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ABSTRACT

Tiemannite (HgSe) is considered the end-product of methylmercury (MeHg) demethylation in vertebrates. The biomineralization of HgSe nanoparticles (NPs) is understood to be an efficient MeHg detoxification mechanism; however, the process has not yet been fully elucidated. In order to contribute to the understanding of complex Hg metabolism and HgSe NPs formation, the Hg isotopic signatures of 40 samples of 11 giant petrels were measured. This seabird species is one of the largest avian scavengers in the Southern Ocean, highly exposed to MeHg through their diet, reaching Hg concentrations in the liver up to more than 900 μg g^{-1}. This work constitutes the first species-specific isotopic measurement ($\delta^{202}Hg$, $\Delta^{199}Hg$) of HgSe NPs in seabirds and the largest characterization of this compound in biota. Similar $\delta^{202}Hg$ values specifically associated to HgSe (δ^{202}_{HgSe}) and tissues (δ^{202}_{bulk}) dominated by inorganic Hg species were found, suggesting that no isotopic fractionation is induced during the biomineralization step from the precursor (demethylated) species. In contrast, the largest variations between $\delta^{202}Hg_{bulk}$ and δ^{202}_{HgSe} were observed in muscle and brain tissues. This could be attributed to the higher fraction of Hg present as MeHg in these tissues. Hg-biomolecules screening highlights the importance of the isotopic characterization of these (unknown) complexes.

KEYWORDS

Mercury, seabirds, isotopic fractionation, HgSe nanoparticles, MeHg demethylation

1 INTRODUCTION

Mercury (Hg) is a globally distributed pollutant present in polar and sub-polar areas through long-range atmospheric transport (Fitzgerald et al., 1998). Once in the marine environment, inorganic mercury (iHg) can be methylated by microbial activity to form methylmercury (MeHg) (Fitzgerald et al., 2007; Kaschak et al., 2013; Mason, 2013). MeHg bioaccumulates in the tissues of aquatic organisms, and biomagnifies through the food chain resulting in predators at the top of the trophic chain to accumulate large amounts of Hg in their tissues (Mason et al., 1995).

Seabirds in the Southern Hemisphere showed specific patterns of accumulation, distribution and biotransformation of Hg (Albert et al., 2019; Mills et al., 2020; Renedo et al., 2021). Giant petrels are among the top scavengers of these latitudes, feeding on high trophic level species, which leads to the very high concentrations of Hg in their tissues (Renedo et al., 2021). Their great longevity, of up to 50 years old (Foote et al., 2011), coupled with their high trophic level makes them a species of great ecotoxicological interest, as they can bioaccumulate large quantities of toxic elements, especially Hg, which is ingested through the diet as MeHg. Hepatic demethylation is considered a key detoxification pathway of MeHg in seabirds (Kim et al., 1996; Thompson and Furness, 1989) together with its excretion in feathers (Albert et al., 2019). Demethylation involves selenium (Se) (Khan and Wang, 2010), an essential element which presents a high affinity to inorganic mercury (iHg), resulting ultimately in the formation of insoluble tiemannite nanoparticles (HgSe NPs). Although the liver is the key organ for Hg bioaccumulation, HgSe NPs have been found in other organs and tissues of fish, marine mammals and seabirds, including kidneys, muscle and brain (Gajdosechova et al., 2016; Manceau et al., 2021c, 2021a).

Hg isotopic analysis in biological samples is a powerful tool to trace Hg trophic sources and metabolic pathways in wildlife (Bolea-Fernandez et al., 2019; Feng et al., 2015; Li et al., 2020; Masbou et al., 2018; Perrot et al., 2016) and in humans (Du et al., 2018; Sherman et al., 2015, 2013). Most of these studies have been carried out on mammalian or fish tissues (Bolea-Fernandez et al., 2019; Feng et al., 2015; Le Croizier et al., 2020; Masbou et al., 2018) and only a few of these studies have focused on seabirds (Manceau et al., 2021b; Poulin et al., 2021; Renedo et al., 2021, 2020, 2018a, 2017). Hg stable isotopes can undergo both mass-dependent

fractionation (MDF) and mass-independent fractionation (MIF). Odd Hg isotope MIF is related to the processes of photoreduction and photodemethylation of Hg mainly in the photic zone, thus allowing traceable marine food source to prey-predator relationships (Kwon et al., 2013; Le Croizier et al., 2020; Renedo et al., 2018c; Sherman et al., 2013). In contrast, the metabolic distribution of Hg in the body can be traced through MDF (Bolea-Fernandez et al., 2019; Feng et al., 2015). This powerful tool has been successfully exploited to track Hg distribution in the tissues of three different seabirds species, revealing that hepatic MeHg demethylation is followed by internal tissue redistribution of the residual MeHg enriched in heavier Hg isotopes (Renedo et al., 2021).

The main aim of this study was to provide new insights into the biomineralization of MeHg into HgSe NPs by the specific isotopic characterization of this inert Hg compound. For this purpose, a large set of giant petrel samples (for a total of 40 tissues) corresponding to 11 individuals of different ages were studied. Complementary analytical techniques have been combined, such as Hg speciation at the chemical and molecular level, as well as Hg and Se measurements between solid and soluble fractions of tissues. The current work represents the first study on Hg isotopic characterization specifically associated to NPs in (sea)birds. Hg isotopic analyses in blood, as well as the comparison between Hg isotopic composition in HgSe NPs and bulk tissues, shedding light about different distribution routes between tissues of seabirds.

2 MATERIAL AND METHODS

2.1. Ecological characteristics of giant petrels

The avian model corresponds to the two sibling species of giant petrels, the northern (*Macronectes halli*) and southern (*Macronectes giganteus*) giant petrels, which are the dominant scavengers of the Southern Ocean (Rheindt and Austin, 2005; Salomon and Voisin, 2010). Giant petrels are large birds that can reach over two meters of wingspan (Carlos and Voisin, 2008). During the reproduction period, adult males feed mostly on land on carrion, seabirds and pinnipeds, while females mainly forage at sea, preying on fish, squid and krill (Thiers et al., 2014).

2.2. Sampling set and individual characteristics

Eleven dead individuals of giant petrels were opportunistically collected. Four of them were *M. halli* (all males) and were collected in the Kerguelen archipelago (Southern Indian Ocean) in 2014. The other four birds were *M. giganteus*. One specimen was collected in the Kerguelen Archipelago in 2014 (male) and three were collected in Adélie Land (Antarctic continent) in 2019 (1 female and 2 males). The age of these individuals could not be precisely determined, but one of the seabirds collected in Adélie Land was a fully-fledged chick (*viz.* less than 1 year old). In addition to this, samples of three other adults *M. giganteus* (see details in Renedo et al., 2021) from the same region were also considered in the present study. The samples from Adélie Land were in good shape for adults who died after a collision against an electric pole, but the chick (P-P1) was very emaciated suggesting it dies from starvation. For all the others, the cause of death is unknown, in general they were in good condition. All of them were collected freshly after their death. Internal tissues were sampled, weighed, and stored individually in plastic bags. Liver, kidneys and pectoral muscle were collected from all the eight birds, while brain tissue was sampled for the three *M. giganteus* collected in 2019. Blood samples were obtained by collecting clotted blood from heart atria. After dissection, all the samples were stored at -20°C. All details relating to the complete sampling set are provided in Table S1. All the tissues used in this work were homogenized with an ultra-turrax® and a portion was freeze-dried for analytical purposes.

2.3. Determination of total Hg, Se and Hg species

2.3.2. Determination of Hg and Se concentrations

Freeze-dried and homogenized tissue samples (0.05 - 0.10 g) were digested in 3 mL of Trace Metal Grade HNO_3 (Fisher scientific, Illkirch, France) in an Ultrawave microwave digestion system (Milestone, Sorisole, Italy) at 220°C for 10 min. Fresh-frozen blood samples were digested directly without freeze-drying. The measurement of total Hg (THg) in the digests was performed by cold vapor - atomic fluorescence spectrometry (CV-AFS PS Analytical 10.025, Kent, UK) by reduction with 3% (m/v) $SnCl_2$ (Scharlab, Barcelona, Spain) in 10% (v/v) HCl (J.T.Baker, Fisher scientific, Illkirch, France).The samples were then diluted in HCl 5% (v/v) for analyses and the quantification was performed by standard calibration. For Se analyses, the resulting digests were appropriately diluted with Milli-Q water and analyzed by ICP-MS. Method protocols were validated using certified reference materials (BCR-464, NIST-1947 and DOLT-5). The information relative to the quality assurance of the Hg analyses of Hg and Se is found in Table S2.

2.3.3. Determination of iHg and MeHg species

Between 0.05-0.10 g of freeze-died samples was added to glass vessels together with 3 mL of 25% (m/v) tetramethylammonium hydroxide *(*TMAH) and a magnetic stir bar. For blood, Hg species were directly extracted from the freshly frozen samples. Vials were closed with a Teflon cap and placed in the microwave. The focused microwave assisted extraction of the samples was performed using a Discover SP-Microwave (CEM Corporation, Matthews, NC). The extraction was carried out using a 1-min ramp up to 75ºC followed by 3 min at 75ºC with constant stirring. Inorganic mercury (iHg) was measured by CV-AFS after dilution of the extract in HCl 5% (v/v) (Davis et al., 2007; Davis and Long, 2011; Renedo et al., 2021). For the determination of total Hg, 50 µL of a 0.1N (0.05 mol/L cBr_2) potassium bromide/bromate solution (Merck, Darmstadt, Germany) was added to 10 mL of the diluted digest in 5% (v/v) HCl. The CV-AFS system was coupled to an oxidation unit (PS Analytical 10.820, Kent, UK) equipped with a UV lamp so that all samples with potassium bromide/bromate were subjected to on-line UV irradiation to facilitate immediate oxidation of MeHg and subsequent measurement of THg. The concentration of MeHg was determined by the subtraction of iHg from the THg concentration (Aranda et al., 2009; Kaercher et al., 2005). This procedure was validated using BCR-464 and TORT-2 as shown in Table S2.

2.4. Determination of Hg biomolecular species by SEC-ICP-MS

The aqueous soluble protein fraction was extracted from fresh samples (approximately 0.1g) by ultra-probe sonication (30 s at 100W power) in 3 mL of 100 mM ammonium acetate (pH 7.4) followed by centrifugation, as described elsewhere (Pedrero et al., 2011). The obtained cytosolic biomolecules were separated by size exclusion chromatography (SEC) using a Superdex 200 300/10 column (10mm ID×300mm length ×13 µm particle size) (GE Healthcare, Uppsala, Sweden). A HPLC Agilent 1100 (Agilent, Wilmington, DE) equipped with a binary HPLC pump and an autosampler was coupled to an Agilent ICP-MS 7500ce (Yokogawa Analytical Systems, Tokyo, Japan). The supernatant was analysed by injection of 100 µL fractions. Isocratic elution was performed at 0.7 mL min^{-1} with a mobile phase of 100 mM ammonium acetate at pH of 7.4 (Pedrero et al., 2011; Pedrero Zayas et al., 2014).

2.5. Extraction of HgSe nanoparticles

Isolation of HgSe NPs was carried out by using an adaptation of the method described elsewhere (Bolea-Fernandez et al., 2019; Gajdosechova et al., 2016). In brief, after defatting, a soft acid treatment with formic acid was applied to the samples. The resulting extract was centrifuged by using 50 kDa cut-off filters (Amicon Ultra). The filter was abundantly washed with Milli-Q water until total removal of soluble Hg and Se was achieved (Gajdosechova et al., 2016). Nanoparticles were then recovered by centrifugation for 3 min at 1000 x g. The collected NPs were then digested in 3 mL HNO_3, using the procedure described in section 2.3. This selective extraction protocol is not considered to be quantitative, due to losses in the cut-off filtration steps. The procedure however has been successfully used for Hg isotopic characterization previously since no isotopic fractionation of this solid species is induced during the sample treatment (Bolea-Fernandez et al., 2019).

2.6. Hg stable isotope analysis

Hg isotopic ratios were measured using a multicollector-(MC)-ICP-MS (Nu Instruments, Wrexham, UK) coupled with continuous flow cold vapor generation (CVG) on the digested samples (see above). The isotopes ^{198}Hg, ^{199}Hg, ^{200}Hg, ^{201}Hg, ^{202}Hg, ^{203}Tl, ^{204}Hg, and ^{205}Tl were simultaneously measured in the Faraday cups L2, L1, Ax, H1, H2, H3, H4 and H5, respectively. In order to correct the instrumental mass-bias, NIST SRM 997 thallium standard solution in 2% (v/v) of HNO_3 (Optima grade, Fisher scientific, Illkirch, France) was nebulized continuously through a desolvation unit (DSN) and the calculation was performed using the exponential law model (Bergquist and Blum, 2007; Blum and Bergquist, 2007; Perrot et al., 2010). The sample standard bracketing (SSB) approach was employed for the calculation of isotope ratios relative to the NIST 3133-iHg standard solution. Mass dependent fractionation (MDF) is reported as recommended by Bergquist and Blum (Bergquist and Blum, 2007) relative to the NIST 3133 Hg solution using Eq. 1:

$$\delta^{xxx}Hg = \left(\frac{xxx/^{198}Hg_{sample}}{xxx/^{198}Hg_{NIST\ 3133}} - 1\right) * 1000\ ‰ \qquad \text{Eq. 1}$$

where xxx is the studied isotope. Mass-independent fractionation (MIF) of Hg is reported using Eq. 2 as the difference between the theoretical value predicted by MDF and the measured values as Δ^{199}Hg and Δ^{201}Hg in ‰, according to the protocol suggested elsewhere (Blum and Bergquist, 2007):

$$\Delta^{xxx}_{v}Hg = \delta^{xxx}Hg - (\delta^{202}Hg \times \beta_{xxx}) \quad \text{Eq. 2}$$

where βxxx is the kinetic mass-dependence scale factor that depends on the isotopes (0.2520 for ^{199}Hg, 0.5024 for ^{200}Hg, 0.7520 for ^{201}Hg and 1.493 for ^{204}Hg) (Blum and Bergquist, 2007). All the samples and standards were measured at a concentration of 1 ng g^{-1} of Hg in a 10% (v/v) HNO_3/2% (v/v) HCl solution, to avoid any bias caused by differences in concentration. The external reproducibility of the measurements is expressed as ±2SD for 35 measurements of the NIST RM 8610 (former UM Almadén) as 0.09 and 0.05 ‰ for δ^{202}Hg and Δ^{199}Hg, respectively. All the values for the isotopic composition of the NIST RM 8610 and the certified reference materials BCR- 464, NIST 1947 and TORT-2 analysed in this work are presented in Table S3.

3 RESULTS AND DISCUSSION

3.1. Hg concentrations in giant petrel tissues

The total mercury (THg) concentrations in the tissues of giant petrels are among the highest ever reported for seabirds, up to 933 µg g^{-1} dry weight (d.w. here and in all reported concentration values) (Table S4). However, THg was highly variable among tissues for each individual and among individuals considering a single tissue. Nonetheless, the liver always showed the highest concentrations among tissues with Hg hepatic concentrations ranging from 99 to 933 µg g^{-1} (mean value of 419 ± 317, n=9) for adult giant petrels. These values are in agreement with previously reported values for giant petrels from New Zealand (Stewart et al., 1999). As liver is the main storage organ for Hg in vertebrates, hepatic concentrations provide an indirect indication of the age of animals (Bolea-Fernandez et al., 2019; Gajdosechova et al., 2016). Thus, our sampling covers a large range of ages allowing us to consider if this factor influences the detoxification

process of MeHg. The only chick of the study (P-P1) presents the lowest Hg levels in all the tissue types. The Hg level in liver of the chick individual (1.1 µg g^{-1}) is consistent by the process of bioaccumulation with age. In kidneys, THg concentrations were higher for most of the adult samples ranging from 7.8-481 µg g^{-1} (mean value of 85 ± 140, n=10) than the already previously reported concentrations for petrels and other large seabirds such as albatrosses (Bocher et al., 2003; Cipro et al., 2014; Kim et al., 1996; Stewart et al., 1999). In muscles, Hg concentrations vary from 1.0 to 52 µg g^{-1} (mean value of 18 ± 20, n=9) in adults (0.10 µg g^{-1} for the chick), and are among the highest reported for seabirds (Renedo et al., 2021). In the case of blood, the two adults from the Antarctic continent (P-10, P-11) exhibit concentrations around 0.5 µg g^{-1} (wet weight), which was fivefold higher than the concentration found in the chick (P-P1) from the same location. A linear correlation (Pearson's correlation coefficient ρ= 0.8971) was found between THg concentrations in liver and muscle (Figure S1), which could indicate that Hg bioaccumulation occurs in both tissues along time, Hg being mainly retained in the liver but its excess being directly transferred towards the muscles as suggested by previous studies (Manceau et al., 2021c; Renedo et al., 2021).

Despite the high variability of MeHg content among individuals (Table S4), likely due to their age difference (Gajdosechova et al., 2016), the percentage of organic Hg was higher in brain (34-74%) than in liver and kidneys (2-34%). Regarding muscles, in the adults with lower Hg concentrations (P10 and P11, both from the Antarctic continent), the MeHg fraction reaches up to 71%. In contrast, in the case of individuals showing higher liver THg concentrations, the percentage of MeHg in the muscles was much lower (8-30%), suggesting that the proportion of MeHg decreases with age together with an increase of THg concentrations. Such a trend was already reported for top of the marine food chain marine mammals including seals and toothed whales (Bolea-Fernandez et al., 2019; Dehn et al., 2005; Gajdosechova et al., 2016).

3.2. Hg isotopic composition of tissues: Understanding Hg (re)distribution

The high $\delta^{202}Hg_{bulk}$ variability in the different tissues analyzed contrasts with the quite homogenous $\Delta^{199}Hg_{bulk}$ values obtained whatever the tissue (Table S5). Thus, MIF ($\Delta^{199}Hg_{bulk}$) ranges from 1.13 to 1.73 ‰ among individuals, except in the case of the chick, which present the

lowest values of the study. Considering the whole data set, the $\Delta^{199}Hg/\Delta^{201}Hg$ slope is 1.13 ± 0.01 (r = 0.968), similar to slope values reported for other marine top predators and fish (Figure S2) (Le Croizier et al., 2020; Masbou et al., 2018; Renedo et al., 2018b, 2021). The reference $\Delta^{199}Hg/\Delta^{201}Hg$ slopes for MeHg photodemethylation processes (1.36 ± 0.02) and iHg photoreduction (1.00 ± 0.02) (Bergquist and Blum, 2007) are also presented in Figure S2. The values of $\Delta^{199}Hg$ for giant petrel tissues agree with those of blood in skua chicks (1.14 ± 0.05 ‰) and subantarctic penguins (1.16 ± 0.05 ‰), which were correlated with latitude (Renedo et al., 2020). Interestingly, MIF values in the single chick collected on the Antarctic continent (P-P1) ranged from 0.88 to 1.1 ‰ for internal tissues, being slightly lower than for the adults. Giant petrel chicks are fed with prey caught near the colony by their parents. Hence, the MIF difference is likely due to its exclusively Antarctic food sources that are characterized by low $\Delta^{199}Hg$ values (Renedo et al., 2020). In contrast, adults sampled in the Antarctic Continent may reflect an integration of Hg from local prey during the breeding season and Hg prey from lower latitude during the wintering season (Renedo et al., 2021).

MDF ($\delta^{202}Hg_{bulk}$) values ranged widely from -1.48 up to 3.05 ‰ (Table S5). In all the individuals, the highest $\delta^{202}Hg$ values correspond to blood samples. The specific differences of $\delta^{202}Hg$ values between tissues (Figure 1) is attributed to the strong dependence on the ratio of methylated and demethylated Hg (Feng et al., 2015; Perrot et al., 2016; Renedo et al., 2021). MeHg demethylation induces mass dependent fractionation, resulting in an enrichment of the inorganic Hg species in lighter isotopes whilst the residual MeHg is enriched in heavier ones (Perrot et al., 2016; Rodríguez-González et al., 2009). The highest MDF values in blood, also observed in other animal studies, is understood as a consequence of the transport of residual MeHg (enriched in heavier isotopes from incomplete demethylation processes) by the blood stream (Bolea-Fernandez et al., 2019; Li et al., 2020; Ma et al., 2018; Renedo et al., 2021).

Regarding brain, it is generally enriched in heavier isotopes ($\delta^{202}Hg$ 0.03-1.77 ‰) in comparison to liver and kidneys (Table S5), suggesting residual MeHg from the blood streams subsequently accumulate in this organ. In the case of muscles, two different trends are identified (Figure 1, Table S5) according to the hepatic Hg concentration (associated to the age of the animals).

Muscles of giant petrel individuals with the highest THg hepatic concentrations (250 - 933 µg g^{-1}) are enriched in lighter isotopes ($\delta^{202}Hg$) in comparison to liver and kidneys, contrasting with muscles of the other group of animals (THg hepatic concentrations lower than 250 µg g^{-1}). Even though Hg isotopic characterization in animal's internal tissues is not extensive, the trend observed in the mentioned samples is in agreement with previous studies on marine mammals and seabirds (Bolea-Fernandez et al., 2019; Li et al., 2020; Renedo et al., 2021) and is attributed to the higher proportion of MeHg in muscles than in the liver. As previously discussed, this organomercurial species is expected to be enriched in heavier isotopes compared to the demethylated ones, assuming preferential demethylation of the lighter Hg isotopes (Perrot et al., 2016; Rodríguez-González et al., 2009).

Noticeably, the giant petrel individuals with the highest THg hepatic concentrations (oldest ones) exhibit lighter $\delta^{202}Hg$ ($\delta^{202}Hg_{bulk}$) values in muscles than in liver and kidneys (Tables S4 and S5). Similarly, a recent investigation in marine mammals reported a decrease of $\delta^{202}Hg$ values in the muscles of the oldest group in comparison to the juvenile one (a shift of 0.8 ‰ between both groups) (Bolea-Fernandez et al., 2019). Atypically, muscles of those giant petrels contained mainly iHg (70-93%). This particular enrichment of muscles in demethylated Hg species is the most likely reason why muscles of these old seabirds contain lighter Hg isotopes, highlighting that demethylation occurs effectively in this tissue as previously reported (Manceau et al. 2021c). Furthermore, several pathways and factors could also influence the stable isotope signature. One of them is the MeHg excretion by molting, a well-known excretion strategy in birds (Bearhop et al., 2000) when MeHg is massively remobilized from internal tissues towards the growing feathers (Renedo et al., 2021). Thus, between 70–90% of the Hg load of the birds is excreted into the new plumage during molting (Agusa et al., 2005; Braune, 1987). In addition, the ageing process intensely impacts, among others, muscle metabolism (Burger, 1993; Wone et al., 2018) and could result in MeHg mobilization (Cuvin-Aralar and Furness, 1991). Moreover, specifically in migrating birds, the consumption of energy stores during migratory fasting could contribute to MeHg mobilization and higher MeHg circulating levels (Seewagen et al., 2016). Therefore, the atypical muscle Hg speciation and isotopic composition in the oldest giant petrels could be the result of the different mechanisms mentioned above.

3.3. Hg isotopic composition of HgSe nanoparticles (tiemannite)

The present work is the first to isolate and measure the Hg isotopic composition of HgSe NPs in seabird tissues. The specific Hg isotopic composition associated to HgSe NPs ($\delta^{202}Hg_{HgSe}$) was compared with the bulk ($\delta^{202}Hg_{bulk}$) value (Figure 2, Table S5). In general, in individuals exhibiting the highest THg hepatic concentrations (250-933 µg g^{-1}), a match for both MDF and MIF, was observed in liver and kidneys between THg ($\delta^{202}Hg_{bulk}$) and HgSe NPs ($\delta^{202}Hg_{HgSe}$) (typically represented in Figure 2.A for seabird P-1). The specific isotopic signature in isolated HgSe NPs found in the muscles of this set of individuals was also quite similar to the isotopic values in the whole tissue. In the other set of individuals (THg hepatic concentrations lower than 250 µg g^{-1}), the $\delta^{202}Hg$ difference between THg and HgSe NPs showed dissimilar trends depending on tissues. Contrary to muscles and brain, most of HgSe NPs in kidneys are enriched in heavier isotopes ($\delta^{202}Hg$ shift up to 0.56 ‰) in comparison to the bulk (Figure 2.D, Table S5) while no tendency is identified in the liver of these individuals. The mentioned pattern, specifically observed in kidneys, could be related to a physiological peculiarity in birds, where a renal portal system allows this organ to be bypassed (Meredith and Johnson-Delaney, 2010). Considering that HgSe NPs is the final product of MeHg breakdown, the observed ^{202}Hg-enrichment of this solid species in liver and kidneys is unexpected. The previous report of Hg isotopic characterization in HgSe is limited to long-finned pilot whales (five livers and two muscles) (Bolea-Fernandez et al., 2019). It is therefore interesting to note a total agreement in the obtained trends in livers and muscles of both animals belonging to two classes of vertebrates.

In the giant petrel group exhibiting the highest Hg hepatic concentrations, there is a perfect match between the isotopic signature of THg ($\delta^{202}Hg_{bulk}$) and those in HgSe NPs ($\delta^{202}Hg_{HgSe}$) for the liver, kidneys and muscles. In those samples, iHg species were the dominant ones (Table S4), with HgSe constituting 95 ± 5, 61 ± 8 and 35 ± 15% in liver, kidneys, and muscle, respectively according to X-ray absorption near edge structure (XANES) analyses (Manceau et al., 2021c). In addition to HgSe NPs, another iHg complex exhibiting a molar ratio Hg:Se (1:4) has been reported by these authors. This complex was described as mercury selenocysteinate, $Hg(SeCys)_4$ which is considered to be precursor of tiemannite. $Hg(SeCys)_4$ was found to be present in liver, kidneys,

and muscles representing 5 ± 2, 35 ± 10 and 61 ± 13% of the THg, respectively (Manceau et al., 2021c). In each tissue, the THg isotopic composition results from the contribution of different Hg species as outlined in Eq 3:

$$\delta^{202}Hg_{bulk} = f_{MeHg\text{-}R} \times \delta^{202}_{MeHg\text{-}R} + f_{iHg} \times \delta^{202}_{iHg} \quad \text{Eq. 3}$$

where f is the fraction of each species in the bulk tissue and the contribution of the iHg species could be expressed as follows in Eq 4:

$$\delta^{202}Hg_{iHg} = f_{HgSe} \times \delta^{202}_{HgSe} + f_{Hg:Se\ (1:4)} \times \delta^{202}_{Hg:Se\ (1:4)} \quad \text{Eq. 4}$$

In the described set of samples with the highest Hg concentrations in the liver, the $\delta^{202}Hg_{bulk}$ and δ^{202}HgSe are identical (Table S5) irrespectively of the proportion of both inorganic species (HgSe and $Hg(SeCys)_4$). Therefore, we propose that the mentioned inorganic species share a similar δ^{202}Hg signature. This finding suggests that no isotopic fractionation seems to be induced during the biomineralization (HgSe NPs) step from the precursor species Hg:Se (1:4).

On the other hand, the largest shifts between δ^{202}Hg in bulk and the one specifically associated to HgSe NPs are found in muscles and brain (Table S5). The observed variations could be attributed to the higher fraction of Hg present as MeHg in these two tissues. The organomercurial compound is expected to be specifically enriched in heavier isotopes, since MeHg demethylation is followed by internal tissue redistribution of the residual MeHg enriched in heavier Hg isotopes (Bolea-Fernandez et al., 2019; Li et al., 2020; Ma et al., 2018; Renedo et al., 2021).

The brain was the organ exhibiting the highest MeHg percentage (Table S4), where HgSe and Hg:Se (1:4) have been also reported (Manceau et al., 2021c). The specific isotopic signature of the organic compound (δ^{202}_{MeHg}) could be estimated by considering; *i.* the proportion of these inorganic species (Table S4 and XANES data) and *ii.* assuming HgSe and Hg:Se (1:4) share a similar MDF pattern (δ^{202}HgSe in Table S5). The obtained δ^{202}MeHg values vary between 1.98 and 2.94 ‰ for the investigated individuals, confirming an enrichment in lighter isotopes of the demethylated iHg with respect to MeHg (Figure 3). The isotopic measurement in each individual species is undoubtedly the best approach to determine the species-specific isotopic signature. The obtained (estimated) values are in good agreement with the shift (~3 ‰ δ^{202}Hg) between

MeHg and iHg species measured by Gas Chromatography coupled to MC-ICPMS, the single study so far that has measured both Hg species in animals (Perrot et al., 2016).

Chemical speciation and (total) Hg isotopic analyses have been combined in several recent studies for the estimation of Hg species-specific isotopic signature (Feng et al., 2015; Li et al., 2020; Poulin et al., 2021). The mentioned approach has provided new and valuable information on Hg in biota, however it should be emphasized that Hg speciation has been simplistically reduced to inorganic and MeHg species, without considering the binding of Hg with biomolecules and proteins (Pedrero et al., 2016).

3.4. New insights on Se-mediated MeHg breakdown

Despite the great interest related to the understanding of MeHg transformation into the bioinert HgSe NPs (Cid-Barrio et al., 2020), the mechanism is still not fully characterized. According to our results, it seems that the limiting step in the detoxification of MeHg leading to the formation of HgSe NPs is the cleavage of the C-Hg bond. The mentioned MeHg demethylation step is associated with a MDF shift varying between 2 and 3 ‰ (Figure 3), in total agreement with species-specific isotopic values reported in the literature (Perrot et al., 2016; Poulin et al., 2021; Rodríguez-González et al., 2009). This route would be mediated by Se-biomolecules (Asaduzzaman and Schreckenbach, 2011; Khan and Wang, 2010)), finally leading to HgSe precipitation in the tissues (Palmisano et al., 1995). The hypothesis that this stage is thermodynamically favorable (Banerjee et al., 2015) could explain the absence of measurable isotopic fractionation between the two inorganic species, the (demethylated) Hg intermediate complex and the HgSe NPs. Deeper investigation focused on Se speciation in giant petrel tissues has revealed that selenoneine is the main Se-compound whatever the considered tissue (authors' unpublished data). The potential involvement of selenoneine in Hg detoxification has been evoked in several works (Achouba et al., 2019; Yamashita et al., 2013). One of the most advanced hypothesis is the mediation of selenoneine in the cleavage of Hg−methyl bonds, based on the demonstration of such capability by 1-methyl-1,3-dihydro-2H-benzimidazole-2-selone, H(sebenzimMe) a structural analogue of the selenoneine (Palmer and Parkin, 2015). The

resulting MeHg demethylation compound presents a molar ratio Hg:Se of 1:4, (Palmer and Parkin, 2015) as the one reported on giant petrel samples (Manceau et al., 2021c).

The complexity of Hg metabolization, including MeHg demethylation, is unquestionable. In general, Hg speciation is mainly limited to the discrimination between inorganic and organic species, with no consideration of Hg linked to proteins and other biomolecules (Pedrero et al., 2016). In the set of samples investigated, a lower Hg proportion was associated to the water-soluble fraction (Table S4). The screening by size exclusion chromatography of the water extractable fraction of internal tissues of giant petrels reveals the presence of several Hg compounds (Figure 4). The diversity of (unknown) Hg species detected in the water-soluble fraction strongly suggests the complexity of Hg metabolic pathways and its interactions with biomolecules in living organisms. The structural identification of Hg metabolites is limited so far to few examples as hemoglobin (-MeHg) and metallothioneins (-iHg) in marine mammals (Pedrero et al., 2011; Pedrero Zayas et al., 2014), peptide complexes in plants (Krupp et al., 2009) and ethylmercury adduct formation of human serum albumin and β-lactoglobulin (Trümpler et al., 2009) due to the great analytical challenges they represent. Despite the significant advances on the understanding of Hg in biota from this and other recent studies (Bolea-Fernandez et al., 2019; Gajdosechova et al., 2016; Manceau et al., 2021c; Pedrero et al., 2012; Pedrero Zayas et al., 2014; Poulin et al., 2021), species-specific isotopic characterization of Hg compounds, including Hg-biomolecules is mandatory to go further on the characterization of Hg metabolic pathways.

4 CONCLUSIONS

In this study, for the first time HgSe NPs species-specific (Hg) isotopic characterization has been carried out in seabirds. The isotopic signature of this compound, which is considered to be the end-product of MeHg demethylation, was determined in numerous tissues including liver, muscle, kidney and brain tissues of individual giant petrels. The Hg isotopic composition matching of HgSe NPs (measured) and other inorganic species (estimated) indicates that no isotopic fractionation appears to be induced during the HgSe NPs biomineralization step from the precursor-demethylated species. The comparison between species-specific Hg isotopic composition of MeHg (estimated) and demethylated species, suggests that a shift of 2-3 ‰ δ^{202}Hg could be

associated to the cleavage of the C-Hg bond. On the other hand, the screening of water-extractable Hg binding proteins from different tissues reveals the presence of several (unknown) Hg species, probably involved on MeHg detoxification. Advances in analytical chemistry allowing isolation and/or characterization by hyphenated techniques (e.g. HPLC-MC-ICPMS) will be crucial to accurately determine the Hg isotopic composition in these species and understand their role on Hg metabolization.

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Figure captions

Figure 1. δ^{202}Hg plotted against the percentage of MeHg for the 41 tissue samples of giant petrels. Grey markers correspond to isotopic values reported elsewhere (Renedo et al., 2021) in a pilot study for three giant petrels (PGA01, PGA02 and PGA03). White-filled markers correspond to isotopic values of the youngest individual (P-P1).

Figure 2. Δ^{199}Hg vs δ^{202}Hg values for liver, kidneys and muscle of giant petrels: A) P-1, B) P-11, C) P-P1 and D) P-10. Dotted arrows correspond to the shift between δ^{202}Hg values from THg to HgSe NPs in different organs. Uncertainties in the measurement of the isotopic ratios are expressed as 2SD on the secondary standard RM 8610.

Figure 3. Simplified diagram for the estimation of MDF ($\delta^{202}Hg$) values of MeHg according to the new insights found for the demethylation/biomineralization of MeHg into HgSe NPs in brain of three giant petrels (PGA01, PGA02 and PGA03).

Figure 4. Typical ^{202}Hg chromatogram obtained by SEC-ICP-MS for the water-soluble fraction of: A) liver and B) kidneys of adult giant petrels.