequency after removing noise from MEG raw data using the latest denoise script. Abnormalities in these parameters were more frequent in patients with certified MD than in control subjects.

We also analyzed fractional anisotropy (FA) values by using a tract-based spatial statistics (TBSS) technique in 72 control subjects and 12 patients with MD. White matter changes in specific human brain regions, including the cerebellum, calcarine area, thalamus, corpus callosum, and the occipital and parietal white matter were significantly more frequently observed in patients with MD.

We observed improvements in ataxia, pain, numbness, and tremor in patients with MD treated with rTMS. We propose that the evaluation of M1 excitability using rs-fMRI can be effective to determine the optimal TMS stimulation conditions for cerebellar ataxia. We also continued botulinum toxin therapy for spasticity in fetal-type patients with MD.

Activities

In recent years, political measures have been undertaken to facilitate the rehabilitation of patients with MD. The Department of Clinical Medicine actively organizes events on MD, in cooperation with related organizations. In addition, we organized conventional rehabilitation activities, including daycare facilities for fetal-type patients with MD, rehabilitation technical schools, and care technical

schools. We also examined the usefulness of vibration therapy for pain relief and for spasticity associated with various chronic diseases of the nervous system, including MD. Furthermore, we introduced a robot suit, HAL (Hybrid Assistive Limbs), which assists patients during gait training.

To examine the effectiveness of home care support for patients with MD and their families, we conducted a project titled "Home support model study, including care prevention" (FY 2006–2008). Subsequently, the projects "Community development project for home care support, including healthcare practice" (FY 2009–2011) and "Community welfare promotion business for supporting MD victims" (FY 2012) were carried out to develop strategies for application of these concepts in the community. After the completion of these projects, we have continued supporting welfare activities in the cities of Minamata and Izumi.

Since pathological tissue specimens of MD autopsy cases are extremely valuable, we plan to digitize the pathological slides for permanent preservation and to publish digitized pathological slides on the website for students and doctors training in pathology.

Additionally, we started to examine the difference in moment-to-moment changes in activities of daily living (ADL) by the clinical entities of MD, to evaluate ADL in chronic MD objectively.

The following section includes an outline of the clinical practice conducted by this group in FY 2018:

[Activities theme and summary]

(1) Rehabilitation programs for patients with Minamata disease and dissemination of information on care and rehabilitation

Atsushi Nakamura

(Department of Clinical Medicine)

We continued to provide outpatients with rehabilitation, in the form of daycare, two days a week. To improve their quality of life (QOL), we conducted vibration stimuli, repetitive facilitation therapy, gait training with HAL, and repetitive peripheral magnetic stimulation. The combined vibration stimuli, repetitive facilitation therapy, repetitive peripheral magnetic stimulation, and gait training with HAL were effective for a patient with fetal-type MD. The manuscript on a case with FMD whose gait movements were improved by an introduction of HAL was accepted in a peer-reviewed rehabilitation journal. We conducted a survey on daily life performance and psychological factors in 10 patients with fetal or infantile type MD.

Annual workshops on rehabilitation and assistance techniques are held in order to improve techniques used by specialized regional staff for patients. This year, the themes of organized workshops included "Neural mechanisms of posture and walking and neurorehabilitation" as a rehabilitation technique and "How to choose and how to choose welfare tools" as a care technique. The questionnaire received from the attendees suggested that the two workshops had been well received. We believe that a workshop is a useful outreach forum to share information on care and rehabilitation techniques that can be implemented in the community.

(2) Community development project for home care support, including health care practice

Masaaki Nakamura

(Department of Clinical Medicine)

We carried out a study titled "Home support model study, including care prevention" (FY 2006–2008) to identify support methods, including rehabilitation,

prompting improvement of ADL for aging MD patients and their families. Following this project, we undertook two other projects titled “Community development project for home care support, including healthcare practice” (FY 2009–2011) and “Community welfare promotion business for supporting Minamata disease victims” (FY 2012), to develop methods to implement these concepts in the community. Eventually, we continued implementing educational activities in the cities of Minamata and Izumi, to improve occupational therapy opportunities. Through this support, we strengthened the connection between our institute and the local community.

When we improved the program of the crafts classroom based on a questionnaire, we received high satisfaction levels from the participants in a survey. We also examined the cognitive function in the older persons of Minamata city using the Odor Stick Identification Test for Japanese.

Furthermore, we publicized the work of the crafts classroom using the homepage and Facebook page of NIMD.

(3) Information dissemination using Minamata disease pathology specimens

Masumi Marumoto
(Department of Basic Medical Sciences)

Our institute has a duty to preserve many pathological tissue specimens of MD, which are extremely valuable, permanently; however, technical difficulties exist due to the fading of pathological tissue slides with time. Therefore, our objectives are to digitize pathological slides for permanent preservation and to publish their digitized versions on the website for students and doctors learning pathology.

(4) Examination of moment-to-moment changes in ADL by the clinical entities of Minamata disease

Liu Xiao Jie
(Department of Clinical Medicine)

Although a rapid decrease in physical activity and ADL occurs in patients with MD, an objective evaluation of ADL has not performed until now. The purpose of this study is to clarify the difference in moment-to-moment change in ADL by the clinical entities of MD using a comparison of ADL in the course of 5 years. During this year, we obtained baseline data for ADL from 21 patients with MD.

3. Exposure and Health Effects Assessment Group

Generally, two subpopulations are susceptible to MeHg exposure: those who are exposed to high Hg levels and those who are more sensitive to its effects. The exposure and health effects assessment group conducts epidemiological surveys in Taiji-cho, Wakayama Prefecture, where the population has been exposed to high MeHg concentrations. The group's research also concerns segments of the population particularly sensitive to MeHg exposure, namely fetuses or people suffering from various diseases, to contribute to appropriate risk assessment of MeHg exposure.

[Research theme and summary]

(1) Effect of glucose metabolism disorders on methylmercury toxicokinetics and toxicity (Fundamental research)

Megumi Yamamoto

(Department of Environment and Public Health)

1) We submitted results obtained up to 2017, including: 1.1) semiquantitative evaluation of neurobehavioral disorder in KK-Ay diabetic model mice associated with MeHg exposure by the Dynamic Weight Bearing (DWB) test; 1.2) detection of brain and peripheral nerve tissue lesions with the macrophage marker CD204; and 1.3) analysis of THg and MeHg in the brain using a simplified MeHg analysis method for biological samples. The research data were published as articles in peer-reviewed journals (Yoshimoto et al., J. Toxicol. Sci. 2016; and J. Appl. Toxicol. 2019 Feb; 39 (2): 221-230).

2) To elucidate the toxicokinetics of MeHg under pathological conditions of glucose metabolism disorders, an experimental study was conducted. MeHg (three doses) was administered to 12-week-old KK-Ay mice and BL/6 normal mice for 4, 7, 11, or 14 days. THg analysis in blood and tissues (brain, kidney, liver, and pancreas) was completed.

3) To elucidate the effects of MeHg in mothers and children with glucose metabolism disorders, research plan applications were approved by the ethical committees of each related organization, and joint research was initiated. In total, 28 samples (hair and blood) were collected, and currently, THg analysis is being conducted in the obtained samples.

(2) Research on the tissue localization of mercury and selenium in the mammals (Fundamental research)

Masumi Marumoto

(Department of Basic Medical Science)

MeHg and Se cannot be visualized with conventional histopathological techniques. MeHg is slowly biotransformed to inorganic Hg in animals and humans exposed to it. Se is a known antagonist of MeHg toxicity; however, the underlying mechanisms are largely unknown and the histopathological distribution of Se has not been clarified. Therefore, the objective of this study is to reveal Hg and Se tissue distribution using an X-ray probe microanalyzer (EPMA).

(3) Health risk assessment of high methylmercury exposure derived from whale (Fundamental research)

Masaaki Nakamura

(Department of Clinical Medicine)

We searched for proteins associated with MeHg toxicity to elucidate the reason why clear adverse effects of MeHg exposure were not seen in Taiji-cho adult inhabitants. We performed two-dimensional gel electrophoresis using plasma samples from four subjects with high Hg levels and four subjects with low Hg levels (two men and two women per group; all inhabitants of Taiji-cho). Using mass spectrometry analysis, we identified 14 proteins with statistically significant differences in the concentration between

high and low Hg level samples. Among them, we focused on three proteins (TTR, adiponectin, and ApoE). In plasma samples from 120 Taiji-cho residents, TTR and adiponectin were positively and negatively correlated with Hg, respectively.

We have been performing a detailed analysis on the survey concerning the effect of MeHg exposure on child development, carried out at the Taiji-cho multi-purpose center from 2012 to 2017.

We also collected 68 umbilical cord samples to examine the sequential changes and late effects of prenatal MeHg exposure in Taiji-cho residents.

(4) Studies on fetal exposure to methylmercury and coexisting elements with mercury (Fundamental research)

Mineshi Sakamoto
(Department of Environment and Public Health)

1) Brain MeHg uptake in fetal, neonate, weanling, and adult rats: risks at different developmental stages

Fetuses and neonates are highly susceptible to MeHg toxicity, but little is known about the relative uptake of MeHg from blood to the developing brain. We hypothesized that the speed and amount of MeHg uptake vary by developmental stage. The objective of the present study was to investigate time-course alterations in MeHg uptake by different organs, especially the brain, at various life stages following a single MeHg injection. We measured time-course changes in Hg concentrations in the brain of fetal, neonate, weanling, and adult rats after administering an injection of 0.08 μg (0.4 nmol) Hg/g MeHg. In the first experiment, MeHg was subcutaneously injected to pregnant dams on embryonic days 17, 18, 18.5, 19, 19.5, or 20, and Hg concentrations in tissues were measured in both mothers and fetuses on embryonic day 21 (1 day before parturition). Brain Hg levels in fetuses peaked 2 days after injection and were approximately 1.5 times higher than those in mothers. In the second experiment, the same MeHg dose was injected subcutaneously to male rats on postnatal days 1 (neonates), 35 (weanlings), or 56 (adults). Hg concentrations in tissues were measured 1, 2, 3, 4, 5, and 6 days after the injection. Brain Hg levels peaked most rapidly in neonates and were approximately 1.5 times higher than levels in weanlings or adults. Peak Hg levels in the brain and the Hg brain/blood ratio 24 h after injection were the highest in fetuses and neonates and decreased with developmental stage. Throughout the prenatal and postnatal experiments, the highest levels in brain Hg were observed in fetuses, followed by levels in neonates. The Hg brain/blood ratio in fetuses was approximately twice the ratio in mothers, followed by the ratio in neonates, and decreased with developmental stage. The present findings suggest that fetuses and newborns are particularly vulnerable to MeHg exposure and uptake by the brain.

2) Hg speciation of preserved historical sludge: risk from sludge trapped under the reclaimed land in Minamata Bay, Japan

A large amount of MeHg was directly discharged into Minamata Bay and resulted in the MD. Then, Hg-rich sludge (exceeding 25 μg/g dry basis) was trapped under reclaimed land of the bay. We conducted Hg speciation of the preserved historical sludge, as well as current sediments, to evaluate potential MeHg pollution risks from sludge leakage. THg dry basis was 0.18 μg/g for the control (n = 1), 6.1 μg/g for the current sediment (0.83–12.2, n=5), and 241 μg/g for the preserved sludge (22.4–3620, n=4); MeHg concentrations were 0.41%, 0.12% (0.051–0.21), and 0.031% (0.014–0.049), respectively. For all samples, the MeHg% decreased exponentially with increase in THg. The extractability of THg into seawater was much lower than that of MeHg. The main chemical form of Hg in the preserved sludge was confirmed to be stable β-Hg sulfide via X-ray absorption fine structure (XAFS) analysis and transmission electron microscopy linked with energy-dispersive X-ray

spectroscopy (TEM-EDX). Results suggest that, although THg is extremely high in the preserved sludge, the risk of MeHg pollution due to accidental leakage from the reclaimed land into Minamata Bay is low.

(5) Total mercury, methylmercury, and nutrients in edible deep-sea fish. (Fundamental research)

Kimiko Izumo
(Department of Environment and Public Health)

Deep-sea fish have attracted attention recently as new foodstuffs in Japan. Several deep-sea fishes have a relatively high Hg concentration. However, fishes are also a source of nutrients, such as Se and DHA, which protect against MeHg toxicity. To determine both the risks and the benefits of fish consumption, we measured THg, MeHg, Se, and ω-3 polyunsaturated fatty acids in deep-sea fishes, in which existing data are scarce. In addition, we examined the usefulness of gills, which are normally not considered food and can be collected without damaging the fish meat, to estimate MeHg concentration in the edible parts of fish.

This year, we determined the relationship between THg concentration in the muscle and in the gill in Marbled rockfish (*Sebastiscus marmoratus*); the gill was used as a control. THgrockindividual. THg concentration in the gill showed a strong correlation with THg concentration in the muscle of the same individual. This result suggests that, at least in the Marbled rockfish that is a kind of demersal fish, it is possible to estimate Hg concentration in the edible part by measuring THg concentration in the gill. Next year, we plan to measure THg, MeHg, Se, and ω-3 polyunsaturated fatty acids and to determine concentration relationships between tissues in deep-sea fishes.

4. Social and Information Service Group

The Social and Information Service Group, Minamata Disease Archives, along with the Minamata Disease Municipal Museum, and the Environmental Education and Intelligence Center have been focusing on disseminating information and educational activities to deepen the understanding of MD and of environmental problems.

In addition, based on an agreement for Comprehensive Cooperation with Minamata City (2015), we submitted a policy proposal book to Minamata city. (No. 1703281 official document of NIMD dated on March 28, 2017)

We have been engaged in activities to enhance our presence as a research institute. We will continue to maintain the cooperative relationship with Minamata City even after fiscal year 2019 and aim to conduct research that contributes to the region.

Further, we have been involved in organizing the MD-related documents stored by our center, in carrying out historical research, and in transmitting risk information, including regarding health effects, after scientific verification. Through these activities, we aim to return profit to the society.

The outline of each research project and activity of our group in fiscal year 2018 is as follows.

[Research theme and summary]

(1) Sociological Study on the Social Environment of Patients with Fetal-Type Minamata Disease (Fundamental research)

Rie Harada

(Department of International Affairs and Research)

Minamata Disease Archives interviewed patients with MD, their families, caregivers, medical staff, supporters, etc. from fiscal 2003 to 2014. From the data of about 90 cases, we will extract information about patients with fetal-type MD, analyze and describe the actual conditions of social environment, medical care, nursing care, and social life around them.

So far, papers on medical research and test results concerning patients with fetal-type MD, and works drawn by supporters, journalists, writers, etc., have been published. However, few insights on the inner world of patients with fetal-type MD are available from the perspective of the patients themselves.

Therefore, we will analyze the data from the viewpoint of fetal patients as much as possible, and we will focus on their wishes, goals, and the kind of life they want to live. If patients are not able to achieve the desired quality of life, we will search for the inhibiting factors. We will summarize our findings as case studies using a sociological perspective and auxiliary documents.

(2) Epidemiological studies on methylmercury exposure and associated health risks (Fundamental research)

Akane Anai

(Department of International affairs and Research)

In this study, previously obtained data were used to examine “simple methods for estimating methylmercury exposure” and “long-term health risks and health effects due to mercury exposure”. Data were obtained from 481 participants in 2017 and they were analyzed with a “simple method for estimating methylmercury exposure”. There was a significant correlation between total hair Hg and MeHg amount

estimated from questionnaires of seafood intake frequency. This suggested that the questionnaire can be used to estimate MeHg exposure. Furthermore, we have proceeded with data analysis of residents' health survey conducted by Minamata City in the 1970's and with examination of questionnaires about health status changes in elderly people in Minamata area.

[Activities theme and summary]

(1) Transmission of information on Minamata disease, and organization of documents and materials in the "Minamata Disease Archives"

Rie Harada

(Department of International Affairs and Research)

In the exhibition facility, we updated the projector on the ground floor Bardsview space. In addition, a brochure case was installed at the entrance, and an information counter was installed in the open space on the second floor.

Regarding exhibition planning, we displayed exhibition panels of the Network of museums for pollution studies in the lobby on the second floor (October). The theme was "Why do we learn from pollution now?" In small exhibition room on the first floor, the exhibition on the Minamata Convention on Mercury has been continued since last year.

The auditorium is not only sponsored by the NIMD but is also used for lectures and environmental studies by other organizations throughout the year.

As an information dispatch, we held a lobby exhibition in Kumamoto City jointly with the Minamata Disease Municipal Museum, and the Environmental Education and Intelligence Center, and introduced our research (August-September).

As executive committee member, we organized two subcommittees for the 6th Pollution Museum Joint Forum in Tokyo, which was held in December. Furthermore, we participated in field surveys conducted in July and November.

We took part in the Minamata Convention on Mercury Commemoration Forum 2018-19 held in Kumamoto City in February and exhibited at the venue.

In regard to document maintenance, we continued to work on cataloging MD-related documents, digitizing documents, and releasing the catalog.

In cooperation with the Minamata Disease Health Care Affairs Division of Kumamoto Prefecture, the Minamata Disease Municipal Museum of Minamata City, and the Minamata Disease Museum of NPO Soshinsha we partially integrated catalog data of materials stored in each organization. Furthermore, we initiated trial operation of the database (February).

In addition, based on the agreement for comprehensive cooperation with Minamata City, realizing the policy proposal submitted to the Mayor is a newly added task for the Minamata Disease Archives.

(2) Information service using hair mercury analysis

Masaaki Nagano

(Department of Basic Medical Science)

In 2018, 498 hair samples were collected from visitors at National Institute for Minamata Disease, Minamata Disease Archives, and from other organizations. The total Hg levels of the samples were measured. The analytical results were sent to each participant.

5. Nature Environment Group

The aim of our research group is to understand Hg cycle and the methylation of inorganic Hg in various natural environments including land, water, and atmosphere on local (Minamata Bay area), regional, and global scale. To achieve this goal, our group implements integrated studies including field surveys, monitoring, and laboratory experiments for environmental Hg. In addition, a multi-collector ICPMS for measuring Hg isotopes with high precision was installed to obtain more detailed information on Hg emission sources and chemical reaction processes. Moreover, we act to obtain valuable data for the effectiveness of the Minamata Convention by collaborating with domestic and foreign research scientists.

Three new studies on Hg isotopes, Hg methylation by microorganisms, and bioaccumulation of Hg in the Minamata Bay were started during this fiscal year. The outlines of our all studies in this fiscal year are described below.

［Research theme and summary］

(1) Research on mercury exchange in air –sea interfaces and accumulation for marine wildlife of mercury around Japanese Islands using atmospheric mercury monitoring network (Project research)

Kohji Marumoto

(Department of Environment and Public Health)

We continued to monitor Hg in the air and rainwaters of Minamata, Fukuoka, Hirado, Omaezaki, and Minamai-Awaji. Hg concentrations in rainwater showed no significant differences in comparison with past several years. Gaseous elemental Hg (GEM) and particulate bound Hg (PBM) concentrations in the air of the Fukuoka city were lower both this and last year. We found that both atmospheric Hg pollution events frequency and maximum concentration decreased annually. We will focus on these year-to-year trends by further continuous monitoring.

In this study, we also investigated oceanic and atmospheric Hg behavior, because their interaction is important for global Hg cycling. Total Hg (THg) concentrations in all seawater samples obtained from 20 observation sites in the subarctic region of the North Pacific Ocean during the last year were measured. In addition, methyl Hg (MeHg) concentrations in western and central parts of this region were also determined. The results showed that THg and MeHg concentrations are lower in the surface layer than in the mesopelagic zones; furthermore, both dissolved gaseous Hg (DGHg) and MeHg concentrations achieved local maximum values at the depth of subsurface chlorophyll maximum (SCM). DGHg and MeHg are easily produced by anaerobic bacteria activity in hypoxic conditions. At the SCM layer, the particle density is extremely high, because of phytoplankton abundance though high dissolved oxygen concentrations by force of photosynthesis by phytoplankton. Thus, DGHg and MeHg concentrations apparently increased due to the formation of local anoxic condition among particles in the seawater.

Contrarily, MeHg concentrations were low in subsurface layers except for the SCM layer. This is due to MeHg photochemical decomposition and uptake into marine organisms such as phytoplankton. Therefore, we investigated MeHg uptake into phytoplankton by incubation experiments. The results

revealed that MeHg uptake rate into phytoplankton were extremely fast and were consistent with the vertical profiles of MeHg concentrations in various sea areas.

The data obtained in this study are important for understanding regional Hg cycling in the North Pacific Ocean. Furthermore, they are useful for updating the multi-media global Hg cycling model developed by the National Institute for Environmental Studies, which continues to collaborate with our research group.

(2) Research on the behavior of mercury in the aquatic environment of Minamata Bay and its surrounding sea area (Fundamental research)

Akito Matsuyama
(Department of International Affairs and Research)

1) Dissolved total Hg and dissolved MeHg annual average concentrations were 0.27 ± 0.03 ng/L and 0.02 ± 0.001 ng/L, respectively (5 measurements / year). The dissolved total mercury annual average concentration at Eco Park in the Minamata reclamation area was 0.69 ± 0.50 ng/L (3 measurements / year).

2) A small environmental incubator experiment for mercury methylation in seawater was carried out; it was based on a basin plan for a combination experiment with salinity 1.5% and 3.0% and temperature 15℃ and 30℃. In addition to the basic experiment, an experiment that changed temperature in three steps was carried out. Although Hg methylation reactivity level of salinity 1.5% and temperature 30℃ in seawater was relatively high in comparison other combination experiments, MeHg concentration level was low, accounting for only several % of the total mercury concentration. Furthermore, there were no differences in methylmercury concentration in the combination experiments that changed salinity levels in several steps.

3) We obtained 43 core sediment samples and 22 surface sediment samples from Amakusa sea area and Yatsushiro Sea area during the last and current year. Then, 821 sediment samples were obtained for chemical analysis from the core and surface sediment samples. As a result, the average total Hg concentration values for Amakusa Sea area and Yatsushiro Sea area were 0.10 ± 0.04 mg/kg dry and 0.46 ± 0.44 mg/kg dry, respectively. After the repair of the special device for bottom sediment movement observation by seawater flow was completed, an initial observation experiment was carried out in the Minamata Bay. Moreover, to utilize the multi collector (MC-ICP-MS), a new methodology for total Hg condensation as for environmental samples such as sediment, fish, etc. was developed to replace current globally used methodology. The new methodology for total mercury in environmental sample is based on the NIMD method using dithizone instead of the purge and trap method by sulfuric acid and permanganate.

(3) Development of atmospheric mercury monitoring method for rapid and simple screening in mercury emission sources and their surrounding areas (Fundamental research)

Kohji Marumoto
(Department of Environment and Public Health)

In this study, the use of a quartz crystal microbalance for Hg (QCM-Hg) as a simple Hg area monitor and a personal monitor under relatively high Hg concentrations conditions, such as volcanic and geothermal areas, gold mining areas, and gold dealing

shops, was examined. To evaluate QCM-Hg as a personal Hg monitor, we investigated the relationships between QCM-Hg oscillations and Hg amounts on its gold sensor and Hg accumulation into living organisms through an in vivo experiment. The results showed that Hg concentrations in rat nasal mucosa, olfactory bulb, and lungs correlate positively with Hg amounts on the sensor, but not with the oscillation. Local metrological data revealed that rat breath moisture and ambient air largely affect QCM-Hg oscillation. However, we found that QCM-Hg is useful as a personal Hg exposure monitor when Hg concentration on its sensor is measured after exposure. Furthermore, to investigate the use of QCM-Hg as an area monitor, Hg concentrations in the atmosphere of gold mining areas and gold dealing shops in Brazil were measured using QCM-Hg and a conventional atomic absorption monitor for Hg. As previously reported for the last fiscal year, the frequency of QCM-Hg vibration became plateau in the condition with extremely high Hg concentrations (More than 100 μg m^{-3}). In gold dealing shops, Hg concentrations in the room air were less than or comparable to about 5-20 μg m^{-3}. Under these conditions, the absolute oscillation degree increased linearly and its slope changed correspondingly with the variation of Hg concentrations. The cost of QCM-Hg is cheaper than that of a conventional Hg monitor. Thus, this study revealed that QCM-Hg can be used as a simple and cost-effective Hg real time monitor, although there is a limit on its measuring ability depending on Hg concentrations.

(4) Fabrication of standard gaseous oxidized mercury generator and its application to fundamental studies of atmospheric chemical reactions related to methylmercury (Fundamental research)

Satoshi Irei

(Department of Environment and Public Health)

The aim of this project is to fabricate a commercially available VOC permeation device to generate standard gas for gaseous elemental or oxidized Hg (GEM and GOM), and then to apply it for the investigation of their homogeneous and heterogeneous chemical reactions under simulated atmospheric conditions. The project is also designed to evaluate the precision and accuracy of on-going field measurements of atmospheric Hg by Tekran 2537/1130.

A permeation device, Dynacalibrator 345 purchased last year, had many technical issues during this year. Specifically, the program and the inside mass flow controller of Dynacalibrator unexpectedly malfunctioned. The repair of both of these defects required shipping the device to the manufacturer overseas.

When the device functioned properly, pilot tests for heterogeneous GEM uptake by acidified water were conducted. Briefly, 0.5 L of acidified pure water by either nitric or sulfuric acid (adjusted to pH = 3) was poured gently into a 2.5 L rectangular shaped glass container, at both ends of which a half inch glass tubing was attached. Through the tubing at one end, a defined concentration of GEM from Dynacalibrator (50 ng m^{-3}, approximately 25 times higher concentration than the atmospheric GEM) was then introduced into the space above the solution in the container at the flow rate of 2 L min^{-1}. During the experiment, another end of the container was opened to the fume hood and temperature and relative humidity of the gas was monitored at this end. After 2.5 ~ 6 h of exposure, the solution was quickly siphoned into a cleaned polypropylene volumetric

cylinder and hydrochloric acid was spiked to a final concentration of 10% hydrochloric acid mixture to stabilize Hg in the solution. A portion of the sample solution was then subjected to conventional Hg analysis: a reduction technique by tin chloride, followed by gold amalgam trap and cold-vapor atomic absorption spectroscopic analysis.

Measurement results demonstrated that Hg uptake by acidified water with nitric or sulfuric acid was 71 and 94 pg per 500 mL, respectively. Based on the GEM concentration and the temperature of the gas, the collision rate of GEM to the surface of the acidified solution was estimated. Comparison of these numbers revealed the uptake coefficients (the number taken up by the solution divided by the number of GEM collision to the surface) were 7×10^{-6} and 22×10^{-6} for the nitric and sulfuric acid solution, respectively. These numbers were very low relative to the reported uptake coefficient of GOM by dried salt surface, 3.3×10^{-4}. Although the uptake was very low, the atmospheric lifetime of GEM under the condition of 10 μm droplet size with 100 droplets cm^{-3} was estimated to be 5 days, approximately. Further careful and detailed investigation will be needed for a more convincing evaluation.

(5) Source apportionment of mercury in the air and marine sediment based on stable isotopic composition (Fundamental research)

Satoshi Irei

(Department of Environment and Public Health)

This project is to (1) apportion the sources of total Hg found in the marine sediment of Yatsushiro Sea (run under the RS-18-11 project); (2) evaluate fractionation in stable Hg isotopic compositions in atmospheric GEM under the simulated atmospheric oxidations; and (3) develop a novel fast sampling technique for isotopic measurements of atmospheric GEM.

This year, most time was spent to troubleshoot many unexpected problems our new multi-collector inductively coupled plasma mass spectrometer (MC-ICP-MS) had. Examples include instability of thallium and/or mercury signal, plasma, MS tuning, and gas flows. These defects occurred and disappeared suddenly and irregularly. With technical assistance from the manufacturer and collaborating scientists several problems were identified (malfunctioning mass flow controller, nebulizer, etc.), but there are still unsolved issues. Solving these problems is currently a top priority to allow producing high quality data.

When MC-ICP-MS was functioning properly, a reference solution (NIST SRM 8610) and several marine sediment samples (two samples from Minamata bay and one sample from Amakusa, ~ 40 km north from Minamata bay) were analyzed. Results of SRM analysis demonstrated insignificant differences (± standard errors) between our results and the recommended by NIST values for δ^{199}Hg, δ^{200}Hg, δ^{201}Hg, δ^{202}Hg, andδ^{204}Hg of -0.01 ± 0.06‰, -0.03 ± 0.05‰, -0.05 ± 0.09‰, 0.0 ± 0.1‰, and 0.0 ± 0.1‰, respectively. Measurement results for the marine sediment showed that in these isotopic compositions there were no significant differences between the two Minamata samples. However, there were small, but significant differences between the averaged isotopic compositions of the Minamata samples and those of the Amakusa sample, implying different source(s) of total Hg in marine sediment. Further detailed studies are needed.

For the laboratory studies of isotopic fractionation, a reaction device had already been built. Several pilot

experiments were performed; however, they were unsuccessful. More well-designed experiments will be performed next year. To develop a fast sampling method, new big gold amalgam traps, theoretically 100 times larger in cross-section than the usual gold amalgam trap, were built and sampling and extraction tests are currently on-going.

(6) Metagenomic study of microbial functional genes involved in Hg methylation in the coastal environments

(Fundamental research)

Yuya Tada

(Department of Environment and Public Health)

In this study, we estimated the concentration of dissolved total Hg (THg) and MeHg, and investigated the microbial functional genes involved in Hg methylation (*hgcAB* genes, and genes associated with the reductive acetyl-CoA pathway) using a metagenomic approach in Minamata Bay. The dissolved THg concentrations in Minamata Bay were 0.34～0.60 ng/L in July and 0.42～0.47 ng/L in October. MeHg concentration accounted for 0.8~1.0% of THg (0.0034～0.0049 ng/L in July and 0.0035～0.0042 ng/L in October, respectively). The relative abundance of *hgcA* sequences ranged from 0 to 0.3 genes / million (M) sequences in July, and from 0.3 to 0.6 genes / M sequences in October. The *hgcB* abundances reached 534 genes / M sequences at 0 m depth in July, and 585 genes / M sequences at 0 m depth in July. Completion ratio of the KEGG modules associated with the reductive acetyl-CoA pathway were 60 to 70% in July, and 70 to 80% in October, respectively. Phylogenetic analysis revealed that the *hgcA* sequences detected in the 10 m depth in October were closely related to the *Desulfatibacillum*-like one, which is known as an anaerobic sulfate-reducing bacterium. These data suggest that anaerobic microbes could be involved in microbial Hg methylation in this region.

(7) Study of food web structures of fishes as top predators and Hg bioaccumulation in Minamata Bay (Fundamental research)

Kenji Yoshino

(Department of Environment and Public Health)

To investigate mercury bioaccumulation pathway to top predators in the food webs of Minamata Bay, total Hg contents (hereafter THg) and carbon and nitrogen stable isotope ratios had been measured for fish collected from Minamata Bay. However, the data had not been sufficiently summarized for statistical analyses. Moreover, there had been no information on the species composition and abundance of subtidal macrobenthos, which link primary producers to fish in food chains. In this study, I firstly summarized the past data on THg and isotope signatures of fish in Minamata Bay and statistically analyzed the effect of trophic position and food sources on Hg bioaccumulation. Linear mixed effects model confirmed that the main pathway of Hg bioaccumulation to fish was benthic web derived from microphytobenthos, which were qualitatively asserted by the predecessor. Secondary, I have started quantitative sampling of subtidal macrobenthos seasonally and have measured THg and carbon and nitrogen isotope ratios of the subsamples. Fish samples and food web bases (i.e., substitutes of potential primary producers) were also newly collected seasonally to construct food web structures. Here, I present the results for summer and fall. Food web structures in both summer and fall were overall

fueled by microphytobenthos, which was consistent with the results described above. The contribution of pelagic phytoplankton was extremely low in both macrobenthos and fish. The macrobenthic community was very poor in abundance and was mainly composed of small annelids, such as Lumbrinerid, Sigalionid, and Glycerid worms. Bivalves were rare and no Gammarid amphipods were collected. While it was difficult to analyze macrobenthos THg, because of their low density and small size, annelids, especially in Lumbrinerid worms, often showed relatively high THg compared with other species such as crabs and shrimps. The results suggest that annelids were a main source of Hg transfer to demersal fish in Minamata Bay.

6. International contribution Group

Environmental pollution by Hg is spreading, and is a particularly serious problem in developing countries. To decrease the risk of environmental pollution, the Minamata convention was adopted by 140 participating nations. Therefore, the International Contribution Group in NIMD plans cooperation with foreign researchers to study Hg and to exchange information via the NIMD forum. Moreover, based on the needs of developing countries facing Hg pollution, we use our experience, knowledge, and the latest technology in cooperation with JICA. In addition, we developed a new simple Hg analysis methodology that is focused on MeHg, which is positioned in the Minamata convention as a commitment of the MOYAI initiative. Finally, a summary of the studies and duties of the international contribution group in 2018 are reported.

[Research theme and summary]

(1) Development of a simple method for the determination of monomethyl mercury in the least-developed countries (Project research)

Koichi Haraguchi

(Department of International Affairs and Research)

Our research project consists of two major parts: 1) development of a simple method for the determination of Hg compounds; and 2) development of reference materials to evaluate the accuracy of Hg analysis for human biomonitoring. These are part of the MOYAI initiatives aimed at supporting the least developed countries (LDCs) in their efforts to monitor Hg. The drinking water standards, directions, and guidelines intended for human consumption are proposed by various countries and organizations, such as the Ministry of Health, Labor and Welfare, Japan, the Council of The European Union, and WHO. Commonly used methods for Hg contamination determination in drinking water require several steps for extraction. One of the main problems in drinking water examination is the operation of a complex preprocess of Hg extraction. An analytical method, based on gold extraction and thermal decomposition amalgamation atomic absorption spectrometry, has been developed that can extract and quantify inorganic Hg in drinking water. This method involves only a few steps: Hg extraction with gold coated silica and its separation. The method was validated by spiked test analyzing various drinking water samples (commercial bottled water, spring water, river water, and tap water). The performance of the method suggested that sub-ng amount of inorganic Hg present in a few milliliter drinking water samples can be measured. This new method, therefore, represents a simplified tool for monitoring Hg in drinking water. Furthermore, the method requires few reagents, making it particularly attractive for the examination of drinking water at a remote site in LDCs.The purpose of the development of new human urine and hair reference materials is to support human biomonitoring for general populations with exposure to elemental mercury (Hg^0) and methylmercury (MeHg), respectively. Hg^0 and inorganic Hg exposure can result from dental amalgams, inhalation of indoor air, broken thermometers, and fluorescence lights. MeHg exposure can result from seafood consumption. The requirements for certified reference materials to assess human Hg are growing with an increasing body of

research from surveys to evaluate the effectiveness of the Minamata Convention. We collected human urine and hair samples from Japanese males and Vietnamese males, respectively, without occupational exposure to Hg compounds. The urine was immediately sterilized by ^{80}Co radiation, filtered, and blended. We prepared 4,000 bottles of urine, each containing 4 mL. Inter-bottle homogeneity was confirmed by measuring the concentrations of THg and cadmium in the material and by subsequent statistical analysis. The hair was incrementally pulverized with 3 mills with liquid nitrogen. The homogenized hair powder was divided 3 g each into 1,200 bottles and sterilized by ^{80}Co irradiation. Values for THg and MeHg, as well as for other trace elements (Cu, Zn, As, Se), were presented. We are characterizing property values to develop reference materials using a collaboratively studied method. Hair reference material will be distributed in fiscal year 2019. The present reference materials will be of value to the parties to the Minamata Convention to assure the analytical quality of their monitoring.

(2) Assessment of methylmercury exposure in Vietnamese (Fundamental research)

Megumi Yamamoto
(Department of Environment and Public Health)

We formulated a research proposal on “Assessment of fetal exposure to methylmercury in Vietnam” with the Hanoi University of Public Health. Then, we conducted a demonstration of a dietary questionnaire using a hair sample and fish model to staff of the Hanoi University of Public Health. Furthermore, we negotiated with a hospital of obstetrics and gynecology in Hanoi, which is a candidate to collect biological samples (hair, nails, and blood) using this research proposal. However, multiple problems related to Vietnamese customs (selection of a birthing hospital, timing of hospitalization and discharge from the hospital of pregnant women, collection timing of biological samples, etc.) regarding sample collection exist, so revisions of the research plan and further negotiations are undergoing. We also investigated the pretreatment conditions of blood samples for Hg analysis by thermal vaporization atomic absorption spectrometry.

[Activities theme and summary]

(1) Examination of hair mercury in areas concerned with Hg pollution around the world (Other activities)

Masatake Fujimura
(Department of Basic Medical Science)

We performed Hg analysis of hair samples and assessed the survey information including gender, age, occupation and residence in areas of Brazil (State of Mato Grosso) and the Philippine (Island area) concerned with Hg pollution (729 samples). Hg hair levels data indicated that Hg pollution was very low in the above-mentioned areas. This year, we presented our prior findings at a conference.

(2) Cooperation of research in the international organization

Mineshi Sakamoto
(Department of Environmental and public health)
(WHO Collaboration Centre)

(Dispatch)

Researchers performed 11 dispatches in seven countries: Poland, Italy, Indonesia, Canada, the United States, Vietnam, and Brazil. We conducted presentations at international conferences,

collaborative research on Hg, surveys, and Hg values measurements after Hg exposure. One researcher participated in the 2nd Collaborating Meeting for Japanese WHO Collaborating Centres. There are, in total, 35 WHO Collaborating Centres in Japan by the end of April 2018, which are approved and recognized by the WHO/WPRO in various fields as “Environment and Health”, “Infectious Diseases”, “NCDs and Chronical Diseases”, “NCDs (Others) and Maternal and Child Health”, “Health System”, and “Others”. One researcher participated in the third Regional Forum of WHO Collaborating Centres in the Western Pacific, November 22-23, 2018 held in Ho Chi Minh City, Vietnam. The objectives of the Forum were: 1. To share good practices and reflect on progress since the second forum in 2016; 2. To strengthen and promote innovative collaboration and networking mechanisms; 3. To identify opportunities to maximize contribution of WHO CCs towards WHO support at the country level.

(Invitation)

We invited one researcher from Indonesia to conduct a collaborative project.

(JICA and other Trainings)

We conducted 3 JICA trainings and presented lectures at the third SAKURA Science Plan, and Vietnam International Internship training. In total, 12 lectures with 182 participants from 31 countries were held.

(3) NIMD Forum and International Workshop

Mineshi Sakamoto
(Department of Environmental and public health)

NIMD Forum 2018 (International mercury problems and NIMD contribution) took place in the Minamata Disease Archives, Minamata on November 1-2, 2018. Speakers: 12 (USA 1, Gahna 1, Uruguay 1, Vietnam 1, Nicaragua 1, and Japan 7, including 5 speakers from NIMD); the audience consisted of approximately 80 individuals.

7. Publications and Scientific meetings

［**International Journals**］

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［**International meetings**］

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