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1 # Microbial cysteine degradation is a source of hydrogen
2 # sulfide in oxic freshwater lakes

3

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23 # Abstract

24 The sulfur-containing amino acid cysteine is abundant in the environment including in
25 freshwater lakes. Biological degradation of cysteine can result in hydrogen sulfide (H_2S), a toxic
26 and ecologically relevant compound that is a central player in biogeochemical cycling in aquatic
27 environments, including freshwater lakes. Here, we investigated the ecological significance of
28 cysteine in oxic freshwater lake environments, using model systems of isolated cultures,
29 controlled growth experiments, and multi-omics. We screened bacterial isolates enriched from
30 natural lake water for their ability to produce H_2S when provided cysteine. In total, we identified
31 29 isolates that produced H_2S and belonged to the phylum proteobacteria Bacteroidetes,
32 Proteobacteria and Actinobacteria. To understand the genomic and genetic basis for cysteine
33 degradation and H_2S production, we further characterized 3 freshwater isolates using whole-
34 genome sequencing, and quantitatively tracked cysteine and H_2S levels over their growth ranges:
35 *Stenotrophomonas maltophila*, *Stenotrophomonas bentonitica* (Gammaproteobacteria) and
36 *Chryseobacterium piscium* (Bacteroidetes). We observed a decrease in cysteine and increase in
37 H_2S, and identified genes involved in cysteine degradation in all 3 genomes. Finally, to assess
38 the presence of these organisms and genes in the environment, we surveyed a five-year time
39 series of metagenomic data from the same isolation source at Lake Mendota and identified their
40 presence throughout the time series. Overall, our study shows that sulfur-containing amino acids
41 can drive microbial H_2S production in oxic environments. Future considerations of sulfur
42 cycling and biogeochemistry in oxic environments should account for H_2S production from
43 degradation of organosulfur compounds.
44

45 # Importance

46 Hydrogen sulfide (H_2S), a naturally occurring gas with biological origins, can be toxic to living
47 organisms. In aquatic environments, H_2S production typically originates from anoxic (lacking
48 oxygen) environments such as sediments, or the bottom layers of lakes. However, the
49 degradation of sulfur-containing amino acids such as cysteine, which all cells and life forms rely
50 on, can be a source of ammonia and H_2S in the environment. Unlike other approaches for the
51 biological production of H_2S such as dissimilatory sulfate reduction, cysteine degradation can

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52 occur in the presence of oxygen. Yet, little is known about how cysteine degradation influences
53 sulfur availability and cycling in freshwater lakes. In our study, we found that multiple bacteria
54 originating from a freshwater lake can not only produce H_2S, but do so in oxic conditions.
55 Overall, our study highlights the ecological importance of oxic H_2S production in natural
56 ecosystems, and necessitates a change in our outlook of sulfur biogeochemistry.
57

58 Introduction

59

60 In most natural environments, the production of hydrogen sulfide gas (H_2S) is usually attributed
61 to defined groups of bacteria and archaea (1, 2) , and occurs primarily in anoxic environments.
62 During the process of dissimilatory sulfate reduction, sulfate acts as a terminal electron acceptor,
63 and is converted to hydrogen sulfide. However, other pathways for H_2S production exist, namely
64 assimilatory sulfate production, in which H_2S contributes to cell growth and increased biomass,
65 and the conversion of sulfur-containing amino acids such as cysteine which can lead to
66 production of pyruvate, ammonia, and H_2S (3). It is believed that assimilatory sulfate reduction
67 contributes to growth but does not release H_2S from the cell, while dissimilatory sulfate
68 reduction and cysteine degradation can contribute to growth and release ecologically relevant
69 nitrogen and sulfur compounds into the ecosystem.
70

71 The sulfur cycle is composed of several assimilatory and dissimilatory pathways, which interact
72 in complex ways through biotic and abiotic factors. Sulfur cycling in freshwater ecosystems can
73 have significant ecological significance, especially in places where strong redox gradients exist
74 (4). For example, in high arctic lakes, sulfur-compounds are suggested to serve as
75 biogeochemical hubs (5). Cysteine, a sulfur containing amino acid, has been proposed to be an
76 overlooked carbon source (6) and is an overlooked source of sulfur. Additionally, seston
77 (moving water that contains both living and nonliving organisms) contains organosulfur
78 containing lipids which settle into the sediments, and contributes to the sulfur pool in lakes such
79 as Lake Superior (7).
80

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81 In seasonally stratified lakes consisting of two layers of oxygenated warm water (epilimnion)
82 floating atop colder anoxic waters (hypolimnion), H_2S is often abundant in the hypolimnion (8,
83 9), due to the presence of anoxic sediment and an anoxic water column. However, an overlooked
84 player in the pool of available H_2S is the utilization of organosulfur compounds such as cysteine
85 by microbes. Cysteine is required for the production of proteins and is important for protein
86 structure. It is one of the two amino acids (methionine being the other) that contains a sulfur
87 group; however, the sulfhydryl group on cysteine is more reactive and can lead to the formation
88 of H_2S. Like all amino acids, cysteine also contains an amine group that will form ammonia once
89 the molecule is degraded. As such, the degradation of cysteine by microbes leads to the
90 production of H_2S. H_2S is ecologically relevant because it can be toxic to plants and animals.
91 During periods of anoxia, H_2S can accumulate to levels beyond the threshold for living
92 organisms, and can cause massive fish kills (10). Unlike other sources of H_2S, cysteine
93 degradation could occur under oxic conditions, thereby expanding the environmental scope of
94 this sulfur pool. In addition, cysteine has been found to be able to be degraded under oxic
95 conditions in the laboratory, but little information exists on the natural prevalence of this process
96 in lakes. We expect that H_2S production in oxic environments (during the mixed water column
97 periods of the year, and throughout the stratified period in the mixed epilimnion) could result
98 from cysteine utilization by microbes.
99
100 In this study, we investigated the prevalence of organosulfur degradation in aquatic ecosystems,
101 using both laboratory and genomic evidence, to inform our understanding of oxic sulfur cycling
102 in freshwater lakes (Figure 1). First, we grew bacterial isolates enriched from Lake Mendota's
103 oxic epilimnion to quantify H_2S and ammonia production, which informs the potential for
104 organosulfur degradation in an oxygenated aquatic environment. We found 18 isolates producing
105 H_2S under oxygenic conditions. We selected three H_2S-producing isolates for detailed
106 characterization using full-genome sequencing and chemical analyses to track cysteine and H_2S
107 production over their growth: *Stenotrophomonas maltophila*, *Stenotrophomonas S. bentonitica*
108 (Gammaproteobacteria) and *Chryseobacterium piscium* (Bacteroidetes). In all three isolates,
109 cysteine decreased when H_2S increased over their exponential growth curve under oxic
110 conditions. Finally, we contextualized our laboratory results using a time-series of metagenomic
111 data from the same isolation source (Lake Mendota, WI), in order to inform the temporal

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112 importance of organosulfur degradation. We found that genes for cysteine utilization were
113 present and abundant throughout the time-series suggesting that the ability to degrade cysteine is
114 widely distributed in Lake Mendota.
115

116 Results

117 Isolates capable of H_2S production in oxic conditions

118

119 To answer the question of whether bacteria could produce H_2S in the presence of oxygen, we
120 grew pure culture isolates originally isolated from the water column of temperate eutrophic Lake
121 Mendota. We grew the isolates under control and treatment conditions (addition of cysteine), and
122 tracked H_2S production after 24 hours **(Figure 2, Table S1)**. Using qualitative H_2S
123 measurements, we found that 18 isolates produced H_2S when grown with cysteine. We
124 performed 16S rRNA sequencing on the 29 isolates that produced H_2S. Isolates that produced
125 both H_2S and ammonia were identified as *Stenotrophomonas rhizophila*, *Stenotrophomona*
126 *maltophilia* (Betaproteobacteria), *Citrobacteria gillenii* (Gammaproteobacteria) and
127 *Chryseobacterium sp.* (Bacteroidetes), whereas those producing H_2S but not ammonia were
128 identified as *Pseudomonas arsenicoxydans, Pseudomonas mandelii, Pseudomonas migulae,*
129 *Pseudomonas thivervalensis,* and *Microbacterium flavescens*.
130

131 Detailed microbiological, chemical and genomic characterization in selected isolates

132

133 Next, we selected 3 isolates (#43, #13 and #66) representing distinct species that produced H_2S
134 for further characterization. These detailed characterizations include OD_{600}-based growth rates,
135 and paired quantitative measurements of cysteine and H_2S. The addition of cysteine resulted in
136 concommital production of H_2S over time (**Figure 3A, Table S2**). All organisms used L-cysteine
137 preferably to D-cysteine (**Figure 3B, Table S3**).
138

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139 Next, we also performed full-genome sequencing using combined short-read and long-read
140 sequencing on these 3 isolates (**Table 1**). We performed full-genome sequencing because
141 functional information such as gene content cannot be predicted reliably from 16S rRNA
142 sequencing of the isolates alone. The full genome of Isolate 43 was assembled into a single
143 circular genome, with estimated completeness of 100%, and taxonomically assigned to
144 *Stenotrophomonas maltophila*. Unlike the 16S rRNA sequence which assigned it to *S.*
145 *rhizophila*, the full-genome was actually closer to *S. maltophila*. The full genome of Isolate 13,
146 could be assembled into 2 long contigs, but also with completeness of 100% and taxonomically
147 assigned to *S. bentonitica*. The *Chryseobacter* genome was assembled in one circular genome,
148 and 100% complete, and assigned to *Chryseobacterium piscium*. Overall, the 16S rRNA
149 amplicon sequencing performed prior agreed with full-genome sequencing assignment in some
150 cases, and in others, the whole-genome sequencing assignment allowed finer taxonomic
151 resolution (such as in the case of Isolate 13), and overall provides more information about the
152 genetic content of the genomes. The genomic content was then used to inform how or why H_2S
153 might be produced in oxic environments, as shown in the laboratory experiment.
154
155 Using genome-level gene annotations of the 3 isolates, we identified the presence of genes
156 involved in cysteine utilization. We identified genes involved in the degradation of cysteine to
157 ammonia, pyruvate and H_2S: metC, malY, tnaA, cysM, cysK (these 5 start from L-cysteine), and
158 dcyD (which uses D-cysteine as a starting substrate) (**Table S4**). However, we note that these
159 genes may have other enzymatic activities, such as cysteine biosynthesis instead of degradation
160 (**Table S5**). (**Figure 4**)
161
162 Leveraging the full-genomic content of the 3 isolates (**Table S6**), we proposed a cellular map of
163 these isolates (**Figure 5A**). All three isolates had genes related to central carbon metabolism:
164 including the TCA cycle, glycolysis, gluconeogenesis, the pentose phosphate pathway and the
165 glyoxylate cycle. They could both generate fatty acids using fatty acid biosynthesis, and utilize
166 fatty acids through the beta-oxidation pathway. As expected, they had genes for the cysteine
167 metabolism, including cysteine biosynthesis pathways from homocysteine and serine, as well as
168 other amino acid utilization, such as methionine degradation pathway.
169

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170 In spite of these similarities, the three isolates also have distinguishing characteristics amongst
171 them (**Table S6, Figure 5B**). For example, while all have genes for sulfur oxidation (sulfur
172 dioxygenase), genes for thiosulfate oxidation were present in the two *Stenotrophomonas* isolates
173 but not *Chryseobacterium*. Isolate #66 (*Chryseobacter piscium*) contained a urease suggesting
174 the use of organic nitrogen in the form of urea but this was absent in the two *Stenotrophomonas*
175 isolates.
176
177 Finally, many more genes for sugar utilization were identified in the two *Stenotrophomonas*
178 isolates in the comparison to *Chryseobacterium*.

179 **Presence of cysteine-degrading organisms and genes in a five-year metagenomic**
180 **environmental time-series**
181
182 To put these laboratory results and lab-grown organisms into a natural environment context, we
183 leveraged previously published genomic time-series of Lake Mendota spanning 2008-2012 in the
184 oxygenated epilimnion to search for the presence of cysteine degradation genes in metagenomic
185 data.
186
187 First, we searched the time-series to see if organisms in our study were also present in the time
188 series. To do this, we linked the 16S rRNA sequences of the isolated organisms to the assembled
189 metagenomes from the time series. We found that while the 16S rRNA sequences were also
190 present in the time series (**Table S7, S8, S9**), and broadly distributed over time, these scaffolds
191 were not part of binned genomes. Therefore, little information about these isolates would be
192 gathered from metagenomic data only. As such, the full-genome sequencing we performed was
193 particularly helpful in understanding the full genomic structure of the H_2S-producing organisms.
194
195 Second, we searched for the 6 genes associated with cystine degradation and production of H_2S
196 (**Table S5**) (in binned and unbinned scaffolds). In total, we searched over 22 million amino acid
197 sequences and identified 1882 hits to 5 genes, since no dcyD homologs (involved in the D-
198 cysteine to pyruvate, ammonia and hydrogen sulfide degradation pathway) were found (**Figure**
199 **6, Table S10**). cysK and malY were the genes with most corresponding matches at any time

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200 point, followed by metC and cysM. Only 2 scaffolds contained tnaA. Overall, after correcting for
201 the genome size, there was no visible temporal trend of the genes, although genes were found
202 throughout the 5-year time series.
203
204 Among these cysteine-degrading gene sequences, several were identified in binned MAGs
205 (**Figure 6B, Table S11**), therefore taxonomy could be assigned to them. Overall, 139 genes were
206 distributed in genomes of Actinobacteria, Bacteroidetes, Chloroflexota, Cyanobacteria,
207 Planctomycetes, Proteobacteria, and Verrucomicrobia, which are common freshwater bacteria,
208 and broadly distributed in freshwater lakes. tnaA was only present in Bacteroidetes, but other
209 genes were more broadly distributed.
210
211

212 # Discussion

213

214 **Different types of H_2S production: the fate of cysteine and the origin of the H_2S**

215 The H_2S producing isolates identified by us fell into two groups, ammonia-producing and
216 ammonia-consuming, when grown in media with cysteine. We hypothesize that those that
217 produced ammonia and H_2S in the presence of cysteine, but not under "controlled" conditions
218 were those that were potentially contributing to the H_2S pool. However, it is possible that the
219 isolates that consume ammonia also conduct the cysteine degradation pathway, but do not
220 excrete ammonia from the cell. Instead, since ammonia is an important biological compound, it
221 could be used by the organism instead of being released into the media and measured.
222
223 We do not believe that this occurs with H_2S, and that most, if not all, of the isolates that produce
224 H_2S in the presence of oxygen will excrete it from the cell. H_2S is toxic to organisms that
225 undergo aerobic cellular respiration (11). There is evidence that some bacteria use H_2S as a
226 protective compound against antibiotics, but it also creates a large amount of stress on the cell
227 making it useful only in extreme situations (12). While it is possible that organisms may use H_2S

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228 internally as a source of sulfur, we did not identify any sulfide quinone oxidoreductases,
229 flavocytochrome c dehydrogenases, or other genes for the oxidation or transformation of H_2S.
230 All 3 isolates were obligate aerobes based on laboratory assays.
231
232 Of the 20 amino acids, little is known about the cysteine in the environment. One of the
233 difficulties in studying the fate of cysteine in oxic environmentsis that it can be oxidized into
234 cystine (13), which *E.coli* has been shown to uptake (14). In a study of *E.coli* K-12 that lacked a
235 cysteine transporter, cysteine was found to be able to enter the cell through transporters
236 dedicated to other amino acids that worked best when no amino acids alternatives other than
237 cysteine were present in the medium (15). It is likely that the majority of H_2S produced by the
238 cell in the three isolates originates from cysteine given the demonstrated reduction in cystine
239 concentrations when the isolates are grown with added cysteine (**Figure 3**), coupled with the
240 release of H_2S and production of ammonia.

241 **Genomic structure of the H_2S-producing isolates**

242
243 Overall, the three isolates selected for whole genome sequencing revealed genes for cysteine
244 degradation into H_2S. Based on laboratory studies, they were able to produce H_2S in the presence
245 of oxygen. All genomes were obligate aerobes, which brings interesting questions about the life
246 history of these organisms.
247
248 Little information is known about the ecology of *Stenotrophomonas maltophilia*,
249 *Stenotrophomonas Bentonitica*, and *Chryseobacterium piscium* in the natural environment. *S.*
250 *bentonitica* was originally characterized in bentonite formations, and was predicted to have high
251 tolerance to heavy metals (16), and has been observed in arctic seawater (17). *C. piscium* was
252 also isolated from a fish in the arctic ocean (18), but its ecological significance in the oceans
253 remains unknown. This previously described *C. piscium* strain LMG 23089 was not reported to
254 produce H_2S yet our genetic analyses suggest that it has the enzymatic machinery to degrade
255 cysteine.
256

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257 One possible explanation for this discrepancy is that LMG 23089 was previously grown on SIM
258 medium to test H_2S, which is lower resolution than the modern H_2S probes which measure µM
259 concentrations, and because SIM medium uses thiosulfate as a sulfur source. As a side test on
260 isolate #66, H_2S was not produced when thiosulfate was provided, but H_2S was produced when
261 cysteine was provided.
262
263 One particular finding of this study was that none of the 6 genes searched for cysteine
264 degradation into H_2S and ammonia was common to *all* three isolates, despite all three isolates
265 showing the same cysteine-decrease, ammonia-increase and H_2S-increase over time. This could
266 be explained by alternative, perhaps less straightforward pathways for H_2S production. One
267 pathway is led by a gene named cystathionine gamma-lyase ("CTH" or "CSE"). In some bacteria
268 and mammals, this enzyme is involved in H_2S production (19). A HMM search for this enzyme
269 showed that it was present in Isolate #13, 43 and #66. While it was not initially included in the
270 initial methods and study, this could hint to another commonality among oxic H_2S producing
271 organisms.

272 **Challenges associated with measuring oxic H_2S production from organosulfur in the**
273 **environment**
274
275 Extrapolating these laboratory results to widespread distribution of organosulfur degradation in
276 the natural environment necessitates several steps, namely because of the major gaps that exist
277 concerning the sulfur cycle in freshwater lakes, and because bridging the gap between
278 cultivation-based, omics-based (20) , and field-based experiments is needed. Foremost, the
279 identity, distribution, and availability of organosulfur compounds broadly across lakes globally is
280 currently mostly unknown. Cysteine is notoriously difficult to measure, and many previous
281 studies characterizing the amino acid composition of the water column only measure the sulfur-
282 containing organosulfur compound taurine (21, 22).
283
284 Organic sulfur in the form of cysteine is an important organosulfur amino acid, and is important
285 in protein folding and function (23). As such, there is a difference in the fates of cysteine when it
286 exists bound in cell walls, versus when cysteine is free in the water column and available for

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287 degradation by bacteria. While cysteine has been shown to contribute to the carbon pool and
288 carbon flow in lakes (6), more quantitative field measurements are necessary to support whether
289 cysteine also serves as a sulfur pool. Yet, other forms of organosulfur have important
290 significance in aquatic environments. In marine environments for example DMSP
291 (dimethylsulfoxonium propionate) is a critical component of the marine organosulfur cycle (24).
292
293 Additionally, current differences between computational gene homology searches versus *in vivo*
294 enzymatic functions are challenging to assess for the genes responsible for the degradation of
295 cysteine into pyruvate, ammonia and H_2S. One reason is that the enzymatic activity of the gene
296 has mostly been described in model organisms such as *E. coli*, and it has been shown that gene
297 activity can be induced by genetic factors, or environmental factors such as metals (25). At least
298 6 genes have been proposed to have this enzymatic activity, yet, each gene may serve different
299 functions *in situ*, and it is difficult to assert directionality of enzymatic function based on
300 metagenomic or genomic analyses only. To this end, the isolated bacterial strains from this
301 study, which are non-model organisms, and originate from the natural freshwater lake
302 environment, may be used for further detailed biochemical, physiological, and microbiological
303 studies. Further characterization of these bacterial isolates using gene-knockoutsand gene
304 expression studies may inform the functional activity of these genes in nature.
305

306 **Implications of oxic H_2S production by microbes in freshwaters**

307

308 This study demonstrates that the production of H_2S by microbes in lake ecosystems occurs in the
309 presence of oxygen, using evidence from pure culture bacterial isolates, and screening of long-
310 term time series of metagenomic lake data. By combining lake-to-laboratory experiments, we
311 show that multiple bacterial strains spanning Gammaproteobacteria, Betaproteobacteria,
312 Actinobacteria and Bacteroidetes are all able to produce H_2S under oxic conditions, and at
313 temperatures that would be ecologically relevant for surface lake water during the summer.
314 Surface water temperatures in Lake Mendota can reach up to 27°C, and the top few meters of
315 water surface are saturated in oxygen. Long-term ecological data shows such trends. Worldwide,
316 maximum lake surface temperature can range between 23 to 31°C (26) (**Table S12**).

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317

318 Unlike dissimilatory sulfate reduction, cysteine utilization by bacteria to generate ammonia,
319 pyruvate and H_2S, is not dependent on sulfate as an initial reaction substrate. Increased sulfate
320 concentrations are shown to lead to higher sulfide reduction rates in shallow eutrophic
321 freshwater, the sulfur originating from algal decay for example (27). While Lake Mendota is a
322 low-iron and high sulfate lake (28), not all lakes have elevated sulfate levels, and therefore, H_2S
323 production might previously not have been thought of as relevant to study. However, sulfur-
324 containing amino-acids can have many origins. In lakes, concentrations of amino acids (free and
325 combined) often reflect the input and outputs of the lake (29, 30). For example, amino acids
326 (although cysteine was not measured) contribute a detectable amount to the nitrogen cycle, and
327 are facilitated by bacterial activity (30).

328

329 There are several implications for aerobic H_2S production in oxygenated aquatic ecosystems. In
330 the carbon cycle, once aerobic methanotrophs were identified as early as 1900's, further aerobic
331 methanotrophs from various phyla of bacteria were found to be ubiquitous in aquatic ecosystems,
332 and have had implications for the cycling of methane, a potent greenhouse gas that is 25X more
333 potent than CO_2. Likewise, the discovery of aerobic bacterial methanogenesis (31), contributes to
334 resolving the "methane paradox", in which methane was often measured in oxygenic surface
335 waters, yet the molecular paradigm explaining it previously was strictly known to occur in
336 anoxic environments. In these two cases, the observation of the processes (methanogenesis and
337 methane consumption) that were typically thought to occur in anoxic environments only, but in
338 oxic environments now, are crucial for the understanding of the carbon (biogeochemical cycle).
339 In a similar vein, much remains to be discovered about the sulfur cycle. In lakes, H_2S has
340 typically been associated with anoxic environments, and from processes that are oxygen-
341 sensitive such as dissimilatory sulfate reduction. Here, we show hydrogen-sulfide production to
342 not only occur in oxic conditions but also by several guilds of bacteria, and in detectable
343 quantities.

344

345 Freshwater lakes which are dimictic can become stratified in temperature and oxygen during the
346 summer, and oxygen concentrations vary throughout the year. In the fall and spring, oxygen is
347 abundant, and cysteine degradation into H_2S could be a relevant process for the sulfur pool, and

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348 the fluxes of H_2S to the atmosphere could be significant since wind is prevalent. Under ice
349 during the winter, where oxygen is plentiful, H_2S could be produced but could be consumed or
350 oxidized, but gases would be trapped under ice. During summer, the anoxic hypolimnion and
351 sediments are known sources of H_2S due to dissimilatory sulfate reduction, but density gradients
352 would prevent H_2S from reaching the atmosphere. However, during the summer, the oxygenated
353 mixed epilimnion could be sources of H_2S through organosulfur degradation. If we consider the
354 importance of oxic hydrogen sulfide production, which could occur year-round, the pool of H_2S
355 and the scope of sulfur transformations may be greater than anticipated, if we focus solely on the
356 anoxic hypolimnion (**Figure 7**). Future work aiming to understand the broader distribution of
357 sulfur-containing amino acids and other organosulfur compounds in freshwaters, their fates and
358 transformations, as well as their contribution to H_2S production, will inform global sulfur
359 biogeochemical cycling.
360

361 Methods

362

363 Enrichment cultures of isolates from a temperate freshwater lake

364 Lake Mendota (43°06′24″ N 89°25′29″ W) is a temperate eutrophic lake in South-Central
365 Wisconsin, in Madison, WI, USA. Lake Mendota is part of the Long-Term Ecological Research
366 Network North Temperate Lakes (NTL-LTER, https://lter.limnology.wisc.edu/about/lakes).
367 Lake Mendota encounters annual stratification and annual seasonal anoxia in the hypolimnion.
368 Lake water was collected on September 14, 2018 from an integrated water sample (0m to 12m)
369 from Lake Mendota at the Deep Hole location (43°05'54", 89°24'28"), where the maximum
370 depth is 23.5m. The lake water was collected in pre-acid washed 2L sampling bottles using a
371 flexible PVC tubing, and brought back on shore within hours for immediate processing. The
372 water samples were collected during stratification and the oxygenated epilimnion. Serial dilution
373 was performed and grown on PCB (plate count media broth) agar media, at room temperature
374 (~21°C), in the lab. The PCB media was made of: 1L water, 5g/L of yeast extract, 10g/L of

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375 tryptone and 2g/L of dextrose/D-glucose. If grown on solid media, 10g of agar per 1L media was
376 added. Enrichment resulted in about 60 isolates.
377

378 **Screening for cysteine degradation into H_2S and ammonia**

379 Isolates were able to grow on PCB and R2A media. R2A media is a culture medium for bacteria
380 that typically grow in water. It is less “nutrient rich” than PCB media, and therefore slightly
381 closer to natural lake water. For the screening of the isolates for H_2S production, we grew them
382 on R2A media. Each isolate had two treatments: grown in R2A media without cysteine for the
383 control, and grown in R2A media with amended cysteine as the treatment.
384

385 R2A media consisted of (1L of water): 0.5g of casein, 0.5g of dextrose, 0.5g of starch, 0.5g of
386 yeast extract, 0.3g of K_2HPO_4, 0.3g of sodium pyruvate, 0.25g of peptone, 0.25g of beef extract,
387 0.024g of $MgSO_4$, and autoclave. To make the same media for plates, we added 15g of agar
388 before autoclaving. For controls, isolates were grown in the media without cysteine amendments.
389 For “treatments”, 2mM cysteine was added.
390

391 To assess the amount of cysteine degradation into H_2S and ammonia, we screened each 60
392 isolates on whether they produce H_2S and/or ammonia. To test H_2S production, we grew the
393 strains individually in liquid media, and used lead acetate test strips (Fisher Scientific, USA) to
394 qualitatively assess H_2S production. A darkening of the strip shows that H_2S was produced. To
395 test ammonia concentrations after 24 hours, we measured samples at time zero, and 24 hours
396 using Ammonia Salicylate Reagent Powder Pillows and Ammonia Cyanurate Reagent Powder
397 Pillows (Hatch Reagents) and used spectrophotometry at the 655nm wavelength.
398

399 **Identification of H_2S producing bacteria using 16S rRNA sequencing**

400 Colony PCR and DNA extractions were conducted using the EtNa Crude DNA Extraction and
401 ExoSAP-ITTM PCR Product Cleanup protocols on the isolates that tested positive for producing
402 H_2S (10). Full length 16S rRNA products were generated for sequencing using universal 16S
403 rRNA primers (27f, 1492r). DNA concentration yields were measured using the qBit dsDNA HS

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404 assay kit (QuBit). DNA was sequenced at the University of Wisconsin Madison Biotechnology
405 Center (Madison, WI, USA) The program 4Peaks (32) was used to clean the base pairs by
406 quality-checking followed by homology search using BLASTn against the NCBI Genbank
407 database (accessed December 2019) (33) to identify the sequences.

408 **Detailed characterization of 3 hydrogen-sulfide producing isolates**

409 We selected 3 isolates that could aerobically produce H_2S for further detailed characterization.
410 We selected these isolates because some of the 18 isolates had identical 16S rRNA sequences,
411 therefore we chose isolates that had distinct 16S rRNA sequences for full-genome sequencing.
412 Additionally, using 16S rRNA sequencing of the isolate, one only was assigned to
413 *Stenotrophomonas sp.*, and we believed that full-genome sequencing would enable us to get a
414 higher taxonomic confirmation and more complete information.
415
416 We performed DNA extraction using the PowerSoil Powerlyzer kit (Qiagen) without protocol
417 modifications, and sent the genomes for full genome sequencing at Microbial Genome
418 Sequencing Center (MIGS) (Pittsburg, PA) for combined short read illumina and long read
419 nanopore sequencing. The data was processed by MIGS to assemble the short-reads (Illumina
420 Next Seq 2000) and long-reads (Oxford Nanopore Technologies) into full-genomes. Quality
421 control and adapter trimming was performed with bcl2fastq (Illumina) and porechop
422 (https://github.com/rrwick/Porechop) for Illumina and ONT sequencing respectively. Hybrid
423 assembly with Illumina and ONT reads was performed with Unicycler (34)**.** Genome annotation
424 of the 3 isolates was done with Prokka v.1.14.5(35), using the --rfam setting.
425
426 Genome completeness and contamination were estimated using CheckM v.1.1.3 (36)
427 *lineage_wf*. Taxonomic classification was conducted using GTDB-tk v.0.3.2 (37) with the
428 database release r95. The full-genome taxonomic classification agreed with the prior 16S rRNA
429 sequencing results, but we were further able to identify Isolate 43 as *Stenotrophomonas*
430 *bentonitica*. We ran METABOLIC-G v.4.0 (38) to identify genes associated with cysteine
431 degradation and other metabolic pathways.
432

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433 Growth measurements of the three isolates were measured using OD600 with a
434 spectrophotometer, with measurements every 1 hour. The isolates were grown in R2A broth
435 media, shaken in an incubator at 27°C. Aliquots were collected over the growth range for
436 cysteine measurements. A H_2S microsensor (Unisense) was used to measure H_2S over time.
437

438 **Methods to measure Cysteine**

439

440 Cysteine concentrations were measured as cystine, as described in (39) (https://osf.io/9k8a6/).
441 One of the reasons for measuring cystine instead of cysteine is that in oxic environments,
442 cysteine is oxidized rapidly into cystine (13, 14). Additionally, unless LC-MS is used, cysteine
443 can be difficult to measure directly. Samples were diluted 5:4:1 Sample:DI H_2O:DMSO and left
444 at room temperature for at least 24 hours. Chromatographic analysis was performed on an
445 Agilent 1260 Infinity IIwith a Agilent Zorbax Eclipse Plus C18 RRHT 4.6x50mm, 1.8µm, with
446 Guard column. Column temperature was maintained at 40°C using an Agilent 1260 TCC
447 (G1316A).
448

449 Gradient elution was performed using Mobile Phase A (MPA) consisting of 10mM Na_2HPO_4,
450 10mM Sodium Tetraborate Decahydrate, in DI H_2O, adjusted to pH 8.2 with HCl, filtered to
451 0.45µm. Mobile Phase B (MPB) consisted of 45:45:10 Acetonitrile:Methanol:DI H_2O. Gradient
452 used for elution was as follows: 0 minutes 98% MPA, 2% MPB; 0.2 minutes 98% MPA, 2%
453 MPB; 6.67 minutes 46% MPA, 54% MPB; 6.77 minutes 0% MPA, 100% MPB; 7.3 minutes 0%
454 MPA, 100% MPB; 7.4 minutes 98% MPA, 2% MPB; 8 minutes 98% MPA, 2% MPB. Flow rate
455 was 2.0mL/min. Pump used was a Agilent Infinity Series G1311B Quat Pump. Pre-column
456 derivatization was performed using an Agilent 1260 ALS (G1329B) with an injector program.
457 Detection was performed using an Agilent 1260 Infinity II MWD (G7165A) at 338nm with
458 10nm bandwidth. Reference was 390nm with 20nm bandwidth.
459 Recovery was tested during method development. Recoveries of Cystine ranged from 87.2-
460 101.5%, with an average of 92.1%.

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461 **Methods to measure H_2S using a microsensor**

462 Aliquots of at least 1mL were taken from cultures at desired times after inoculation. H_2S
463 concentrations were measured by suspending the H_2S probe (Unisense) in the aliquot and leaving
464 it in place until measurement stabilized. Measurements were manually edited to exclude data
465 gathered while the probe was stabilizing in the sample.
466

467 **Generation of Metagenome-assembled genomes**

468 Sequencing of the Lake Mendota time series for 2008-2012 was previously done at the Joint
469 Genome Institute (40), containing 97 times points (and therefore 97 metagenomic datasets).
470 Metagenomics data was processed in-house. In summary, each metagenome was quality filtered
471 using fastp (41), and individually assembled using metaSPAdes. Each metagenome was mapped
472 to each individual assembly using BBMap v38.07 (42) version with 95% sequence identity
473 cutoff. Differential coverage mapping to all samples was used to bin contigs into metagenome-
474 assembled genomes (MAGs) using Metabat2 v.2.12.1 (43). Bins were quality assessed with
475 CheckM v.1.1.2 (36), dereplicated with dRep v.2.4.2 (44), and classified with GTDB-tk v.0.3.2
476 (45). This resulted in a total of 116 MAGs from Lake Mendota (**Table S13**)
477 (https://osf.io/qkt9m/).
478

479 **Searching for the presence of the three cultured isolates presence in the Lake Mendota**
480 **time-series**

481 To determine whether the isolates that were cultured in the laboratory were present in the natural
482 environment, we performed a homology search using Blastn v.2.6.0+ (33) of the 16S rRNA,
483 using an e-value threshold of 1e-6. 16S rRNA sequences were queried against a custom database
484 of the scaffolds in the assembly (-db), with a further search against the binned scaffolds in the
485 116 MAGs (-db). The comprehensive database containing all scaffolds of the assembly
486 comprised 16,599,321 scaffolds, whereas the final set of MAGs contained 28,395 scaffolds.
487

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488 **Searching for cysteine genes and isolates presence in time-series**

489

490 Genes for cysteine degradation were identified using HMMsearch v3.1b2 (46) . HMMs were
491 downloaded from KoFam (47), accessed May 2020). The KO numbers for the six cysteine
492 degradation genes are: metC (K01760), cysK (K01738), cysM (K12339), malY (K14155), tnaA
493 (K01667), and dcyD (K05396) (**Table S4**). Our HMM files are available in **Supplementary File**
494 **1** but are the same published by KoFAM, with the modification of manual addition of the TC
495 threshold. HMM-based homology searches were conducted on the 97 Lake Mendota
496 metagenomes assemblies as described above.

497

498 Data availability

499 The 16S rRNA sequences for the 29 H_2S producing isolates, the full genome sequences
500 (nucleotides and amino acids) for isolates #13, #43 and #66 are available on OSF:
501 https://osf.io/g25eq/ during the peer-review process. The sequences will be deposited in NCBI
502 prior to publication.

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521

522 **Contributions**

523

524 P.Q.T, S.C.B., J.C.H, K.K and K.A contributed to study design and conceptualization. P.Q.T,
525 S.C.B, J.C.H and K.K conducted experiments on the isolates. J.C.H performed the chemical
526 analyses of the isolates. P.Q.T, S.C.B and J.C.H conducted genomic analyses. P.Q.T, S.C.B and
527 E.A.M conducted metagenomic analyses. P.Q.T, S.C.B and J.C.H analyzed the data and
528 generated figures. P.Q.T, S.C.B, J.C.H and K.A drafted and edited the manuscript. All authors
529 provided feedback and suggestions.

530

531

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669 # List of Main and Supplementary Figures & Tables

670 **List of figures**

671 **Figure 1.** Overview of methods of our study.
672 **Figure 2.** H_2S and ammonia production screening of the isolates
673 **Figure 3.** H_2S utilization, L-cysteine and D-cysteine utilization by the 3 isolates over time.
674 **Figure 4.** Diagram showing presence and absence of cysteine degradation genes in the 3 isolates.
675 **Figure 5.** Cellular diagram of genes and pathways common to all three isolates.
676 **Figure 6.** Cysteine utilization/degradation genes in the Lake Mendota Time Series (LMTS)
677 assemblies, and in the MAGs from that assembly.
678 **Figure 7**. Conceptual model of the potential importance of the cysteine degradation in a
679 freshwater lake water column, with respect to oxygen levels and seasonality.
680

681 **List of Tables**

682 **Table 1.** Description of the 3 isolates (genome size, taxonomy, etc.)
683

684 **List of Supplementary Tables**

685 **Table S1.** Description of all initial isolates used in screening
686 **Table S2.** Quantitative measurement of H_2S over time in the 3 isolates
687 **Table S3.** Quantitative measurement of cystine over time in the 3 isolates.
688 **Table S4.** Description of the 6 genes involved in the degradation of L-cysteine or D-cysteine into
689 pyruvate, ammonia and hydrogen sulfide
690 **Table S5.** Detailed list of all potential enzymatic activities of genes in the cysteine degradation
691 and biosynthesis pathways.
692 **Table S6.** METABOLIC table: Full genomic content of the 3 isolates
693 **Table S7.** HMMsearch table output of 16S rRNA amplicons of the 28 isolates that produce H2S
694 from Figure 1 against the Lake Mendota Time Series assembly.
695 **Table S8.** HMMsearch table output of 16S rRNA amplicons of the 28 isolates that produce H2S
696 from Figure 1 against the metagenome-assembled-genomes from the LMTS.
697 **Table S9.** HMMsearch table output of 16S rRNA amplicons of the 28 isolates that produce H2S
698 from Figure 1 against the SSU (16S small ribosomal subunit) of the metagenome-assembled-
699 genomes from the LMTS.

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700 **Table S10.** Cysteine hits the Assembly.

701 **Table S11.** Cysteine hits to MAGS

702 **Table S12.** MAG characteristics binned from LMTS.

703 **Table S13.** Global minimum and maximum lake surface temperatures (ref. Sharma et al., 2015)

704

705 **List of Supplementary Files**

706 **File S1.** HMM profiles.zip

707

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708 # Tables

709 **Table 1.** Genome characteristics of 3 selected isolates that produce H2S in the presence of
710 oxygen and cysteine

711

Name	Genome Size (bp)	Completeness[1]	GC content	Taxonomy[2]	NCBI Project ID	Number of contigs
Isolate13-LM-B-02-08	4,188,104	100%	66.8%	d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Xanthomonadales;f__Xanthomonadaceae;g__Stenotrophomonas;**s__Stenotrophomonas maltophilia_O**	TBD	1
Isolate43-LM-B-01-03	4,325,715	100%	66.5%	d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Xanthomonadales;f__Xanthomonadaceae;g__Stenotrophomonas;**s__Stenotrophomonas bentonitica**	TBD	2
LM_BA_5.2	1,375,102	100%	33.7%	d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Flavobacteriales;f__Weeksellaceae;g__Chryseobacterium;**s__Chryseobacterium piscium**	TBD	7

712

713 [1] Using CheckM (See Methods) [2] Using GTDB-tk (See Methods)

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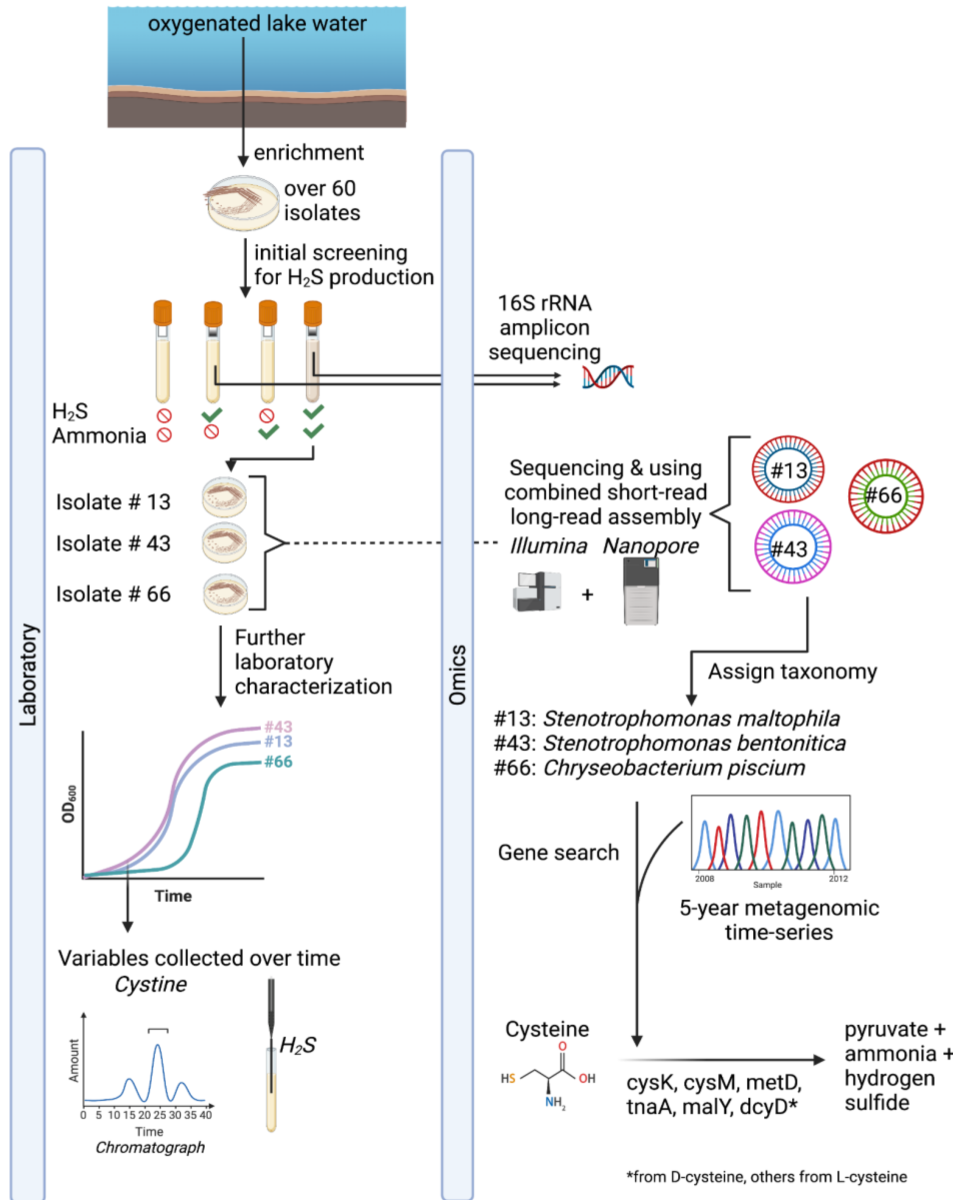
714 # Figures



715
716
717 **Figure 1. Methods overview of our study.** Isolates were enriched from oxygenated lake water.
718 Isolates were screened for H_2S and ammonia production, and those that produced H_2S were
719 selected for 16S rRNA sequencing. Then based on the results, three isolates were selected for
720 whole-genome sequencing using a combination of short and long-reads. Genome
721 characterization of functional potential and taxonomic classification was conducted on the
722 isolates. Genes involved in cysteine degradation were searched in the isolates, and a five-year
723 metagenomic time series of Lake Mendota (2008-2012).

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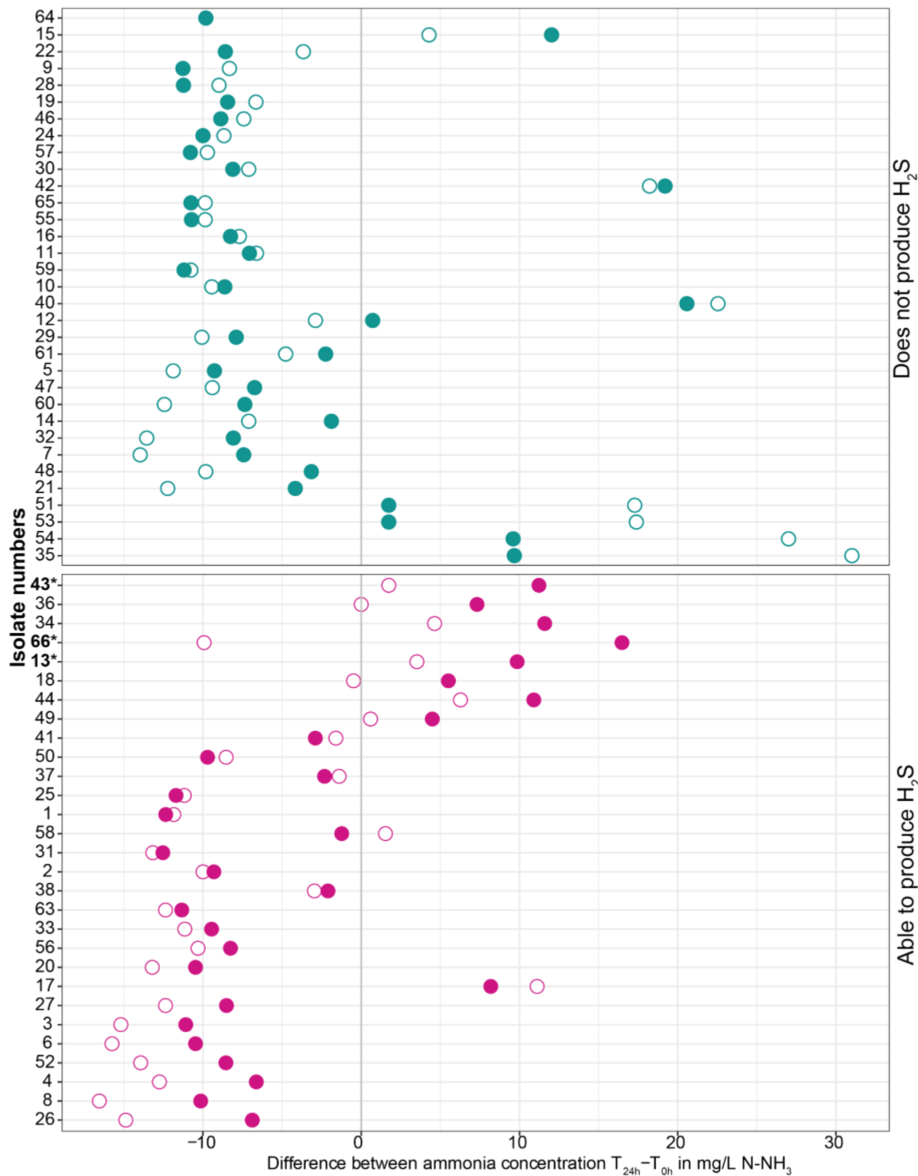

724

725 **Figure 2. Qualitative production of hydrogen sulfide among microbial isolates enriched**
726 **from a freshwater lake water column.** Filled circles are isolates when grown with cysteine, and
727 open circles are isolates when grown without cysteine. The vertical line represents values
728 corrected for the control (natural ammonia production/consumption in the negative control). All
729 points to the right of the vertical lines indicate a production of ammonia, and all points to the left
730 of the vertical lines refer to those that consumed ammonia after 24 hours. The isolates #43, #13
731 and #66 were selected for further analysis.

732

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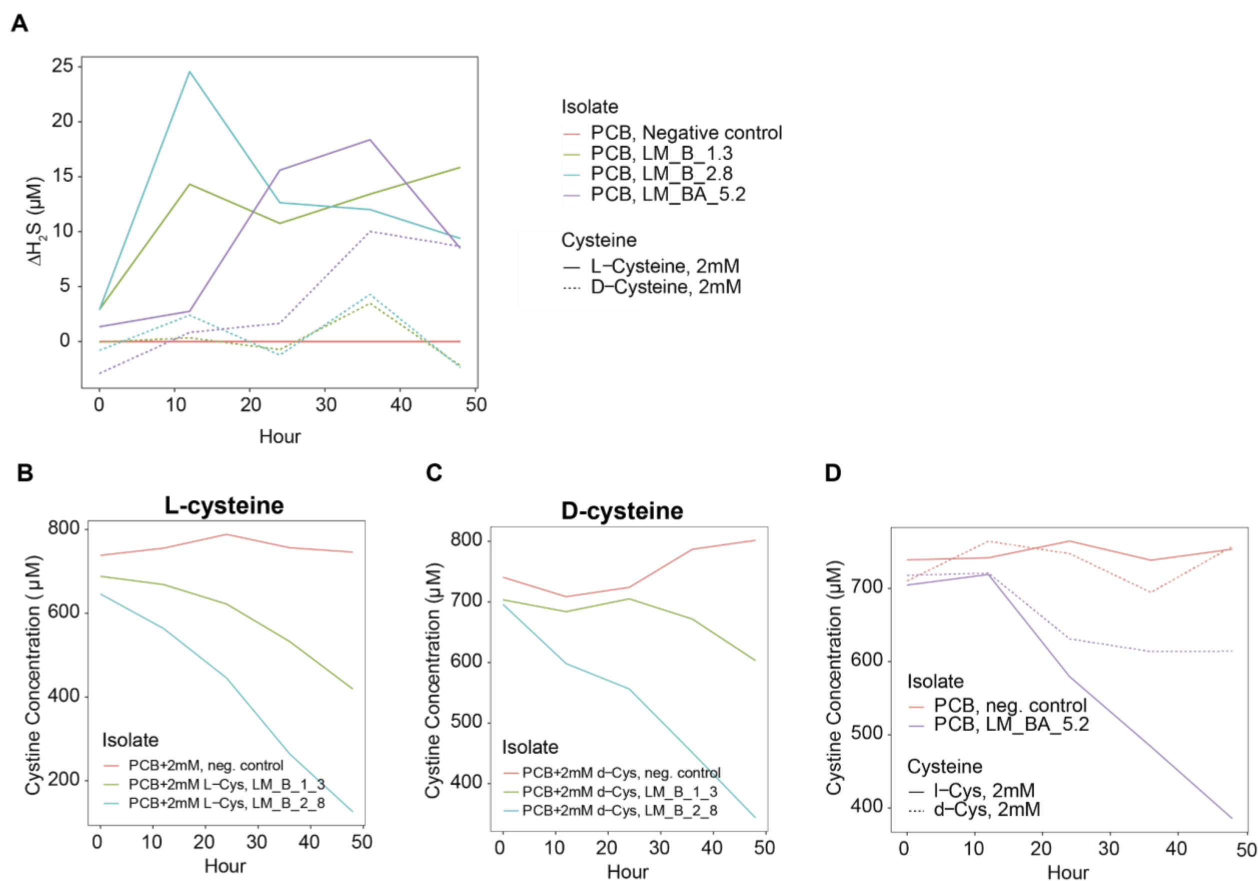

733

734 **Figure 3. Further characterization of the three isolates and demonstration of cysteine**
735 **degradation and H_2S production.** A. Higher amounts of H_2S was produced by the three isolates
736 over 50 hours compared to negative controls. B, C, D. Identification of different forms of
737 cysteine that can be degraded. L-cysteine decreased in all isolates compared to the control (B,
738 D). D-cysteine also decreased over time in all except the negative control, however, the net
739 amount decreased was less compared to L-cysteine. Cysteine concentrations were measured as
740 Cystine as described in the methods. Because Isolate #13 and #43 were assessed in another
741 experiment than #66, but using the exact same instruments and methods, plots are separated by
742 HPLC runs. Due to large sample volume it was not possible to test all isolates and conditions in
743 one HPLC run.

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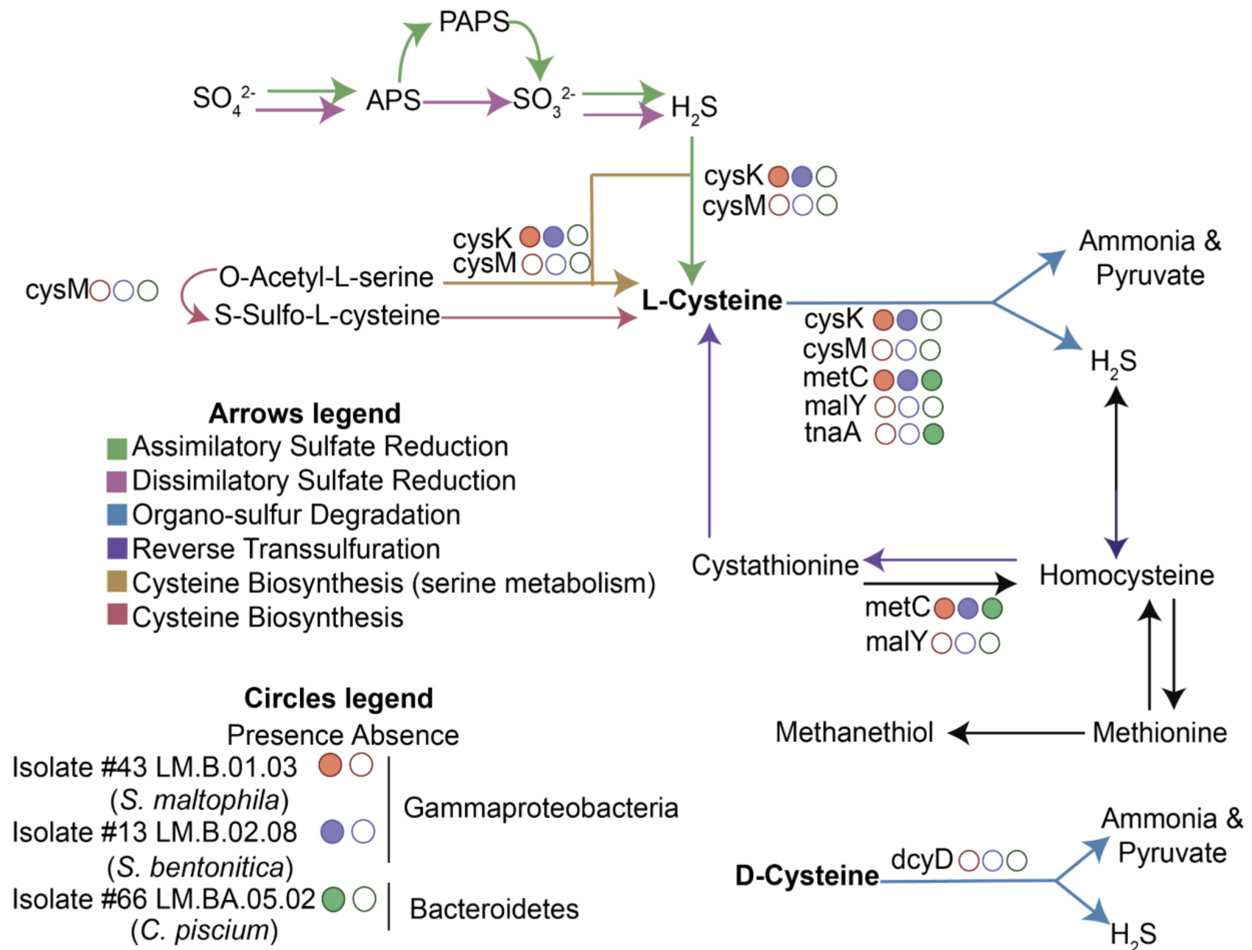

745

746 **Figure 4. Diagram showing pathways for sulfur and organosulfur metabolism**. Several
747 pathways for hydrogen sulfide and cysteine production exist in microorganisms. The
748 presence/absence of key genes (cysK, cysM, malY, cysM, metC, tnaA, sseA, aspB, and dcyD
749 along the blue arrow) in the three isolate's genomes are shown by filled (present) circles, and
750 clear (not present) circles.

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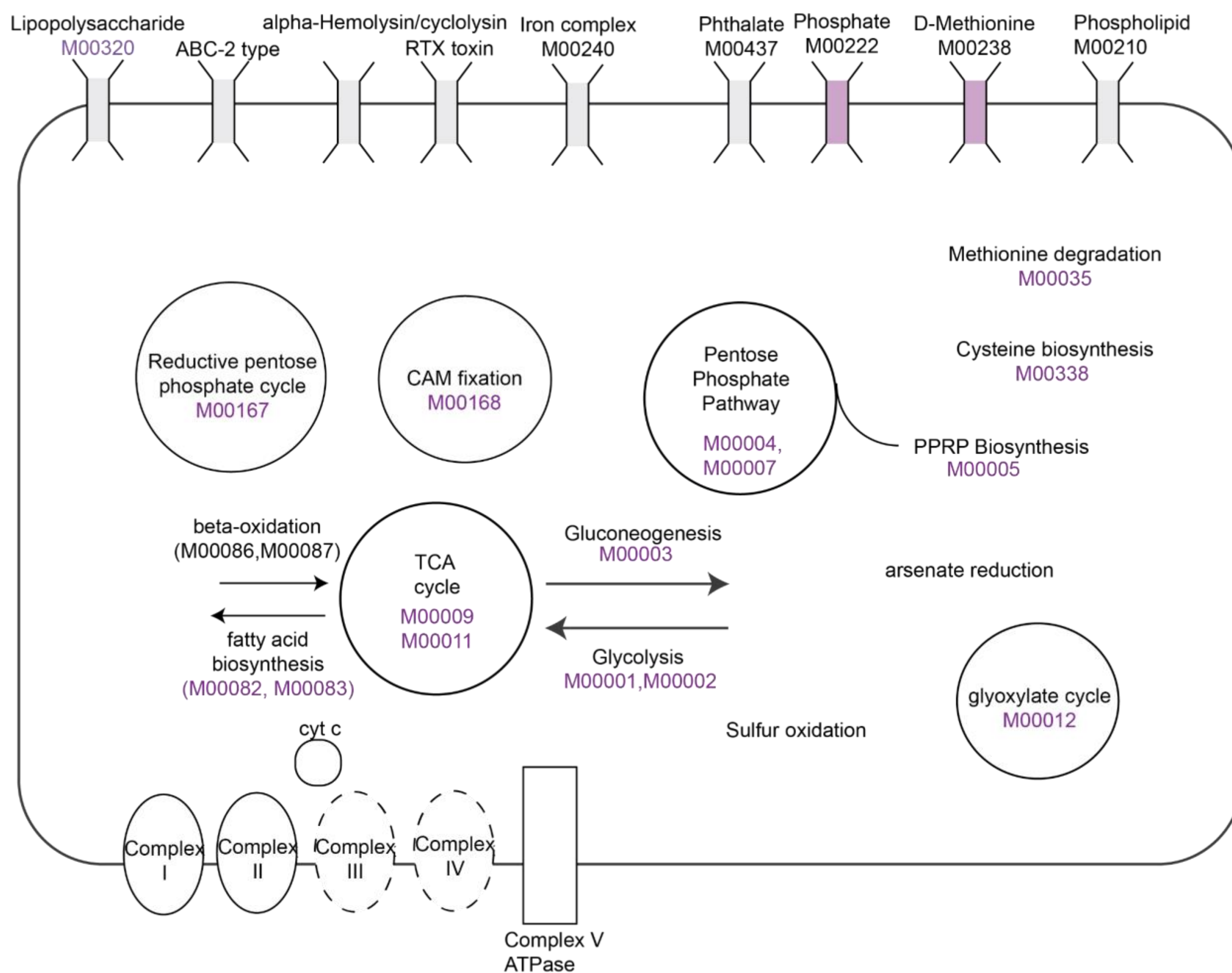

754

755 **Figure 5A. Cellular map showing the pathways, genes, cycles and transporters which were**
756 **common to all three isolates.** A complete list is found in **Table S6**. The KEGG module
757 identifiers are listed in purple whenever relevant.

758

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759

760

761 **Figure 5B. Heat map showing selected metabolic functions and pathways in the three**

762 **isolates.** A complete list is found in **Table S6**.

763

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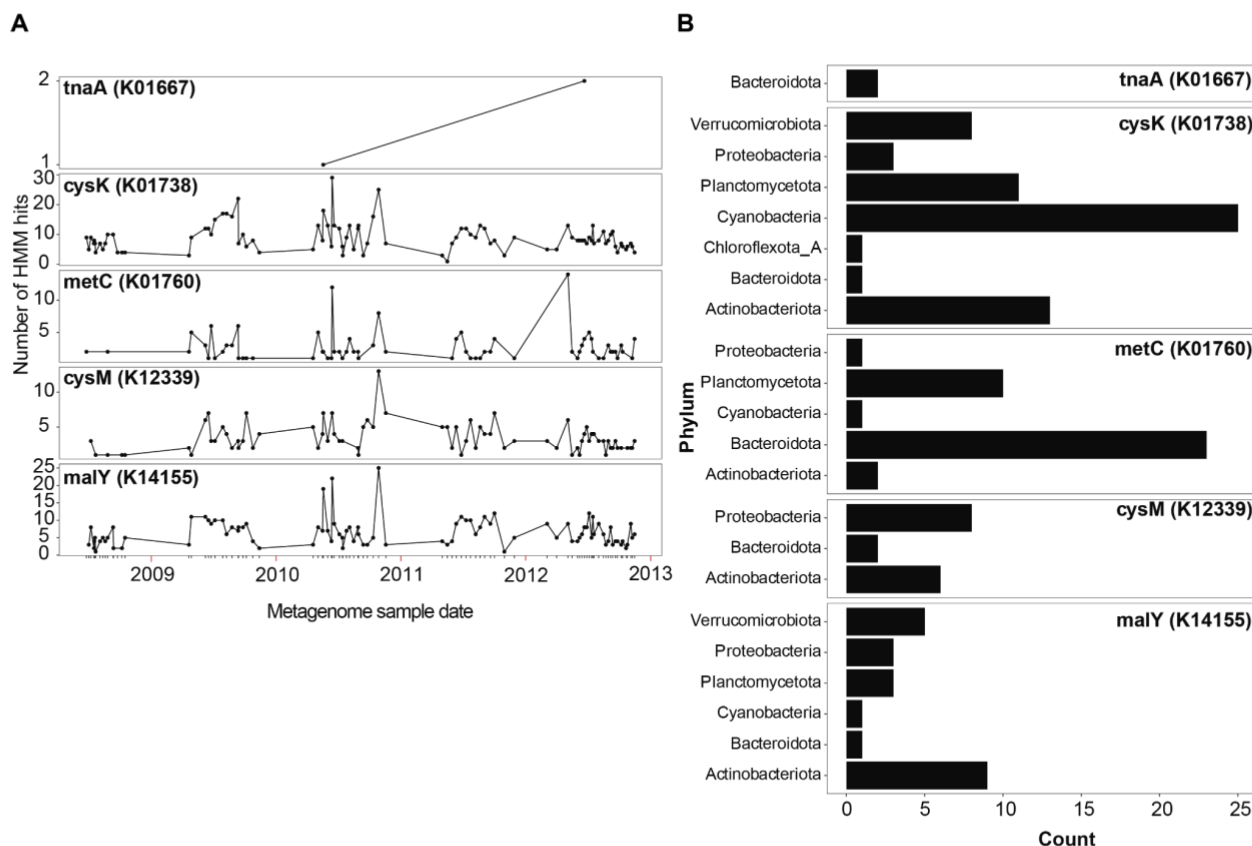

765 **Figure 6.** A. **Number of amino acid coding sequences matching one of the cysteine genes**
766 **searched (5 genes) which may have the enzymatic activity of degrading cysteine into**
767 **ammonia, pyruvate and hydrogen sulfide**. We also searched for dcyD (6th gene) but did not
768 identify it in any sample. B. **Presence of these 5 genes in the binned metagenome-assembled**
769 **metagenomes of the same time series**.

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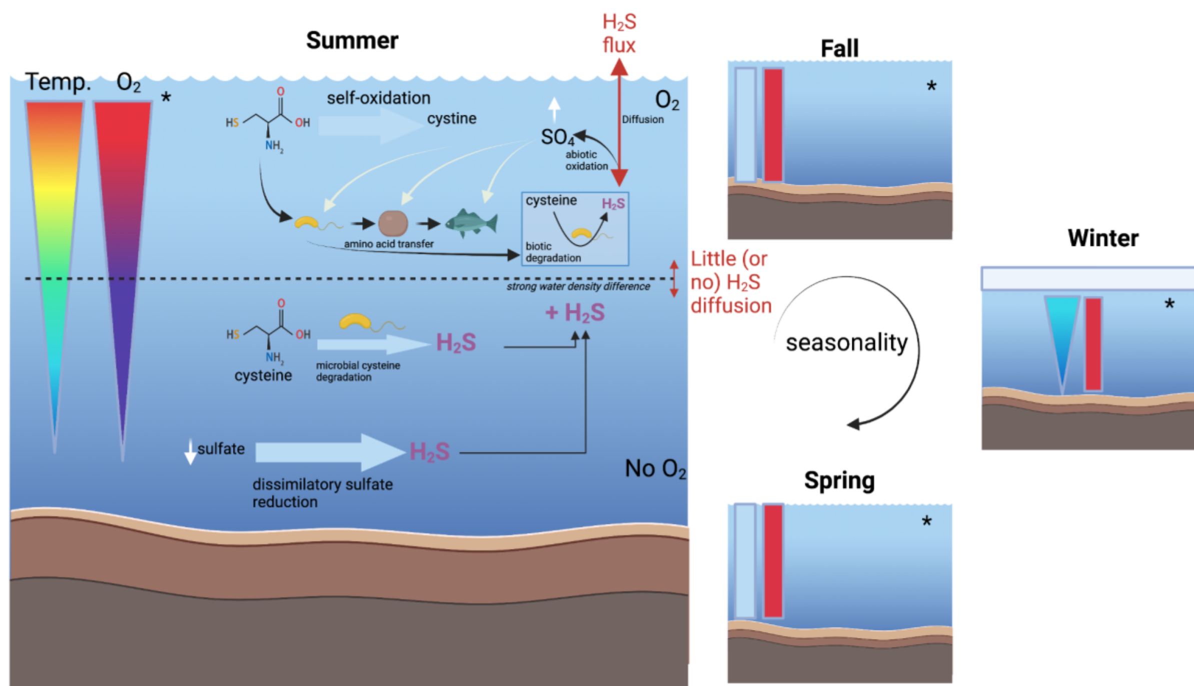

771

772 **Figure 7. Conceptual figure showing the potential relevance of cysteine degradation in a**
773 **freshwater lake environment, with respect to oxygen availability and seasonality.**
774 Oxygenated seasons and part of the lake water columns are shown with an asterix. Significant
775 research gaps include cysteine concentrations in the natural environment over time, H_2S fluxes
776 across different layers in the lake water column, and contribution of different H_2S sources in the
777 hypolimnion.