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1 **Divalent mercury in dissolved organic matter is bioavailable to fish and**
2 **accumulates as dithiolate and tetrathiolate complexes**

3

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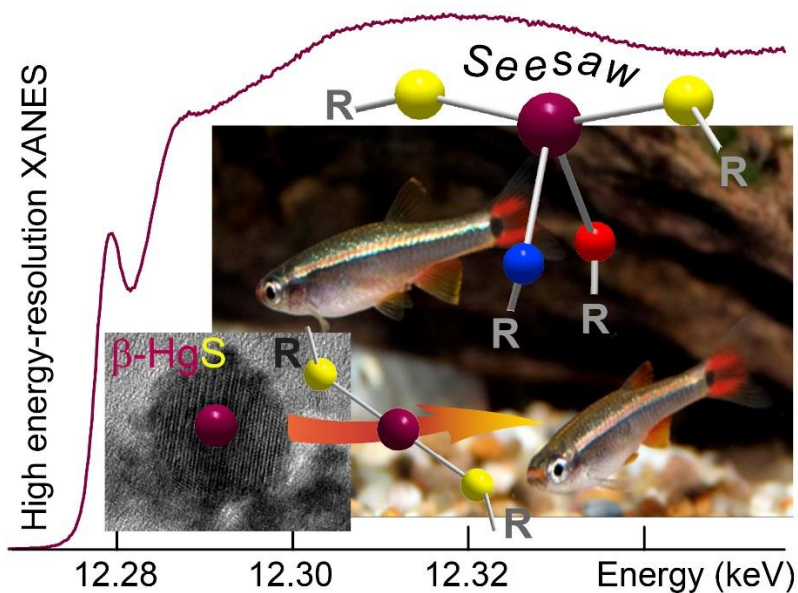
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18

19 **Keywords:** Mercury, fish, DOM, speciation, metacinnabar, XANES

20



21

22 **ABSTRACT**

23 The freshwater cyprinid *Tanichthys albonubes* was used to assess the bioavailability of
 24 divalent mercury (Hg(II)) complexed in dissolved organic matter (DOM) to fish. The fish acquired
 25 0.3 to 2.2 $\mu\text{g Hg/g}$ dry weight after eight weeks in aquaria containing DOM from a *Carex* peat with
 26 complexed mercury at initial concentrations of 14 nM to 724 nM. Changes in the relative
 27 proportions of dithiolate $\text{Hg}(\text{SR})_2$ and nanoparticulate $\beta\text{-HgS}$ in the DOM, as quantified by high
 28 energy-resolution XANES (HR-XANES) spectroscopy, indicate that $\text{Hg}(\text{SR})_2$ complexes either
 29 produced by microbially-induced dissolution of nanoparticulate $\beta\text{-HgS}$ in the DOM or present in
 30 the original DOM were the forms of mercury that entered the fish. In the fish with 2.2 $\mu\text{g Hg/g}$, 84
 31 $\pm 8\%$ of Hg(II) was bonded to two axial thiolate ligands and one or two equatorial N/O electron
 32 donors ($\text{Hg}[(\text{SR})_2+(\text{N/O})_{1-2}]$ coordination), and 16% had a $\text{Hg}(\text{SR})_4$ coordination, as determined
 33 by HR-XANES. For comparison, fish exposed to Hg^{2+} from 40 nM HgCl_2 contained 10.4 $\mu\text{g Hg/g}$ in
 34 the forms of dithiolate ($20 \pm 10\%$) and tetrathiolate ($23 \pm 10\%$) complexes, and also Hg_xS_y clusters
 35 ($57 \pm 15\%$) having a $\beta\text{-HgS}$ -type local structure and a dimension that exceeded the size of
 36 metallothionein clusters. There was no evidence of methylmercury in the fish or DOM within the
 37 10% uncertainty of the HR-XANES. Together, the results indicate that inorganic Hg(II) bound to

38 DOM is a source of mercury to biota with dithiolate $\text{Hg}(\text{SR})_2$ complexes as the immediate species
39 bioavailable to fish, and that these complexes transform in response to cellular processes.

40

41 INTRODUCTION

42 Mercury (Hg) is a potent neurotoxin in animals and humans that bioaccumulates in aquatic
43 food webs mainly as organic methylmercury (MeHg). Although dietary ingestion of MeHg is the
44 primary exposure pathway in fish, exposure also occurs by intake of waterborne forms of MeHg
45 and inorganic (Hg(II)) mercury, both of which can rapidly contaminate an entire ecosystem. Field
46 and laboratory experiments showed that 10% to 38% of total mercury accumulated in fish can
47 originate directly from water.¹⁻⁶ The relative importance of the food and water entryways may be
48 seasonal, with a water source dominating in the spring and fall, for example reaching up to 80%
49 of the total MeHg accumulated in yellow perch (*Perca flavescens*), and a food source dominating
50 in the summer.⁷ In previous experiments conducted with free ionic mercury species (MeHg⁺,
51 Hg²⁺), mercury was considered to enter fish tissues through gills. However, mercury is strongly
52 bound to dissolved organic matter (DOM) in aquatic systems,⁸ and therefore may not be readily
53 available for respiratory uptake. Mercury was less bioavailable to fish and zooplankton in the field
54 and in laboratory experiments when complexed to DOM.⁹⁻²² Similarly, complexation to DOM has
55 also been shown to reduce the toxicity of Zn to rainbow trout⁹ and the toxicity of both Zn and Cd
56 to the microalga *Pseudokirchneriella subcapitata*.²³

57 In contrast, other studies have reported that Hg concentration in fish increases with the
58 amount of dissolved organic carbon (DOC).^{13, 24-29} In aquatic invertebrates from Arctic tundra
59 lakes, mercury bioaccumulation was promoted at [DOC] < 8.8 mg C/L, but inhibited above this
60 concentration.³⁰ Bioaccumulation, toxicity, and uptake level of Cd by aquatic organisms all have
61 been shown to increase in the presence of DOM in freshwater and seawater.^{9, 31-33} Lastly, under

62 anaerobic conditions, *Desulfovibrio desulfuricans* bacteria were able to methylate Hg complexed
63 to DOM,³⁴ indicating that at least some of the complexed Hg is easily bioavailable.

64 The molecular structure of any metal cation in the DOM must play a principal role in the
65 processes that lead to bioavailability. Inorganic Hg in DOM occurs in two dominant forms: a linear
66 dithiolate complex (Hg(SR)₂) and nanoparticulate metacinnabar (β-HgS_{NP}).³⁵⁻³⁷ These forms may
67 be altered under oxygenated biotic conditions, for example as a result of the microbial production
68 of extracellular low-molecular-weight (LMW) thiols that might extract Hg from the larger
69 molecules of DOM.³⁸

70 The forms of inorganic Hg in DOM that are bioavailable to fish were assessed by exposing
71 *Tanichthys albonubes*, which belongs to the Cyprinidae family like *Denio rerio* (zebrafish), to DOM
72 pre-equilibrated with Hg(II) in aquaria. After eight weeks, the molecular structures of Hg in the
73 DOM and fish were determined by high energy-resolution X-ray absorption near-edge structure
74 (HR-XANES) spectroscopy.^{37, 39} The initial concentration of DOM was fixed to 29.6 mg C/L, and
75 the Hg concentration in the initial DOM was varied from 28 to 1453 μg Hg/g dry DOM producing
76 14 to 724 nM Hg(II) in the aquaria. For comparison, dissolved organic carbon (DOC)
77 concentrations range from 10 to 80 mg/L in peatland pore waters from North America,⁴⁰ and
78 from 60 to 200 mg/L in coastal lagoons in Rio de Janeiro State, Brazil.^{41, 42} The mercury
79 concentrations are representative of contaminated water, beginning just above the 10 nM
80 maximum contaminant level (MCL) for inorganic mercury in primary drinking water.⁴³ Changes
81 from the initial β-HgS_{NP}: Hg(SR)₂ ratio in the DOM and characterization of the molecular structure
82 of mercury in the fish would indicate processes that may release Hg from the DOM, making it
83 bioavailable. The forms of mercury in the fish potentially could be biomarkers of exposure to
84 inorganic mercury sourced from DOM in aquatic systems.

85

86 MATERIALS AND METHODS

87 A detailed description of the experimental and computational methods is given in the Supporting
88 Information (SI).

89 **Preparation of Hg-DOM.** The DOM was extracted from a *Carex* peat.^{44, 45} Divalent mercury
90 was added at pH 6 to obtain initial concentrations of 28, 270, and 1453 $\mu\text{g Hg/g}$ dry DOM
91 (hereafter called DOM_i-28, DOM_i-270 and DOM_i-1453), and the Hg-DOM solutions were aged in
92 the dark for six months following the protocol described previously for Hg(II) complexed to Elliott
93 Soil humic acid.³⁷

94 **Fish exposure to Hg(II)-DOM and dissolved HgCl₂.** Fifty-five adult *T. albonubes* were
95 placed in each of 12 separate aquaria containing aerated chlorine-free water at pH 7.7 ± 0.05 and
96 maintained at 21 ± 4 °C, and six experimental conditions were replicated. Three were controls:
97 C₀, without DOM and without Hg(II); C_{DOM}, with DOM and without Hg(II); and C_{Hg}, without DOM
98 and with 8 $\mu\text{g/L Hg}^{2+}$ (40 nM Hg) from a HgCl₂ stock solution (> 99.5% purity, Merck). For the
99 other three, the stock Hg-DOM suspensions containing 570 mg DOM/L were diluted in the aquaria
100 to a final concentration of 100 mg DOM/L, or 29.6 mg DOC/L yielding calculated initial Hg
101 concentrations of 14 nM or 2.8 $\mu\text{g/L}$ (DOM_i-28), 135 nM or 27 $\mu\text{g/L}$ (DOM_i-270), and 724 nM or
102 145 $\mu\text{g/L}$ (DOM_i-1453).⁴⁶ Total Hg concentrations were measured in water ($n = 4$, 2 per duplicated
103 aquarium) at the start of the experiment (t_0) and after one (t_1), two (t_2), four (t_4), and eight (t_8)
104 weeks (t_8) for all aquaria and in whole fish ($n = 6$, 3 per duplicated aquarium) at t_1 , t_2 , t_4 and t_8
105 for all aquaria except C₀. Mercury concentration was determined in six fish at t_0 from the
106 acclimatization tank. After eight weeks, whole fish from the DOM_i-1453 and C_{Hg} experiments
107 were lyophilized and analyzed by HR-XANES spectroscopy. Freeze-drying a frozen tissue does not
108 change the speciation of the metal.^{47, 48} The possibility of methylation in the Hg-DOM experiments

109 was tested by comparison to a separate experiment in which fish were exposed to MeHg (C_{MeHg}),
110 as described previously.⁴⁹

111 **Hg Analyses.** Mercury in fish was quantified with an AMA-254 mercury analyzer (Altec,
112 Prague) (fish) and in DOM with a DMA-80 (Milestone, Dual-Cell).

113 **HR-XANES spectroscopy.** Mercury L₃-edge HR-XANES spectra were measured at 10-15 K
114 with high-reflectivity analyzer crystals⁵⁰ on beamline ID26 at the European Synchrotron
115 Radiation Facility (ESRF). Data were analyzed against a large database of spectra for mercury
116 minerals (α -HgS, β -HgS, β -HgS_{NP}), Hg(II) complexes in natural organic matter, and Hg(II) and
117 methylmercury (MeHg) model complexes with thiolate ligands.^{36, 37, 39, 51, 52} All reference spectra
118 were considered as a basis for identification, but only diagnostic spectra are discussed herein.

119 **Geometry optimization of model Hg complexes.** The geometries of structural models for
120 the Hg complexes were optimized at a high level of molecular orbital theory (MP2/TZVP-ecp)
121 using ORCA 3.0.3⁵³ and a computational scheme tested previously on the modeling of the
122 structure and stability of monomeric Hg–thiolate complexes,^{36, 37, 54} and herein on the
123 Hg(Cysteamine)₂ complex (Figure S1).

124

125 RESULTS

126 **Transfer of Hg(II) from DOM to fish.** Chemical analyses of fish tissue show that Hg(II)
127 initially bound to DOM became bioavailable (Table 1). Whole fish exposed to initial [Hg_{DOM}] of 2.8,
128 27, and 145 $\mu\text{g/L}$ (14 to 724 nM) contained corresponding amounts of 0.33, 1.06, and 2.20 μg
129 Hg/g dry weight fish after eight weeks. Fish in the control C_0 aquaria with neither Hg nor DOM
130 contained $0.022 \pm 0.007 \mu\text{g Hg/g wet weight}$ ($0.09 \pm 0.03 \mu\text{g Hg/g dry weight}$). The amounts of
131 mercury in the fish after eight weeks corrected for the amount in the control fish (C_0) were 1.9,
132 0.88, and 0.32 %, respectively, of the total final mass of Hg present in aquaria in both water and

133 fish. The fraction of Hg(II) transferred to fish was much higher in the C_{Hg} aquaria with initial
134 $[HgCl_2]$ of 8 $\mu g/L$ (40 nM), reaching 50% after eight weeks (Table S1). Total Hg in whole fish
135 exposed to Hg-DOM ranged from 3 to 21% of the amount in fish in the C_{Hg} experiment with $HgCl_2$
136 (10.4 μg Hg/g fish) and was 3 to 24 times higher than the amount in the control C_0 experiment.
137 Although the transfer percentages in the Hg-DOM experiments are low, they confirm that this pool
138 of mercury can become bioavailable.

139 A higher percentage of Hg was bioavailable from DOM that contained lower amounts of Hg
140 (Table 1). Conservative values for bioconcentration factors (BCFs), defined as the ratio of the
141 chemical concentration in the fish (on a wet weight basis) to the initial concentration in water,
142 were 27 in the $[Hg_{DOM}] = 2.8 \mu g/L$ experiment and decreased to 8.3 and 4.4 in the $[Hg_{DOM}] = 27$
143 and 145 $\mu g/L$ experiments, respectively. These BCFs follow the expected ecotoxicological rule for
144 pollutant uptake by organisms (Table S2).

145 After eight weeks, the concentration of Hg in the DOM decreased from 28 to 2 $\mu g/g$ (DOM_{i-}
146 28, DOM_{f-2}), 270 to 27 $\mu g/g$ (DOM_{i-270} , DOM_{f-27}), and 1453 to 240 $\mu g/g$ (DOM_{i-1453} , DOM_{f-240})
147 (Table 1). Given that the amounts of Hg acquired by the fish were relatively small compared to
148 the system totals, these large changes resulted from both an increase in the quantity of DOM over
149 time, from food addition and production of biogenic DOM in the aquaria, and from loss of the
150 initial DOM and its associated Hg to the filtration apparatus (SI and Table S3). Neither change in
151 the amount of DOM in the systems was quantified.

152 **Changes in proportions of molecular structures of Hg in DOM.** The initial β -
153 $Hg_{SNP}:Hg(SR)_2$ ratio in the $[Hg_{DOM}] = 2.8 \mu g/L$ experiment (DOM_{i-28}) decreased considerably after
154 eight weeks (DOM_{f-2}) (Figure 1a). The DOM_{i-28} spectrum was modeled as a linear combination
155 of 88% β - Hg_{SNP} and 12% $Hg(SR)_2$ (± 8 at. %) compared to 37% β - Hg_{SNP} and 63% $Hg(SR)_2$ (± 10
156 at. %) in DOM_{f-2} (Figures 1b and 1c and Table S4). This result suggests that nanoparticulate β -

157 HgS in DOM_i-28 was the main source of the Hg that became bioavailable, and also that new
158 Hg(SR)₂ complexes may have been generated from the β-HgS. To demonstrate that the shift in
159 species proportions required the presence of living organisms, the DOM_i-28 stock solution was
160 diluted to the same concentration as in the fish aquaria, and aged at ambient temperature in air-
161 equilibrated water for eight weeks, in the presence or absence of light. Spectra for these samples
162 were similar to each other and to that of the original DOM_i-28 (Figure S2a) showing that the
163 change in β-HgS:Hg(SR)₂ ratio was related to the presence of the fish.

164 In contrast to DOM_f-2, the changes in the ratio of β-HgS_{NP} to Hg(SR)₂ were smaller in DOM_f-
165 27 and DOM_f-240 compared to the initial values (Figure S3) despite the observation that fish
166 accumulated three times (DOM_f-27) and seven times (DOM_f-240) as much Hg as in the DOM_f-2
167 experiment (Table 1). The smaller variations in these ratios are directly related to the lower
168 proportions of Hg transferred to fish (0.88 and 0.32%, respectively, of the Hg at t₈).

169 **Form of Hg in fish from Hg-DOM experiments.** Fish from the DOM_i-1453 experiment were
170 examined by HR-XANES because the concentration of Hg was above the detection limit of 0.5 μg
171 Hg/g³⁹ (Table 1). The spectrum (Fish-DOM) is distinct from those of the initial (DOM_i-1453) and
172 final (DOM_f-240) DOM samples, and from those of fish contaminated with MeHg (Fish-MeHg from
173 the C_{MeHg} experiment) and Hg²⁺ (Fish-Hg from the C_{Hg} experiment) (Figures 2a and 2b). Also, the
174 molecular structure of Hg in Fish-DOM is not a simple mixture of the structures in fish exposed to
175 MeHg and HgCl₂ (Figure 2c).

176 The Fish-DOM spectrum was most similar to those for Hg(II) complexed to L-glutathione
177 (Hg(GSH)₂, with GSH = γ-Glu-Cys-Gly) and to L-cysteine (Hg(Cys)₂) at pH 7.5 from the spectral
178 database^{36, 37, 39, 51} (Figure 3a). Two component fits of Hg(GSH)₂ or Hg(Cys)₂ with Hg(Cys)₄
179 decreased the fit residuals (NSS) (Figure 3b) and both fits match the normalized X-ray absorption
180 to within about 2% (Figure 3c). The species proportions are 88% Hg(GSH)₂ + 12% Hg(Cys)₄ or

181 80% $\text{Hg}(\text{Cys})_2 + 20\% \text{Hg}(\text{Cys})_4$ with uncertainties of $\pm 8\%$. Therefore, on average about 84% of
182 the Hg in fish is two-coordinate ($\text{Hg}(\text{SR})_2$) and 16% is four-coordinate ($\text{Hg}(\text{SR})_4$).

183 The molecular structure of Hg in the fish is discerned by considering how glutathione and
184 cysteine bond to Hg according to geometry optimization by *ab initio* post-Hartree-Fock
185 computations.^{36, 54} In $\text{Hg}(\text{GSH})_2$, Hg(II) is bonded approximately linearly to the cysteinyl sulfur
186 atoms at 2.33 Å from the two γ -Glu-Cys-Gly peptides and surrounded in trans-equatorial position
187 by a carboxyl oxygen from a Gly residue at 2.62 Å, the backbone carbonyl oxygen at 2.88 Å from
188 the Gly-Cys peptide bond (Gly-NH-CO-Cys) of the same GSH molecule, and an amide group (-NH)
189 at 3.01 Å from the second GSH molecule (Figure 4a and Video S1). The shorter Hg-O distance is
190 well below the sum of the van der Waals radii of 3.07 Å for the Hg-O pair, whereas the longer Hg-
191 O distance approaches it, and the Hg-N distance is close to the value of 3.10 Å for the Hg-N pair.
192 The two O atoms are close enough to Hg to be considered secondary bonding interactions, but the
193 N atom is not. The complex is a double-ring chelate with one ring of six members and the other
194 seven. The coordination of Hg is $\text{Hg}[(\text{SR})_2 + \text{O}_2]$, and the geometry is disphenoidal, or seesaw (i.e.,
195 an octahedron without two cis-equatorial ligands).⁵⁵ The S-Hg-S angle is bent to 167.2° by the
196 carboxyl oxygen (Figures 4a), which on the HR-XANES spectrum is seen as a decrease in the near-
197 edge peak intensity (Figures S4 and S5a).

198 The structure of the $\text{Hg}(\text{Cys})_2$ complex depends on pH, which changes the protonation of the
199 amine groups. The structure ranges from a nearly linear $\text{Hg}(\text{SR})_2$ coordination with weak Hg-OOC
200 contacts at 3.06-3.15 Å at pH 3 ($\text{Hg}(\text{SR})_2$ coordination) to a bis five-membered ring chelate ($\text{Hg}[5$ -
201 $\text{S}/\text{N}\text{-ring}]_2$) with Hg coordination of $\text{Hg}(\text{SR} + \text{NH}_2)_2$ and disphenoidal geometry, like when bonded
202 to GSH, at pH 11.5 (Figure S4). However, it is unlikely that both amine groups deprotonate and
203 fold simultaneously to form a double-ring chelate,^{39, 55-57} and therefore at pH 7.5, near
204 physiological conditions, the Hg coordination is most likely dominantly $\text{Hg}[(\text{SR})_2 + \text{NH}_2]$. The

205 structure of Hg(GSH)₂, which is bent by a carboxyl oxygen, and the structure of Hg(Cys)₂, which
206 is bent by an amine group, give nearly identical HR-XANES traces (Figure S5a). We conclude that
207 Hg is bonded in the Fish-DOM dithiolate complex to two cysteinyl sulfur atoms and to one or two
208 nucleophilic donors, such as amine and amide nitrogen and carboxyl and carbonyl oxygen. The
209 dithiolate Hg coordination in fish is denoted Hg[(SR)₂+(N/O)₁₋₂].

210 The Hg(Cys)₄ complex, synthesized at pH 11.9, has four thiolate ligands at 2.52 Å,⁵¹ a distance
211 ~0.17 Å longer than that in Hg(Cys)₂. In HR-XANES, the post-edge absorption shifts to lower
212 energy because of the increase in bond length in agreement with the Natoli rule⁵⁸ and, the near-
213 edge peak disappears because of the tetrahedral coordination. Both features occur in the Fish-
214 DOM spectrum. Although β-HgSNP also has four-coordinate Hg(II), and therefore these same
215 spectral features, there is no modulation at 12300 eV and above in the Fish-DOM spectrum
216 indicative of the Hg-Hg pairs in β-HgSNP (Figures 1 and S2). Thus, Hg(Cys)₄, with coordination
217 denoted Hg(SR)₄, is considered a good representation of the tetrahedral coordination of Hg in
218 fish.

219 **Form of Hg in fish from the Hg(Cl)₂ experiment.** The Fish-Hg spectrum has features like
220 those of β-HgSNP including a bumpy profile at high energy indicative of Hg-Hg pairs (Figure S6a).
221 Linear least-squares fits identified β-HgSNP as the first component, Hg(Cys)₄ as the second and
222 Hg(Cys)₂ at pH 7.5 as the third, immediately followed by Hg(GSH)₂ (Figure S6a). A two-component
223 fit with 83% β-HgSNP + 17% Hg(Cys)₄ decreased NSS by 35% compared to the β-HgSNP fit, and a
224 three-component fit with 57±15% β-HgSNP + 23±10% Hg(Cys)₄ + 20±10% Hg(Cys)₂ pH 7.5
225 decreased NSS by an additional 51%. Replacing Hg(Cys)₂ with Hg(GSH)₂ reduced NSS by an
226 additional 49%, confirming that the two model complexes are indistinguishable. Both three-
227 component best-fit residuals are as low as 1% (Figure S6b).

228 **Form of Hg in fish from the methylmercury experiment.** The spectrum for MeHg
229 complexed to L-cysteine at pH 7.5 provided the best match to the Fish-MeHg spectrum, as
230 expected⁵⁹ (Figure S7a). HR-XANES spectra of the MeHgCys complex as a function of pH and
231 corresponding geometry optimizations show that the amine group is folded at pH ≥ 4.5 to form
232 an aminothiolate ring chelate as in Hg(Cys)₂, except that the complex is a mono-cysteinate
233 (MeHg(SR+NH₂) coordination) (Figures S4e and S7). In comparison, the spectrum of MeHg
234 complexed to DOM (Nordic Aquatic fulvic acid: NAFA-MeHg) has a deeper near-edge minimum
235 and is shifted to the right in the range 12282-12286 eV (Figure S7b). Like in the comparison of
236 Fish-DOM and pH 3 Hg(Cys)₂ spectra (Figure S5a), and the evolution of the MeHgCys spectra with
237 pH (Figure S4d), this shift results from the lack of secondary N/O ligands in NAFA-MeHg. Thus,
238 secondary coordination to N/O ligands, which occurs in Fish-MeHg, offers a means to differentiate
239 a dithiolate complex in living (Hg[(SR)₂+(N/O)₁₋₂] coordination) and detrital (Hg[(SR)₂
240 coordination) organic matter.⁵¹

241 In the C_{MeHg} experiment, no differences were observed within experimental noise of the HR-
242 XANES measurements between spectra from gills, brain, liver and muscle, indicating that the
243 binding environment is MeHg(SR+N/O) in all tissues, and that no demethylation occurred during
244 the 28 days of experiment, even in liver⁶⁰⁻⁶⁵ (Figure S7c). Thus, lack of an intense near-edge peak
245 in Fish-DOM and Fish-Hg is direct evidence that these fish did not contain detectable
246 methylmercury, implying that little to no MeHg formed in the DOM or biofilms in the oxygenated
247 aquaria.

248

249 **DISCUSSION**

250 **Experimental parameters and the environment.** The initial DOM concentrations in the
251 experiments are characteristic of natural aquatic environments, and the initial Hg concentrations

252 represent environments contaminated with point sources of mercury. In natural waters away
253 from point sources, the concentration of dissolved Hg is between 0.2 and 15 ng/L, and most often
254 below 5 ng/L.^{40, 66} The experimental Hg concentration ranged from 2.8 to 145 µg/L, starting just
255 above 2 µg/L which is the MCL for Hg in primary drinking water. With these higher initial
256 concentrations of Hg in the aquaria and the eight-week experimental duration, fish obtained
257 enough Hg for HR-XANES spectral acquisition within a reasonable timeframe. For example, the
258 good quality Fish-DOM spectrum was obtained in only 6 hours using high-reflectivity analyzer
259 crystals⁴⁸ on fish that acquired 2.1 µg Hg/g (2.1 ppm Hg) in the DOM_i-1453 experiment.

260 The amounts of mercury in the fish from the Hg-DOM experiments (0.33 to 2.1 µg Hg/g),
261 bracket the amount of about 1 µg/g observed in wild fish from impacted areas⁶⁶⁻⁶⁸ that acquire
262 mercury over much longer periods. Despite a higher Hg/C ratio in the Hg-DOM experiments (94
263 to 4900 µg Hg/g C) than is typical of the natural uncontaminated environment (0.006 to 0.5 µg
264 Hg/g C calculated with the experimental concentration of DOC), the mass fractions of total Hg
265 transferred to the fish are in excellent agreement with results of similar experiments performed
266 with midge larvae in which the Hg concentration was 0.1 µg/L and Hg/C ratios (0.1 to 1 µg Hg/g
267 C) overlapped the natural range. The mass fraction of total Hg transferred to fish was 21% in the
268 HgCl₂ experiment and 0.33% to 2.1% in the Hg-DOM experiments. In experiments with *Chaoborus*
269 larvae, uptake of inorganic Hg(II) and MeHg decreased from 20% without DOM to less than 1%
270 (Hg(II)) and 5% (MeHg) with DOM.¹⁷

271 **Source and transfer of Hg from DOM to fish.** The reduction of β-Hg_{SNP} from 88% to 37%
272 and associated increase of Hg(SR)₂ from 12% to 63% in the DOM_i-28/DOM_F-2 samples (Figure 1)
273 suggests that β-Hg_{SNP} provided the Hg that became bioavailable to fish. However, because
274 nanoparticulate β-HgS that had formed abiotically in DOM^{37, 69} was not observed in the fish, the
275 β-Hg_{SNP} must have dissolved and been transferred to fish in another form, probably through

276 exchange of bithiolate-bound Hg at the surface of the gill epithelium.

277 Although the solubility of crystalline β -HgS is low (K_{sp} of $10^{-36.8}$ for the reaction $\text{HgS}_{(s)} + \text{H}^+ =$
278 $\text{Hg}^{2+} + \text{HS}^-$ at 25°C)⁷⁰, the nanoparticulate β -HgS in the DOM would be less stable, or more soluble,
279 than larger crystals⁷¹ due to excess surface free energy. During the initial aging of the Hg-DOM
280 solutions, the DOM molecules likely adsorbed to nascent particles and slowed their crystal
281 growth,⁷²⁻⁷⁵ and/or prevented their aggregation.⁸⁴ However, microbial communities in observed
282 DOM-biofilm assemblages in the aquaria could have produced small organic molecules that
283 promoted dissolution of the β -HgS_{NP}. Periphytic phototrophic microorganisms, such as algae, can
284 increase the bioavailability of Hg in such biofilms through the exudation of low-molecular-weight
285 (LMW) thiols, including thioglycolic acid, cysteine, and glutathione,³⁸ which are known to
286 participate in ligand-promoted dissolution of metal sulfides including nanoparticulate phases.⁷⁶
287 Graham et al.⁷⁷ observed that Hg was more available for methylation by anaerobic bacteria in the
288 presence of DOM when the solution was slightly supersaturated with respect to β -HgS, indicating
289 that dissolution of nanoparticulate β -HgS in such systems is possible. Under oxic conditions,
290 however, added Hg(II) was more bioavailable to a bioreporter bacterium after short pre-
291 equilibration times ($\leq 3\text{h}$) with DOM than when DOM was absent or when Hg(II) and DOM had
292 pre-reacted for 24 h.⁷⁸ A reaction time of 24 h would have been sufficient to form β -HgS_{NP},^{37, 79}
293 especially in the Suwannee River DOM used in ref. ⁷⁸ because it is as rich in reduced sulfur as
294 peat,⁴⁵ and in this case, nanoparticulate β -HgS may not have dissolved. Those results also suggest
295 that Hg(II) associated with smaller nutrient carbon-bearing molecules was able to enter the
296 bacterial cells.⁷⁸

297 We suspect that Hg(SR)₂ moieties in the DOM probably were also a source of Hg to the fish,
298 because in the DOM_i-1453 experiment this species unambiguously declined in proportion relative
299 to β -HgS_{NP} after eight weeks in the aquaria (Figure S3). DOM naturally contains low molecular

300 weight molecules. For example, 36% of the mass of Suwannee River fulvic acids can pass through
301 a dialysis membrane of 100 to 500 Da molecular weight cutoff,⁸⁰ and aqueous peat extracts can
302 permeate through human skin and exert physiological effects.⁸¹ Also, larger DOM molecules could
303 be rapidly broken down by the activity of microbial heterotrophic communities naturally present
304 in the skin and digestive tract of fish. Upon degradation, DOM could release bioavailable thiol-
305 bound Hg(II) macromolecules, as has been shown for other metals.^{82, 83} DOM can interact with
306 cell membranes,²³ increasing their permeability to passive uptake of neutral chemical species. In
307 various freshwater organisms, DOM is taken up directly via epithelia,⁸⁴ and upon exposure to
308 dried DOM, several nonspecific organic transporters were induced in the nematode
309 *Caenorhabditis elegans*.⁸⁵ Once internalized, DOM can migrate to organs or organelles and
310 provoke stress response reactions such as lipid peroxidation,⁸⁶ biotransformation activities,⁸⁵
311 and induction of chemical defense proteins such as Hsp70.⁸⁷

312 No MeHg was observed by HR-XANES in either DOM or the fish after eight weeks, presumably
313 because of the oxygenated conditions prevailing in the aquaria and the low concentration of
314 sulfate (8 mg/L). Even in anaerobic conditions, elevated sulfate is necessary for net methylation
315 of mercury by sulfato-reducing microorganisms.⁸⁸

316 **Binding environments of Hg in fish.** We conclude that the mercury entered the fish as a low
317 molecular weight molecule in a Hg(SR)₂ coordination structure derived either from the DOM or
318 from microbial exudates that dissolved β-HgS_{NP} in the DOM. Internally, this mercury was
319 transformed to other coordinating environments. In the fish exposed to Hg(II)-spiked DOM, the
320 dominant species (84 ± 8% of total Hg equal to 2.2 μg/g) is a dithiolate complex with
321 Hg[(SR)₂+(N/O)₁₋₂] coordination. The minor species, formed with the remaining 16 ± 8% Hg, is a
322 tetrathiolate Hg(SR)₄ complex. The fish exposed to HgCl₂ (Fish-Hg), which contained 10.4 μg
323 Hg/g, has as its dominant species a third Hg_xS_y form of larger nuclearity (56 ± 15%), with the two

324 mononuclear complexes in similar lesser proportions ($20-23 \pm 10\%$). The coordination
325 environment of Hg in the three species is discussed below with the goal of indicating possible
326 internal biological pathways for the transformations.

327 *The Hg[(SR)₂+(N/O)₁₋₂] coordination.* Divalent mercury is bonded in the dithiolate complex to
328 two cysteinyl sulfur atoms at ~ 2.35 Å and to one or two electron donors at 2.5-3.0 Å. The
329 secondary bonds are nearly perpendicular to the RS-Hg-SR bond axis, and cause the RS-Hg-SR
330 angle to bend more, the shorter the bonds.^{39, 89} The geometry optimizations show that the
331 disphenoidal configuration can be obtained with both N and O ligands of different functionalities
332 and molecular conformations. Besides the Hg((SR)₂+O₂) coordination from Hg(GSH)₂ and the
333 Hg(SR+NH₂)₂ coordination from Hg(Cys)₂, two intramolecular disphenoidal geometries, both
334 consistent with the HR-XANES results, were modeled. The Hg(Cys+GlyCysGly) model (Figure
335 S8a) features a tetracoordinate double chelate with the cysteinyl NH₂ group at 2.57 Å and the
336 carbonyl oxygen of the cysteine residue from the tripeptide at 2.73 Å. The oxygen of this
337 functional group is electron rich because of its lone pairs and the C=O π bond, both favoring a
338 nucleophilic attack on the positive mercury center. The Hg(GlyCysGly)₂ model (Figure S8b)
339 features a tetracoordinate complex with the two O-terminal carboxylic groups at 2.55 and 2.57 Å.
340 Disphenoidal geometry also may occur intermolecularly with side chain amine and guanidyl NH
341 (e.g. from arginine^{39, 90, 91}) groups via protein folding and ligand docking.⁹²⁻⁹⁴

342 Natural population analysis (NPA^{95, 96}) shows that the partial atomic charge, hence the
343 nucleophilicity, of the O and N donors decreases in the following order: NH₂ (-0.9 e) > COO⁻ (-0.8
344 e) > C=O (-0.7 e) > NH (-0.6 e) (Figures S1, S4 and S8). Amine and carboxyl groups are more likely
345 to bond Hg than an amide. Their higher binding strength is reflected in the geometry
346 optimizations by shorter Hg-NH₂ and Hg-COO⁻ bond distances and smaller S-Hg-S angles.
347 However, other factors are involved in the stability of a macromolecular complex, such as the

348 conformation of the chelate, inter- and intra-molecular packing forces and interactions of the
349 metal with other moieties (e.g., hydrogen bonds). For example, in plants Hg is selectively bonded
350 to the thiol peptide phytochelatin PC2 over GSH, although the GSH concentration largely exceeds
351 the PC2 concentration in the cytosol.⁹⁷⁻¹⁰¹ PC2 is a GSH dimer with the amino acid sequence (γ -
352 Glu-Cys)₂-Gly. The strong affinity of Hg for PC2 is explained by the formation of a bis six-
353 membered ring chelate Hg[6-S/O-ring]₂ with the thiolate sulfur and carbonyl oxygen atoms from
354 each cysteine residue (Hg(SR+O)₂ coordination (Figure S9)).⁵¹ The peptide forms a scaffold for the
355 Hg complex: the two thiolate donors bind Hg like crab claws and the Cys- γ Glu-Cys molecular cage
356 is stabilized by one hydrogen bond between an amide proton and a carbonyl oxygen
357 (>NH...O=C<), as is customary for the secondary structure of proteins. The calculated Hg-O
358 distances are 2.65 Å and 2.95 Å and the SR-Hg-SR bond angle 164.0°, compared to d(Hg-O) = 2.62
359 Å and 2.88 Å and an angle of 167.2° for Hg(GSH)₂. As a result, the Hg(GSH)₂ and Hg(PC2)
360 complexes have similar HR-XANES spectra (Figure S5b).

361 In animal and bacterial cells, Hg(II) is most commonly bonded to the consensus CXXC motif
362 (single-letter amino acid code, where X can be any amino acid) of metalloproteins with the
363 Hg[(SR)₂+(N/O)₁₋₂] coordination. Examples include metallochaperones and metal-transporting
364 ATPases MerP,¹⁰² MerA,⁹⁴ and Atx1 and Ccc2.^{103, 104} Interestingly, the chain length of the XX
365 sequence (X = Ser, Gly, Ala,...) from the highly conserved motif GMTCCXXC found in
366 metalloproteins¹⁰⁵ is close to the γ Glu length from the CXC motif of PC2.^{48, 51} Thus, the CXXC motif
367 can cause a distortion from linearity in the same way as Hg(PC2), Hg(Cys)₂ and Hg(GSH)₂. For
368 example, the S-Hg-S bond angle is 167° in Atx1.¹⁰³ We conclude that the Hg(Cys)₂ and Hg(GSH)₂
369 models used in our HR-XANES analysis are good representations of the secondary bonding
370 environment of dithiolated Hg in fish, whether intermolecular or intramolecular. Despite
371 uncertainty on its exact nature, for energetic reasons Hg is more likely bonded in a claw setting

372 CXXC site of a protein.

373 *The Hg(SR)₄ coordination.* A high Cys/Hg ratio and non-physiological alkaline pH are required
374 to form the Hg(SR)₄ coordination to low-molecular-weight ligands.^{54, 57, 106} With proteins,
375 monomeric tetrathiolate coordination has been described only in Hg-substituted rubredoxins,
376 which are iron-sulfur electron-transfer proteins in sulfur-metabolizing bacteria and archaea.¹⁰⁷⁻
377 ¹⁰⁹ The rarity of this coordination may be explained by the thermodynamic preference for Hg(SR)₃
378 over Hg(SR)₄ coordination from pH 4.8 to 10.6 in biological systems when the thiols are
379 structurally connected.⁵⁴ The trigonal Hg(II)-SR complex is observed for instance in MerR¹¹⁰ and
380 the Hah1 metallochaperone.⁹² In Hg(Hah1), Hg is covalently bonded to three sulfur atoms at 2.3-
381 2.5 Å and weakly bonded to a fourth at 2.8 Å. We did not find any evidence for the trigonal
382 coordination in fish, as modeled in our database with Hg(D-Pen)₃⁵⁶ and [NEt₄][Hg(SC₆H₁₁)₃].¹¹¹

383 Four-fold coordination with sulfur atoms occurs, however, in polynuclear structures with
384 thiolate ligands in metallothioneins (Hg_x(SR)_y)^{48, 112} or with sulfide ligands in β-HgS.¹¹³ The
385 second structure model was favored in recent studies on the binding of Hg(II) to *Escherichia coli*
386 and *Bacillus subtilis* under aerobic conditions using EXAFS and XANES spectroscopy.^{114, 115} The
387 authors identified the Hg(SR)₄ coordination and inferred that particulate β-HgS_(s) precipitated
388 from the reaction of biogenic sulfide with Hg(SR)₂.

389 Here, we favor the metallothionein (MT) interpretation because if β-HgS nanoparticles
390 existed they should have been detectable by high energy-resolution XANES at liquid helium
391 temperature. Fish metallothioneins have two metal binding domains. The α domain can bind four
392 Hg atoms in a Hg₄Cys₁₁ cluster and the β domain three Hg atoms in a Hg₃Cys₉ Hg₃Cys₉ cluster
393 (Figure 4b).^{48, 116} In each cluster the Hg(Cys)₄ tetrahedra are connected through their apices with
394 a β-HgS-type core structure (Figure 4c). Metallothionein nanoclusters with nuclearity of only
395 three to four are vanishingly small compared for example to β-HgS nanocrystals of 3-5 nm in

396 diameter observed by HRTEM in natural organic matter.³⁷ An α -Hg₄Cys₁₁ cluster has on average
397 2.5 Hg-Hg pairs at 4.1-4.4 Å and a β -Hg₃Cys₉ cluster only 2, compared to 12 in β -HgS.¹¹³ Both
398 clusters lack any Hg-Hg pairs beyond 7 Å. Because of the structural disorder and reduced size of
399 a MT Hg_x(SR)_y core, the same HR-XANES fine structures that are associated with Hg-Hg pairs as
400 seen in the β -HgS_{NP} reference are smeared together and cannot be distinguished. A
401 metallothionein HR-XANES spectrum with a polynuclear core has a bell-shaped top edge with no
402 distinct modulation of the absorption signal: it appears like the spectrum from the Hg(Cys)₄
403 reference, even though some Hg-Hg pairs are present.^{48, 51}

404 *The Hg_xS_y coordination.* Based on XANES calculations,^{117, 118} a β -Hg_xS_y cluster size of at least 1
405 nm is required to produce the Hg-Hg multiple scattering events at the origin of the fine structures
406 in the HR-XANES spectrum of Fish-Hg (Figure S6a). This minimum size corresponds to a
407 stoichiometry of β -Hg₇S₁₆, which exceeds the maximum nuclearity of Hg₄S₁₁ for the MT clusters.
408 Therefore, the Hg_xS_y coordination of Fish-Hg is most likely from particulate β -HgS which could
409 form in the reducing environment of the cytosol by the reaction of Hg(SR)₂ with hydrogen
410 (mono)sulfide (H₂S/HS⁻).^{114, 115}

411 The absence of compelling evidence for nanoparticulate β -HgS in Fish-DOM can be explained,
412 at least in part, by the fact that fish from the DOM experiment had only 20% of the Hg that was in
413 fish from the HgCl₂ experiment. The dithiolate and tetrathiolate Hg species, which coexist in both
414 Fish-DOM and Fish-Hg, are probably more representative of the forms of inorganic Hg at
415 environmental concentrations. Lastly, although the same, or closely related, β -HgS-type
416 nanoparticles occur in DOM and in Fish-Hg, the DOM nanoparticles most likely transformed
417 externally to Hg(SR)₂ prior to mercury incorporation in fish tissue. Furthermore, the two mercury
418 sulfides formed by different reaction pathways. In Hg-DOM, the β -HgS nanocrystals were
419 produced under aerated conditions from Hg(SR)₂ by a dealkylation reaction,^{37, 69} whereas in fish

420 the β -Hg_xS_y clusters were likely formed by reaction of Hg(SR)₂ with biogenic sulfide.^{114, 115}
421 Although most mercury in fish tissue is methylmercury,⁶⁶ the presence in fish of any of the
422 structures observed in the Fish-DOM or Fish-Hg spectra would be a biomarker of an inorganic Hg
423 source and may have potential for forensic applications.

424

425 **ASSOCIATED CONTENT**

426 **Supporting Information**

427 The Supporting Information is available free of charge on the ACS Publications website at DOI:
428 Supplemental information on materials and methods, Supplementary Tables and Figures,
429 Cartesian coordinates of the Hg(Cys)₂ and Hg(GSH)₂ complexes, Hg₄(SMe)_{11- α} and Hg₃(SMe)_{9- β}
430 clusters, and HR-XANES spectra (PDF). Video S1 showing the structures of Hg(GSH)₂.

431

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436 **Notes**

437 The authors declare no competing financial interests.

438

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446

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772

773 LEGENDS TO FIGURES

774 **Figure 1.** (a) Mercury L_3 -edge HR-XANES spectra of the initial aged Hg-DOM (28 $\mu\text{g Hg/g DOM}_i$,
775 purple) and the final Hg-DOM from aquaria with fish after eight weeks (2 $\mu\text{g Hg/g DOM}_f$, blue).
776 (b) Comparison of the final Hg-DOM spectrum with spectra from Hg linearly complexed to thiol
777 ligands from the humic acid fraction of the DOM ($\text{Hg}(\text{SR})_2$ complex³⁶) (green) and from
778 nanoparticulate β -HgS (metacinnabar) (black). The $\text{Hg}(\text{SR})_2$ reference was obtained by reacting
779 $\text{Hg}(\text{NO}_3)_2$ with the *Carex* peat HA isolate for 15 h to minimize formation of nanoparticulate β -
780 HgS ³⁷, and β - HgS_{NP} was synthesized at room temperature (RT) from $\text{Hg}(\text{L-Cys-OEt})_2$ complex
781 aged for 80 days in contact with air.³⁷ The increase in proportion of $\text{Hg}(\text{SR})_2$ is detected in plots
782 (a) and (b) by an increase of the near-edge peak at 12279.5 eV, a direct indicator of Hg(II) linearly
783 coordinated to two thiol ligands,^{36, 37} and a shift to higher energy of the trailing edge of the
784 spectrum (arrows in (a)). Tetrahedral bonding of mercury to four sulfur atoms in β - HgS_{NP} is seen
785 as a shoulder instead of a peak on the rising part of the spectrum. (c) Linear least-squares fit
786 (orange) to the Hg-DOM_f spectrum (blue) with $63 \pm 10\%$ $\text{Hg}(\text{SR})_2$ and $37 \pm 10\%$ nanoparticulate
787 β -HgS. The normalized sum-squared residual (NSS) is the normalized difference between two
788 spectra expressed as $\Sigma[(y_{\text{exp}} - y_{\text{fit}})^2] / \Sigma(y_{\text{exp}}^2)$. Top, ball-and-stick representation of the Hg-thiolate
789 complex and polyhedral representation of the β -HgS structure.

790

791 **Figure 2.** (a) Mercury L_3 -edge HR-XANES spectrum of fish (Fish-DOM) exposed to Hg-DOM_i =
792 1453 $\mu\text{g Hg/g}$ with spectra from the initial and final DOMs. (b) HR-XANES spectra of fish exposed

793 to DOM_i-1453, dietary MeHg, and HgCl₂ (8 μg/L). The Fish-MeHg spectrum has an intense near-
794 edge peak at 12279.8 eV and the Fish-Hg spectrum has a weak peak, more like β-Hg_{SNP} (Figure
795 1b). (c) Linear least-squares fit (orange) to the Fish-DOM spectrum (green) with a mixture of the
796 Fish-MeHg and Fish-Hg spectra shows that Hg has a unique chemical form in the fish exposed to
797 the Hg-DOM.

798

799 **Figure 3.** Mercury coordination in fish contaminated by Hg-DOM (DOM_i-1453) derived from Hg
800 L₃-edge HR-XANES. (a) A one-component fit to all reference spectra identified the dithiolate
801 complexes Hg(GSH)₂ and (Hg(Cys)₂) at physiological pH as the closest model compounds to the
802 binding environment of Hg in fish (Hg[(SR)₂+(N/O)₁₋₂] coordination, Figures 4a and S9). (b) A
803 two-component fit further identified the tetrathiolate complex Hg(Cys)₄ as secondary model
804 species. (c) Fitting residuals were used to evaluate the magnitude of the resultant uncertainty of
805 the determined Hg coordinations. The residual of the Hg(Cys)₂ + Hg(Cys)₄ reconstruction is close
806 to experimental noise and has a smaller NSS value than the Hg(GSH)₂ + Hg(Cys)₄ reconstruction
807 (1.06×10^{-4} vs 1.28×10^{-4}).

808

809 **Figure 4.** (a) Geometry-optimized model of the molecular structure of the Hg(GSH)₂ complex
810 (Video S1). Hg(II) is bridged to two primary SR⁻ thiolate ligands (one from each GSH molecule)
811 and two secondary O ligands from the same GSH molecule forming an intramolecular double-ring
812 chelate. The Hg(GSH)₂ complex is further stabilized by an extended intermolecular H-bond
813 network involving the protonated amino groups (-NH₃⁺) and deprotonated carboxyl groups of the
814 two glutamate residues. The two H-bridged peptides form a molecular scaffold which binds Hg(II)
815 in a pseudo claw-setting environment. (b) Geometry-optimized Hg₃(SMe)₉ and Hg₄(SMe)₁₁
816 clusters featuring the inorganic core structure of the Hg-β and Hg-α domains in vertebrate
817 metallothionein. (c) {Hg₃S₉} and {Hg₄S₁₀}^{95, 119} motifs of the β-HgS structure¹¹³ showing the
818 similarity of the polyhedral associations with the metallothionein clusters. See Ref.⁴⁸ for details.
819 MP2/TZVP-ecp optimization. Bond lengths, in angstroms, and bond angles are in black. Atomic
820 charges, in units of elementary charge e and calculated by natural population analysis (NPA^{95, 119}),
821 are in blue. Dark red, Hg; yellow, S; blue, N; red, O; gray, C; light gray, H. Cartesian coordinates of
822 the Hg(GSH)₂ model are provided in the SI.

823

TABLE 1. Samples codes and concentration of Hg in the initial and final Hg(II)-spiked DOM and in fish

Experiment ^a	Initial Hg ^b [μg/g DOM]	Initial Hg ^c in water [μg/L]	Final Hg ^b [μg/g DOM]	Final DOM sample code	[Hg] in water (μg/L) at t ₈	Whole fish Hg ^d [μg/g] at t ₈	Fraction of Hg in fish at t ₈ [%] ^e
DOM _i -28	28 ± 3	3 ± 6	2 ± 1	DOM _f -2	1.3 ± 0.3	0.33 ± 0.03	1.9 ± 0.4
DOM _i -270	270 ± 3	31 ± 5	27 ± 1	DOM _f -27	11.5 ± 0.5	1.06 ± 0.08	0.88 ± 0.07
DOM _i -1453	1453 ± 8	124 ± 6	240 ± 1	DOM _f -240	70 ± 2.8	2.20 ± 0.14	0.32 ± 0.02
HgCl ₂ (C _{Hg})		5.2 ± 0.6			1.1 ± 0.2	10.4 ± 0.3	50 ± 9

^aValues represent averages from duplicate experimental aquaria. ^bConcentrations measured on freeze-dried DOM. ^cConcentration of Hg in unfiltered water. ^dConcentration based on dry weight. ^eCalculation performed by dividing the total amount of accumulated Hg in whole fish by the total amount of Hg present in the aquaria (35 L), both at t₈. The first quantity was obtained by multiplying the total number of fish at t₈ (n = 46) by the corrected concentration of Hg in whole fish at t₈ and the average dry fish weight of 80.5 ± 20 mg. The corrected concentration of Hg in whole fish at t₈ is the concentration accumulated between t₀ and t₈ and therefore calculated by subtracting from the whole fish Hg concentration at t₈ that of control fish at t₀ equal to 0.09 ± 0.03 μg/g dry weight.

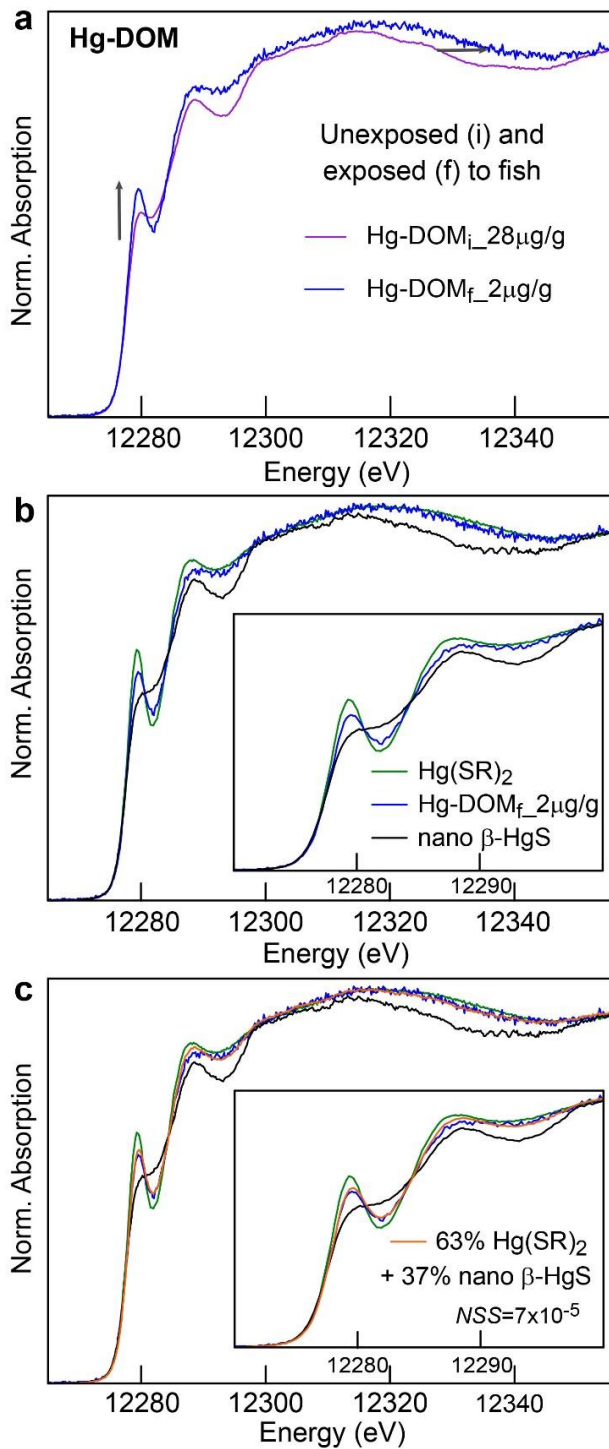
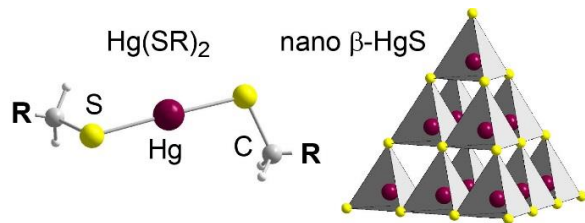


Figure 1

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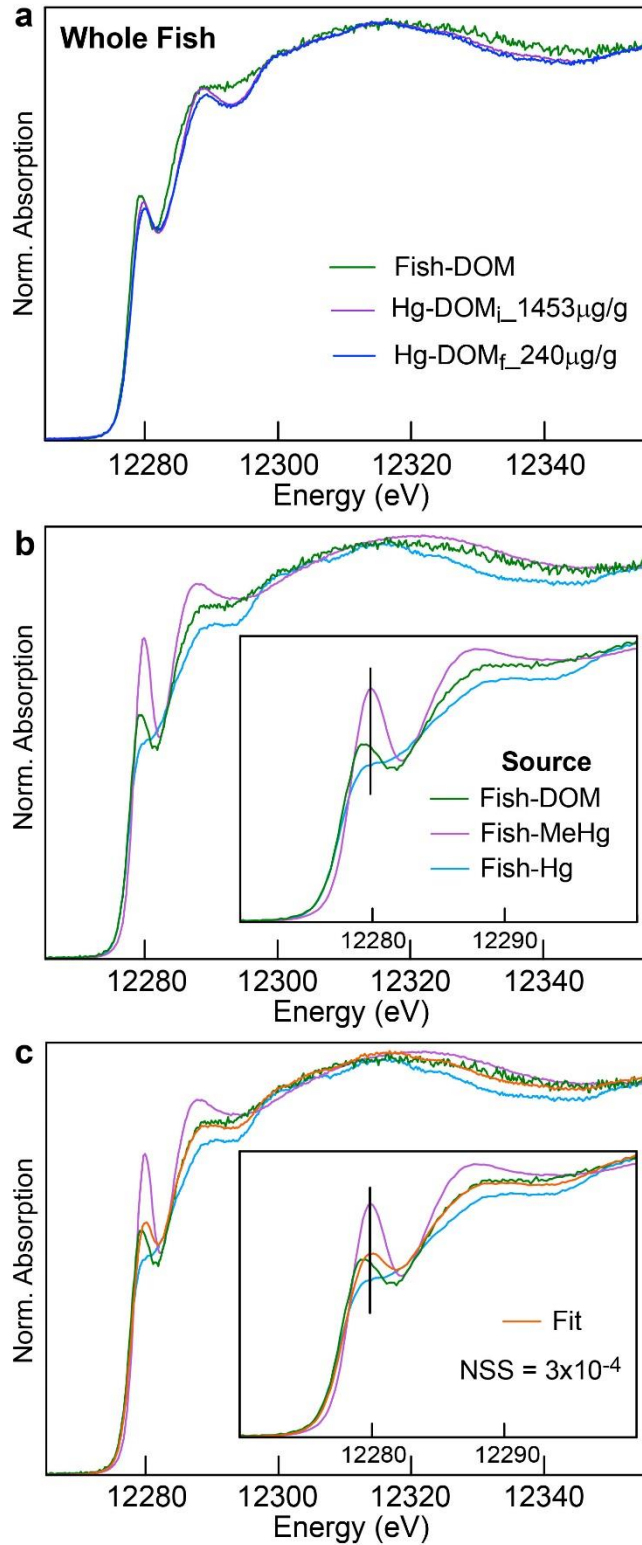


Figure 2

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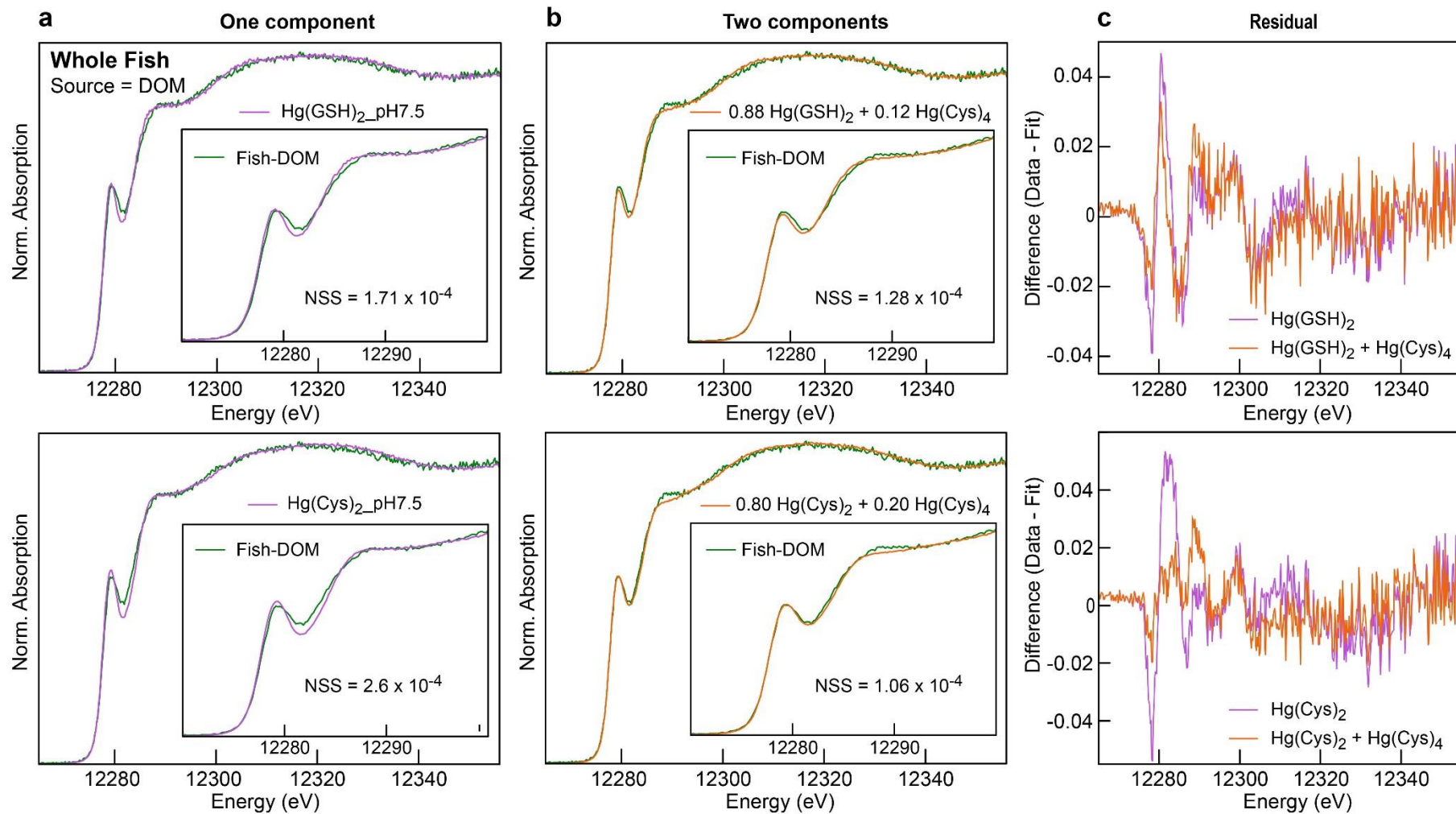
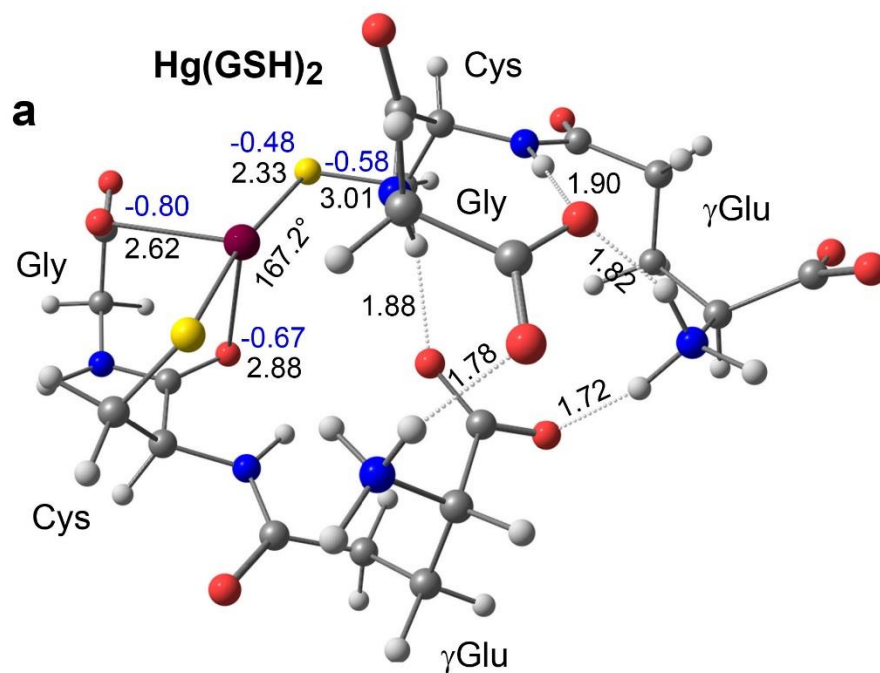
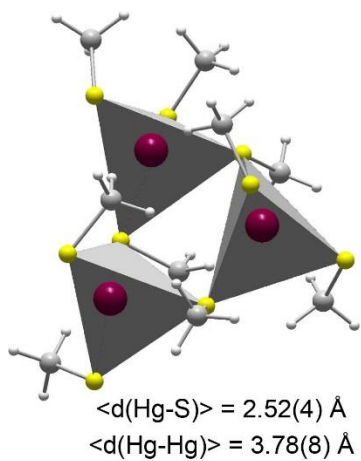


Figure 3

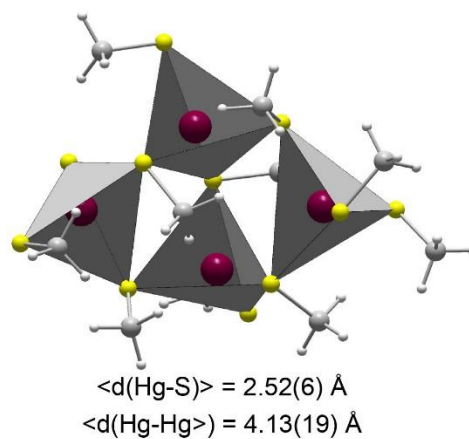
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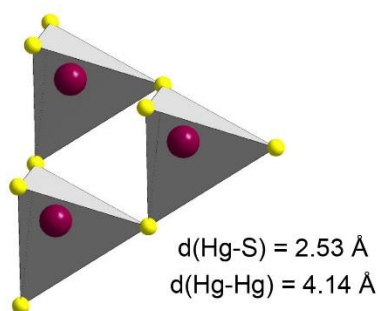
b **Hg₃S₉ β domain**



Hg₄S₁₁ α domain



c **Hg₃S₉ β -HgS**



Hg₄S₁₀ β -HgS

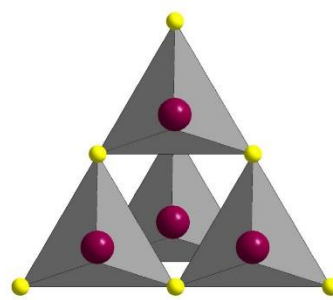


Figure 4

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