**Overview**

Sunlight drives complex biogeochemical cycles in darkly stained lakes that are found at high density in boreal and sub-arctic regions. Anoxygenic phototrophic microbes thrive in such systems when they are deep enough to thermally stratify. We hypothesize that extracellular electron transfer mediates a tight coupling between such phototrophs and anaerobic heterotrophs, and that abundant humic substances derived from peat serve as needed electron shuttles. This has major implications for carbon and energy budgets in lakes that are projected to emit ~100 tera-grams-C by 2100 due to climate warming and thawing permafrost (Walter Anthony et al 2016).

Our team is linked to the North Temperate Lakes Long Term Ecological Research site and our proposed work is underpinned by 20 years of data collected from humic bog lakes in northern Wisconsin. The primary study site is darkly stained and is characterized by sharp gradients of temperature, light, oxygen, and sulfide. Conditions are highly reducing 1-m below the oxycline and methanogens thrive in the sediment. A dense plate of green sulfur bacteria forms in the summer and appears to fuel daily and seasonal fluctuations in redox status. We propose that this activity is in turn supported by electron donor regeneration by respiring heterotrophs, with humic substances cycling between reduced and oxidized states. Thus, light is driving a diurnal cycle of reducing equivalent consumption and production, decreasing the net reducing equivalents available to fuel methanogenesis. This reasoning is the motivation behind three hypotheses we propose to test here using a combination of field work and lab-based experiments: H1: Sunlight modulates overall redox conditions within and beyond the zone of anoxygenic phototroph activity, with consequences for geochemical speciation over cm-scale distances. H2: Gene expression varies synchronously across day-night cycles, providing the physiological basis for diurnal rhythms in redox cycles. H3: Partnerships between electron-uptaking anoxygenic phototrophs and electron-dispensing heterotrophs are responsible for ambient redox cycling and their partnership is modulated by the specific available electron donors and terminal electron acceptors.

**Intellectual Merit**

Dissecting and interpreting complex microbial assemblages is a major grand challenge in biology. Cryptic elemental cycles are especially difficult to study and inadequately accounted for in ecosystem-scale mass balances and models. We address a critical knowledge gap related to the emergent effects of light and microbes on redox cycling in humic lakes. The projected impact of humic lakes on climate warming feedbacks makes the work especially urgent.

**Broader Impacts**

Our work has clear and broad ramifications for carbon and energy budgets in aquatic ecosystems that are critical hubs for biogeochemical processing in boreal and sub-arctic regions. The project will provide training for one graduate student and one postdoctoral researcher, as well as undergraduate scholars. All team members supervising students will participate in the popular Research Mentor Training seminar facilitated by the UW-Madison Delta Program. We will disseminate our work to other scientists through standard routes of publication and presentation at conferences. Our integration with the NTL-LTER provides support for outreach activities that annually reach 100's of community members in both rural northern Wisconsin and the Madison area. We will construct "bog batteries" that are simple microbial fuel cells inoculated with lake water that can produce electricity, for the annual Trout Lake Station and Hasler Lab Open Houses. In preparation, the graduate student will participate in the Wisconsin Idea STEM Fellows program that provides training in high-impact outreach practices through a semester-long cohort model. Funds are requested to support this training.

## 1. Problem Statement and Intellectual Merit

New evidence for the role of extracellular electron transfer (EET) in biogeochemical cycles continues to challenge paradigms of energy flow through microbial communities. We know that in the absence of oxygen, heterotrophs capable of EET will often partner with chemolithotrophs to mineralize organic matter (Kappler and Bryce, 2017; Kelly and Wood, 2013). In dimly lit systems occupied by **anoxygenic photolithotrophs (AOPs),** externally derived energy (light) can power such transformations (Berg et al., 2016; Ha et al., 2017) and support even more complex interaction networks by serving as an electron sink in partnership with the EET-heterotrophs (**Figure 1**). This leads us to the overarching question guiding the proposed work:

*How do anoxygenic phototrophs regulate complex redox cycles in pelagic systems?*

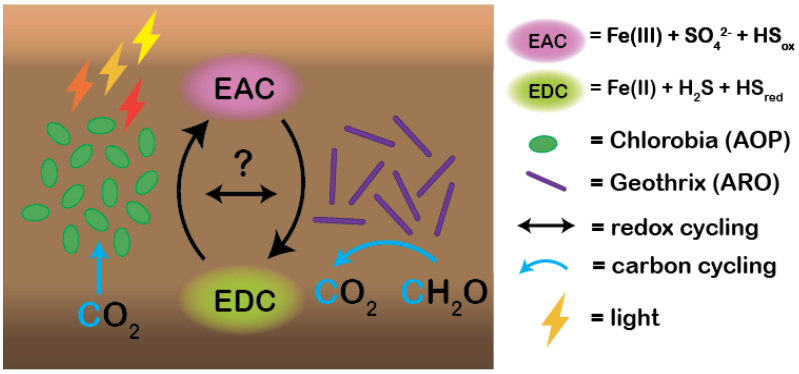



**Figure 1**: Hypothesized major processes mediated by AOPs and AROs, focused on energy and carbon. EAC = electron accepting capacity, EDC = electron donating capacity, HS = humic substances, ARO = anaerobic respiratory organism. We note that sulfur in other redox states (e.g. $S^o$, thiosulfate) could also be involved instead of $SO_4^{2-}$. Acronyms also defined in **Table 1**.

Untangling these partnerships is critical to understanding the implications of EET for energy flow through ecosystems. However, it is challenging to tease apart the individual contributions of each member and to discern how EET binds them together. Electron flow through such tightly coupled syntrophies can involve cryptic cycles (Hansel et al., 2015; Kappler and Bryce, 2017) and/or as yet unrecognized molecular structures in microbial outer membranes (Ha et al., 2017; Lovley and Holmes, 2022). Further, humic substances in the organic matter pool can provide additional electron exchange routes by serving as both donor and acceptor, potentially in a regenerative cycle (Klüpfel et al., 2014; Lau and del Giorgio, 2020). A renewed interest in the role of **light as a driver of these processes** (Avetisyan et al., 2019; Berg et al., 2016; Gupta et al., 2019; Peng et al., 2019; Schmidt et al., 2020; Tsuji et al., 2020) creates a new dimension of complexity. Creative combinations of field- and lab-scale electrochemistry experiments, enrichment and axenic cultures, and 'omics tools can begin to "decryptify" such interactions.

Our team brings together the unique skill set needed to do this. McMahon and Roden have shown that EET is likely driving energy and carbon flow in a well-studied bog lake using genome-resolved metagenomics (He et al., 2019; Olmsted et al., 2023) and have data pointing to connections between AOPs and electrogens. Qin has expertise in the kind of bioelectrochemistry systems used to measure EET in microbial communities (Qin et al., 2016; Qin and He, 2017).

Solving the challenge of dissecting complex networks comprised of microbes, abiotic redox reactions, and light in model systems will bring us closer to defining the rules governing energy flow through globally distributed ecosystems. We will address this knowledge gap in a freshwater lake study system that represents water bodies on earth predicted to release 103-129 Tg-C

annually by 2100 in the form of $CO_2$ and $CH_4$ (Walter Anthony et al., 2016). Many such small lakes are darkly stained with high humic acid concentrations, causing them to thermally stratify. How do pelagic microbes in these lakes control redox conditions and associated carbon processing pathways in the water column? Our proposed work is organized around explicit hypotheses addressing this question:

*H1: Sunlight modulates overall redox conditions within and beyond the zone of AOP activity, with consequences for geochemical speciation over cm-scale distances*

*H2: Gene expression varies synchronously across day-night cycles, providing the physiological basis for diurnal rhythms in redox cycles*

*H3: Partnerships between electrotrophic AOPs and electrogenic heterotrophs are responsible for redox cycling and their partnership is modulated by the specific available electron donors and terminal electron acceptors (TEAs)*

The need to understand how microbial communities are "wired" is reflected in the NSF's focus on illuminating the Rules of Life. Communities structured by intimate interactions are the norm across nearly all ecosystems, including soil, freshwater, marine, and host-associated. This project will shed light on such interactions in a study system that is an analog for a rapidly increasing number of greenhouse gas point sources (thermokarst and bog lakes).

## 2. Background and Rationale

Redox stratification in spatially structured ecosystems is textbook environmental microbiology. This is especially true of lakes that are reminiscent of Winogradsky columns. **Why, then, should we be interested in studying them further?** Continuous discoveries of unexpected physiologies and diversity is a proverbial theme in environmental microbiology. Here we propose that anoxygenic phototrophs (AOPs) (principally green sulfur bacteria) play a previously underappreciated role in regulating redox profiles in stratified humic lakes. This has potentially major consequences for our ability to quantify and predict fundamental geochemical cycling and specifically greenhouse gas emissions.

**Table 1- Acronym definitions***

| | |
|---|---|
| AOP | Anoxygenic Phototrophs |
| ARO | Anaerobic Respiratory Organisms |
| (c)(D)OM | (colored)(Dissolved) Organic Matter |
| CV | Cyclic Voltammetry |
| EAC/EDC | Electron Accepting/Donating Capacity |
| EET | Extracellular Electron Transfer |
| GSB | Green Sulfur Bacteria |
| HS | Humic Substances |
| MAG | Metagenome-Assembled Genome |
| ORP | Oxidation Reduction Potential |

** We recommend printing this table to decode acronyms throughout the text, as there are many*

**Our study system: why bog lakes?** Northern peatlands are estimated to store ~500-1,000 gigatons of carbon (Nichols and Peteet, 2019; Yu, 2012). They are a major source of methane emissions to the atmosphere and their impact on the global carbon cycle is expected to increase as permafrost thaw accelerates (Schuur et al., 2015; Wauthy et al., 2018). Ponds that form in thawing peat are often ombrotrophic, darkly stained, and acidic (pH 4-5), resembling bog lakes found in more temperate regions (Peura et al., 2020; Vonk et al., 2015). Even relatively shallow ponds (~3.5 m) are dark enough to thermally stratify in the summer months. Intermittent stratification can promote anaerobic processes in the lower water column, with implications for coupled biogeochemical cycles. The fate of aquatic organic matter originating in the peat is complex (Joshi et al., 2021; Treat et al., 2014) but the moieties supporting electron transfer (either

biotic or abiotic) in humic-rich DOM can cycle reversibly like a "battery" that can be charged by microbial respiration (Klüpfel et al., 2014) and discharged by abiotic (or possibly biotic) oxidation. Methane release when other terminal electron acceptors (TEAs) have all been reduced is of particular concern due to climate warming feedbacks (Hugelius et al., 2019). Curiously, peat-derived organic matter serving as a TEA quenches methane production (Gao et al., 2019; Valenzuela and Cervantes, 2021).

Darkly stained lakes have almost intractably complex geochemical cycles. Photochemical oxidation near the surface leads to oxidative radical formation, DOM mineralization, and Fe reduction/oxidation through combinations of abiotic and biotic processes (Cory and Kling, 2018; Kappler et al., 2021). In anoxic regions, cryptic cycles involving S, DOM, Fe, and potentially other metals such as Mn make disentangling the redox transformations formidable. *We emphasize that in this proposed work we are not aiming to explicitly dissect these cycles but instead to parse out the effect of AOPs on the total electron donating (EDC) and electron accepting capacity (EAC) of the water.* The exact chemical transformations are for another day. We focus here on the biological processes that impact the EDC and EAC pools.

**AOPs exploit and create sharp environmental gradients.** The tendency of AOPs to form dense plates at specific depths in stratified lake water columns has been of interest to microbiologists and limnologists for many decades. Their role in the carbon and sulfur cycles is well documented in textbooks. In darkly stained lakes GSB exploit red light from above and electron donors generated below (**Figure 2**) (Parkin and Brock, 1981a; Rossi et al., 2013; Taipale et al., 2009). As autotrophs, they can impact lake-scale productivity and carbon burial (Parkin and Brock, 1981b). As diazotrophs (nitrogen fixing), they also support the nitrogen cycle (Fernandez et al., 2020; Overmann, 2006). Purple bacteria are generally less abundant overall in these systems due to rapid extinction of appropriate wavelengths with depth.

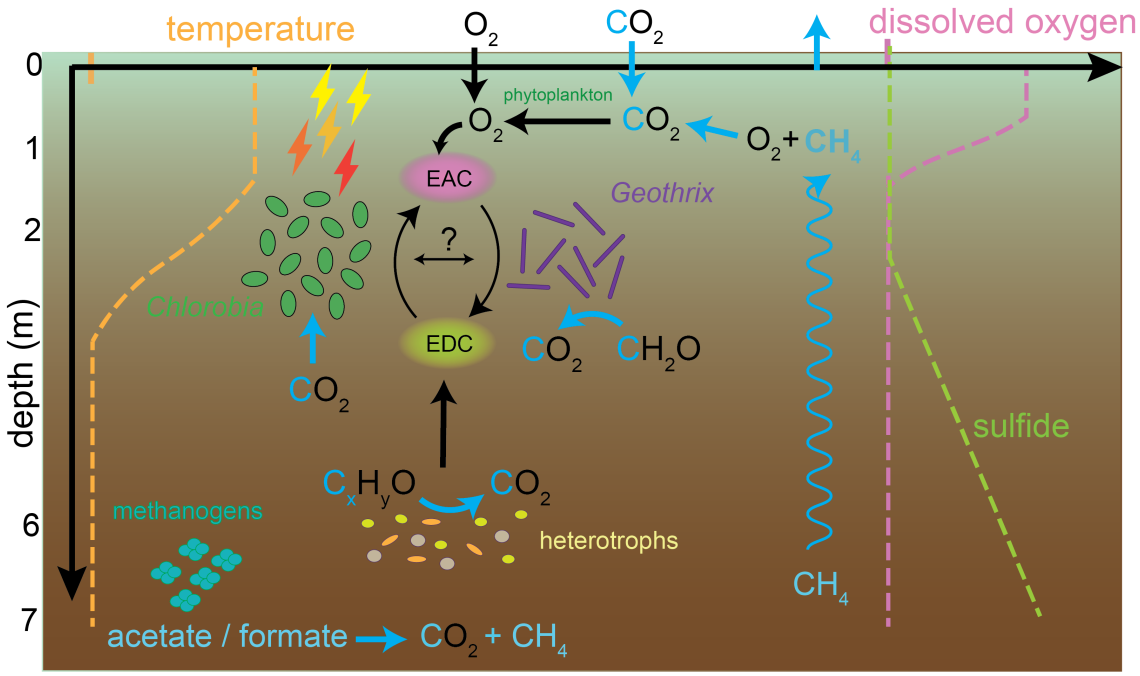



**Figure 2.** Biologically mediated processes relating to carbon and energy cycling in Trout Bog Lake with emphasis on $CO_2$, $CH_4$, EDC/EAC, and microbes most relevant to our proposed work.

Ample culture-based work has shown that sulfur cycling can support GSB growth when co-cultured with S-reducers and supplemented with acetate (Biebl and Pfennig, 1978). The famous "*Chlorochromatium aggregatum*" aggregates are actually comprised of GSB and a colorless member of the *Comamonadaceae* that are physically attached and hypothesized to share a quinone pool (Liu et al., 2013; Overmann and Schubert, 2002). Recent genomic evidence of electron donor specialization in GSB suggests that some might also use Fe(II) as a source of reducing equivalents (Crowe et al., 2014; Tsuji et al., 2020) during growth under free-living conditions (Heising et al., 1999; Lambrecht et al., 2021). GSB were implicated in close coupling of light-driven anoxygenic Fe(II) oxidation to microbial Fe(III) reduction in the water column of meromictic Lake Cadagno (Berg et al., 2016), suggesting the possibility for rapid "cryptic" Fe redox cycling in stratified ferruginous lakes. GSB from such environments encode a homolog of Cyc2, an outer membrane monoheme cytochrome typically found in Fe(II) oxidizers (Castelle et al., 2008; McAllister et al., 2020) and proposed to support photoferrotrophy in the GSB *Chlorobium* (Lambrecht et al., 2021). Given the proposed ability of Cyc2 to transfer electrons from Fe(II), it is not unreasonable to predict that it can extract electrons from quinone moieties in humic substances (Lau and del Giorgio, 2020). The role of humic substance redox cycling in pelagic ecosystems such as these has, to our knowledge, not been considered.

**Electron donating microbes add to the mix.** Sulfate reducers and other anaerobic respiring heterotrophs are abundant in these lakes (Linz et al., 2018; Peura et al., 2020) and maintain highly reduced conditions below the oxycline. A surprising number of organisms in our lake encode in their genomes the machinery that probably enables EET between microbial cells and extracellular substrates, such as redox-active metals and both particulate and dissolved humic substances (He et al., 2019; Olmsted et al., 2023). Canonical *Geothrix*-like organisms are especially abundant in the GSB plate of our system, suggesting they play a key role in regenerating EDC for the *Chlorobium*. Such metabolic handoffs have been shown in pure culture experiments, sometimes involving EET via electrodes (Badalamenti et al., 2014, 2013; Ha et al., 2017). In (co)cultures of GSB from ferruginious environments, GSB cannot be grown without an Fe(III)-reducing *Geobacter*-like partner (Lambrecht et al., 2021). Our overarching question is based on these interactions and we propose that they bind together electro**GENS** (ARO) and electro**TROPHS** (AOPs), with ecosystem-scale implications.

**Ecosystem-scale implications.** Taken together, the tightly knit cooperation between AOPs and AROs in stratified humic lakes has the potential to influence whole-lake metabolism in wholly unappreciated ways. Anoxygenic carbon fixation is supported by AROs that regenerate EDC for use by AOPs (**Figure 2**). *In situ* productivity measurements show that $^{14}CO_2$ incorporation into AOP biomass represents a small fraction of overall productivity when compared to oxygenic fixation in the upper layer because algal carbon is rapidly turned over by grazers (Kuuppo-Leinikki and Salonen, 1992; Parkin and Brock, 1981b). However, AOP sedimentation contributes 20-% of total carbon sedimentation across the growing season (Parkin and Brock, 1981b). This challenges the notion that AOPs and AROs form a "closed loop" of carbon cycling since the sedimentation shunts carbon out of the photic zone. Furthermore, the sedimented AOP biomass is available for conversion to methane in the highly reducing hypolimnion. Thus, the AOP-ARO coupling could enhance methane production at a whole-lake scale.

On the other hand, we have preliminary transport-reaction model-based evidence for a substantial role for AOPs in **quenching** methane production (see Preliminary Data). Light-driven microbial oxidation of HS, Fe, and S species during AOP bloom development during the summer

is likely to lead to net EAC generation, whose use by AROs will attenuate methane production within the water column, and ultimately limit the downward flux of labile organic matter that would otherwise drive methanogenesis in the sediments. Model calculations suggest that modest levels of AOP activity could reduce water column methane production ca. 4-fold compared to what would take place in the absence of such activity. In addition, given that lake destratification and bulk mixing begins relatively soon (within a month) after the summer AOP maximum, a large fraction of the accumulated biomass is likely to be oxidized through aerobic respiration rather than ARO activity. In this manner, the seasonal onset and accumulation of AOP biomass can exert a major impact on lake carbon cycling during the period of maximum ecosystem metabolism.

## 3. Intellectual Merit

Anoxygenic phototrophs thriving in dimly lit waters of stratified lakes have been studied by environmental microbiologists for decades. However, genome-based insights point to a possible unrecognized role in biogeochemical cycling with implications for ecosystem scale carbon and energy flow. The study system is a proxy for boreal and permafrost thaw ponds with high colored DOM (cDOM) concentrations and potential for rapid carbon mobilization into greenhouse gases. We aim to unravel the connections between microbes that are carbon and electron sinks, and those that generate reduced chemical species while mineralizing organic matter. The proposed work will test hypotheses derived from prior work using genome-resolved metagenomics, high-resolution field sensors, and computational models. This approach can serve as a conceptual model for integrating 'omics, biogeochemistry, and mathematical representations of complex networks involving diverse microbial functional guilds and cryptic geochemical cycles.

## 4. Hypotheses and Objectives

How do anoxygenic phototrophs regulate complex redox cycles in dimly lit pelagic ecosystems? To answer this question, we will test three hypotheses using a combination of field (*in situ*), modeling, and laboratory experiments **(Figure 3**). These include sensors deployed at key depths collecting measurements at high frequency, manual profiles to delineate distinct microbial niches, 'omics tools to reveal how community members respond to light availability, and laboratory experiments with electrochemical reactors to probe the role of light with both native and constructed consortia.

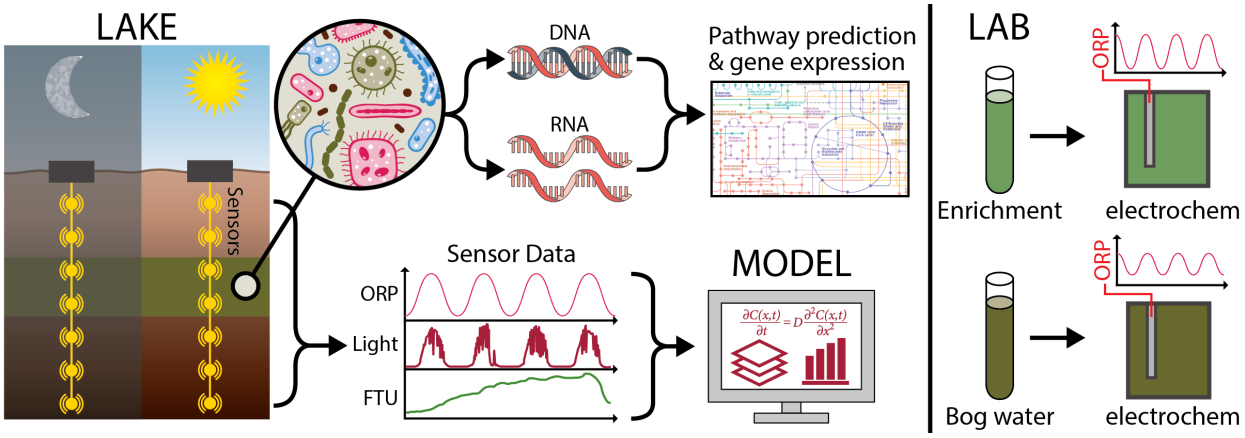



**Figure 3.** Conceptual diagram of the proposed work.

***Hypothesis 1: Sunlight modulates overall redox conditions within and beyond the zone of AOP activity, with consequences for geochemical speciation over cm-scale distances***

We will test H1 by measuring *in situ* water column profiles across diurnal cycles. The goal is to characterize seasonal scale and sub-daily microbial niche dynamics with respect to water column redox conditions and light.

**Objective 1.1:** Autonomously measure temperature, sunlight, dissolved oxygen, oxidation-reduction-potential, turbidity, pH, and fluorescence hourly May-November.

**Objective 1.2:** Measure bulk EAC/EDC, humic substance redox status, light conditions, and productivity across three day-night cycles, focusing on key transition points in light availability.

***Hypothesis 2: Gene expression varies synchronously across day-night cycles, providing the physiological basis for diurnal rhythms in redox cycles***

We will test H2 with targeted metatranscriptomics experiments at key depths across a day-night cycle. The goal is to use gene expression as a "sensor" to probe the redox conditions experienced by the resident microbes.

**Objective 2.1:** Manually sample water at the AOP peak and 2 m below the peak four times across a 24-hour period. (2 depths x 4 times x 3 replicates = 24 metatranscriptomes and 2 metagenomes).

**Objective 2.2:** Map metatranscriptomes to draft genomes recovered from metagenomes, to infer gene expression with a focus on key organisms (AOPs and AROs) and functions.

***Hypothesis 3: Partnerships between electrotrophic AOPs and electrogenic heterotrophs (AROs) are responsible for redox cycling and the partnership is modulated by the specific available electron donors and terminal electron acceptors***

We will test H3 in the laboratory by cultivating AOPs and AROs in an electrochemical reactor to measure redox cycling under controlled conditions of light and terminal electron donor/acceptor compositions. The factorial experimental design will dissect the parameters that determine electron flow, under less complex conditions than found in the lake and with perturbations.

**Objective 3.1:** Incubate fresh bog water communities in reactors under different light regimes, and with different combinations of Fe, S, HS, and DOM. Use electrochemical techniques to define bulk EAC/EDC cycling under each condition and through time.

**Objective 3.2:** Isolate or enrich strains from the lake to repeat Objective 3.1 with pure or reduced complexity cultures.

## 5. Preliminary Results

Trout Bog Lake is a primary study site in the North Temperate Lake Long Term Ecological Research program (NTL LTER) (**Table 2**). The NTL LTER maintains a 40+ year time series of limnological variables and a buoy that logs high-frequency measurements of water temperature profiles with depth, dissolved oxygen, meteorological variables, and phytoplankton pigments. McMahon's group maintains a 20+ year time series of microbial community samples that have

**Table 2.** Typical values for key parameters in Trout Bog Lake. The pH varies with depth and time. Sulfide was measured at 5 m in mid-July 2017.

| Parameter | Value |
|---|---|
| Max depth | 7 m |
| Surface area | 10,100 $m^2$ |
| pH ranges | 3.25 – 4.80 |
| Average DOC | 20 ppm |
| Total Fe | 500 ppb |
| Sulfide | 1 mM |

yielded **53 publications based on the NTL Microbial Observatory, to date**. The lake is easily accessible by a small rowboat stored on site, 4 miles from the Trout Lake Field Station (46.042, -89.685). It is deep, dark, and sheltered enough to stratify strongly during the ice-free period. Weekly routine samples during the ice-free season are paired with manual profiles collected with a multimeter that measures temperature, dissolved oxygen, oxidation-reduction potential, pH, and turbidity at cm-scale depth intervals.

*Water column physical/chemical conditions vary at multiple time scales*. Immediately following ice thaw in late April or early May, thermal stratification intensifies and conditions below the thermocline become highly reduced over a period of days to weeks. A turbidity peak gradually develops at ~2 m with maximum density in August. Our prior work showed that this corresponds to a "plate" of GSB (*Chlorobium*) (Berg et al., 2021) as has been observed in similar lakes around the globe (Garcia et al., 2021; Peura et al., 2012; Taipale et al., 2011; Tsuji et al., 2020). On sub-daily time scales we observe expected oscillations in dissolved oxygen between the surface and ~ 1 m due to oxygenic photosynthesis. Curiously, we also detect diurnal variations in oxidation reduction potential (ORP) at 2 m (**Figure 4**). **We hypothesize that electron "consumption" by the *Chlorobium* increases the ORP during daylight and respiration by AROs decreases the ORP at night.**

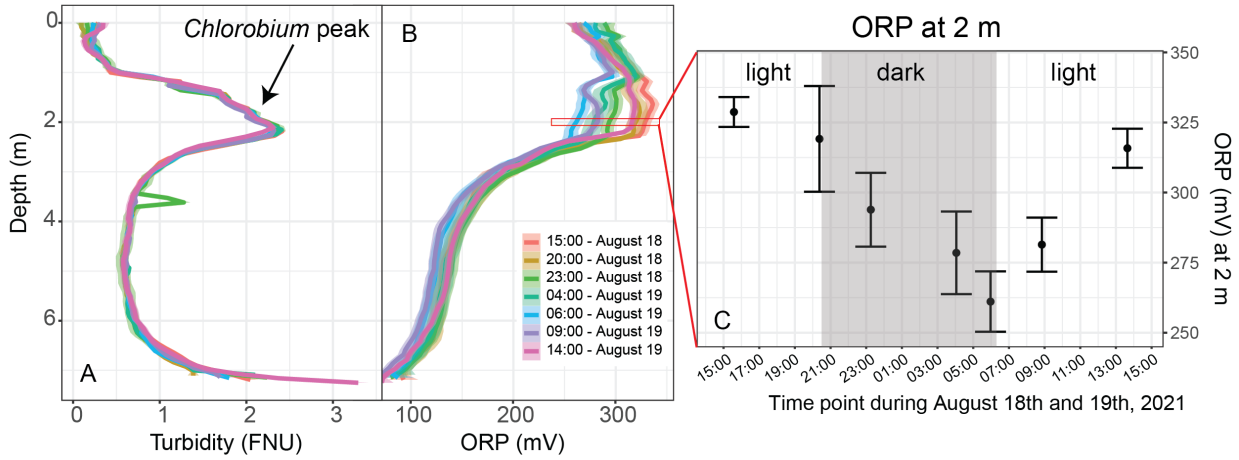



**Figure 4**. Diurnal variation in ORP profiles showing more oxidizing conditions during daylight and more reducing conditions at night. (A) Turbidity profile showing *Chlorobium* plate near 2 m. (B) ORP sensor profile collected across two days. (C) ORP at 2 m across the two days.

We found that, year to year, the peak in turbidity consisted primarily of *Chlorobium*-like taxa, comprising 15–30% of the total bacteria, and *Geothrix*-like taxa at 10–15%, based on shotgun metagenomic reads mapped to a collection of reference metagenome-assembled genomes (MAGs) from the same lake (Bendall et al., 2016; Linz et al., 2018).

*Genomes recovered from Trout Bog and similar lakes are enriched in EET genes.* We analyzed 102 MAGs from Trout Bog and discovered that genes putatively involved in one or multiple types of EET were found in 42% of the MAGs (He et al., 2019). These included genes encoding putative extracellular multiheme cytochromes, porin-multiheme cytochrome *c* protein complexes, and what appeared to be homologs of Cyc2 (an outer membrane monoheme

cytochrome *c* typically found in Fe(II) oxidizers such as *Sideroxydans* and *Zetaproteobacteria*) (Keffer et al., 2021; Liu et al., 2012). These corresponded to organisms well known for their ability to exchange electrons with extracellular donors and acceptors, such as redox-active metals and DOM containing quinone structures (e.g. *Geothrix*), but also many taxa that have previously not been identified as "EET organisms" (e.g. *Methylotenera*). *Chlorobium*-MAGs encoded three Cyc2 homologs, one from Cluster 1 and two from Cluster 3 (as defined by Keffer *et al.* 2021). Cyc2 has been proposed to support photoferrotrophy in *Chlorobia* that carry it (Crowe et al., 2017; Lambrecht et al., 2021; Tsuji et al., 2020). This strategy for acquiring electrons for autotrophic growth could also be characterized as EET since it requires electron transfer directly from a donor, onto a fused cytochrome-porin on the outer membrane (He et al., 2017).

We found similar results while analyzing a large metagenomic dataset of 36 small lakes that include 11 bog lake systems (Olmsted et al., 2023). The proportion of MAGs with EET genes (47%) was positively correlated to dissolved organic carbon (DOC). All together, these results led us to hypothesize that EET is substantially more important for carbon and energy flow in the lake than previously known, with both novel "electro**TROPHIC**" and "8hermos8enic" taxa regulating redox status at multiple spatial and temporal scales.

*EET genes are expressed in situ*. We have tantalizing results from metatranscriptomes that show EET genes are expressed in the Trout Bog hypolimnion (2-6 m, one time point in triplicate) (Olmsted et al., 2023). All three *cyc2* copies in the dominant *Chlorobium* were expressed, with one (Cluster 3) mapping 100x as many reads as the other Cluster 3 and ~20x as many as the Cluster 1. Three outer surface porin-cytochrome *c* complex homologs were clearly expressed in the *Geothrix*, with one *mtrC* homolog mapping significantly more reads than the others. Transcripts from many other EET-related genes (e.g. multi-heme cytochromes) associated with diverse MAGs were also detected. The work proposed here was inspired by these findings.

*Transport-reaction modeling points to ecosystem-scale impacts.* We implemented a model coupling physical transport by molecular diffusion and relevant biogeochemical reactions (**Figure 5**). Turbulent diffusivity driven by advection is negligible below the top of the thermocline due to strong stratification and the sheltered lake location (Read and Rose, 2013).

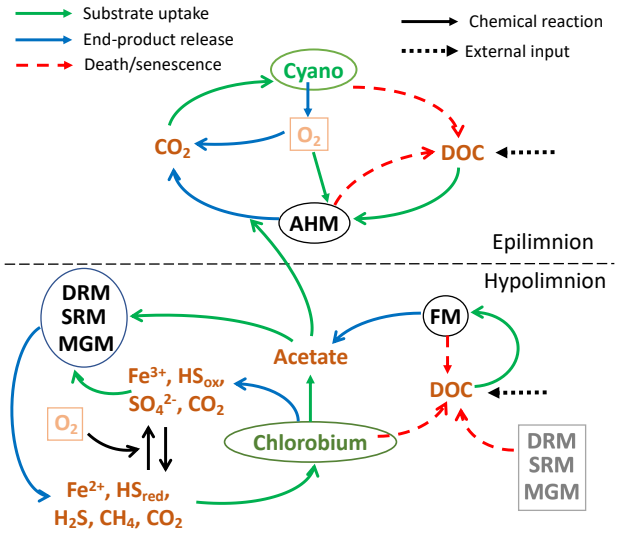
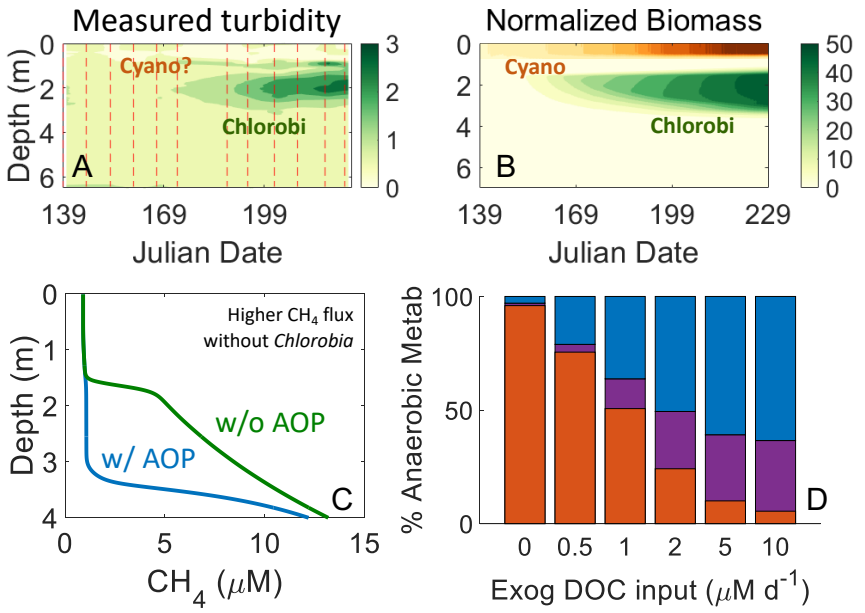



**Figure 5**. Flow diagram of coupled microbial metabolism and chemical cycling in the bog transport-reaction model. Primary dependent variables are shown in bold, with **microbial populations in black** and **chemical compounds in orange**. Greyed variables in boxes ($O_2$; DRM, SRM, MGM) are used to depict fluxes not easily incorporated into the flow diagram. **Cyano** = Cyanobacteria; **AHM** = Aerobic Heterotrophic Microbes; **DRM** = Dissimilatory $Fe^{3+}$ and $HS_{ox}$ Reducing Microbes; **SRM** = Sulfate Reducing Microbes; **MGM** = Methanogenic Microbes; **FM** = Fermentative Microbes. $HS_{red}$ = reduced HS; $HS_{ox}$ = oxidized HS.

We included oxidation/reduction reactions for Fe, $H_2S/SO_4$, and humic-DOM; methanogenesis when TEAs were fully reduced; and aerobic respiration in the epilimnion. Total AOP activity was scaled to the turbidity gradient. Kinetic parameters were estimated based on literature values. Differential equations to describe growth and substrate use were implemented for functional microbial groups while accounting for the fraction of reducing equivalents used for growth versus energy generation (Rittmann and McCarty, 2020). Although this model is only preliminary, requiring further parameter refinement, we used it to consider different community activity scenarios. We simulated a full growing season with and without AOP activity. Without AOP participating in oxidation reactions, redox conditions were fully reduced and stable through space and time. With AOPs included in the model, oxidized products accumulated in the *Chlorobium* plate with substantial influence on methane production in the hypolimnion (**Figure 6**). This is because the model predicts reducing equivalent consumption and sequestration into AOP biomass, quenching ARO activity and ultimately inhibiting methanogenesis. **This illustrates the potential for a critical role for AOPs in modulating greenhouse gas release from humic bog lakes.**

**Figure 6.** (A) Observed turbidity during summer 2019, indicating **Chlorobia** and possible **Cyano** peaks; (B) simulated growth of **Cyano** and **Chlorobi** biomass during the summer (dissimilatory $Fe^{3+}$, $HS_{ox}$ and $SO_4^{2-}$ reduction active and 1 μM $d^{-1}$ exogenous DOC input); (C) simulated water column $CH_4$ profiles **with** and **without** APO; (D) % anaerobic metabolism mediated by **methanogenic** and **non-methanogenic** pathways; **magenta** bars indicate *additional* $CH_4$ production, and *reduced* non-methanogenic metabolism *without* APO. activity




## 6. Research Approach

As described above, our prior work in this system shows that:

- A dense plate of *Chlorobium*-like AOPs forms just below the oxycline during summer stratification in Trout Bog.
- Redox conditions within the AOP plate vary diurnally.
- Microbial genomes encode and express a high density of genes potentially involved in EET and the density within a population correlates with lake DOC (color).
- AOPs may influence whole-lake metabolism with implications for net heterotrophy and greenhouse gas emissions.

Based on this, we hypothesize that AOP activity substantially expands the zone within which rapid redox cycling of HS, Fe, S compounds takes place just below the thermo- and oxycline (**Figure 2)**. Specifically, oxidation of reduced humic substances ($HS_{red}$), $Fe^{2+}$, and reduced sulfur coupled to anoxygenic photosynthesis regenerates oxidized humic substances ($HS_{ox}$), $Fe^{3+}$, and $S^o/SO_4^{2-}$ that are used as electron acceptors for AROs, including dissimilatory humic/metal-reducing organisms that utilize EET as previously inferred for the TB hypolimnion (He et al., 2019). This partnership between AOPs and AROs exerts previously unappreciated controls on whole-lake metabolism.

### *Objective 1: Defining spatial and temporal variation of physicochemical conditions*

Trout Bog and many other temperate or boreal humic lakes thermally stratify for most of the ice-free season (Read and Rose, 2013; Vonk et al., 2015), often mixing completely only in the fall. These physical dynamics create the diverse microbial niches supporting the phenomena we are studying. *In situ* sensors and manual water-column profiling will be used to examine physical structure, redox gradients, water chemistry on daily to seasonal time scales, providing a high-resolution view of system dynamics in time and space.

#### *Objective 1.1: High temporal resolution profiling with sensors and 2x manual profiling*

Trout Bog is already equipped with a buoy that supports high-frequency sensors to log water column temperature profiles at 0.5 m intervals, dissolved oxygen at 0.5 m, meteorological parameters, and chlorophyll-a. We will add a sensor to continuously monitor cDOM fluorescence, since this metric revealed off-set diurnal cycles at different depths in a previous study (Watras et al., 2015), presumably because of either pH or redox condition changes (Cory and McKnight, 2005). A separate buoy will be equipped with electrodes positioned at key points in the water column (**Figure 7**). During a period of three weeks in July-August 2023 we will collect daily profiles at 11:00 am using a hand-held multimeter to measure temperature, dissolved oxygen, oxidation reduction potential, and turbidity. This is the time period when the AOP plate is fully developed. During four consecutive days we will collect full depth-profiles hourly. This may be repeated as needed to obtain a sub-daily time series that is as complete as possible.

#### *Objective 1.2: High spatial resolution profiling with manual sample collection*

We will fully characterize the geochemical conditions at cm-scale depth resolution at key timepoints across the stratified season (**Figure 7**), and every six hours over three days during the same intensive profiling period described for Obj. 2 below. These sampling efforts will also be paired with those in Obj. 1.1. Water samples will be collected using a peristaltic pump with weighted tubing to control cm-resolved positioning and maintain ambient anoxic conditions. Sub-samples will be preserved for geochemical analyses to be conducted in the Water Science and Engineering Laboratory at UW-Madison as described (Peterson et al., 2020). These include total and dissolved organic carbon, Fe, and Mn, as well as SUVA, $H_2S$, and $SO_4$. We will measure bulk EDC/EAC using mediated electrochemical analysis with diquat dibromide and 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid), respectively (Aeschbacher and Sander, 2010).

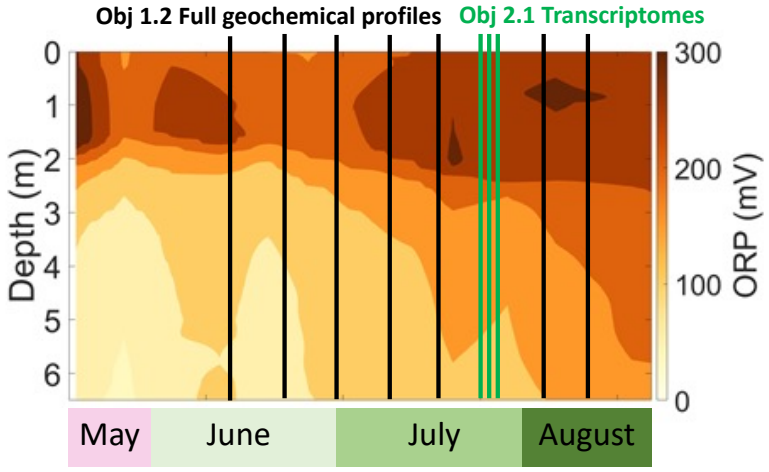



**Figure 7.** ORP in 2019 with planned full geochemical profiles (black, Obj 1.2) and intensive meta-transcriptome sampling (green, Obj 2.1). We hypothesize that coupled AOP and ARO activity manipulate the redox conditions in the thermocline (~ 1-2.5 m)

***Objective 1.3: Determine if GSB in Trout Bog Lake are free-living or physically associated with ARO using scanning electron microscopy.***

Aggregates containing GSB and a colorless partner from freshwater are relatively well-studied (Overmann and Schubert, 2002). The colorless partner is often obligately syntrophic, likely acquiring vitamins from the GSB who surround the partner as an "epibiont" (Kyndt et al., 2020; Müller and Overmann, 2011). Several studies report a physical connection between the two, forming "periplasmic tubes" that are hypothesized to allow direct sharing of the quinone pool (Liu et al., 2013). This could be one possible explanation for our observations in Trout Bog, but fluorescence activated cell sorting of the *Chlorobium* peak based on bacteriochlorophyll fluorescence did not yield any obvious partners in these enriched metagenomes (Berg et al., 2021). However, to confirm that the Trout Bog *Chlorobium* are free-living, we will perform scanning electron microscopy from the mature turbidity maximum in August 2024 using established protocols (Wanner et al., 2008). The Newcomb Imaging Center in the Botany Department at UW-Madison has an FEI Quanta 200 microscope and offers hands-on training.

***Objective 2: Measure differences in genome-wide gene expression through time***

Metatranscriptomics can be used as a "sensor" for a microbe's response to environmental conditions. Changes in gene expression reflect changing conditions such as diurnal variation in light and redox conditions (though we acknowledge that not all gene expression is regulated this way). Functional gene expression profiles provide clues on which carbon sources are used by AROs (Beier et al., 2015; Durham et al., 2015) and the suite of EET genes used by both AOPs and AROs. We hypothesize that *Chlorobium* gene expression changes over light/dark cycles.

***Objective 2.1: Collect time-series samples across three days***

Our prior work studying diurnal changes in gene expression (Linz et al., 2020) positions us well to carry out targeted sample collection for metatranscriptomic sequencing. We will collect samples at two depths in triplicate at four time points (5 am, 11 am, 5 pm, 11 pm) during three consecutive 24-h periods in late July when the turbidity peak is fully developed (**Figures 6 and 7**). Depths will be chosen to match the *Chlorobium* peak usually at 2.0 m and also 2 m below the peak. Based on contemporary physical-chemical profile data (Obj. 1), we will select one 24-h period for metatranscriptomic analysis, totaling 24 samples with replicates. Paired metagenomes

will be also sequenced (one per depth). Additional time points and/or depths will be analyzed if sequencing costs decrease in the next two years, allowing us to stretch the budgeted funds further.

***Objective 2.2: Recover metagenome-assembled genomes (MAGs) and map metatranscriptomes to evaluate gene expression***

Metagenomes will be assembled using metaSPAdes (Nurk et al., 2017), binned based on differential coverage and kmer frequency with MetaBAT2 (Kang et al., 2019), and dereplicated using dRep (Olm et al., 2017) at a 98% average nucleotide identity cutoff to create "metagenomic operational taxonomic units (mOTUs)" (Ruscheweyh et al., 2021). Alternative assemblers and binners will be evaluated as needed and bins will be refined in Anvi'o (Eren et al., 2021). The resulting MAGs will be added to our existing curated collection of MAGs and SAGs (single amplified genomes) from Trout Bog (Linz et al., 2018) to use as reference genomes for mapping with bowtie2 (Langmead and Salzberg, 2012) or any pipelines newly available at the time of analysis. McMahon's group has extensive experience with evaluating different analysis pipelines for both metagenome and metatranscriptome analysis in a variety of aquatic and engineered ecosystems using combinations of custom python/R/shell scripts and published packages (Linz et al., 2020; McDaniel et al., 2021).

Total RNA will be extracted from triplicates following the addition of an internal standard, which allows for quantifying the absolute abundance of transcripts in samples for comparing transcript abundance among samples, (Gifford et al., 2014; Linz et al., 2020) and shipped to the QB3 Genomics sequencing facility at UC-Berkeley for rRNA depletion, reverse transcription, and sequencing via Illumina NovaSeq. Metatranscriptome datasets will be competitively mapped to our Trout Bog MAG/SAG database using Kallisto (Bray et al., 2016). Average transcript concentration for each gene will be calculated after normalizing based on the internal standard coverage and gene length. We will evaluate correlations in gene expression dynamics within and across mOTUs, accounting also for lags as observed in our previous diurnal metatranscriptomics work (Linz et al., 2020).

**Potential pitfalls and contingency plans**

Metatranscriptomics is often viewed as a fishing expedition. **However, in this case we have specific predictions about what we will find, that are aligned with our hypotheses.** In the event that we do not detect evidence for diurnal changes in EET-related gene expression (offset between electrogens and electrotrophs), we will mine the metatranscriptomes for other diurnal patterns that provide clues to metabolic regulation controlled by redox conditions (e.g. carbon processing, $N_2$ fixation). At minimum, the datasets will provide additional evidence for metabolic capabilities that we have already inferred from MAGs (Linz et al., 2018), which will be of tremendous value for our ongoing studies of carbon and energy cycling in the lake. Our preliminary results and published work (Linz et al., 2020), however, are a firm footing for Obj. 2.2.

***Objective 3: Probe electrogen and electrotroph behavior using electrochemistry***

Our project focuses on the distinct roles of AOPs (electrotrophs) and AROs (electrogens), as well as the emergent phenomenon of bulk redox conditions cycling diurnally. To probe these roles and consequences, we will use electrochemical cells to conduct experiments under controlled conditions in the laboratory. This will build upon similar prior work conducted with cocultures of

green sulfur bacteria (*Chlorobium*) + purple non-sulfur bacteria (*Rhodopseudomonas*) from freshwater sediments (Schmidt et al., 2020), and *Chlorobium* + *Geobacter* (Badalamenti et al., 2014).

***Objective 3.1: Incubate fresh bog water consortia with different electron donors. Use electrochemical techniques to measure bulk EAC/EDC cycling under each condition and through time.***

We will construct anaerobic bioreactors to incubate consortia while measuring redox potential under manipulated conditions. A high-sensitivity platinum electrode (Aux.:Pt; Ref.:Ag) (Metrohm) will be used to measure and log ORP over time at sub-minute time scales.

Badalamenti *et al.* (2014) proposed that *Chlorobium* were storing glycogen in the light and fermenting it to acetate in the dark. In their experiment, *Geobacter* consumed the acetate and respired with an anode poised at -0.35 V vs Ag/AgCl. Sulfide was added periodically to replenish the electron source for *Chlorobium*. In our experiments, whole bog water collected from the *Chlorobium* plate will be used with an anode suspended in the water, to monitor changes in the EDC/EAC pool with a high-sensitivity platinum electrode (Aux.:Pt; Ref.:Ag) (Metrohm) over time at sub-minute time scales. Different combinations of electron donors will be added to determine how bulk redox conditions fluctuate under light/dark cycles, both in magnitude and duration. Triplicate experiments will be conducted over only 4-5 light/dark cycles (72-96 hrs) to exclude effects of biofilms forming on the electrode. Experiments will be conducted in an anaerobic chamber equipped with light emitting diode panels emitting at 660-770 nm to minimize the activity of purple non-sulfur bacteria that are present at low abundance in the turbidity peak.

We will add the following electron donors/shuttles to the anolyte at time zero, individually in separate experiments: Fe(II), $Na_2S$, thiosulfate, AQDS (a simple model humic substance), and commercially available model humic substances (i.e. Pahokee Peat and Suwannee River). We will try several concentrations of electron donating equivalents (e.g. 1 mM, 5 mM, 10 mM, 20 mM), expecting ORP to decrease in the dark due to AROs regenerating the EDC, and increase in the light when AOPs consume electrons.

***Objective 3.2: Isolate or enrich strains of Chlorobium and Geothrix from the lake***

We will attempt to isolate *Chlorobium* and *Geothrix* (or *Geobacter*) from Trout Bog Lake water using the approach described by Badalamenti et al (2013, 2014) and (Heising et al., 1999). A synthetic medium will be designed for further enrichment/isolation of both types of organisms, based on water chemistry characteristics that are well established by the NTL-LTER. Notably, the water has very low ionic strength compared to classical basal media. Media will include N-(3,4-dichlorophenyl)-N-dimethylurea (DCMU) to inhibit oxygenic phototrophs.

Pre-enrichment will be carried out in 50-mL serum bottles filled with lake water under an atmosphere of 80%$N_2$ /20%$CO_2$. For *Chlorobium*, we will also try pre-enriching with 80%$H_2$ /20%$CO_2$, since genomic analysis shows that the *Chlorobium* should be able to use $H_2$ as an electron donor (Garcia et al., 2021; Tsuji et al., 2020). For *Chlorobium,* $NH_4Cl$ will be omitted to impose $N_2$-fixing conditions (we have detected genes (Fernandez et al., 2020) and transcripts (unpublished) from *Chlorobium* $N_2$-fixing machinery *in situ*). Serum bottles will be incubated in an anaerobic chamber with light source with > 700 nm to select against purple bacteria. If enrichment is successful, isolation attempts will be carried out in agar shake tubes. For *Geothrix*/*Geobacter*,

basal freshwater medium will contain ferrihydrite and acetate (Coates et al., 1999). We will try both suspended culture in serum bottles and in bioelectric cells using an anoxic anode chamber and aerobic cathode chamber as described (Bond and Lovley, 2003; Holmes et al., 2004) using a range of different potentials (Torres et al., 2009). Enrichments in liquid and on electrodes will be screened using 16S rRNA gene amplicon sequencing.

Enrichment and isolation efforts are fraught with risk, particularly with anaerobes. However, co-PI Roden has extensive experience with culturing Fe-oxidizing bacteria and PI McMahon is an instructor for the Marine Biological Laboratory Microbial Diversity Course where she teaches cultivation strategies for anoxygenic phototrophs (both serum bottles and agar shakes). Co-PI Qin regularly operates anoxic bioelectric cells to enrich for anode-respiring microbes. We are also recruiting a postdoc with expertise in AOP isolation (Verena Nikeleit from Prof Andreas Kappler's laboratory).

**Potential pitfalls and contingency plans**

Obtaining pure cultures of such fussy organisms can be difficult and usually requires many months or even years to perfect the culture conditions. Thus, we acknowledge that Obj 3.2 is high risk. However, the enrichment process will also yield valuable information that should confirm what we observe in Obj 3.1. We argue that the potential reward of having pure cultures with which to perform more mechanistic experiments in the future, is worth the effort.

## 7. Broader Impacts

The **students** and **postdoctoral researcher** supported by this award will receive training in field work, bench work, and bioinformatics. The graduate student and postdoc will travel to the Trout Lake field station in the summer to perform intensive experiments, maintain sensor arrays, and supervise undergraduate researchers. They will participate in a Research Mentor Training seminar facilitated by the UW-Madison Delta program to build mentoring skills (4x2-hr sessions in a cohort model) (Pfund et al., 2015). One undergraduate student is supported per summer by a local fellowship to work at the field station. They maintain our long-term time series, project-related routine sampling (e.g. high-resolution profiles), and have an independent project. Past students have developed microbial fuel cells with different configurations and substrate additions, the data from which have directly informed our proposed work. At least one undergraduate will also participate during the academic year, supported by campus- or department-level fellowships and/or for independent study credits. McMahon is passionately committed to evidence-based approaches to training and mentoring. She regularly facilitates a Research Mentor Training seminar for faculty with a structured curriculum she co-developed with Pfund *et al.* (2015).

A significant component of this work includes maintaining bog water and/or cultures under anoxic conditions. Culturing AOPs and *Geothrix*-like organisms is not a trivial undertaking. The graduate student will attend either the Hopkins Course at Stanford University or the Microbial Diversity course at the Marine Biological Laboratory in Woods Hole during the first year, to gain skills in culturing anaerobes. Notably, McMahon is an instructor at the Microbial Diversity course at least through 2023 and will recommend the student to serve as a teaching assistant in subsequent years, during the early part of the curriculum (since field work requires the student be in Wisconsin during late July/August). The postdoc we hope to recruit for the project took the course in 2022, where she met McMahon.

The Center for Limnology has vigorous outreach and education programs in which we have participated for many years. The Trout Lake field station near Trout Bog has an annual open house in August that regularly draws hundreds of visitors (250 in 2021 and 400 in 2022). During the past 3 open houses (excluding 2020) we have created an interactive display featuring fuel cells colonized by Trout Bog microbes, highlighting what we call the "Bog Batteries". In fact, it was an open house in 2018 that first prompted our hypotheses about the effects of light on the communities. The PhD and undergraduate students running the display noticed that current production increased when clouds passed overhead, shading the cultures. **This is a powerful example of outreach being tightly integrated with research questions.** The fuel cells were featured in the Lakeland Times, a local newspaper.

We will continue to refine our display and will participate in open houses each year of the project (and beyond). The graduate students supported on this project will join the Wisconsin Idea STEM Fellows program at the UW-Madison. Researchers in the Fellows program participate in multiple training workshop sessions as an interdisciplinary cohort. Workshops focus on best-practice models to **design, implement, and assess** interactive face-to-face activities that support appreciation and understanding of current science research. Following the workshops, researchers are supported by staff on the UW-Madison Broader Impacts Team in continued development of their interactive stations and their commitment to participate in a minimum of three public engagement with science events. We will also host a station at a Saturday Science event at the Wisconsin Institutes for Discovery, which is tightly integrated with the Fellows program.

**8. Results of Prior Support**

McMahon: NSF-CBET-1935173. $329,608 (5/2020-4/2023) "Unrecognized microbial sources of methyl mercury in freshwater lakes" Progress on this award was delayed by the pandemic due to lack of access to the field and lab. **Intellectual Merit:** We carried out metagenomic and metatranscriptomic sequencing on samples paired with detailed biogeochemical analyses to examine the relationship between putative mercury methylators and key terminal electron acceptors found in the stratified water column. Incubation experiments were also performed with known methylation inhibitors to dissect the anaerobic food web that sustains methylation. Two manuscripts are in preparation. A fruitful ongoing collaboration with the Mercury Laboratory allowed us to test some of our hypotheses along a sulfate gradient in the Florida Everglades and a paper is in press describing these results (Peterson et al., 2023). We also participated in creating a consensus protocol for recovery of methylation genes from metagenomes (Capo et al., 2022). **Broader Impacts:** Three graduate students were trained and two are currently postdoctoral researchers in new labs. Three undergraduate students were trained and all three are currently PhD students at other universities. An additional two undergraduate students will be attending graduate school in Fall 2023. McMahon presented results from the project at the Clean Lakes 101 Breakfast for the Clean Lakes Alliance in Madison, WI.

Qin: NSF-CBET-2219089. $1,687,899 (09/2022-08/2026) "ECO-CBET: Modular electrochemical processes for simultaneous nitrogen recovery and carbon dioxide mitigation". **Intellectual merit:** The goal of this work is to develop modular electrochemical processes to tackle carbon, nutrient, and water challenges in livestock manure systems. **Broader impacts:** This project will introduce transformative concepts for more efficient electrochemical resource recovery and $CO_2$ mitigation and engage underserved rural communities. There are no publications to report yet.

Roden: No NSF support in the past five years.

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(Products from prior work conducted by our team members are in bold)

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