

1 **Environmental predictors of electroactive bacterioplankton**

2 **in small boreal lakes.**

3

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19 ABSTRACT

20 Extracellular electron transfer (EET) by electroactive bacteria in anoxic soils and
21 sediments is an intensively researched subject, but EET's function in planktonic ecology has
22 been less considered. Following the discovery of an unexpectedly high prevalence of EET genes
23 in a bog lake's bacterioplankton, we hypothesized that the redox capacities of dissolved organic
24 matter (DOM) enrich for electroactive bacteria by mediating redox chemistry. We developed the
25 bioinformatics pipeline FEET (Find EET) to identify and summarize EET proteins from
26 metagenomics data. We then applied FEET to several bog and thermokarst lakes and correlated
27 EET protein occurrence values with environmental data to test our predictions. Our results
28 provide evidence that DOM participates in EET by bacterioplankton. We found a similarly high
29 prevalence of EET genes in most of these lakes, where oxidative EET strongly correlated with
30 DOM. Numerous novel clusters of multiheme cytochromes that may enable EET were identified.
31 Taxa previously not considered EET-capable were found to carry EET genes. We conclude that
32 EET and DOM interactions are of major ecological importance to bacterioplankton in small
33 boreal lakes, and that EET, particularly by methylotrophs and phototrophs, should be further
34 studied and incorporated into both conceptual and quantitative methane emission models of
35 melting permafrost.

36

37 INTRODUCTION

38 Aquatic microbes influence Earth's atmosphere directly and indirectly through
39 fermentation, respiration, photosynthesis, and other metabolism all requiring the transfer of
40 electrons. Water provides a medium for integrating solutes involved in these processes, yielding
41 high metabolic turnover at rates that make water bodies hubs for the landscape's biogeochemical
42 processing. We present evidence that in small freshwater boreal lakes planktonic metabolism
43 may often be more connected than previously realized—electroactively through extracellular
44 electron transfer (EET). EET is the conduction of electrons through the outermost membranes of
45 cells, allowing metabolism like respiration to make use of solute or solid electron acceptors
46 without having to transport them into the cell.

47 In and on structured environments such as rocks, soils, sediments, and conductive
48 surfaces there is ample evidence to demonstrate that, ubiquitously, electroactive organisms can
49 be found using EET to connect internal electrochemistry to the external environment (Coates *et*
50 *al.*, 2002; Roden *et al.*, 2010; Tang *et al.*, 2019; McAllister *et al.*, 2020; Keffer *et al.*, 2021;
51 Rowe *et al.*, 2021) or to other bacteria (Hegler *et al.*, 2008; Shi *et al.*, 2016; Beyenal *et al.*, 2017;
52 Ishii *et al.*, 2018). This led studies to focus on electron transfer to solid-phase substances.
53 However, the ability of bacteria to reduce soluble electroactive substances is well-known, and
54 this raises the possibility of their use as substrates for EET rather than as mediators or electron
55 shuttles to a solid substrate (Lovley *et al.*, 1991; Bond and Lovley, 2005; Li *et al.*, 2019). For
56 example, a surprisingly high number of genes encoding multiheme cytochromes (MHCs) and
57 other putative EET proteins were found in bacteria inhabiting the water column of Trout Bog
58 Lake, a humic lake in WI, USA, and researchers pointed to the high electron accepting capacity
59 of the dissolved organic matter (DOM) as being a possible explanation (He *et al.*, 2019). Smaller

60 water bodies have a higher surface area to volume ratio, so they tend to maintain a higher DOM
61 concentration. Trout Bog Lake is also influenced by a dense Sphagnum mat surrounding the
62 open water, which leaches DOM with high humic content (Maizel *et al.*, 2017) that we expect to
63 support the high electron accepting capacity. While some EET proteins in Trout Bog might be
64 relics of ancient metabolisms, their abundance across genera instead suggests active use. The
65 aforementioned observations and the scarcity of studies on EET in the water columns of lakes
66 led to our suspicions that there may be more electroactive metabolism(s) in small water bodies
67 than currently realized.

68 External electron acceptors like Fe(III), other metals, and complex forms of DOM are
69 known substrates for heterotrophic EET as a terminal electron acceptor for respiration, in other
70 words, electrogenesis or reductive EET (redEET) (Lovley *et al.*, 1999; Lipson *et al.*, 2010;
71 Roden *et al.*, 2010). All such acceptors might be used at some frequency planktonically in Trout
72 Bog. EET can also be used to access external electron donors (Ross *et al.*, 2011; Rowe *et al.*,
73 2015), for example, as an electron source for reducing CO₂ into biomass. Phototrophic isolates
74 like some *Rhodopseudomonas palustris* strains are capable of electrotrophy or oxidative EET
75 (oxiEET), accepting extracellular electrons from Fe(II) and electrodes, and some have been
76 shown to be capable of growing by cryptically cycling photoreduced iron–organic matter
77 complexes (Guzman *et al.*, 2019; Peng *et al.*, 2019). Cryptic cycling, defined as transformations
78 unobserved due to quick turnover, likely occurs any time there is a regenerable redox-active
79 substance, whether *via* photoreduction, across steep oxygen gradients in stratified systems, or *via*
80 commensal metabolisms. EET may enable cryptic electron cycling directly and through
81 otherwise inaccessible extracellular chemicals like some DOM. However, DOM-involved EET

82 has remained largely unconsidered with a few notable exceptions (Berg *et al.*, 2016; Lau *et al.*,
83 2017; He *et al.*, 2019; Li *et al.*, 2020) in planktonic systems.

84 A metagenomic study showed that dominant planktonic phototrophs of the class
85 Chlorobia in Trout Bog contain multiple copies the extracellular Fe(II)-oxidizing protein Cyc2
86 (He *et al.*, 2019), much like many of its cultured and uncultured and globally dispersed relatives
87 (He, Barco, *et al.*, 2017; Garcia *et al.*, 2021). However, it is difficult to determine what these
88 uncultured Chlorobia are predominantly using for an electron donor because of their various
89 oxidoreductases. They could potentially use sulfide, hydrogen, Fe(II), or reduced DOM via
90 cryptic cycling of any or all of these substances. If, as it seems in Trout Bog, other lakes have
91 phototrophic or chemotrophic electrotrophs living alongside electrogens and especially if DOM
92 is a substrate or mediator for electrotrophs, then there may be an unconsidered regime of often-
93 cryptic metabolism occurring in small lakes all across the globe.

94 Regardless of whether the electron exchange inferred from the high abundance of
95 oxidative and reductive EET proteins in Trout Bog bacteria occurs directly with DOM or through
96 DOM–metal cycling, questions remain: Are other similar bodies of water as inundated with
97 EET-capable microbes, and is it related to DOM concentrations or quality? How might
98 planktonic metabolisms such as anoxygenic photosynthesis, respiration, and others connect
99 through EET? What environmental parameters lead to favorable conditions for planktonic EET
100 metabolism—DOM quantity, iron, sulfur? To get a foothold on these questions, we performed a
101 broad metagenomics analysis.

102 We hypothesized that DOM enriches for planktonic electroactive microorganisms in
103 small boreal lakes by acting as a substrate and mediator for EET (Fig. 1). We therefore predicted
104 that one would observe a significant positive correlation between a given lake’s DOC (dissolved

105 organic carbon, a measure of DOM quantity) and the propensity of bacteria in that lake to house
106 EET genes. To test this hypothesis, we correlated metagenomic and environmental
107 characteristics of non-sediment, planktonic samples from 36 small boreal freshwater lakes, such
108 as bog lakes and thermokarst lakes, in relation to EET genes and their prevalence across the
109 inhabiting diversity of bacteria.

110

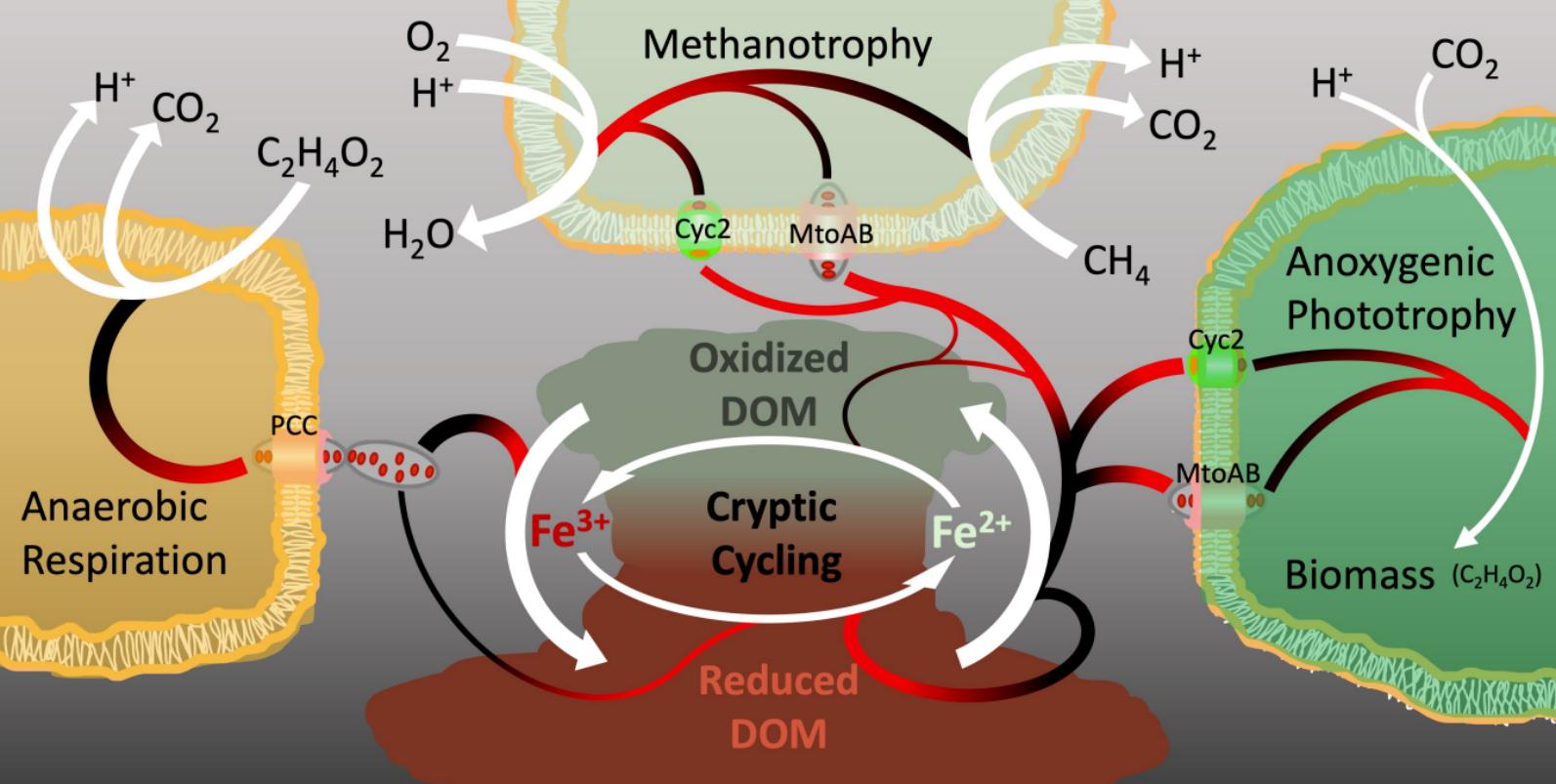


Figure 1. Conceptualized cryptic electron cycling and putative role in methane metabolism in small boreal lake water columns. Chemical transformations are shown with white arrows. Electrons flow from black to red.

111 RESULTS

112 **Putative EET proteins were found in roughly half of all bacterial genomes analyzed**
113 **from planktonic boreal lake samples.** We compiled a dataset of 5569 Metagenome-Assembled
114 Genomes (MAGs), each over 50% complete and less than 10% contaminated, from 190
115 metagenomic assemblies from the water columns of 36 small boreal lakes in order to compare
116 the planktonic representation of EET genes in lakes of similar yet contrastable features. These
117 included thermokarst ponds of various ages from three to 60 years since thaw, sheltered bog
118 lakes characterized by *sphagnum* moss growth in a surrounding bog mat, and a range of other
119 small boreal lakes (Supp. Tbl. 1). The original DNA samples were collected from various water
120 column depths representing niches defined by light, temperature, and redox status. The MAGs
121 were clustered by 95% ANI (average nucleotide identity) to form 2552 metagenomic Operational
122 Taxonomic Units (mOTUs) (Buck *et al.*, 2021). We then defined “OTUs” to be lake-specific (i.e.
123 counting each mOTU once per lake), yielding 3030 OTUs.

124 We developed the computational workflow “Find EET” (FEET) to identify putative EET
125 protein-encoding genes within genomes (hereafter called EET+ organisms, acknowledging that
126 the actual EET capability of each organism must be verified experimentally). The workflow is
127 based on the bioinformatics tool FeGenie (Garber *et al.*, 2020) but adds an automated version of
128 the outer-surface MHC and porin–cytochrome *c* protein complex (PCC)-identification approach
129 described previously (He *et al.*, 2019). Among the 3030 OTUs from the full dataset, 50%
130 contained at least one protein found by FEET, and 40% had two or more. We note that a few of
131 FeGenie’s Hidden Markov Model (HMM) (Baum and Petrie, 1966) hits should not be counted as
132 promising EET evidence when found by themselves, including DFE variants and FoxABCYZ

133 (Deng *et al.*, 2018; Garber *et al.*, 2020), which leaves 47% of OTUs with at least one protein that
134 may enable EET. However, these numbers may be higher given more complete genomes.

135 The ability to transfer electrons to substances outside the cell is likely to co-occur with
136 other strategies used for redox cycling. Thus, we asked which other inorganic electron donors
137 and acceptors might be accessible to the EET+ organisms. The bioinformatic pipeline
138 METABOLIC uses HMMs to make such predictions (Zhou *et al.*, 2022) and showed that EET
139 was not the only method for redox metabolism, in the form of non-EET oxidoreductases (Supp.
140 Tbl. 3), genomically available to about 98% of our EET+ bacterial OTUs. However, EET was
141 the only identified inorganic pathway for either oxidative or reductive redox metabolism for one
142 or both sides of an OTU's redox metabolism in 11% of these OTUs (Supp. Tbl. 6).

143 **The capacity for EET in water columns of small boreal lakes is significantly**
144 **correlated to metabolic and environmental factors.** All future mentions of correlation are
145 positive unless stated otherwise. The variation in overall number and diversity of EET genes
146 found in each lake made us curious about which organismal and environmental characteristics
147 were associated with EET capacity. To quantify the propensity of a system to select for EET+
148 organisms, we calculated the ratio of EET+ OTUs relative to the total number of OTUs found in
149 each lake. We also calculated the average number of EET proteins per OTU as a proxy for the
150 propensity of a system to enrich for proficiency, redundancy, or flexibility in EET metabolism.
151 By both proxies, oxiEET in water columns of boreal lakes was strongly and significantly
152 correlated to DOM concentration (Fig. 2A). Methane concentration was slightly but significantly
153 correlated to the average number of redEET proteins per OTU and to the ratio and number of
154 oxiEET proteins. We observed several other expected and unexpected correlations (Fig. 2A,
155 Supp. Fig. 5&6), as discussed below.

A

TotalEETRatio	.105 (30)	.407 (30)	.972 (32)	.002 (28)	.112 (36)	.379 (23)	.028 (36)	.047 (36)	.225 (22)	.615 (23)	.000 (27)
TotalEET/OTU	.000 (30)	.078 (30)	.106 (32)	.040 (28)	.000 (36)	.291 (23)	.000 (36)	.562 (36)	.412 (22)	.915 (23)	.325 (27)
RedNoNEETRatio	.096 (30)	.202 (30)	.057 (32)	.173 (28)	.069 (36)	.933 (23)	.000 (36)	.562 (36)	.365 (22)	.572 (23)	.544 (27)
RedNoNEET/OTU	.004 (30)	.095 (30)	.039 (32)	.422 (28)	.002 (36)	.310 (23)	.000 (36)	.696 (36)	.154 (22)	.837 (23)	.895 (27)
OxiEETRatio	.021 (30)	.914 (30)	.185 (32)	.000 (28)	.168 (36)	.971 (23)	.255 (36)	.003 (36)	.365 (22)	.632 (23)	.001 (27)
OxiEET/OTU	.021 (30)	.876 (30)	.331 (32)	.000 (28)	.277 (36)	.575 (23)	.098 (36)	.000 (36)	.643 (22)	.962 (23)	.006 (27)
NitrMHCEETRatio	.531 (30)	.026 (30)	.160 (32)	.479 (28)	.019 (36)	.168 (23)	.155 (36)	.068 (36)	.327 (22)	.011 (23)	.718 (27)
NitrMHCEET/OTU	.133 (30)	.965 (30)	.014 (32)	.481 (28)	.306 (36)	.743 (23)	.000 (36)	.505 (36)	.007 (22)	.127 (23)	.497 (27)
NovelEETRatio	.531 (30)	.026 (30)	.160 (32)	.479 (28)	.019 (36)	.168 (23)	.155 (36)	.068 (36)	.327 (22)	.011 (23)	.718 (27)
NovelEET/OTU	.002 (30)	.001 (30)	.077 (32)	.405 (28)	.000 (36)	.512 (23)	.015 (36)	.036 (36)	.700 (22)	.411 (23)	.854 (27)

C

TotalEETRatio	.031 (36)	.037 (36)	.200 (36)	.104 (36)	.349 (36)	.004 (36)	.189 (36)	.189 (36)	.481 (36)	.523 (36)	.001 (36)	.001 (36)	.595 (36)	.634 (36)	.035 (36)	.008 (36)	.074 (36)	.166 (36)	.373 (36)	.697 (36)	.018 (36)	.025 (36)	.373 (36)	.700 (36)
TotalEET/OTU	.002 (36)	.038 (36)	.149 (36)	.544 (36)	.710 (36)	.003 (36)	.170 (36)	.170 (36)	.181 (36)	.128 (36)	.072 (36)	.191 (36)	.687 (36)	.939 (36)	.047 (36)	.460 (36)	.540 (36)	.832 (36)	.052 (36)	.144 (36)	.005 (36)	.005 (36)	.059 (36)	.165 (36)
RedNoNEETRatio	.000 (36)	.003 (36)	.270 (36)	.379 (36)	.291 (36)	.012 (36)	.083 (36)	.083 (36)	.030 (36)	.024 (36)	.005 (36)	.030 (36)	.504 (36)	.332 (36)	.001 (36)	.045 (36)	.087 (36)	.487 (36)	.197 (36)	.256 (36)	.014 (36)	.083 (36)	.128 (36)	.209 (36)
RedNoNEET/OTU	.001 (36)	.010 (36)	.071 (36)	.706 (36)	.550 (36)	.023 (36)	.164 (36)	.164 (36)	.045 (36)	.031 (36)	.125 (36)	.248 (36)	.807 (36)	.596 (36)	.016 (36)	.392 (36)	.424 (36)	.953 (36)	.077 (36)	.087 (36)	.019 (36)	.046 (36)	.069 (36)	.097 (36)
OxiEETRatio	.184 (36)	.556 (36)	.232 (36)	.113 (36)	.287 (36)	.000 (36)	.777 (36)	.777 (36)	.894 (36)	.757 (36)	.060 (36)	.062 (36)	.355 (36)	.623 (36)	.452 (36)	.348 (36)	.177 (36)	.335 (36)	.916 (36)	.596 (36)	.017 (36)	.003 (36)	.941 (36)	.502 (36)
OxiEET/OTU	.143 (36)	.888 (36)	.421 (36)	.106 (36)	.457 (36)	.000 (36)	.629 (36)	.629 (36)	.938 (36)	.754 (36)	.091 (36)	.170 (36)	.215 (36)	.480 (36)	.471 (36)	.691 (36)	.340 (36)	.621 (36)	.705 (36)	.756 (36)	.009 (36)	.001 (36)	.735 (36)	.767 (36)
NitrMHCEETRatio	.018 (36)	.080 (36)	.635 (36)	.479 (36)	.426 (36)	.408 (36)	.868 (36)	.868 (36)	.529 (36)	.402 (36)	.026 (36)	.178 (36)	.731 (36)	.525 (36)	.032 (36)	.212 (36)	.436 (36)	.541 (36)	.026 (36)	.072 (36)	.704 (36)	.917 (36)	.037 (36)	.094 (36)
NitrMHCEET/OTU	.271 (36)	.826 (36)	.467 (36)	.753 (36)	.989 (36)	.449 (36)	.190 (36)	.190 (36)	.913 (36)	.945 (36)	.214 (36)	.244 (36)	.957 (36)	.817 (36)	.621 (36)	.644 (36)	.232 (36)	.065 (36)	.000 (36)	.010 (36)	.143 (36)	.469 (36)	.000 (36)	.006 (36)
NovelEETRatio	.018 (36)	.080 (36)	.635 (36)	.479 (36)	.426 (36)	.408 (36)	.868 (36)	.868 (36)	.529 (36)	.402 (36)	.026 (36)	.178 (36)	.731 (36)	.525 (36)	.032 (36)	.212 (36)	.436 (36)	.541 (36)	.026 (36)	.072 (36)	.704 (36)	.917 (36)	.037 (36)	.094 (36)
NovelEET/OTU	.132 (36)	.094 (36)	.099 (36)	.613 (36)	.412 (36)	.727 (36)	.706 (36)	.706 (36)	.402 (36)	.391 (36)	.583 (36)	.915 (36)	.853 (36)	.852 (36)	.297 (36)	.777 (36)	.822 (36)	.674 (36)	.093 (36)	.271 (36)	.229 (36)	.290 (36)	.157 (36)	.377 (36)

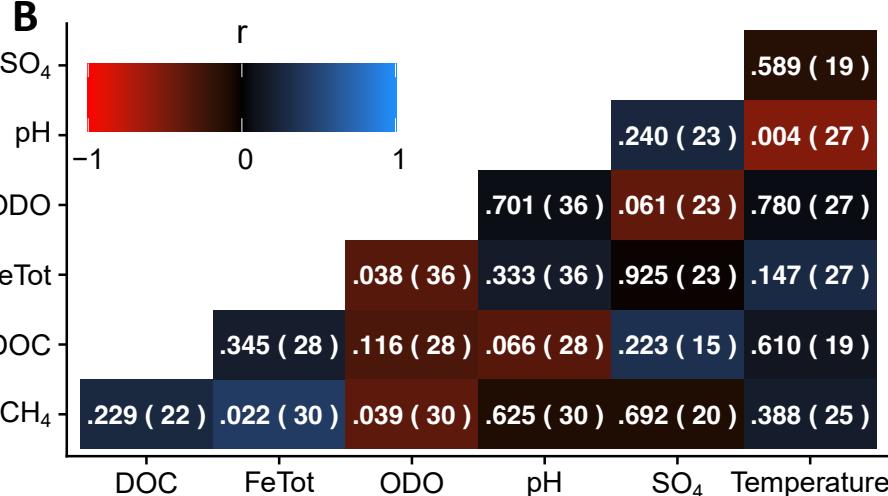
B

Figure 2. Correlations between environmental parameters (B) and EET values (A) and other oxidoreductase values (C) of 36 boreal lakes.

Categories of protein values are delineated by "Ratio" or "/OTU" respectively standing for the ratio of OTUs with at least one of the given kind of protein or the average per OTU. Other values represent averages over available samples and data. For bog lakes involved in Long Term Ecological Research (Iter. limnology.wisc.edu), recent (2018–2020) environmental data was subsampled by depth. Heatmap color represents Pearson correlation coefficient, r . Unadjusted p-values are out of $N = 25$ available corelates. Yellow text indicates a significance of $p < 0.05$ when adjusted by the Benjamini-Hochberg method. Statistical values and heatmaps were generated with the program R. Non-EET oxidoreductases were evaluated using METABOLIC (Zhou *et al.*, 2022) whereas EET proteins were evaluated with the FEET pipeline. Category titles mean as follows: "TotalEET" = any putative EET protein; "RedNoNEET" = EET expected for reductive metabolism but excluding putative nitrate or nitrite reductase MHCs; "OxiEET" = EET expected to involve oxidative metabolism; "NitrMHCEET" = nitrate or nitrite reductases but only those that are identified as MHCs; "Novel" = outer membrane MHCs that were not classified as nor were 80% similar to any known EET protein; "C1Oxi" = single carbon compound oxidation; "NitrRedNonMHC" = Nitrate or Nitrite reductases that were not identified by FEET to be MHCs; "FeTot" = total iron and/or Fe(II) + Fe(III) measurements; "ODO" = optical dissolved oxygen; "DOC" = dissolved organic carbon. Included HMMs and full category descriptions are listed in Supplementary Table 3.

156 **We observed putative EET proteins in most small boreal lake methylotrophs,**
157 **especially methanotrophs, and these often coincided with carbon and nitrogen fixation**
158 **proteins.** Of the 121 Methylococcales OTUs (taxonomically expected methanotrophs) 83%, or
159 88% of those that were EET+, housed Cyc2 or another putative oxiEET protein. Of the 113
160 *Methylophilaceae* OTUs (expected methylotrophs) 58%, or 65% of EET+, had oxiEET. One
161 possible usage of electrons is to support carbon and nitrogen fixation. Of the Methylococcales
162 with oxiEET, 51% harbored nitrogen fixation marker proteins, and another 8% had carbon
163 fixation markers, whereas only 35% of the few without oxiEET had N-fixation markers and none
164 had C-fixation markers. Only one *Methylophilaceae* OTU had a N-fixation marker, and it did not
165 have oxiEET. Of those with oxiEET, however, 12% did have C-fixation indicator proteins. Of
166 the 16% of EET+ Methylococcales that had multiple redEET proteins as defined (Supp. Tbl. 4),
167 these proteins were generally DFE variants or MtrB with MtoA; so, neither specifically indicates
168 EET-mediated respiration (Hartshorne *et al.*, 2009; Liu *et al.*, 2012). The porin MtrB is defined
169 as both redEET and oxiEET because, with our methods, it indistinguishable from the MtoB porin
170 (Supp. Tbl. 4). Only two *Methylophilaceae* had methane monooxygenases, and both were EET+.
171 Of Methylococcales with oxiEET proteins 82% had a methane monooxygenase, and of those
172 without oxiEET, 60% had a methane monooxygenase (Supp. Tbl 6). We note that some MAGs
173 within *Methylophilaceae* and Methylococcales were reported as EET+ (Tanaka *et al.*, 2018; He
174 *et al.*, 2019; Tsuji *et al.*, 2020; Yang *et al.*, 2020 – respectively found: *Methylococcus capsulatus*
175 redEET, *Methylotenera* oxiEET, *Methylomonadaceae* oxiEET, *Methylophilus* redEET).

176 **EET proteins in small boreal lakes were found in bacterial groups not yet**
177 **documented to exhibit electroactivity.** Historically familiar lake bacteria including
178 *Polynucleobacter* and *Limnohabitans* (Newton *et al.*, 2011) and several other taxa not previously

179 considered to be capable of EET were represented by OTUs with proteins that strongly indicate
180 EET metabolism (Fig. 3, Supp. Tbl. 2). Three Nanopelagicales (aciI Actinobacteria) out of 358
181 Actinobacteria OTUs had between one and three EET proteins. As noted above, we were not the
182 first to identify EET+ members of Methylococcales and *Methylophilaceae*, but we also identified
183 additional EET+ taxa like *Methyloimonas*, *Methylovulum*, *Methylopumilus*, and other
184 *Methylococcaceae*. All the EET+ taxa are summarized in Supplementary Table 2 (Ehrenreich
185 and Widdel, 1994; Suzuki *et al.*, 2006; Croal *et al.*, 2007; Qiu *et al.*, 2008; Karthikeyan *et al.*,
186 2012; Johnson *et al.*, 2014; Li *et al.*, 2015; Lino *et al.*, 2015; Yu *et al.*, 2015; He, Barco, *et al.*,
187 2017; He, Stevens, *et al.*, 2017; Eichorst *et al.*, 2018; Jiang-Hao *et al.*, 2019; Kjeldsen *et al.*,
188 2019; Yang *et al.*, 2020; Koskue *et al.*, 2021), including more that have not been previously
189 considered capable of EET.

190 **606 novel putative EET Multiheme Cytochrome protein clusters were identified in**
191 **taxa previously known and unknown to be electroactive (Fig. 3).** We refer hereafter to these
192 EET MHC clusters as “novel clusters.” For each novel cluster (Supp. Tbl. 5) we have no
193 evidence for homology with each other, MHC nitrate nor nitrite reductases, nor any known EET
194 protein, using a cutoff of 20% amino acid sequence identity. This cutoff was chosen as a coarse
195 balance between maintaining likely semblance in structure or function within clusters yet having
196 a useful level of grouping for analysis. Taxa in which novel clusters were found include the
197 following (numerically summarized in Supp. Tbl. 2, Fig. 3, Supp. Fig. 1).

198 Members of the phylum Myxococcota (Supp. Tbl. 2) comprised the top several taxa with
199 the highest average number of novel clusters, closely followed by Geobacterales,
200 Desulfocapsaceae, and then Geothrix. Accompanying the Myxococcota novel clusters was a mix
201 other EET proteins like DFE variants, MtrC, ExtABC, CbcL, and Cyc2. Notably, EET has been

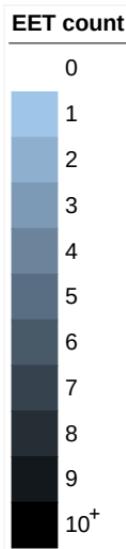
202 experimentally demonstrated in the Myxococcota member Anaeromyxobacteraceae (Marshall *et*
203 *al.*, 2009).

204 Members of Planctomycetota including Phycisphaerae and especially Planctomycetes,
205 the class that includes annamox bacteria which were very recently shown to be capable of EET
206 (Shaw *et al.*, 2020), were found containing one or several novel clusters and often with no other
207 identifiable EET protein, though occasionally—and more so with Phycisphaerae—DFE variants
208 or CbcL. However, none of the Planctomycetota were found to have characteristic annamox
209 proteins, HzoA or HzsA.

210 The cosmopolitan and freshwater-specific Nanopelagicales contributed OTUs carrying
211 novel clusters. The common freshwater genus *Limnohabitans* contributed OTUs with one novel
212 cluster accompanied by either Cyc2 or MtoA and MtrB. The cosmopolitan anoxic phototroph
213 Chlorobia contributed an OTU with a novel cluster and Cyc2. Numerous other instances of novel
214 clusters distributed in taxa are summarized (Supp. Tbl. 5).

215
216

Tree scale: 1



p_Patescibacteria
p_Chloroflexota
p_Actinobacteriota
p_Armatimonadota
p_Cyanobacteria
p_Elusimicrobiota
p_Firestonebacteria
p_Omnitrophota
p_Planktomycetota
p_Verrucomicrobiota
p_Gemmatimonadota
c_Chlorobia
p_Bacteroidota
p_Acidobacteriota
p_Bdellovibrionota
p_Myxococcota
p_Desulfuromonadota
p_Campylobacterota
c_Alphaproteobacteria
c_Gamaproteobacteria
o_Methylcoccales
f_Methylphilaceae
Other

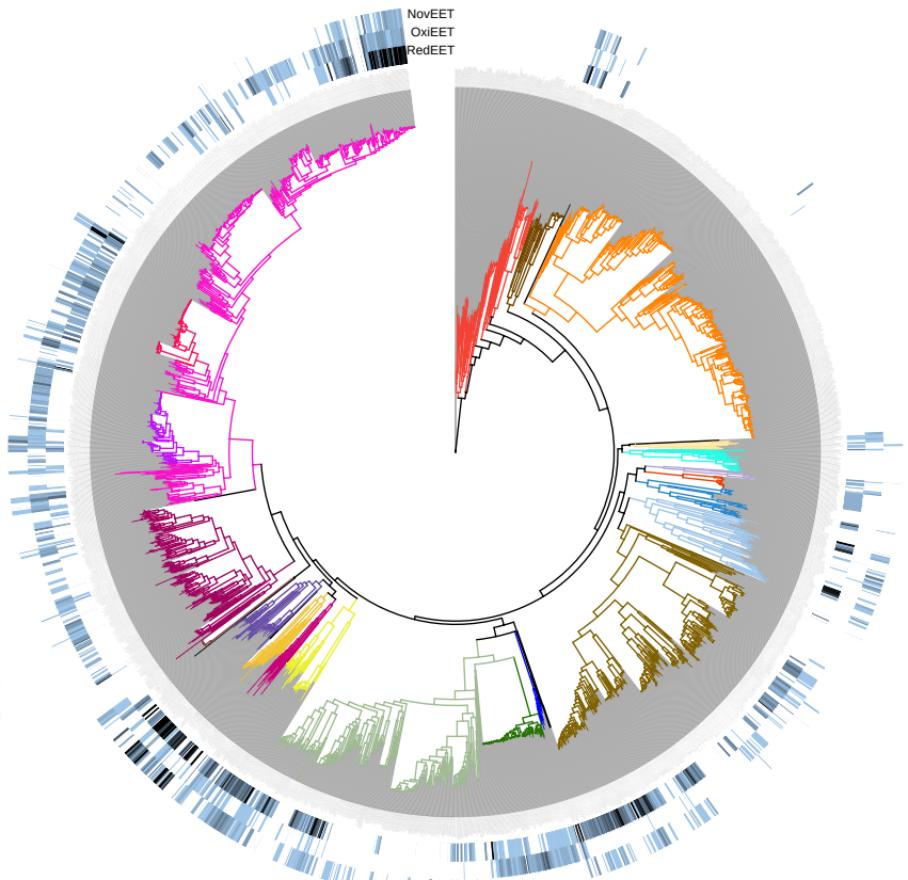


Figure 3. Putative novel (NovEET), oxidative (OxiEET), and reductive (RedEET) EET protein counts across phylogenetic tree of small boreal lake bacteria. OxiEET and redEET protein counts do not include MtrB and only include those specifically in the oxidative or reductive categories as designated (Supp. Tbl. 4). A 5040 amino acid-long sequence alignment of concatenated GTDB-tk markers genes found in the 2536 out of 2552 mOTUs which did not have identical marker sequences was used by RAxML-HPC BlackBox (Kozlov *et al.*, 2019) on CIPRES (Miller *et al.*, 2010) to generate a tree file set to use optimal bootstrapping. Tree visualization was performed using iTOL v5 (Letunic and Bork, 2021). GTDB-tk-assigned taxonomic level is designated by “p_” for Phylum-level and so on, and taxa colors are listed in clockwise order from the root.

217 DISCUSSION

218 **We have provided evidence that DOM mediates EET in small boreal lakes.**

219 We hypothesized that DOM serves as both an extracellular electron donor and acceptor in
220 lakes with high DOM concentrations, not simply as an electron acceptor for redEET as proposed
221 for humic substances acting as a substrate or electron shuttle in sediment and soil (Heitmann *et*
222 *al.*, 2007; Roden *et al.*, 2010) and lakes (Lau *et al.*, 2017). In support of this hypothesis, our
223 prediction—that DOC will correlate to the propensity of bacteria to house EET proteins—was
224 true, but not for all forms of EET. While DOM can be an optional receiver of electrons from
225 many respiration-linked EET proteins traditionally associated with iron reduction (Lovley *et al.*,
226 1999; Gralnick and Newman, 2007), DOM has not yet been demonstrated to be a direct donor
227 for autotrophs which would be expected to require net electron input (*i.e.* obtained from
228 extracellular donors) to reduce CO₂ into biomass. Yet, oxiEET is significantly correlated with
229 DOC while redEET is significantly correlated with iron, and not the reverse (Fig. 2A). Though
230 these correlations may suggest DOM acts as a direct electron donor for oxiEET, as we propose,
231 this inference cannot be made without acknowledging the correlations may also be due to DOM
232 supporting oxiEET organisms in other ways. DOM could promote oxiEET by promoting the
233 activity of redEET both as an electron-shuttling agent for dissimilatory Fe(III) reduction and as
234 DOM is fermented into small organic acids that can be respired, fueling redEET and thereby
235 regenerating Fe(II) for oxiEET. Also, cryptic photo-regeneration of Fe(II)-ligands as evidenced
236 previously (Caiazza *et al.*, 2007; Van Trump *et al.*, 2013) likely adds to materials available for
237 electrotrophs. As expected and reflected in its correlation to redEET, iron in high concentrations
238 will likely (depending on pH) have an insoluble, oxidized fraction and will also increase Fe(II)-
239 ligand-induced DOM photolysis (Caiazza *et al.*, 2007), both of which likely enrich for redEET.

240 The ability of electrotrophs to use reduced forms of DOM as electron donors may reflect
241 structural aspects of oxiEET proteins. For example, in the known oxiEET systems, an analogue
242 for the redEET protein, MtrC—an extracellular protein which may span the negatively charged
243 lipopolysaccharide layer (Edwards *et al.*, 2020)—is lacking or not identified which may be due
244 to the propensity of Fe(II) to be soluble only in this reduced form and therefore able to diffuse
245 through the lipopolysaccharide layer. As Fe(II) is oxidized, precipitating Fe(III) can present
246 physiological challenges for some bacteria through encrustation, though perhaps not as much at
247 low pH like in many of these small boreal lakes as contemplated previously (Hegler *et al.*, 2010;
248 Bryce *et al.*, 2018). In contrast, we expect DOM for the most part to be soluble in either reduced
249 or oxidized forms. We do indeed observe a correlation between low pH and DOC (Fig. 2B), both
250 of which are correlated to oxiEET (Fig. 2A). It is this combination of factors that may allow
251 pelagic electrotrophs access to DOM and iron's redox chemistry. These factors seem particularly
252 important for photolithoautotrophs using oxiEET. When using DOM or Fe(II) as extracellular
253 electron donors to fix CO₂ into biomass (Fig. 1), they would also require a net assimilation of
254 hydrogen, perhaps as protons aided by low pH, so we speculate. Indeed, in the dataset (Fig. 3,
255 Supp. Fig. 3), other than expected methanotrophs as we later discuss, possible and probable
256 phototrophs in Chlorobia, Alphaproteobacteria, and Gammaproteobacteria comprised much of
257 the small lake OTUs with oxiEET proteins. OxiEET was also found within a variety of
258 organoheterotrophs, which corresponds with previous research. The ubiquity of anaerobic
259 respiring organisms in soils and sediments that are capable of coupling DOM use as an
260 extracellular electron donor to the reduction of inorganics like nitrates has been previously noted
261 (Coates *et al.*, 2002; Roden *et al.*, 2010), likely using oxiEET proteins.

262

263 **EET in the water column has implications for methane emissions.**

264 Only eight out of 5569 MAGs analyzed here were predicted to encode machinery for
265 methanogenesis, among only 21 archaeal MAGs that passed the quality cutoffs. This, along with
266 dissolved methane measurements in similar systems (Rissanen *et al.*, 2021; van Grinsven *et al.*,
267 2021), suggests that the majority of methanogenesis is occurring in sediments rather than the
268 water column. The observed correlation between iron and methane, both of which are negatively
269 correlated with dissolved oxygen (DO) (Fig 2B), is unsurprising because methanogenesis occurs
270 typically when available iron and other electron acceptors are in the reduced state and Fe(II) is
271 more soluble than Fe(III). Considering this and that iron correlates as it should to redEET, there
272 may be noncausal correlation between redEET and methane. However, oxiEET appears to be
273 independently correlated to methane concentrations (Fig. 2A&B, Supp. Fig. 5&6), and a simple
274 explanation for this is that methanotrophs with oxiEET proteins are responsible.

275 However, the observed ubiquity of methylotrophs with oxiEET mechanisms is surprising
276 considering methane or methyl groups are rather electron-rich, and an electron-intake mechanism
277 like oxiEET would seem redundant unless many of these organisms are frequently not using
278 single carbon compounds as the only electron source. One possibility is that the metabolism of
279 these methanotrophs is more flexible than the classically understood methane oxidation model
280 (Chistoserdova and Lidstrom, 2013). One might suspect that some require additional electron
281 intake for carbon fixation or even nitrogen fixation. At least for Methylococcales with oxiEET,
282 about 60% had nitrogen fixation genes or carbon fixation genes, but this explanation overlooks
283 how the other 40% might use oxiEET (Supp. Tbl. 6). Considering the aforementioned ubiquity
284 and that most Methylococcales did indeed have some form of methane or methyl
285 monooxygenase, we suggest planktonic methanotrophic EET plays a role in regulating

286 greenhouse gas emissions by supplementing metabolism when methane is limiting and therefore
287 growing or sustaining their population until methane is not limiting. In particular, they may use
288 oxiEET for energy generation by coupling to the reduction of nitrogen compounds, especially
289 considering 82% of Methylococcales and 40% *Methylophilaceae* with oxiEET also have at least
290 one reductase for nitrate, nitrite, or nitric oxide (Supp. Tbl. 6). Otherwise, they might couple
291 oxiEET to oxygen reduction when operating in a microaerophilic zone.

292 Methylococcales and *Methylophilaceae* oxiEET genes were typically Cyc2 and/or the
293 MHC MtoA. MtoA was nearly always accompanied by the outer membrane porin MtrB—which
294 is not distinguished from MtoB in FEET (Garber *et al.*, 2020), consistent with Methylococcales
295 observed in Rissanen *et al* (2021). Because MtoA, though likely used for oxiEET, has been
296 observed to operate in redEET (Liu *et al.*, 2012), one might suspect they can couple redEET to
297 methane oxidation like in Yang *et al* (2020). However, we observed no difference in the
298 proportion of methylotrophs with Cyc2 or MtoA regardless of sample depth, including
299 Methylococcales well below the oxycline, and if being used primarily for redEET coupled to
300 methane oxidation, one may expect MtoA to be less common than Cyc2 in methylotrophs lower
301 in the water column where there is more methane.

302 Furthermore, we found the strength of the correlation between temperature and oxiEET
303 (Fig. 2A) surprising because temperature affects the rate of all metabolism, not just oxiEET.
304 Perhaps this correlation is due to oxiEET's role in autotrophy and its particular temperature
305 dependencies (García-Carreras *et al.*, 2018). Whatever the underlying mechanisms, this suggests
306 that as thermokarst continue to warm (Zandt *et al.*, 2020), oxiEET may become more prevalent.
307 Furthermore, if this is due to methanotroph activity, EET may play an even larger role in
308 controlling methane emissions.

328

329 Much like in sediments and soils, boreal lake EET is often connected to the sulfur and
330 nitrogen redox cycles.

331 Our results suggest that while EET is sometimes the only pathway available for redox
332 metabolism, generally it is accompanied by non-EET oxidoreductases, as reflected in the strong
333 positive correlations in Figure 2C. These include proteins enabling more commonly recognized
334 energy generation modes such as nitrate respiration, sulfide oxidation, and ammonia oxidation.
335 Notably, 84% of OTUs with identified respiratory pathways, EET or otherwise, had no identified
336 inorganic oxidation pathway, implying that organotrophy dominates the microbial diversity of
337 small lakes with high DOM. Still, similar couplings of EET to oxidoreductases have been
338 observed in non-planktonic habitats (Osorio *et al.*, 2013; Kawaichi *et al.*, 2018). Building on
339 such previous studies, we suggest that planktonic EET can cooccur or alternate with and/or
340 couple to sulfur and nitrogen redox transformations.

341 As an example, oxiEET was more often found in small lakes where a higher proportion
342 of organisms were capable of some manner of inorganic oxidation using donors such as
343 ammonia or nitrite, and in particular sulfur, sulfide, sulfite, or thiosulfate—suggesting that
344 bacteria may often alternate between or concurrently use oxiEET and such metabolisms.
345 RedEET is more often found in lakes with a high proportion of organisms with proteins for
346 nitrate/nitrite reduction, but also especially oxidation of sulfur forms (Fig. 2C)—suggesting
347 redEET often cooccurs or alternates with nitrate/nitrite respiration yet, as observed previously in
348 Lovely and Phillips (1994), couples to sulfur oxidation. The novel EET clusters (Supp. Tbl. 5) in
349 particular may be involved with nitrite respiration, sulfate respiration, and/or iron metabolism,
350 based on correlations to both oxidoreductases and environmental conditions conducive to these
351 transformations (Fig. 2C).

352 Given the frequency of EET possibly being used as an additional redox source and the
353 frequency of possible redox couplings to EET, we propose that EET may typically be a principal

354 mode of microbial metabolism in small boreal lakes. However, to investigate precisely how often
355 EET functions as a principal rather than as a backup metabolic mode in these systems, future
356 studies based on transcriptome profiles, sensitive *in situ* electrochemical analyses, and/or pure
357 cultures will be necessary.

358

359 **EET protein count reflects the expected ecology and metabolism of OTUs with implications**
360 **for planktonic taxa in small boreal lakes.**

361 The distribution of EET proteins in genomes across the full diversity of MAGs in the
362 dataset confirmed some expectations but also yielded some surprises (Fig. 3, Supp. Fig. 1–4).
363 For example, OTUs from the phylum Cyanobacteria, other than one OTU in the class
364 Vampirovibrionia, had no identifiable EET proteins which makes sense given their abundant
365 electron donor, H₂O. Regarded thus far as non-photosynthetic, Vampirovibrionia are not
366 representative of the classically understood Cyanobacteria nor as well studied (Grettenberger *et*
367 *al.*, 2020). This raises questions about how or why Vampirovibrionia carry the oxiEET proteins
368 Cyc2 and FoxY. The widely distributed Candidate Phylum Radiations (CPR), Paceibacteria and
369 Saccharimonadia, were never found harboring EET proteins. While notable, this is not
370 unexpected considering the observed tendency of CPR to lack even some of the most conserved
371 bacterial components (Hug *et al.*, 2016).

372 We also noted the lack of EET proteins in the class Actinobacteria. Of the 647
373 Actinobacteria MAGs, in only three did we find putative EET proteins. Considering that most
374 Actinobacteria are organoheterotrophs relying on respiration for energy metabolism, we might
375 have expected to find EET genes in this lineage simply because the capacity for EET is so
376 markedly enriched across most genomes in our dataset. One possibility is that we may have had

377 a search bias against finding EET in gram-positive bacteria like the class Actinobacteria because
378 the study of gram-positive EET is relatively new with more to uncover mechanistically. At the
379 time of analysis, the included EET proteins shown to confer EET in gram-positive bacteria were
380 PplA, EetAB, FoxABC, and sulfocyanin (Garber *et al.*, 2020). Supporting the possibility of this
381 bias, in the same phylum Actinobacteriota but gram-negative class Thermoleophilia (Zarilla and
382 Perry, 1986), eight out of 19 MAGs had EET proteins like CbcL homologues, DFE_0448, and
383 outer surface MHCs.

384 Moderate numbers of oxiEET proteins seem associated with carbon assimilation while
385 high copy numbers seem associated with energy generation. Organisms with oxidative EET
386 protein counts two through six typically fell within Chlorobia, Alphaproteobacteria, and
387 Gammaproteobacteria, most of which were expected to be methylotrophic or phototrophic based
388 on their taxonomy (Fig. 3, Supp. Fig. 3, Supp. Tbl. 6). Some, particularly those with oxiEET
389 count greater than six were actually expected to also respire (Chaudhuri and Lovley, 2003; Jangir
390 *et al.*, 2016; Gagen *et al.*, 2019). However, a low number of oxiEET proteins, one to three, was
391 associated with taxa expected to use a wide variety of metabolic strategies (Supp. Tbl. 2).

392 High numbers of redEET proteins seemed associated with energy generation through
393 respiration as expected, though with some caveats (Fig 3, Supp. Fig. 4). For example, counts 1-6
394 represented a great variety of metabolisms, even including Chlorobia, a class expected to be
395 obligately photoautotrophic. However, some evidence exists for substantial dark metabolism
396 based on fermentation (Badalamenti *et al.*, 2014) or respiration (Badalamenti *et al.*, 2013). In
397 Gammaproteobacteria, those with over 12 redEET proteins were in classes that include some
398 photoheterotrophs. Some Phycisphaerae were found with high numbers of redEET, mostly DFE
399 variants or CbcL, and they were rather rarely—only three out of 139 OTUs—found containing

400 any putative oxiEET protein. One OTU in Bacteriovoracia, a prominent class whose known
401 members are predatory, was found with 16 reductive EET proteins (Supp. Tbl. 4), four CbcL and
402 12 DFE variants, and one oxidative protein MtoA. Other OTUs in the similarly predatory class,
403 Bdellovibrio (Bratanis *et al.*, 2020; Ezzedine *et al.*, 2020), were found with several of both
404 oxidative and reductive EET proteins. Thus, whether they respire oxidized cellular components
405 or extract electrons, predatory bacteria may similarly gain virulence using EET like some
406 mammalian pathogens (Light *et al.*, 2018).

407

408 CONCLUSION

409 In support of our hypothesis that DOM in aquatic systems can act as a substrate and
410 mediator for EET-based metabolism, we discovered that Trout Bog is far from unique in its
411 EET-enriched ecology (He *et al.*, 2019); high proportions of putative electroactive bacteria and
412 high numbers of EET proteins are present in small lakes across the globe. We also found that
413 environmental propensity for EET correlates to DOC and other environmental and metagenomic
414 values. Our findings have implications for small lake metabolism, green-house gas emissions,
415 and our general understanding of bacterial ecology.

416 We have discovered potential roles for unconsidered proteins and added to our
417 understanding of EET's place in the environment by providing an overview of correlative
418 relationships between EET and environmental conditions in small lakes, and by considering
419 likely and possible resulting ecological interactions. Future modeling of methane emissions from
420 thermokarst sediments, as permafrost continues to melt and form new ponds, should consider the
421 roles of cryptic cycling and EET generally in the overlying water column. One likely role
422 includes the mediation by planktonic methanotrophic bacteria where increased temperature, low
423 pH, microbially derived DOM, and possibly pond age may increase the vibrance of the
424 methanotrophic population beyond that expected from increased methane. Similar factors are
425 also likely to increase the influence of bacteria using oxidative EET for anoxygenic
426 photosynthesis. As reflected in our analysis, such influence likely extends from the role that
427 DOM plays in enabling cryptic redox cycling, sustaining bacterial populations that respire or
428 generate oxidized DOM that can then be (re-)respired, coupled to organotrophy and/or
429 chemotrophy, thereby outcompeting methanogens in the water column for small organic
430 fermentation products.

431 Though EET allows some bacteria to use solid-state surfaces, we show here that EET
432 likely plays important roles even planktonically. This dataset analysis does not include large
433 lakes which themselves often have areas of anoxia where EET may or may not be relevant, nor
434 does it include representation of small lakes in tropical areas. There are also more recently
435 discovered EET systems that are not fully represented in our analysis. Our findings set the stage
436 for future studies to investigate these possibilities. Such studies may benefit from the use of
437 cyclic voltammetry, *in situ* electrical measurements, and metatranscriptomics to investigate
438 metabolic activity and to further define electroactive metabolism in lakes.

439

440 EXPERIMENTAL PROCEDURES

441 *Sample Site Characteristics*

442 The 36 small boreal lakes included in this dataset all have less surface area than 2.1 km². Some
443 are thermokarst melt pond ranging from old, middle-aged, and young (Peura *et al.*, 2020). Some
444 are acidic bog lakes with sphagnum moss growing over the edges. The rest are small lakes with a
445 variety of surroundings and characteristics (Supp. Tbl. 1). The dataset includes lakes in Canada,
446 Wisconsin and Alaska USA, Sweden, and Finland. All samples included in this study were from
447 the water column and not from the sediment.

448 *Metagenomic Data Collection.*

449 MAGs (Supp. Tbl. 6) from some lakes, as denoted (Supp. Tbl. 1), were generated and made
450 publicly available by collaborators (Buck *et al.*, 2021), from which we used one metagenome per
451 each of 186 samples. Other MAGs generated by the following methods were assembled from
452 numerous samples from Long Term Ecological Research, and they comprise four of the
453 metagenomes that are publicly available (<https://osf.io/qkt9m/>). Each was quality filtered with
454 fastp 0.20.0 (Chen *et al.*, 2018) and individually assembled with metaSPAdes v3.9.0 (Bankevich
455 *et al.*, 2012). Each metagenome was mapped to each individual assembly using BBmap from the
456 BBTools v38.07 suite with a 95% sequence identity cutoff (Bushnell *et al.*, 2017). Differential
457 coverage from mapping to all samples was used to bin contigs into metagenome-assembled
458 genomes using MetaBAT2 v2.12.1 (Kang *et al.*, 2015). Bins were quality assessed with checkM
459 v1.1.2 (Parks *et al.*, 2015) and dereplicated with dRep v2.4.2 (Olm *et al.*, 2017). All taxonomies
460 were assigned by GTDB-tk v0.3.2 (Chaumeil *et al.*, 2020).

461 *Finding EET Pipeline (FEET)*

462 To find and summarize EET protein-encoding genes this pipeline combines a homology-based
463 search tool, FeGenie (Garber *et al.*, 2020) modified with additional HMMs (Supp. Tbl. 4), with
464 the following more generalized approach that may allow for the identification of novel EET
465 genes. MHCs participate in EET in at least two forms, 1) as extracellular or outer membrane
466 MHCs to interact with extracellular substrates (such as OmcE, OmcS, and OmcZ), and 2) as a
467 periplasmic MHC inserted into a porin to form a PCC (such as MtrABC and PioABC). FEET
468 searches for both cases. FEET first uses python to find MHCs using the following regular
469 expressions for heme binding motifs: [C.CH], [C..CH], [C...CH], and [C.....[!=C]..CH]
470 (<http://www.python.org>). We set FEET's adjustable parameters so that for FEET to call an
471 MHC, the amino acid sequence must contain at least three [C..CH] motifs and at least five total
472 aforementioned motifs. It then must be predicted by Cello V2.5 (<http://cello.life.nctu.edu.tw/>)
473 likely to be an extracellular or outer membrane protein. Otherwise, it could be a periplasmic
474 protein within eight open reading frames of a beta-barrel outer membrane porin (BB-OMP)
475 predicted by a HMM or another extracellular or outer membrane-predicted MHC protein. The
476 BB-OMPs in PCCs were also counted as EET proteins. All protein predictions were
477 crosschecked between methods using FEET scripts. FEET pipeline performed here excludes a
478 search for e-pili because at the time of analysis we lacked a way to distinguish PilA used as e-pili
479 over Type IV secretion, however it has been since added to FeGenie, and future usage of FEET
480 would also include it and any other recent additions. The pipeline and output is publicly available
481 on Github (<https://github.com/McMahonLab/FEET.git>).

482 *FEET Performance*

483 FEET pipeline performs as expected, finding EET proteins in model EET organisms and not
484 finding EET proteins in model non-EET organisms. In order to ground-truth the FEET tool, we

485 used it to analyze reference genomes of well-studied model organisms that are known or
486 expected to contain EET proteins, including *Geobacter sulfurreducens* PCA – GenBank
487 accession number GCA_000007985.2 and *Shewanella oneidensis* MR – GenBank accession
488 number GCA_000146175.2. We also checked some genomes from organisms expected not to be
489 capable of EET, including, *Chlorobaculum tepidum* TLS – GenBank accession number
490 GCA_000006985.1, *Synechocystis* sp. PCC 6803 – GenBank accession number
491 GCA_001318395.1. FEET found 54 EET proteins in *G. sulfurreducens* including one or more
492 copies of CbcABL, OmcS, Cyc2, OmcFSZ, DFE_0461, DFE_0462, DFE_0463, DFE_0449,
493 DFE_0448, ExtABCD, ImcH, Geobacter-associated PCCs, MtrA, MtoA, and 12 unidentified
494 outer surface MHCs. FEET found 14 EET proteins in *S. oneidensis* including multiple copies of
495 MtrABC, a DFE_0448, and a DFE_0465. *C. tepidum*, *Synechocystis* sp., and *E. coli* did not
496 contain any identified EET proteins.

497 *Novel Sequence Curation and Crosschecking*

498 Novel EET MHC amino acid sequences were clustered by 80 percent identity to infer homology
499 using CD-hit (Li and Godzik, 2006) software with all the pipeline's EET proteins as well as
500 representative nitrate or nitrite reductase MHCs from the METABOLIC V3 pipeline (Zhou *et al.*,
501 2022). Sequence identities were crosschecked with all other oxidoreductase HMM matches
502 (Supp. Tbl. 3) from METABOLIC and the top NCBI BLAST (<https://www.ncbi.nlm.nih.gov/>)
503 results (Supp. Tbl. 5). METABOLIC relies on unique HMMs as well as HMMs from TIGRFAM
504 (Haft, 2003) and KEGG (Kanehisa, 2000) databases.

505 *Statistical Analysis*

506 All Pearson correlation statistics were generated using R (R Core Team, 2014). Lake-wide
507 statistical values for proteins are based on the presence/absence of OTUs and not normalized by

508 read depth. Not normalizing to the read depth overrepresents uniquely binned populations as
509 opposed to the dominating bacteria. Lake-wide averages and standard errors for environmental
510 data represent all available samples' accompanying metadata except for bog lakes involved in
511 Long Term Ecological Research (lter.limnology.wisc.edu) recent (2018–2020) environmental
512 data was subsampled by lake depth. For example, “Depth” (Supp. Tbl. 1, Fig 2A) represents the
513 average sample depth for most lakes. Only MAGs over 50% complete and less than 10%
514 contaminated as evaluated by CheckM (Parks *et al.*, 2015) were considered, and the most
515 complete and least contaminated MAG from a given lake was selected to represent the OTU in
516 that lake. Except in Supplementary Figures 1–4, taxa bar sizes were based on MAGs and
517 normalized to the number of samples taken from each MAG’s lake. This was to circumvent
518 protein count discrepancies between MAGs in a given lake belonging to the same mOTU.
519 Decisions to remove certain potentially available bog, thermokarst, or other lakes’ metagenomic
520 datasets from the analyses were based on either not having the required DOC data, being a lake
521 of surface area over 2.5km², or, in the case of one Canadian thermokarst pond labeled “F5,”
522 because of especially limited metagenomic data, retaining 36 bodies of water.

523 *Graphical Analysis*

524 Heatmaps and bar charts were generated using R’s ggplot2 package (Wickham, 2009). The
525 phylogenetic tree was generated using the 5040 amino acid-long sequence alignment output from
526 GTDB-tk of concatenated GTDB-tk markers genes found in the 2536 out of 2552 mOTUs which
527 did not have identical marker sequences. This alignment was used to generate tree files with
528 RAxML-HPC BlackBox (Kozlov *et al.*, 2019) on CIPRES (Miller *et al.*, 2010) using the default
529 optimal bootstrapping. Tree file and EET metadata visualization was performed using iTOL v5

530 (Letunic and Bork, 2021).

531

532 FIGURE LEGENDS

533 **Figure 1.** Conceptualized cryptic electron cycling and putative role in methane metabolism in
534 small boreal lake water columns. Chemical transformations are shown with white arrows.

535 Electrons flow from black to red.

536 **Figure 2.** Correlations between environmental parameters (B) and EET values (A) and other
537 oxidoreductase values (C) of 36 boreal lakes. Categories of protein values are delineated by
538 “Ratio” or “/OTU” respectively standing for the ratio of OTUs with at least one of the given kind
539 of protein or the average per OTU. Other values represent averages over available samples and
540 data. For bog lakes involved in Long Term Ecological Research (lter.limnology.wisc.edu), recent
541 (2018–2020) environmental data was subsampled by depth. Heatmap color represents Pearson
542 correlation coefficient, r. Unadjusted p-values are out of “(N)” available corelates. Yellow text
543 indicates a significance of p<.05 when adjusted by the Benjamini-Hochberg method. Statistical
544 values and heatmaps were generated with the program R. Non-EET oxidoreductases were
545 evaluated using METABOLIC (Zhou *et al.*, 2022) whereas EET proteins were evaluated with the
546 FEET pipeline. Category titles mean as follows: “TotalEET” = any putative EET protein;
547 “RedNoNEET” = EET expected for reductive metabolism but excluding putative nitrate or nitrite
548 reductase MHCs; “OxiEET” = EET expected to involve oxidative metabolism; “NitrMHCEET”
549 = nitrate or nitrite reductases but only those that are identified as MHCs; “Novel” = outer
550 membrane MHCs that were not classified as nor were 80% similar to any known EET protein;
551 “C1Oxi” = single carbon compound oxidation; “NitrRedNonMHC” = Nitrate or Nitrite
552 reductases that were not identified by FEET to be MHCs; “FeTot” = total iron and/or Fe(II) +
553 Fe(III) measurements; “ODO” = optical dissolved oxygen “DOC” = dissolved organic carbon.
554 Included HMMs and full category descriptions are listed in Supplementary Table 3.

555 **Figure 3.** Putative novel (NovEET), oxidative (OxiEET), and reductive (RedEET) EET protein
556 counts across phylogenetic tree of small boreal lake bacteria. OxiEET and redEET protein counts
557 do not include MtrB and only include those specifically in the oxidative or reductive categories
558 as designated (Supp. Tbl. 4). A 5040 amino acid-long sequence alignment of concatenated
559 GTDB-tk markers genes found in the 2536 out of 2552 mOTUs which did not have identical
560 marker sequences was used by RAxML-HPC BlackBox (Kozlov *et al.*, 2019) on CIPRES
561 (Miller *et al.*, 2010) to generate a tree file set to use optimal bootstrapping. Tree visualization
562 was performed using iTOL v5 (Letunic and Bork, 2021). GTDB-tk-assigned taxonomic level is
563 designated by “p_” for Phylum-level and so on, and taxa colors are listed in clockwise order
564 from the root.

565 SUPPLEMENTARY FIGURE LEGENDS

566 **Supplementary Figure 1.** Taxonomic proportions (A) and number (B) of OTUs with specific
567 counts of novel putative EET proteins in small boreal lakes. These include MHC that genes do
568 not cluster by CD-hit within 80 percent identity of any known EET HMM match in dataset of 36
569 bog and thermokarst lakes. Counts include some unidentified porins near outer surface MHCs.
570 Taxonomies were assigned by the GTDB-tk database. Figures were generated in R using
571 ggplot2. Column “X” includes any count including zero, representing the dataset’s taxonomy.
572 Proportions less than 0.005 are not labeled. Some lower taxa were separated out from class
573 level. Taxa with numerical GTDB-tk designations were combined at a higher taxonomic level.
574 Full results are in Supplementary Table 6.

575 **Supplementary Figure 2.** Taxonomic proportions (A) and number (B) of OTUs with specific
576 counts of all EET proteins in small boreal lakes. Taxonomies were assigned by the GTDB-tk
577 database. Figures were generated in R using ggplot2. Column “X” includes any count including

578 zero, representing the dataset's taxonomy. Proportions less than 0.005 are not labeled. Some
579 lower taxa were separated out from class level. Taxa with numerical GTDB-tk designations were
580 combined at a higher taxonomic level.

581 **Supplementary Figure 3.** Taxonomic proportions (A) and number (B) of OTUs with specific
582 counts of oxidative EET proteins in small boreal lakes. Taxonomies were assigned by the
583 GTDB-tk database. Figures were generated in R using ggplot2. Column "X" includes any count
584 including zero, representing the dataset's taxonomy. Proportions less than 0.005 are not labeled.
585 Some lower taxa were separated out from class level. Taxa with numerical GTDB-tk
586 designations were combined at a higher taxonomic level.

587 **Supplementary Figure 4.** Taxonomic proportions (A) and number (B) of OTUs with specific
588 counts of reductive EET proteins in small boreal lakes. Taxonomies were assigned by the
589 GTDB-tk database. Figures were generated in R using ggplot2. Column "X" includes any count
590 including zero, representing the dataset's taxonomy. Proportions less than 0.005 are not labeled.
591 Some lower taxa were separated out from class level. Taxa with numerical GTDB-tk
592 designations were combined at a higher taxonomic level.

593 **Supplementary Figure 5.** Correlations between and EET values from 36 boreal lakes.
594 Categories of protein values are delineated by "Ratio" or "/OTU" respectively standing for the
595 ratio of OTUs with at least one of the given kind of protein or the average per OTU. Heatmap
596 color represents Pearson correlation coefficient, r. Unadjusted p-values are out of "(N)" available
597 correlates. Yellow text indicates a significance of $p < .05$ when adjusted by the Benjamini-Hochberg
598 method. Statistical values and heatmaps were generated with the program R. Full EET protein category
599 descriptions are listed in Supplementary Table 3.

600 **Supplementary Figure 6.** Correlations between environmental parameters and non-EET
601 oxidoreductase values of 36 boreal lakes. Categories of protein values are delineated by “Ratio”
602 or “/OTU” respectively standing for the ratio of OTUs with at least one of the given kind of
603 protein or the average per OTU. Environmental values represent averages over available samples
604 and data. For bog lakes involved in Long Term Ecological Research (lter.limnology.wisc.edu),
605 recent (2018–2020) environmental data was subsampled by depth. Heatmap color represents
606 Pearson correlation coefficient, r. Unadjusted p-values are out of “(N)” available corelates.
607 Yellow text indicates a significance of $p < .05$ when adjusted by the Benjamini-Hochberg method.
608 Statistical values and heatmaps were generated with the program R. Non-EET oxidoreductases
609 were evaluated using METABOLIC (Zhou *et al.*, 2022). Included HMMs and full category
610 descriptions are listed in Supplementary Table 3.

611 SUPPLEMENTARY TABLE LEGENDS

612 **Supplementary Table 1.** Lake-wide information. This table includes lake characteristics, lake-
613 wide EET protein values and METABOLIC protein values and standard deviations when
614 available.

615 **Supplementary Table 2.** Unique taxa characteristics and references. This table includes average
616 EET protein values for each unique taxa. It also contains our designation of the quality of
617 evidence that each unique taxa contains members suspected capable of EET. These designations
618 occur under the header “EET Evidence Quality” with following category keywords “obviously”
619 (some members contain several promising EET genes as defined in Supplementary Table 3),
620 “likely” (highest suspect members contain one or two promising EET genes), “possibly” (highest
621 suspect members contain two or three DFE variants or FoxABCYZ proteins but no other more
622 promising EET genes), and “unknown” (contains just one of such “possible” EET genes). This

623 table also contains whether each unique taxa has been found in a previous study to have or likely
624 have EET capabilities under the header “Previous EET evidence” with the following keyword
625 meanings: “yes” (we found studies that show EET in organisms in the genus or species), “no”
626 (we did not find studies including genus or species doing EET; or taxonomic level is not defined
627 enough to know), and “related”(organisms in the same order or family but not the particular
628 genus or species were found in a previous study with EET).

629 **Supplementary Table 3.** Abbreviations and descriptions. In addition to abbreviations, this table
630 lists the names of proteins for which HMMs were used in METABOLIC for each category.

631 **Supplementary Table 4.** Bit scores for FeGenie Hidden Markov Models and observed redox
632 association. This table also includes additional conditionals for whether each HMM was
633 considered as promising evidence of EET capability.

634 **Supplementary Table 5.** Novel cluster information. This table includes each unique novel
635 cluster sequence, its percent identity to the reference sequence, and its number of heme motifs.
636 Also included is which open reading frame the sequence represents, and what taxa it was found
637 in.

638 **Supplementary Table 6.** Information unique to each metagenome assembled genome (MAG).
639 This includes to what lake, sample, and mOTU each MAG belongs, its GTDB-tk taxonomy,
640 genetic characteristics, and FEET and METBOLIC proteins.

641

642 ACKNOWLEDGEMENTS

643 We give special thanks to Moritz Buck, Sarahi L. Garcia, Leyden Fernandez, Gaëtan
644 Martin, Gustavo A. Martinez-Rodriguez, Jatta Saarenheimo, Jakob Zopfi, Stefan Bertilsson, and
645 Sari Peura for early access to and setup with their dataset (Buck *et al.*, 2021) to support this
646 “pandemic project.” We thank the University of Wisconsin (UW)-Trout Lake Station, the UW
647 Center for Limnology, and the John and Patricia Lane Award program for their invaluable
648 support. We thank the U.S. National Science Foundation North Temperate Lakes Long-Term
649 Ecological Research site (NTL-LTER DEB-1440297; DEB-0702395) for providing funding of
650 the Microbial Observatory for long-term sampling of Lake Mendota and Trout Bog. We thank
651 the U.S. Department of Energy Joint Genome Institute for sequencing and assembly (CSPs 394
652 and 2796). This research was also performed in part using the Wisconsin Energy Institute
653 computing cluster, which is supported by the Great Lakes Bioenergy Research Center as part of
654 the U.S. Department of Energy Office of Science. We are also thankful for fellowships provided
655 through the department of Bacteriology at UW-Madison. We declare no conflicts of interest.

656

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