Reply on "Mercury Isotope Fractionation by Internal Demethylation and Biomineralization Reactions in Seabirds: Implications for Environmental Mercury Science": Principles and limitations of source tracing and process tracing with stable isotope signatures

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Reply on "Mercury Isotope Fractionation by Internal Demethylation and Biomineralization Reactions in Seabirds: Implications for Environmental Mercury Science": Principles and limitations of source tracing and process tracing with stable isotope signatures

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In Manceau et al. (2021),[1] we measured the mass-dependent fractionation (MDF) of mercury (Hg) stable isotopes, noted as δ^{202}Hg, in 22 biological tissues from eight giant petrels (*Macronectes* spp.) previously characterized for Hg speciation using state-of-the-art X-ray absorption spectroscopy and transmission electron microscopy.[2] The tissues contained three dominant Hg species (Sp_i), a methylmercury cysteinate (MeHgCys) complex, a Hg-tetraselenolate ($Hg(Sec)_4$) complex, and mercury selenide (HgSe) grains, and their fractions (f(MeHgCys), $f(Hg(Sec)_4)$, and f(HgSe)) in each tissue were known. The δ^{202}Hg value of each tissue ($\delta^{202}Hg_t$) is a weighted sum of the δ^{202}Hg of each Hg species expressed as follows:

$$\delta^{202}Hg_t = \sum_{i=3} f\left(Sp_i\right)_t \times \delta^{202}Sp_i \qquad (1)$$

in which $\delta^{202}Sp_i$ are species- and tissue-specific δ^{202}Hg values.

If each Hg species occurs in every tissue with a tissue-specific $\delta^{202}Sp_{i,t}$ value, the system of 22 linear equations has 66 unknowns. Such a linear system is underdetermined and has infinitely many solutions. However, using an inversion algorithm, we found that the degree of freedom of the petrel system could be reduced from 44 (some of the tissues had only one or two species) to 3 with identical species-specific $\delta^{202}Sp_i$ signatures between different tissues of the same bird and across the eight individuals. The $\delta^{202}Hg_t$ values of the 22 tissues were described as a weighted sum of three species-specific $\delta^{202}Sp_i$ values:

$$\delta^{202}Hg_t = \sum[f\left(MeHgCys\right)_t \times \delta^{202}MeHgCys]$$

$$+[f\left(Hg(Sec)_4\right)_t \times \delta^{202}Hg(Sec)_4]+[f\left(HgSe\right)_t \times \delta^{202}HgSe] \qquad (2)$$

using δ^{202}MeHgCys = 2.69 ± 0.04‰, $\delta^{202}Hg(Sec)_4 = -1.37 \pm 0.06$‰, and δ^{202}HgSe = 0.18 ± 0.02‰. Further, δ^{202}Hg of MeHg (δ^{202}MeHg) values were measured directly on a subset of 9 tissues using an established distillation and anion-exchange chromatography method.[3] The majority of the MeHg-specific isotope measurements were in agreement with the calculated values (within the analytical uncertainty of 0.16‰). However, one feather had δ^{202}MeHg = 3.05‰ which was attributed to differences in dietary sources or feeding ranges that were integrated over time in the bird.[1] Also, one muscle had a δ^{202}MeHg = 0.90‰ that was anomalous and is discussed in detail below. While there may be limitations to MeHg-specific isotope measurements, all data were presented and discussed in Manceau et al. (2021)[1] for transparency. Wiederhold and Jiskra[4] comment on the validity of the data analysis approach and contend that the anomalous δ^{202}MeHg outlier values can be explained using a closed-system Rayleigh fractionation model. We challenge these critiques below and detail a conceptual diagram that is appropriate for isotope analyses and interpretations for organisms with continuous dietary inputs, internal transformations of Hg, and depuration.

First, Wiederhold and Jiskra[4] comment that the petrel linear isotope system is mathematically underdetermined. Actually, the regression analysis was statistically robust and the 22 $\delta^{202}Hg_t$ values were recalculated within experimental uncertainty from the reduced linear system (Eq. 2). The observed linearity in the isotope system in the petrels is consistent with observed two-component isotope systems that documented MeHg demethylation to inorganic Hg in waterbirds[5] and the brain of whales.[6] Further, similar isotopic results were obtained in a companion article on long-finned pilot whales.[7] In this companion study, the $\delta^{202}Hg_t$ values of 89 tissues (excluding blood) from 28 individuals reported in the independent studies of Bolea-Fernandez et al. (2019)[8] and Li et al. (2020)[6] were analyzed by mathematical inversion. It was also found that three $\delta^{202}Sp_i$ values were sufficient to describe the species-averaged $\delta^{202}Hg_t$ values between tissues of the same individual and across individuals sampled at different geographical locations and times. The highly consistent observations between the giant petrels and long-finned pilot whales indicate that the isotopic fractionation due to *in vivo* demethylation and biomineralization reactions occurs in diverse vertebrates, isotopic signatures of the chemical Hg species are homogenized within individuals, and localized isotope effects due to these reactions in tissues are not apparent.

Second, Wiederhold and Jiskra[4] point out the discrepancy between the calculated (2.69 ± 0.04‰) and two measured δ^{202}MeHg values (3.05‰, 0.90‰). This was the first study to compare mathematical estimates of Hg isotope end-members based on Hg speciation from X-ray absorption spectroscopy with direct measurements of MeHg-specific isotope values. Given the complexities of each technique, perfect agreement between measured and calculated values was not expected. Giant petrels are wild animals that live across expansive ranges and have diverse prey items, and yet the measured δ^{202}MeHg isotope values were overall well constrained.[1] We agree with Wiederhold and Jiskra[4] that the measured δ^{202}MeHg value of 0.90‰ is puzzling, but it is anomalous compared to the other measurements. Determining if this data point is truly an analytical anomaly is difficult, but the feather tissue from the same petrel containing 100% MeHgCys had a δ^{202}MeHg value consistent with the mathematical estimate. Importantly, the muscle tissue with the anomalous δ^{202}MeHg value of 0.90‰ had 40±8% $Hg(Sec)_4$ and 60±8% HgSe, and the MeHgCys species was below the spectroscopic detection limit (<3%). Therefore, this tissue did not contribute to the calculation of δ^{202}MeHgCys, as was detailed in the study.

Third, Wiederhold and Jiskra[4] propose that a Rayleigh fractionation model can explain the anomalous δ^{202}MeHg values quantified in the petrel tissues. Unfortunately, there are oversights with the proposed use of a Rayleigh fractionation model by Wiederhold and Jiskra[4] and they egregiously misrepresented the data in Manceau et al. (2021).[1] The Rayleigh fractionation model assumes a closed system, where a finite reservoir of reactant (in this case MeHg) becomes enriched in heavier isotopes as lighter isotopes are demethylated to the product pool. In the birds, however, a constant influx of dietary MeHg replenishes the reactant pool, MeHg undergoes stepwise demethylation and biomineralization reactions, HgSe is accumulated,[2] both MeHg and $Hg(Sec)_4$ are excreted in waste, and MeHg is depurated in feathers. Figure 1 presents a schematic representation of the birds, modified from Hayes (2002),[9] which serves as a model to guide isotope interpretations and applies to other vertebrates (e.g., marine mammals). The continuous input of MeHg and loss terms of MeHg and $Hg(Sec)_4$ in the birds, as well as internal exchange between tissues via the circulatory system, likely explain the consistency in species-specific measurements of the MeHg isotope pool across different petrels; these processes are not accounted for in the Rayleigh fractionation model. If the finding of a steady-state isotopic fractionation of MeHgCys → $Hg(Sec)_4$ demethylation and $Hg(Sec)_4$ → HgSe biomineralization reactions in birds[1] and whales[7] is novel, its hypothesis is not. Bolea-Fernandez et al. (2019)[8] observed that δ^{202}Hg was uniform in the blood of 7 juvenile and 7 adult whales due to the homogenization of the isotopic composition of MeHg throughout the body. This interpretation is

reinforced by a previous study showing a rapid shift in Hg isotope values of fish tissues to values of dietary MeHg replenishment.[10]

We assess the validity of a Rayleigh fractionation model in Figure 2a, which presents the measured δ^{202}MeHg values versus the fraction of MeHg in tissues (f_{MeHg}) of the petrels from Manceau et al. (2021)[1] against the theoretical Rayleigh fractionation model from Wiederhold and Jiskra.[4] The measured δ^{202}MeHg values are relatively invariant across a wide range of f_{MeHg} (aside from the outlier of 0.90‰), and δ^{202}MeHg values at low f_{MeHg} never exceeded δ^{202}MeHg at high f_{MeHg} (counter to the Rayleigh fractionation model). These observations in petrels are consistent with the measurement of δ^{202}MeHg values of other bird[5] and seal tissues[11] with varying degrees of demethylation (i.e., f_{MeHg}), as shown in Figure 2b. The observed invariance in measured δ^{202}MeHg data across multiple organisms and studies is inconsistent with the use of a Rayleigh fractionation model in this system, seen by comparing the Wiederhold and Jiskra model (red line and data)[4] to the measurement of the petrels (Figure 2a).

Given the lack of transparency in the analysis provided by Wiederhold and Jiskra,[4] we can only surmise the source of the data points they present in their Figure 1b that reside precisely on the theoretical Rayleigh fractionation line. As such, Wiederhold and Jiskra[4] first used the anomalous δ^{202}MeHg value of 0.90‰ as the starting pool (red diamond in Figure 2a) and assigned a $f_{MeHg} = 1.0$ despite the measured value of $f_{MeHg} \leq 0.03$. Next, from the "starting pool," the theoretical Rayleigh fractionation was modeled (shown as a solid line in Figure 2a), and the other data points presented were assigned fictious f_{MeHg} values (red circles in Figure 2a). Simply, the measured paired δ^{202}MeHg and f_{MeHg} values from Manceau et al. (2021)[1] are not presented in Figure 1b of Wiederhold and Jiskra.[4] Further, the range of δ^{202}MeHg values presented by Wiederhold and Jiskra[4] were as high as ~4.0‰, exceeding those reported in Manceau et al. (2021),[1] and the source of the isotope enrichment factor used in their model simulation is incorrectly cited. While it is important to consider the potential of Rayleigh fractionation in environmental matrices, this model does not explain the outliers in the dataset and the consistency of δ^{202}MeHg across multiple tissues. In summary, the premise that bulk δ^{202}Hg signatures of giant petrel tissues can be described as weighted sums of three species-specific δ^{202}MeHgCys, δ^{202}Hg(Sec)$_4$, and δ^{202}HgSe values is fully validated using the direct measurements of δ^{202}MeHg.[1]

The holistic approach suggested by Wiederhold and Jiskra[4] holds merit as it highlights current gaps in the literature associated with Hg stable isotope applications in birds and mammals. Specifically, further examination is warranted on the dietary variability of the MeHg pool in wildlife,[12]

and the isotopic modeling of open systems (e.g., Figure 1) where multiple products are produced via steady-state reactions (e.g., demethylation and biomineralization) and select products are depurated (e.g., MeHg, $Hg(Sec)_4$) and others accumulated (e.g., HgSe). These gaps, however, do not directly address any of the points made in an attempt to dismiss the findings of our study. The mechanistic examination of internal demethylation and biomineralization pathways could be overlooked by simplifying these findings to a mass balance within the organism, emphasizing the need for continued isotope research that applies complementary analytical methods (X-ray absorption spectroscopy, species-specific mercury stable isotope, and protein-level screening)[13] to advance mechanistic understanding and modeling. Furthermore, while the multi-dimensional nature of Hg isotopes (MDF and mass independent fractionation) is an important consideration in source tracking and assessing Hg isotope fractionation *in vivo*, as noted by Wiederhold and Jiskra,[4] there was no evidence that individual petrels had dissimilar exposures to Hg processing effects (e.g., photochemical demethylation noted by $\Delta^{199}Hg$) or differing atmospheric sources (noted by $\Delta^{200}Hg$).[14] Although much remains to be known on the internal cycling of Hg within mammals and birds, the petrels presented a unique opportunity to examine an organism where internal demethylation and biomineralization reactions are responsible for as much as a 3.5‰ difference in tissue $\delta^{202}Hg$ values. This work opens the door for new methods and techniques to examine internal processing of Hg in biota.

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Figure caption

Figure 1. Schematic representation of an open isotope system with a continuous input of MeHg ("reactant") to the bird, the bird being a "reaction chamber" where isotopic fractionation occurs due to stepwise demethylation and biomineralization reactions. HgSe accumulates in the bird ("accumulated product"), and MeHg and $Hg(Sec)_4$ are excreted in waste and/or depurated (product streams). Modified from Hayes (2002).[9]

Figure 2. (a) Direct comparison of the measured δ^{202}MeHg versus the fraction of MeHg (f_{MeHg}) from Manceau et al. (2021)[1] for giant petrel tissues and the theoretical Rayleigh fractionation model presented by Wiederhold and Jiskra[4] (red line and data points, extracted using OriginLab (Version 2121b, OriginLab Corporation, Northampton, MA, USA). The source of the x and y data points from Wiederhold and Jiskra[4] is unknown. (b) Comparison of the measured δ^{202}MeHg versus f_{MeHg} for Clark's grebe (*Aechmophorus clarkia*) and Forster's tern (*Sterna forsteri*) tissues from Poulin et al. (2021)[5] and seal tissues from Perrot et al. (2016).[11] Measured data in plots a and b demonstrate that δ^{202}MeHg is largely invariant across a wide range of f_{MeHg} and is inconsistent with a Rayleigh fractionation model. For tissues from Manceau et al. (2021),[1] $f_{MeHg} = 0.03$ was used for samples at the spectroscopic detection limit for MeHgCys.

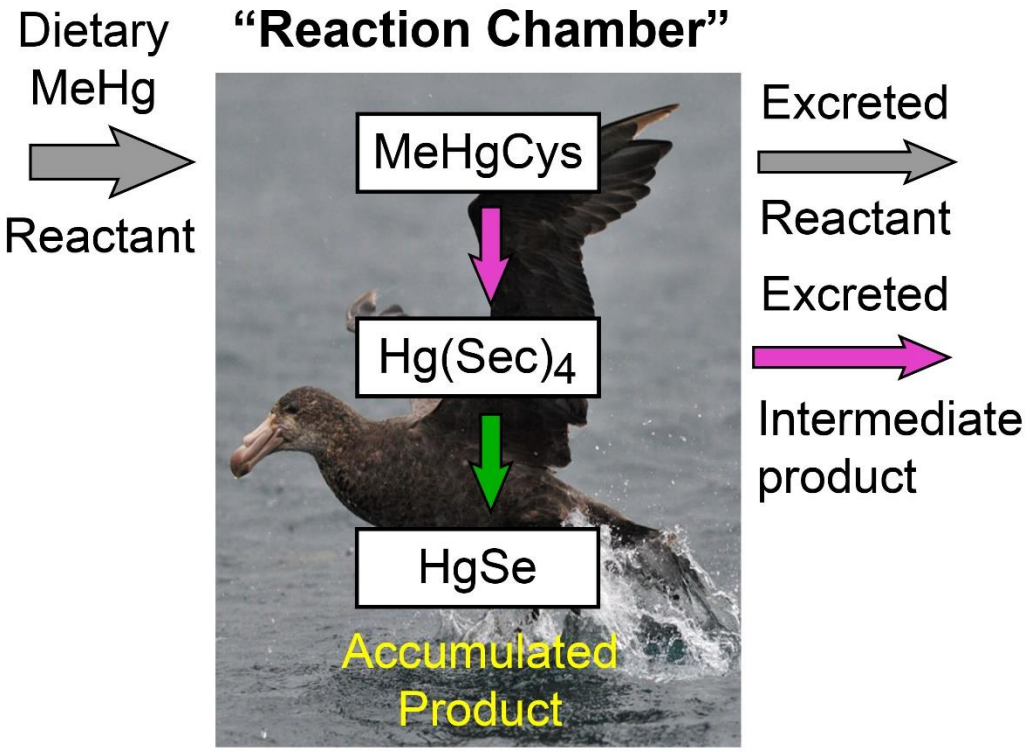
Dietary
MeHg
Reactant
"Reaction Chamber"
MeHgCys
Hg(Sec)4
HgSe
Accumulated
Product
Excreted
Reactant
Excreted
Intermediate
product

Figure 1

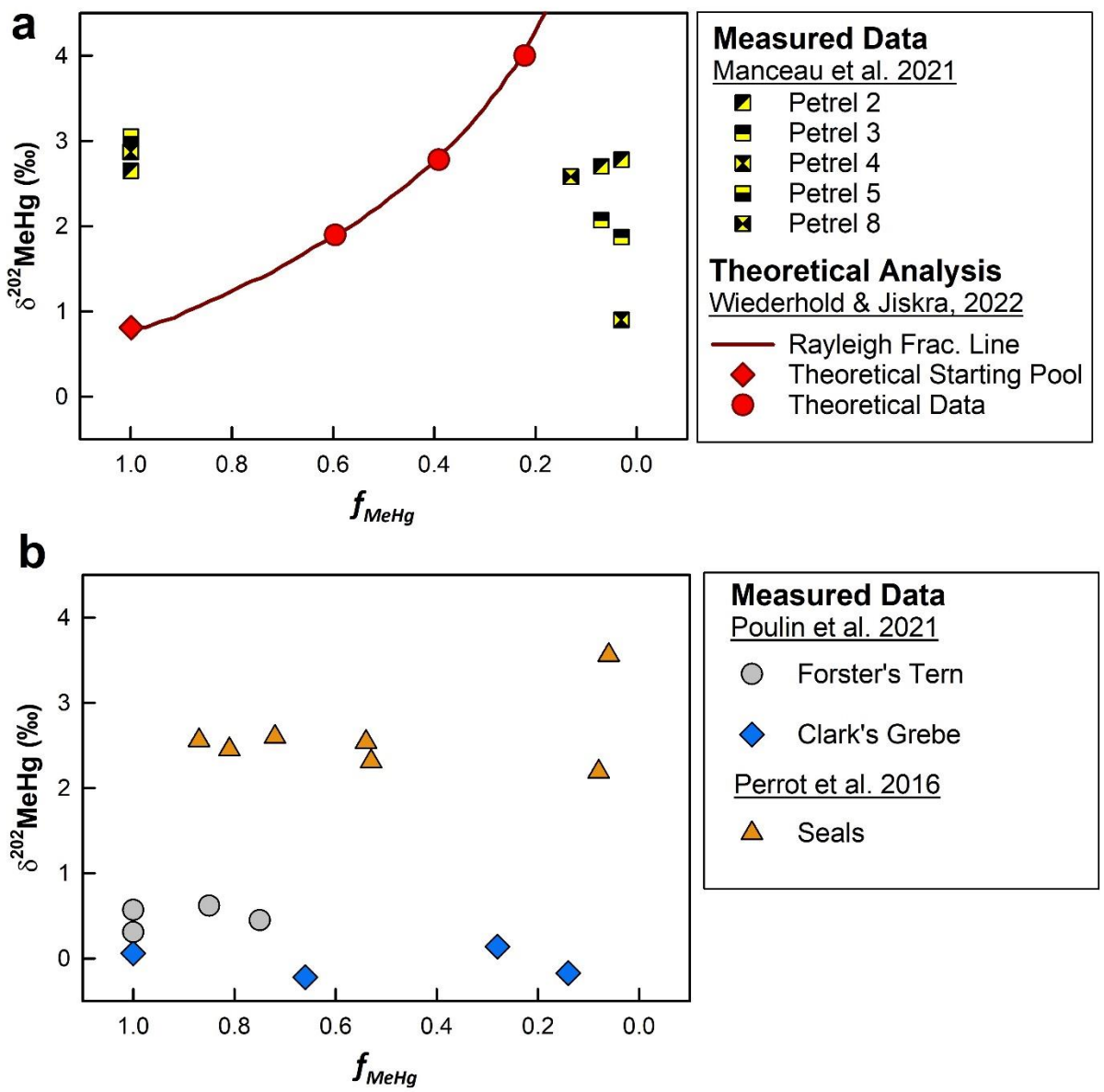
a
δ202MeHg (‰)
fMeHg
Measured Data
Manceau et al. 2021
Petrel 2
Petrel 3
Petrel 4
Petrel 5
Petrel 8
Theoretical Analysis
Wiederhold & Jiskra, 2022
Rayleigh Frac. Line
Theoretical Starting Pool
Theoretical Data
b
δ202MeHg (‰)
fMeHg
Measured Data
Poulin et al. 2021
Forster's Tern
Clark's Grebe
Perrot et al. 2016
Seals

Figure 2