Master Ciencias Analíticas y Bioanalíticas
Universidad de Oviedo

Master Sciences Analytiques pour le Vivant et l'Environnement
Université de Pau et des Pays de l'Adour

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Pau, June 2014

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CONTENTS

1 INTRODUCTION

1.1 Mercury: toxicity and presence in the environment

Mercury is a chemical element represented by the symbol Hg from the Greek "hydrargyros", meaning silver water. Its atomic number is 80, its atomic mass 200.59 g mol^{-1} and it has seven stable isotopes, with the following distribution: ^{196}Hg (0.15%), ^{198}Hg (9.97%), ^{199}Hg (16.87%), ^{200}Hg (23.10%), ^{201}Hg (13.18%), ^{202}Hg (29.86%) and ^{204}Hg (6.87%). This is a transition metal which appears in liquid form under ordinary conditions of temperature and pressure and also vaporizes easily (due to its relatively high vapour pressure for being a metal).

Due to its physicochemical properties, Hg naturally occurs in all types of environmental compartments and under different chemical forms: elemental mercury (Hg^0), divalent inorganic form (Hg^{2+} or IHg), and organic forms including monomethylmercury (CH_3Hg or MeHg) and dimethylmercury ($(CH_3)_2Hg$ or DMHg).

Hg causes high toxic effects on animal and human health and it is considered as a powerful neurotoxin especially in these last organometallic forms. It is very irritating to skin, eyes and the respiratory tract and it can lead to poisoning. Its negative effect is exacerbated due to its capability of binding proteins and biomolecules.

Human exposure to Hg compounds can occurs by ingestion, respiratory tract or skin contact. Although it was used in the past as a cure of syphilis and various infections, Hg is nowadays considered as a priority and global pollutant especially due to its accumulation in aquatic food chains. MeHg is recognized by its capability of bioaccumulation and biomagnification along the trophic webs. In fact, fish consumption remains the major contributor to Hg contamination in humans and is generally used as a reliable criteria for the risk assessment associated to Hg exposition.[1]

The first known large scale Hg poisoning took place in Minamata Bay, Japan, in 1956 by the release of MeHg in the wastewater of the Chisso factory where Hg was used as a catalyst for the production of acetaldehyde. This disaster caused more than 1000 deaths during 36 years and nearly 2 million people suffered from a neurological syndrome, so called Minamata disease.

Although Hg natural sources such as soils, rocks, volcanoes or biomass can be involved in the remobilization of Hg at a global scale, the main source of this metal comes from the anthropogenic activities. It is the result of the heavy exploitation of Hg in mining, in metallurgical field and in the process of fossil fuels combustion. Coal combustion during the Industrial Revolution greatly enhanced the natural cycle of Hg in environmental compartments. Hg alloys easily with many metals like gold, silver and tin and these alloys are called amalgams. Since the Ancient times, the exploitation of Hg natural reservoirs as cinnabar (its mineral form, HgS) is being used for the extraction of gold in mines. Extensively used in South America by Spanish colonizers

for precious metal recovery, it is estimated that nearly 200000 tonnes of mercury were released to the environment from 1550 to 1850. [2] Nowadays, the main Hg extraction mines all around the world are located in the periphery of the Pacific Ocean (principally in Peru and Mexico) and between Mediterranean and Himalayan areas as Spain (Almadén), Italy, Slovenia, Algeria and China. [3]

Due to its chemical properties, Hg is present in all compartments, transported over long distances and accumulated in the environment, where once methylated, it biomagnifies. These facts associated to the anthropogenic pressure, which have increased concentrations in many systems, make Hg become a ubiquitous contaminant through global atmospheric, oceanic circulation and biological turnover. As a result of its severe toxicity, Hg is considered a global pollutant of major concern for humans and animals.

1.2 Mercury in aquatic systems: bioaccumulation in organisms

The presence of Hg in aquatic systems can represent an important source of pollution for the atmosphere due to volatilization of the gaseous form Hg^0, for sediments as IHg and for trophic webs through the MeHg accumulation.

Different Hg species are located among the dissolved, colloidal and particulate phases, bounded to different ligands that form a variety of complexes. In anoxic environments, sulfurs (S^{2-}) are the most abundant species and when they are absent, Hg forms complexes preferentially with the organic matter. However, chlorinated complexes ($HgCl_x^{2-x}$) and hydroxides ($Hg(OH)_x^{2-x}$) are prevalent in oxygenated slightly-eutrophic environments. This fact involves a different Hg speciation distribution between marine and freshwater systems.

1.2.1 Hg methylation by bacteria in sediments

Multiple transformations of Hg as oxidation-reduction, methylation-demethylation and photochemical reactions take place in aquatic systems. Although the main part of these transformations is influenced by biotic processes, it has been demonstrated that abiotic methylation processes are responsible for the presence of a non-negligible part of MeHg in the environment. [4] [5] [6] [7] [8] MeHg production is principally associated to sediments from estuaries and lakes, coastal zones or oceans. The percentage of MeHg in surface sediments will be useful as a proxy for methylation inputs in some environments.[3]

It is well known that biotic methylation processes of Hg in aquatic systems are mainly carried out by microorganisms such as sulphate and iron reducing bacteria (called SRB and FRB, respectively). SRB are recognized as the principal Hg methylators in anoxic environments however, not all SBR present Hg methylation capacities and the methylation yields are variable between strains. [9]

Hg metabolic methylation processes remain still unknown and it has been suggested that they could be a protective mechanism against Hg toxicity in some microorganisms.[10] In the case of SRB, Hg methylation seems to require the

activation of acetyl coenzyme A synthetase and the transfer of a methyl group via the methylcobalamin (CH_3CoB_{12}), which is a molecule able to transform the methyl group into a carbanion:

$$CH_3CoB_{12} + H_2O + Hg^{2+} \rightarrow CH_3Hg^{+} + H_2OCoB12$$

MeHg production by bacteria is influenced by many environmental factors such as pH, temperature, redox conditions and the presence of sulphates and dissolved organic matter. All of them affect the speciation and availability of mercuric ions and the activity of methylating microbial populations. Hence, each microbial organism has its own specific predispositions or mechanisms for Hg methylation.[11]

1.2.2 Bioaccumulation and biomagnification of Hg in aquatic organisms

Biomagnification is defined as the increasing concentration of a contaminant at successively higher trophic levels of a food web. Therefore it is the result of trophic transport when the absorption of the contaminants occurs faster than their depuration. [12] Heavy metals can enter cells by different routes, such as membrane transport or passive diffusion of neutral chemical species. [13]

As it has been said before, Hg is a ubiquitous environmental pollutant with severe risks for health (especially MeHg). Although extremely low Hg concentrations are found in the dissolved water phase (order of $ng \cdot L^{-1}$ or $pmol \cdot L^{-1}$, with an average between 1 and 5% of MeHg), high Hg accumulation levels are reached in aquatic systems. Bioaccumulation involves two processes: firstly, the uptake from water into biota at lower trophic levels; and secondly, the transfer between trophic levels that are influenced by chemical speciation. Moreover, Hg biomagnifies through the food chain and its main Hg exposition route for population is the continuous fish consumption. In fact, top predators can accumulate high levels of Hg, principally as MeHg (>95%). [14]

It is widely recognized that the accumulation of Hg in fish is a result of dietary intake [15] [16] and therefore, it rises with the level of the organism in the food webs. For example piscivorous fish, which are positioned at a higher trophic level, have higher accumulation rates of Hg than omnivorous and planktivorous. Hg accumulation can also be influenced by the contribution from the water via respiration. This process would depend on numerous environmental parameters which control MeHg accumulation and bioavailability in aquatic systems. [17]

Hg concentration levels in organisms are also determined by other factors such as environmental factors (pH, salinity, DOC or contamination level) or physiological conditions of different organisms (size, weight, age and assimilation capabilities) [12] Every living organism has also its own characteristics concerning the biochemical functions which could affect the uptake through the biological barriers, storage processes in tissues and cells, and depuration/excretion mechanisms. [18] This fact enhances the variety of Hg chemical species and distribution among the different compartments.

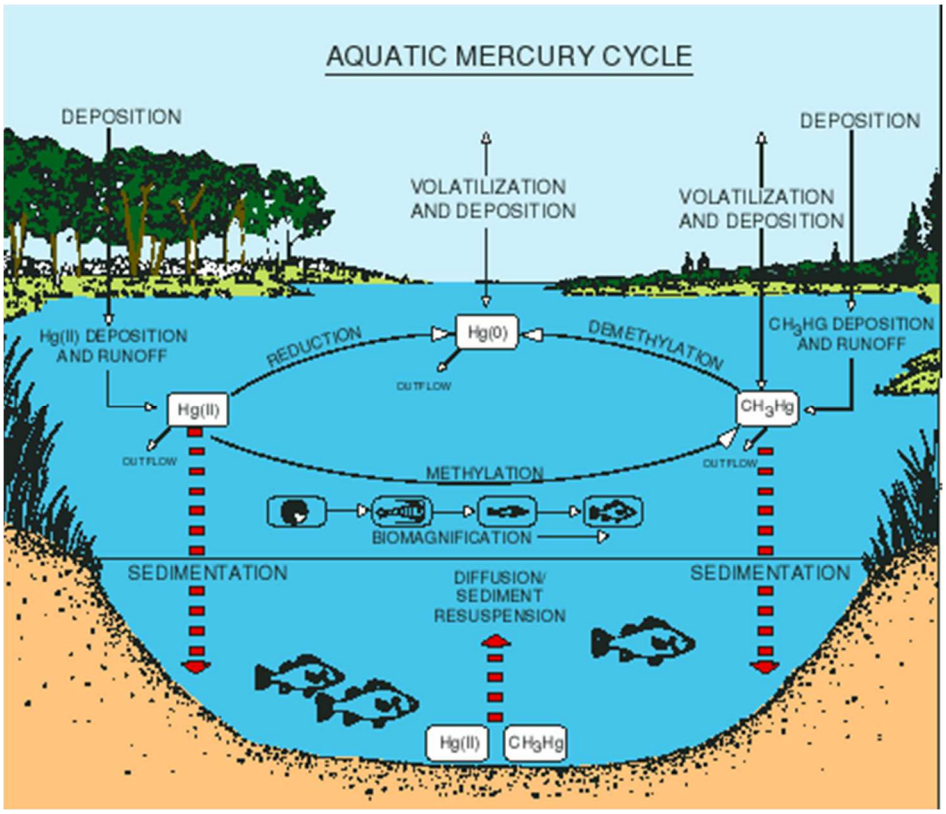

Figure 1: Scheme of Hg transformation and bioaccumulation in aquatic systems (USGS Science for a Changing World) [19]

The metabolism of Hg has not been completely understood yet but some evidences have already been established. After ingestion, Hg is transported through the blood to all organs and tissues and MeHg is the species preferentially accumulated.

Muscles are in terms of weight the largest compartment of the total body mass of fishes (>60%) [15] and it is directly associated with the risks of human contamination due to fish consumption. In addition, muscle has been considered in several studies as a significant site of Hg accumulation (especially of MeHg) because of its high storage capacities and slow depuration rates.

Brain is able to accumulate large quantities of Hg, especially in its methylated form as it occurs in skeletal muscle. This fact elucidates the neurotoxicity associated with MeHg contamination. As it was well-known in previous studies [20] [21], long-term dietary exposure of fish to MeHg cause a lack of coordination, diminished responsiveness, and swimming activity and lesions in the brain. Gonzalez et al. [13] analysed 13 genes in brain tissue of zebra fish to investigate the genetic response after MeHg dietary exposure. Despite a high level of MeHg bioaccumulation in this organ, a lack of genetic response was obtained, so they could also prove the neurotoxicity induced by MeHg.

Previous studies on marine mammals suggest that liver plays a detoxification role on Hg metabolism by demethylation of MeHg. IHg, the main Hg species in this organ, has been identified binding metalothioneins (MT), cysteine-rich proteins able to chelate and trap metals. [11] [13] [15] It has been understood as a detoxification mechanism as well as the formation of tiemannite (HgSe), an insoluble compound found in form of granules, likely to reduce Hg bioavailability.

1.2.3 Examples of Hg bioaccumulation in fishes of contaminated areas

As it has been said before, the main source of Hg contamination is the anthropogenic activity issued principally from gold mining practices and metallurgical industry.

One of the areas where several Hg contamination studies have been made is French Guiana, where the mining exploitation has been the cause of a severe pollution. Total Hg concentrations measured in Suriname River waters in this area were between 1 and 10 $ng \cdot L^{-1}$. One of the highest Hg concentration found in fish muscles in this study corresponded to the piscivorous species, and was about 6141 $ng \cdot g^{-1}$. [15]

Another example of Hg pollution is Pearl River Delta in China, attributed to sewage sludge relating to chlor-alkali industry. Levels of MeHg in this river reached 1.4 $ng \cdot L^{-1}$ and concentrations in freshwater fish ranged from 280 to 580 $ng \cdot g^{-1}$. [22]

Another study carried out in Bangladesh showed that total Hg concentrations in marine fish were generally low, 10 $ng \cdot g^{-1}$ for omnivorous fish to 370 $ng \cdot g^{-1}$ for a large predatory fish. [23]

1.3 Molecular speciation of mercury: State of the art

Coupling Gas Chromatography (GC) with Inductively Coupled Plasma Mass Spectrometry (ICP-MS) using isotope dilution analysis is a well-established method for speciation studies. It is an efficient technique to precise quantification but also to follow metabolic transformations. The first publications dealing with mercury speciation using stable isotopes were reported mainly by Hintelmann et al. [24]

Demuth and Heumann et al. [25] and Rodríguez Martín-Doimeadios et al. [26] studied the possible transformations of MeHg and IHg into elemental Hg during the derivatization step for the analysis of aqueous samples by GC. An optimization of this derivatization step (buffer solution, pH and $NaBEt_4$ concentrations) was proposed and a spiking procedure was also designed in order to ensure complete isotope equilibration between the added enriched species and the naturally occurring ones in the sample.

Monperrus et al. [27] reported a method for the simultaneous speciation analysis of tin and mercury in biological samples combining ICP-MS with the use of two different isotopically enriched species of two different elements (^{201}Hg-enriched methylmercury and ^{117}Sn-enriched tributyltin). The developed methodology was employed for the

analysis of a biological certified reference material composed of oyster tissue (CRM 710).

The use of species-specific isotopic tracers for IHg and MeHg was also used in another study concerning the simultaneous determination of the methylation and demethylation potentials of pure culture of isolated sulfate-reducing bacterial (SRB) strains using low Hg species concentration levels ^{199}IHg and ^{201}MeHg. [9]

One research study carried out by LCABIE about the identification of the biomolecules binding Hg [10] used the isotopic pattern deconvolution approaches for analysis of Hg speciation by GC-ICP-MS. This technique was confirmed to allow the quantification of both Hg species concentrations and transformation factors, as methylation and demethylation, affecting the two isotopic tracers during the analytical procedure.

Accordingly, the analysis of Hg speciation by GC-ICP-MS using species-specific spiking isotope dilution is a righteous method which provide valuable information to elucidate the metabolic pathways of Hg.

1.3.1 Introduction to isotope dilution

Isotope dilution consists on the measurement of isotope ratios in samples where the isotopic composition has been intentionally modified by the addition of a known amount of an isotopically enriched solution of the same element, what is called spike. Thanks to this method it is possible to quantify the concentration of the naturally occurring species or elements, with higher precision and accuracy. [28] [29]

Figure 2 shows the principle of this technique for an element containing two stable isotopes: *a* and *b*. The isotope *a* is clearly more abundant in the sample whereas the spike is enriched specifically in isotope *b* so that the isotope ratio in the mixture "sample + spike" will be intermediate between both, always depending on the amount of spike added and the previous concentration of the sample.

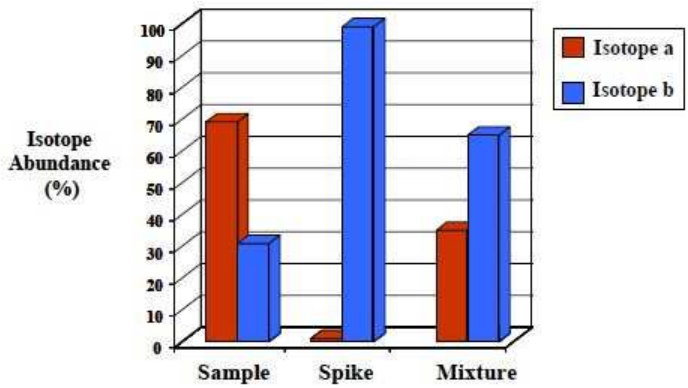

Figure 2: Principle of the technique using isotope enriched spike solutions

The isotope dilution equation can be expressed in different ways, Figure 3 details the principle of the isotope dilution technique and the equation that was used in this study.

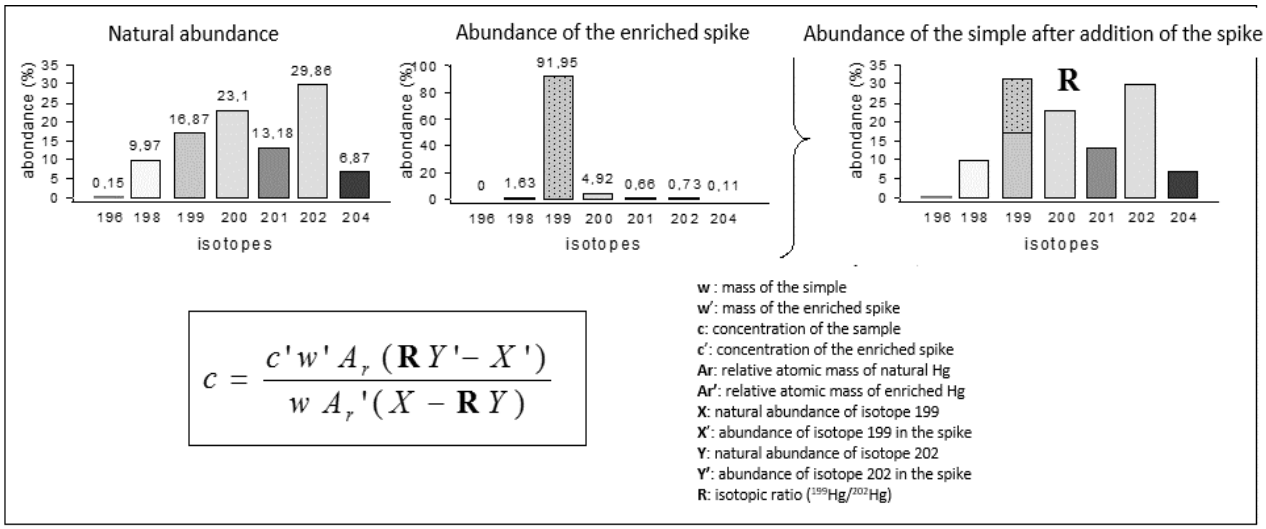

Figure 3: Explanation of isotope dilution equation used for Hg speciation

The application of isotope dilution analysis for elemental speciation is performed under species-specific spiking mode. This mode consists on the use of a spike solution containing the species to be analysed in an isotopically labelled form and requires that the composition and structure of the species is exactly known.

Several isotopes can be chosen for labelling so that each compound of interest can be enriched in a different isotope of the element. Therefore, this technique allows the determination of several species of the same element and provides new forms of quantitation and correction of species interconversion reactions.

The spike solution is added to the sample at the beginning of the analytical procedure and then, a mixture of the added enriched species and the naturally occurring ones in the sample is achieved. [28]

1.4 Isotopic fractionation

Isotope fractionation can occur during physical, chemical and biological processes. During biochemical reactions, isotopes of an element are distributed between different chemical species. In chemical reactions lighter isotopes react faster than heavier isotopes so this kinetic phenomenon has a mass-dependent nature. However, some isotopes of an element can also fractionate due to mass-independent mechanisms under specific conditions. The isotope fractionation depends on the relative mass difference between the isotopes and also on the behaviour of the element in physical processes or chemical reactions. [29]

Both mass-dependent and mass-independent isotope fractionation have been demonstrated to be an efficient tool to follow the trace of Hg processes and sources in the environment. [30]

1.4.1 Isotope fractionation: State of the art

Currently most of the measures of isotopic composition of Hg are carried out with Multicollector-Inductively Coupled Plasma Mass Spectrometer (MC-ICP-MS) able to measure the isotopic ratios with high precision and accuracy. It is the most suitable type of instrumentation which provides several advantages as sensitivity, selectivity and a high ionization efficiency. In fact, it presents the ability to analyse elements with a high ionization energy, which is the case of Hg. [29]

During the last decade, several studies have shown that Hg isotopic signatures allow the identification of Hg pollution sources in the environment. However, the application of isotopic speciation using MC-ICP-MS for metabolic studies has gained great importance in the last years.

Recent studies [31] [32] [33] have demonstrated that combining measurements of stable isotopic ratios of Hg in biological samples from marine or freshwater aquatic ecosystems can be useful to study bioaccumulation and trophic transfer of Hg. Moreover, it has been established that Hg isotope signatures in human hair provide qualitative and quantitative information on MeHg sources related to human exposure via diet. [34]

One of the objectives for research projects, including RIMNES, is to focus on the trophic chain by analysing bedrock, sediments, biofilms, fish and rice samples in order to understand Hg isotopes fractionation before absorption of MeHg by human.

The measurement of Hg stable isotopes by MC-ICP-MS is therefore an efficient and well-established method to evaluate Hg transformations in metabolism of aquatic organisms.

1.4.2 Mass-dependent fractionation (MDF)

As it was previously mentioned, several isotopes of the element can fractionate because in chemical reactions lighter isotopes react faster than the heavy ones due to the less dissociation energy of light isotopes. This phenomenon is managed by the nuclear mass isotope effect causing MDF as a result, the products of reactions are richer in light isotopes.

Processes such as diffusion, metabolic transformations or high temperature equilibrium processes involve MDF.

Isotope composition is reported using delta notation (δ) which is the per mil (‰) deviation from a reference material:

$$\delta^{xxx} = \left[\frac{(^{xxx}Hg/^{198}Hg)_{\ unknown}}{(^{xxx}Hg/^{198}Hg)_{\ SRM\ 3133}} - 1\right] * 1000$$

where xxx is the mass of each Hg isotope between ^{199}Hg and ^{204}Hg. NIST SRM 3133 is recommended as a common reference material and δ^{202}Hg is suggested as the preferred ratio for reporting MDF measurements.[30] The difference is multiplied by 1000 and expressed in units of per mil (‰) to deal it easily and facilitate laboratory comparisons.

1.4.3 Mass-independent fractionation (MIF)

Some processes origin an unusual behaviour, referred as mass-independent or "anomalous" isotope fractionations. This means that there is not a linear relationship between the range of fractionation observed and the mass difference between the isotopes considered, so one or more isotopes show an additional effect on top of the well understood mass-dependent fractionation.

This phenomenon has also been observed for some metals including Hg, but its effects are still less understood. The processes producing MIF are related with photo-reduction processes. [29]

Mercury MIF is reported using Δ which is the deviation of the measured isotope ratio from the theoretical ratio predicted by MDF. MIF is the difference between the measured δ^{xxx}Hg and the theoretically predicted δ^{xxx}Hg,

$$\Delta^{xxx}Hg \approx \delta^{xxx}Hg - (\delta^{202}Hg * \beta_{xxx})$$

where xxx is the mass of the Hg isotope and β_{xxx} is the kinetic or equilibrium fractionation factor appropriate for that isotope.

Two mass-independent mechanisms are responsible of isotope fractionation: the nuclear volume effect (NVE) and the magnetic isotope effect (MIE).

NVE depends on the nuclear volume and nuclear charge radius. As the nucleus of isotopes differ on the number of neutrons, they will have different masses, shape and size. The nucleus of nuclides with an odd number of neutrons are often smaller than expected based on the mass difference relative to those of the nuclides with an even number of neutrons. The resulting difference between the isotopes regarding to their density and shape of the electron cloud implies differences in the efficiency of their participation in chemical reactions. The NVE is negligible for light elements but becomes significant for heavy elements.

MIE is an effect of light exposure. Nuclides characterized by an odd number of protons or neutrons are characterized by a non-zero nuclear spin. The more probable cause of the large MIF observed in photochemical reactions and fish is the MIE.

The controls on expression of MIE and variable $\Delta^{199}Hg/\Delta^{201}Hg$ are not clearly understood. MIE is a complex phenomenon and depends on several factors, including hyperfine coupling, lifetime of the radical pair, coupling strength of the radical pair, spin–orbital coupling, diffusion factors, and the solvent cage (space) in which the reaction occurs. [30]

Mass independent fractionation provides the element with a very specific isotopic signature. That is the reason why it is a very interesting application to environmentally important elements as Hg, so that it can reveal the sources and conversions of such a complex biogeochemical cycle.

Large positive MIF (~5‰) signatures are observed in aquatic organisms. Due to the similarities in the $\Delta^{199}Hg/\Delta^{201}Hg$ ratio and the magnitude and direction of MIF between MeHg photodegradation experiments and MIF anomalies in fresh-water fish obtained by Bergquist and Blum [35], it was suggested that the fish signatures were derived from photochemical processing of the MeHg prior to incorporation into the food web.

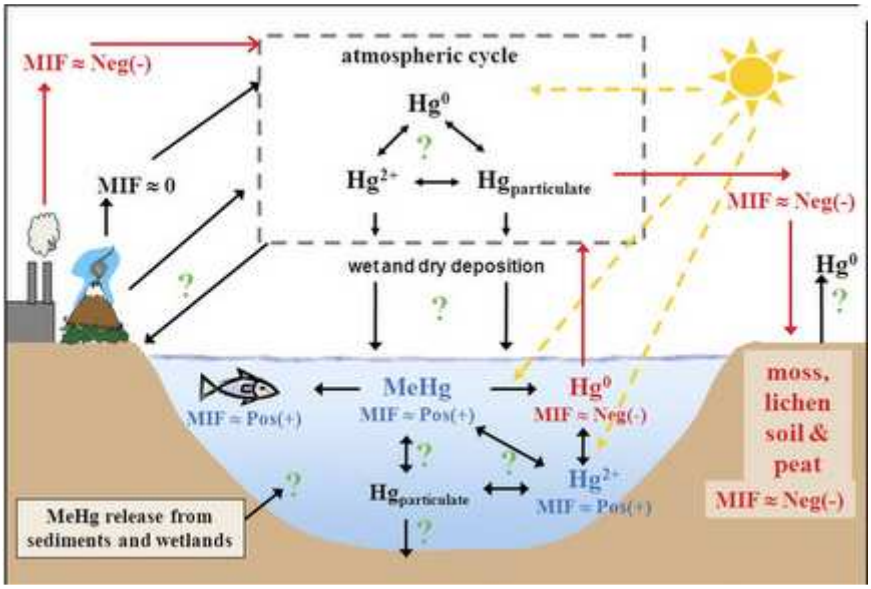

Figure 4: Hg cycle following positive and negative MIF isotopic signatures (Bergquist and Blum. 2009) [30]

Photoreduction of IHg and photodegradation of MeHg in the presence of dissolved organic matter resulted in large MIF of the odd isotopes, which were preferentially retained in the aqueous phase as IHg species.[35]

1.5 Presentation of the study "RIMNES-Experience on microcosms"

1.5.1 RIMNES project

The RIMNES project (Mercury Isotopes Fractionation and Notch/apoptosis biomarkers: new tracers linking Environment and Health) is part of the French scientific Programme CESA of ANR (Agence Nationale de la Recherche). The main objective of the whole

project RIMNES is to provide innovative techniques for geochemical and biological studies to work on an interdisciplinary problem between Environment and Health. The first aim is to understand the process of transformation of mercury in the environment. The general objectives of RIMNES are:

- to use the Hg isotope fractionations to trace and quantify the natural and anthropogenic sources in environmental compartments, from the sediment and the soil, from the aquatic and terrestrial food chain to human hair of exposed communities,
- to study Hg toxicity impact on fish and human cells by genomic and cell biology approaches, and
- to design new MeHg biomarker assays for biomonitoring applications

Two complementary scientific approaches are planned:

1. ***a "reductional approach":*** experimental studies in the laboratory by feeding adult male zebra fish (*Danio rerio sp.)* with MeHg and IHg enriched food of known Hg isotopic composition, compared with not contaminated fish, and

2. ***a field study*** with natural environmental samples collected in both impacted by Hg polluting activities and pristine areas:

    - the Oyapock River basin in French Guiana, comparing a gold-mining area (around Camopi) and a pristine one upstream, in Trois Sauts, and
    - the Guizhou Province in China, studying Wanshan characterized by Hg mining activities, Guiyang, an urban zone, and a remote area, Leigong.

1.5.2 Participants

- Géosciences Environnement Toulouse (GET-OMP)
- Environnements et Paleoenvironnements Océaniques et Côtiers (EPOC)
- Laboratoire de Chimie Analytique Bio-Inorganique et Environnement (LCABIE-IPREM)
- Laboratoire d'Aérologie (LA-OMP)
- Parc Amazonien de Guyane (PAG)
- Institute of Geochemistry, Chinese Academy of Sciences (IGCAS)

1.5.3 Presentation of the study: Investigation of the fate of IHg and MeHg in zebrafish by speciation and isotopic analyses

The experimental study described in this report takes part of RIMNES Project. The specific objective is to investigate and compare the fate of MeHg and IHg in zebra fish *(Dario rerio* sp*)* by Hg speciation and isotopic fractionation analyses. Different fish organs (liver, brain and muscle) were analyzed after dietary exposure of food enriched with mercury species (MeHg or IHg) and the experiments were conducted with different durations (0, 7, 25 and 62 days).

Figure 5: Feeding zebra fish (Source: Novartis Global)

In parallel, a second objective of this project is to better understand the mechanisms of action of Hg species in fish and in human cells. This study implies the investigation of the impact of MeHg contamination from aquarium and Oyapock river fish through genomic and cell biology approaches.

2 MATERIAL AND METHODS

2.1 Fish exposure conditions

The life conditions during the experiment consisted in keeping the fishes in different tanks of at least 1 L of oxygenated water per fish, at a temperature of 24ºC with a neon photoperiod of 12h/12h. The tanks were kept away from natural lightning in order to limit Hg photoreduction of food and faeces. The water was daily renewed with ½ of the volume changed to avoid direct contamination.

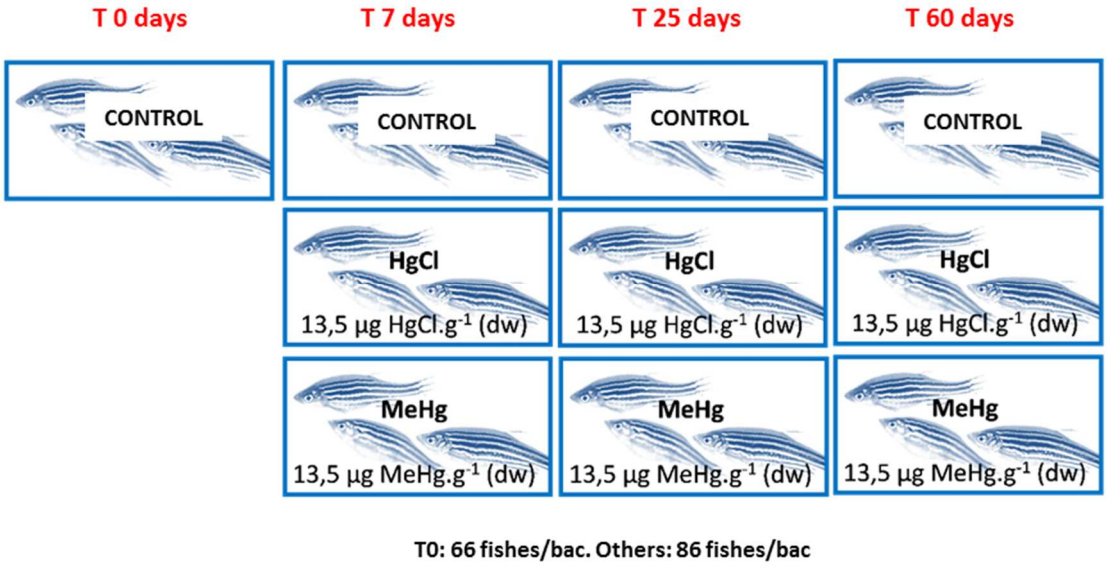

Figure 6: Scheme of the Hg-contaminated diet for Zebra fish during the experiment

For the development of the experience 66 fishes were sacrificed on day 0 (T0), 258 fishes on days 7 (T7), 25 (T25) and 62 (T62), of which 66 fishes corresponded to the different exposure conditions (control, MeHg and IHg), as described in Figure 6. Also faeces were collected at several moments during the fish exposure. The food provided to the fishes was analyzed before and after enrichment with IHg and MeHg in order to precisely characterize the Hg levels of exposure.

The specific experimental conditions are based on previous studies carried out with zebra fish, [13] an average of temperature between 23,6 ± 0,4 ºC and pH conditions between 7,8 ± 0,1. Fishes were fed with a controlled quantity of artificial food (corresponding to 3 % of the fish weight) twice a day in order to avoid stress caused by shortage of food, which could involve negative consequences on the animals and affect their genetic response.

Before all the analysis, the samples of food, organs and faeces of zebra fish were previously dried frozen.

2.2 Analysis of Total Mercury concentrations: AMA-254

Total Hg concentrations in food and organs were previously performed by the laboratory EPOC, also participant of the Project RIMNES. Total Hg contents in faeces were measured at the LCABIE.

2.2.1 AMA-254 Instrumentation

2.2.1.1 Principle

The total mercury analyzer model used in our laboratory is the AMA -254 (LECO Corp, MI, USA). This device is based on the principle of atomic absorption. It is composed of a catalyst that decomposes the solid sample by combustion in an oxygen-rich environment (850 ° C) in electronically controlled ovens. The mercury present in the sample is atomized and transported to a gold trap were Hg is caught. This mercury is desorbed from the gold trap by heating and the generated stream is transported to the detection chamber of an atomic absorption spectrophotometer where the mercury content is quantified. Thanks to the existence of two measuring cells, Hg is measured at two concentration ranges and appropriate range is used to determine the Hg concentration.

Figure 7: Mercury analyzer AMA-254 and nickel platform/boat

2.2.1.2 Analytical Procedure

A small amount of dry sample (10-20 mg), previously ground, is inserted in a nickel platform/boat (Figure 7) and weighed.

To control the accuracy of the device, a reference material was analysed. Figure 77 and Figure 8 describe the AMA-254 equipment and the operating scheme.

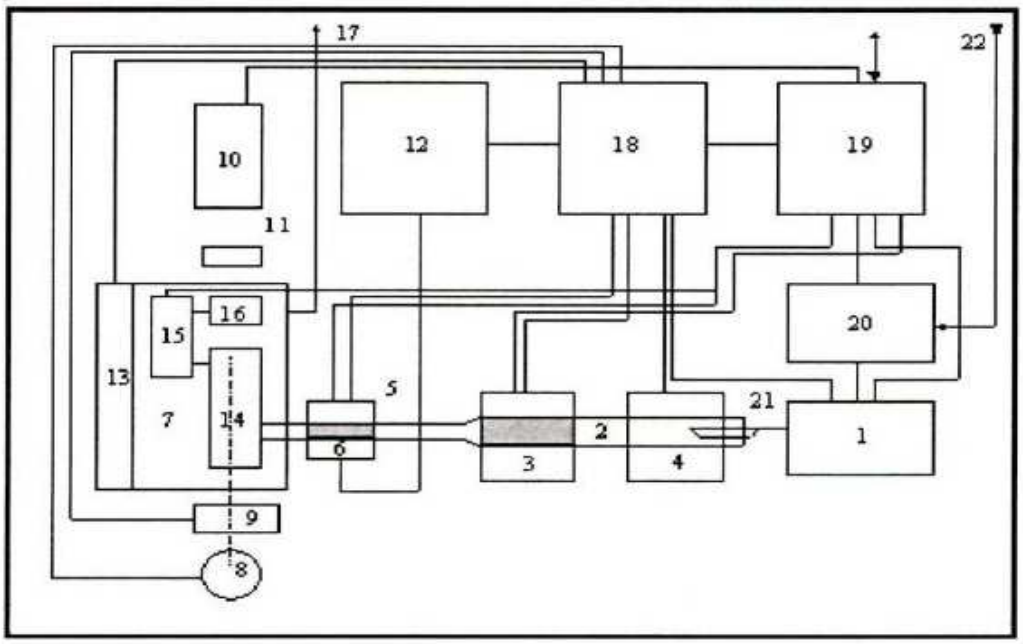

Figure 8: Scheme of the operating system of AMA-254

1. Automatic introduction system
2. Catalytic Tube
3. Catalytic oven (900 º C)
4. Decomposition oven (750 º C)
5. Amalgamator (trap gold)
6. Oven golden trap
7. Support optical cells
8. Mercury lamp
9. Shutter
10. Photomultiplier

20. Flow oxygen regulator
21. Plate for sample transport

2.3 Analysis of mercury speciation: GC-ICP-MS

2.3.1 Sample preparation

2.3.1.1 Mineralisation

In order to preserve the Hg speciation, samples were digested with Tetramethylammonium hydroxide (TMAH). The alkaline digestion is carried out by a CEM Microwave system (Discover SP-D, CEM Corporation) coupled to an autosampler Explorer 4872 96 (USA).

Samples are introduced in Pyrex vessels of 10 ml (**¡Error! No se encuentra el origen de la referencia.**) and 5 ml of 25% TMAH solution (Sigma Aldrich) are added. The extraction is carried out by 1 min of warming up to 75 ºC and 3 min at 75ºC. Sample solution is homogenised during the digestion by a magnetic stir bar.

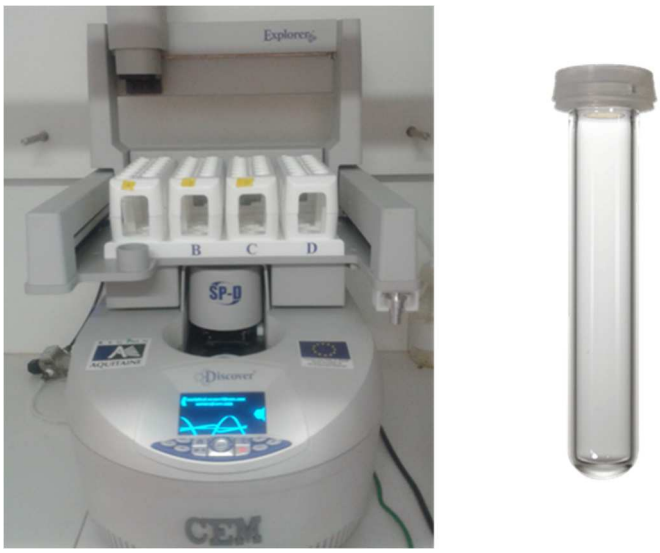

Figure 9: CEM Microwave (Discover SP-D) and CEM Pyrex vessels

2.3.1.2 Derivatization

The second step of the sample preparation for Hg speciation analyses by GC-ICP-MS consist of a derivatization reaction in order to produce ethylated or propylated forms of Hg to be separated by GC. In this study, digested samples were derivatized by propylation. Then the derivatized species are concentrated in an organic solvent (isooctane) before their injection into the GC column.

The use of enriched stable isotopes for Hg speciation to determinate the amount of methylated Hg and inorganic Hg in fish samples is based in the addition of enriched species: ^{199}Hg-enriched IHg^{2+} and ^{201}Hg-enriched $MeHg^{+\cdot}$ In this way, the natural isotopic composition of the sample is altered and this allows us to quantify the Hg species in our samples.

Quantification of Hg species was carried out by reverse isotope dilution analysis by adding the adequate amount of natural IHg and MeHg NIST standards.

Hg species were propylated by using sodium tetrapropylborate ($NaBPr_4$) and extracted in isooctane by manual shaking during five minutes. Extracts were stored at -20°C before analysis by GC-ICP-MS. The same methodology was previously performed in the laboratory for Hg speciation and species-specific isotope composition. [5][10] [27] [36][37]

Preparation of buffer (Sodium acetate) and $NaBPr_4$ is detailed in Annexes 2.

In general, samples were analyzed in triplicate except in the case of liver and brain corresponding to control conditions due to the lower mass amount and Hg concentration.

An external calibration curve was made from Hg- natural abundance standard solutions of both species, MeHg and IHg of 10 ppb in order to estimate the Hg concentration before isotopic dilution analyses.

For validation of our results fish Certified Reference Materials were analysed: ERM-CE-464, DOLT-4 and TORT-2.

Analyses of abundance and reverse standards were also performed in order to characterize our isotopically enriched spike solutions. Abundance analyses allow to determine the abundance of ^{199}IHg and ^{201}MeHg introduced in the samples. The isotopic abundance of isotopes 199 and 201 in the ^{199}IHg and ^{201}MeHg tracers was 98 and 92%, respectively. In both cases the abundance of ^{202}Hg was lower than 1.5%. The exact concentrations of ^{199}IHg and ^{201}MeHg of the working solutions were determined by reverse isotope dilution analysis. Reverse analysis are achieved using natural standards of MeHg and IHg, which are added to the spike solutions. This strategy allows us to calculate the real concentration of our spike solutions based on the isotope dilution equation.

2.3.1.3 Principle

Derivatized Hg compounds are volatilised at 250°C in the injector of the GC system and separated under helium gas flow and by the application of a temperature gradient program, as described in Table 1.

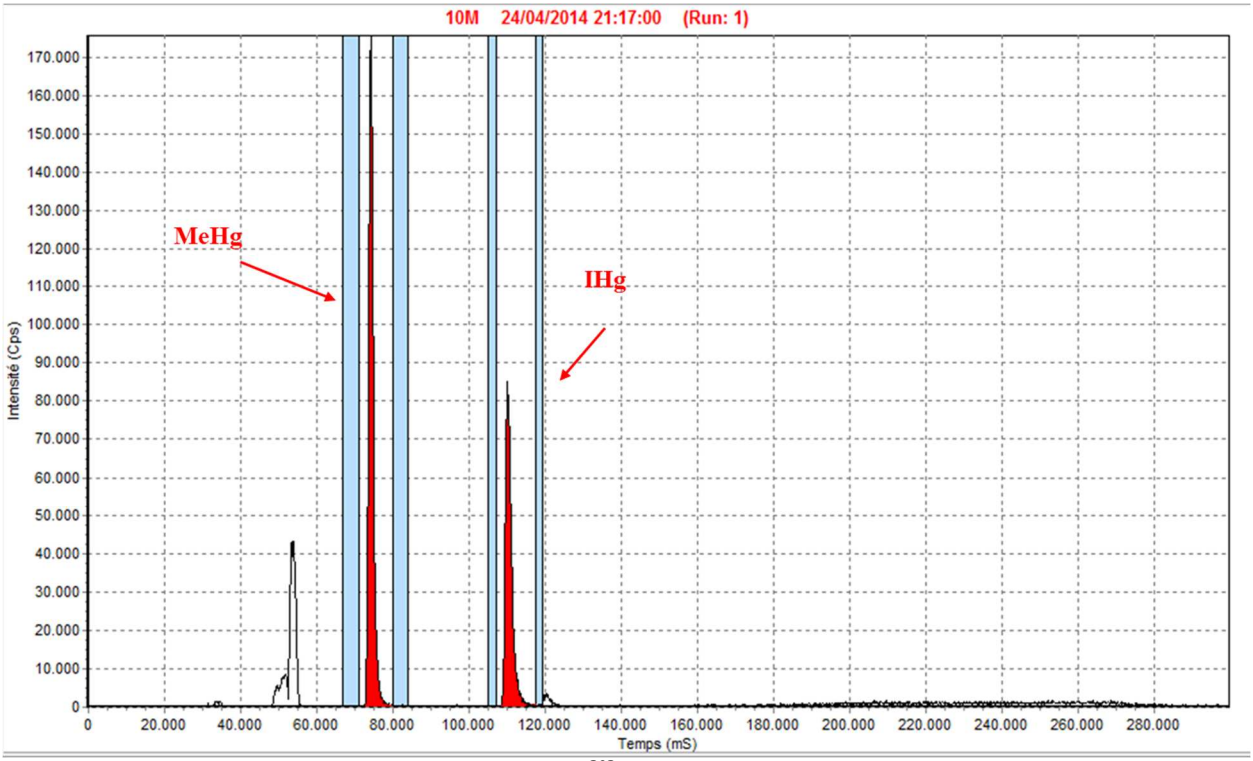

Figure 10: Example of chromatogram for isotope ^{202}Hg of a Zebra fish muscle in IHg conditions during 7 days (TST, peak integration program)

Mass bias correction during the analysis was achieved by measurement of ^{203}Tl and ^{205}Tl isotopes, from a liquid 0.5 ppb solution, simultaneously introduced during the ICP-MS acquisition.

Table 1: Operating parameters of the GC-ICPMS device

GC conditions	
Injection	2µl in Splitless mode at 250°C
Carrier gas	He (25 mL min^{-1})
Column	MXT1 Restek (30m lgth, 0.53mm ID, 1.0 µm df)
Oven temperature program	80°C (0.5min)→ 60°C/min→ 250°C (1min)
ICP-MS Detection	
Forward power	1250 W
Neb gas	Ar (0.6 L min^{-1})
Make-up gas	Ar (0.25 L min^{-1})
Isotopes monitored	^{196}Hg, ^{198}Hg, ^{199}Hg, ^{200}Hg, ^{201}Hg, ^{202}Hg, ^{204}Hg, ^{203}Tl, ^{205}Tl
Dwell time	30 ms

2.3.1.4 Equipment

GC device used for our speciation analysis was a Trace Ultra GC (Thermo Scientific, USA). Splitless injection mode was configured, because the analyte is present in very low concentration so that 100% of the sample is introduced. The ICP-MS used was a XSeries II (Thermo Scientific, USA) hyphenated to the GC system with the commercial Thermo Scientific transfer line, continuously heated at 250°C. A double inlet ICP-MS torch was used, in order to connect both GC line and a nebulisation chamber.

Figure 11: GC-ICPMS used for Hg Speciation analyses at LCABIE

2.4 Analysis of Hg isotopic composition: MC-ICP-MS

Hg isotopic composition have been performed by Cold Vapor Generation coupled to the MC-ICP-MS (Nu Instruments). For this purpose, samples were strongly digested in order to transform all Hg species under the IHg form and then to reduce them in Hg° with stannous chloride ($SnCl_2$). $SnCl_2$ and Hg vapour are transferred to the plasma for online detection.

Inorganic mercury reference material (NIST-SRM-3133) was used as bracketing standard and NIST-SRM-997 thallium solution for instrumental mass bias correction. A series of external standards (BCR-CRM-464 tuna fish, DOLT4 dogfish liver and UM Almadén) with previously reported δ and Δ values were used for method validation.

2.4.1 Sample preparation

The elemental analysis of various matrices such as environmental samples requires a prior digestion before analysis. Zebra fish samples were digested in acid using a HPA (high pressure asher, Anton Paar) mineralisation system.

Analysis Procedure

Samples are prepared in a Clean Room to avoid potential contamination. Zebra fish organs are introduced into the quartz vials (around 1 g) and 3 mL of HNO_3 are added to start the digestion. Quartz vials are covered with a quartz cap and carefully sealed with Teflon film to avoid losses of acid allowing at the same time the exit of CO_2. The quartz cup is also fixed around with Teflon film. After this step samples are ready to be introduced in the HPA. (Figure 12)

The program followed for the digestion reaches a high pressure and temperature (130 bars and 300ºC). The ramp temperature follows these steps:

Room temperature: 80ºC – 120 ºC – 300 ºC – 80 ºC
(2ºC/min) (2,5 h) (1 h)

After digestion quartz vessels are rinsed with 1 mL of ultrapure Milli-Q water twice in order to recover all the mercury. In some cases a minimum volume of H_2O_2 was added to reduce matrix effects.

Figure 12: HPA-S High Pressure Asher and Quartz vessel

2.4.2 MC-ICP-MS Instrumentation

The MultiCollector Inductively Coupled Plasma Mass Spectrometer allows to measure the slight difference between isotopic abundances.

One of the advantages of MC-ICP-MS comparing to other techniques used for getting information about isotopic composition of the elements (for example Thermal Ionization Mass Spectrometry TIMS) is its ability to analyse even elements with a high

ionization energy such as Hg thanks to the high ionization efficiency of the ICP source. Furthermore, it works at atmospheric pressure, which allows the introduction of samples in liquid or gas form.

However measuring isotope ratios with high precision requires leading with additional issues as instrumental mass bias, which is quite significant in this case.

All the samples and standards introduced into MC-ICP-MS were analysed at Hg concentration of 1 ppb in a matrix of HNO_3 (10%) and HCl (2%).

2.4.2.1 Principle

In classical introduction mode, samples are introduced in the nebulizer and transformed into an aerosol which is transported through a nebulization chamber where the smallest particles are directed to the plasma.

When the ions exit from the plasma, they are focalised firstly by a lens system, transferred to an electrostatic field and to a magnetic field. The detector is composed of several Faraday cups where ions are discriminated simultaneously with regards to its ratio m/z.

A Faraday cup (Figure 13) is a detector who measures electrical current of the ions or electrons arriving to it. The arrival of positive ions to the detector creates an induced current of electrons. Then, the signal is amplified by a preamplifier and measured by a voltmeter.

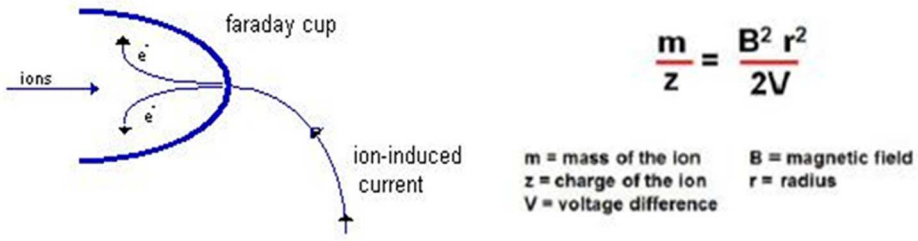

Figure 13: Scheme of mechanism of ion-current induction in a Faraday cup and equation for the quantification of ions separation with m/z ratio

Hg isotopic composition was determined by measurement of the most abundant Hg stable isotopes (^{198}Hg, ^{199}Hg, ^{200}Hg, ^{201}Hg, ^{202}Hg, and ^{204}Hg).

2.4.2.2 Parameters affecting accuracy

<u>Mass bias correction</u>

Mass bias is an important factor to consider. This phenomenon consists on a preferential transmission of certain ions usually due to two factors:

- The space charge effect: it is the main processes causing mass-bias in the instrument. Bigger charged ions will have priority comparing to the small ones because their trajectory will be more stable. For example a small positively charged ion is affected by a much bigger positive ion because the trajectory of the smaller one will also be affected by the repulsion of charges.

- Vacuum effect: at the interface a vacuum is produced by the vacuum pump that allows the ions to pass through the plasma to the spectrometer, which is at different pressure conditions. Due to this vacuum effect, bigger ions trajectory is less affected because of the higher mass. This effect can be noticed even between isotopes of the same element with consecutive masses, such as ^{13}C and ^{12}C. It is very important to correct this since minority ions (~ 1%) will be discriminated against major ions (~ 99%).

Two mass bias corrections are used simultaneously to improve the precision and accuracy of the results: internal and external correction.

In the case of internal mass-bias correction, NIST-SRM-997 thallium solution was used as internal standard for the correction of instrumental mass bias. Tl is used because its fractionation in the instrument is quite similar to Hg and it is located in the same mass range of the analyte. The solution is introduced continuously and the $^{205}Tl/^{203}Tl$ ratio is monitored. Then the instrumental mass-bias is calculated using an exponential law.

$$\beta_{Tl} = \ln\left(\frac{\left(\frac{^{205}Tl}{^{203}Tl}\right)_{measured}}{\left(\frac{^{205}Tl}{^{203}Tl}\right)_{certified}}\right) * \frac{1}{\ln\left(\frac{M_{205}}{M_{203}}\right)}$$

The external mass bias correction used is the standard-sample bracketing technique in which a certified isotopic standard is measured before and after each sample. The bracketing of the sample was made by the inorganic mercury standard NIST SRM 3133.

Another secondary standard UM-Almadén is introduced during the analysis to validate the measurements. This secondary standard is prepared with metallic Hg mined from Almadén (Spain).

Preparation of NIST-SRM-997, NIST 3133, UM and $SnCl_2$ is detailed in Annexes 3.

2.4.2.3 Equipment

A Nu Plasma HR MC-ICP-MS (Nu Instruments, UK) was employed for isotopic analysis. A cold vapor generator was hyphenated to produce efficient Hg vapor flow. A DSN Desolvating Nebuliser System was used for the introduction of Tl standard. A

solution of $SnCl_2$ (3%) is used as a reducing agent and mixed online with Hg standards and samples to generate volatile elemental Hg.

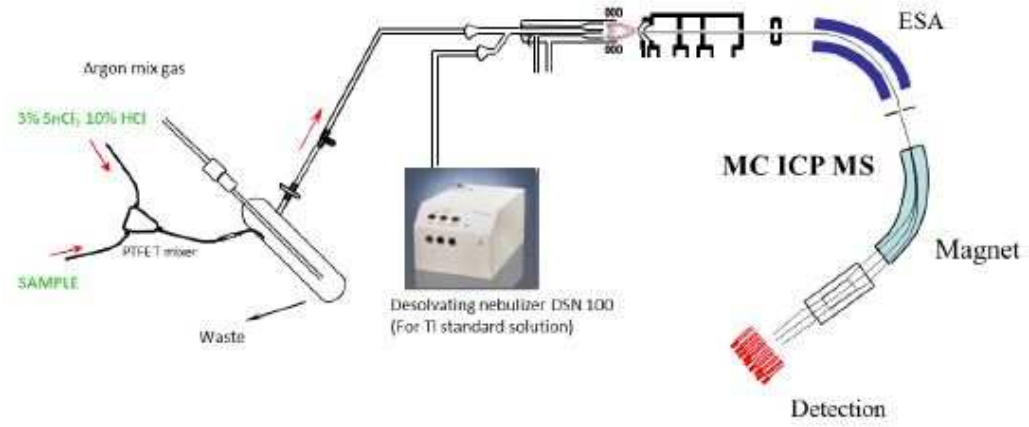

Figure 14: Scheme of the MC-ICP-MS operating system with Tl introduction (Desolvation nebulizer DSN 100)

Figure 15: Nu Plasma MC-ICPMS used for isotopic analyses (Nu Instruments Limited, Wrexham UK)

3 RESULTS

3.1 Hg concentrations

As it was said previously, total Hg concentrations in food and organs were performed by the laboratory EPOC, participant in Project RIMNES. Total Hg contents in faeces were measured at the LCABIE.

All the samples were dry-frozen before all the analysis, therefore the concentrations obtained are expressed in $ng \cdot g^{-1}$ of dry weight.

Total Hg concentrations in water are shown in Table 2. Total Hg concentrations in food, organs and faeces are shown in Table 3.

Concerning the amount of total Hg concentrations in the water contained in the aquariums, we can observe a progressive increase of Hg concentrations with time for all the conditions. However, a much higher concentration of Hg is perceived under MeHg and IHg dietary exposure conditions comparing to the control. The higher concentration of Hg is achieved under MeHg conditions after 62 days of experiment and corresponds to more of twice the concentration obtained under IHg conditions at the same period.

As expected Hg level increases with the exposure time in the analysed organs of animal exposed to IHg and MeHg, meanwhile it remains stable in control conditions. The highest values in brain, muscle and liver corresponds to a dietary exposure to MeHg. On the contrary, on faeces the highest values corresponds to the ingestion of IHg.

The distribution of Hg among the organs was different depending on the speciation in the food pellets. Liver was the target organ when zebra fish were exposed to IHg, followed by brain and muscle, reaching up to 3756±282 ng g^{-1} after 62 days. In contrast, brain of animals exposed to MeHg exhibits the highest Hg concentration. After 62 days of incubation, the level in this organ reaches 113850±42318 $ng \cdot g^{-1}$, which is 1.8 and 3.8 times higher than the values at this time for liver and muscle, respectively. A similar distribution trend (brain>muscle>liver) is observed in the “control” animals.

In contrast, faeces show a different tendency comparing to the fish organs. Concentrations on total Hg under IHg-enriched food are almost twice higher than the total Hg amount present under MeHg contamination.

No results are presented for Hg concentrations in faeces corresponding to control conditions. Considering the low concentration and the reduced amount of samples the priority was given to analysis of Hg isotopic composition.

Table 2: Results of total mercury (THg) concentrations in water during experiment.

CONDITION		Time of esposure (days)	THg ($ng \cdot L^{-1}$)		
Control conditions	WATER	7	1.4	±	0.1
		25	1,2	±	0,3
		62	3,1	±	0.8
MeHg conditions	WATER	7	35,4	±	0.6
		25	60	±	2
		62	176	±	5
IHg conditions	WATER	7	15	±	1
		25	54	±	1
		62	75	±	3

Table 3: Results of total mercury (THg) concentrations in food, muscle, liver, brain and faeces of *Danio rerio* during experiment.

CONDITION		Time of esposure (days)	THg ($ng \cdot g^{-1}$)		
Control conditions	FOOD		52	±	1
	MUSCLE	0	363	±	5
		7	368	±	3
		25	372	±	9
		62	578	±	72
	LIVER	0	216	±	31
		7	178	±	19
		25	216	±	21
		62	355	±	16
	BRAIN	0	405	±	120
		7	313	±	50
		25	378	±	83
		62	628	±	114
	FAECES	7		ND	
		25		ND	
		62		ND	
MeHg conditions	FOOD		13654	±	348
	MUSCLE	0	363	±	5
		7	7544	±	300
		25	23518	±	2388
		62	32630	±	1651
	LIVER	0	216	±	31
		7	7281	±	139
		25	42413	±	4665
		62	63995	±	10730
	BRAIN	0	405	±	120
		7	15087	±	2273
		25	40657	±	970
		62	113850	±	42318
	FAECES	7	2462	±	212
		25	12591	±	2442
		62	14095	±	2678

IHg conditions	FOOD		13588	±	731
	MUSCLE	0	363	±	5
		7	481	±	1
		25	583	±	28
		62	788	±	64
	LIVER	0	216	±	31
		7	2229	±	303
		25	1950	±	232
		62	3756	±	282
	BRAIN	0	405	±	120
		7	625	±	35
		25	1123	±	132
		62	2908	±	132
	FAECES	7	18722	±	3217
		25	33785	±	2359
		62	25358	±	1232

Results expressed as mean value±SD (n= 3, 3 organisms pooled).SD means standard deviation. ND means not determined.

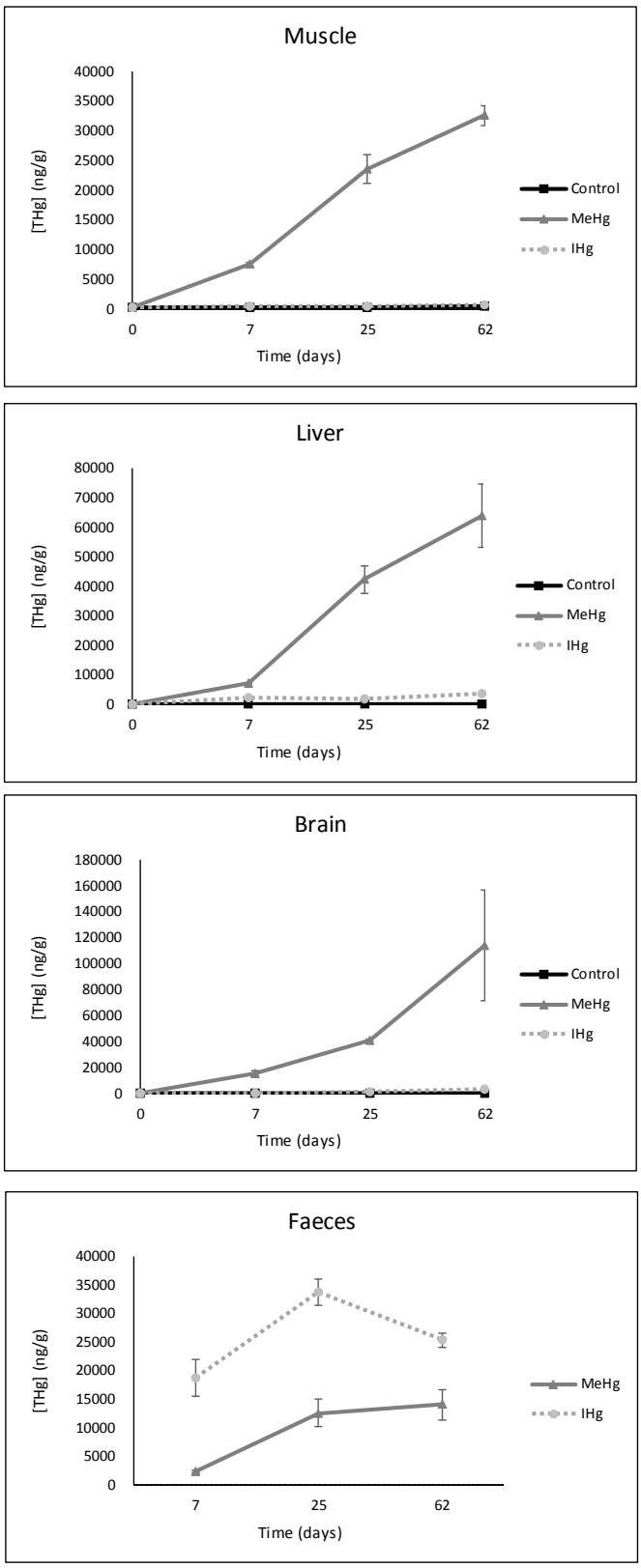

Figure 16: Time course evolution of THg concentrations in muscle, liver, brain and faeces of *Danio rerio* for the 3 experimental conditions.

3.2 Hg speciation

Hg speciation (GC-ICP-MS) analyses were performed in muscle, brain, liver and faeces of zebra fish, as well as in the food provided to the animals and in the water of the aquariums. In general, the analysed samples correspond to a pool of three individuals.

The method was validated by the analyses of fish's organs and tissues reference materials: TORT-2, DOLT-4, ERM-CE-464. (Table 4)

Table 4: MeHg concentrations (as Hg ng g^{-1}) in Certified Reference Materials CRM

CRM	MeHg Experimental value, as Hg ($ng \cdot g^{-1}$)	MeHg Certified value, as Hg ($ng \cdot g^{-1}$)
TORT-2	147 ± 3	152 ± 13
DOLT-4	1196 ± 24	1330 ± 120
ERM-CE-464	4403 ± 183	5500 ± 170

Results expressed as mean value±SD. SD means standard deviation.

In Table 5 total Hg concentration in aquarium's water is shown. As it can be observed, concentration increases with the incubation time. Regarding Hg speciation, the percentage of MeHg in water slightly increases on control and MeHg exposure conditions and it decreases noticeably on IHg ones.

Table 5. Hg species concentration in water of the different aquariums

Condition	time	MHg ng L^{-1}	IHg ng L^{-1}	THg ng L^{-1}	MHg/THg %
control	7	0.4±0.1	1.1±0.1	1.4±0.1	29
	25	0.3±0.1	0.9±0.2	1.2±0.3	26
	62	1.6±0.5	1.7±0.2	3.1±0.8	52
MeHg	7	28±1	8.1±0.4	35.4±0.6	77
	25	49±2	10.2±0.1	60±2	83
	62	155±7	21±2	176±5	88
IHg	7	2.4±0.4	13±1	15±1	16
	25	1.1±0.1	53±1	54±1	2
	62	1.3±0.3	74±3	75±3	2

Results expressed as mean value±SD (n= 9).SD means standard deviation.

Hg concentration in the food pellets (Table 7) before enrichment was 52±16 $ng \cdot g^{-1}$, principally constituted by MeHg (80%). The addition of IHg and MeHg results on total Hg concentration of 13000 $ng \cdot g^{-1}$, where MeHg represent 1 % and 95%, respectively.

Bioaccumulation factor (BAF) was calculated in the different organs (Table 6) as the ratio of total Hg concentration in the organ to the total Hg concentration in the diet. The

extent of Hg bioaccumulation is noticeable different depending on the feeding conditions, being much higher when exposed to MeHg. Under MeHg conditions the highest bioaccumulation take place in the brain followed by liver. In contrast, the exposure to IHg leads to a higher accumulation in liver than brain. In both cases muscle exhibit the lowest accumulation.

Table 6: Bioaccumulation factor for the different organs under IHg and MeHg enriched diet conditions at different sampling times

Organ	0 days		7 days		25 days		62 days	
	MeHg	IHg	MeHg	IHg	MeHg	IHg	MeHg	IHg
MUSCLE	0.02	0.02	0.55	0.03	1.72	0.04	2.39	0.06
LIVER	0.01	0.02	0.53	0.16	3.12	0.14	4.69	0.28
BRAIN	0.03	0.03	1.10	0.05	2.98	0.08	8.34	0.21

Table 7 and Figure 17 show Hg species concentrations ($ng \cdot g^{-1}$) in muscle, liver, brain and faeces under different dietary conditions after 0, 7, 25 and 62 days of exposure. Under control conditions, MeHg was the principal species in all the analysed organs. The highest values are observed in muscle, where it represents more than 90%. In liver and brain the Hg species distribution is quite similar, constituted by approximately 70% of MeHg.

Diet enrichment on MeHg leads to an increase of such species with the incubation time in all the analysed organs. It represents the main species in all cases (higher than 90%), being preferentially accumulated in brain followed by liver. Under this diet condition, Hg concentration in faeces is much lower than in the analysed organs, being excreted as MeHg.

Concerning IHg diet enrichment, the concentration of this species increases in all the organs with the exposure time. In contrast to MeHg conditions, the distribution of IHg and MeHg changes over the time, and IHg becomes the main species in all the organs. MeHg is progressively reduced to 50% and 10% of the total Hg concentration in muscles and brain, respectively, after 62 days. In liver, the organ which accumulates the highest amount of Hg under IHg dietary conditions (3568±262 $ng \bullet g^{-1}$ at 62 days), the percentage of MeHg decreases sharply from 76% to 7% after 7 days of exposure, remaining constant until the end of the experiment. Hg is almost exclusively excreted as IHg at concentration levels approximately 10 times higher than in the fish analysed organs.

It should be noticed that MeHg concentration under IHg-enriched diet have similar values to the control for all the organs, likely related to the MeHg contained in the (control) pellets.

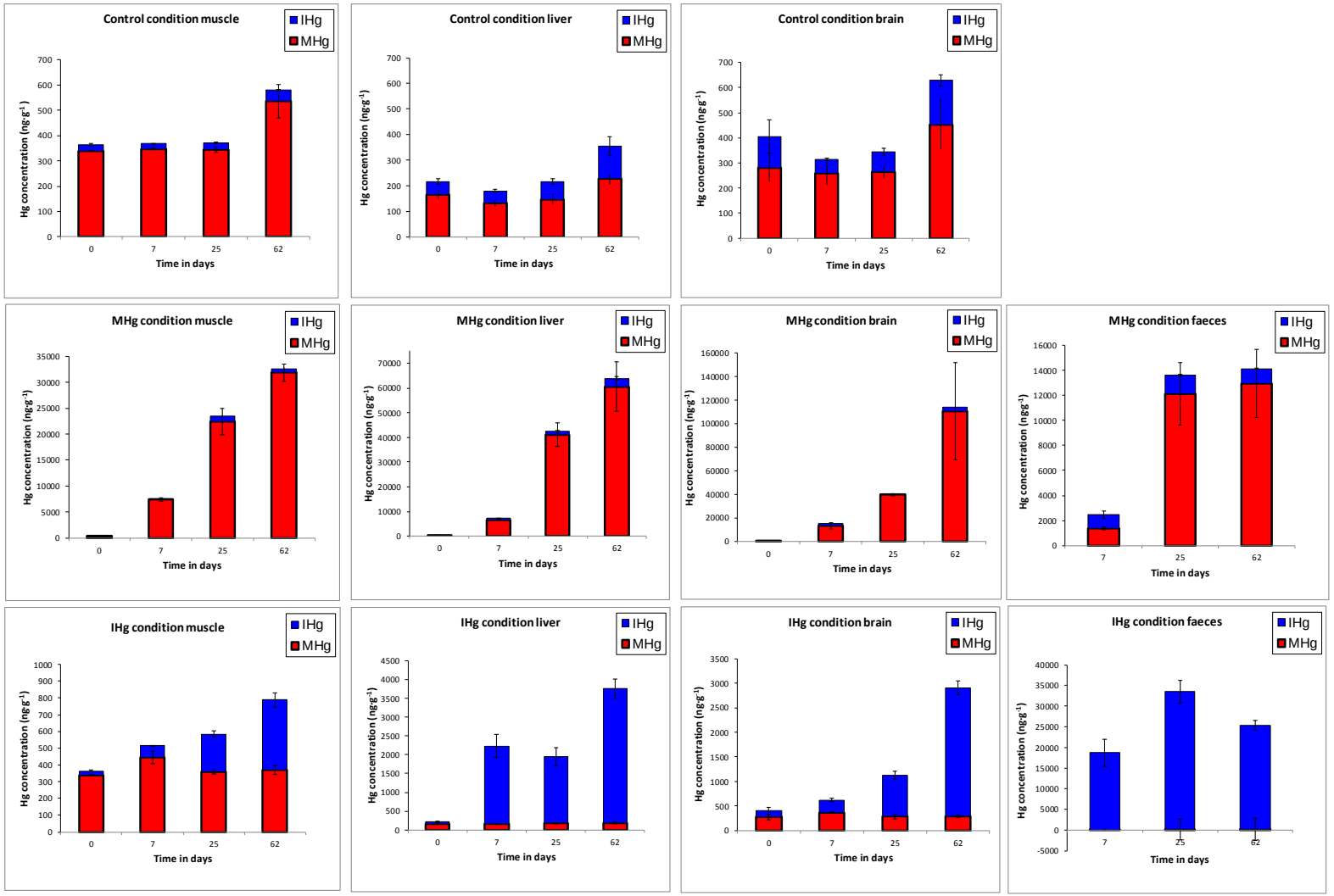

Figure 17: Hg species concentrations (ng·g^{-1}) in muscle, liver, brain and faeces under different dietary conditions after 0, 7, 25 and 62 days of exposure.

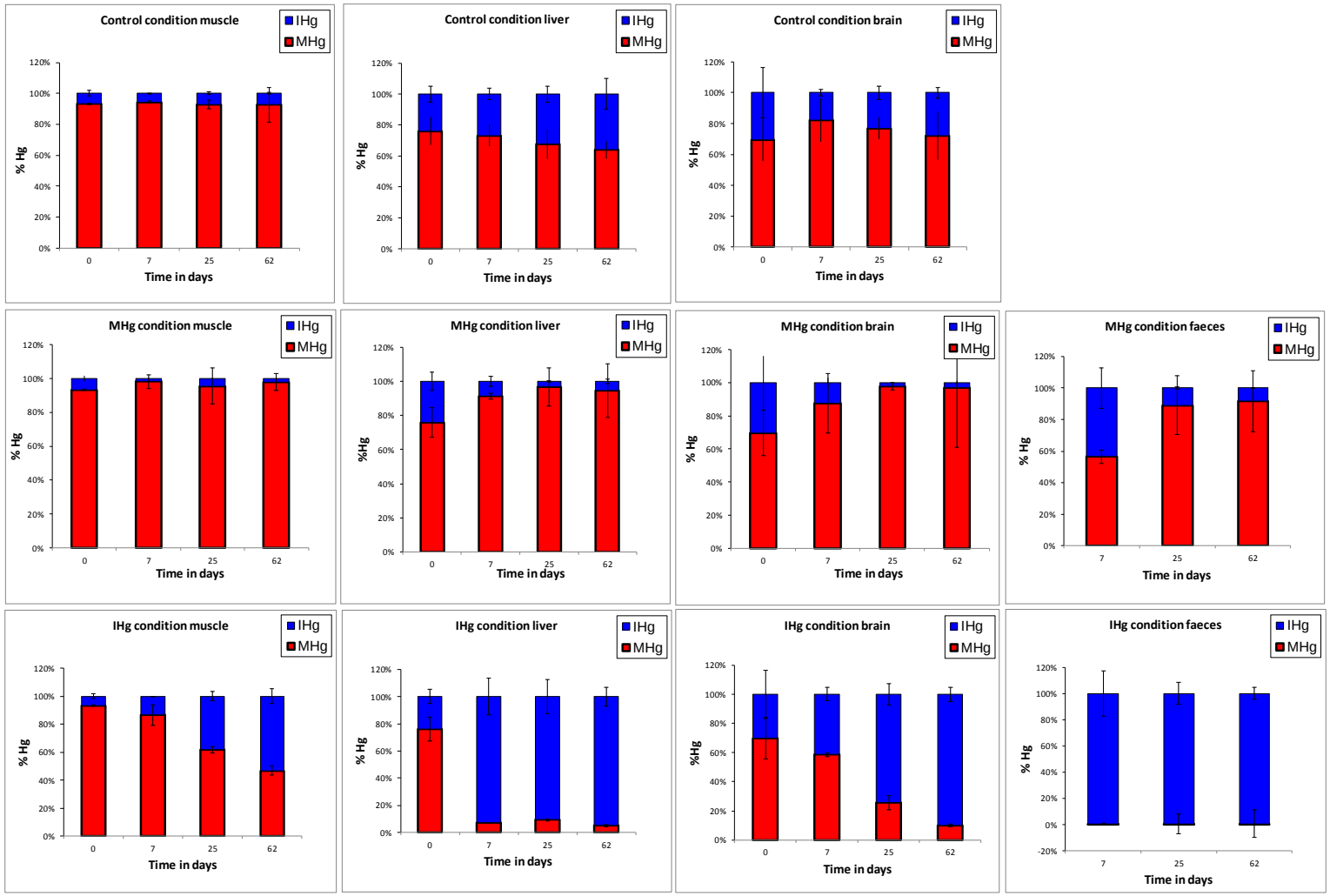

Figure 18: Hg species distribution expressed as % of total Hg in muscle, liver, brain and faeces under different dietary condition after 0, 7, 25 and 62 days of exposure.

3.3 Hg isotopic composition

Hg isotopic composition was determined by measurement of the most abundant Hg stable isotopes (^{198}Hg, ^{199}Hg, ^{200}Hg, ^{201}Hg, ^{202}Hg, and ^{204}Hg) by cold-vapor MC-ICP-MS. Mass dependent fractionation (MDF) is reported as δ^{202} Hg in permil (‰) related to NIST SRM 3133 and Mass Independent fractionation (MIF) is reported as Δ^{199}Hg. The standard deviations are expressed as 2SD.

Regarding Hg isotopic composition in control organisms (Table 7), MDF and MIF values of the control food (δ^{202} Hg: 0.08 ± 0.15, Δ^{199}Hg: 1.13 ± 0.12) are preserved on muscle, liver and brain despite the increasing of total Hg at 62 days. Much lighter δ^{202} Hg values (approx. -2.5‰) are observed on the faeces. Regarding MIF in the excretion, Δ^{199}Hg values are also lighter (approx. -0.8 ‰) than in the food pellets.

In the MeHg spiked food experiment (Figure 21), MDF and MIF values of the different organs and faeces quickly shift (7 days) to the values of the spiked food (δ^{202} Hg: -0.84 ± 0.11, Δ^{199}Hg: 0.03 ± 0.07). The isotopic composition is preserved during the rest of the exposure period.

Regarding organisms exposed to IHg the isotopic signature in muscle and brain samples during the first 7 days of exposure remains stable. A decrease of both MDF and MIF values on the mentioned samples is observed after 25 days of incubation but even at 62 days the isotopic signature in such organs is higher than in the spiked food. In the case of liver, the isotopic composition exhibits a different trend than in muscle and brain. The lowest δ^{202} Hg (-0.79 ± 0.07) value is observed after 7 days of exposition, The MIF trend also differs than the other organs of animals grows under the same conditions. In this case there is a sharply decrease (approx.-1.2 ‰) of the initial Δ^{199}Hg value. Concerning the Hg isotopic composition of the faeces, it remains almost constant from 7 to 62 days, exhibiting the same values of the supplied IHg-enriched food.

Table 7: Results of MeHg, IHg and THg concentrations (ng·g-1), δ^{202} and Δ^{199} (‰) values obtained in skeletal muscle, liver, brain and faeces of zebra fish after 0, 7, 25 and 62 days of different contaminated conditions (control, MeHg-food and IHg-food) and food concentrations.

CONDITION		Time of esposure (days)	MMHg (ng·g^{-1})	IHg (ng·g^{-1})	THg (ng·g^{-1})	THg by AMA-254 (ng·g^{-1})	% MeHg	δ^{202} (‰)	Δ^{199} (‰)
Control conditions	FOOD		42 ± 1	10 ± 1	52 ± 1	60 ± 10	80 ± 2	0,08 ± 0,15	1,13 ± 0,12
	MUSCLE	0	339 ± 2	24 ± 6	363 ± 5	363 ± 5	93 ± 2	0,12 ± 0,12	1,28 ± 0,10
		7	347 ± 3	21 ± 1	368 ± 3	368 ± 3	94,3 ± 0,1	0,23 ± 0,13	1,24 ± 0,05
		25	344 ± 10	27 ± 3	372 ± 9	372 ± 9	93 ± 1	0,23 ± 0,07	1,25 ± 0,08
		62	536 ± 65	43 ± 3	578 ± 72	578 ± 72	93 ± 1	0,10 ± 0,16	1,40 ± 0,04
	LIVER	0	164 ± 19	52 ± 12	216 ± 31	149 ± 17	76 ± 2	-0,04 ± 0,16	1,16 ± 0,06
		7	130 ± 13	48 ± 7	178 ± 19	171 ± 20	73 ± 1	0,07 ± 0,16	1,13 ± 0,06
		25	146 ± 21	70 ± 12	216 ± 21	168 ± 9	68 ± 6	0,02 ± 0,16	1,08 ± 0,06
		62	226 ± 20	129 ± 36	355 ± 16	271 ± 20	64 ± 9	-0,06 ± 0,16	1,17 ± 0,06
	BRAIN	0	281 ± 55	123 ± 66	405 ± 120	286 ± 38	70 ± 10	0,08 ± 0,16	1,08 ± 0,06
		7	257 ± 44	56 ± 6	313 ± 50	213 ± 9	82 ± 1	ND	ND
		25	265 ± 25	79 ± 14	378 ± 83	279 ± 16	70 ± 13	0,15 ± 0,16	1,04 ± 0,06
		62	453 ± 97	175 ± 22	628 ± 114	481 ± 67	72 ± 3	-0,01 ± 0,16	1,16 ± 0,06
	FAECES	7	ND	ND	ND	ND	ND	-1,16 ± 0,16	0,26 ± 0,06
		25	ND	ND	ND	ND	ND	-3,99 ± 0,16	0,37 ± 0,06
		62	ND	ND	ND	ND	ND	-2,46 ± 0,16	0,67 ± 0,06

CONDITION		Time of esposure (days)	MMHg ($ng \cdot g^{-1}$)	IHg ($ng \cdot g^{-1}$)	THg ($ng \cdot g^{-1}$)	THg by AMA-254 ($ng \cdot g^{-1}$)	% MeHg	δ^{202} (‰)	Δ^{199} (‰)
MMHg conditions	FOOD		12936 ± 298	719 ± 50	13654 ± 348	11580 ± 450	0,0 ± 0,0	-0,84 ± 0,11	0,03 ± 0,07
	MUSCLE	0	339 ± 2	24 ± 6	363 ± 5	295 ± 38	93 ± 2	0,12 ± 0,12	1,28 ± 0,10
		7	7398 ± 290	146 ± 10	7544 ± 300	7490 ± 476	98 ± 2	-0,93 ± 0,16	0,11 ± 0,05
		25	22430 ± 2498	1088 ± 110	23518 ± 2388	22576 ± 4606	95 ± 1	-0,87 ± 0,11	0,07 ± 0,04
		62	31869 ± 1638	762 ± 193	32630 ± 1651	28777 ± 3739	98 ± 1	-0,89 ± 0,14	0,05 ± 0,09
	LIVER	0	164 ± 19	52 ± 12	216 ± 31	149 ± 17	76 ± 2	-0,04 ± 0,16	1,16 ± 0,06
		7	6655 ± 126	626 ± 209	7281 ± 139	12528 ± 2181	91 ± 3	-0,79 ± 0,16	0,06 ± 0,07
		25	41097 ± 4743	1376 ± 231	42413 ± 4665	30979 ± 4980	97 ± 1	-0,96 ± 0,09	0,08 ± 0,03
		62	60591 ± 10005	3404 ± 801	63995 ± 10730	46703 ± 6825	95 ± 1	-1,05 ± 0,08	0,02 ± 0,04
	BRAIN	0	281 ± 55	123 ± 66	405 ± 120	286 ± 38	70 ± 10	0,08 ± 0,16	1,08 ± 0,06
		7	13216 ± 2728	1871 ± 455	15087 ± 2273	11565 ± 2444	88 ± 5	-0,86 ± 0,30	0,13 ± 0,07
		25	39851 ± 867	806 ± 172	40657 ± 970	42267 ± 11127	98 ± 10	-0,90 ± 0,19	0,09 ± 0,08
		62	110653 ± 41090	3197 ± 1228	113850 ± 42318	82426 ± 4957	97,2 ± 0,4	-0,94 ± 0,19	0,08 ± 0,05
	FAECES	7	1390 ± 102	1072 ± 314	2462 ± 212	1948 ± 42	56 ± 3	-0,53 ± 0,16	0,07 ± 0,06
		25	12121 ± 2509	1470 ± 66	12591 ± 2442	14346 ± 2791	96 ± 5	-0,87 ± 0,16	0,01 ± 0,06
		62	12936 ± 2717	1159 ± 58	14095 ± 2678	18913 ± 2321	92 ± 4	-0,89 ± 0,16	0,11 ± 0,06

CONDITION		Time of esposure (days)	MMHg ($ng \cdot g^{-1}$)	IHg ($ng \cdot g^{-1}$)	THg ($ng \cdot g^{-1}$)	THg by AMA-254 ($ng \cdot g^{-1}$)	% MeHg	δ^{202} (‰)	Δ^{199} (‰)
IHg conditions	FOOD		128 34	13461 311	13588 731	11920 540	0,0 ± 0,0	-0,54 ± 0,13	-0,049 ± 0,005
	MUSCLE	0	339 ± 2	24 ± 6	363 ± 5	295 ± 38	93 ± 2	0,12 ± 0,12	1,28 ± 0,10
		7	444 ± 38	69,6 ± 0,3	481 ± 1	424 ± 41	92,4 ± 0,1	0,13 ± 0,11	1,39 ± 0,12
		25	359 ± 13	224 ± 19	583 ± 28	562 ± 33	62 ± 2	-0,14 ± 0,14	0,80 ± 0,01
		62	369 ± 27	419 ± 43	788 ± 64	780 ± 35	47 ± 2	-0,22 ± 0,12	0,60 ± 0,09
	LIVER	0	164 ± 19	52 ± 12	216 ± 31	149 ± 17	76 ± 2	-0,04 ± 0,16	1,16 ± 0,06
		7	162 ± 7	2067 ± 304	2229 ± 303	2881 ± 387	7 ± 1	-0,79 ± 0,07	0,10 ± 0,01
		25	179 ± 19	1771 ± 245	1950 ± 232	1079 ± 165	9 ± 2	-0,42 ± 0,10	0,13 ± 0,10
		62	187 ± 32	3568 ± 262	3756 ± 282	3457 ± 1069	5 ± 1	-0,22 ± 0,08	0,03 ± 0,13
	BRAIN	0	281 ± 55	123 ± 66	405 ± 120	286 ± 38	70 ± 10	0,08 ± 0,16	0,07 ± 0,06
		7	365 ± 7	260 ± 29	625 ± 35	432 ± 83	58 ± 2	0,29 ± 0,16	0,83 ± 0,06
		25	288 ± 53	835 ± 81	1123 ± 132	1013 ± 269	26 ± 2	0,04 0,16	0,28 ± 0,06
		62	296 ± 26	2612 ± 140	2908 ± 132	3028 ± 99	10 ± 1	-0,16 0,16	0,08 ± 0,06
	FAECES	7	77 ± 7	18645 ± 3224	18722 ± 3217	19884 ± 1926	0,4 ± 0,1	-0,60 ± 0,16	0,01 ± 0,06
		25	174 ± 31	33300 ± 2818	33785 ± 2359	31906 ± 1884	0,5 ± 0,1	-0,64 ± 0,16	-0,06 ± 0,06
		62	186 ± 59	25172 ± 1173	25358 ± 1232	23383 ± 1994	0,7 ± 0,2	-0,49 ± 0,16	-0,04 ± 0,06

Hg concentration results expressed as mean value±SD. MDF and MIF results expressed as mean value±2SD (n= 3, 3 organisms pooled).SD means standard deviation. ND means not determined.

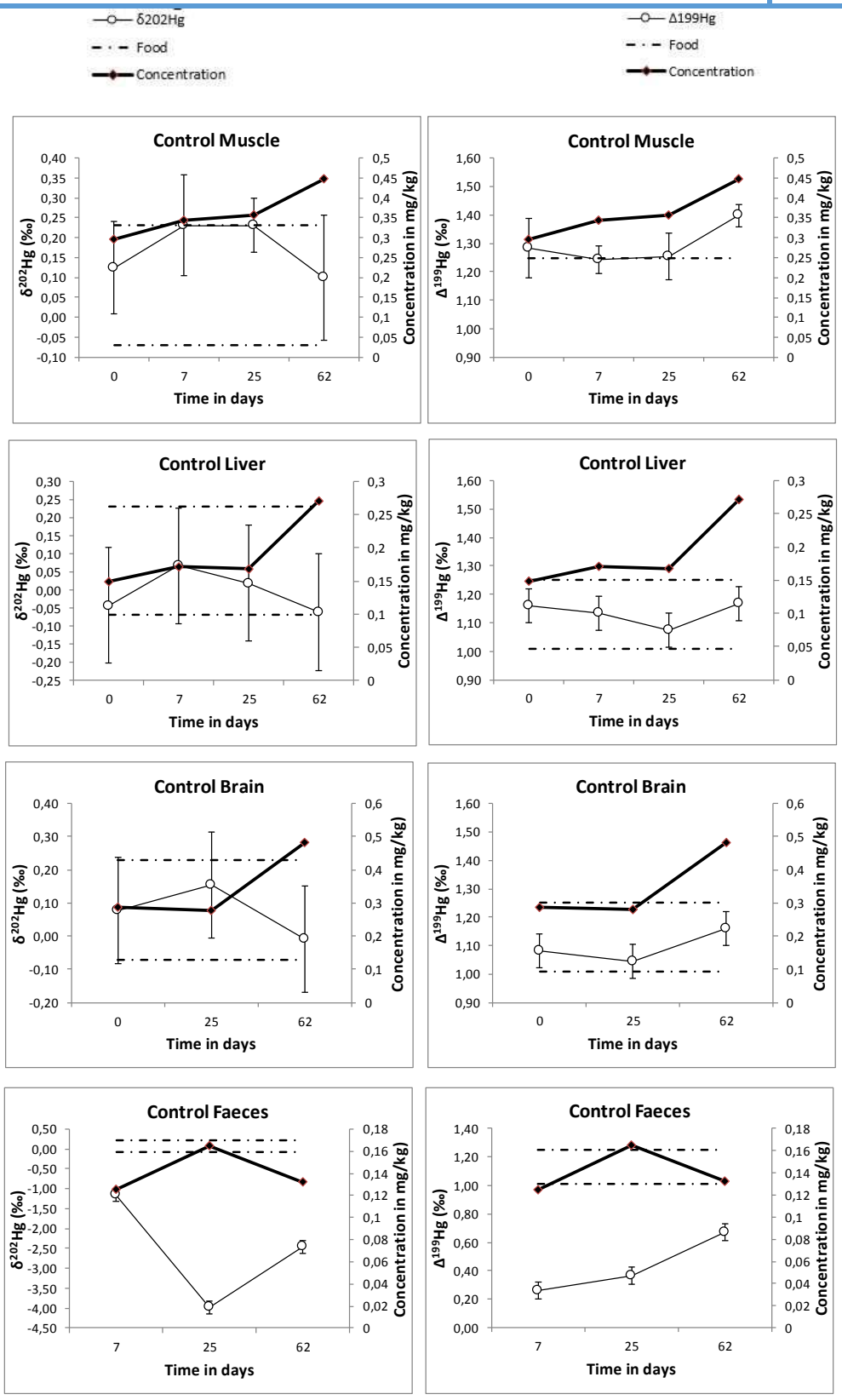

Figure 19: MDF (left panel) and MIF (right panel) variations in control samples. Dash lines represent the isotopic composition of the control food.

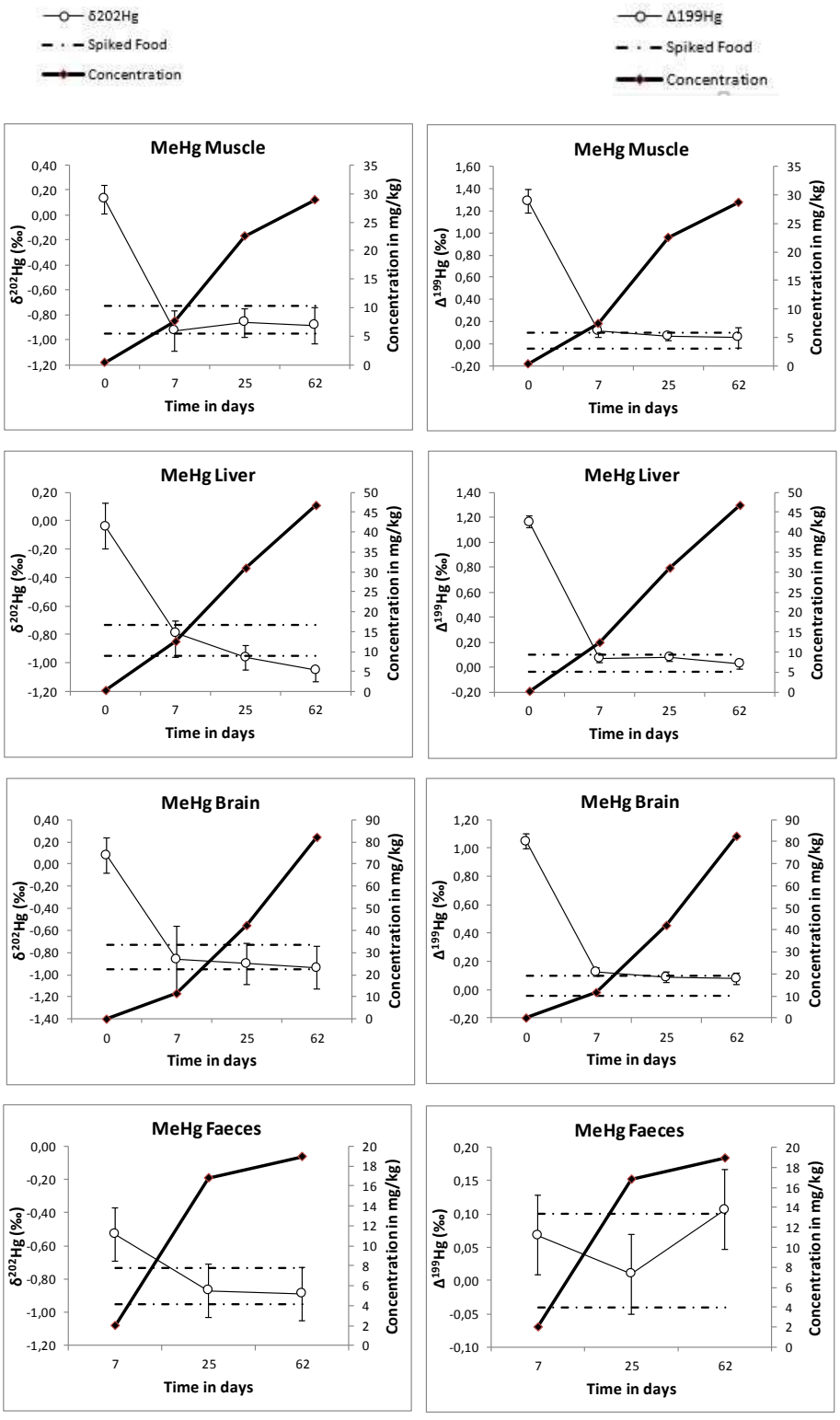

Figure 20: MDF (left panel) and MIF (right panel) variations during MeHg incubations. Dash lines represent the isotopic composition of MeHg spiked food.

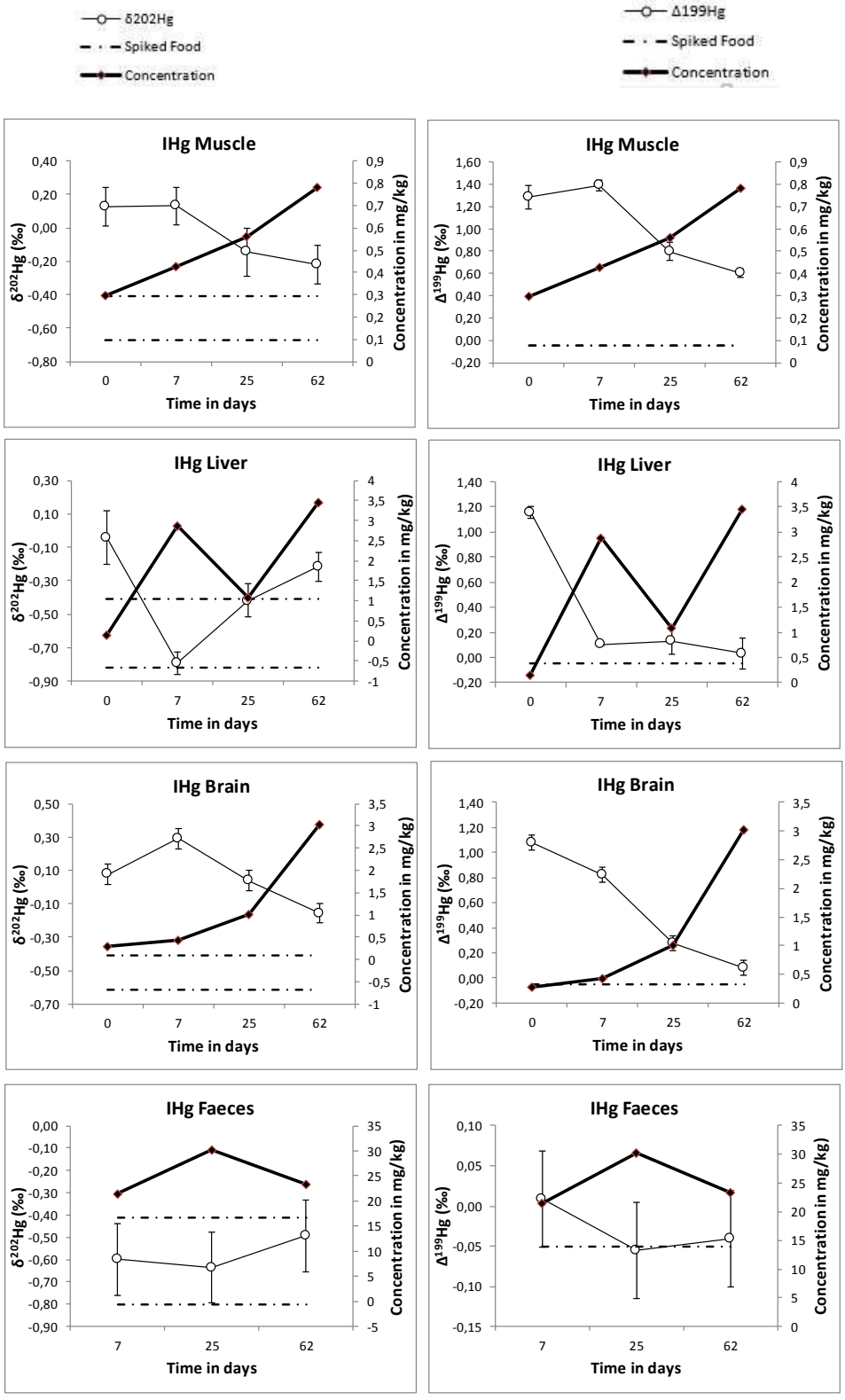

Figure 10: MDF (left panel) and MIF (right panel) variations during IHg incubations. Dash lines represent the isotopic composition of IHg spiked food.

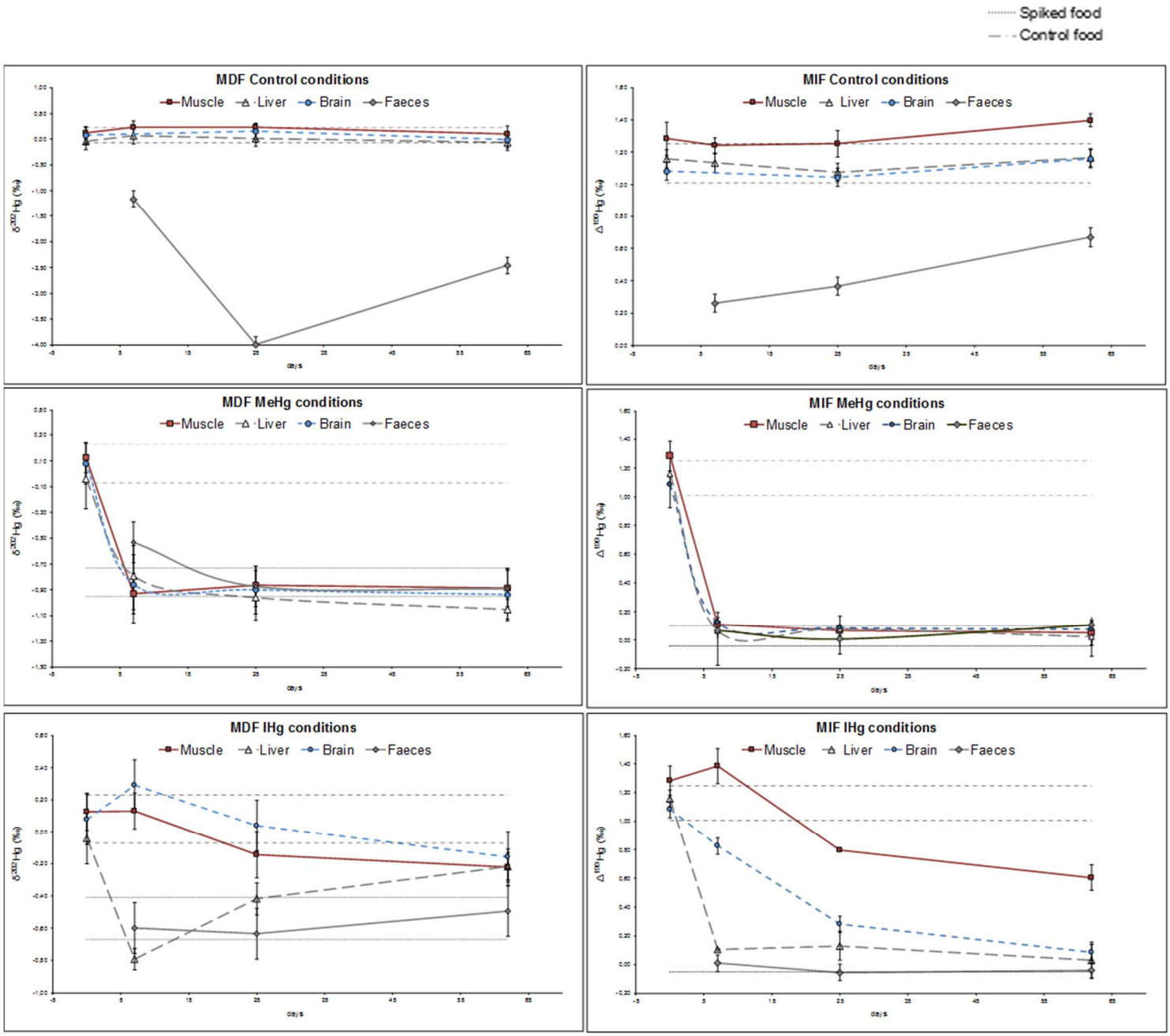

Figure 22: Isotopic composition (MDF right panel, MIF left panel) on muscle, brain, liver and faeces of animals exposed to different conditions

4 DISCUSSION

Regarding Hg concentrations in the aquarium's water, MeHg amount in water experiments a remarkable decrease under IHg exposure conditions while it increases on control and MeHg conditions. The observed trends can be attributed to the release of Hg soluble compounds from the provided food.

The comparison of total Hg concentration on organs of animals exposed to IHg and MeHg shows that IHg was less accumulated than the organic form. The organ which presents a higher amount of Hg under control and MeHg exposure conditions is brain. However, liver is the target organ under IHg dietary exposure conditions. Under MeHg conditions, the highest concentrations are found in the brain followed by the liver, and the Hg amount for all the organs is almost exclusively composed of MeHg. Liver presents the highest concentrations of Hg under IHg exposure conditions, then brain and finally muscle. In this case, the percentage of MeHg is noticeably decreased over time of exposure for all the three organs. These results are confirmed by the BAF calculated for animals exposed to MeHg and IHg conditions.

The analysis of faeces expose a higher amount of Hg under IHg conditions than MeHg exposure. They also reveal that Hg concentrations excreted by animals exposed to IHg are 10 times higher than in the rest of the organs. It contrasts with the low Hg proportion excreted under control and MeHg conditions. Hg levels found in the faeces under both conditions prove an efficient excretion of IHg and a high bioaccumulation rate of Hg organic species and agree with the conclusions obtained from total Hg and isotopic results.

The highest concentration of Hg was obtained on brain at 62 days of MeHg-enriched diet exposure. This result is in good agreement with the ones obtained by González et al. [13] Regarding the bioaccumulation factor, the brain seems to be the organ that accumulates more Hg for control and MeHg conditions, this fact could be related to the recognised neurotoxic effects of this element.

Total Hg concentration in the different organs of animals exposed to MeHg-diet are in good agreement with values previously reported by Gonzalez et al [13] under analogous conditions. Despite the similarity of total Hg levels, the percentage of MeHg in brain at the initial time is 30% lower in the present study. The most noticeable differences are related to Hg species distribution in liver, since MeHg represent more than 90% after 7 days of exposure in our experiments, and the previously reported value vary from 66 to 36% [13] during the same exposure period. In contrast, a surprising result has been obtained in liver samples. This fact contrasts with previous studies where the percentage of MeHg clearly decreases over time in this organ as a result of MeHg demethylation, resulting in a less toxic Hg species. [13] The high percentage of MeHg, that in addition remains stable during the exposure time, does not evidence hepatic MeHg demethylation. A possible hypothesis that could explain this fact is that the input of MeHg provided by diet was higher than the metabolization output so that the detoxification occurs more slowly and MeHg is demethylated over longer time periods.

Under MeHg contamination we can observe a progressive accumulation tendency with time in the three organs where Hg is present almost exclusively in the methylated form. After ingestion, MeHg is transported through the blood to all organs and tissues so the distribution in the different internal compartments produces high concentrations of MeHg in the skeletal muscle, also due to its high storage capacities and slow depuration rates.[15] Isotopic signature of MeHg-enriched food is reflected on the different organs after 7 days, what can be attributed to a quick assimilation of MeHg. The rapid equilibration of the Hg isotopic composition of the internal organs to the new MeHg food source was also shown by Kwon et al. [38]. In this study marine fish called greater Amberjack (*Seriola dumereli*) were fed with blackfin tuna (*Thunnus atlanticus*) and brown shrimp (*Farfantepenaeus aztecus*). After 10 days of tuna dietary exposure, the isotopic signature of all the organs shifted closed to the tuna values except for one muscle sample, which presented a delayed response so that the isotopic value did not shift to the food value until after 30 days. In the case of brown shrimp consumption, liver, brain and muscle shifted towards the food isotopic composition after 10, 30 and 50 days, respectively. It can be supposed that the assimilation rates are probably different depending on the fish species, Hg concentrations or the age of the exposed animals. In conclusion, the efficient assimilation of MeHg diet is reflected by the homogenous isotopic composition of food and organs under MeHg conditions, as it was observed in previous studies. [38] [39]

A particular trend is observed on the kinetic isotopic signature of liver under the IHg conditions. According to the total Hg distribution, liver seems to be the target organ after 7 days of exposure, exhibiting the lowest δ^{202}Hg (-0.8‰).

Liver accumulates the highest amount of IHg after 62 days. It should be noticed that the percentage of MeHg decreases sharply from 76% to 7% in 7 days of exposure and it remains constant until the end of the experiment. It is coherent with the results obtained from isotopic fractionation, where liver presents the lowest δ^{202}Hg at 7 days of incubation. This evidence also agrees with the mercury accumulation presented in the liver in previous studies where the most part in the inorganic form. [13] [15] As we can observe with BAF calculations, liver is the target organ for Hg accumulation in IHg diet, which could also support the existence of a demethylation process in this organ. It has been repeatedly observed in Hg elimination studies in fish [40] [41], that a reduction in total Hg concentrations in the visceral organs is followed by the redistribution of Hg to the storage organs (muscle).

This fact could be reasoned from the isotopic signature under IHg conditions, where a subsequent enrichment on heavier isotopes in liver after 25 days could be due to an initial accumulation followed by a redistribution to muscle and brain. The total concentration in liver at 7 days is the highest in the organism at this sampling time, therefore this is consistent with the isotopic composition.

The results obtained from isotopic analysis show that faeces present lighter MDF values so it can be assumed that a similar isotopic fractionation is produced by Hg demethylation in fish intestines, followed by preferential trophic transfer of the remaining MeHg (higher δ^{202}Hg) to the internal organs with excretion of IHg product

(low δ^{202}Hg) from the body, as it was proposed by Kwon et al. The higher total amount of Hg excreted by fishes exposed to IHg comparing to the other conditions and the results of Hg speciation, where faeces are exclusively composed of IHg, are also consistent with an efficient excretion of IHg and a high bioaccumulation rate of Hg organic species.

It can be confirmed that dietary habits have a notorious influence on Hg accumulation. The ingestion of food artificially enriched in Hg species is a determining factor in the organotropism in fish, as it was observed in previous studies. [15] [16] [17] [39] [38]

5 CONCLUSIONS

The combination of Hg speciation and isotopic analyses in organs (skeletal muscle, liver and brain) and faeces of zebra fish brings significant information about uptake and distribution of Hg species. Dietary habits seemed to be a determining factor on Hg accumulation and concentration in fish. MeHg was more extensively and rapidly bioaccumulated than IHg, what was reflected on the homogenous Hg isotopic composition in the organs.

Hg species distribution in organs of animals exposed to both conditions also reveals the differences on their metabolization. MeHg is the major species in all organs and excretion in animals exposed to such species. In contrast the dietary-IHg is the dominant species in liver and brain.

In general terms, noticeable differences on the isotopic patterns are observed under IHg and MeHg conditions.

The combination of speciation and isotopic composition is therefore an efficient tool not only for the identification of Hg transformations and sources in the environment, but also to elucidate metabolism in organisms.

6 PERSPECTIVES

The main perspectives of this work are the combination of our results with the genomic study (Real time RT-PCR, SAGE) performed by EPOC in the frame of RIMNES project to study Hg impact on fish and the investigation of isotopic fractionation at different levels of the aquatic trophic chain.

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8 ANNEXES

8.1 Annexes 1: Hg isotopic composition

Table 8: Results of δ202, Δ199, δ200 and Δ201 values (‰) obtained in skeletal muscle, liver, brain and faeces of zebra fish after 0, 7, 25 and 62 days of different contaminated conditions (control, MeHg-food and IHg-food).

CONDITION		Time of esposure (days)	$^{202}\delta$ (‰)			Δ^{199} (‰)			$^{200}\delta$ (‰)			Δ^{201} (‰)		
Control conditions	FOOD		0,08	±	0,15	1,13	±	0,12	0,04	±	0,12	0,93	±	0,17
	MUSCLE	0	0,12	±	0,12	1,28	±	0,10	0,12	±	0,11	1,03	±	0,13
		7	0,23	±	0,13	1,24	±	0,05	0,15	±	0,07	1,01	±	0,04
		25	0,23	±	0,07	1,25	±	0,08	0,16	±	0,09	1,04	±	0,02
		62	0,10	±	0,16	1,40	±	0,04	0,08	±	0,09	1,15	±	0,03
	LIVER	0	-0,04	±	0,16	1,16	±	0,06	-0,004	±	0,09	0,87	±	0,06
		7	0,07	±	0,16	1,13	±	0,06	0,07	±	0,09	0,90	±	0,06
		25	0,02	±	0,16	1,08	±	0,06	0,08	±	0,09	0,87	±	0,06
		62	-0,06	±	0,16	1,17	±	0,06	0,01	±	0,09	0,93	±	0,06
	BRAIN	0	0,08	±	0,16	1,08	±	0,06	0,05	±	0,09	0,91	±	0,06
		7	ND			ND			ND			ND		
		25	0,15	±	0,16	1,04	±	0,06	0,11	±	0,09	0,86	±	0,06
		62	-0,01	±	0,16	1,16	±	0,06	0,02	±	0,09	0,89	±	0,06
	FAECES	7	-1,16	±	0,16	0,26	±	0,06	-0,59	±	0,09	0,19	±	0,06
		25	-3,99	±	0,16	0,37	±	0,06	-2,00	±	0,09	0,30	±	0,06
		62	-2,46	±	0,16	0,67	±	0,06	-1,21	±	0,09	0,55	±	0,06
MMHg conditions	FOOD		-0,84	±	0,11	0,03	±	0,07	-0,44	±	0,02	0,003	±	0,11
	MUSCLE	0	0,12	±	0,12	1,28	±	0,10	0,12	±	0,11	1,03	±	0,13
		7	-0,93	±	0,16	0,11	±	0,05	-0,48	±	0,11	0,05	±	0,02
		25	-0,87	±	0,11	0,07	±	0,04	-0,42	±	0,09	0,05	±	0,04
		62	-0,89	±	0,14	0,05	±	0,09	-0,46	±	0,10	0,04	±	0,09
	LIVER	0	-0,04	±	0,16	1,16	±	0,06	-0,004	±	0,09	0,87	±	0,06
		7	-0,79	±	0,16	0,06	±	0,07	-0,41	±	0,08	0,02	±	0,07
		25	-0,96	±	0,09	0,08	±	0,03	-0,47	±	0,10	0,03	±	0,05
		62	-1,05	±	0,08	0,02	±	0,04	-0,56	±	0,05	0,01	±	0,06
	BRAIN	0	0,08	±	0,16	1,08	±	0,06	0,05	±	0,09	0,91	±	0,06
		7	-0,86	±	0,30	0,13	±	0,07	-0,42	±	0,17	0,09	±	0,02
		25	-0,90	±	0,19	0,09	±	0,08	-0,47	±	0,13	0,04	±	0,04
		62	-0,94	±	0,19	0,08	±	0,05	-0,47	±	0,10	0,03	±	0,10
	FAECES	7	-0,53	±	0,16	0,07	±	0,06	-0,27	±	0,09	0,03	±	0,06
		25	-0,87	±	0,16	0,01	±	0,06	-0,47	±	0,09	0,06	±	0,06
		62	-0,89	±	0,16	0,11	±	0,06	-0,42	±	0,09	0,06	±	0,06
IHg conditions	FOOD		-0,54	±	0,13	-0,049	±	0,005	-0,26	±	0,15	0,03	±	0,17
	MUSCLE	0	0,12	±	0,12	1,28	±	0,10	0,12	±	0,11	1,03	±	0,13
		7	0,13	±	0,11	1,39	±	0,12	0,11	±	0,05	1,14	±	0,09
		25	-0,14	±	0,14	0,80	±	0,01	-0,09	±	0,03	0,66	±	0,03
		62	-0,22	±	0,12	0,60	±	0,09	-0,08	±	0,07	0,48	±	0,10
	LIVER	0	-0,04	±	0,16	1,16	±	0,06	-0,004	±	0,09	0,87	±	0,06
		7	-0,79	±	0,07	0,10	±	0,01	-0,40	±	0,05	0,09	±	0,10
		25	-0,42	±	0,10	0,13	±	0,10	-0,23	±	0,05	0,05	±	0,02
		62	-0,22	±	0,08	0,03	±	0,13	-0,11	±	0,02	0,08	±	0,05
	BRAIN	0	0,08	±	0,16	0,07	±	0,06	0,05	±	0,09	0,91	±	0,06
		7	0,29	±	0,16	0,83	±	0,06	0,14	±	0,09	0,66	±	0,06
		25	0,04		0,16	0,28	±	0,06	0,03	±	0,09	0,24	±	0,06
		62	-0,16		0,16	0,08	±	0,06	-0,08	±	0,09	-0,01	±	0,06
	FAECES	7	-0,60	±	0,16	0,01	±	0,06	-0,34	±	0,09	0,004	±	0,06
		25	-0,64	±	0,16	-0,06	±	0,06	-0,35	±	0,09	-0,02	±	0,06
		62	-0,49	±	0,16	-0,04	±	0,06	-0,24	±	0,09	-0,01	±	0,06

Results expressed as mean value±2SD (n= 3, 3 organisms pooled).SD means standard deviation.

8.2 Annexes 2: Preparation of buffer and $NaBPr_4$ solutions

For Hg analyses the buffer solution must be at a pH value of 3,9 to ensure a total derivatization and extraction of the Hg present in the samples. The concentration of the solution is 0,1 M of Sodium acetate ($Na^+CH_3COO^-$) in acetic acid (CH_3COOH). Table 9 shows the amount of solvent and solute required for the buffer solution preparation in a total volume of 500 ml.

Table 9: Preparation of acetate buffer for mercury analyses

MERCURY			Acetate Na	Acetic Acid
0,1M - pH 3,9 - 500ml				
			Mass (g)	Volume (ml)
Concentration acid	0,085	0,042	2,549	**2,428**
Concentration acetate	0,015	0,008	**1,027**	

The derivatization step requires to prepare a 1% $NaBPr_4$ solution (w/w) on a daily basis. The solution is stored at +4°C in the dark, after use.

8.3 Annexes 3: Preparation of NIST 3133, UM and $SnCl_2$

For the preparation of the standard bracketing solution, a concentration of 2 ppm of NIST 3133 is needed (360µL in 180µL of acid).

UM (Almadén) solution is prepared in a concentration of 1 ppm (50µL of CRM in 50mL of water)

$SnCl_2$ solution is prepared in a concentration of 3% (30g of $SnCl_2$, 100 mL of HCl in a total volume of 1000 mL in Milli-Q water).

8.4 Annexes 4: Washing procedure of material used

Ultra trace analysis based on isotopes measurements (isotopic dilution and isotopic composition) require the use of dedicated procedures to avoid possible contamination of the samples and cross contamination of the material. Therefore, it is mandatory to follow a washing protocol for the decontamination of all the material used during the experimental part and to discriminate “enriched isotopes” from “natural isotopes” activities.

Washing procedure for glass and quartz vials, CEM tubes and Teflon liners involves the following steps:

- First wash in an concentrated detergent (RBS T105) 1 hour in an ultrasonic bath
- Rinse with Milli-Q water
- Second wash in HNO_3 (10%) for 1 hour in an ultrasonic bath
- Rinse with Milli-Q water
- Third wash in HNO_3 (10%) for 1 hour in an ultrasonic bath
- Rinse with Milli-Q water
- Forth wash in HCl (1%) for 1 hour in an ultrasonic bath.
- Rinse with Milli-Q water
- Drying step under laminar flow hood

For plastic parts (ring and septum) of CEM tubes:

- First wash in an concentrated detergent (RBS 105) 1 hour in an ultrasonic bath
- Rinse with Milli-Q water
- Second wash in Milli-Q water for 1 hour in an ultrasonic bath
- Rinse with Milli-Q water
- Third wash in Milli-Q water for 1 hour in an ultrasonic bath
- Rinse with Milli-Q water
- Forth wash in Milli-Q water for 1 hour in an ultrasonic bath
- Rinse with Milli-Q water
- Drying step under laminar hood