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**Reply to the comment on “New insights into the biomineralization of mercury selenide nanoparticles through stable isotope analysis in giant petrel tissues” by A. Manceau, J. Hazard. Mater. 425 (2021) 127922. doi: 10.1016/j.jhazmat.2021.127922**

Silvia Queipo-Abad, Zoyne Pedrero, Claudia Marchán-Moreno, Khoulood El Hanafi, Sylvain Bérail, Warren Corns, Yves Cherel, Paco Bustamante, David Amouroux

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1 **Reply to the comment on “New insights into the biomineralization of**  
2 **mercury selenide nanoparticles through stable isotope analysis in giant**  
3 **petrel tissues” by A. Manceau, J. Hazard. Mater. 425 (2021) 127922. doi:**  
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5 **Silvia Queipo-Abad<sup>a</sup>, Zoyne Pedrero<sup>a\*</sup>, Claudia Marchán-Moreno<sup>a</sup>, Khouloud El**  
6 **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>,**  
7 **David Amouroux<sup>a</sup>**

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

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

13 <sup>c</sup> Centre d’Etudes Biologiques de Chizé, UMR 7372 CNRS - La Rochelle Université,  
14 79360 Villiers-en-Bois, France

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

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

18

19 \*Corresponding author: Zoyne Pedrero

20 E-mail address: zoyne.pedrerozayas@univ-pau.fr

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

24

25 **Keywords:** Mercury, Seabirds, Isotopic fractionation, HgSe nanoparticles, MeHg  
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)<sub>4</sub>, 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)<sub>4</sub> 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:

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

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)<sub>4</sub> 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)<sub>4</sub> 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 <sup>202</sup>Hg isotope is*  
109 *actually fractionated during the Hg(Sec)<sub>4</sub> → 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.

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

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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<sup>[26]</sup>=P3<sup>[2]</sup>; Individual 2: P3<sup>[26]</sup>=P4<sup>[2]</sup>; Individual 3: P5<sup>[26]</sup>=P8<sup>[2]</sup>; Individual 4: P8<sup>[26]</sup>=PGA03<sup>[2,25]</sup>