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1 **Molybdate inhibits mercury methylation capacity of *Pseudodesulfovibrio hydrargyri* BerOc1 regardless of**  
2 **the growth metabolism**

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13

14 **Abbreviations:** sulfate-reducing bacteria, SRB; mercury, Hg; inorganic mercury, Hg(II); monomethylmercury,

15 MeHg; sulfate adenylyltransferase, Sat enzyme; adenosine 5'-phosphosulfate, APS; Ct: Cycle threshold.

16

17 **Abstract**

18 Molybdate inhibits sulfate respiration in sulfate-reducing bacteria (SRB). It is used as inhibitor to indirectly  
19 evaluate the role of SRB in mercury methylation in the environment. Here, the SRB *Pseudodesulfovibrio*  
20 *hydrargyri* BerOc1 was used to assess the effect of molybdate on cell growth and mercury methylation under  
21 various metabolic conditions. *Geobacter sulfurreducens* PCA was used the non-SRB counterpart strain with the  
22 ability to methylate mercury. While PCA growth and methylation are not affected by molybdate, 1 mM of  
23 molybdate inhibits BerOc1 growth under sulfate respiration (50% inhibition) but also under fumarate respiration  
24 (complete inhibition). Even more surprising, mercury methylation of BerOc1 is totally inhibited at 0.1 mM of  
25 molybdate when grown under sulfate or fumarate respiration with pyruvate as electron donor. As molybdate is  
26 expected to reduce cellular ATP level, the lower Hg methylation observed with pyruvate could be the consequence  
27 of lower energy production. Although molybdate alters the expression of *hgcA* (mercury methylation marker) and  
28 *sat* (involved in sulfate reduction and molybdate sensitivity) in a metabolism-dependent manner, no relationship  
29 with mercury methylation rates could be found. Our results show, for the first time, a specific mercury methylation  
30 inhibition by molybdate in SRB.

31

32 **Keywords:** Sulfate respiration; fumarate respiration; sulfate-reducing bacteria; methylmercury methylation;  
33 molybdate inhibition; mercury methylation.

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## 36 **Introduction**

37 Mercury (Hg) is a toxic metallic element that poses significant environmental and public health concerns. While  
38 all forms of mercury are considered hazardous, methylated forms, such as monomethylmercury (MeHg), are the  
39 most alarming (Hong Young-Seoub, 2012). MeHg is neurotoxic, primarily produced by microorganisms. This  
40 organic form of Hg is bio-accumulated and bio-amplified in food webs putting at risk humans and wildlife (Chen  
41 et al., 2008; Ma et al., 2019). The predicted membrane-attached methyltransferase HgcA and the ferredoxin-like  
42 protein HgcB are so far the only identified proteins implicated in the transformation of inorganic mercury Hg(II)  
43 into MeHg (Cooper et al., 2020; Parks et al., 2013; Smith et al., 2015). HgcA is thought to transfer the methyl  
44 group to Hg(II) via its corrinoid cofactor, while HgcB acts as an electron donor via its Fe-S cluster to re-activate  
45 the catalytic cycle. *hgcAB* cluster is currently used as a bioindicator of Hg methylation in a large number of  
46 ecological studies (e.g., Bouchet et al., 2018; Podar et al., 2015; Vigneron et al., 2021; Villar et al., 2020).

47 Hg methylation process is associated with the activity of iron reducing bacteria and methanogens (Bravo et al.,  
48 2018; Gilmour et al., 2018), along with the activity of sulfate reducing bacteria (SRB). The Hg methylation activity  
49 of the SRB group has been extensively studied (e.g., Gilmour et al., 1992; Goñi-Urriza et al., 2020; King et al.,  
50 2000; Ranchou-Peyruse et al., 2009). The SRB belongs to the group of sulfate reducing prokaryotes capable of  
51 utilizing sulfate as terminal electron acceptor in order to perform the dissimilatory reduction of sulfate to sulfide  
52 (Barton and Fauque, 2009), the so called sulfate respiration. The sulfate respiration is initiated by the ATP-  
53 dependent activation of sulfate via the sulfate adenylyltransferase (ATP sulfurylase or Sat enzyme), to generate  
54 adenosine 5'-phosphosulfate (APS) and inorganic pyrophosphate (Keller and Wall, 2011). After the activation,  
55 APS is reduced to bisulfite with the APS reductase AprBA (Meyer and Kuever, 2008). The produced bisulfite is  
56 further reduced to (bi)sulfide by the dissimilatory (bi)sulfite reductase DsrAB (Ferreira et al., 2022; Oliveira et al.,  
57 2008).

58 Oxyanion analogs of sulfate, such as molybdate ( $\text{MoO}_4^{2-}$ ), tungstate, selenate, chromate, arsenate, etc, are selective  
59 inhibitors of sulfate reduction activity (Carlson et al., 2021; Taylor and Oremland, 1979). Molybdate, for instance,  
60 has been widely used as a specific inhibitor of sulfate respiration in different physiological (e.g., Biswas et al.,  
61 2009; Tucker et al., 1997) and ecological studies (e.g., Bouchet et al., 2018; Kleikemper et al., 2002; Lee et al.,  
62 2014). Studies on selected SRB strains suggested that the effect of molybdate is partially due to the inhibition of  
63 cellular sulfate transport (Newport and Nedwell, 1988). However, since molybdate did not affect sulfate  
64 accumulation (Cypionka, 1989), it is unclear whether molybdate inhibits sulfate transport. The inhibition of the  
65 sulfate-respiring organisms by molybdate, and other oxyanions of the group VI elements, is also thought to be

66 linked to an intracellular decrease of ATP level (Taylor and Oremland, 1979). The Sat enzyme is thought to be  
67 one of the targets in this inhibition, since Sat catalyzes in the presence of molybdate the formation of an unstable  
68 APS analog (adenosine 5'-molybdophosphate), leading to a fast ATP hydrolysis and a non-productive ATP  
69 cycling. However, a  $\Delta sat$  mutant of *Desulfovibrio vulgaris* Hildenborough remained sensitive to molybdate (Zane  
70 et al., 2020). Notwithstanding, strain lacking both Sat and a newly identified YcaO-like protein become resistant  
71 to molybdate (Zane et al., 2020). Although the mode of action of YcaO-like protein in the SRB-inhibition by  
72 molybdate remains unknown, the authors hypothesized that it is similar to that of the Sat enzyme (Zane et al.,  
73 2020).

74 The inhibition of the sulfate reduction by molybdate is widely used to evaluate the ecological role of SRB in the  
75 Hg methylation process in ecosystems (e.g., Bouchet et al., 2018; Fleming et al., 2006; Gentès et al., 2013; Hines  
76 et al., 2012). However, SRB are metabolically versatile, and may shift from one metabolism to other in the presence  
77 of molybdate, in order for microorganisms to remain active. Interestingly, previous study conducted by our group  
78 in the Lake Titicaca hydrosystem (Bolivian Altiplano) showed that sulfate reducers were still active in biofilm and  
79 periphyton upon the addition of molybdate, while the Hg methylation process was inhibited by more than 90%  
80 (Bouchet et al., 2018). The sulfate reducers remained active in the presence of molybdate, probably because they  
81 switched from sulfate respiration to another growth metabolism, as suggested by the observed absence of H<sub>2</sub>S  
82 production (Bouchet et al., 2018). The effect of molybdate on Hg methylation in this study is perplexing, especially  
83 considering that Hg methylation potential in SRBs has been found to be higher under non-sulfidogenic growth  
84 conditions than under sulfidogenic growth conditions in cell culture studies (Barrouilhet et al., 2022; Compeau  
85 and Bartha, 1985) as well as in ecological studies (Compeau Geoffrey C. and Bartha Richard, 1987; Gilmour and  
86 Henry, 1991). Indeed, under non-sulfidogenic growth, Hg is expected to be more bioavailable for methylation  
87 (Benoit J. M. et al., 2001; Benoit et al., 2001). Moreover, molybdate can be complexed to sulfides in a sulfide-rich  
88 environment (i.e. sulfate respiration) (Biswas et al., 2009; Tucker et al., 1997). Consequently, the rate of mercury  
89 methylation may vary depending on whether molybdate is utilized under sulfidogenic growth conditions as  
90 opposed to non-sulfidogenic growth conditions, such as fumarate respiration. The molybdate-induced inhibition  
91 of Hg methylation in a microbial community where sulfate reducers are still active (Bouchet et al., 2018) is not  
92 fully understood. In such a complex structure, it is certainly difficult to tease apart the mode of action of molybdate,  
93 especially whether it directly or indirectly affects the growth mode of SRB or their Hg methylating activities.  
94 Likewise, molybdate induced-inhibition of Hg methylation of non-sulfate reducers in such a complex environment  
95 is difficult to decipher.

96 In this work, the SRB and the Hg methylating model bacterium, *Pseudodesulfovibrio hydrargyri* BerOc1 was used,  
97 to assess the impact of molybdate on cell growth and the Hg methylation process in three different growth  
98 conditions, including sulfidogenic growth conditions (two under sulfate respiration) and non-sulfidogenic growth  
99 (fumarate respiration). To further understand the mechanisms of molybdate inhibition, the expression level of *hgcA*  
100 gene involved in Hg methylation (Parks et al., 2013), *sat* and *ycaO* genes involved in molybdate-sensitivity (Zane  
101 et al., 2020) were investigated when molybdate inhibition was induced. Additionally, the Non-SRB and the iron-  
102 reducer *Geobacter sulfurreducens* PCA, which is also capable of Hg methylation, was employed to evaluate  
103 whether the specific effect of molybdate on Hg methylation could be extended to non-SRB strains.

## 104 **Experimental procedures**

### 105 *Strains and culture conditions*

106 Two Hg-methylating strains were used in this study; the sulfate reducing bacterium *P. hydrargyri* BerOc1  
107 (Ranchou-Peyruse et al., 2018) and the iron reducing bacterium, *G. sulfurreducens* PCA (Lin et al., 2014). *P.*  
108 *hydrargyri* BerOc1 is a model organism for studying the mechanisms of mercury methylation at the cell level  
109 (Bakour et al., 2023; Barrouilhet et al., 2022; Goñi-Urriza et al., 2015; Isaure et al., 2020). This bacterium can use  
110 lactate and pyruvate as electron donor for sulfate respiration (Ranchou-Peyruse et al., 2018) and pyruvate as  
111 electron donor for fumarate respiration (Barrouilhet et al., 2022). *G. sulfurreducens* PCA is capable of using acetate  
112 as electron donor in fumarate respiration (Caccavo F et al., 1994). Strains were cultivated under anoxic conditions  
113 in a common minimal medium (adapted from DSMZ), supplemented with different substrates in respect to their  
114 growth conditions. The medium contained the following (in g.L<sup>-1</sup>, unless otherwise indicated): NH<sub>4</sub>Cl, 0.25; NaCl,  
115 10; KH<sub>2</sub>PO<sub>4</sub>, 0.2; MgSO<sub>4</sub>, 0.4; MgCl<sub>2</sub>, 0.4; CaCl<sub>2</sub>·2H<sub>2</sub>O, 0.1; KCl, 0.5; Hepes, 10 mM; trace element SL12 (1  
116 mL.L<sup>-1</sup>; composed of (in g.L<sup>-1</sup>): H<sub>3</sub>BO<sub>3</sub>, 0.3; FeSO<sub>4</sub>·7H<sub>2</sub>O, 1.1; CoCl<sub>2</sub>·6H<sub>2</sub>O, 0.19; MnCl<sub>2</sub>·2H<sub>2</sub>O, 0.05; ZnCl<sub>2</sub>,  
117 0.042; NiCl<sub>2</sub>·6H<sub>2</sub>O, 0.024; Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O, 0.018; and CuCl<sub>2</sub>·2H<sub>2</sub>O, 0.002 (modified from (Overmann et al.,  
118 1992)), and 1 mL.L<sup>-1</sup> selenite/tungstate solution (composed of NaOH, 0.5 g; Na<sub>2</sub>SeO<sub>3</sub>, 2 mg and Na<sub>2</sub>WO<sub>4</sub>·2H<sub>2</sub>O,  
119 4 mg for 1 liter of water at pH 7.0). After autoclaving, the culture media were flushed with N<sub>2</sub> gas and supplemented  
120 with 1 mL.L<sup>-1</sup> of filtered V7 vitamin solution (Pfennig and Trüper, 1992) containing (in mg.L<sup>-1</sup>) biotin, 2; para-  
121 aminobenzoate, 10; thiamin, 10; pantothenate, 5; pyridoxamin, 50; cobalamin, 20 and nicotinate, 20. *P. hydrargyri*  
122 BerOc1 was grown at 37°C on either sulfate or fumarate respiration. For the sulfate reducing growth condition,  
123 the medium was supplemented with either Na-sulfate (40 mM) and Na-pyruvate (40 mM) or with Na-sulfate (40  
124 mM) and Na-lactate (40 mM) as substrates. For the fumarate respiration condition, the medium was supplemented

125 with Na-fumarate (40 mM) and Na-pyruvate (40 mM). *G. sulfurreducens* PCA was grown at 30°C on fumarate  
126 respiration in medium supplemented with Na-acetate (10 mM) and Na-fumarate (50 mM) as substrates.

127 For all experiments described below, the strains were cultivated for 24h in sulfate respiration and 48h in fumarate  
128 respiration. Once grown, the cultures were opened in the anaerobic chamber and diluted two times by adding fresh  
129 culture medium and spiked with 0.05 µM of Hg(II) (from HgCl<sub>2</sub>) or 0.0047 µM of MeHg (from CH<sub>3</sub>HgCl). As  
130 shown previously, no toxic effect was observed for *P. hydrargyri* BerOc1 (Isaure et al., 2020) and PCA (Wang et  
131 al., 2013) at these Hg concentrations. Then, 10 ml of culture were distributed in 10 ml Bellco tubes containing the  
132 appropriate concentrations of molybdate (Na-MoO<sub>4</sub>) (ranging from 0 to 2 mM for *P. hydrargyri* BerOc1 and from  
133 0 to 10 mM for *G. sulfurreducens* PCA) and were sealed with Teflon stoppers. Cells were collected for initial time  
134 measurements (T<sub>0</sub>) and stored until analysis, as described below. Then, tubes were incubated for 10h for *P.*  
135 *hydrargyri* BerOc1 in sulfate reducing conditions and 24h in fumarate respiration for both *P. hydrargyri* BerOc1  
136 and *G. sulfurreducens* PCA. Cell growth was monitored following optical density (OD) at 600 nm until the end of  
137 the growth. Experiments were performed in triplicates. For each molybdate concentration, a control was performed  
138 without HgCl<sub>2</sub>. At the end of the experiments, tubes were homogenized and samples were collected for final time  
139 measurements (T<sub>f</sub>) and stored for each analysis (MeHg and Hg(II) production, quantification of cell number,  
140 metabolite productions and gene expressions), as described below.

#### 141 *Quantification of bacterial cell numbers*

142 The cell numbers in each culture were determined by flow cytometry. Briefly, 40 µl of filtered formaldehyde  
143 (38%) were added to 800 µl of cultures at T<sub>0</sub> and T<sub>f</sub>, and stored at -80°C, until needed. Defrosted samples were  
144 labelled with Sybr Green 10X (Invitrogen) in a dark incubation during 15 min, and counted using Trucounts beads  
145 (BD), then injected in the cytometer (BD Accuri C6 cytometer, TBMCore) with Trucounts beads (BD) as internal  
146 standard. The minimal medium used was also injected in the cytometer to determine background noises and  
147 subtract it from the sample measurements.

#### 148 *Measurement of MeHg and Hg(II) productions*

149 Similar methods were performed to measure MeHg/Hg(II) production. To measure either the MeHg or Hg(II)  
150 produced in the samples, 500 µl of cell culture from each sample were collected and added to 500 µl of nitric acid  
151 6N at T<sub>0</sub> and T<sub>f</sub>. The MeHg/Hg(II) produced was detected using gas chromatography coupled to inductively  
152 plasma mass spectrometry (GC-ICP-MS) (Monperrus et al., 2008). A derivatization of the samples was performed  
153 to produce species of volatile ethylated Hg, easily separated via gas chromatography. Samples were then

154 transferred into vials containing 5 ml of water and 5 ml of acetic acid/acetate 0.1 M pH 3.9 buffer. Species specific  
155 isotope dilution analysis was used to accurately quantify Hg(II) and MeHg in culture medium. To this end,  
156 appropriate concentrations of Hg isotopes ( $^{199}\text{HgII}$  and  $^{201}\text{MeHg}$ ) were added to obtain an isotopic ratio close to 1  
157 and the pH was adjusted between 3.85 and 4.05. After adjusting the pH, 500  $\mu\text{l}$  of isooctane and 80  $\mu\text{l}$  of  
158 tetraethylborate were added and samples were subjected to constant shaking for 20 min. The organic phase was  
159 collected and injected in the GC-ICP-MS. Chromatograms obtained were treated with the software PlasmaLab. To  
160 calculate the MeHg/Hg(II) produced by strains, MeHg/Hg(II) concentrations measured at the initial time were  
161 subtracted from the concentrations obtained at the final time. The detection limit of Hg compounds for GC-ICP-  
162 MS analytical method were 0.01 ng/g and 0.042 ng/g for iHg and MeHg, respectively. In all the tested conditions,  
163 the average concentration of MeHg found at the end of the incubations in the controls without Hg(II) were 0.05  
164 +/- 0.01 ng/g. The percentage of recovery of mercury species was 75.08% +/- 10.86% in average.

#### 165 *Measurement of electron donors/acceptors and their produced metabolites*

166 The consumption of electron donor and acceptor and the metabolite produced during the growth of *P. hydrargyri*  
167 BerOc1 grown in the presence of 0, 0.01 and 0.1 mM of molybdate, were analyzed using ion chromatography.  
168 Calibration curve was performed with sulfate, pyruvate, lactate, fumarate, acetate and succinate at concentration  
169 values of 0, 2, 4, 6, and 10 mg/L. 500  $\mu\text{l}$  of cell culture from each sample were collected at T0 and Tf, filtered  
170 using syringe filters IC Millex® (0.20  $\mu\text{m}$ ) and stored at 4°C, until analysis. Samples were diluted 1000 times to  
171 obtain a salt concentration of 10 mg.L<sup>-1</sup>. The liquid phase was analyzed on a Dionex ICS-1100 ion chromatography  
172 (ThermoFisher Scientific) equipped with a column Dionex ionpac AS11 HC with a pre-column Dionex AG11-HC  
173 used to remove metal contamination from samples and a DS6 heated conductivity detector (ThermoFisher  
174 Scientific). 125  $\mu\text{l}$  of samples were injected. KOH was the eluent used with a gradient from 1 mM to 30 mM during  
175 the passage of the sample. Chromeleon program was used to analyze the data.

176 For sulfides quantification, 100  $\mu\text{l}$  of cell cultures from each sample were added to 400  $\mu\text{l}$  of 2% zinc acetate at  
177 T0 and Tf to trap the sulfides produced. Sulfide concentrations were quantified by a colorimetric method as  
178 describe by Cline (CLINE, 1969). A standard curve was carried out with a sulfide solution, and along with the  
179 samples, were quantified by the addition of 150  $\mu\text{l}$  of DMPD (N,N-dimethylparaphenylenediamine sulfate) at 0.2%  
180 (m/v) and 10  $\mu\text{l}$  of iron chloride at 10% (m/v). Samples were incubated 20 min at room temperature in the dark.  
181 The absorbance was measured at 670 nm in a microplate reader (Synergy HTX multi-mode reader (BioTek)).

#### 182 *Quantification of gene expression level by qRT-PCR*



183 To study the impact of molybdate on the expression of *hgcA*, *sat* and *ycaO* genes, *P. hydrargyri* BerOc1 strain  
184 was grown as described above, then the cell cultures (grown as previously described) were exposed to 100 ppb of  
185 HgII and different concentrations of molybdate (0, 0.01 and 0.1 mM). After 1h, the OD<sub>600</sub> was measured and 5 ml  
186 of culture were collected. RNA protect (sodium acetate-saturated phenol: acetate (1M) pH 5.5 in 5% of saturated  
187 phenol and 95% ethanol) was added to each sample (at 0.6 of OD<sub>600</sub>) at 1.25:10 ratio. Samples were vortexed and  
188 incubated 5 min at room temperature, then centrifuged 10 min at 8000 g at 4°C. Pellets were stored at -80°C, until  
189 RNA extraction. RNA extractions were performed using EXTRACT-ALL® Kit (Eurobio) according to the kit  
190 protocol guidelines. DNA contaminations were removed using TURBO DNase RNase-free™ Kit (Ambion).  
191 Purified RNA was quantified with Quant-iT RNA Assay Kit (Invitrogen™). RNA was diluted to obtain a  
192 concentration of 20 ng.µl<sup>-1</sup>. cDNA was synthesized using M-MLV-RT (Invitrogen). Expression of *hgcA*, *sat* and  
193 *ycaO* genes, were quantified by quantitative Reverse-transcription PCR (qRT-PCR) in a CFX Connect Real-Time  
194 PCR Detection System using the DyNAmo™ ColorFlash SYBR® Green qPCR Kit (Finnzyme). 1 µl of cDNA  
195 was added with the appropriate primers in a final volume of 20 µl at 0.1 µM for *sat* and *ycaO* and 0.4 µM for  
196 *hgcA*. Primers used 1) for *hgcA* gene were peg.1401(*hgcA*)\_DNDF1 (GTGCGGCTTCAAAGTGATCT) and  
197 peg.1401(*hgcA*)\_DNDR1 (GAATATCTCCACCGGGATGA), 2) for *sat* gene were BerOc1\_sat\_F  
198 (CTACTGCTACAAGTGCGACG) and BerOc1\_sat\_R (TGCATCTTGACCTCGACCTT) and 3) for *ycaO* gene,  
199 were BerOc1\_YcaO\_F (AAGGATTTCTCGCTGGGCTA) and BerOc1\_YcaO\_R  
200 (TCTGGTCAAGCTCGGTGAAT). The housekeeping gene *gyrB* was used for normalization (Goñi-Urriza et al.,  
201 2015) with *gyrBF1* (CCTCGAAGAAGGTGTTTCAGC) and *gyrBR1* (CCAAGAAGCTGGTCCAGAAG)  
202 primers. Real time PCR was carried out with an initial denaturation of 7 min at 95°C, followed by 45 cycles of 10  
203 sec at 95°C, and 20 sec at 60 °C. Double delta Ct (Cycle Threshold) procedure using *gyrB* expression as reference  
204 was used to determine gene's induction level. PCR efficiency using each primer's pairs varied between 97% and  
205 104%.

## 206 *Statistical analysis*

207 The non-parametric Kruskal-Wallis test and the Dunn test for pairwise comparisons were performed using R  
208 software.

## 209 **Results**

210 *Effects of molybdate on the growth of P. hydrargyri BerOc1 and G. sulfurreducens PCA*

211 The effect of molybdate on cell growth was evaluated in the sulfate-reducing bacterium, the SRB *P. hydrargyri*  
212 BerOc1, which is capable of Hg methylation (Ranchou-Peyruse et al., 2018). The non-sulfate reducing bacterium,  
213 *G. sulfurreducens* PCA, which is also capable of Hg methylation (Lin et al., 2014), was used to evaluate the effect  
214 of molybdate in a non-SRB strain (Fig. 1, Table 1 and Table S1). *P. hydrargyri* BerOc1 was grown in sulfate  
215 reducing metabolism in the presence of i) sulfate and pyruvate or ii) sulfate and lactate as substrates or in fumarate  
216 respiration with iii) fumarate and pyruvate as substrates. *G. sulfurreducens* PCA was grown in fumarate respiration  
217 in the presence of fumarate and acetate as substrates. As shown in Figure 1, 0.01 mM of molybdate has almost no  
218 effect on the biomass production of *P. hydrargyri* BerOc1 in all the tested conditions (Kruskal-Wallis test,  $p >$   
219 0.05). However, starting from 0.05 mM of molybdate, the biomass production of this bacterium decreases with the  
220 increase of molybdate concentrations in sulfate respiration as well as in fumarate respiration. Surprisingly, the  
221 biomass production of *P. hydrargyri* BerOc1 is reduced to less than 50% at 0.1 mM molybdate, as compared to 0  
222 mM, in fumarate respiration (Fig. 1, inset). The growth is then completely inhibited at 1 mM in fumarate respiration  
223 (Dunn test  $p = 0.0135$ ), while complete inhibition of biomass production is observed in sulfate-reducing  
224 metabolisms at 2 mM. The differences are statistically significant compared to 0 mM molybdate for both lactate  
225 and pyruvate as electron donors ( $p < 0.05$ , Kruskal-Wallis test). For all the concentrations tested, molybdate has no  
226 impact on the biomass production of *G. sulfurreducens* PCA (Fig. 1), nor on the growth rate, even up to 10 mM  
227 (Table S1).

#### 228 *Molybdate inhibits Hg(II) methylation in P. hydrargyri BerOc1*

229 The Hg methylating capacity of *P. hydrargyri* BerOc1 and *G. sulfurreducens* PCA, was evaluated in the presence  
230 of molybdate. The MeHg production was reported in ng of MeHg produced/cell (Fig. 2 and table S2).  
231 Interestingly, Hg(II) methylation is inhibited in the SRB *P. hydrargyri* BerOc1 grown in some of the tested  
232 conditions upon addition of molybdate, with a more pronounced difference at 0.05 mM of molybdate ( $p < 0.05$ ,  
233 Kruskal-Wallis test). Although the cell growth is still observed at 0.1 mM of molybdate in fumarate respiration  
234 (Fig.1 inset), the production of MeHg is completely inhibited at this concentration (Fig. 2, inset). Similar results  
235 are observed for *P. hydrargyri* BerOc1 cells grown in sulfate respiration in the presence of pyruvate as electron  
236 donor. The production of MeHg is much less inhibited by molybdate under the sulfate-reducing condition with  
237 lactate as the electron donor (Fig. 2). However, the molybdate-inhibition in the latter condition becomes complete  
238 when molybdate concentration reaches 2 mM, a concentration at which no bacterial growth is observed. For the  
239 iron-reducing bacterium *G. sulfurreducens*, molybdate does not affect the production of MeHg even up to 10 mM  
240 of molybdate (Fig. 2, table S2). The MeHg produced/cell represents the mean of all cellular states and account for

241 the viable as well as the non-viable cells, that can partially exist across all the growth conditions. The rate of MeHg  
242 production have been calculated and showed the same trends as the MeHg produced/cell (Table S2).

243 To investigate whether molybdate inhibition of MeHg production is directly due to a decrease of Hg(II)  
244 methylation or indirectly due to an increase of MeHg demethylation, the impact of molybdate on MeHg  
245 demethylation was also investigated in *P. hydrargyri* BerOc1 (Table S2). Similarly to the MeHg production  
246 experiments, the production of Hg(II) was quantified after the exposition of the cells to increasing molybdate  
247 concentrations as well as to 0.0047  $\mu\text{M}$  of MeHg (not toxic for *P. hydrargyri* BerOc1 (Isaure et al., 2020)). No  
248 relationship between molybdate concentration and MeHg demethylation could be found (Table S2). These results  
249 showed that molybdate inhibition of Hg(II) methylation in *P. hydrargyri* BerOc1 is not due to an increase of the  
250 cellular MeHg demethylation.

#### 251 *Effect of molybdate on the consumed substrates and the produced metabolites*

252 As described above, molybdate inhibits the growth and the Hg methylation activity of *P. hydrargyri* BerOc1 in all  
253 the tested metabolisms, although the rates of the inhibition vary. To ensure that the growth of *P. hydrargyri* BerOc1  
254 in the presence of molybdate corresponds to the initially tested metabolism and is not due to a cellular metabolism  
255 circumvention (caused by a switch of the growth to other metabolisms (*e.g.* fermentation)), the concentrations of  
256 the provided substrates (electron donors and acceptors) and the expected produced metabolites of each condition  
257 were separated and quantified at the final time of the growth (Tf) (Table 1, Fig. 3). Metabolites were only measured  
258 in conditions where molybdate does not affect (or only slightly affect) the growth of *P. hydrargyri* BerOc1.

259 In fumarate respiration, the pyruvate (electron donor) and the fumarate (electron acceptor) were all consumed at  
260 Tf in all the tested molybdate concentrations (Table 1). The concentration of succinate (from fumarate reduction)  
261 and acetate (from pyruvate oxidation), corresponding to the metabolites generated during fumarate respiration  
262 were produced as expected (Tf ranging from 30 to 40 mM), since one mole of fumarate (when it is reduced to  
263 succinate) accepts two electrons from one mole of pyruvate (Table 1). Low sulfide concentrations were also  
264 measured in fumarate respiration and were due to the initial addition of  $\text{Na}_2\text{S}$  as sulfur source in the culture medium  
265 (Fig. 3).

266 In sulfate respiration, the electron donor and acceptor when using lactate (electron donor) and sulfate (producing  
267 APS and bisulfite as electron acceptors) were partially consumed at Tf in the tested conditions, probably due to a  
268 surplus of substrates compared to fumarate respiration. Acetate (from lactate oxidation) and sulfides (from sulfate  
269 reduction), corresponding to the metabolites produced in this condition, were produced at concentrations range of

270 20-25 mM for acetate and 11-14 mM for sulfide in all the molybdate conditions, in accordance with the metabolism  
271 tested (Table 1, Fig. 3). When pyruvate and sulfate are used in sulfate respiration, pyruvate is totally consumed,  
272 producing the expected amount of acetate (ranging from 30 to 40 mM) and less sulfides (ranging from 5 to 8 mM)  
273 are produced (Table 1, Fig. 3), compared to the presence of lactate and sulfate. Two molecules of lactate are needed  
274 to reduce one molecule of sulfate in sulfate respiration, whereas four molecules of pyruvate are needed for this  
275 reduction (Keller and Wall, 2011). This explains the higher consumption of the electron donor (pyruvate) and the  
276 lower production of sulfides when using pyruvate and sulfate compared to those when using lactate and sulfate.

277 *Molybdate altered the expression level of hgcA and sat genes of P. hydrargyri BerOc1*

278 The expression level of *hgcA* gene involved in Hg methylation (Parks et al., 2013) and *sat/ycaO* genes involved in  
279 molybdate-sensitivity (Zane et al., 2020) were monitored (Fig. 4) in *P. hydrargyri* BerOc1 culture exposed to  
280 different molybdate concentrations.

281 Interestingly, the *hgcA* gene exhibited higher expression value ( $p < 0.05$ , Kruskal-Wallis test) when exposed to 0.1  
282 mM of molybdate during fumarate respiration, as well as during sulfate respiration when lactate was used as the  
283 electron donor (Fig. 4A). No significant variations in *hgcA* fold change were observed during sulfate respiration  
284 when pyruvate was used ( $p > 0.05$ , Kruskal-Wallis test), despite molybdate's inhibition of Hg methylation under  
285 this condition (Fig. 2). This suggests once again that the cellular response to molybdate in Hg methylator organisms  
286 like *P. hydrargyri* BerOc1 is variable and dependent on cellular metabolism.

287 The *sat* gene is expressed in *P. hydrargyri* BerOc1 under all the tested conditions, including fumarate respiration  
288 (Fig. S1). When the cells were grown in fumarate respiration, no significant fold change was observed in the *sat*  
289 gene expression in the presence of molybdate ( $p > 0.05$ , Kruskal-Wallis test). However, for both sulfate-reducing  
290 conditions, the expression of *sat* gene was inhibited by molybdate (Fig. 4B). The molybdate inhibition of *sat* gene  
291 increase with higher molybdate concentrations ( $p < 0.05$ , Kruskal-Wallis test, when 0 mM is compared to 0.1 mM  
292 molybdate conditions). For *ycaO* gene expression (Fig. 4C), molybdate did not change the expression level of this  
293 gene ( $p > 0.05$ , Kruskal-Wallis test) in any of the tested metabolic conditions.

## 294 **Discussion**

295 In this study, we investigated the effect of molybdate on bacterial growth and Hg methylation using a SRB model  
296 (*P. hydrargyri* BerOc1) and a non-SRB model (*G. sulfurreducens* PCA). The results showed that molybdate  
297 inhibits the growth of the Hg methylating SRB *P. hydrargyri* BerOc1, regardless of the metabolic growth

298 conditions. While it is generally acknowledged that molybdate inhibits the sulfate metabolism of SRB (Biswas et  
299 al., 2009; Newport and Nedwell, 1988; Zane et al., 2020), molybdate inhibition of pyruvate fermentation in *D.*  
300 *vulgaris* Hildenborough have been previously reported (Zane et al., 2020, p. 2). Additionally, molybdate inhibits  
301 the growth of *Desulfovibrio desulfuricans* on sulfate-free lactate medium, when in co-culture with a methanogen  
302 (Pak K.-R. and Bartha R., 1998). Here, we demonstrated for the first time that molybdate inhibits the growth of *P.*  
303 *hydrargyri* BerOc1 during fumarate respiration, further indicating that molybdate inhibition is not specific to  
304 sulfate respiration. Molybdate inhibition of SRB involves Sat and YcaO-like proteins (Zane et al., 2020). As  
305 describes earlier, these proteins are likely involved in the formation of an unstable APS analogue in the presence  
306 of molybdate, leading to a non-productive ATP cycling. In this work, we showed that the expression of *sat* gene  
307 is constitutive in all the tested conditions, including fumarate respiration. Sat enzyme is involved in the activation  
308 of sulfate in both sulfate dissimilatory and assimilatory pathways (Abdulina et al., 2020) and may explain the  
309 constitutive expression of the *sat* gene even in non-sulfidogenic growth condition. If the Sat protein is active during  
310 fumarate respiration, the APS analog can still be formed, potentially leading to non-productive ATP cycling, and  
311 induce molybdate inhibition in this growth condition as well.

312 Interestingly, the sensitivity to molybdate was higher in fumarate respiration (complete inhibition at 1 mM) than  
313 in sulfate respiration (complete inhibition at 2 mM). It is known that molybdate in a sulfide-rich environment (i.e.  
314 sulfate respiration) may form molybdenite [MoS<sub>2(s)</sub>] compounds which appear to occur in the periplasm and  
315 accumulate extracellularly or at the cell surface (Biswas et al., 2009; Tucker et al., 1997). This molybdate  
316 speciation could limit molybdate bioavailability and reduce the molybdate inhibitory effect during sulfidogenic  
317 growth conditions. Furthermore, when monitoring the expression of *sat* and *ycaO* genes involved in molybdate  
318 sensitivity (Zane et al., 2020), we observed that the expression of *sat* gene was inhibited at 0.1 mM in both sulfate-  
319 reducing conditions. However, its expression remained unchanged during fumarate respiration. The inhibition of  
320 *sat* gene during sulfidogenic growth conditions may reduce the inhibitory effect of molybdate on the growth of *P.*  
321 *hydrargyri* BerOc1 by mitigating the effect of the ATP 'futile' cycle.

322 Remarkably, the data presented in this work showed that molybdate inhibits Hg methylation process in *P.*  
323 *hydrargyri* BerOc1, even at concentrations where growth is not fully inhibited. The metallic ions zinc and cadmium  
324 (Schaefer et al., 2014) as well as copper at high concentrations (Lu et al., 2018) were shown to inhibit Hg  
325 methylation. In the case of the zinc inhibition, it was suggested that Hg uptake might occurs via an active zinc  
326 transporter leading to a competition for uptake between the two metals (Schaefer et al., 2014). A similar  
327 competition between the uptake of molybdate and Hg may occur. However, the competition between Hg and other

328 metal uptakes has been proposed for a decade but never been demonstrated. An alternative mechanism involving  
329 cellular ATP content could be also considered. Indeed, the Hg methylation process is energy-dependent (Schaefer  
330 et al., 2011) and the generation of an ATP ‘futile’ cycle via molybdate may harm the production of MeHg. In this  
331 study, we observed a higher inhibition of mercury methylation induced by molybdate when using pyruvate as an  
332 electron donor (complete inhibition at 0.1 mM of molybdate) compared to lactate (complete inhibition at 2 mM of  
333 molybdate). This disparity could be attributed to the difference in energy produced when using lactate versus  
334 pyruvate. While the oxidation of lactate coupled with sulfate reduction seems to generate low ATP via substrate  
335 phosphorylation (Peck, 1960), lactate metabolism leads to hydrogen production through hydrogenase activity  
336 (Berg et al., 1991), likely compensating for the lower energy yield. An overproduction of the [Ni-Fe] hydrogenase  
337 1 was observed also under molybdate-stressed condition in *Desulfovibrio alaskensis* G20 (Nair et al., 2015).  
338 Consequently, under molybdate stress, sulfate respiration with lactate could be energetically more favorable for  
339 cell growth due to the enhanced hydrogenase activity. This could potentially reduce the inhibitory effect of  
340 molybdate on MeHg production when using lactate as electron donor under sulfidogenic growth conditions.

341 In ecological studies, the observed decrease of Hg methylation after addition of molybdate (Bouchet et al., 2018)  
342 is probably a result of molybdate-induced inhibition of this process in SRBs, regardless of whether they are under  
343 sulfidogenic or non-sulfidogenic metabolisms. Indeed, the inhibition of Hg methylation by molybdate observed in  
344 our study aligns with the findings in biofilms and periphyton from the Lake Titicaca hydrosystem (Bouchet et al.,  
345 2018). Despite the fact that sulfate reducers remain active in the presence of molybdate, Hg methylation in Lake  
346 Titicaca was inhibited, providing further support that molybdate inhibits this process in sulfate reducers.

347 The data presented in this work also showed that the inhibition of Hg methylation in the presence of molybdate is  
348 not linked to an inhibition of *hgcA* gene expression (involved in Hg methylation (Parks et al., 2013)). *hgcA*  
349 expression was on the contrary higher when Hg methylation is completely inhibited during fumarate respiration  
350 and sulfate respiration when using lactate as electron donor. Although the link between *hgcA* expression and MeHg  
351 production was not demonstrated before (Goñi-Urriza et al., 2015), the increase seen in the expression of *hgcA*  
352 could be a cellular response to overcome the decrease seen in the Hg methylation rate in the presence of molybdate.  
353 Indeed, the decrease in MeHg production may increase the concentration of Hg(II) in the cell. This overexpression  
354 of *hgcA* could potentially serve to increase the rate of Hg methylation as a mechanism of resistance against the  
355 toxicity associated with Hg(II). *arsR* gene of the ArsR/SmtB family of metal-sensory transcriptional regulators  
356 (Saha et al., 2017), is found upstream *hgcAB* in multiple Hg methylating SRB (Goñi-Urriza et al., 2020) and is

357 responsive to arsenic and homocysteine (Gionfriddo et al., 2022). It will be interesting to investigate in future  
358 studies the role of ArsR in *hgcAB* regulation in respect to molybdate and its inhibitory effect.

359 As expected molybdate does not inhibits the growth of the iron-reducing and the Hg methylating bacterium *G.*  
360 *sulfurreducens* PCA in fumarate respiration. This resistance might be due to the absence of *sat* and *ycaO* genes,  
361 involved in molybdate sensitivity (Zane et al., 2020) in the genome of *G. sulfurreducens* PCA. Additionally,  
362 molybdate does not inhibit the Hg methylation process of *G. sulfurreducens* PCA. Ecological studies indicate that  
363 molybdate only partially inhibits the Hg methylation activity of certain anoxic sediments. It has been suggested  
364 that iron-reducing bacteria, like *G. sulfurreducens* PCA, might contribute to this 'molybdate-independent'  
365 methylation process (Fleming et al., 2006). This is consistent with our findings, where we observed no inhibition  
366 of growth or Hg methylation activity with a pure culture of *G. sulfurreducens* PCA.

### 367 **Conclusions**

368 Overall, this study demonstrates that molybdate inhibits the growth of *P. hydrargyri* BerOc1 during both sulfate  
369 and fumarate respiration, with a higher level of inhibition observed during fumarate respiration. Even more  
370 interesting, molybdate was shown to inhibit mercury methylation in *P. hydrargyri* BerOc1. This inhibition may be  
371 linked to cellular energy production, which is inherently metabolism-dependent. The data presented in this work  
372 highlight the importance of studying molybdate-induced inhibition in regards to *sat* coupled to *hgcA* and Hg  
373 methylation in order to advance our knowledge regarding the players involved in MeHg production at the  
374 environmental level.

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377

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## 579 **Competing Interests**

580 The authors have no relevant financial or non-financial interests to disclose.

## 581 **Author Contributions**

582 RG, DS, BKH and MSGU conceptualized the work, designed experiments and analyzed the data. DS performed  
583 experiments. ET, CG and MRP helped setting up experiments and data analysis of GC-ICP-MS, qRT-PCR and

584 ion chromatography, respectively. DS and BKH wrote the manuscript and RG and MSGU helped achieving the  
585 final draft of the manuscript. All authors participated in the critical thinking of the data and edited the manuscript.

#### 586 **Ethics approval**

587 Not applicable. This study does not involve human participants.

#### 588 **Consent to participate**

589 Not applicable.

#### 590 **Consent to publish**

591 Not applicable.

#### 592 **Figure and table legends**

593 Figure 1: Effect of molybdate on the growth of *P. hydrargyri* BerOc1 and *G. sulfurreducens* PCA. *P. hydrargyri*  
594 BerOc1 was grown on sulfate respiration with sulfate and lactate (black triangle), sulfate and pyruvate (black  
595 square) or on fumarate respiration with fumarate and pyruvate (black circle) as substrates. *G. sulfurreducens* PCA  
596 was grown on fumarate respiration in presence of acetate and fumarate (white square). For each condition, strains  
597 were grown in presence of increasing molybdate concentrations (0, 0.01, 0.05, 0.1, 1 and 2 mM) and 0.05  $\mu$ M of  
598 Hg(II). The biomass production is reported in percentage while 100% of biomass production corresponds to the  
599 biomass production of strains in the condition with no molybdate. The inset shows the % of biomass production  
600 values at 0, 0.01, 0.005 and 0.1 mM of molybdate. Experiments were performed in triplicate.

601 Figure 2: Effect of molybdate on Hg(II) methylation in *P. hydrargyri* BerOc1 and *G. sulfurreducens* PCA. MeHg  
602 production is expressed in ng/cell of *P. hydrargyri* BerOc1 grown with sulfate and lactate (black triangle), sulfate  
603 and pyruvate (black square) or fumarate and pyruvate (black circle) as substrates. *G. sulfurreducens* PCA was  
604 grown in presence of acetate and fumarate (white square). For each condition, strains were grown in presence of  
605 increasing molybdate concentrations (0, 0.01, 0.05, 1 and 2 mM) and 0.05  $\mu$ M of Hg(II). The inset shows the %  
606 of MeHg production values at 0, 0.01, 0.005 and 0.1 mM of molybdate. Experiments were performed in triplicate.

607 Figure 3: Sulfide's production in *P. hydrargyri* BerOc1 and *G. sulfurreducens* PCA. Sulfide production (mM) was  
608 measured at Tf in *P. hydrargyri* BerOc1 grown in sulfate (Suf/Lact and Suf/Pyr) and fumarate respiration  
609 (Fum/Pyr) and in *G. sulfurreducens* PCA grown in fumarate respiration (Acet/Fum). Fum: fumarate, Pyr: pyruvate,  
610 Sulf: sulfate, Lact: lactate, Acet: acetate.

611 Figure 4: Expression of genes involved in Hg methylation (*hgcA*) and molybdate sensitivity (*sat* and *ycaO*) in *P.*  
612 *hydrargyri* BerOc1. Real-time RT-PCR was performed on cultures of *P. hydrargyri* BerOc1 grown on sulfate  
613 respiration with lactate (Sulf/Lact) or pyruvate (Sulf/Pyr) as electron donor, as well as on fumarate respiration with  
614 pyruvate (Fum/Pyr). The expression level of *hgcA* (A), *sat* (B) and *ycaO* (C) genes were monitored, in the presence  
615 of increasing molybdate concentrations (0, 0.01, and 0.1 mM) and 0.05  $\mu$ M of Hg(II). Data were normalized using  
616 *gyrB* gene expression as a housekeeping gene in the same condition, following double delta Ct method. Error bars  
617 represent the standard deviations of three independent replicates, each one measured three times.

618 Table 1: The growth parameters of *P. hydrargyri* BerOc1 (BerOc1) are presented in biomass production ( $\Delta$ OD<sub>600</sub>)  
619 and  $\mu$  (h<sup>-1</sup>). 40 mM of the substrates were added at the initial time of the experiment. The values of the electron  
620 acceptor and donor are reported in mM of the measured substrate at Tf. The metabolites produced from the electron  
621 acceptor and donor are reported in mM of the produced metabolites at Tf. Fum; fumarate, Pyr; pyruvate, Sulf;  
622 sulfate, Lact; lactate, Sulfi; sulfide, Acet; acetate, Succ; succinate, NA : not applicable, ND : not determined.

### 623 **Supplementary Materials**

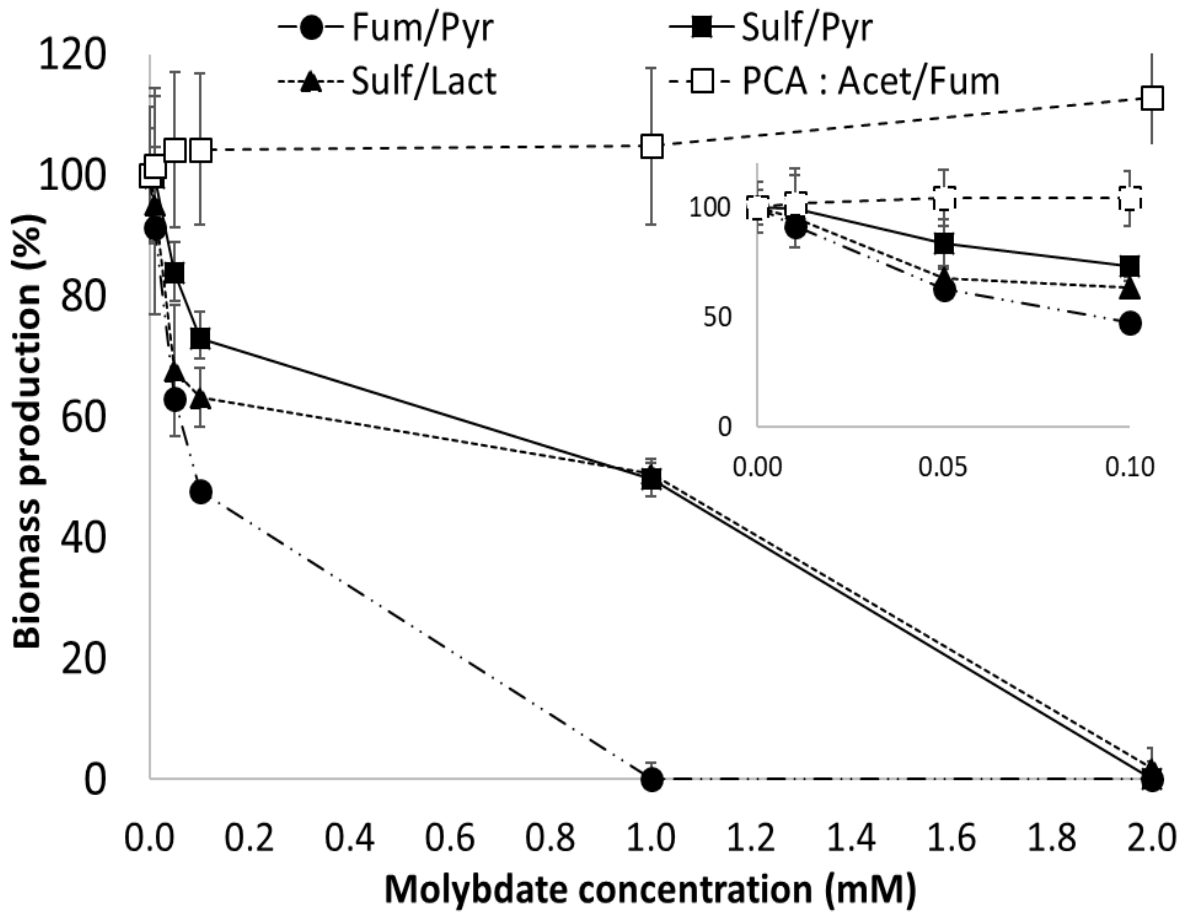
624 Figure S1: Expression of *P. hydrargyri* BerOc1 *sat* gene at 0 mM of molybdate.

625 Table S1: Growth parameters of *G. sulfurreducens* PCA.

626 Table S2: Quantification of the Hg(II) methylation (MeHg production) and MeHg demethylation (Hg(II)  
627 production).

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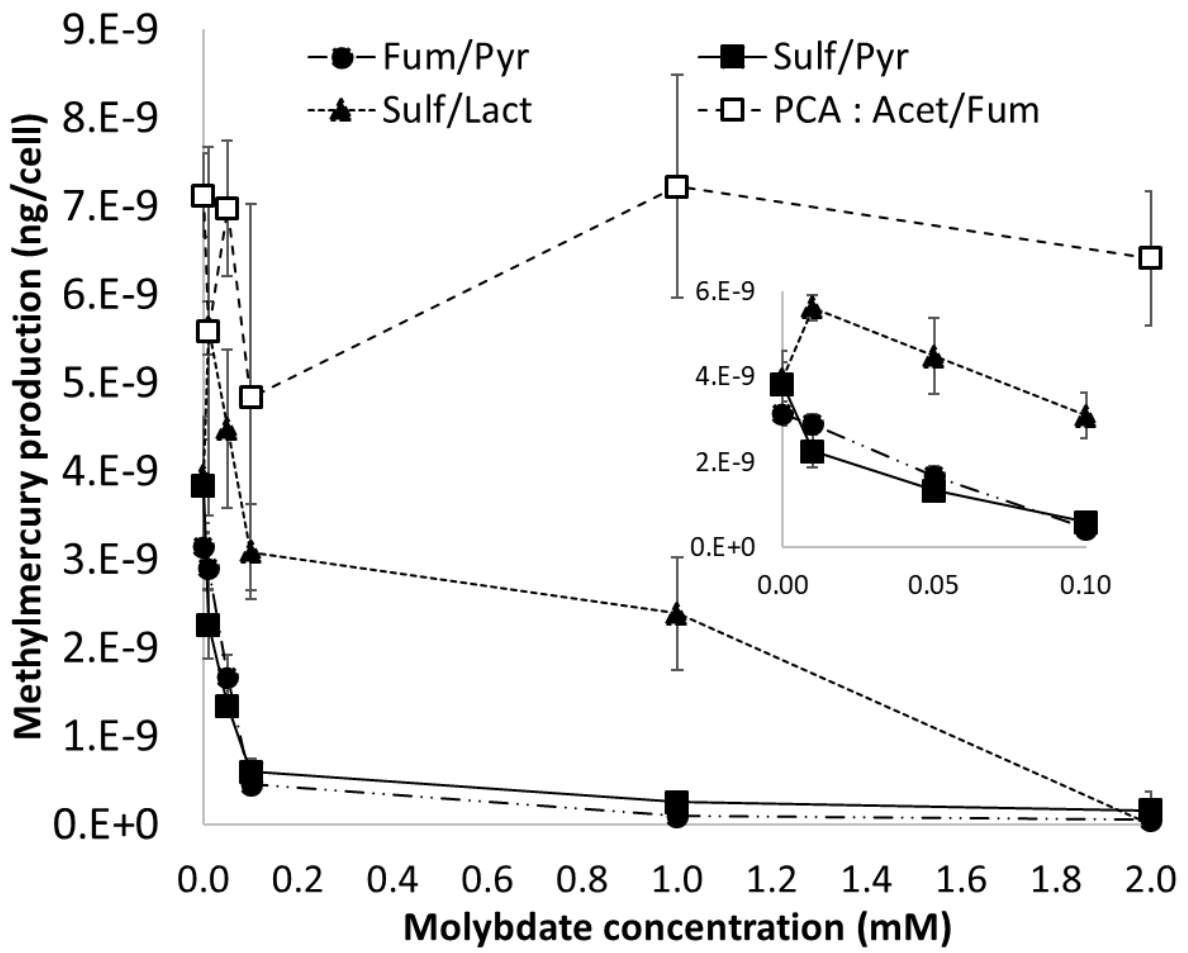
629 Figure 1



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632 Figure 2

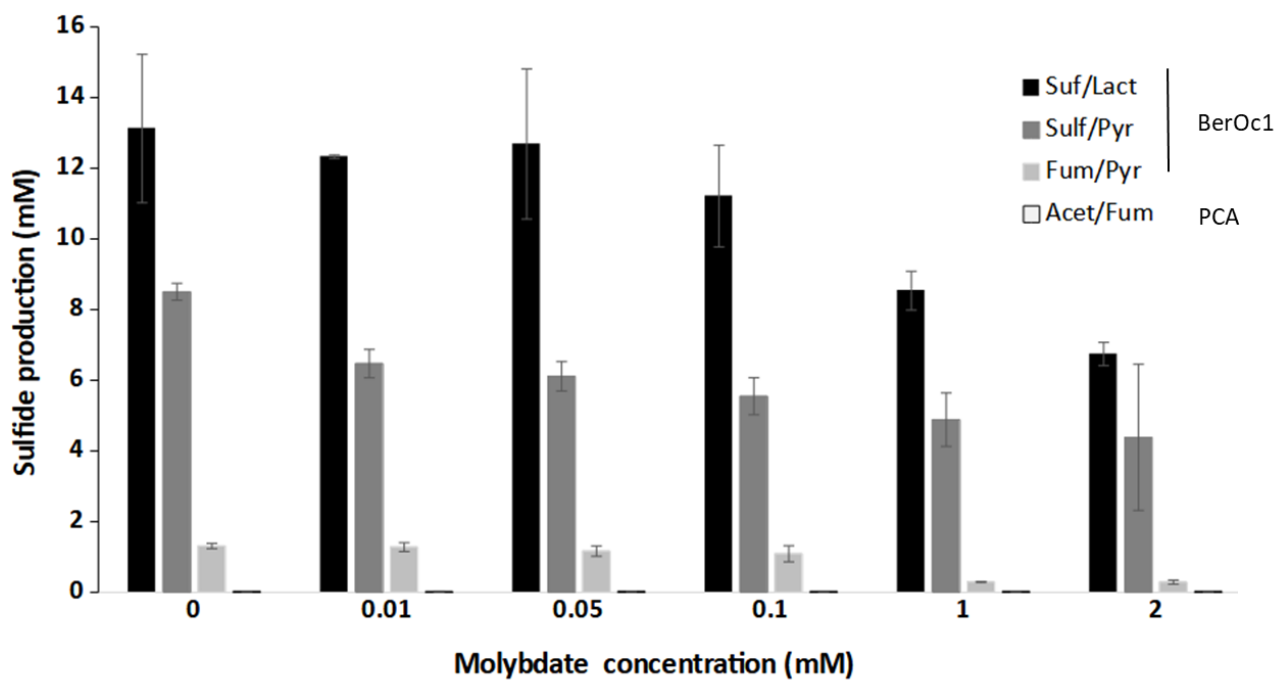


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636 Figure 3



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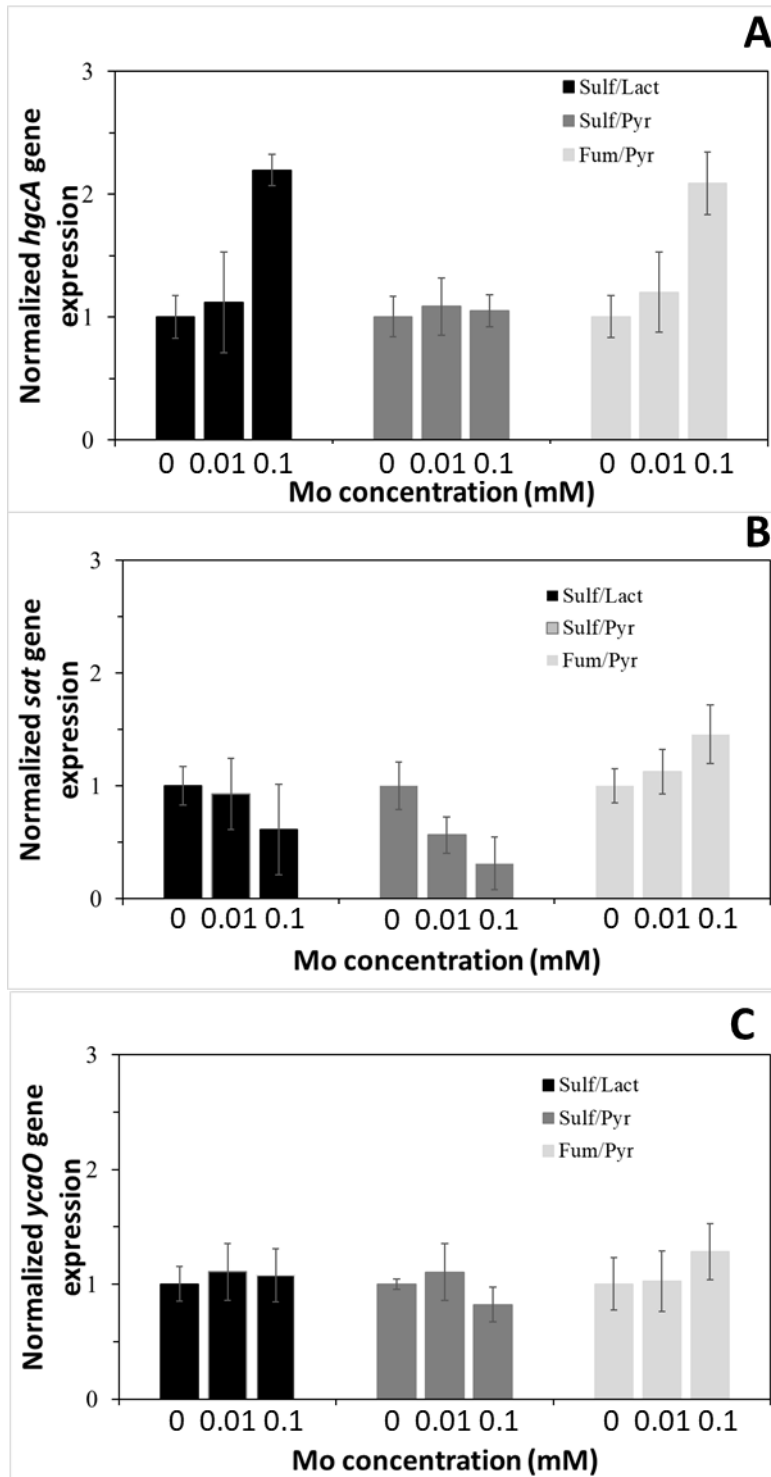
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656 Table 1

	Mo (mM)	Growth parameters		Metabolite measurements			
		Biomass production ( $\Delta OD_{600}$ )	$\mu$ ( $h^{-1}$ )	Electron donor mM at Tf	Electron acceptor mM at Tf	Metabolite produced from the electron donor (mM at Tf)	Metabolite produced from the electron acceptor (mM at Tf)
BerOc1 Fum/Pyr	0	0.23 ± 0.001	0.04 ± 0.001	Pyr : 0	Fum : 0	Acet : 41.80 ± 1.59	Succ : 34.62 ± 2.22
	0.01	0.21 ± 0.003	0.04 ± 0.002	Pyr : 0	Fum : 0	Acet : 39.27 ± 1.74	Succ : 35.94 ± 1.65
	0.05	0.15 ± 0.002	0.02 ± 0.001	ND	ND	ND	ND
	0.1	0.11 ± 0.003	0.02 ± 0.001	Pyr : 0	Fum : 0	Acet : 30.63 ± 1.86	Succ : 38.49 ± 0.82
	1	NG	NG	-	-	-	-
	2	NG	NG	-	-	-	-
BerOc1 Sulf/Pyr	0	0.16 ± 0.005	0.07 ± 0.002	Pyr : 0	sulf : 35.15 ± 4.93	Acet : 37.91 ± 1.29	Sulfi : 8.50 ± 0.24
	0.01	0.16 ± 0.003	0.06 ± 0.004	Pyr : 0	sulf : 36.16 ± 5.22	Acet : 31.92 ± 2.04	Sulfi : 6.47 ± 0.40
	0.05	0.13 ± 0.003	0.04 ± 0.002	ND	ND	ND	ND
	0.1	0.11 ± 0.003	0.03 ± 0.001	Pyr : 0	sulf : 36.34 ± 5.23	Acet : 32.78 ± 1.42	Sulfi : 5.55 ± 0.52
	1	0.08 ± 0.003	0.02 ± 0.001	ND	ND	ND	ND
	2	NG	NG	-	-	-	-
BerOc1 Sulf/Lact	0	0.09 ± 0.007	0.05 ± 0.001	Lact : 11.65 ± 0.22	Sulf : 29.73 ± 0.40	Acet : 23.00 ± 0.51	Sulfi : 13.12 ± 2.10
	0.01	0.08 ± 0.009	0.04 ± 0.003	Lact : 10.94 ± 0.58	Sulf : 30.15 ± 1.32	Acet : 24.88 ± 2.71	Sulfi : 12.33 ± 0.05
	0.05	0.06 ± 0.005	0.03 ± 0.004	ND	ND	ND	ND
	0.1	0.05 ± 0.006	0.03 ± 0.001	Lact : 14.85 ± 0.44	Sulf : 32.70 ± 0.36	Acet : 19.46 ± 0.96	Sulfi : 11.21 ± 1.44
	1	0.04 ± 0.002	0.02 ± 0.002	ND	ND	ND	ND
	2	NG	NG	-	-	-	-

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