NIMD Annual Report 2020

（April 2020 to March 2021）

National Institute for Minamata Disease

Ministry of the Environment

Japan

Table of contents

Report on Research and Other Activities in the Fiscal Year 2020

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: 1. to understand the initial signs of MeHg poisoning, 2. to evaluate MeHg toxicity, 3. to develop methods to protect against MeHg-induced disorders, and 4. to develop new treatment options for MeHg poisoning. To achieve these objectives, our study group applied techniques from the fields of biochemistry, molecular biology, and pathology in cell cultures and animal models.

This group conducted the following research during the fiscal year (FY) 2020.

[Research theme and summary]

1. Fundamental research on neurotoxic mechanism of methylmercury and its prevention and treatment

(Project research)

Masatake Fujimura

(Department of Basic Medical Sciences)

(1) Research on differential cytotoxic effects underlying MeHg neurotoxicity

We observed that synapses in the maternal brain were reconstituted in female rats exposed to MeHg during pregnancy. We published our findings in a peer-reviewed article. Moreover, *in vitro* experiments showing that hippocampal neurons were more resistant to MeHg toxicity than cerebrocortical neurons corroborated comparative *in vivo* observations. The results also suggested that the mechanism underlying the resistance to MeHg toxicity was related to the antioxidant capacity.

(2) Discovery of biomarkers indicating individual susceptibility to MeHg neurotoxicity

We demonstrated that blood thiol antioxidant ability is a reliable biomarker to predict MeHg toxicity in a rat model of MeHg poisoning. Our observations were published in a peer-reviewed article.

(3) Basic research on neuropathic pain triggered by MeHg and a related treatment option

An *in vivo* study revealed that MeHg poisoning triggered neuropathic pain in rats. Our results also suggested that the underlying mechanism for the onset of MeHg-triggered neuropathic pain was related to tactile/pain complex synapse formation in the somatosensory cortex. The research results were presented at meeting of academic societies and submitted for publication. Furthermore, we demonstrated that the drug gabapentin had a therapeutic effect on MeHg-triggered neuropathic pain.

(4) Joint research with external research institutes

We collaborated with external research institutes on MeHg toxicity-related research (endoplasmic stress, epigenome, etc.) that are not investigated at our research center, and we presented the results at meetings of academic societies.

(5) Other

We compiled a review article mainly based on original peer-reviewed papers (10 reports) about methylmercury toxicity and oxidative stress, which had been published over the past 15 years. In addition, we gave a presentation at an academic society meeting on our previous research titled "Recovery effect of vibration stimulation on muscle atrophy".

2. Study on reducing the health risk of methylmercury by food ingredients

(Fundamental research)

Masaaki Nagano

(Department of Basic Medical Science)

The aim of this study is to reduce the health risk of MeHg by utilizing the function of food ingredients. So far, we demonstrated that the daily intake of wheat bran or fructooligosaccharides significantly reduced the tissue mercury concentration in mice exposed to MeHg.

In FY 2020, we examined the adsorption of MeHg by dietary fibers (pectin, sodium alginate and indigestible dextrin), and also evaluated the combination effect of wheat bran and fructooligosaccharides on the tissue

concentration and excretion of mercury in mice.

3. Research on the methylmercury-induced alteration of protein function and its protective factors

(Fundamental research)

Takamitsu Unoki

(Department of Basic Medical Sciences)

Redox (reduction-oxidation) balance is an essential concept for clarifying the mechanism underlying the toxic effects of MeHg on the nervous system. In our previous study, we elucidated the mechanism for maintaining redox homeostasis by reactive sulfur species (RSS), the low molecular weight molecules with releasable sulfane sulfur. Sulfane sulfur easily transfers to protein thiols to form the reactive persulfide/polysulfide species. MeHg can covalently modify protein thiols, causing their dysfunction and thereby exerting deleterious effects. Our research aims to assess the physiological role of sulfane sulfur-mediated modification of protein thiols in the nervous system during MeHg exposure.

In FY 2020, we developed a gel shift assay and a pull-down assay combined with an alkylating reagent, which are novel methods for detecting the sulfane sulfur-mediated modification of protein thiols. The level of protein-bound sulfane sulfur was increased in rat serum albumin when treated with the RSS donor compound Na_2S_4. In addition, when Na_2S_4-treated rat brain samples were incubated with various electrophiles, including MeHg, multiple protein bands showed mobility shifts in electrophoresis. These results suggest that the sulfane sulfur transfer from RSS to protein thiols is diminished by MeHg.

4. Development of a sensor for the methylmercury toxicity and research on the mechanism of the methylmercury neurotoxicity

(Fundamental research)

Akio Sumioka

(Department of Basic Science)

Exposure to MeHg induces oxidative stress in the brain and consequently causes neurodegeneration. However, the mechanisms underlying the cellular specificity and a developmental stage dependency of MeHg toxicity remain unclear. Therefore, an investigation of MeHg neurotoxicity requires the monitoring of the brain at an appropriate cellular damage stage with translocation of MeHg and exertion of its toxic effects. Consequently, we developed a new sensor vector for MeHg toxicity and studied synaptic and axonal dysfunction mediated by MeHg.

(1) Development of a sensor vector for MeHg toxicity

To monitor the processes involved in MeHg-induced neuronal degeneration, we developed a sensor vector for detecting MeHg toxicity quickly. The genetically engineered vector system utilized deficient selenocysteine (SeC) insertion triggered by MeHg exposure during selenoprotein translation.

In FY 2020, we improved the sensor vector system by substituting the cDNA of a fluorescent protein with that of luciferase. As a result, the luciferase had a 100 times higher signal-to-noise ratio than the fluorescent protein. Furthermore, we designed a sensor vector that MeHg-dependently increased the signal. The new sensor vector contained the KRAB transcription depressor domain fused to the TetR DNA-binding domain via an artificial SeC residue linker. The new construct increased the signal intensity in a MeHg-dependent manner.

During the following reporting period, we will validate the MeHg sensor vector and prepare a generation of MeHg sensor mice. For the validation, the MeHg sensor vector will be compared with other toxin sensors, and the sensitivity of the sensor vectors for MeHg and the other oxidants will be evaluated. The present sensor vector systems are composed of transcription factors associated with the SeC expression vector and the responsive luciferase expression vector. For preparing the sensor mice, these cDNAs will be combined into one vector.

(2) Research on MeHg-mediated neurotoxicity

Our research on MeHg-mediated neurotoxicity is focused on axons and synapses as neuronal-specific

structures.

In animal models, the exertion of MeHg toxicity needs to be monitored. To solve this problem, primary cerebellum granule cells were prepared from mice pups. We observed that MeHg exposure induced the translocation of AMPA-type glutamate receptors (AMPAR) from the detergent-soluble extra-synaptic fraction to the detergent-insoluble postsynaptic density fraction and reduced the myelin basic protein as an axonal marker protein. Additionally, we found that the MeHg-mediated AMPAR translocation did not depend on the traditional calcium signal of the *N*-methyl-D-aspartate receptor.

The tau protein is an axonal marker, and oxidative stress is a major risk factor for tau pathology. Therefore, tau toxicity was evaluated in the presence of various oxidants, including MeHg, by transiently expressing the tau protein in COS-7 cells. The tau toxicity test detected oxidant-dependent sensitivity. Moreover, in transgenic mice with hyperphosphorylation of tau protein, increased sensitivity to MeHg was confirmed.

During the following reporting period, we will investigate the effects of MeHg on synapses and verify neuronal excitotoxicity. Furthermore, axonal toxicity will be examined by analyzing tau protein aggregation and degradation.

2. Medical practice・Welfare・Society Group

Research

Minamata disease (MD) is an intoxication of the central nervous system caused by ingestion of seafood from the methylmercury (MeHg)-contaminated areas of Japan. The diagnosis of MD is usually not difficult in typical and severe cases; however, it is difficult in mild cases. Thus, objective methods are critical to clinically diagnose atypical or mild symptoms in the chronic stages of MD. We have been evaluating the brain function in MD using magnetoencephalography (MEG) and magnetic resonance imaging (MRI).

In addition, effective therapies are needed for intractable chronic pain, tremor, ataxia, and spasticity, as they affect the quality of life of patients with MD. We started a clinical study of repetitive transcranial magnetic stimulation (rTMS) therapy for neuropathic pain.

To create a detailed plan for regional revitalization in the area for MeHg pollution victims, we started a multidirectional analysis of issues related to MeHg pollution victims.

The research conducted by our group during FY 2020 is outlined below.

[Research theme and summary]

1. Research on health effects of methylmercury exposure in humans and therapeutic development

(Project research)

Masaaki Nakamura

(Department of Clinical Medicine)

To develop an objective evaluation protocol using MEG and MRI, the somatosensory-evoked fields and morphological features of MD patients were compared with those of control subjects from the Kumamoto district (not polluted with MeHg). Using MEG, the early cortical somatosensory processing as indexed by N20m amplitude, the reproducibility of N20m at single-trial responses, and the evoked gamma-band oscillations in the primary sensory cortex were compared between control and MD. As a result, we found that MD and control could be distinguished with a sensitivity of 60% or more. In addition, we have been developing the script quantitating the extent of the network of the somatosensory area.

We compared the brain network between control and MD using resting-state functional MRI. Fourteen patients with MD (6 adult-type, 5 child-type, and 3 fetal-type MD) and 39 age-matched control patients were included in this study. In MD, the functional connectivity of the caudate nucleus/thalamus and the cerebellum or occipital lobe and temporal lobe was decreased, whereas that of cerebellar hemispheres and the occipital lobe or temporal lobe and pallidum was increased.

In 2020, the research subject (single comparative study confirming the effectiveness of magnetic stimulation for neuropathic pain including Minamata Disease Patients) was approved by the Kyushu University Certified Institutional Review Board for Clinical Trials. We also performed an interview survey concerning the life history of 13 MD patients.

2. Area studies about the regional revitalization in Minamata City and neighboring cities

(Fundamental research)

Rie Harada

(Department of International Affairs and Research)

We analyzed the research theme from various angles by starting from the following four perspectives: (1) Understand the actual situation of social damage among MD patients, (2) clarify issues of community welfare, (3) conduct “Gimotogaku” (area studies) as know-how for regional revitalization, and (4) as a pioneering and experimental initiative, verify community development through art.

The research target is based on MD patients, and the plan is to expand to the patients' families, communities, specialized institutions, and the entire region.

During FY 2020, we mainly focused on (1) and (2).

(1) An oral survey was conducted based on the Pregnancy/Childbirth/Miscarriage/Stillbirth Survey (Itai, 2003) in the Fukuro district of Minamata City and Tsunagi Town.

(2) The "Community Welfare Needs Survey in Minamata City" was conducted in collaboration with the Minamata City Council of Social Welfare.

(3) This task is in the pre-survey stage.

(4) This survey included participant observation, such as cooperating with the planning of the Tsunagi Art Museum.

We completed survey (2). The outline of the community-based welfare needs survey was as follows. The questionnaire survey was conducted targeting 5 districts (1,021 households) in Minamata City to survey the actual conditions of carers (people who provide long-term care). The collection rate of the questionnaire was 24.49%.

A total of 90% of the caregivers who answered the questionnaire were in a situation where they continued to take care while receiving support mainly from relatives, but 10% answered that they were not supported by anyone. In addition, 40% of the caregivers answered that "there are no people, institutions, or contact points that can be trusted and consulted," and 12% felt isolated.

We found that support from specialized consultation desks and institutions is an urgent issue for caregivers who feel isolated because there is no one around to support them.

Next, we visited the respondents who answered that they could cooperate with the interview. In addition, we conducted hearings with welfare organizations and local organizations in Minamata City to clarify the issues of community welfare in Minamata City.

Activities

To examine the effectiveness of home care support for patients with MD and their families, we initially 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 conducted to develop strategies for the application of these concepts in the community. After completing these projects, we have continued supporting welfare activities in the cities of Minamata and Izumi.

In addition, we organized conventional rehabilitation activities, including daycare facilities for fetal-type patients with MD, along with annual workshops on rehabilitation and assistance techniques. We also examined the benefit of vibration therapy for pain relief and spasticity associated with various chronic diseases of the nervous system, including MD. Furthermore, we introduced a robot suit, a walking assistant machine, and the General Therapeutic Electrical Stimulator, all of which assist patients during gait training.

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

Based on the comprehensive cooperation agreement with Minamata City, we also supported the survey related to regional revitalization, including "Moyainaoshi" in collaboration with Minamata City.

The following section includes an outline of the clinical practice conducted by this group in FY 2020.

[Activity theme and summary]

1. Local welfare support service

Masaaki Nakamura
(Department of Clinical Medicine)

We conducted a study titled "Home support model study,

including care prevention" (FY 2006–2008) to identify support methods, including rehabilitation and improvement of ADL for aging patients with MD 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 MD victims" (FY 2012) to develop methods for implementing these concepts in the community. Subsequently, we continued implementing educational activities in the cities of Minamata and Izumi to provide an opportunity for occupational therapy. Through this support, we strengthened the connection between our institute and the local community.

We improved the craft class program based on responses to a questionnaire. As a result, we received high satisfaction levels from the participants in a subsequent survey. However, the participation decreased sharply due to the COVID-19 epidemic in 2020.

Furthermore, we publicized the activities and schedule of the craft class on the homepage and Facebook page of our institute.

2. 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 rehabilitation programs for MD patients to improve their quality of life (QOL). Due to the COVID-19 epidemic, we canceled the workshop and did not perform outpatient rehabilitation at full capacity. However, we continued to support people living alone by calling and making short visits.

In addition to the implemented rehabilitation activities, including vibration stimuli, repetitive facilitation therapy, and gait training with hybrid assistive limb (HAL), we added a non-powered walking assist machine and the General Therapeutic Electrical Stimulator for gait-strengthening exercise. We also introduced the training of communication skills using eye-gaze input and the iPad.

In addition, we visited the Orange Hall, which has been used as a base for health and welfare in the southern part of Minamata City, three times a month. We disseminated information to local residents and provided care prevention services, including physical and cognitive function evaluations, ADL evaluations, and health exercises.

3. Information dissemination using Minamata disease pathology specimens

Masumi Marumoto

(Department of Basic Medical Sciences)

Pathological tissue specimens of autopsy cases of MD are extremely valuable. Our institute has a duty to permanently preserve several pathological tissue specimens of MD. However, permanent preservation of pathological tissue slides is difficult as they fade away over time. Therefore, our objectives are to digitize these pathology slides for permanent preservation and publish the digitized pathology tissue slides on a website for students and doctors learning pathology.

4. Needs survey on the agreement for comprehensive cooperation with Minamata City

Rie Harada

(Department of International Affairs and Research)

Based on the comprehensive cooperation agreement with Minamata City, this activity aims to strengthen the cooperative relationship with Minamata City and support measures related to regional revitalization, including "Moyainaoshi."

As a concrete activity, we will utilize the network and survey results in the region that the Regional Policy Section has created so far to provide information and cooperate in surveys to Minamata City and related organizations and cooperate in surveys conducted by these entities.

In FY 2020, we summarized the "Community Welfare Needs Survey in Minamata City" in a report. The survey results were integrated into the "3rd Minamata City Community Welfare Plan/Community Welfare Activity

Plan" formulated by Minamata City and the Minamata City Council of Social Welfare.

Moreover, we provided information on measures for vacant houses and support for migrants in the Minamata City Regional Promotion Section, and we collaborated to inspect vacant houses and interview vacant house owners and migrants.

Results of hearings for migrants were published in the Minamata special feature of the magazine "Inakagurashi." In addition, the content of each hearing is provided on the migration portal site newly opened by Minamata City.

We will continue to cooperate with the Minamata City Council of Social Welfare and the Minamata City Regional Promotion Section from the next fiscal year onward.

3. Risk Assessment Group

High-risk groups for MeHg exposure include populations exposed to high concentrations of Hg and groups with high sensitivity to Hg. The risk assessment group will comprehensively study the assessment of human exposure to Hg and the effects on health caused by environmental pollution. We will conduct both epidemiological and experimental studies, assessing various confounding factors, such as selenium (Se), to elucidate the exposure, risk assessment, and health effects of MeHg, especially in populations exposed to high concentrations of MeHg and in vulnerable populations, such as fetuses, children, and those with diseases.

[Research theme and summary]

1. Studies on coexisting of mercury and selenium in Minamata disease and effects of methylmercury to fetus and breast-feeding infants

(Fundamental research)

Mineshi Sakamoto

(Special advisor to the Director-General)

1) Evaluation of total mercury concentrations analyzed by dithizone colorimetric method at the time of Minamata disease.

The Hg concentrations in preserved samples of shellfish (*Hormomya mutabilis*) meat and organs of patients analyzed by the dithizone colorimetric method tended to indicate lower concentrations than those obtained by the atomic absorption analysis. The values analyzed by the dithizone method were especially lower at the high Hg concentration range. These results suggested that the analysis of total Hg concentration analyzed by the dithizone method at that time may have underestimated the MeHg exposure levels.

2) Reanalysis of mercury and selenium in Minamata disease-related samples (JSPS19K12353).

Increased Se concentrations were confirmed in all historical samples, such as the preserved sludge near the outlet to Minamata Bay, organs of cat No. 717, the muscle of a red sea bream, and shellfish meat. The elevated Se concentration in Minamata Bay sludge was exceptionally higher than the levels observed in the biological samples, suggesting that a relatively high amount of Se might have been excreted in the bay. During the following reporting period, we will analyze Se levels and Hg/Se molar ratios in organs of patients. Furthermore, we will conduct an animal study using rats exposed to high MeHg concentrations for an extended period to examine the effects of MeHg itself on Se levels.

3) Methylmercury exposure during the vulnerable stage of the cerebrum in postnatal developing rats.

The developing brain is known to be sensitive to the toxic effects of MeHg. This study showed the specific effects of toxic levels of MeHg on neurobehavior, neurodegeneration, and selenoenzyme activity in the cerebrum of infant rats, suggesting that MeHg exposure to infant rats is useful for predicting the effects of MeHg on the cerebral growth spurt in humans (M. Sakamoto et al. *Environ. Res.* 2020 Sep.).

2. Improvement of the exposure assessment system for groups at high risk of methylmercury exposure

(Fundamental research)

Megumi Yamamoto

(Department of Environment and Public Health)

1) To elucidate the toxicokinetics of MeHg in the pathogenesis of glucose metabolism disorders, we administered a single dose of MeHg (3 doses) to KK-Ay type 2 diabetic mice and BL/6 normal mice (male, 12 weeks). We analyzed total Hg in blood sampled over time (4, 7, 11, and 14 days) and in target organs (brain, kidney, liver, pancreas). Relevant pharmacokinetic parameters (Vd/F, K_{el}, $t_{1/2}$, AUC, CL/F, K_p value: tissue/plasma Hg value, $K_{p'}$ value: tissue/whole blood Hg value) were calculated using the mouse study data. Under the experimental conditions, the absorption of

MeHg into blood cells, brain, liver, kidney, and pancreas, and its efflux from each tissue showed a trend: KK-Ay>BL/6. These results were submitted for publication and accepted *(J. Appl. Toxicol.* 2020 Oct. *in press*).

2) To elucidate the maternal transfer of MeHg to the pup in the pathogenesis of glucose metabolism disorder, we investigated a mouse model of glucose metabolism disorder in females. So far, there are very few reports on diet-induced obesity (DIO) mouse models of glucose metabolism disorder in females. In this study, we conducted a comparative experiment in which mice were fed a normal diet and a high-fat diet of our choice. As a result, we established a DIO mouse model after a 12-week feeding period based on an evaluation using markers of glucose metabolism disorder. We are currently using this mouse model to investigate the gestation and delivery relative to the exposure and evaluation system of MeHg.

3) Research on Hg in biological samples (hair, blood, placental tissue, and umbilical cord tissue) of mothers and infants with glucose metabolism disorder (gestational diabetes and pregnancy complicated by diabetes) is being conducted in collaboration with the University of Occupational and Environmental Health, and the total Hg content in these samples is being analyzed (FY 2018: 26 samples; FY 2019: 41 samples; FY 2020: 12 samples; total: 79 samples).

3. Exposure assessment of mercury and technology transfer in developing countries

(Fundamental research)

Megumi Yamamoto

(Department of Environment and Public Health)

1) For the survey among pregnant women in Hanoi, Vietnam, we summarized the 48 food frequency questionnaires (FFQs) obtained from respondents recruited by the end of FY 2019.

2) Information on the gold mining area in Indonesia was obtained as a potential study site for Hg exposure assessment.

3) We contributed an introduction of our research on the Hg analysis method as a tool for exposure assessment to the official journal of the Japan Society for Analytical Chemistry, "Bunseki" (January 2021 issue).

[Activity theme and summary]

1. Information service using hair mercury analysis

Masaaki Nagano

(Department of Basic Medical Science)

During FY 2020, 142 hair samples were collected from visitors to the National Institute for Minamata Disease (NIMD), the Minamata Disease Archives, and other organizations. We measured the total Hg levels in these samples and sent the analytical results to each individual.

4. Nature Environment Group

The aim of our research group was to understand the Hg cycle and the methylation of inorganic Hg in various natural environments, including air, land, water, and biota at the local (the Minamata Bay area), regional, and global scale. Therefore, our group implemented integrated studies, including field surveys, monitoring, and laboratory experiments for environmental Hg. Especially in analyzing biota, we are extensively studying marine ecosystems from the microorganisms and primary producers, such as phytoplankton, up to top predators. In addition, an MC–ICPMS (multi-collector–inductively coupled plasma mass spectrometry) for measuring Hg isotope ratios with high precision was conducted to obtain more detailed information on emission sources and chemical reactions involving Hg. Moreover, we are working toward obtaining valuable data for the effective implementation of the Minamata Convention by collaborating with other domestic and foreign research scientists. We are also actively conducting the outreach related to Hg research and general sciences and technologies as education for elementary and junior high school students, along with other high school students.

The outlines of our research during FY 2020 are presented below.

［Research theme and summary］

1. Study on factors influencing the vertical distribution in speciated mercury in the ocean

(Project research)

Kohji Marumoto

(Department of Environment and Public Health)

The aim of this study is to understand the factors affecting the vertical distribution of Hg in ocean environments and to develop a simple model for estimating the concentrations of Hg species according to these factors. This study is divided into four subtopics, as described below.

a) Quantitative estimation of the Hg flux across air-sea interfaces.

b) Investigation of the vertical and horizontal distribution of Hg species, including mono-methyl Hg (MMHg), di-methyl Hg (DMHg), elemental Hg [Hg(0)], and inorganic Hg [Hg(II)] in the ocean.

c) Quantitative evaluation of the coefficients related to the rate of MMHg intake into the phytoplankton and the partitioning between seawater and plankton in surface and subsurface layers.

d) Quantitative evaluation of the fate of DMHg and MMHg under the high-water pressure in mesopelagic zones.

In FY 2020, we conducted the field observation and laboratory experiments for the subtopics a) and b). Under subtopic a), we established a continuous observation system for Hg flux using a gas–liquid equilibrator. Real-time observation data on Hg fluxes were obtained by a test installation under the research vessel (R/V) Shinsei-Maru and R/V Kaimei. However, we also identified some problem areas for the automatic operation of the system. Under subtopic b), we measured the total Hg (T-Hg) and MeHg (MeHg: DMHg + MMHg) levels in seawater samples collected during a cruise of R/V Kaimei in the region of the Izu–Ogasawara Trench in 2019. The dissolved (T-Hg) concentrations were lower in the surface and subsurface layers than in the mesopelagic layers. Almost no traces of MeHg were detected in the surface layer, whereas MeHg concentrations remained almost constant, starting from the subsurface below 100 m to deeper layers. The Hg research in the same sea area was conducted in 2020, and approximately 500 samples were obtained. In addition, the concentrations of dissolved gaseous Hg (DGHg) were measured onboard. Under subtopic c), the laboratory incubation experiments with oceanic diatoms in the presence of MMHg were carried out. The result showed that the amount of MMHg intake into the diatom and the partitioning coefficients between seawater and the planktons were increased by rising

temperature. This observation indicated that the process of MMHg intake into diatoms is not passive adsorption but an active absorption driven by the metabolism of the phytoplankton.

2. Research on the behavior of mercury in soil, water, and sediment

(Fundamental research)

Akito Matsuyama

(Department of International Affairs and Research)

I have introduced this topic as a new subject and conducted research on the following three subtopics, as outlined below.

a) Study of Hg methylation characteristics in seawater

This study has been continuously conducted since the mid-term plan 2015. The T-Hg concentration was unstable in time course incubation experiments using raw Minamata bay seawater. Therefore, Minamata Bay seawater was pretreated by filtration using a 0.8 μm membrane filter. Using pretreated seawater in the incubation experiments, the T-Hg concentration barely varied regardless of the changes in seawater temperature and salinity, compared with the concentration data from the previous experiment using raw seawater. However, we observed an instant production of dissolved MeHg at the beginning of the incubation, but the MeHg concentration remained at a low level throughout the experiment.

b) Understanding the characteristic changes in Minamata Bay sediments associated with fluctuations in seawater quality in the Minamata Bay

Surface sediments from Minamata Bay were used in the following experiments. The Hg elution experiments were carried out at three different seawater temperatures and salinities. The elution method was based on public method No. 46 established by the Ministry of the Environment. The elution experiment showed that variations in the eluted content of dissolved T-Hg were associated with differences in the two seawater parameters. Especially fluctuations in salinity ware associated with differences in the eluted content of dissolved T-Hg.

c) Understanding the origin of mercury in the sediment and the distribution characteristics of mercury in the sediment accumulated in the Yatsushiro sea

This study has been continuously conducted since the mid-term plan 2015. It assessed and compared simulation results using the Delft 3D system and plane distribution results of T-Hg concentrations in Yatsushiro sea surface sediments. We initially developed a new method that can resort sediment particles by size using dispersion and gravity sedimentation. The method was applied to several sediment samples that were taken from vertical sediment cores collected in the Amakusa sea area outside of Minamata Bay. Interestingly, the T-Hg concentration variations depended on the particle size, the original vertical depth within the sediment core, and the sampling site location. Moreover, the same sediment samples were subjected to Hg isotope analysis using MC–ICPMS. A total of 48 sediment samples were analyzed. Currently, the analysis results are under consideration. In comparison with last year, mechanical condition of MC–ICPMS for obtaining of good data was improved, it was generated consistent results.

3. Bioprotection from methylmercury toxicity by complex formation with selenium in large marine animals

(Fundamental research)

Masumi Marumoto

(Department of Basic Medical Sciences)

MeHg uptake occurs via food consumption. In the body, the MeHg is gradually converted into inorganic Hg. However, the ability to mineralize Hg varies among animal species. It is known that selenium (Se), an essential trace mineral, plays a major role in Hg mineralization, but the mechanisms underlying these processes are still unknown. So far, we found that differences in the tissue distribution of inorganic Hg and Se depended on the animal species based on pathological research using an X-ray probe microanalyzer (EPMA). Because the EPMA can determine which cell types contain Hg and Se in various organs, we will be able to assess the intra-tissue

distribution of Hg and Se in large marine animals, especially in cetaceans and marlins. In 2020, we focused on the relationship between the body length of cetaceans and the T-Hg concentration in various organs.

4. Research on primary producer dynamics and mercury bioaccumulation in fish via food chains

(Fundamental research)

Kenji Yoshino

(Department of Environment and Public Health)

Typically, the phytoplankton functions as the main primary producer in coastal ecosystems. It fuels not only surface production but also benthic production by sinking to the bottom layer. Meanwhile, stable isotope analyses of macrobenthos and demersal fish from the Minamata Bay demonstrated that the microphytobenthos on the sediment surface highly contributed to subtidal benthic production. Because the Hg content is generally higher in the sediment than in the water, the high dependence of benthic production on the microphytobenthos may cause the relatively high Hg bioaccumulation in demersal fish observed at Minamata Bay. However, there is little information about the primary producers, such as seasonal fluctuations of the density, stable isotope ratios, and Hg content. I investigated the seasonal dynamics of surface phytoplankton and the structure of the water body. In 2020, salinity stratification was formed in July after the rainy season, and the phytoplankton bloomed in surface water as a result. The surface phytoplankton bloom eventually disappeared when the stratification was disrupted in October 2020. In 2020, disastrous precipitation during the rainy season might have created anomalous conditions, and the nutrient levels during the mixed period were not oligotroph. I also attempted to collect pure microphytobenthos from the surface sediment using phototaxis. High amounts of surface sediment samples had to be collected several times to obtain sufficient amounts of microphytobenthos for stable isotope and Hg content analyses. I found that the favorable photic condition for an optimal phototaxis response was achieved at approximately 30 μmol/s of photosynthetically active radiation (PAR), but more purification is needed to obtain representative microphytobenthos samples by removing sediment contamination as much as possible.

5. Characterization of stable isotopic composition of mercury at the emission source

(Fundamental research)

Satoshi Irei

(Department of Environment and Public Health)

The objective of this research project is to characterize and report the initial stable isotopic compositions of Hg at emissions to gain insight into the global Hg cycle using the analysis of stable isotopes at their natural abundances. In FY 2020, we aimed to establish an analytical method for Hg in commercial goods and report the stable isotopic compositions.

Due to strict governmental regulation of Hg use in Japan, types of commercially available goods containing Hg are very limited, including mostly fluorescent tubes and thermometers. Therefore, we focused on analyzing these items in FY 2020. We obtained 9 new thermometers and more than 80 used fluorescent tubes. However, the analysis of the majority of fluorescent tubes was not successful; thus, this report included the successful analysis of 13 fluorescent tubes. Bulk elemental Hg was collected from the thermometers by breaking the tips. Then, a drop of liquid elemental Hg from each sample was spiked to 20 mL of 40% reverse aqua regia (nitric acid: hydrochloric acid = 2:1) in a 20 mL glass vial. The vials were capped and stored until the drop of Hg was completely dissolved. Then, the samples were analyzed using cold-vapor generation with MC-ICPMS for the quantitative and stable isotope analysis of Hg. For the fluorescent tube analysis, we found that the tubes contained gaseous elemental mercury (GEM) and Hg adsorbed to the inner tube wall. Therefore, we sampled and analyzed those separately. Specifically, each fluorescent tube was inserted into a 5 L Ziploc plastic bag together with a PFA tube (¼ in o.d.), which was connected to a gold

amalgam Hg trap. After sealing the bag, the tube was cracked, and GEM evolving from the tube was sampled by the Hg trap. The captured GEM was dissolved in 100 mL 40% reverse aqua regia using the plastic bag extraction we previously established. After removing GEM, the cracked tube glass was smashed into small pieces, and the Hg adsorbed to the tube wall was dissolved with a mixture of concentrated sulfuric, nitric, and perchloric acids in a 1 L beaker.

The analytical tests showed that most of the sampled Hg was dissolved in the acid solutions. The dissolution was confirmed by observing the disappearance of the metallic Hg drop in the solution, quantitatively analyzing the residual GEM collected from the plastic bag extraction, or observing the constant Hg concentrations of the sulfuric acid mixture over a defined period. Results of five stable isotope ratios measurements [$^{199}Hg/^{198}Hg$, $^{200}Hg/^{198}Hg$, $^{201}Hg/^{198}Hg$, $^{202}Hg/^{198}Hg$, and $^{204}Hg/^{198}Hg$ ratios expressed in δ notation defined as δ = (isotope ratio of the sample)/(isotope ratio of the reference substance – 1)] showed that all the isotope ratios of the thermometers were within the range of 0.53‰, while those of GEM in the fluorescent tubes and the Hg adsorbed to the tube wall varied within the range of 1.8‰–11‰ and 1.4‰–6.3‰, respectively.

6. Study of microbes involved in mercury speciation in the ocean

(Fundamental research)

Yuya Tada

(Department of Environment and Public Health)

To evaluate the Hg speciation (methylation and demethylation) by microorganisms in the ocean, we analyzed the concentration of dissolved T-Hg and MeHg in seawater, and we assessed the microbial functional genes involved in Hg methylation and demethylation (*hgcAB* and *merB*, respectively) using a metagenomic approach for samples from the Seto Inland Sea (SIS) of Japan. In the western part of SIS (especially the Bungo Channel), the dissolved T-Hg concentration was in the range of 0.0004–0.0011 nM, and the MeHg concentration represented 0.2%–10.6% of T-Hg. The proportion of MeHg was relatively high in the deepest sampling layer (at 90 m depth) in the southern part of the Bungo Channel. The *hgcAB* and *merB* genes were also detected in this region. However, there was no correlation between the abundance of these genes and the Hg concentrations. Phylogenetic analysis of *hgcA* showed that Euryarchaeota (including methanogens), *Nitrospira*, and *Nitrospina* (including nitrite-oxidizing bacteria) were potentially involved in the Hg methylation. In the *merB* analysis, the actinobacterial lineage was dominant (57% of all *merB* sequences), suggesting that this lineage could contribute to the MeHg demethylation process. Compared with our previous metagenome data from the eastern part of SIS, we concluded that microbial Hg speciation could involve distinct phylogenetic lineages that differed between the eastern and western parts of SIS.

7. Study on factors affecting the long-term trend of atmospheric mercury concentrations in the Asian-Pacific region

(Fundamental research)

Kohji Marumoto

(Department of Environment and Public Health)

The main purpose of this study is to obtain the continuous observation data on Hg in the air and wet depositions with high accuracy and precision by utilizing the effectiveness evaluation of the Minamata Convention. In addition, we try to understand the factors affecting the variation of their concentrations, such as seasonal variations and the contribution of emission sources. To date, regular monitoring of the atmospheric Hg has been continued at Minamata and Fukuoka, and the wet Hg deposition fluxes have been regularly measured at Minamata, Hirado, Fukuoka, and Omaezaki to ensure continuous monitoring. However, monitoring of the wet Hg depositions at the sites excluding Minamata was discontinued for the maintenance of the sampler from

April to December 2020. On the other hand, a new observation program for wet Hg depositions at Matsue and Tsukuba was started in September 2020. To obtain reliable and comparable data on wet Hg deposition, our laboratory joined the inter-calibration program under the auspices of the U.S. Geological Survey, and a collaborative study with the National Central University of Taiwan was started.

In addition, continuous monitoring of atmospheric Hg at the north side of Mt. Aso was conducted in FY 2020 to investigate the influence of current volcanic activity. The monitoring data at Mt. Aso indicated that the atmospheric Hg concentration increased when the wind was blowing from the south, indicating the effect of the volcanic gas containing Hg. Typically, at this site, the concentrations were substantially decreased, especially during the daytime. However, the factors potentially involved in this phenomenon are still being considered.

[Activity theme and summary]

1. Implementation of seawater quality monitoring in the Minamata Bay and support of various regional activities around the Minamata area

Akito Matsuyama
(Department of International Affairs and Research)

I have introduced this topic as a new subject and conducted research on the following two subtopics, as outlined below.

a) Mercury and seawater quality monitoring in the Minamata Bay

Hg and seawater quality monitoring in the Minamata Bay was conducted three times in 2020 (May, August, November). To carried out the monitoring, three monitoring stations has been set up in Minamata Bay (Hadakase, Wannou, and Koijishima). Hg monitoring around the revetment wall of the Minamata landfill site was carried out two times in 2020 (May and September). Our results indicated that the annual average of the dissolved T-Hg concentration was 0.30 ± 0.07 ng/L, and the annual average of the dissolved MeHg concentration was 0.04 ± 0.02 ng/L. The annual average of the dissolved T-Hg concentration at the revetment wall of the Minamata landfill site was 1.7 ± 0.3 ng/L.

b) Supporting various regional activities around the Minamata area (Subtitle: Development of an efficient oyster farming technology in the Minamata Bay)

Previous surveys indicated that the concentration of nutrients was higher in the Minamata River estuary than in other areas. Therefore, an oyster farming raft was installed in the Minamata River estuary during the second half of February 2020.

Moreover, to compare oyster growth, two additional oyster farming rafts were simultaneously installed in the sea area (Fukuro Bay, Marushima Shinko), and the differences in oyster growth were examined in 2020. The oysters in the estuary grew steadily until around May 2020, but barnacles were covering the scallop shells. Thus, although the oysters in the experimental farming area of the estuary were growing well at the beginning of the experiment, their growth deteriorated sharply due to the barnacles. In addition, the Minamata River was flooded by heavy rain in southern Kyushu in early July 2020, and the aquaculture raft positioned at the mouth of the river was destroyed and washed away. Then, a new oyster farming raft with a subsidence bridge structure was set up in August, and the experiment was restarted. However, although the oyster farming experiment in the estuary was restarted in August 2020, the data from the three experimental sites were not uniform, and the experiment could not be evaluated accurately.

2. Outreach activities related to science and technology research for elementary and junior high school students

Masumi Marumoto
(Department of Basic Medical Sciences)

Researchers and research institutes are required to disseminate scientific knowledge and research results to the public. Minamata City, where the NIMD is located, is known as a town where Minamata Disease occurred, but elementary and junior high school students have little knowledge of Hg itself. Therefore, we will hold classes at

elementary and junior high schools in Minamata City to provide correct knowledge about Hg. In addition, to emphasize interactivity, a question box will be set up after the on-site lesson to avoid one-sided presentations from researchers; hence, interaction with feedback will be possible by answering questions from children. At the same time, we will contribute to the local community by proactively responding to requests in addition to the on-site lessons on Hg. During FY 2020, we conducted on-site lessons, set up question boxes, and created answers at elementary schools in the Minamata area.

5. International Contribution • Information Group

Environmental pollution due to Hg, that which is the causative substance of Minamata disease, is spreading, and environmental pollution is particularly serious in developing countries of around the world. To decrease the risk of Hg pollution to for the human body humans and the environment, the Minamata Convention was adopted by 140 participating nations in October 2013 in Minamata, and it came into effect in August 2017. Based on this background, the international contribution group in at the NIMD have planned has promoted mutual cooperation with foreign researchers for to participate in studies on Hg and the exchange of information via the NIMD forum. Moreover, based on the needs of the developing countries facing Hg pollution, we use our experience, knowledge, and the latest technology by obtaining establishing the mutual cooperation of via the Japan International Cooperation Agency (JICA). In addition, we developed a new and simple Hg analysis methodology that is focused on MeHg, and it is positioned in the Minamata Convention as a commitment to the MOYAI initiative. A summary of the studies and activities of the international contribution group in during 2020 is reported provided below.

[**Research theme and summary**]

1. Development of a simple method for the determination of mercury and reference materials

(Project research)

Koichi Haraguchi

(Department of International Affairs and Research)

The Minamata Convention on Mercury was launched, and it draws attention to Hg exposure linked to fish consumption and gold mining. Our research project consists of two major parts: 1) the development of a simple method for the determination of Hg compounds, and 2) the development of reference materials to evaluate the accuracy of Hg analysis for biomonitoring in humans. These tasks represent a part of the MOYAI initiatives by providing technical assistance to promote the effective implementation of the Minamata Convention.

Guidelines for water intended for human consumption have been proposed by various countries and organizations. The commonly used methods for the determination of Hg contamination in drinking water require several steps for extraction. One of the main problems in drinking water examination is the operation of a complex preprocess for Hg extraction. A regent-free analytical method based on the high affinity of Hg and Au and the thermal decomposition atomic absorption spectrometry was applied to analyze contaminated groundwater and brackish water. The water data were in good agreement with the values obtained by conventional methods. The performance of the method suggested that sub-nanogram amounts of inorganic Hg present in a few milliliters of a water sample can be measured. This highly sensitive method, which requires few reagents, is particularly attractive for the examination of drinking water in developing countries and at remote sites.

Two certified reference materials (CRMs), NIMD-02 and NIMD-03, were developed for the analysis of Hg in human urine. Certified values of total Hg in NIMD-02 and NIMD-03 with the expanded uncertainties (coverage factor, k = 2) were 0.292 ± 0.057 μg L^{-1} and 4.80 ± 0.56 μg L^{-1}, respectively. NIMD-02 also contained cadmium at a concentration of 0.154 ± 0.030 μg L^{-1}. Extended uncertainties were estimated by sample homogeneity, long- and short-term stabilities, and characterization using measurements performed by collaborating laboratories. A short-term stability test for 4 weeks at 37 °C was conducted considering overseas shipment. We found that the CRMs can be transported without a cold supply chain within 4 weeks to resource-limited places.

[**Activity theme and summary**]

1. Examination of hair mercury in areas at risk of mercury pollution from around the world

Masatake Fujimura

(Department of Basic Medical Science)

As a result of advertising the measurement of hair Hg at our research center through an English website advertisement, we received inquiries about hair Hg measurement from research institutes (icddr, b and TEC) in Bangladesh and Costa Rica, and we started hair collection by local collaborators.

Bangladesh has an E-waste (electronic waste) dumping area, and there is concern about human exposure due to Hg spills from E-waste. Initially, it was planned to collect hair from 400 participants. However, due to the spread of the COVID-19 pandemic, only 40 people are currently collecting hair. Therefore, we plan to extend the hair collection period until next year to measure hair Hg.

Costa Rica has an illegal gold mining area, and there is concern about human exposure due to the outflow of Hg used in gold mining. Although the region had planned to collect hair samples from local residents, there is no ongoing effort due to the expansion of the COVID-19 pandemic. Therefore, the future of this project is still undecided.

In addition, we submitted a paper as a collaborative research effort about the results of a previous survey on the status of human Hg exposure in the state of Mato Grosso, Brazil.

2. NIMD forum and international workshop

Akito Matsuyama
(Department of International Affairs and Research)

We planned to hold the NIMD forum in the Minamata Disease Archives on November 14, 2020. The main theme was the regional regeneration of a polluted city and the role of the citizens, enterprises, and the administration. This forum was planned to be different from the conventional style, which mainly includes researchers focusing on domestic and foreign Hg research. Hence, a new NIMD forum style was proposed. We envisioned that the participants were not only researchers but also Minamata citizens with interest in this topic. However, our activities were severely limited by the COVID-19 pandemic that started in the middle of January 2020 in Japan. Consequently, the NIMD forum originally planned to be held in 2020 was canceled due to the COVID-19 pandemic, which affected domestic affairs and international activities.

3. Research cooperation in the international organization

Akito Matsuyama
(Department of International Affairs and Research)

Our activities were very limited due to the COVID-19 pandemic, which started in the middle of January 2020 in Japan. Hence, the work plan for 2020, which was the first year of the mid-term plan, was changed. Furthermore, overseas dispatch and acceptance of training activities for foreign researchers were not implemented.

However, online meetings via personal computers, online training, and participation in international meetings were implemented. Several specific activities are listed below.

- Participation in a premeeting on September 4, 2020, for the International Conference on Mercury as a Global Pollutant (ICMGP) to be held in South Africa (Cape Town, September 2022): One researcher from NIMD was elected as a preparatory committee member for the Health area and attended the meeting.
- Attendance of the 9th Annual Asia Pacific Mercury Monitoring Network Partners Meeting: One researcher from NIMD gave an oral presentation about our activities (September 8, 2020).
- One researcher from NIMD gave a lecture for students at the Tsukuba University (November 18–20, 2020). The lecture was titled “Environmental pollution due to Hg in the world.”
- Currently, NIMD is deeply related to the Minamata Convention via Japanese government and UNEP. Related to this activity was a UNEP meeting with 106 participants from 12 countries (in South Asia and other regions). Three researchers from NIMD attended this conference and gave oral lectures.

4. Transmission of information on MD and organization of

documents and materials in the “Minamata Disease Archives”

Rie Harada
(Department of International Affairs and Research)

NIMD is involved in collaborative research with the Minamata High School Super Global High School (SGH), and related results are exhibited in the Minamata Disease Archives (April-July, 2020).

Preparations for the new exhibition are ongoing in 2021. Other scheduled programs will be postponed or canceled due to COVID-19.

The Minamata Disease Archives has actively collected data and materials associated with MD and Hg. These data and materials will be utilized for presently ongoing research.

5. Activities as a WHO Collaborating Center

Megumi Yamamoto
(Department of Environment and Public Health)

The NIMD was designated as a WHO Collaborating Center for “Studies on the Health Effects of Mercury Compounds” (World Health Organization Western Pacific Regional Office: WPRO) in September 1986 (Ref. # JPN-49). The institute has been conducting surveys and research activities on the health effects of Hg. The main roles of NIMD as a WHO Collaborating Center are as follows: (1) conduct surveys in areas with potential Hg contamination, analyze Hg samples, and assess Hg exposure and contamination; (2) disseminate information on the dangers of Hg and the risks posed by Hg through publications and other means; (3) provide training in Hg analysis and exposure assessment for researchers and technicians in countries with Hg-related environmental problems; (4) assess human and environmental exposure to Hg in locations where Hg-related public health problems are of concern and provide preventive and curative measures and responses. In FY 2020, we mainly carried out the following tasks:

1) WHO CC annual report: Prepared and processed an annual report on the activities of the WHO CC for January to December 2020.
2) WHO CC designation renewal: Prepared and processed renewal documents for the re-designation of NIMD in the next quadrennium (January 2021-December 2024)
3) We contributed an article on the role and activities of NIMD, which was published in the spring 2021 issue of “Medemiru WHO,” the official journal in the Friends of WHO Japan (to be published on the website in mid-October 2021).

6. Publications and Scientific meetings

［**International Journals**］

Fujimura M, Usuki F: Pregnant rats exposed to low level methylmercury exhibit cerebellar synaptic and neuritic remodeling during the perinatal period. Arch. Toxicol, 2020; 94: 1335-1347.

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GJ: Neuropathology associated with exposure to different concentrations and species of mercury: A review of autopsy cases and the literature. Neurotoxicology, 2020; 78: 88-98.

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Sakamoto M: Health hazard of mercury and its countermeasures. UNEP Online Training Programme #1: Role of monitoring laboratory for national mercury management, Web meeting, 2020. 12.（Invited lecture）

Fujimura M, Usuki F, Nakamura A: Methylmercury induces allodynia through activation of inflammatory microglia in spinal cord and subsequent stimulation in somatosensory cortex od rats. 60th Society of Toxicology, Virtual event, 2021. 3.

Irei S: Laboratory ad field studies for the sources and fate atmospheric gaseous elemental mercury. 2nd International Conference on Chemistry and Nanoscience 2021, virtual meeting, 2021. 3.