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1 **New insights into the biomineralization of**  
2 **mercury selenide nanoparticles through stable**  
3 **isotope analysis in giant petrel tissues**

4 **Silvia Queipo-Abad<sup>a</sup>, Zoyne Pedrero<sup>a\*</sup>, Claudia Marchán-Moreno<sup>a</sup>, Khouloud El**  
5 **Hanafi<sup>a</sup>, Sylvain Bérail<sup>a</sup>, Warren T. Corns<sup>b</sup>, Yves Cherel<sup>c</sup>, Paco Bustamante<sup>d,e</sup>,**  
6 **David Amouroux<sup>a</sup>**

7 <sup>a</sup> Université de Pau et des Pays de l'Adour, E2S UPPA, CNRS, IPREM, Institut des  
8 Sciences Analytiques et de Physico-chimie pour l'Environnement et les matériaux, Pau,  
9 France.

10 <sup>b</sup> PS Analytical, Arthur House, Crayfields Industrial Estate, Main Road, Orpington, Kent  
11 BR5 3HP, UK

12 <sup>c</sup> Centre d'Etudes Biologiques de Chizé, UMR 7372 CNRS - La Rochelle Université,  
13 79360 Villiers-en-Bois, France

14 <sup>d</sup> Littoral Environnement et Sociétés (LIENSs), UMR 7266 CNRS - La Rochelle  
15 Université, 2 rue Olympe de Gouges, 17000 La Rochelle, France

16 <sup>e</sup> Institut Universitaire de France (IUF), 1 Rue Descartes, 75005 Paris, France

17  
18 \*Corresponding author: Zoyne Pedrero

19 E-mail address: [zoyne.pedrerozayas@univ-pau.fr](mailto:zoyne.pedrerozayas@univ-pau.fr)

20 Université de Pau et des Pays de l'Adour, E2S UPPA, CNRS, IPREM, Institut des  
21 Sciences Analytiques et de Physico-chimie pour l'Environnement et les matériaux, Pau,  
22 France

25 **ABSTRACT**

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

41 **KEYWORDS**

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

43

44

## 1 INTRODUCTION

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

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

65 Hg isotopic analysis in biological samples is a powerful tool to trace Hg trophic sources and  
66 metabolic pathways in wildlife (Bolea-Fernandez et al., 2019; Feng et al., 2015; Li et al., 2020;  
67 Masbou et al., 2018; Perrot et al., 2016) and in humans (Du et al., 2018; Sherman et al., 2015,  
68 2013). Most of these studies have been carried out on mammalian or fish tissues (Bolea-  
69 Fernandez et al., 2019; Feng et al., 2015; Le Croizier et al., 2020; Masbou et al., 2018) and only  
70 a few of these studies have focused on seabirds (Manceau et al., 2021b; Poulin et al., 2021;  
71 Renedo et al., 2021, 2020, 2018a, 2017). Hg stable isotopes can undergo both mass-dependent

72 fractionation (MDF) and mass-independent fractionation (MIF). Odd Hg isotope MIF is related to  
73 the processes of photoreduction and photodemethylation of Hg mainly in the photic zone, thus  
74 allowing traceable marine food source to prey-predator relationships (Kwon et al., 2013; Le  
75 Croizier et al., 2020; Renedo et al., 2018c; Sherman et al., 2013). In contrast, the metabolic  
76 distribution of Hg in the body can be traced through MDF (Bolea-Fernandez et al., 2019; Feng et  
77 al., 2015). This powerful tool has been successfully exploited to track Hg distribution in the tissues  
78 of three different seabirds species, revealing that hepatic MeHg demethylation is followed by  
79 internal tissue redistribution of the residual MeHg enriched in heavier Hg isotopes (Renedo et al.,  
80 2021).

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

## 90 **2 MATERIAL AND METHODS**

### 91 **2.1. Ecological characteristics of giant petrels**

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

98

99      **2.2.    Sampling set and individual characteristics**

100    Eleven dead individuals of giant petrels were opportunistically collected. Four of them were *M.*  
101    *halli* (all males) and were collected in the Kerguelen archipelago (Southern Indian Ocean) in 2014.  
102    The other four birds were *M. giganteus*. One specimen was collected in the Kerguelen  
103    Archipelago in 2014 (male) and three were collected in Adélie Land (Antarctic continent) in 2019  
104    (1 female and 2 males). The age of these individuals could not be precisely determined, but one  
105    of the seabirds collected in Adélie Land was a fully-fledged chick (*viz.* less than 1 year old). In  
106    addition to this, samples of three other adults *M. giganteus* (see details in Renedo et al., 2021)  
107    from the same region were also considered in the present study. The samples from Adélie Land  
108    were in good shape for adults who died after a collision against an electric pole, but the chick (P-  
109    P1) was very emaciated suggesting it dies from starvation. For all the others, the cause of death  
110    is unknown, in general they were in good condition. All of them were collected freshly after their  
111    death. Internal tissues were sampled, weighed, and stored individually in plastic bags. Liver,  
112    kidneys and pectoral muscle were collected from all the eight birds, while brain tissue was  
113    sampled for the three *M. giganteus* collected in 2019. Blood samples were obtained by collecting  
114    clotted blood from heart atria. After dissection, all the samples were stored at -20°C. All details  
115    relating to the complete sampling set are provided in Table S1. All the tissues used in this work  
116    were homogenized with an ultra-turrax® and a portion was freeze-dried for analytical purposes.

117

118      **2.3.    Determination of total Hg, Se and Hg species**

119              **2.3.2.    Determination of Hg and Se concentrations**

120    Freeze-dried and homogenized tissue samples (0.05 - 0.10 g) were digested in 3 mL of Trace  
121    Metal Grade HNO<sub>3</sub> (Fisher scientific, Illkirch, France) in an Ultrawave microwave digestion system  
122    (Milestone, Sorisole, Italy) at 220°C for 10 min. Fresh-frozen blood samples were digested directly  
123    without freeze-drying. The measurement of total Hg (THg) in the digests was performed by cold  
124    vapor - atomic fluorescence spectrometry (CV-AFS PS Analytical 10.025, Kent, UK) by reduction  
125    with 3% (m/v) SnCl<sub>2</sub> (Scharlab, Barcelona, Spain) in 10% (v/v) HCl (J.T.Baker, Fisher scientific,  
126    Illkirch, France). The samples were then diluted in HCl 5% (v/v) for analyses and the quantification  
127    was performed by standard calibration. For Se analyses, the resulting digests were appropriately  
128    diluted with Milli-Q water and analyzed by ICP-MS. Method protocols were validated using  
129    certified reference materials (BCR-464, NIST-1947 and DOLT-5). The information relative to the  
130    quality assurance of the Hg analyses of Hg and Se is found in Table S2.

131 **2.3.3. Determination of iHg and MeHg species**

132 Between 0.05-0.10 g of freeze-died samples was added to glass vessels together with 3 mL of  
133 25% (m/v) tetramethylammonium hydroxide (TMAH) and a magnetic stir bar. For blood, Hg  
134 species were directly extracted from the freshly frozen samples. Vials were closed with a Teflon  
135 cap and placed in the microwave. The focused microwave assisted extraction of the samples was  
136 performed using a Discover SP-Microwave (CEM Corporation, Matthews, NC). The extraction  
137 was carried out using a 1-min ramp up to 75°C followed by 3 min at 75°C with constant stirring.  
138 Inorganic mercury (iHg) was measured by CV-AFS after dilution of the extract in HCl 5% (v/v)  
139 (Davis et al., 2007; Davis and Long, 2011; Renedo et al., 2021). For the determination of total  
140 Hg, 50 µL of a 0.1N (0.05 mol/L  $\text{cBr}_2$ ) potassium bromide/bromate solution (Merck, Darmstadt,  
141 Germany) was added to 10 mL of the diluted digest in 5% (v/v) HCl. The CV-AFS system was  
142 coupled to an oxidation unit (PS Analytical 10.820, Kent, UK) equipped with a UV lamp so that all  
143 samples with potassium bromide/bromate were subjected to on-line UV irradiation to facilitate  
144 immediate oxidation of MeHg and subsequent measurement of THg. The concentration of MeHg  
145 was determined by the subtraction of iHg from the THg concentration (Aranda et al., 2009;  
146 Kaercher et al., 2005). This procedure was validated using BCR-464 and TORT-2 as shown in  
147 Table S2.

148 **2.4. Determination of Hg biomolecular species by SEC-ICP-MS**

149 The aqueous soluble protein fraction was extracted from fresh samples (approximately 0.1g) by  
150 ultra-probe sonication (30 s at 100W power) in 3 mL of 100 mM ammonium acetate (pH 7.4)  
151 followed by centrifugation, as described elsewhere (Pedrero et al., 2011). The obtained cytosolic  
152 biomolecules were separated by size exclusion chromatography (SEC) using a Superdex 200  
153 300/10 column (10mm ID×300mm length ×13 µm particle size) (GE Healthcare, Uppsala,  
154 Sweden). A HPLC Agilent 1100 (Agilent, Wilmington, DE) equipped with a binary HPLC pump  
155 and an autosampler was coupled to an Agilent ICP-MS 7500ce (Yokogawa Analytical Systems,  
156 Tokyo, Japan). The supernatant was analysed by injection of 100 µL fractions. Isocratic elution  
157 was performed at 0.7 mL min<sup>-1</sup> with a mobile phase of 100 mM ammonium acetate at pH of 7.4  
158 (Pedrero et al., 2011; Pedrero Zayas et al., 2014).

159 **2.5. Extraction of HgSe nanoparticles**

160 Isolation of HgSe NPs was carried out by using an adaptation of the method described elsewhere  
161 (Bolea-Fernandez et al., 2019; Gajdosechova et al., 2016). In brief, after defatting, a soft acid  
162 treatment with formic acid was applied to the samples. The resulting extract was centrifuged by  
163 using 50 kDa cut-off filters (Amicon Ultra). The filter was abundantly washed with Milli-Q water  
164 until total removal of soluble Hg and Se was achieved (Gajdosechova et al., 2016). Nanoparticles  
165 were then recovered by centrifugation for 3 min at 1000 x g. The collected NPs were then digested  
166 in 3 mL HNO<sub>3</sub>, using the procedure described in section 2.3. This selective extraction protocol is  
167 not considered to be quantitative, due to losses in the cut-off filtration steps. The procedure  
168 however has been successfully used for Hg isotopic characterization previously since no isotopic  
169 fractionation of this solid species is induced during the sample treatment (Bolea-Fernandez et al.,  
170 2019).

171 **2.6. Hg stable isotope analysis**

172 Hg isotopic ratios were measured using a multicollector-(MC)-ICP-MS (Nu Instruments,  
173 Wrexham, UK) coupled with continuous flow cold vapor generation (CVG) on the digested  
174 samples (see above). The isotopes <sup>198</sup>Hg, <sup>199</sup>Hg, <sup>200</sup>Hg, <sup>201</sup>Hg, <sup>202</sup>Hg, <sup>203</sup>Tl, <sup>204</sup>Hg, and <sup>205</sup>Tl were  
175 simultaneously measured in the Faraday cups L2, L1, Ax, H1, H2, H3, H4 and H5, respectively.  
176 In order to correct the instrumental mass-bias, NIST SRM 997 thallium standard solution in 2%  
177 (v/v) of HNO<sub>3</sub> (Optima grade, Fisher scientific, Illkirch, France) was nebulized continuously  
178 through a desolvation unit (DSN) and the calculation was performed using the exponential law  
179 model (Bergquist and Blum, 2007; Blum and Bergquist, 2007; Perrot et al., 2010). The sample  
180 standard bracketing (SSB) approach was employed for the calculation of isotope ratios relative  
181 to the NIST 3133-iHg standard solution. Mass dependent fractionation (MDF) is reported as  
182 recommended by Bergquist and Blum (Bergquist and Blum, 2007) relative to the NIST 3133 Hg  
183 solution using Eq. 1:

184 
$$\delta^{xxx}Hg = \left( \frac{^{xxx/198}Hg_{sample}}{^{xxx/198}Hg_{NIST\ 3133}} - 1 \right) * 1000 \text{ ‰} \quad \text{Eq. 1}$$



185 where  $xxx$  is the studied isotope. Mass-independent fractionation (MIF) of Hg is reported using  
186 Eq. 2 as the difference between the theoretical value predicted by MDF and the measured values  
187 as  $\Delta^{199}\text{Hg}$  and  $\Delta^{201}\text{Hg}$  in ‰, according to the protocol suggested elsewhere (Blum and Bergquist,  
188 2007):

$$189 \quad \Delta_{\nu}^{xxx}\text{Hg} = \delta^{xxx}\text{Hg} - (\delta^{202}\text{Hg} \times \beta_{xxx}) \quad \text{Eq. 2}$$

190 where  $\beta_{xxx}$  is the kinetic mass-dependence scale factor that depends on the isotopes (0.2520  
191 for  $^{199}\text{Hg}$ , 0.5024 for  $^{200}\text{Hg}$ , 0.7520 for  $^{201}\text{Hg}$  and 1.493 for  $^{204}\text{Hg}$ ) (Blum and Bergquist, 2007). All  
192 the samples and standards were measured at a concentration of  $1 \text{ ng g}^{-1}$  of Hg in a 10% (v/v)  
193  $\text{HNO}_3/2\%$  (v/v) HCl solution, to avoid any bias caused by differences in concentration. The  
194 external reproducibility of the measurements is expressed as  $\pm 2\text{SD}$  for 35 measurements of the  
195 NIST RM 8610 (former UM Almadén) as 0.09 and 0.05 ‰ for  $\delta^{202}\text{Hg}$  and  $\Delta^{199}\text{Hg}$ , respectively. All  
196 the values for the isotopic composition of the NIST RM 8610 and the certified reference materials  
197 BCR- 464, NIST 1947 and TORT-2 analysed in this work are presented in Table S3.

198

### 199 **3 RESULTS AND DISCUSSION**

#### 200 **3.1. Hg concentrations in giant petrel tissues**

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

211 process of MeHg. The only chick of the study (P-P1) presents the lowest Hg levels in all the tissue  
212 types. The Hg level in liver of the chick individual ( $1.1 \mu\text{g g}^{-1}$ ) is consistent by the process of  
213 bioaccumulation with age. In kidneys, THg concentrations were higher for most of the adult  
214 samples ranging from  $7.8\text{-}481 \mu\text{g g}^{-1}$  (mean value of  $85 \pm 140$ ,  $n=10$ ) than the already previously  
215 reported concentrations for petrels and other large seabirds such as albatrosses (Bocher et al.,  
216 2003; Cipro et al., 2014; Kim et al., 1996; Stewart et al., 1999). In muscles, Hg concentrations  
217 vary from  $1.0$  to  $52 \mu\text{g g}^{-1}$  (mean value of  $18 \pm 20$ ,  $n=9$ ) in adults ( $0.10 \mu\text{g g}^{-1}$  for the chick), and  
218 are among the highest reported for seabirds (Renedo et al., 2021). In the case of blood, the two  
219 adults from the Antarctic continent (P-10, P-11) exhibit concentrations around  $0.5 \mu\text{g g}^{-1}$  (wet  
220 weight), which was fivefold higher than the concentration found in the chick (P-P1) from the same  
221 location. A linear correlation (Pearson's correlation coefficient  $\rho= 0.8971$ ) was found between  
222 THg concentrations in liver and muscle (Figure S1), which could indicate that Hg bioaccumulation  
223 occurs in both tissues along time, Hg being mainly retained in the liver but its excess being directly  
224 transferred towards the muscles as suggested by previous studies (Manceau et al., 2021c;  
225 Renedo et al., 2021).

226 Despite the high variability of MeHg content among individuals (Table S4), likely due to their age  
227 difference (Gajdosechova et al., 2016), the percentage of organic Hg was higher in brain (34-  
228 74%) than in liver and kidneys (2-34%). Regarding muscles, in the adults with lower Hg  
229 concentrations (P10 and P11, both from the Antarctic continent), the MeHg fraction reaches up  
230 to 71%. In contrast, in the case of individuals showing higher liver THg concentrations, the  
231 percentage of MeHg in the muscles was much lower (8-30%), suggesting that the proportion of  
232 MeHg decreases with age together with an increase of THg concentrations. Such a trend was  
233 already reported for top of the marine food chain marine mammals including seals and toothed  
234 whales (Bolea-Fernandez et al., 2019; Dehn et al., 2005; Gajdosechova et al., 2016).

### 235 **3.2. Hg isotopic composition of tissues: Understanding Hg (re)distribution**

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

239 lowest values of the study. Considering the whole data set, the  $\Delta^{199}\text{Hg}/\Delta^{201}\text{Hg}$  slope is  $1.13 \pm 0.01$   
240 ( $r = 0.968$ ), similar to slope values reported for other marine top predators and fish (Figure S2)  
241 (Le Croizier et al., 2020; Masbou et al., 2018; Renedo et al., 2018b, 2021). The reference  
242  $\Delta^{199}\text{Hg}/\Delta^{201}\text{Hg}$  slopes for MeHg photodemethylation processes ( $1.36 \pm 0.02$ ) and iHg  
243 photoreduction ( $1.00 \pm 0.02$ ) (Bergquist and Blum, 2007) are also presented in Figure S2. The  
244 values of  $\Delta^{199}\text{Hg}$  for giant petrel tissues agree with those of blood in skua chicks ( $1.14 \pm 0.05$  ‰)  
245 and subantarctic penguins ( $1.16 \pm 0.05$  ‰), which were correlated with latitude (Renedo et al.,  
246 2020). Interestingly, MIF values in the single chick collected on the Antarctic continent (P-P1)  
247 ranged from 0.88 to 1.1 ‰ for internal tissues, being slightly lower than for the adults. Giant petrel  
248 chicks are fed with prey caught near the colony by their parents. Hence, the MIF difference is  
249 likely due to its exclusively Antarctic food sources that are characterized by low  $\Delta^{199}\text{Hg}$  values  
250 (Renedo et al., 2020). In contrast, adults sampled in the Antarctic Continent may reflect an  
251 integration of Hg from local prey during the breeding season and Hg prey from lower latitude  
252 during the wintering season (Renedo et al., 2021).

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

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

267 Muscles of giant petrel individuals with the highest THg hepatic concentrations (250 - 933  $\mu\text{g g}^{-1}$ )  
268 are enriched in lighter isotopes ( $\delta^{202}\text{Hg}$ ) in comparison to liver and kidneys, contrasting with  
269 muscles of the other group of animals (THg hepatic concentrations lower than 250  $\mu\text{g g}^{-1}$ ). Even  
270 though Hg isotopic characterization in animal's internal tissues is not extensive, the trend  
271 observed in the mentioned samples is in agreement with previous studies on marine mammals  
272 and seabirds (Bolea-Fernandez et al., 2019; Li et al., 2020; Renedo et al., 2021) and is attributed  
273 to the higher proportion of MeHg in muscles than in the liver. As previously discussed, this  
274 organomercurial species is expected to be enriched in heavier isotopes compared to the  
275 demethylated ones, assuming preferential demethylation of the lighter Hg isotopes (Perrot et al.,  
276 2016; Rodríguez-González et al., 2009).

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

### 296 3.3. Hg isotopic composition of HgSe nanoparticles (tiemannite)

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

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

324 and muscles representing  $5 \pm 2$ ,  $35 \pm 10$  and  $61 \pm 13\%$  of the THg, respectively (Manceau et al.,  
325 2021c). In each tissue, the THg isotopic composition results from the contribution of different Hg  
326 species as outlined in Eq 3:

$$327 \quad \delta^{202}\text{Hg}_{\text{bulk}} = f_{\text{MeHg-R}} \times \delta^{202}_{\text{MeHg-R}} + f_{\text{iHg}} \times \delta^{202}_{\text{iHg}} \quad \text{Eq. 3}$$

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

$$330 \quad \delta^{202}\text{Hg}_{\text{iHg}} = f_{\text{HgSe}} \times \delta^{202}_{\text{HgSe}} + f_{\text{Hg:Se (1:4)}} \times \delta^{202}_{\text{Hg:Se (1:4)}} \quad \text{Eq. 4}$$

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

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

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

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

353 Chemical speciation and (total) Hg isotopic analyses have been combined in several recent  
354 studies for the estimation of Hg species-specific isotopic signature (Feng et al., 2015; Li et al.,  
355 2020; Poulin et al., 2021). The mentioned approach has provided new and valuable information  
356 on Hg in biota, however it should be emphasized that Hg speciation has been simplistically  
357 reduced to inorganic and MeHg species, without considering the binding of Hg with biomolecules  
358 and proteins (Pedrero et al., 2016).

359

#### 360 **3.4. New insights on Se-mediated MeHg breakdown**

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

379 resulting MeHg demethylation compound presents a molar ratio Hg:Se of 1:4, (Palmer and Parkin,  
380 2015) as the one reported on giant petrel samples (Manceau et al., 2021c).

381 The complexity of Hg metabolization, including MeHg demethylation, is unquestionable. In  
382 general, Hg speciation is mainly limited to the discrimination between inorganic and organic  
383 species, with no consideration of Hg linked to proteins and other biomolecules (Pedrero et al.,  
384 2016). In the set of samples investigated, a lower Hg proportion was associated to the water-  
385 soluble fraction (Table S4). The screening by size exclusion chromatography of the water  
386 extractable fraction of internal tissues of giant petrels reveals the presence of several Hg  
387 compounds (Figure 4). The diversity of (unknown) Hg species detected in the water-soluble  
388 fraction strongly suggests the complexity of Hg metabolic pathways and its interactions with  
389 biomolecules in living organisms. The structural identification of Hg metabolites is limited so far  
390 to few examples as hemoglobin (-MeHg) and metallothioneins (-iHg) in marine mammals (Pedrero  
391 et al., 2011; Pedrero Zayas et al., 2014), peptide complexes in plants (Krupp et al., 2009) and  
392 ethylmercury adduct formation of human serum albumin and  $\beta$ -lactoglobulin (Trümpler et al.,  
393 2009) due to the great analytical challenges they represent. Despite the significant advances on  
394 the understanding of Hg in biota from this and other recent studies (Bolea-Fernandez et al., 2019;  
395 Gajdosechova et al., 2016; Manceau et al., 2021c; Pedrero et al., 2012; Pedrero Zayas et al.,  
396 2014; Poulin et al., 2021), species-specific isotopic characterization of Hg compounds, including  
397 Hg-biomolecules is mandatory to go further on the characterization of Hg metabolic pathways.

#### 398 **4 CONCLUSIONS**

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



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

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## 670 **Figure captions**

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

675

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

680

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

684

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