ReadME for statistical analysis scripts and data frames to accompany the publication:

Nitrate Treatment Suppresses Mercury Demethylation in Coastal Estuarine Sediment

(Calvin et al. in prep)

Abstract

The sediment used to produce the data provided here was collected at the University of California Natural Reserve System's Younger Lagoon Reserve, doi: (10.21973/N3894D). Sampling sites included Beach Zone (BZ; 36.949380 N, -122.067624 W), at the southern edge of the lagoon, characterized by sandy sediment and a close connection to the ocean, and East Fork (EF; 36.951483 N, -122.066275 W), at the northern boundary of the lagoon, characterized by organic-rich, clay sediment and spatial proximity to agricultural sites. Sediment for experimental microcosm incubations was sampled in October 2020 and October 2022 and was subsequently spiked with Hg isotope enriched tracers and nitrogen amendments to investigate the putative effects of agricultural fertilizers on mercury methylation dynamics. Treatments were not significantly different than the control for potential Hg methylation rate constants (k_{meth}) in 2020 nor in 2022. Molybdate, a known inhibitor of biotic sulfate reduction, led to lower k_{meth} values for some replicates at both BZ and EF, though high variability between biological replicates precluded a statistically significant result. A suppression of k_{meth} under molybdate within some microcosms suggests that Hg methylation in the system was dominated by sulfate reducing bacteria. Treatments with two concentrations of nitrate in 2022 microcosms led to no change in k_{meth} compared to the control, while both high and low nitrate treatment resulted in significantly suppressed potential methylmercury demethylation rates (k_{demeth}).

Files:

Script:

1. **ANOVA for 2020 Microcosms 8.26.25.R**

Dataframes for use with ANOVA for 2020 Microcosms 8.26.25.R:

2. **East Fork kmeth Data 2020 8.25.25.csv**
3. **Beach Zone kmeth Data 2020 8.25.25.csv**

Script:

4. **ANOVA for 2022 Microcosms 8.10.25.R**

Dataframes for use with ANOVA for 2022 Microcosms 8.10.25.R:

5. **kmeth Microcosms 2022 8.10.25_for_code.csv**
6. **kdemeth Microcosms 2022 8.9.25_for_code.csv**

Descriptions of each file:

1. **ANOVA for 2020 Microcosms 8.26.25.R** provides code to conduct an analysis of variance (ANOVA) on potential mercury methylation rate constants (k_{meth}; Equation 1) from microcosm experiments conducted in October 2020. The script also contains code to produce a boxplot figure to visualize the k_{meth} values (Figure 1). The experiments investigated k_{meth} in native sediment collected at two sites, Beach Zone and East Fork , within the University of California Natural Reserve System's Younger Lagoon Reserve. The ANOVA was used to examine whether statistically significant effects relative to the 48-hour control existed when amendments were added (Table 1). The identity and the concentrations of amendments used are given below (Table 2). A summary of mean k_{meth} and standard deviation by site and treatment are provided (Table 3). Table 4 indicates the column headers of both the Beach Zone and East Fork data frames and provides a description of the contents of each cell.

2. **East Fork kmeth Data 2020 8.25.25.csv** contains calculated k_{meth} values from East Fork sediment microcosms and is meant to be read into the script called ANOVA for 2020 Microcosms 8.26.25.R.
3. **Beach Zone kmeth Data 2020 8.25.25.**csv contains calculated $_{kmeth}$ values from Beach Zone sediment microcosms and is meant to be read into the script called ANOVA for 2020 Microcosms 8.26.25.R.
4. **ANOVA for 2022 Microcosms 8.10.25.R** provides code to conduct an analysis of variance (ANOVA) on potential mercury methylation rate constants (k_{meth}; Equation 2) and potential methylmercury demethylation rate constants (k_{demeth}; Equation 3) from microcosm experiments conducted in October 2022. The script contains the code to calculate the k_{demeth} values while the k_{meth} values are already calculated prior to the data being added to **kmeth Microcosms 2022 8.10.25_for_code.csv.** The 2022 microcosms were follow up experiments to those conducted in 2020. The 2022 microcosms included sediment sampled from Beach Zone and focused on the use of two concentrations of nitrate amendments and a $Me^{198}Hg$ tracer to determine k_{demeth} in addition to the $^{200}Hg(II)$ tracer used for determining k_{meth}. The script also contains code to produce a boxplot figure to visualize the k_{meth} and k_{demeth} values (Figure 2). The ANOVA was used to examine whether statistically significant effects relative to the 48-hour control existed when amendments were added (Table 5). Tukey's Honest Significant Differences (HSD) was used as a post hoc test (Table 6). The identity and the concentrations of amendments used are given in Table 7. A

summary of mean k_{meth} and k_{demeth} and standard deviations by treatment at Beach Zone are provided (Table 8).

5. **kmeth Microcosms 2022 8.10.25_for_code.csv** contains calculated k_{meth} values from Beach Zone sediment microcosms and is meant to be read into the script called **ANOVA for 2022 Microcosms 8.10.25.R**. Table 9 provides a description of the column header labels and cell contents of the k_{meth} data frame.
6. **kdemeth Microcosms 2022 8.9.25_for_code.csv** contains $Me^{198}Hg$ concentration data for calculating k_{demeth} values from Beach Zone sediment microcosms and is meant to be read into the script called **ANOVA for 2022 Microcosms 8.10.25.R**. Table 10 provides a description of the column header labels and cell contents of the k_{demeth} data frame.

Table 1. Results of Analysis of Variance (ANOVA) for 2020 Microcosm k_{meth} at BZ & EF

Location	Test	Test Statistic	Degrees of freedom	p-value
Beach Zone	ANOVA	3.8 (F)	4, 9	0.04*
East Fork	ANOVA	3.7 (F)	4, 10	0.04*

*While the p-values of analyses at either location indicate statistically significant differences ($\alpha = 0.05$), none were detected with Tukey's Honest Significant Difference post-hoc tests. Results of individual contrasts shown within script comments.

Table 2. Location & Concentrations of Amendments for October 2020 Microcosms

Treatment	Concentration at EF (mM)	Concentration at BZ (mM)
Molybdate	39	37
Nitrate	117	106
Ammonium	107	99
Nitrate + Molybdate	107, 39	106, 37
48 hour control	Overlying water only	Overlying water only

*When two numbers are given, the first refers to the nitrate concentration and the second to the molybdate concentration.

Table 3. k_{meth} from EF and BZ Averaged by Treatment Group for 2020 Microcosms

Treatment Group	Location	k_{meth} Group Mean (% per day)	Standard Deviation (% per day)	Number of replicates
Control	Beach Zone	1.0	0.79	3
Nitrate	Beach Zone	2.4	1.2	3
Ammonium	Beach Zone	1.7	1.4	3
Molybdate	Beach Zone	0.21	0.11	2*
Molybdate + Nitrate	Beach Zone	0.27	0.10	3
Control	East Fork	0.23	0.11	3
Nitrate	East Fork	0.21	0.01	3
Ammonium	East Fork	0.24	0.10	3
Molybdate	East Fork	0.08	0.06	3
Molybdate + Nitrate	East Fork	0.09	0.02	3

*One outlier was removed after Dixon's Q-test.

Table 4. Description of Contents of Data Frames for 2020 Microcosms

Column header label	**group**	**pg.per.gram**	**kmeth.per.day**	**kmeth.percent.per.day**
Description of the cell contents	Treatment or control group label.	Picograms of 202MeHg from 202Hg(II) spike divided by the mass of dry sediment distilled.	Potential Hg methylation rate constant as calculated by the equation given in section **Equation 1.**	Values in the kmeth.per.day column multiplied by 100.

Equation 1. The following equation was used to calculate potential Hg methylation rate constants (k_{meth}) for 2020 microcosms with units of percent per day:

$$k_{meth} = (-\ln(1-[Me^{202}Hg]_{48})/[^{202}Hg]_0)/time)$$

$[Me^{202}Hg]_{48}$ is the amount of excess $Me^{202}Hg$ measured in the sample of microcosm sediment in pg/g after 48 hours. Excess refers to $Me^{202}Hg$ generated from the spike of $^{202}Hg(II)$ added as an isotopically-enriched tracer which is resolved with matrix algebra from the background (ambient) pool of $Me^{202}Hg$. $[^{202}Hg]_0$ is the amount of $^{202}Hg(II)$ spiked into the microcosm in pg/g dry sediment and time is the length of incubation (2 days). The

^{202}Hg(II) concentration was 4460 pg ^{202}Hg(II)/g dry sediment at BZ and 8420 pg ^{202}Hg(II)/g at East Fork. Multiplying by 100 gives units of percent day^{-1}.

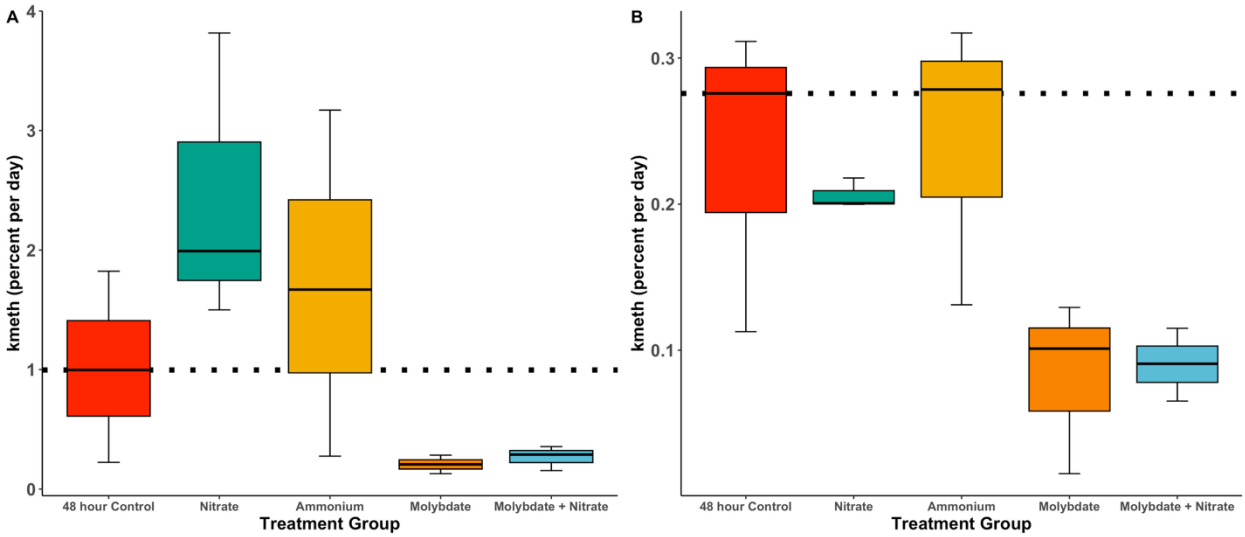

Figure 1. Boxplots of k_{meth} at Beach Zone (A) and East Fork (B) from microcosm experiments amended with nitrate, ammonium, molybdate, and molybdate plus nitrate in October 2020. Median k_{meth} value is depicted as a black line within each box. Whisker edges portray the data's maximum and minimum for a given condition. No statistically significant differences were found relative to the 48-hour control for either site. Note the difference in scale of the y-axis between locations, indicating higher potential Hg methylation rate constants at Beach Zone than East Fork. Horizontal black dotted lines represent the median of each 48-hour control and are included for reference.

Table 5. ANOVA results for October 2022 k_{meth} and k_{demeth} values

Rate Constants Evaluated	Location	Test	Test Statistic	n	Degrees of freedom	p-value
k_{meth}	Beach Zone	ANOVA	1.3 (F)	13	2,10	0.3
k_{demeth}	Beach Zone	ANOVA	8.0 (F)	13	2,10	0.008*

*Post hoc testing was conducted for k_{demeth} values only as the p-value of the omnibus test was significant for k_{demeth} but not for k_{meth}.

Table 6. 2022 Microcosm k_{demeth} Tukey's Honest Significant Differences p-values

Site	Post-hoc Test	Comparison	p-value
Beach Zone	Tukey's HSD after ANOVA	Low Nitrate-Control	0.035
Beach Zone	Tukey's HSD after ANOVA	High Nitrate-Control	0.010
Beach Zone	Tukey's HSD after ANOVA	High Nitrate-Low Nitrate	0.96

Table 7. Concentrations of Nitrate Amendments for Beach Zone 2022 Microcosms

Treatment	Concentration within BZ Porewater
Low Nitrate (micromolar)	337
High Nitrate (millimolar)	106
48 hour control	Overlying water only
Instantaneous Control	Overlying water only

Table 8. k_{meth} and k_{demeth} averaged by treatment group for Beach Zone 2022 microcosms

Treatment Group	k_{meth} Group Mean (% per day)	Standard Deviation (% per day)	k_{demeth} Group Mean (% per day)	Standard Deviation (% per day)	Number of replicates
48 hour control	4.1	1.7	32	11	5
Low Nitrate (micromolar)	3.7	0.59	15	2.1	3
High Nitrate (millimolar)	3.0	0.35	13	5.1	5

Equation 2. The following equation was used to calculate potential Hg methylation rate constants (k_{meth}) for 2022 microcosms with units of percent per day:

$$k_{meth} = (-\ln(1-[Me^{200}Hg]_{48})/[^{200}Hg]_0)/time)$$

$[Me^{200}Hg]_{48}$ is the amount of excess $Me^{202}Hg$ measured in the sample of microcosm sediment in ng/g after 48 hours. Excess refers to $Me^{200}Hg$ generated from the spike of $^{200}Hg(II)$ added as an isotopically-enriched tracer which is resolved with matrix algebra

from the background (ambient) pool of $Me^{200}Hg$. $[^{200}Hg]_0$ is the amount of $^{200}Hg(II)$ spiked into the microcosm in ng/g dry sediment and time is the length of incubation (2 days). The mean average $^{200}Hg(II)$ concentration at Beach Zone was 5.5 +/-0.1 ng $^{200}Hg(II)$/g dry sediment. Multiplying by 100 gives units of percent day^{-1}.

Equation 3. Potential demethylation rate constants (k_{demeth}) were calculated from the following equation for 2022 microcosms:

$$k_{demeth} = (-\ln([Me^{198}Hg]_{48}/[Me^{198}Hg]_0)/time)*100$$

$[Me^{198}Hg]_{48}$ is the amount of excess $Me^{198}Hg$ measured in the sample of microcosm sediment in ng/g after 48 hours. Excess refers to $Me^{198}Hg$ from the added $Me^{198}Hg$ isotopically-enriched tracer which is resolved with matrix algebra from the background (ambient) pool of $Me^{198}Hg$. $[Me^{198}Hg]_0$ is the mean amount of excess $Me^{198}Hg$ measured in instantaneous control (IC) microcosms (n=5) in ng/g. IC microcosms were created under the same conditions as the 48-hour control, but were frozen immediately upon creation. The mean concentration of $Me^{198}Hg$ in IC microcosms was 1.1 +/-0.17 ng/g dry sediment. Time is the length of incubation (2 days). Multiplying by 100 gives units of percent day^{-1}.

Table 9. Description of Contents of Data Frame for 2022 Microcosms kmeth values

Column header label	Sample ID	Treatment	kmeth	percent.per.day
Description of the cell contents	ID of microcosm from which sediment was distilled.	Treatment or control group label.	Potential Hg methylation rate constant as calculated by the equation given in section **Equation 2.**	Values in the kmeth column multiplied by 100 to give rate constants in units of per day.

Table 10. Description of Contents of Data Frame for 2022 Microcosms kdemeth values

Column header label	Sample ID	Treatment	198MeHg.ng.g
Description of the cell contents	ID of microcosm from which sediment was distilled.	Treatment or control group label.	An average of analytical replicates (n=2 or 3) of excess $Me^{198}Hg$ in nanograms per gram dry sediment. To be read into the script for calculating kdemeth based on **Equation 3.**

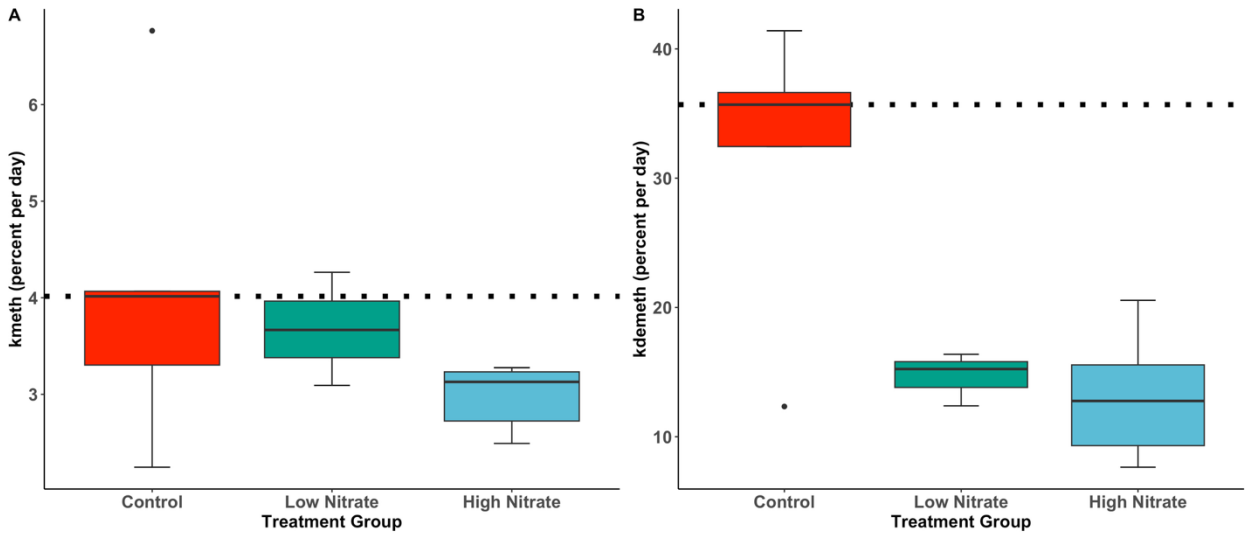

Figure 2. Boxplots of kmeth (A) and kdemeth (B) from microcosm experiments at Beach Zone amended with nitrate in October 2022. Low nitrate treatment and high nitrate treatments correspond to nitrate concentrations of 337 µM and 106 mM, respectively. No statistically significant difference was observed in methylation potential (kmeth) with nitrate treatment, though demethylation potential (kdemeth) was significantly suppressed by either nitrate amendment. Black dashed line indicates the median kmeth or kdemeth of the 48-hour control for each assay.

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