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Mercury methylation and methylmercury demethylation in boreal lake sediment with legacy sulphate pollution†

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Sulphate and dissolved organic matter (DOM) in freshwater systems may regulate the formation of methylmercury (MeHg), a potent neurotoxin that biomagnifies in aquatic ecosystems. While many boreal lakes continue to recover from decades of elevated atmospheric sulphate deposition, little research has examined whether historically high sulphate concentrations can result in persistently elevated MeHg production and accumulation in aquatic systems. This study used sediment from a historically sulphate-impacted lake and an adjacent reference lake in northwestern Ontario, Canada to investigate the legacy effects of sulphate pollution, as well as the effects of newly added sulphate, natural organic matter (NOM) of varying sulphur content and a sulphate reducing bacteria (SRB) inhibitor on enhancing or inhibiting the Hg methylation and demethylation activity (K_{meth} and K_{demeth}) in the sediment. We found that K_{meth} and MeHg concentrations in sulphate-impacted lake sediment were significantly greater than in reference lake sediment. Further adding sulphate or NOM with different sulphur content to sediment of both lakes did not significantly change K_{meth}. The addition of a SRB inhibitor resulted in lower K_{meth} only in sulphate-impacted sediment, but methylation was not entirely depressed. Methylmercury demethylation potentials in sediment were consistent across lakes and experimental treatments, except for some impacts related to SRB inhibitor additions in the reference lake sediment. Overall, a broader community of microbes beyond SRB may be methylating Hg and demethylating MeHg in this system. This study reveals that legacies of sulphate pollution in boreal lakes may persist for decades in stimulating elevated Hg methylation in sediment.

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Environmental significance

Atmospheric sulphate loading can stimulate the production of methylmercury, a neurotoxin that biomagnifies in aquatic ecosystems. Atmospheric sulphate deposition has been declining in many parts of the world for decades, but how this decline affects methylmercury in lake sediment is not well understood. This research demonstrated that 25 years after stopping experimental sulphate inputs to a lake, methylmercury production in the sediment is still 10-fold higher than in a reference lake. The sediment, however, cannot be further stimulated to produce methylmercury *via* additions of more sulphate or different types of natural organic matter. Importantly, this study contrasts with studies in other ecosystems, like wetlands, where methylmercury concentrations and production decline quite rapidly when sulphate deposition declines.

1. Introduction

Mercury (Hg) pollution is a continuing global-scale problem with serious ecosystem and human health concerns, including well-documented impacts on the central nervous system.[1–4] The methylmercury (MeHg) form is of greatest concern because it is significantly more bioaccumulative than inorganic Hg(II) and poses the greatest risk of exposure to consumers of contaminated foods such as fish.[5] Understanding what biogeochemical factors regulate the transformation of inorganic Hg(II) into MeHg is therefore critical in assessing Hg-related risk to human or other mammalian consumers, especially in aquatic ecosystems.

The methylation of inorganic Hg(II) is primarily driven by anaerobic microbial activity under anoxic and suboxic saturated conditions, as occurs in lake sediment, wetlands and flooded soils.[6–8] The *hgcAB* gene pair that enables methylation capabilities is relatively widely dispersed amongst anaerobic microbes,[9,10] but MeHg production in natural settings is most commonly biogeochemically associated with sulphate

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reduction and the activity of sulphate reducing bacteria (SRB).[6,11–13] Additionally, sulphur can have more complex roles in regulating Hg methylation *via* sulphur–dissolved organic matter (S–DOM) complexation and subsequent effects on Hg bioavailability to methylating microbes.[14–16]

Dissolved organic matter can either lessen or enhance Hg bioavailability for methylation, depending on the character of DOM and biogeochemical conditions.[17–19] For example, large Hg–DOM complexes may be difficult to diffuse through cell membranes, thus reducing Hg bioavailability for methylation.[19,20] In contrast, low molecular mass DOM compounds can facilitate microbial Hg uptake for methylation through active transport of Hg–DOM complexes into cells.[21,22] Sulphur (S) containing functional groups in DOM are particularly strong binding sites for inorganic Hg(II), and therefore also strongly affect Hg bioavailability.[15,23,24] Moreover, higher sulphur content and more aromatic DOM are more effective at enhancing Hg bioavailability because they can more effectively inhibit the aggregation and precipitation of more bioavailable Hg–sulphur–DOM nanoparticles at favorable aqueous sulphide concentrations.[15,16,18,25] Still, whether DOM composition, particularly as it relates to sulphur content, significantly stimulates or inhibits Hg methylation in natural settings is not yet well understood.

Sulphur pollution leads to acidification and elevated sulphate concentrations in lake water.[26] Low pH is usually associated with stimulated Hg methylation in the lake bottom sediment–water interface areas, probably due to increased bioavailable Hg species.[20,27–29] In freshwater sediment and in wetlands, elevated sulphate inputs usually increase MeHg production[11,30] whereas decreasing sulphate inputs can lead to relatively rapid declines in MeHg concentrations.[31] In other systems, high sulphate content does not always lead to elevated MeHg concentrations or Hg methylation potentials in lake sediment[8] or wetlands.[7,32] This is likely because of inhibitory effects on Hg bioavailability at high sulphate—and subsequently high sulphide—concentrations.[7,8,18] Interestingly, little to no published research has examined the legacy impacts of sulphate deposition on sulphate driven promotion or sulphide driven inhibition of Hg methylation in lake sediment, decades after sulphate loading decreases or stops. Such research would have important practical significance because many lakes around the world were subjected to decades of elevated sulphate deposition during the past century.[33–35] Following national and international legislation, atmospheric sulphate concentrations and deposition have substantially decreased in many parts of the world, particularly in North America and Europe since the 1980s[36,37] and many of the affected lakes continue to recover.[38–40] Therefore, experimental work to untangle the legacy effects of historic excess sulphate inputs on MeHg production and accumulation in aquatic systems is needed.

The objectives of this study were to explore MeHg production and degradation in boreal lake sediment with historically elevated sulphate loading but where current levels have returned to background levels for a substantial period of time - approximately two decades (Fig. S1a†). Using lake sediment from a historical experimental acidification project at the IISD Experimental Lakes Area (ELA) in Canada, this study investigated how (1) legacy sulphate contamination, (2) new sulphate additions, (3) chemical inhibition of sulphate reduction, and (4) addition of two natural organic matter (NOM) isolates incorporating relatively high and low sulphur content, affect *in situ* Hg methylation and MeHg demethylation in boreal lake sediment.

2. Methods

2.1 Study lakes description

Lake sediment cores were retrieved from two study lakes in the IISD ELA, northwestern Ontario. Lake 223 (N 49.698°, W 93.708°, surface area = 27.3 ha, maximum depth = 14.4 m) is a sulphate-impacted lake that was experimentally acidified to pH 5.1 by addition of sulphuric acid during 1976–1983, and recovered by gradually reducing sulphuric acid additions from 1984 through 1993 until pre-acidification pH values were met (6.5–6.8).[41,42] From 1994–2017, the pH in lake 223 remained stable at values of 6.7–7.1.[43] Sulphate concentrations in lake 223 water increased from an average value of 3 mg L^{-1} before acidification to a peak value of 16 mg L^{-1} in the early 1980s, then returned to pre-experimental levels after approximately the year 2000 (Fig. S1a†). Dissolved organic carbon (DOC) concentrations in lake 223 increased for short periods in the early 1980s but remained at background levels (4–5 mg L^{-1}) after 1985 (Fig. S1c†). In contrast, lake 224 (N 49.690°, W 93.717°, surface area = 25.4 ha, maximum depth = 26.7 m) is an adjacent unmanipulated reference lake where pH values and sulphate and DOC concentrations have remained relatively constant over the same period of time (Fig. S1b and d;† Mills *et al.*, 2002). Lake 224 is located upstream of lake 223, sharing similar limnological characteristics.[41,44] Both lakes are dimictic, with lake water stratified in winter and summer, with turnover in spring and fall.[45,46]

2.2 Sample collection and experimental design

During February 2018, lake sediment cores were collected using a gravity corer (6.7 cm ID) lowered through auger holes in winter ice in areas of maximum depth for each lake. Both lakes had anoxic environments at the maximum depth as demonstrated by low oxygen concentrations in the bottom water (Fig. S4†). Sediment cores were kept in a cooler prior to transport back to a field laboratory facility at the ELA research station later in the same day. In the laboratory, cores were either extruded for THg/MeHg depth profile analyses or for sediment incubation experiments (see below). For THg/MeHg depth profiles, the surface 20 cm of sediment was sectioned at 2 cm intervals and each section was stored individually frozen (−20 °C) in a specimen cup. For incubation experiments, the uppermost 2 cm of sediment was used assuming Hg methylation most actively occurs within anoxic zones near the sediment–water interface[47,48] and because surface sediment are the most relevant for sediment-lake water exchange.[49] The uppermost 2 cm of sediment was scooped from each core and transferred into a serum

bottle that was immediately purged with high purity nitrogen for 2 minutes to replace the head space air prior to closure with a Teflon coated rubber septum and aluminum cap.

In addition to examining sediment geochemical differences between lakes, a number of sediment incubation experiments were conducted to test for significant effects on mercury methylation and demethylation when: (1) sulphate was added to sediment with different sulphate contamination histories; (2) NOM isolates with variable composition and sulphur content were added; (3) molybdate, an SRB inhibitor, was added; and (4) accounting for interactions amongst sulphate and NOM additions. In these experiments, the sediment in serum bottles were manipulated in a factorial design using inputs of sulphate (Na_2SO_4, 300 μM), Suwannee River fulvic acid standard II (SRFA, low sulphur, sulphur% = 0.46%, 600 μm equivalent carbon), Suwannee River natural organic matter (SRNOM, high sulphur, sulphur% = 1.78%), and molybdate (Na_2MoO_4, 500 μM) as a SRB inhibitor. All additive solutions were prepared individually by dissolving the respective chemical in deionized water and used without additional filtration/purification. Both NOM isolates and compositional information including sulphur content were obtained from the International Humic Substances Society (IHSS). Similar NOM solutions (*e.g.* SRHA in Graham *et al.*, 2012, 2013 (ref. 18 and 50)) have previously been used to investigate the impacts of DOM on microbial Hg methylation. All experimental treatments were completed in triplicate. For estimation of methylation and demethylation potentials (see next section), a mixture of enriched Hg isotope solutions (98.29% enriched inorganic $^{200}Hg(II)$ and 84.70% enriched $Me^{201}Hg$) was also added to each serum bottle, by injection through the septum. The isotope solutions were premixed with respective overlying lake water and equilibrated for one hour in the dark prior to addition. The anticipated mass ratio of the enriched inorganic $^{200}Hg(II)$ to $Me^{201}Hg$ in the sediment was 10 : 1. Sediment slurries were well mixed with their additives and incubated in the dark for 24 hours at 13 °C, which is close to summer lake hypolimnetic water average temperatures in the area.[46] Incubations were ended by freezing sediment at −20 °C.

Pore water (within 5 cm of sediment–water interface) from both lakes was extracted using Rhizon® soil solution samplers inserted into sediment cores, with vacuum applied that was equivalent to a fully open 60 mL syringe. Pore water samples were stored at 4 °C in polyethylene tubes for determination of DOC and sulphate concentrations. Lake water samples (both hypolimnetic and epilimnetic layers) were collected from both lakes using a peristaltic pump equipped with HCl acid cleaned Teflon tubing. Samples were stored in 250 mL PETG bottles in a cooler. In the research station laboratory facility, some of the lake water sample was filtered using pre-cleaned, disposable 0.45 μm cellulose nitrate filter units (Nalgene). Filtrate was used to determine DOC and sulphate concentrations (all samples), as well as the DOM composition (hypolimnetic water only). The remaining unfiltered water samples for determination of THg and MeHg concentrations were acidified to 0.5% by volume with trace metal grade HCl and stored refrigerated. Water samples for DOC and sulphate were stored in polyethylene tubes and refrigerated until analysis. The filtered lake water for DOM composition analysis was stored in 500 mL PETG bottles (1.5 L water for each lake) and then freeze–dried for analysis. All samples were processed and analyzed at the University of Toronto Scarborough.

2.3 Chemical analyses

The isotope dilution method was used to measure both MeHg and THg concentrations, according to the approach outlined in ref. 51 and 52. All sediment analyses were conducted on freeze–dried samples. For MeHg analysis, homogenized samples went through a sequential procedure consisting of steam distillation, distillate buffering and ethylation, amalgamation onto Tenax-filled traps, thermal desorption, gas chromatography (GC) separation and inductively coupled plasma mass spectrometry (ICP-MS) analysis. Specifically, 0.2–0.3 g of dry sediment were mixed with dilute copper sulphate ($CuSO_4$), potassium chloride (KCl) and sulphuric acid (H_2SO_4) solution in a Teflon vessel wherein a known amount of enriched $Me^{199}Hg$ internal standard solution was added prior to distillation. The MeHg in the distillate was then pH stabilized with addition of sodium acetate buffer and ethylated by addition of sodium tetraethylborate in a glass bubbler. The ethylated, volatile MeHg was purged by bubbling with high purity nitrogen and amalgamated onto a Tenax trap. Trap contents were thermally desorbed on a stream of high purity argon gas into a GC for separation of Hg species. The separated Hg species were then streamed by argon gas into the hyphenated ICP-MS (7700x, Agilent) for quantification of signal response of each individual Hg isotope. The ratios between the spiked $Me^{199}Hg$ and both excess $Me^{200}Hg$ and $Me^{201}Hg$ (that in excess of natural abundance and therefore attributable to the spikes for methylation and demethylation assays), as well as $Me^{202}Hg$ (used to measure ambient Hg concentrations because it is the mostly naturally abundant Hg isotope) were calculated. The ambient MeHg and newly produced enriched isotopic MeHg derived from the added enriched isotopes were calculated using these ratios and the known mass of $Me^{199}Hg$ added according to calculations described in ref. 52.

For THg analysis, a sequential procedure was employed including acid digestion, bromine monochloride (BrCl) oxidation, tin chloride ($SnCl_2$) reduction, gold trap amalgamation and ICP-MS determination.[53] The last three steps were automated *via* a hyphenated Tekran 2600-ICP-MS system. Specifically, 0.2–0.3 g of dry sample was digested with 10 mL of hot nitric acid. A known amount of enriched inorganic $^{199}Hg(II)$ internal standard solution was added to each sample prior to digestion. The digestion ended once vapors became colourless. The digestate was then diluted by deionized water and oxidized with BrCl solution overnight. The oxidized solution was introduced into an automated Tekran 2600-ICP-MS system where inorganic Hg(II) was reduced by $SnCl_2$ to elemental Hg that was carried by a stream of argon, concentrated onto dual gold traps in series, and desorbed on a stream of argon into the ICP-MS for quantification of the signal response of individual isotopes. Using the same methods for MeHg calculation, THg

concentrations, including ambient THg, excess $T^{200}Hg$ and $T^{201}Hg$, were determined simultaneously.

For both MeHg and THg analyses, a series of quality control (QC) and quality assurance (QA) samples were run, including analyses of blanks, duplicates, spikes and certified standard materials (CRM) (Table 1). Method detection limit (MDL) was calculated as three times the standard deviation of the measured Hg values from blanks. All sample measurements were above MDLs. CRMs included MESS-3 and IAEA-158 for THg and MeHg, respectively.

Water sulphate concentrations were measured using an ion chromatography system (Metrohm 930 Compact IC Flex). Dissolved organic carbon concentrations in lake water were measured with a Shimadzu $V_{CPH/CPN}$ TOC analyzer. Dry mass of each sediment sample was measured using an electronic balance after freeze–drying. Sediment bulk density was calculated as a ratio of dry mass to volume of the sediment.

2.4 Determination of Hg methylation and demethylation potential rate constants

As previously described, to assess the potential for Hg methylation and demethylation, sediment was incubated with both enriched inorganic $^{200}Hg(II)$ and organic $Me^{201}Hg$ equilibrated with lake water, for 24 hours at 13 °C. The incubation duration (24 hours) in this research falls within the expected first-order range of MeHg production and demethylation, and has been found suitable for calculation with first-order reaction equations.[54,55] K_{meth}, the first-order rate constant for Hg methylation potential can be expressed as:

$$K_{meth} = [Me^{200}Hg]_{t24}/([T^{200}Hg] \times t) \text{ (ref. 54 and 55)}$$

where $[Me^{200}Hg]_{t24}$ is the concentration (ng g^{-1}) of newly produced $Me^{200}Hg$ in the sample at the end of incubation, $[T^{200}Hg]$ is the concentration (ng g^{-1}) of the added inorganic $^{200}Hg(II)$ in the sample, and t is incubation time (d).

Simultaneously and on the same sample, K_{demeth}, the first-order rate constant for MeHg demethylation potential can be expressed as:

$$K_{demeth} = -Ln([Me^{201}Hg]_{t24}/[Me^{201}Hg]_{t0})/t \text{ (ref. 54 and 55)}$$

where $[Me^{201}Hg]_{t24}$ indicates the $Me^{201}Hg$ concentration (ng g^{-1}) that remained in the sample at the end of incubation, $[Me^{201}Hg]_{t0}$ is the $Me^{201}Hg$ concentration (ng g^{-1}) at the beginning of incubation represented by $T^{201}Hg$ concentration of the sample (confirmed with GC-ICP-MS that all ^{201}Hg was in methylated form before the experiment, data not shown), and t is incubation time (d).

2.5 DOM composition analysis

Analysis of DOM composition was completed in the Environmental Nuclear Magnetic Resonance (NMR) Centre at the University of Toronto Scarborough using solution-state ^1H NMR spectroscopy. The filtered (0.45 μm) lake water was freeze–dried and further dried over P_2O_5 under vacuum to remove additional water. Dried samples were re-dissolved into deuterium oxide (D_2O, 99.9% D) and sodium deuteroxide (NaOD, 99.5% D, 30 wt% in D_2O) and then centrifuged. The supernatants were then transferred into 1.7 mm NMR tubes[56] for NMR analysis using a Bruker BioSpin Avance III 500 MHz NMR spectrometer equipped with a ^1H–^{15}N–^{13}C TXI 1.7 mm microprobe fitted with an actively shielded Z gradient (Karlsruhe, Germany). ^1H NMR spectra were collected using a PURGE (presaturation utilizing relaxation gradients and echoes) approach to suppress the resonances from water at ~4.7 ppm.[57] The spectra were collected using 256 scans per sample with a recycle delay of 2 s, and 32 K time domain points. The spectra were processed using a zero filling factor of 2 and were apodized by multiplication with an exponential decay corresponding to 0.3 Hz line broadening. The collected NMR spectra were integrated into four main classes of DOM components based on the chemical shift values: materials derived from linear terpenoids (MDLT), 0.6–1.6 ppm; carboxyl-rich alicyclic molecules (CRAM), 1.6–3.2 ppm; carbohydrates and peptides, 3.2–4.5 ppm; and aromatic and phenolic components, 6.5–8.4 ppm.[58,59] The integration of each region was normalized to the total area using Analysis of Mixtures (AMIX; v. 3.9.15) software (Bruker BioSpin, Rheinstetten, Germany) to indicate the relative contribution of each component. Among these four components, the carbohydrates and peptides are the more preferred microbial substrates. The CRAM group of compounds is hypothesized to include more

Table 1 QA/QC data for THg/MeHg measurements

	Sediment THg			Sediment MeHg				
	Ambient	Excess ^{200}Hg	Excess ^{201}Hg	Ambient	Excess ^{200}Hg	Excess ^{201}Hg	Water THg	Water MeHg
Method detection limit	1.18 ng g^{-1} [1.15 ng g^{-1}][a]	0.53 ng g^{-1}	0.12 ng g^{-1}	0.22 ng g^{-1} [0.035 ng g^{-1}]	0.16 ng g^{-1}	0.18 ng g^{-1}	0.08 ng L^{-1}	0.02 ng L^{-1}
Duplicate RSD	1.9 ± 1.4% (n = 4 pairs)	1.7 ± 1.0% (n = 4 pairs)	0.8 ± 0.4% (n = 4 pairs)	9.0 ± 7.2% (n = 7 pairs)	5.3 ± 7.0% (n = 7 pairs)	1.9 ± 1.5% (n = 7 pairs)	0.5% (n = 1 pair)	2.3 ± 2.4% (n = 2 pairs)
Spike recovery	94 ± 10% (n = 5)	—	—	—	—	—	97% (n = 1)	—
CRM (ng g^{-1})	88 ± 2 (n = 6) {91 ± 9}[b]	—	—	1.45 ± 0.05 (n = 9) {1.41 ± 0.4}	—	—	—	—

[a] Numbers in square brackets represent MDLs without excess isotopes. [b] Numbers in braces are the certified values.

persistent molecules in DOM that are derived from cyclic terpenoids.[60]

2.6 Statistical analyses

Normality of data was assessed by Shapiro–Wilk test, *Q*–*Q* plots, density plots, and comparing mean and median values of each sample group to confirm the approximate normal distribution of all samples. Homogeneity of variance among comparisons was verified using Bartlett's test ($p > 0.05$). The differences of K_{meth}, K_{demeth}, MeHg concentrations and the percentage of THg presented as MeHg (MeHg%) in the surface sediment between two lakes were assessed using the Student's *t*-test. The effects of experimental treatments on K_{meth} and K_{demeth} were analyzed using one-way ANOVA, followed by the Tukey post-hoc test to compare the mean values of K_{meth} and K_{demeth} between any two treatments. The statistical differences were deemed significant at $p \leq 0.05$. All statistical analyses were completed using R, version 4.1.1.[61]

3. Results

3.1 Sulphate, DOM, total mercury and methylmercury in lake water

The current sulphate concentration in the sulphate-impacted lake was relatively low and comparable to the concentration in the unmanipulated reference lake (Table 2 and Fig. S1a†). The epilimnetic water DOC concentration in the sulphate-impacted lake was higher than in the reference lake, while hypolimnetic water DOC concentrations were similar in the two lakes. In both lakes, THg and MeHg concentrations were relatively low in epilimnetic waters, but significantly elevated in hypolimnetic waters (Table 2). While hypolimnetic water THg concentrations were comparable between two lakes, both MeHg concentration and MeHg% in hypolimnetic water were approximately four times greater in the historically sulphate-impacted lake (Table 2). The DOM composition in both lakes were similar, with the most abundant compound class within the DOM represented by carbohydrates and peptides (41% and 43% in the sulphate-impacted and reference lake, respectively), followed by CRAM (31% and 33%) and MDLT (25% and 21%) components. The aromatic and phenolic components (3% and 3%) were the least abundant (Table 2 and Fig. S2†).

3.2 Total mercury and methylmercury in lake sediment

Sediment depth profiles illustrated sharp increases for MeHg concentration and MeHg% in the upper 10 cm of the historically sulphate-impacted lake (from average 0.26 to 1.30 ng g^{-1} and 0.28 to 0.81%, respectively), but concentrations were relatively consistent or, for MeHg% specifically, decreased towards the sediment surface within the same depth of sediment in the reference lake (from average 0.46 to 0.75 ng g^{-1} and 0.67 to 0.55%, respectively; Fig. 1). Though the sediment cores were not dated, sediment properties and data from other studies suggest that the surface 10 cm of sediment coincides with the period of time since the start of acidification experiment in 1976.[41,42] Given bulk densities of 0.073 and 0.055 g cm^{-3} in the sulphate-impacted and reference lake, respectively, and sedimentation rates estimated to be 170 g per m^2 per year in the reference lake,[62] it was estimated that the surface 10 cm of sediment represents a time window of approximately the past 40 and 30 years in the sulphate-impacted lake and the reference lake, respectively. These estimations of sediment ages are comparable with results of recent research using freeze sediment cores that were collected in the same lakes and same winter as our study.[42]

In the uppermost 2 cm of sediment, THg concentrations were 160 ± 9 ng g^{-1} (mean ± standard deviation, dry weight, $n = 5$) in the sulphate-impacted lake, which was significantly higher than concentrations in the reference lake sediment (136 ± 16 ng g^{-1}; $t(8) = 2.97$, $p = 0.018$, $n = 5$; Fig. 1). Sediment THg concentrations in both lakes were comparable with values reported for nearby locations, including lakes from the ELA and from northern Minnesota.[48,63,64] In the uppermost 2 cm of sediment, MeHg concentrations and MeHg% in the sulphate-impacted lake were 1.30 ± 0.14 ng g^{-1} and 0.81 ± 0.11%, respectively, which were significantly higher by approximately

Table 2 Concentrations of sulphate, DOC, THg and MeHg and DOM composition in study lakes

							Fraction of DOM components (%)			
	Water depth	Sulphate (mg L^{-1})	DOC (mg L^{-1})	THg (ng L^{-1})	MeHg (ng L^{-1})	MeHg (%)	MDLT	CRAM	Carbohydrates and peptides	Aromatic and phenolic
Lake 223 (sulphate-impacted)	Epilimnetic water	2.67[a]	10.71	0.62–0.62[b]	0.03–0.03	4.1–4.9	—	—	—	—
	Hypolimnetic water	0.31	10.08	3.92–4.25	1.00–1.01	23.8–25.5	25	31	41	3
	Porewater	0.48	17.97	N/A	N/A	N/A	—	—	—	—
Lake 224 (reference)	Epilimnetic water	2.15	5.81	0.35–0.44	0.02–0.02	3.8–5.7	—	—	—	—
	Hypolimnetic water	0.64	9.36	4.26–4.47	0.23–0.27	5.0–6.2	21	33	43	3
	Porewater	0.31	18.87	N/A	N/A	N/A	—	—	—	—

[a] Single sample analyzed for sulphate and DOC at each water depth. [b] Duplicate samples for Hg related analysis, both results shown here.

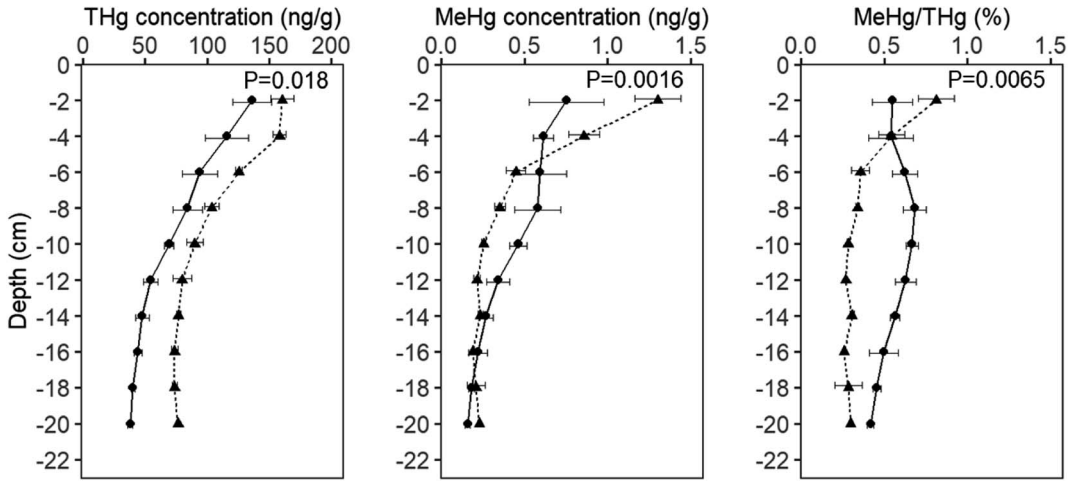

Fig. 1 Depth profiles of THg and MeHg concentrations and MeHg% in sulphate-impacted (triangle, dash line) and reference (dot, solid line) lake sediment. Error bars represent one standard deviation (for the uppermost 2 cm, $n = 5$; other depths, $n = 2$). The p-values denote significant differences in the uppermost 2 cm sediment between two lakes.

1.5 times compared to the values observed in the reference lake (0.75 ± 0.22 ng g^{-1} and $0.55 \pm 0.12\%$; $t(8) = 4.67$, $p = 0.0016$ and $t(8) = 3.65$, $p = 0.0065$ for MeHg concentration and MeHg%, respectively, $n = 5$; Fig. 1). Methylmercury concentrations and MeHg% in this study fell within the middle-to-high end of the range found in nearby relatively pristine lakes.[48,65,66]

3.3 Mercury methylation and demethylation experiments

The mercury methylation potentials (K_{meth}) and methylmercury demethylation potentials (K_{demeth}) in sediment measured in our study were consistent with the ranges reported in other aquatic systems without significant mercury contamination.[67] Overall, there was no statistically significant impact of experimental treatments on K_{meth} in sediment from both lakes, but under control conditions, K_{meth} differed significantly between lakes. In the uppermost 2 cm of sediment, K_{meth} were one magnitude greater ($t(4) = 4.62$, $p = 0.01$, $n = 3$) in the sulphate-impacted lake (0.034 ± 0.008 d^{-1}, mean $\pm$ standard error, dry weight, $n = 3$; Fig. 2a, no-addition) compared to the reference lake (0.003 ± 0.002 d^{-1}; Fig. 2b, no-addition). With additions of sulphate and the SRB inhibitor molybdate to either the sulphate-impacted or reference lake sediment, K_{meth} values did not significantly change ($F(2,6) = 3.98$, $p = 0.08$ and $F(2,6) = 0.81$, $p = 0.49$, respectively for the sulphate-impacted and reference lake sediment, $n = 3$; Fig. 2). It is worth noting that in the sulphate-impacted sediment, when compared to the "No-addition" treatment, a slight reduction in K_{meth}, which bordered on statistical significance ($p = 0.068$, $n = 3$), was observed with molybdate addition, but methylation was not completely inhibited (Fig. 2a). In the reference lake sediment, when sulphate was added, one K_{meth} value was nearly one magnitude higher than the other two replicates, leading to a slightly elevated average K_{meth} compared to the "No-addition" treatment (Fig. 2b). There was no significant change in Hg methylation in either the sulphate-impacted or reference lake sediment upon the addition of SRFA or SRNOM, nor with the combined addition of sulphate + SRFA or sulphate + SRNOM (Fig. 3).

The sediment K_{demeth} were not significantly different between lakes (0.26 ± 0.03 and 0.24 ± 0.03 d^{-1} in sulphate-impacted and reference lake, respectively) or across most

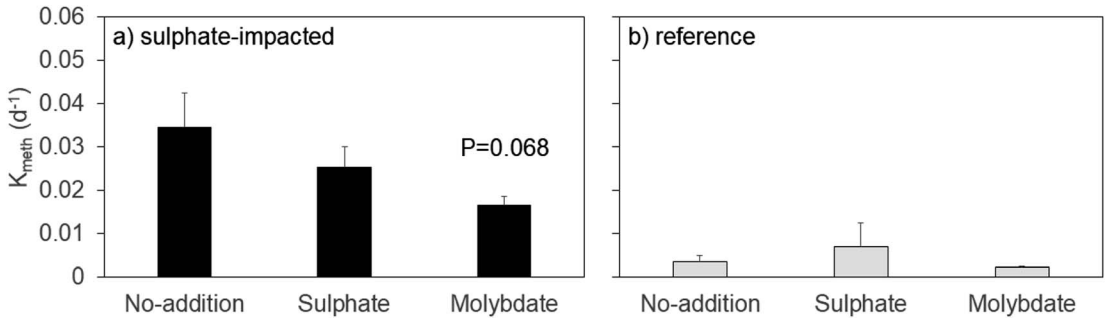

Fig. 2 K_{meth} in (a) sulphate-impacted and (b) reference lake sediment with additions of sulphate (Na_2SO_4, 300 μM) and molybdate (Na_2MoO_4, 500 μM) as compared to no-addition control. Error bars represent one standard error of replicate ($n = 3$) experiments. The p-value denotes the significance between treatments and no-addition within the same lake.

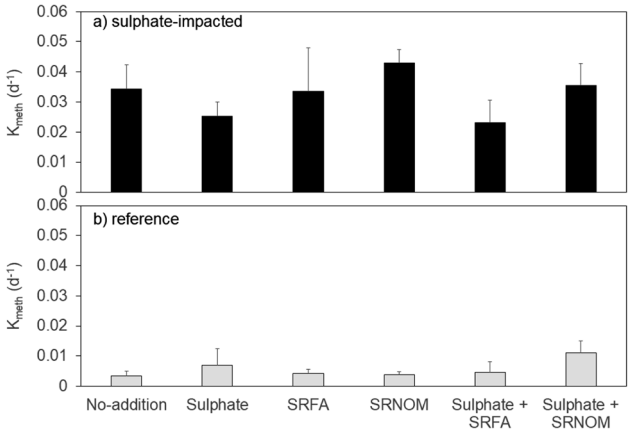

Fig. 3 K_{meth} in (a) sulphate-impacted and (b) reference lake sediment with singular additions of sulphate (Na_2SO_4, 300 μM), SRFA (low sulphur content, 600 μM carbon), SRNOM (high sulphur content, 600 μM carbon) and combined additions of sulphate and SRFA or sulphate and SRNOM as compared with no-addition control. Error bars represent one standard error of replicate ($n = 3$) experiments.

experimental treatments in either lake (Fig. 4 and S3†). Only in the reference lake sediment, with the additions of sulphate and molybdate, was there a significant effect on K_{demeth} ($F(2,6) = 5.36$, $p = 0.046$, $n = 3$). When compared to the "No-addition" treatment in the reference lake sediment, a statistically significant decline in K_{demeth} ($p = 0.04$, $n = 3$) was observed in the molybdate treatment (Fig. 4b).

4. Discussion

4.1 Legacy effects of historical sulphate loading

Historical sulphate loading is the most plausible reason for the significantly elevated MeHg production potential observed in the sulphate-impacted lake sediment relative to the reference lake sediment. Previous research reveals no discernibly increased primary productivity resulting from the experimental acidification in lake 223, the sulphate-impacted lake in our study.[68] Coupled with the similar DOC concentrations and the NMR-based DOM compositions (Fig. S2†) in the two lakes, it suggests that benthic organic matter is not likely the controlling factor for the substantially different K_{meth} between the two lakes. Though further detail on sulphur composition of the sediment (*e.g.*, Pierce *et al.*, 2022 (ref. 69)) was not available as part of this study, the bulk of evidence suggests that a build-up of sulphur and internal cycling in the sediment (due to historical sulphate loading) is the causative factor for the elevated MeHg concentration and production in the sulphate-impacted lake. This is supported by the otherwise immediate proximity of the two lakes, their similar limnological and meteorologic conditions, as well as the similar sulphate concentrations. Sulphate in freshwater lakes is microbially reduced to sulphide in anoxic sediment and can potentially be followed by sulphide reoxidation to sulphate, which can occur both anaerobically and aerobically[70] and therefore support ongoing SRB activity.[70,71] The stratification and turnover cycles of lake water, which occur in the sulphate-impacted lake in our study, can bring in oxygen and sulphate from the epilimnion to the hypolimnion, potentially contributing to *in situ* sulphide reoxidation, as well as continuous SRB activity.[45,46,70] Sulphide can also be incorporated into organic matter, augmenting the quantity of organosulphur compounds in sediment,[72,73] possibly increasing Hg bioavailability and K_{meth} in lake sediment.[15,74] Recent research has shown that sediment organosulphur, upon hydrolysis and mineralization, can contribute substantially to sustaining SRB activity, particularly in low sulphate (*e.g.*, <100 μM) lakes.[75] These reducing–oxidizing cycles involve the majority of the produced sulphide and crucially influence the scale of sulphur burial,[70,76] rendering a longer process of sulphur sequestration and continuing SRB stimulation in sediment. Therefore, initiated by the historical (1976–1993) experimental additions of sulphate,[41] and coupled with an apparently stronger internal sulphur cycling in the sediment, the sulphate-impacted lake sediment appears able to stimulate and maintain robust SRB activity, and possibly elevated Hg bioavailability, for decades after the termination of sulphate inputs.

The elevated MeHg accumulation in the sulphate-impacted lake sediment is also most likely a result of historical sulphate loading. Net MeHg production is a result of dynamic equilibrium between Hg methylation and MeHg demethylation.[6,77] Therefore, in the uppermost 2 cm sediment, the substantially higher K_{meth} in the sulphate-impacted lake, but comparable K_{demeth} between two lakes, is a reasonable

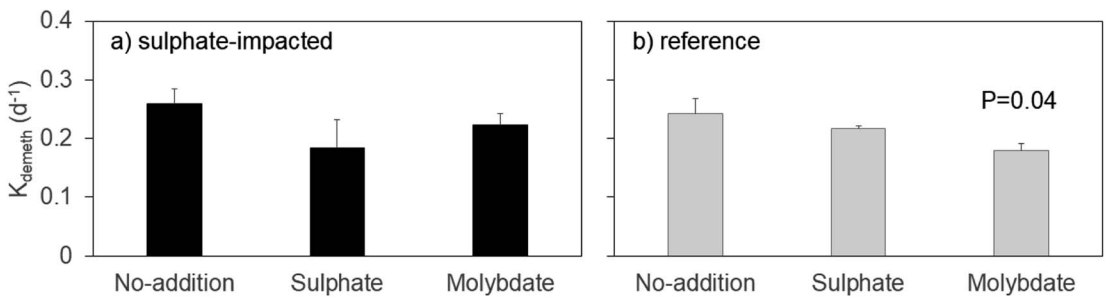

Fig. 4 K_{demeth} in (a) sulphate impacted and (b) reference lake sediment with additions of sulphate (Na_2SO_4, 300 μM) and molybdate (Na_2MoO_4, 500 μM) as compared with no-addition control. Error bars represent one standard error of replicate ($n = 3$) experiments. The p-value denotes the significance between treatments and no-addition within the same lake.

explanation for the observed higher sediment MeHg concentrations and MeHg% in the sulphate-impacted lake relative to the reference lake. Additionally, the contrasting sediment MeHg profiles (Fig. 1) suggest that historically elevated sulphate inputs have chronically shifted and maintained an ongoing dominance of Hg methylation over demethylation. The present study is one of a very few studies (*e.g.* Coleman Wasik *et al.*, 2012 in a wetland setting) to examine the legacy effects of sulphate exposure on Hg methylation in wet systems. In contrast to observations in wetlands where MeHg nearly recovered to control levels relatively quickly (~4 years) once experimental sulphate inputs were ceased,[31] our results suggest that freshwater lakes will need a considerably longer period of time (multiple decades) for natural attenuation to mitigate MeHg production in sediment of previously sulphate-impacted lakes.

4.2 Impact of sulphate addition on K_{meth} in sediment

The statistically consistent K_{meth} in the sulphate-impacted lake sediment upon addition of sulphate suggests that increased loading of sulphate is unable to further promote Hg methylation in lake sediment that has been subject to historical sulphate impacts. This is different from investigations in more sulphate-limited lakes and wetlands, wherein higher levels of MeHg production were stimulated by additions of sulphate.[11,12,30] This contradictory observation was not necessarily unexpected, however, because sulphate stimulation of MeHg production appears to largely be constrained to sulphate-limited freshwater systems. For instance, in systems where sulphate concentrations are not limiting (*e.g.*, impacted by mining of high-sulphur ore or by naturally elevated groundwater sulphate), higher sulphate inputs led to unchanged or lower $K_{meth.}$[7,32] Although the porewater sulphide derived from sulphate reduction can interact with inorganic Hg(II) to form more bioavailable Hg species for Hg methylation,[6,78,79] addition of sulphate to already high sulphate/sulphide systems will lead to production of excess sulphide. When sulphide concentrations exceed the 'Goldilocks zone' wherein bioavailable Hg–S complexes are optimally formed, any potential stimulation of Hg methylation may be offset by reductions in Hg accessibility/bioavailability to microbial methylators, therefore reducing MeHg production.[7,8,18,32] Our results further suggest that in historically sulphate-impacted lake sediment, even if the current freely available porewater sulphate concentrations are low, addition of sulphate will not stimulate Hg methylation. Although direct sulphide measurements were not made in this study, constantly produced sulphide might be available in the sulphate-impacted sediment porewater, attributable to degradation of sulphur-containing DOM[15,80] that derives from internal sulphur cycling of the large amount of historical sulphate loading.[70,72,73]

The statistically unchanged K_{meth} in the reference lake sediment that resulted from sulphate addition was surprising in that a substantial stimulation was expected. The lower sulphate concentrations in the hypolimnetic water and porewater relative to the epilimnetic water in both lakes suggest active sulphate reduction occurring in the anoxic lake bottom areas. The lack of statistical significance is based on high inter-replicate variability, which may suggest that a potentially more complex community of Hg methylators exists in this lake sediment, including microbes that do not utilize sulphate as an electron acceptor.[81–83] The results from samples amended with molybdate, a well-known sulphate reduction inhibitor,[11,84,85] also support this view. Molybdate addition had no effect on K_{meth} in the reference lake sediment, but it did weakly and significantly reduce, though not eliminate, Hg methylation in the sulphate-impacted sediment, implying that microbial methylators other than SRB contribute to Hg methylation in the sediment of both lakes.

4.3 Impact of sulphur content in NOM isolates on K_{meth} in sediment

A lack of significant changes in K_{meth} in the sediment incubation experiments when SRFA and SRNOM solutions were added suggests that NOM with different sulphur content does not significantly affect Hg methylation in these natural systems. It was expected that higher sulphur content NOM would lead to greater MeHg production, as demonstrated in previous studies.[15,18] However, the previous investigations were conducted under highly controlled laboratory conditions using model bacteria as Hg–methylators in culture experiments.[15,18] Our incubation experiments were carried out using boreal lake sediment that encompassed substantially more complex microbial compositions and geochemical conditions. This suggests that sulphur content in NOM affects Hg methylation weakly or not at all under natural sediment conditions.

4.4 Methylmercury demethylation in lake sediment

Our results suggest that historical sulphate loading negligibly alters demethylation capabilities in sediment, especially when compared to the larger influence it has on methylation. The lack of site or experimental differences for K_{demeth} (Fig. 4 and S3†) further supports this and also indicates that demethylation in these systems is less sensitive to alterations of sulphate, as well as NOM inputs. Although MeHg can be effectively demethylated *via* both abiotic and biotic pathways, in light-free lake sediment, microbial demethylation generally dominates.[6,86] The only observed significantly lower K_{demeth} in the reference sediment was in relation to the addition of molybdate, a SRB inhibitor. This result suggests that SRB may play a role in demethylating MeHg in the system, in line with observations in other studies.[87] The incomplete inhibition of K_{demeth} further indicates a diverse microbial community that degrades MeHg, such as including methanogens[88] and iron reducing bacteria,[77] though insufficient data exist to yet support an explicit microbial composition. Conservatively, the relatively consistent K_{demeth} in lake sediment, as opposed to much greater experimental variability in K_{meth}, suggests that the extent of net MeHg production in these lakes is more strongly governed by Hg methylation rates than by demethylation rates, a result in agreement with other studies in boreal soil[89,90] and in Arctic sediment.[91]

5. Conclusions

Our findings demonstrate that a lake's historical sulphate exposure is important when assessing MeHg production. In sediment, the accumulation of MeHg was more clearly a function of methylation processes than of demethylation processes, given the latter did not vary across most experimental treatments or between lakes. Despite 25 years passing since intentional sulphate additions to the experimental lake in this study, Hg methylation potential, MeHg concentration and MeHg% were all significantly higher in the historically sulphate-impacted lake than in the reference lake. Sulphate driven stimulation of Hg methylation therefore appears to be much slower to naturally attenuate in boreal lake sediment compared to wetlands.[31] In the sulphate-impacted lake sediment, adding more sulphate did not further increase the already elevated K_{meth}, suggesting the likelihood of sulphide-driven inhibition of Hg methylation, which is consistent with other studies where sulphate concentrations are not limiting.[8] This study adds new knowledge, having examined a lake where sulphate concentrations were historically elevated but have recovered to background sulphate levels, compared to other research where high sulphate inputs were ongoing and sulphate concentrations remained elevated.[8] An important next step in better understanding the explicit mechanisms of these sulphur–Hg methylation interactions in historically sulphate-impacted lakes would be to further investigate the sediment and pore water sulphur speciation (*e.g.*, Pierce *et al.*, 2022 (ref. 69)), including analysis for the available reduced inorganic sulphur species (*e.g.* chromium reducible sulphur[92]) that play an important role in understanding the relatively slow recovery of sulphur and mercury cycling in the sulphate-impacted lake.

Furthering our understanding of the role of NOM in stimulating or inhibiting Hg methylation remains difficult. The SRFA and SRNOM used in this study have different sulphur contents (0.46% *vs.* 1.78%), but adding either of them did not lead to significantly different K_{meth} or K_{demeth} in the sediment incubation experiments. This result suggests that total sulphur content in NOM cannot predict Hg bioavailability for methylation in these natural systems. Further investigations on the reduced sulphur groups[74] and unsaturated carbon bonds[15,93] may help in a deeper examination of whether and how DOM composition affects Hg bioavailability.

Conflicts of interest

There are no conflicts to declare.

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