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26 demethylation

27 **ABSTRACT**

28 In the comments reported by A. Manceau [1], relating to our recent paper on mercury (Hg)
29 species-specific isotopic characterization in giant petrel tissues [2] two critical questions were
30 raised. Firstly, according to A. Manceau, our method of extraction and isolation of nanoparticles
31 was not able to efficiently isolate mercury selenide nanoparticles (HgSe NPs) and therefore the
32 $\delta^{202}\text{Hg}$ values measured are not species-specific, but rather $\delta^{202}\text{Hg}$ of mixtures of complexes such
33 as MeHgCys, Hg(Sec)₄, and HgSe. Secondly, he suggests that our main findings showing that
34 no isotopic fractionation is induced during the HgSe NPs biomineralization step from the
35 precursor-demethylated species is erroneous because it contradicts the conclusion of two recent
36 articles by A. Manceau and co-workers [3,4]. In this reply we defend our scientific findings and
37 respectively respond to the questions and comments raised by A. Manceau.

38

39 **1. DISCUSSION**

40 Firstly, we address the comment questioning the purity of the isolated HgSe NPs fraction.
41 Manceau is suggesting that this fraction is a mixture of MeHgCys, Hg(Sec)₄ and HgSe, therefore
42 leading to a non-species-specific HgSe NPs isotopic characterization. We would like to point out
43 that the NPs isolation approach, as detailed in the manuscript, is based on the published research
44 by Bolea-Fernández and co-workers for Hg isotopic characterization of Tiemannite in pilot whale
45 tissues [5,6]. We have done slight modifications to their method including a heating step at 85°C
46 for 2 hours with formic acid to remove any organic molecules. Even if a small amount of Hg could
47 be found in the solid residual resulting fraction, it is also a product of Hg (bio)demethylation. In
48 addition, as detailed in our article, the cut-off filter with isolated HgSe NPs fraction was abundantly
49 washed with MQ-water until complete elimination of total soluble Hg was achieved and verified
50 by CV-AFS measurements. Therefore, by using such sample treatment, the absence of the
51 concomitant Hg species mentioned by Manceau is guaranteed and it is unlikely it will modify the
52 interpretation of the obtained data. Manceau supports his criticism regarding HgSe NPs purity,
53 probably based on those values where the $\delta^{202}\text{Hg}_{\text{bulk}}$ is equivalent to species-specific $\delta^{202}\text{Hg}$ of
54 isolated HgSe NPs. However, in more than 50% of the samples the absolute shift $\delta^{202}\text{Hg}$ (Bulk -

55 HgSe NPs) is larger than 0.20 ‰), all having been treated with the same procedure and
56 demonstrating that labile-Hg was removed from that fraction.

57 The second comment addressed by Manceau questions our main hypothesis that HgSe NPs
58 biomineralization from the precursor-demethylated species does not induce Hg isotopic
59 fractionation due to its conflict with two research investigations conducted by himself and his co-
60 workers [3,4]. In this regard, we would like to state that our premise is solidly based on high
61 precision measurements of the Hg isotopic composition in the tissues of 11 giant petrels (40
62 tissues in total, with HgSe NPs extracted from 37 tissues, all except three blood samples). In
63 contrast, the two recent publications by Manceau and co-workers [3,4], based their hypothesis on
64 a mathematical calculation approach which has not yet been experimentally validated by
65 measurements of species-specific Hg isotopic composition.

66 In each tissue, the bulk Hg isotopic composition results from the contribution of different Hg
67 species as outlined in equation 1:

68
$$\delta^{202}\text{Hg}_{\text{bulk}} = \sum_{i=\text{Hg species}} f_i \times \delta^{202}\text{Hg}_i \quad (\text{‰}) \quad \text{Eq. 1}$$

69 where $\delta^{202}\text{Hg}_{\text{bulk}}$ represents the isotopic composition of total mercury in the tissue, f_i is the fraction
70 of i -species in the corresponding tissue, and $\delta^{202}\text{Hg}_i$ represents the species-specific isotopic
71 composition of Hg in i -species. The resolution of this equation requires the knowledge of a series
72 of data that have been over simplified in the aforementioned mathematical works [3,4] with the
73 following assumptions: i) the isotopic composition of each Hg species is only species-dependent,
74 and it does not vary between individuals or between tissues; ii) Hg species in tissues are
75 exclusively limited to three different forms, viz.: MeHgCys, Hg(Sec)₄ and HgSe. Regarding the
76 invariance of Hg isotopic composition, to the best of our knowledge there is no research to date
77 that supports the theory that there is a specific $\delta^{202}\text{Hg}$ value that is solely dependent on the nature
78 of the species. So far, in cases where different Hg species have been extracted, no unique and
79 constant $\delta^{202}\text{Hg}$ value has been obtained for all tissues or in all individuals studied. This has not
80 been experimentally proven neither for MeHg [4,7–9] or HgSe NPs [2,5] in different living
81 organisms. A study developed by our group (IPREM CNRS Pau) and co-workers is the only one
82 to jointly report Hg isotopic compositions for MeHg and inorganic mercury (iHg) [9] in aquatic
83 mammal tissues. These values reflect a large variability between individuals and tissues for the

84 species-specific $\delta^{202}\text{Hg}$ values in beluga whales ($\delta^{202}\text{Hg}_{\text{MeHg}}$ variation of $\sim 3.5\%$) and seals
85 ($\delta^{202}\text{Hg}_{\text{MeHg}}$ variation of $\sim 1.7\%$). This aspect of the mathematical approach has also been recently
86 questioned by Wiederhold and Jiskra [10], which makes it clear that this assumption remains
87 questionable for the seabird tissues. So far the number of studies dealing with Hg species-specific
88 isotopic composition is limited mainly due to the great challenge associated with the
89 extraction/isolation of the different Hg species while preserving the original isotopic pattern [2,5,7–
90 9,11]. The Hg species-specific approach in animal tissues has been mainly applied on the isotopic
91 characterization of MeHg [4,7–9]. Meanwhile Bolea-Fernández and co-workers together with our
92 article [2,5] reported the unique Hg species-specific isotopic composition relative to HgSe NPs.
93 The obtained Hg species-specific isotopic data has been key to obtain information on metabolic
94 processes [2,5,7–9].

95 The comments of Manceau about the $\delta^{202}\text{Hg}$ in HgSe NPs values experimentally determined in
96 our article comes from the differences with the values estimated by a mathematical approach
97 considering exclusively three species of Hg (MeHgCys, Hg(Sec)₄ and HgSe). However, the
98 analysis of the water-soluble fraction from the different seabird tissues by size exclusion
99 chromatography (SEC)-ICP-MS presented in Figure 4 of our recent article [2], evidenced that Hg
100 binds several (unknown) biomolecules that probably play key roles on MeHg demethylation. The
101 unambiguous characterization of Hg binding biomolecules/proteins represents an important
102 analytical challenge, which explain the limited number of publications reporting Hg-metabolites
103 [12–19]. The crucial role of speciation in understanding metabolic processes is undoubtedly an
104 additional dimension to Hg isotopic characterisation. Thus, we consider that a mathematical
105 approach which simplifies the number of Hg species and the possibly large variability of $\delta^{202}\text{Hg}$ in
106 different tissues and individuals, cannot be used in a general way to estimate Hg isotopic values
107 to improve our understanding of the pathways and fate of Hg in biota.

108 Moreover, we would also like to highlight this statement by Manceau: “*that the ²⁰²Hg isotope is*
109 *actually fractionated during the Hg(Sec)₄ → HgSe reaction, and therefore that this isotope can be*
110 *used to trace the Hg metabolic pathways between tissues in a single individual and in different*
111 *animals*”. This argument is independent of whether there is isotopic fractionation at that stage,
112 and even transcends this study, as $\delta^{202}\text{Hg}$ (MDF) has already been used in several works to trace

113 metabolic pathways in living organisms [5,9,20–25]. Even the fact that there is no isotopic
114 fractionation of Hg at this stage characterizes the (metabolic) process, therefore, this statement
115 for us means a trivialization of the scientific results of this research.

116 Additionally, our hypothesis related to the absence of isotopic fractionation during the
117 biomineralization step from the precursor (demethylated) species, can be supported by the
118 combination of $\delta^{202}\text{Hg}$ values measured in giant petrels and estimated with equation 1. The $\delta^{202}\text{Hg}$
119 species-specific experimental data (table 1) reported [2,4,25,26] for MeHg ($\delta^{202}\text{Hg}_{\text{MeHg}}$) and for
120 HgSe NPs ($\delta^{202}\text{Hg}_{\text{HgSe}}$) were combined by applying equation 2 (adapted from equation 1) in the
121 estimation of $\delta^{202}\text{Hg}_{\text{bulk}}$ as follows:

$$122 \quad \delta^{202}\text{Hg}_{\text{bulk estimated}} = f(\text{MeHg}) \times \delta^{202}\text{Hg}_{\text{MeHg}} + f(\text{HgSe}) \times \delta^{202}\text{Hg}_{\text{HgSe}} + f(\text{HgSe (1:4)}) \times \delta^{202}\text{Hg}_{\text{HgSe 1:4}} \quad (\%) \quad \text{Eq.2}$$

123 where $\delta^{202}\text{Hg}_{\text{bulk}}$ represents the estimation of isotopic composition of total mercury in the tissue
124 and f is the fraction of the three main species (MeHg, precursor HgSe (1:4), and HgSe NPs)
125 determined by HR-XANES [26]. The $\delta^{202}\text{Hg}_{\text{HgSe (1:4)}}$ will be considered equivalent to $\delta^{202}\text{Hg}_{\text{HgSe}}$, in
126 line with our observation in the preceding article. The obtained $\delta^{202}\text{Hg}_{\text{bulk estimated}}$ reported in table
127 1 and the measured values (average between $\delta^{202}\text{Hg}_{\text{bulk}}$ measurements in different sections of
128 the tissues) [2,4] show a good agreement when assuming the lack of Hg isotopic fractionation in
129 the biomineralization and approximating the $\delta^{202}\text{Hg}_{\text{HgSe (1:4)}}$ to the $\delta^{202}\text{Hg}_{\text{HgSe}}$.

130 In summary, the complexity of Hg pathways in biota calls for a combination of complementary
131 analytical techniques to contribute to their elucidation. The analytical approaches addressed on
132 this discussion (species-specific isolation, liquid chromatography separation, HR-XANES
133 identification and high precision isotopic analyses) demonstrates the potential of such synergy to
134 go further on the understanding of Hg processes in biota.

135

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234

235 **Table 1.** Estimation of the $\delta^{202}\text{Hg}_{\text{bulk}}$ based on the species-specific $\delta^{202}\text{Hg}$ of the three main species according to HR-XANES measurements. The measured
 236 $\delta^{202}\text{Hg}_{\text{bulk}}$ value for comparison has been calculated as an average of the two published values for these tissues. The $\delta^{202}\text{Hg}_{\text{HgSe (1:4)}}$ has been approximated to
 237 the $\delta^{202}\text{Hg}_{\text{HgSe}}$ experimentally measured in the corresponding tissues.

		$\delta^{202}\text{Hg}_{\text{bulk}}$ (‰) ^[4]	$\delta^{202}\text{Hg}_{\text{bulk}}$ (‰)	Mean $\delta^{202}\text{Hg}_{\text{bulk}}$ (‰)	$\delta^{202}\text{Hg}_{\text{MeHg}}$ (‰) ^[4]	$\delta^{202}\text{Hg}_{\text{HgSe}} = \delta^{202}\text{Hg}_{\text{HgSe (1:4)}}$ (‰) ^[2]	f MeHg (%) ^[26]	f HgSe (1:4) (%) ^[26]	f HgSe (%) ^[26]	$\delta^{202}\text{Hg}_{\text{bulk estimated}}$ (‰)
Individual 1	Kidneys	0.15 ± 0.02	0.11 ^[2]	0.15 ± 0.03	2.70 ± 0.03	-0.08	0.07 ± 0.03	0.32 ± 0.09	0.61 ± 0.08	0.11
	Muscle	-0.76 ± 0.03	-0.48 ^[2]	-0.76 ± 0.20	2.78 ± 0.03	-0.70	-	0.67 ± 0.08	0.33 ± 0.08	-0.70
Individual 2	Liver	0.04 ± 0.04	-0.08 ^[2]	0.04 ± 0.08	1.87 ± 0.03	-0.05	-	0.09 ± 0.06	0.91 ± 0.06	-0.05
	Muscle	-0.57 ± 0.02	-0.51 ^[2]	-0.54 ± 0.04	2.07 ± 0.03	-0.55	0.07 ± 0.03	0.60 ± 0.09	0.33 ± 0.09	-0.37
Individual 3	Muscle	-0.73 ± 0.06	-	-0.73 ± 0.06	0.90 ± 0.04	-	-	0.40 ± 0.08	0.60 ± 0.08	-
Individual 4	Brain	0.50 ± 0.04	0.10 ^[8]	0.30 ± 0.28	2.58 ± 0.04	-0.32	0.13 ± 0.05	0.16 ± 0.10	0.71 ± 0.08	0.06

238

239 $\delta^{202}\text{Hg}_{\text{bulk estimated}} = f_{\text{MeHg}} \times \delta^{202}\text{Hg}_{\text{MeHg}} + f_{\text{HgSe}} \delta^{202}\text{Hg}_{\text{HgSe}} + f_{\text{HgSe (1:4)}} \delta^{202}\text{Hg}_{\text{HgSe 1:4}}$ (‰)

240 Individual 1: P2^[26]=P3^[2]; Individual 2: P3^[26]=P4^[2]; Individual 3: P5^[26]=P8^[2]; Individual 4: P8^[26]=PGA03^[2,25]