Investigation of the Deep Chlorophyll Maximum in Lake Louise, Georgia

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ABSTRACT

Lake Louise, a temperate lake in south-central Georgia, USA, is 6.5 meters deep at its deepest point and approximately 6 hectares in area. Despite being relatively shallow, the lake stratifies each year, typically stabilizing from mid-March until mid-November, with a thermocline located at a depth of 2.0 – 2.5 meters. During the summer, high tannin concentrations in the water also cause a sharp decrease in light levels with less than 0.1% of incident light reaching a depth of 2.5 m. However, spectrophotometric pigment analysis indicates that a distinct deep chlorophyll maximum (DCM) develops at an approximate depth of 3.0 m, well below the photic zone, and contains higher apparent chlorophyll *b* concentrations than the surface waters. Photosynthetic activity at this depth suggests organisms which are capable of compensating for extremely low-light. Preliminary data at 3.0 m indicate an abundance of filamentous, photosynthetic organisms that are visually consistent with cyanobacteria. Chlorophyll spectra from sampling events suggested bacteriochlorophyll *d* as the primary photosynthetic pigment in the DCM, while initial imaging of filters and sequencing of 16s RNA PCR amplicons further indicated the presence of eubacteria in the phyla Chlorobi and Chloroflexi. A *Chloronema*-type trichome has been identified through light micrographs that may be indicative of a novel morphospecies.

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Chapter I

INTRODUCTION

Current lacustrine monitoring programs and trophic classification schemes rely heavily on the determination of primary production in relation to the concentration of photoautotrophic organisms in the epilimnion (Carey, Ibelings, Hoffman, Hamilton, & Brookes, 2012; Nürnberg, 1996; Williamson, Morris, Pace, & Olson, 1999). Such an approach neglects the ecological contribution of hypolimnetic organisms to the ecosystem (Camacho, Erez, Chicote, Florin, Squires, Lehmann, & Bachofen, 2001; Imhoff & Thiel, 2010). According to Chapin, Denoyelles Jr., Graham, & Smith, 2004, organisms responsible for deep chlorophyll maxima (DCM), including bacteria belonging to the phyla of green and purple sulfur, ultimately account for up to 40% of the total photosynthetic productivity during periods of prolonged water column stability (Chapin, Denoyelles Jr., Graham, & Smith, 2004; Crowe, Maresca, Jones, Sturm, Henny et al., 2014; Wetzel, 2001). Some members of *Chlorobium*, a genus of green sulfur bacteria, are known to serve as the epibionts in symbiotic relationship with heterotrophic, motile bacteria, and can be useful detectors of environmental shifts (Kanzler, Pfananes, Vogl, & Overmann, 2005; Overmann & Gemerden, 2000). Yet, despite the abundance and influence of the anoxygenic sulfur and nonsulfur phototrophic bacteria in aquatic ecosystems, studies often fail to sample and report below the photic zones, potentially rendering conclusions about the system of interest incomplete (Chapin et al., 2004; Crowe et al., 2014).

Lake Louise, a moderately dystrophic and seasonally stratified system near Valdosta, Georgia, is the site of ongoing phycological and geological research that has spanned decades. More recently, efforts have been made to establish baselines for future biological and geochemical investigations (Gould, Nienow, Nienow, & Nienow, 2019; Riggs, Pascarella, & Bechler, 2010; Tepper & Hyatt, 2011). In 1995, the ecology of the lake changed and most of the surface was covered by a dense mass of duck weed composed of species of *Spirodela* and *Wolffia* species (Nienow, unpublished observations). The reasons for this change are not known; both the release of nutrients from the surrounding woodlands as the result of the construction of a beaver dam across the outlet and invasive duckweed brought in by illegal fishing activities have been suggested. In early 2002, the duckweed disappeared, again for reasons unknown (Nienow, unpublished observations).

A monitoring program was established with weekly to biweekly sampling from May 2002 to December 2004 in order to determine the short-term impacts in the aftermath of the duckweed bloom. During these investigations, anomalous maxima in chlorophyll *a* (Chl *a*) and chlorophyll *b* (Chl *b*) concentrations at a depth of about 3 meters, near the thermocline and below the 0.1% isophot were observed. Initial microscopic analysis of material recovered from this depth indicated the presence of decaying plant matter, thought to be the remains of the duckweed cover, and a large population of *Planktothrix isothrix* (Skuja) Komárek & Komarkova (Nienow, unpublished data). An initial conjecture attributed the anomalous chlorophyll concentrations to a combination of these two factors. However, the deep chlorophyll *b* maximum returned in 2003 (Figures 1-2), 2004 (Figures 3-4), and again in 2009, long

after winter-mixing had dispersed any remnants of the duckweed and the populations of *P. isothrix* had diminished (Nienow, unpublished observations). The long-term persistence of the Chl *b* DCM suggested the possible emergence of a cyanobacterial population within the genus *Prochlorothrix* Burger-Wiersma, Stal, & Mur (1989).

Although members of *Prochlorothrix* are, thus far, exclusively associated with eutrophic systems (Burger-Wiersma et al., 1989; Pinevich, Velichko, & Ivanikova, 2012), preliminary data indicated that large aggregations of unidentified trichomes coincided with a chlorophyll *b* DCM at a depth of 3 meters. Based on collective literature and work conducted by Bullerjahn and Post (1993), the presence of *Prochlorothrix* in a moderately dystrophic lake would be unprecedented, but not necessarily unfounded. Atypical of traditional cyanobacteria, *Prochlorothrix* do not possess phycobilisomes, instead adapting to their – often anoxic – environments through an association between photosystem I and an unprecedented chlorophyll *a/b* antenna (Bullerjahn & Post, 1993; Post & Arieli, 1997). However, the absence of the phycobilisomes in *Prochlorothrix* represents a photosynthetically disadvantageous adaptation for the extreme low-light conditions of Lake Louise. Further, any penetrant wavelengths of photosynthetically active radiation (PAR) reaching the DCM site are likely to be in the yellow and infrared spectrum, both of which exist outside of the absorptive capabilities of chlorophylls *a* and *b* but are easily harvested by carotenoids and chlorosomes of green sulfur and green non-sulfur bacteria (Blankenship & Matsuura, 2003; Hohmann-Marriott & Blankenship, 2011; Imhoff & Thiel, 2010; Tronrud, Wen, Gay, & Blankenship, 2009). Most notably, the DCM occurs seasonally, developing during thermal stratification and disappearing with turnover events, implying an adherence to a strictly anaerobic lifestyle. Such major inconsistencies

with the *Prochlorothrix* hypothesis necessitated further inquiry and investigation of both microflora and chlorophyll spectra.

New monitoring programs were conducted in 2015 and again in 2017 with the dual purpose of re-establishing the baseline for future studies and resolving the nature of the deep chlorophyll *b* maximum. Additional samples were collected in May 30, 2018, and May 31, 2019 for analysis of the microbial community based on 16s rRNA data (Clarridge III, 2004; Janda & Abbott, 2007).

Chapter II

MATERIALS AND METHODS

Lake Louise

Lake Louise (Figure 5) is a 5.7-hectare lake located approximately 15 kilometers south of Valdosta, Georgia and a few hundred meters east of Interstate Highway 75 (Tepper & Hyatt, 2011). Formed as a result of sinkhole collapse caused by limestone dissolution roughly 9,500 years ago (Tepper & Hyatt, 2011; Watts, 1971), the lake exhibits a maximum depth of 6.5 meters (Hains, 1987), with much of its water supplied via diffusion from surrounding wetlands (Hains, 1987; Tepper & Hyatt, 2011). Adjacent to Lake Louise is another small system, Cathead Lake, which intermittently drains into the Lake Louise basin (Tepper & Hyatt, 2011). Organic-rich gyttja, responsible for impeding groundwater input, is converted to humic acid by resident heterotrophic bacteria through the general mechanisms described by Jansson, Blomqvist, & Jonsson (1996), establishing a pH range of about 5.4–6.0 in the water column. Excess humic acid and tannin concentrations in the water, especially during the summer months, cause the lake to become darkly colored. Although Lake Louise is considered shallow, the steep banks and an overall depth of around 6 meters are morphologically sufficient to allow for thermal stratification between April and October, when air temperatures are consistently above 16^{o} C. During the winter months, Lake Louise is thermally unstable, and may completely mix several times in response to changes in air temperatures as cold fronts

move through the region (Hains, 1987; Tepper & Hyatt, 2011; Nienow, unpublished data).

Previous studies conducted by Miller, Mora, Grissino-Mayer, Mock, Uhle, and Sharp (2006) and Tepper and Hyatt (2011) suggest that major disturbances, including the Great Hurricane of 1780 and the construction of I-75 in the early 1960s, have fundamentally altered the rate of allochthonous nutrient deposition into the lake, thereby forcing a long-term shift in trophic state. In fact, despite the prior distinction of Lake Louise as a dystrophic system due to its dark coloration and low pH, its 'blackwater' quality may actually have an enhancing effect on primary productivity (De Haan, 1974; Hains, 1987; Jones, 1992; Nürnberg, 1996).

Environmental Monitoring

Samples were collected at roughly weekly intervals from May 16 to July 9 in 2015, and from February 10 to December 9 in 2017. Due to the possibility of diel fluctuations in phytoplankton abundance and distribution, all sampling was conducted between 10 and 11:30 a.m. Photosynthetically active radiation (PAR, µmol photons · m^{-2} · s^{-1}) was measured simultaneously at the surface, using an LI-190 quantum sensor, and at 0.1-meter intervals in the water column to a depth of 2 meters, using a spherical quantum sensor (LI-COR SPQA); both sensors were connected to an LI-1400 recorder (LICOR, Inc., Lincoln, Nebraska). Temperature (°C), conductivity (siemens · cm^{-1}), and dissolved oxygen concentrations (DO, mg · L^{-1}) within the water column were measured every 0.1 meters to a depth of 2 meters and at 0.5-meter intervals thereafter to a final depth of 6 meters using a YSI Pro2030 (YSI, Inc., Yellow Springs, Ohio). This system mimics the techniques used during the 2002-2004 monitoring program. A parallel set of

measurements was taken using a YSI EXO1 sonde equipped with sensors for depth, temperature, conductivity, dissolved oxygen, and chlorophyll (relative fluorescence) attached to an EXO hand-held display.

Biological Sample Collection

A series of water samples were collected from Lake Louise during each sampling event in 2015 and 2017 and again on May 30, 2018 and May 31, 2019. Congruent with the 2002–2004 sampling program (Nienow, unpublished data), 2 liters of unfiltered water were collected for pigment analysis at 1-meter intervals from 0–5 meters using a Kemmerer bottle. During this process, 50 mL from each meter of the water column was added to a tube (labeled with the corresponding depth of collection) prepared in advance with 1 mL Lugol's iodine for observation using imaging flow cytometry (Fig. 6). For molecular-based community analysis (Padilla, Ganesh, Gantt, Huhman, Parris, Sarode, & Stewart, 2015), 4 additional liters of water were collected from depths of 1 meter, 3 meters, and 5 meters. These samples were stored in a cold room, maintained at 4°C, for up to 24 hours until filtration. Additional 100-mL aliquots of these samples were separated and fixed with 0.5% glutaraldehyde in 20 mM HEPES buffer (pH 7.0) upon return to the lab. The aliquots were subsequently stored at 4°C for later evaluation using light microscopy.

Pigment Analysis

Duplicate samples from each depth were collected on glass fiber filters with a nominal pore size of 0.7 μm for pigment analysis. Depending on the density of microorganisms in the sample, 100–500 mL aliquots of water for each depth were filtered within 3 hours of sample collection. Flow-through was retained and stored at 4°C for use

in the preparation of sterile enrichment media. Each filter was extracted overnight at 4°C in 3 mL of 90% acetone. Extracts were centrifuged and the absorption spectrum between 350 and 800 nm determined using a PerkinElmer Lambda 35 UV/VIS spectrometer (PerkinElmer, Waltham, Massachusetts). The estimated concentration of chlorophylls *a*, *b*, and *c* from each extract ($\mu g \cdot mL^{-1}$) and the entire sample ($\mu g \cdot L^{-1}$) was determined using the trichroic equations (Equation 1) of Jeffrey and Humphrey (1975) based on absorption at 664 nm, 647 nm, and 630 nm; the absorption at 750 nm was used as a control for turbidity of the sample.

Equation 1: *Trichroic Equation ($\mu g\ chl \cdot mL^{-1}$)*

$$\text{Chlorophyll } a = 11.85 \cdot (A_{664} - A_{750}) - 1.54 \cdot (A_{647} - A_{750}) - 0.08 \cdot (A_{630} - A_{750})$$

$$\text{Chlorophyll } b = -5.43 \cdot (A_{664} - A_{750}) + 21.03 \cdot (A_{647} - A_{750}) - 2.66 \cdot (A_{630} - A_{750})$$

$$\text{Chlorophyll } c = -1.67 \cdot (A_{664} - A_{750}) - 7.6 \cdot (A_{647} - A_{750}) + 24.53 \cdot (A_{630} - A_{750})$$

During 2015, the opportunity arose to analyze extracted pigments using high performance liquid chromatography (HPLC). For this purpose, a third filter was prepared for each depth using twice the volume of water and stored at -80ºC until further processing. Frozen filters were transferred to test tubes with 1–2 mL of sonicated extraction solution (80% acetone, 15% methanol, 5% ddH_2O), capped, and pulverized with a wand sonicator before centrifugation at 3,000 rpm for 2–3 minutes. Tubes were left to incubate overnight at -20°C followed by centrifugation at 3,000 rpm for 5 minutes. Extracted pigments from each sample were then separated using a Prominence HPLC System (Shimadzu Corporation, Kyoto, Japan) following the protocols in Waters, Piehler, Smoak, & Martens (2010). The spectrum of each fraction was analyzed using the software accompanying the instrument (LabSolutions LC/GC, release 5.42 SP2).

Imaging Flow Cytometry and Light Microscopy

Imaging flow cytometry was used to approximate the distribution of microorganisms in the water column. Water samples preserved on-site in Lugol's iodine were analyzed using a FlowCAM® (Fluid Imaging Technologies, Scarborough, Maine) following the basic procedures in Gould et al. (2019). In the present analysis, both 10x and 20x objective lenses were used. To remove large debris and ensure that particles fell within appropriate size ranges for observation, samples were prefiltered through 100 μm nylon mesh for the 10x objective, and through a 35 μm nylon mesh for the 20x objective. Optical filters for each lens were developed using the VisualSpreadsheet software accompanying the instrument. The filters allowed separation of particles imaged for each objective into tentative groups using morphological parameters including size, color, and shape.

To confirm biodiversity estimates from imaging flow cytometry, images generated by FlowCAM were compared to manually captured light micrographs of net phytoplankton and concentrated water samples collected from each depth. For the latter, samples were fixed overnight in 1% glutaraldehyde in 20 mM HEPES, using 90 mL of unfiltered water as the diluent for a 100 mL solution of sample. Each sample was then centrifuged at 2,000 rpm for 30 minutes to improve microbial concentration without disrupting cell morphology. After centrifugation, 95 mL of the supernatant fluid were removed, and the remaining 5 mL were used to resuspend the pellets. Using a slight modification to the Stevenson (1984) protocol, chemically fixed and concentrated material from each depth was semi-permanently mounted on a series of microscope slides

using Taft's syrup medium. Wet mounts of net plankton and permanent slides of concentrated material were observed at 200x and 1000x magnification on an Olympus BX60 compound light microscope (Olympus, Tokyo, Japan) equipped with differential interference contrast optics and a Canon EOS Rebel digital camera (Canon, Tokyo, Japan) configured for remote viewing through open-source Entangle® software. Species counts were conducted by hand.

Isolation of Photosynthetic Organisms

Attempts were made to create uni-algal cultures by serial dilution of whole-water samples collected from each depth. Enrichment solutions composed of BG-11 nutrients in 1 L of filtered water from specified depths in Lake Louise were autoclaved to create stratum-specific nutritional growth media for cyanobacteria. In total, 18 series consisting of 6 ten-fold dilutions of unfiltered Lake Louise water were prepared using 9 mL of sterilized medium. All series were placed in an environmental growth chamber at 18°C with constant illumination. Cultures were gently aerated once per week using an orbital shaker (Forma Scientific 4518) at 20°C and 150 rpm for 4–5 hours.

A medium specially designed to selectively encourage the development of green sulfur bacteria was prepared following methods outlined by Pfennig and Trüger (1981). Salt and trace mineral solutions were combined and diluted to a volume of 1 liter before autoclaving alongside 10 mL of freshly prepared 5% $Na_2S \cdot 9\ H_2O$ solution. After sterilization, 10 mL of B_{12} vitamin solution and 40 mL 5% $NaHCO_3$ were added to the autoclaved medium and dispensed into sterile, screw-capped test tubes. Unfiltered lake water was used to inoculate the medium and tubes were maintained under constant illumination at 20°C in an environmental growth chamber for several weeks. For soft

agar medium, granulated agar was added to salt and mineral media at a concentration of 1.5% (w/v).

Genetic Analysis

Initially, samples for DNA analysis were collected on Isopore® polycarbonate membranes (0.4 μm pore size) via vacuum filtration (Minamoto, Naka, Moji, & Maruyama, 2016). Membranes for 1-meter and 5-meter water samples each filtered 150 mL water from the corresponding depths. However, due to clogging by filamentous organisms, only 100 mL of water from 3 meters could be filtered through the 0.4 μm pore, necessitating an increase in pore size. Thereafter, 150 mL from each depth was filtered through membranes with a pore size of 1.2 μm to both retain the species of interest and account for the collection of larger organisms in the samples (Padilla et al., 2015). Approximately 3.75 L from each depth were ultimately captured across 25 filters, including one 0.4 μm membrane. Filters were stored at -20°C in 1.5 mL screw-capped polyethylene microfuge tubes. The remaining ~250 mL from each sample were stored for 24 to 48 hours at 4°C until used for culturing.

DNA was extracted directly from polycarbonate filters using a hybrid phenol-chloroform and spin-column protocol, designed for gentle cell lysis, removal of PCR-inhibiting organics present in the lake water, and high-yield elution of nucleic acid (Wintzingerode, Göbel, & Stackebrandt, 1997). Additional replicates of the extraction also were run to determine if significant variation existed in the observed concentration of collected DNA when biomaterial was removed from filters first by incubation and vortexing (Smith, Carroll, & Mottice, 1993; Yilmaz, Phlips, & Tillett, 2009).

Cell wall lysis was achieved by placing cells in 1.5 mL Eppendorf tubes and immersing in 1 mL of sterile sodium chloride-Tris-EDTA (STE) buffer (pH 8.0). This was followed by high-speed centrifugation for 5 minutes. After discarding the supernatant fluid, the filters were resuspended in 500 µL STE buffer (pH 8.0) and 20 µL of 25 mg/mL lysozyme solution. After a 5-minute incubation at 37°C, 10 µL of 10% sodium dodecyl sulfate (SDS) was added and the sample was returned to the 37°C water bath for 30 additional minutes. Each sample was then briefly heat-shocked at 65°C for 2 minutes, 50 µL of proteinase K solution (20 mg · mL^{-1}) was added, and the sample was incubated at 65°C for 30 minutes.

After incubation, DNA was extracted with phenol-chloroform in a fume hood (Sambrook, Fritsch, & Maniatis, 1989). An amount of phenol roughly equal to the volume of lysis solution was added to each sample, which was then gently inverted for up to 10 minutes to form an emulsion layer. Upon appearance of the emulsion, the samples were centrifuged at 12,000 x *g* for 3 minutes at room temperature, and the aqueous phase was transferred to a fresh tube. The number of phenol washes and subsequent centrifugations was contingent upon the size of the protein interface. Once clean, the collected aqueous phase received an equal volume of chloroform and was centrifuged again at 12,000 x *g* for another 3 minutes.

The resulting aqueous phase from each sample was transferred to a QIAGEN spin column in a 2 mL wash tube (taken from DNeasy Blood and Tissue Kit) and 200 µL of 70% ice-cold ethanol was added to each column to facilitate binding. Columns were centrifuged for 1 minute at high speed and the flow-through material was discarded. Procedure and materials (buffers AW1 and AW2) from the QIAGEN DNeasy Blood and

Tissue Kit were then used to complete spin column recovery. DNA was eluted using 100 µL sterile Tris-EDTA (TE) buffer (pH 7.8) and stored at -20°C. DNA concentration from each sample was quantified using a Nanodrop 2000 spectrophotometer by ThermoScientific™ (Padilla et al., 2015; Yilmaz et al., 2009).

Universal eubacterial DNA primers (ReadyMade™ 16s rRNA For/Rev; Integrated Technologies) designed for amplification of the 16s rRNA gene were selected (Clarridge, 2004) and PCR conditions were optimized to accommodate aquatic, environmental DNA samples (Bhadbury & Ward, 2009; Caporaso, Lauber, Walters, Berg-Lyons, Lozupone et al., 2011; Castiglioni, Rizzi, Frosini, Sivonen, Rajaniemi et al., 2004). Primers designed by Velichko, Averina, Gavrilova, Ivanikova, & Pinevich (2014) to target the 16s rRNA gene in *Prochlorothrix* were also employed to detect potential *Prochlorothrix* species with higher sensitivity and specificity. For samples using the universal forward and reverse primers, initial melting of DNA at 95°C for 4 minutes was followed by 35 cycles of denaturation at 95°C (30 s), annealing at 50°C (30 s), and extension at 72°C (45 s). An additional 10 minutes of extension at 72°C was allowed before the PCR product from each sample was collected. Samples run using *Prochlorothrix* primers (pctx) followed the PCR protocol outlined by the Velichko cohort (Velichko, Averina, Gavrilova, Ivanikova, & Pinevich, 2014).

All 16s rRNA gene amplicons were visualized using electrophoresis of 1% agarose gels made with 1X Tris-acetate-EDTA (TAE) buffer. To stain the nucleic acid during electrophoresis, ethidium bromide (EtBr) stock (10 mg · mL^{-1}) was added to the agarose solution (2 µL EtBr/100 mL gel) immediately prior to pouring and setting. Depending on the size of the gel, voltage and time were adjusted for optimal results. Small gel volumes

(80 mL) required 95–100 V for 1 hour 15 minutes while large gels (180 mL) were exposed to 125 V for 2 hours 30 minutes. Bands were viewed by ultraviolet light on a Fisher Scientific™ transilluminator to ensure proper amplification.

Cloning and Sequencing

Double-stranded DNA amplicons were integrated into linearized pCR™ 4-TOPO® plasmid vectors using an Invitrogen TOPO® TA cloning® kit. After a 5-minute incubation, the product of the cloning reaction was added to OneShot® chemically competent TOP10 cells of *Escherichia coli*. Cell mixtures were then exposed to a 30 second heat pulse at 42°C and immediately returned to ice to enhance permeability of the plasma membranes for successful transformation. Super optimal broth with catabolite repression (SOC) medium was subsequently added and cell mixtures were shaken at 200 rpm on an orbital shaker for one hour at 37°C.

After shaking, 100 µl of transformed cells were spread on LB-ampicillin agar plates (50 µg/mL ampicillin) containing X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) solution at a concentration of 20 µg/mL. Plates were incubated at 37°C for 24 hours to allow appropriate colony formation before blue/white screening. Resulting white colonies (indicative of a successful insert) from each plate were selected for isolation and grown on new LB-amp plates. Colonies from these plates were further purified by a secondary selection and isolation step to ensure coverage and maintenance of all possible inserts. All collected white colonies were cultured in liquid LB-amp media before plasmids were extracted using an Invitrogen PureLink™ Quick Plasmid Miniprep kit and quantified using the Nanodrop 2000. Before sequencing, plasmids were assessed for successful insertion by digestion with EcoRI followed by gel electrophoresis.

Plasmid DNA samples were sent for sequencing at the Florida State University DNA sequencing facility. A “big-dye” terminator for double-stranded DNA products was used on an Applied Biosystems 3730 Genetic Analyzer. A 16s rRNA nucleotide BLAST of the results against all known bacterial species represented in the database, including the prochlorophytes, was used to evaluate biodiversity of the eubacterial microflora in Lake Louise and potentially identify the species of interest.

In accordance with NIH guidelines for the handling of recombinant nucleic acids, approval was obtained from the Valdosta State University Biosafety Committee to work with chemically transformed *Escherichia coli* for this project (see *Appendix A*).

Chapter III

RESULTS

Environmental Data

Reported environmental measurements for the 2017 Lake Louise sampling session can be found in figures 6–9. In 2017, on average, the summer thermocline in Lake Louise was found between depths of 1.5–3.0 m. At the interface between the lower epilimnion and upper thermocline, temperatures reached upwards of 25°C–30°C, in contrast to temperatures of 12°C–15°C upon nearing the hypolimnion (Fig. 7). Organic material in the water rapidly attenuated the light, resulting in less than 0.1% of incident light at a depth of 3 meters (Fig. 8), but did not appear to significantly influence measures of conductivity (Fig. 9). Importantly, physical disturbance to Lake Louise from Hurricane Irma prematurely mixed the water column to a depth of 3 meters, as is reflected by a surge in DO content at 2–2.5 meters in mid-September 2017 (Gould et al., 2019). Nevertheless, the hypolimnion remained functionally anoxic below 2 meters for most of the year, returning to oxygenated conditions during winter-mixing (Fig. 10).

Data collected in May 2018 and May 2019 fit these general patterns (Figs. 11–13). During both sampling events, the thermocline was established between a depth of 1.5 m–3.5 m, yielding a temperature change of approximately 15°C in less than 2 meters (Fig. 11), and the hypolimnetic concentration of DO remained biologically negligible (<0.1 mg $\cdot$ L^{-1}; Fig. 12). In both 2018 and 2019, < 0.1% of incident light (photon $\cdot$ m^{-2} $\cdot$ s^{-1}) reached

3 meters, and only ≤ 6–12% of light penetrated past a depth of 1 meter (Fig. 13), indicating an extremely shallow photic region within the epilimnion.

Particle counts derived from flow cytometry of the water column indicate that vertical distribution of phytoplankton concentration and phyla varied by orders of magnitude, largely depending on temperature and oxygen availability. Although the detectable microflora community appeared to be mostly composed of chlorophytes and cyanobacteria, other refractile filamentous organisms also dominate towards the lower metalimnion. In 2018 alone, flow cytometry conducted on Lake Louise water samples indicated that the total particle count at a depth of 3 meters was 2.1×10^4 particles $\cdot$ mL^{-1}. At 3 meters, 98.5% of the microorganisms were prokaryotic, 91.2% of which were classified as filamentous. The epilimnion, in contrast, was surprisingly less suitable for planktonic growth than the metalimnion, yielding only 4.6×10^3 particles $\cdot$ mL^{-1}.

Chlorophyll Spectrum and HPLC Analysis

A DCM developed below the photic zone of Lake Louise, from May to September 2017, accumulating at an approximate depth of 3 meters with slight distribution toward the benthic zone (Figs. 14 & 15). Trichromatic chlorophyll equations designed for spectrophotometric analysis in 90% acetone determined chlorophyll *b* as the primary pigment responsible for the composition of the DCM (Fig. 16; Table 1), which accounted for 54.6% of the total chlorophyll extracted from 3 meters. Chlorophyll *a* is most strongly concentrated in the epilimnion with reduced concentrations appearing at the depth of the DCM (Fig. 14). Samples collected from Lake Louise after Hurricane Irma in September 2017 indicated the premature loss of the DCM at 3 meters, due to disturbance-induced limnetic mixing, and improved Chl *a* signal from the surface waters. These data agree

with previous records of DCM disappearance during periods of oxygen restoration via major climatic disturbances or regular seasonal variation.

Table 1. *Spectrophotometry data collected from 2017 – 2019 from Lake Louise. Absorption values in 90% acetone for major chlorophyll pigments and bacteriochlorophyll a shown, along with wavelength of maximum absorption.*

Spectrophotometry Analysis in 90% Acetone						
Date	**Depth**	**A_{663}**	**A_{647}**	**A_{630}**	**A_{750}**	**λ_{Max} (nm)**
06/01/2017	**1 meter**	0.314	0.086	0.068	0.004	665
	3 meters	0.274	0.240	0.070	0.006	655
	5 meters	0.083	0.056	0.021	0.004	656
		A_{663}	**A_{647}**	**A_{630}**	**A_{750}**	**λ_{Max}**
05/30/2018	**1 meter**	0.337	0.098	0.070	0.010	665
	3 meters	1.573	1.350	0.378	0.016	655
	5 meters	0.380	0.359	0.103	0.013	655
		A_{663}	**A_{647}**	**A_{630}**	**A_{750}**	**λ_{Max}**
05/31/2019	**1 meter**	0.906	0.348	0.268	0.098	664
	3 meters	0.591	0.451	0.212	0.098	656
	5 meters	0.432	0.359	0.142	0.058	655

In concordance with observations from 2003, 2004, 2015, and 2017, a strong deep chlorophyll maximum (DCM) was present at approximately 3 meters during both 2018 and 2019 sampling sessions (Figs. 17 & 18). The average concentration of subepilimnetic chlorophyll in Lake Louise at the height of thermal stratification was found to be higher than that of the epilimnion (Table 1), indicating increased rates of primary productivity within the anoxic region of the thermocline.

The trichromatic equation applied to spectrophotometry data for both May 2018 and May 2019 demonstrated that absorbance for chlorophyll *b* (A_{647}) was highest for samples collected at 3 meters, while results for chlorophyll *a* (A_{664}) were less conclusive. Though

it remains true that Chl *a* is the predominant photosynthetic pigment in the surface waters, A_{664} peaks at 3 meters in the 2018 and 2019 samples appeared marginally stronger than those of A_{647} at the same depth (Figs. 17 & 18; Table 1). Spectra of the peaks found at 3 meters were plotted to resolve the issue and revealed major absorptive capacity at wavelengths of 653–655 nm, intermediate to either traditional Chl *a* or Chl *b* red-spectrum peaks in acetone and well outside the known carotenoid absorptive ranges (Tables 1 & 2). The major bacteriochlorophyll *a* (BChl *a*) peaks at 750 nm were not apparent in the spectra. In addition, relative fluorescence data captured by the sonde sensor fail to depict a major chlorophyll maximum below 2 meters deep (Fig. 19), further suggesting the absence of any plant-type pigments in the hypolimnion.

Table 2. *HPLC data collected in 2015. Approximate pigment ID provided based on observed retention times and absorptive capacities.*

HPLC Analysis (May 30, 2015)				
Depth	**Retention Time (min)**	**Concentration**	**Peaks (nm)**	**Pigment ID**
0 meters	10.094	1.817	450, 476	Carotenoid
	12.5	N/A	N/A	N/A
	14.638	1.146	430, 664	Chl *a*
	15.308	3.464	431, 665	Chl *a*
1 meter	10.091	2.818	449, 474	Carotenoid
	12.5	N/A	N/A	N/A
	14.649	2.473	429, 664	Chl *a*
	15.322	2.945	431, 665	Chl *a*
3 meters	10.425	3.18	428, 656	BChl *d*
	12.5	1.743	428, 656	BChl *d*
	14.476	1.033	420, 657	BChl *d*
	15.3	1.279	431, 664	Chl *a*

HPLC analysis on pigment extracts from May 30, 2015 corroborated the spectrophotometric reports (Table 2). Material examined from the surface, 1 meter, and 3 meters all produced concentrated peaks consistent with Chl *a* with a similar retention time (R_t) ≈ 15.3 minutes. However, the 3-meter extract exhibited additional chlorophyll peaks, both with a higher concentration, at R_t = 10.425 minutes and R_t = 12.5 minutes, suggesting a bacteriochlorophyll-type pigment at this depth.

These findings confirmed the differential pigment composition of the water column, demonstrating well isolated populations of Chl *a* (with some carotenoid interference) in the surface waters and mixed concentrations of both Chl *a* and a possible bacteriochlorophyll at 3 meters (Table 2).

Microflora Morphology and Characteristics

The refractile trichomes reported in 2017 as *Prochlorothrix* (Gould et al., 2019) reappeared during both subsequent sampling seasons, most densely collecting at a depth of 3 meters without formal colony formation. Cells appeared a conspicuous gold-green color, cylindrical, and were longer than they were wide, approximately 1–1.5 µm wide by 3–5 µm long (Fig. 20). A sheath surrounded the entire linear trichome, often extending beyond the terminal cells. All observed filaments of the same type appeared to reach a genetically predetermined length.

Species of the shade-tolerant cyanobacterial genera *Planktothrix* and *Planktolyngbya* were also observed in significant numbers in the metalimnion to hypolimnion during thermal stratification (Fig. 21). *Planktothrix* filaments were of variable length, with longer trichomes tending to curve gradually. Bright blue-green cells were nearly isodiametric (6 µm width × 5 µm length) but could be marginally shorter

than wide with generous aerotope distribution. Depending on the filament, slight apical tapering could be observed. *Planktolyngbya* appeared similar in size to the refractile filaments, though individual cell size was often difficult to distinguish due to ambiguous intercellular septa. Firm and highly noticeable sheaths surrounded all *Planktolyngbya* trichomes. Although Lake Louise cyanobacterial blooms *in situ* were difficult to distinguish by color due to reduced water clarity, blue-green mats developed readily in serially diluted BG-11 cultures.

Sequence Data

PCR products generated for cultivation-independent 16s rRNA gene analysis varied in length from those intended to target the whole-gene region for *Prochlorothrix* or the hypervariable regions within specified genera, such as *Chlorobium* and *Chloroflexus*. ReadyMade™ primers (16s rRNA For/Rev; Integrated Technologies) optimized for environmental amplification produced 1.5 kb amplicons, ideally representative of the entire gene region, while *Prochlorothrix*, Chlorobi, and Chloroflexi primers amplified 1.4 kb and 500 bp regions, respectively. Although all primer sets were able to generate product, ReadyMade™ primers demonstrated the highest amplification efficiency while Chloroflexi primers were poorest in performance. In total, 19 plasmid inserts were considered viable for sequencing and 8 offered taxonomically significant insight, of which 7 were sequences generated from the ReadyMade™ 16s rRNA amplicons. A general nucleotide BLAST of the sequenced 16s rRNA gene from one insert revealed strong matches (>99%) to uncultured species of *Chlorobiaceae* with an 85% homology to various species of both *Pelodictyon* and *Chlorobaculum* in the 16s rRNA genetic library from NCBI. Three additional inserts suggested a close lineage with green non-sulfur

bacteria of the genus *Chloroflexus*, although noteworthy alignment (95.16% homology) only occurred in a region of fewer than 100 bp. The remaining inserts implicated species of *Methylocystis*, *Nevskia*, and α/γ-proteobacteria in the overall biological composition at 3 meters. Interestingly, none of the sequences, especially those generated by *Prochlorothrix*-type primers, revealed significant alignment with any cyanobacterial genera, including *Prochlorothrix* and *Planktolyngbya*.

Chapter IV

DISCUSSION

Though DCM have been attributed to BChl *a* found in sulfur bacteria of the families Chlorobiaceae, Ectothiorhodospiraceae, and Chromatiaceae (Blankenship, 2010; Chapin et al., 2004), the calculated chlorophyll concentrations observed during summer stratifications of Lake Louise seemed to suggest that the DCM was the result a high concentration of chlorophyll *b*. In addition, spectra of the extracts did not show the characteristic BChl *a* peak near 750 nm. Because most photosynthetic eubacteria do not produce plant-type pigments (Blankenship, 2010; Overmann, 2001), the spectra were initially hypothesized to be the result of decaying organic material from the macrophyte community that had dominated the lake in previous years. The annual recurrence and maintenance of the Chl *b* DCM at a consistent depth indicated that this initial assumption was false, leading to further inquiry.

Chlorophyll *b* is often utilized by green algae, phototrophic euglenoids, and higher plants for photosynthesis in low-light conditions, therefore a consistent peak in its concentration below the surface waters could, at first, be considered an unremarkable response to a shallow and relatively transparent euphotic zone (Nürnberg & Shaw, 1999). Indeed, euglenoids are frequently observed in the epilimnion in Lake Louise. However, according to FlowCAM data from 2017–2019, the cyanobacteria community occurred more abundantly by several orders of magnitude, with minimized presence of green algae and suppressed the growth of aquatic plants. Notably, the DCM also appeared to be

related to the level of oxygen in the water column, as chlorophyll peaks disappeared when circulation was restored. Because cyanobacteria are generally aerobic organisms, their absence during periods of prolonged oxygen availability seems unlikely. Thus, environmental pressures on the otherwise photoautotrophic cyanobacteria community may have forced temporal shifts to chemoorganotrophic metabolism.

Light microscopy indicated highly variable proportions of microflora throughout the water column, suggesting a direct relationship between organism prevalence and reported pigment concentration. At the site of the reported DCM, refractile, narrow trichomes of apparently fixed length were observed. Conditions within the hypolimnion during thermal stratification appeared to be chemically compatible with the development of a chlorophyll *a*/*b*-producing cyanobacterial strain in the genus *Prochlorothrix* (Burger-Wiersma et al., 1986; Konopka, 1989; Pinevich et al., 2012), and the incidence of any *Prochlorothrix* species in Lake Louise would account for a Chl *a/b* DCM (Bullerjahn & Post, 1993). Importantly, however, the known range of *Prochlorothrix* does not currently extend beyond Lake Loosdrecht in the Netherlands, though some uncultivated genetic markers have been identified in New Orleans, Louisiana (Amaral-Zettler, Rocca, Lamontagne, Dennett, & Gast, 2008). Further, the morphometry and moderately dystrophic state of Lake Louise is strikingly different from that of the hypereutrophic Lake Loosdrecht, providing unsuitable environmental conditions to facilitate a population of *Prochlorothrix*.

Environmental DNA sequence data, while inconclusive, do not indicate the presence of *Prochlorothrix* and suggest instead that members of the green sulfur bacteria (Chlorobiaceae) and the green non-sulfur bacteria (Chloroflexales) may be present in the

DCM. This finding is corroborated by the spectra derived from pigment extracts and HPLC. Thus, it is reasonable to conclude that a case of mistaken identity may be responsible for a skewed spectra analysis (Ritchie, 2018) and a more accommodating equation for the evaluation of pigments by spectrophotometry is required. Even so, determination of chlorophylls through acetone extractions can be easily misinterpreted, as plant-biased equations fail to account for the presence of BChl *a*, which can potentially radically alter concentration estimations for Chl *a* (Ritchie, 2018). In the case of this study, baselines set at 800 nm for detection of BChl *a* did not show signs of absorptivity in the typical anoxygenic photosynthetic range. Equations designed for the detection of major chlorophylls subsequently failed to identify peak maxima, consolidating spectrophotometric results into composite estimations without consideration of less common bacteriochlorophylls. As work is currently being done to craft a more comprehensive equation for efficient and accurate spectrophotometry, solvent efficacy must simultaneously be optimized for precision and pigment removal (Ritchie, 2006; Ritchie 2018).

Ultimately, evidence implicates bacteriochlorophyll *d,* designated by Caple, Chow, & Strouse (1978) as "*Chlorobium* chlorophyll 650," as the most likely pigment associated with the absorption peaks observed from Lake Louise at 3 meters. Though antiquated, Caple's pseudonym accurately reflects the exiguous organisms which produce bacteriochlorophyll *d* (BChl *d*), allowing for some cogent assertions about its biological origin in the study site. First, the utilization of BChl *d*, though restricted to green sulfur and non-sulfur bacteria, is certainly not ubiquitous even within genera, occurring only in some *Chlorobium* species, *Prosthecochloris vibrioformis*, *Chlorobaculum parvum*, and

Chloronema species (Boone, Castenholz, & Garrity, 2001; Imhoff, 2003). Thus, based largely on the inferences drawn from sequence data, the natural conclusion is a genus of green sulfur bacteria. Although this would neatly explain the oxygen-intolerant tendencies derived from the chlorophyll data, generated sequence matches for Chlorobi fall below recommended guidelines for incontrovertible identification as any of the BChl *d*-containing species. Additionally, if present, the green sulfur bacteria remain either too small or overgrown by more competitive organisms for visualization by light microscopy, preventing any photographic consensus with molecular data.

Secondly, if the presence of *Chlorobiaceae* in the water column is insufficient for microscopic visualization, then a more abundantly observed bacteriochlorophyll *d*-containing bacterium is a more likely explanation. Morphology and ecology of the species of interest point towards a species of *Chloronema*, anoxygenic green non-sulfur bacteria that thrive in narrow hydrochemical niches similar to those below the Lake Louise thermocline. *Chloronema* are equipped with chlorosomes, saturated with bacteriochlorophyll *d* as the major light-harvesting pigment (Boone et al., 2001; Hohmann-Marriott & Blankenship, 2011), and are hypothesized to be representative of a large percentage of phototrophic bacteria in freshwater lakes, although their actual geographical distribution remains unclear (Gich, Garcia-Gil, & Overmann, 2001). *Chloronema*, much like their relatives in *Chloroflexus*, demonstrate gliding motility and are often mistaken for cyanobacteria of the genus *Lyngbya*, which may in part account for hyperinflated filamentous particle counts generated by the FlowCAM optical filters.

The Lake Louise *Chloronema* are visually identifiable as such based on their gold-green coloration and strikingly reflective appearance. The documented type species for

the genus *Chloronema* is *Chloronema giganteum* Dubinina & Gorlenko, a gold-green filamentous prokaryote with overlapping habitat and nutrient requirements to the observed species (Boone et al, 2001). However, major dissimilar physical characteristics including cell size and trichome length precludes classification of the purported *Chloronema* species as *C. giganteum*, indicating the presence of a novel morphospecies. The universal lack of success in culturing *Chloronema* to date is, in part, responsible for a widespread lack of genetic information on the organism, restricting scientists to taxonomic assignments based primarily on morphological characteristics (Boone et al., 2001; Gich et al., 2001). Difficulty with culturing and sequencing within the present study may be reflective of the lack of available sequences in genetic databases. It is important, however, to note that despite little physiological information on *Chloronema*, it reportedly demonstrates aerotolerance *in situ*, reverting to heterotrophy in oxygen-rich environments (Boone et al., 2001). The heterotrophic capacity of *Chloronema* during turnover in Lake Louise could potentially manufacture the false narrative of anaerobic organisms, as their photosynthetic mechanisms and light-harvesting pigments are down-regulated.

Cyanobacteria of the genus *Planktothrix* are often ecological parallels to *Chloronema*, capable of absorbing photosynthetically active radiation across the entire visible light spectrum (400–700 nm) and surviving cold-weather mixing by reverting to heterotrophy in the benthos of temperate, ferruginous lakes (Legnani, Copetti, Oggioni, Tartari, Palumbo et al., 2005; Walsby & Jüttner, 2005; Walsby, Schanz, & Schmid, 2006). Coincidentally, *Planktothrix rubescens* shows affinity for the metalimnion of oligotrophic or mesotrophic lake systems, although growth is not benefitted by prolonged

exposure to stratified columns (Dokulil & Teubner, 2012), nor is the red coloration observed in Lake Louise phytoplankton. A re-emergence of *Planktothrix isothrix* is more likely. *Planktolyngbya* species are also consistent with the morphological evidence presented in this study, though not as ecologically convincing as *Planktothrix*. In conjunction with the discrepancies in size and shape of the observed *Chloronema*, it is apparent that the shifting hydrochemical and trophic state of Lake Louise as described by Tepper and Hyatt (2011) may be strongly influencing trichome development and diversity of both filamentous anoxygenic photosynthesizers and cyanobacteria.

In beginning to describe the cohort of microorganisms which exist in the metalimnion and hypolimnion of Lake Louise, the present study ultimately furthers work conducted by Hains in 1987, which characterized the epilimnetic flora to a depth of 2 meters. Here, we introduce a necessary baseline for phytoplankton and chlorophyll concentration in Lake Louise, allowing inferences on the condition and future species richness in other sub-tropical limnetic systems. Although many of these systems exist virtually free of recreational or consumer disturbance (Tepper & Hyatt, 2011; Watts, 1971), natural deviations from standards established by this study potentially serve as functionally relevant guidelines for predicting the rate of change due to factors such as anthropogenic interference. In addition, with average global temperatures on the rise, limnological studies are becoming increasingly important as we try to anticipate the deterioration of freshwater resources in response to human stressors (Parry, Canziani, Paultikof, van der Linden, & Hansen, 2007; Verschuren, Johnson, Kling, Edgington, Leavitt et al., 2002). As minimum daily temperatures gradually increase, the degree of thermal stratification may become stronger (Havens & Jeppesen, 2018; Richardson, D.

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APPENDIX A:

MEMORANDUM OF UNDERSTANDING AND AGREEMENT (MUA) FOR RECOMBINANT DNA EXPERIMENTS (APPROVAL)

BUILDING & ROOM NO(s) Bailey Science Center, Room #2081, #2068, #2047

GRANTING AGENCY ———

GRANT NO. (IF APPLICABLE) ———

TITLE OF GRANT OR PROJECT:
Investigating Deep Chlorophyll Maximum in Lake Louise, GA

A. Describe the experiment involving recombinant DNA techniques. Your description is to be sufficiently complete so as to provide committee members an understanding of what you intend to do and how you will do it. A summary or abstract of your methods and materials section will also be helpful. Please reference this discussion to appropriate NIH Guidelines and/or USDA/APHIS, and EPA regulations.

Refer to attached document

MEMORANDUM OF UNDERSTANDING AND AGREEMENT

FOR RECOMBINANT DNA EXPERIMENTS (Contiued)

B. ASSESSMENT LEVELS OF PHYSICAL AND BIOLOGICAL CONTAINMENT.

1. Describe how you intend to meet physical and biological containment requirements (reference NIH/USDA/EPA guidelines). See attached
2. Will this project involve environmental release? See attached
3. Describe procedures and precautions to be followed in transporting biohazardous agents between laboratories. See attached

C. Agreements

DW I agree to accept responsibility for training of all laboratory workers involved in the project.

DW I agree to comply with all appropriate requirements pertaining to shipment and transfer of recombinant DNA materials.

A. This study aims to identify cyanobacteria in Lake Louise which may be responsible for the unusual accumulation of chlorophyll b in the water column. The following is an excerpt of our methods for the utilization of TOPO TA cloning kits to reproduce 16s rRNA gene amplicons from Lake Louise aquatic samples:

"Bands excised from TAE-agarose gels will be eluted using the Invitrogen PureLink™ Gel Extraction Kit. By exploiting the natural terminal transferase activity of *Taq* polymerase that occurs during PCR, the eluted double-stranded DNA fragments will be integrated into linearized pCR™ 2.1-TOPO® plasmid vectors using an Invitrogen TOPO® TA cloning® kit without the need for ligase. After a 5 minute incubation, the aforementioned cloning reaction is added to OneShot® chemically competent TOP10 *Escherichia coli*. Cell mixtures are then exposed to a 30 second heat pulse at 42°C and immediately returned to ice to enhance permeability of the plasma membranes for successful transformation. Super optimal broth with catabolite repression (SOC) medium is then subsequently added and cell mixtures will be shaken at 200 rpm on an orbital shaker for one hour at 37°C.

After shaking, 100 µl of transformed cells are to be spread on LB-ampicillin agar plates (50 µg/mL ampicillin) containing X-Gal solution in media at a concentration of 20 µg/mL. Plates will be sealed and incubated at 37°C for two days to allow sufficient colony formation before blue/white screening. Resulting white colonies (indicative of a successful insert) from each plate are then selected for isolation and grown on new, sealed LB-amp plates in an incubator. Due to the infidelity of environmental DNA, after another incubation period of 2 days, colonies will then be further purified by a secondary selection and isolation from these plates. All collected white colonies are to be cultured in fluid LB-amp media (without agar) before plasmids are extracted using an Invitrogen PureLink™ Quick Plasmid Miniprep kit. PCR and gel electrophoresis protocols will be run to preliminarily assess plasmids for successful insertion. Plasmids will be subsequently stored in 1.7 mL microfuge tubes at -20°C until sent for sequencing."

(***NIH Guidelines for Research Involving Recombinant or Synthetic Nucleic Acid Molecules, Section III; Section III-D***)

B. In accordance with NIH guidelines, the use of *Escherichia coli K-12* host-vector systems for recombination, with some exceptions, is permissible with BL-1 containment conditions and may be exempt from NIH guidelines. In the case of our study, the plasmid vector (pCR™ 2.1) is linearized and non-conjugative, therein meeting conditions for exemption from NIH guidelines. However, all BL-1 containment and sterilization procedures, in addition to laboratory conditions, will be upheld. **(Section III-F-8, Appendices C-II/G-II-A/G-III-M)**

    a. All surfaces will be disinfected prior to and after work, and in the case of spills (**Appendix G-II-A-1-b**).

    Gloves will be worn when handling all host-vector systems and in the manipulation of genetic material from PCR/gel electrophoresis (**Appendix G-II-A-1-h**).

All contaminated items, including inoculation loops, pipettes, agar plates, liquid media, and microfuge tubes, used in the transfer or transformation of recombinant host *E. coli* will be autoclaved at 121°C for 20 minutes prior to disposal. Plastic inoculation loops will be placed in disinfectant before autoclaving (**Appendix G-II-A-1-c**).

Agar plates and liquid media used for growth of *E. coli* will be sealed with covers (and parafilm, in the case of agar plates) during incubation (**Appendices G-II-A-1-g; G-II-A-2-a**).

b. This project will NOT involve environmental release.

c. All biohazardous materials will be transported between labs in leak-proof containers on wheeled carts to prevent any spills and minimize/eliminate the production of all aerosols.

APPENDIX B:

Charts and Figures

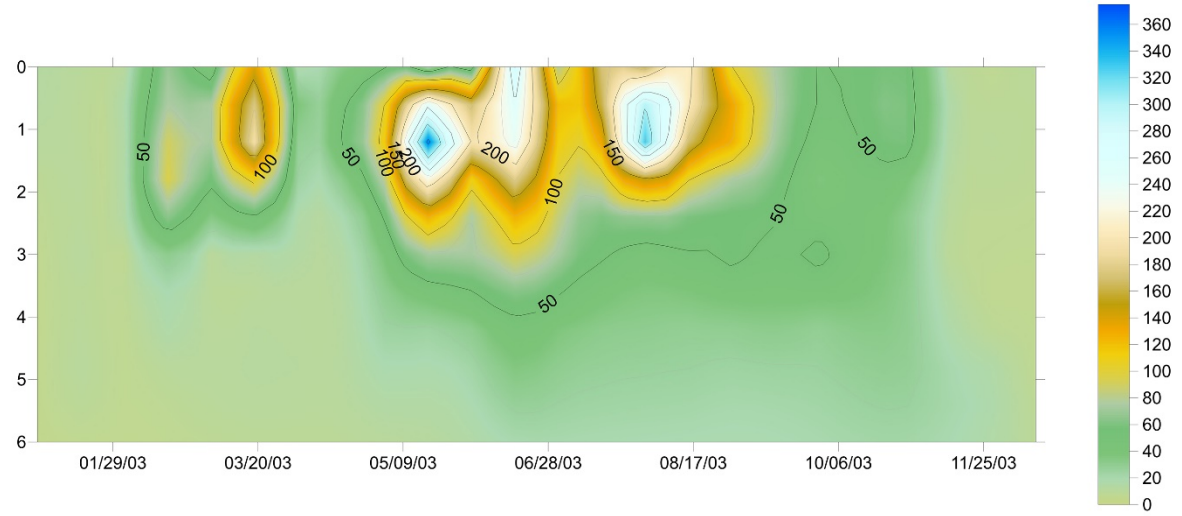

Figure 1. Total chlorophyll *a* pigment (µg · L-1) in samples collected from 0 – 6 meters in Lake Louise during 2003, immediately following the disappearance of *Spirodela* and *Wolffia* species. Chlorophyll *a* is highly concentrated in surface waters during stratification (May – August) but undetectable during periods of mixing.

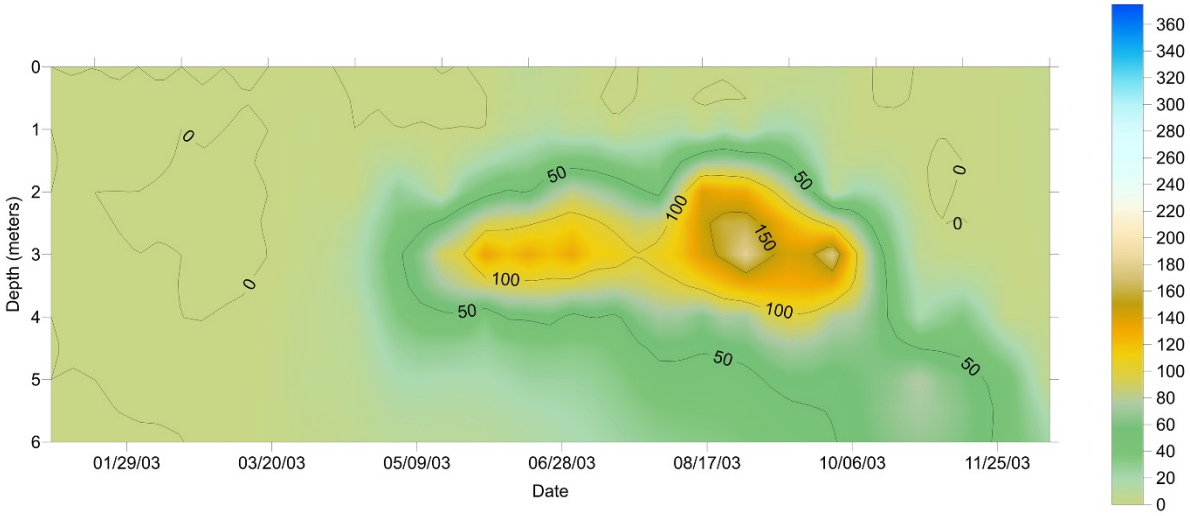

Figure 2. Total chlorophyll *b* pigment (µg · L-1) in samples collected from 0 – 6 meters in Lake Louise during 2003, immediately following the disappearance of *Spirodela* and *Wolffia* species. The majority of the chlorophyll *b* pigment localizes at a depth of 3 meters but is also detectable deeper within the hypolimnion. Chlorophyll *b* disappears during colder months.

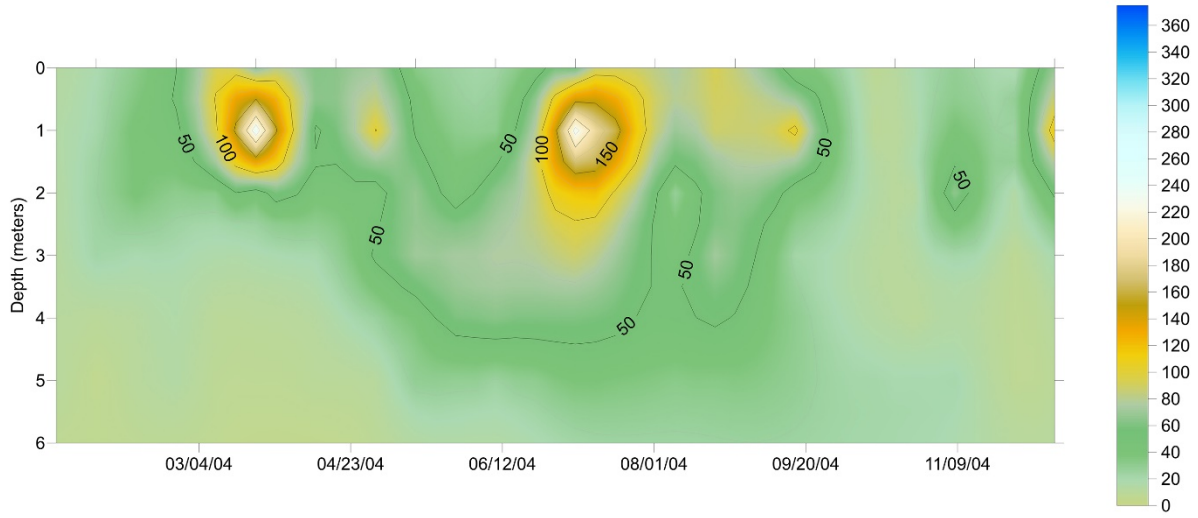

Figure 3. Total chlorophyll *a* pigment (µg · L-1) in samples collected from 0 – 6 meters in Lake Louise during 2004. Chlorophyll *a* concentration is greatly reduced from previous year and remains mostly contained within uppermost 2 meters.

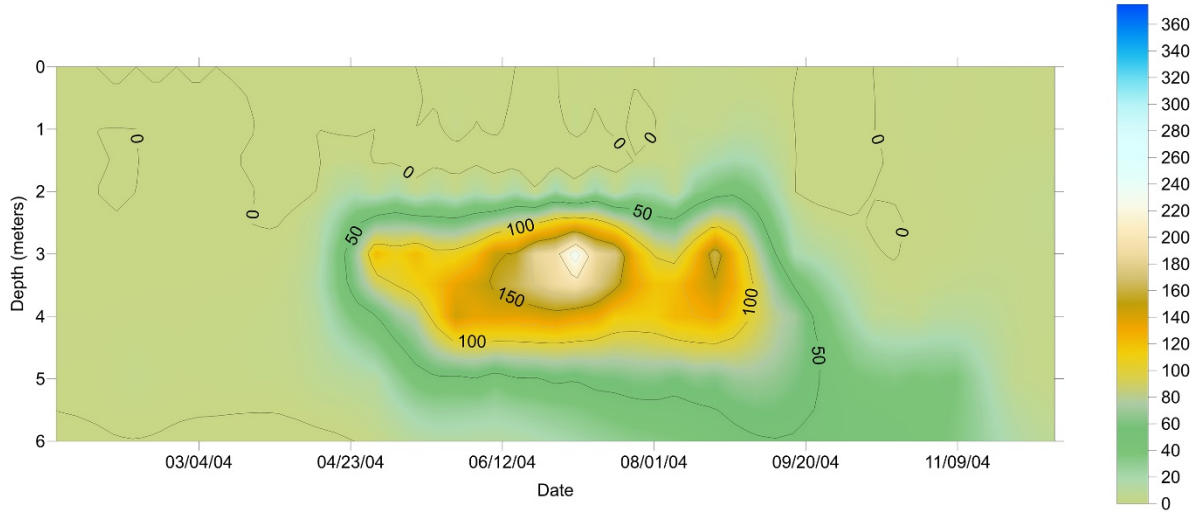

Figure 4. Total chlorophyll *b* pigment (µg · L-1) in samples collected from 0 – 6 meters in Lake Louise during 2004. Chlorophyll *b* reappears during thermal stratification for a second year following the disappearance of duckweed. Chlorophyll *b* remains confined to the lower metalimnion and below and is again susceptible to vertical mixing.

Figure 5. Aerial view of Cathead Lake (*left*; 30°43’22” N, 83°15’48” W) and Lake Louise (*right*; 30°43’30”N, 83°15’21” W) **Map data: Google Earth, Maxar Technologies**

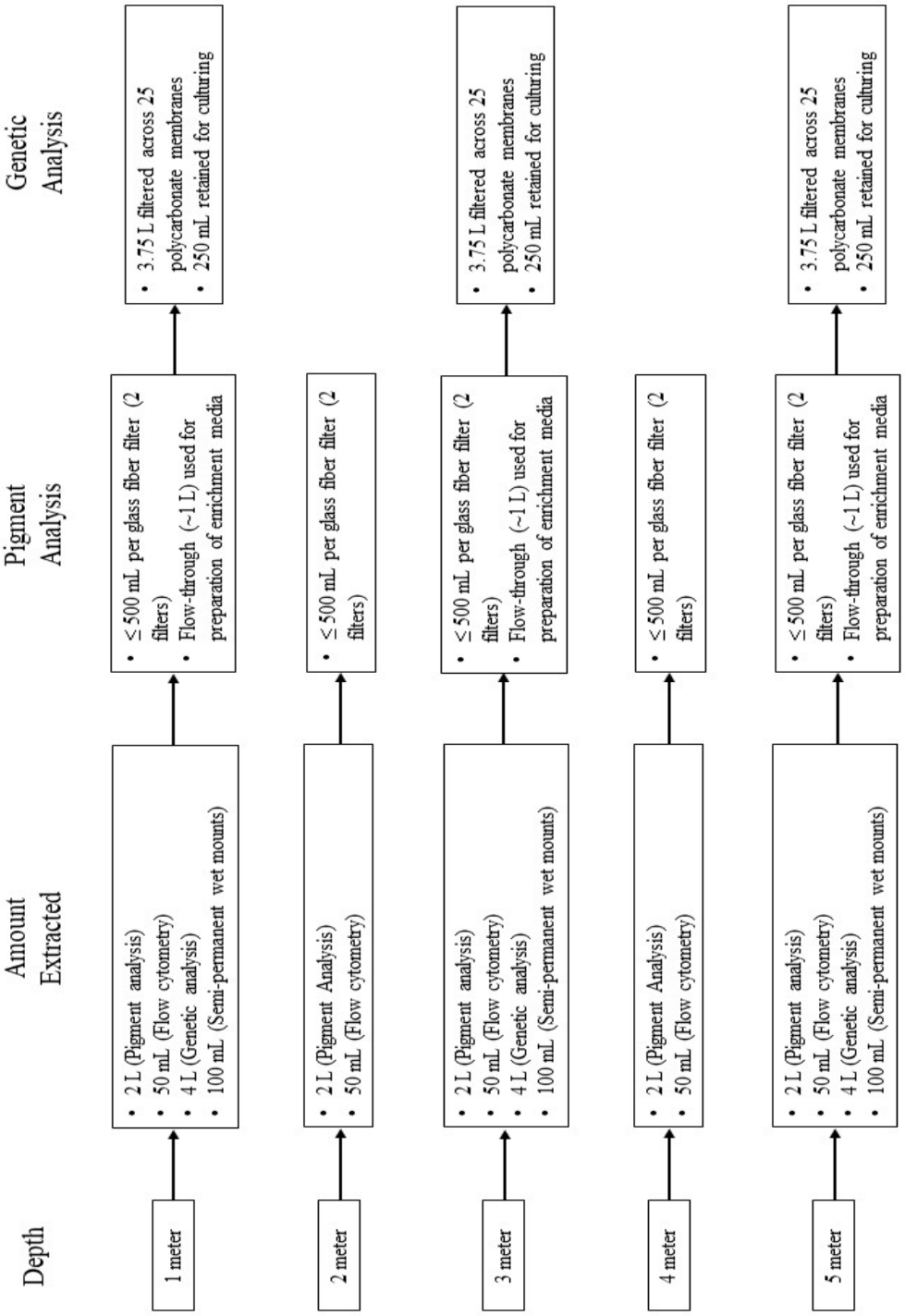

Figure 6. Flow chart demonstrating the processing procedures for biological sample collection.

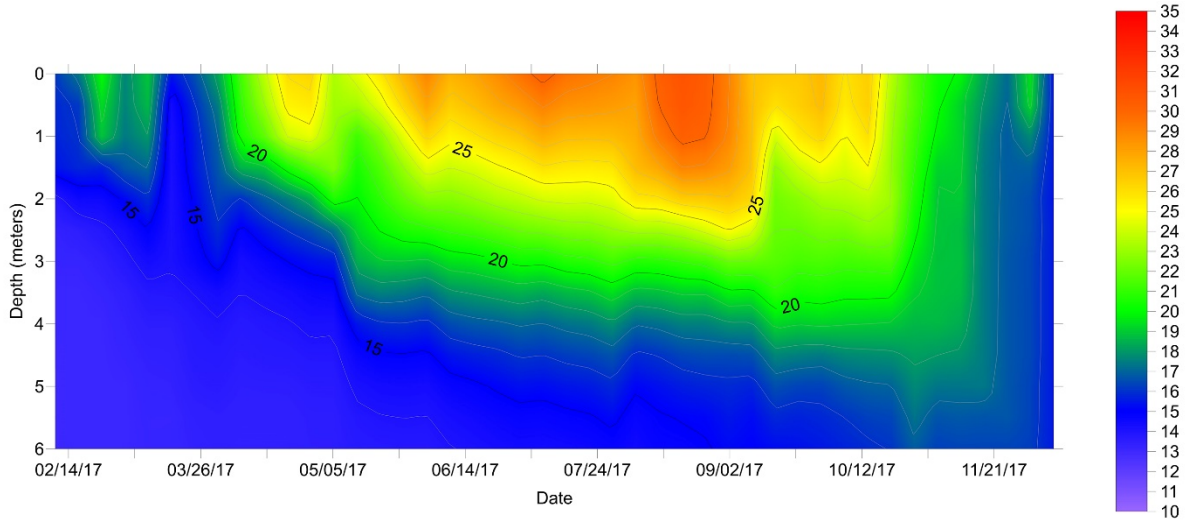

Figure 7. Annual temperature profile (°C) of Lake Louise in 2017. Clear thermal separation begins in late April and persists through October 2017. At the beginning of stratification, the thermocline (1.5 – 3 meters) demonstrates an approximate 15°C drop in temperature. Prolonged column stability results in a broader metalimnion and therefore a less steep temperature gradient as the year progresses. Colder weather results in mixing of water column, regulating heat distribution and causing temperature uniformity from top to bottom in the lake.

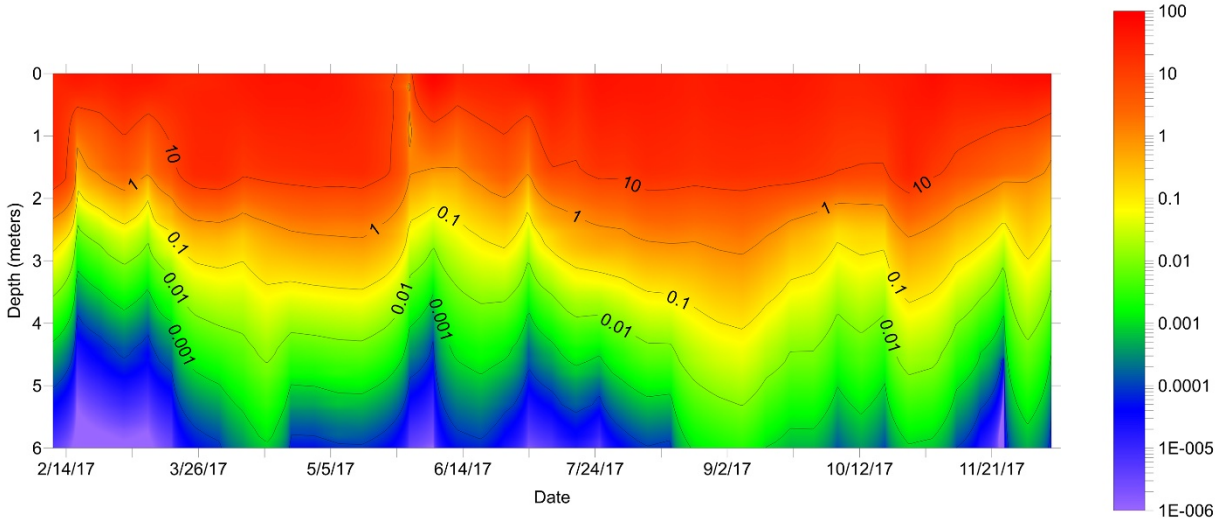

Figure 8. Percentage of incident light (photons · m-2 · s-1) received throughout the water column from February – November 2017. Light is rapidly attenuated at the surface with only 1% penetrating to a depth of 2 meters. Irradiance at 3 meters fluctuates between 0.1 – 0.01% of incident surface light, indicating a low availability of photosynthetically active radiation (PAR).

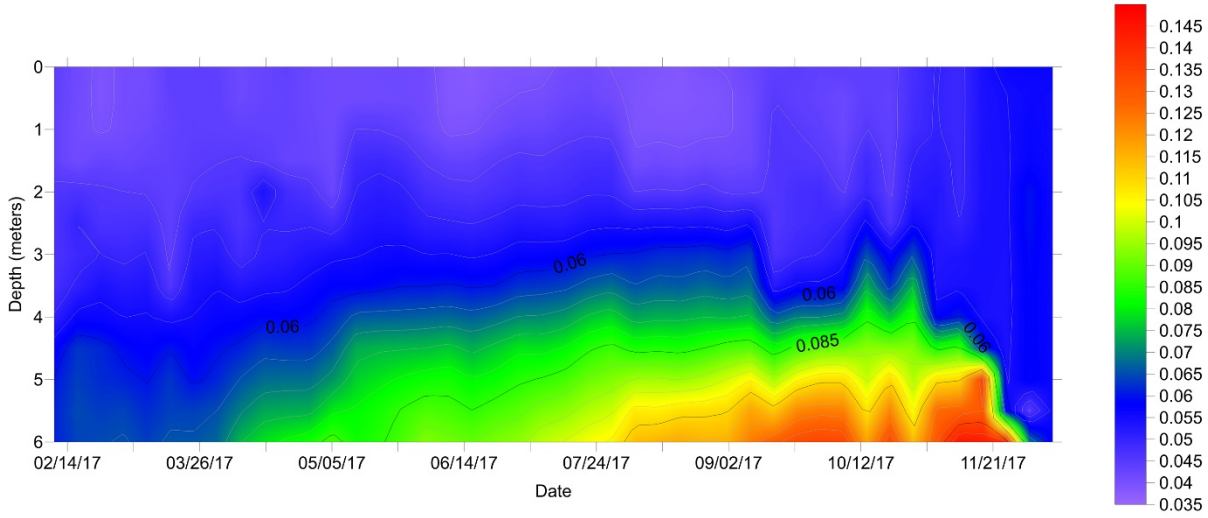

Figure 9. Relative conductivity of Lake Louise (Siemens · cm-1) from February – November 2017. Salt concentrations in the hypolimnion increase as stratification stabilizes, though peak conductivity remains negligible. Highest reading indicative of nearly pure freshwater.

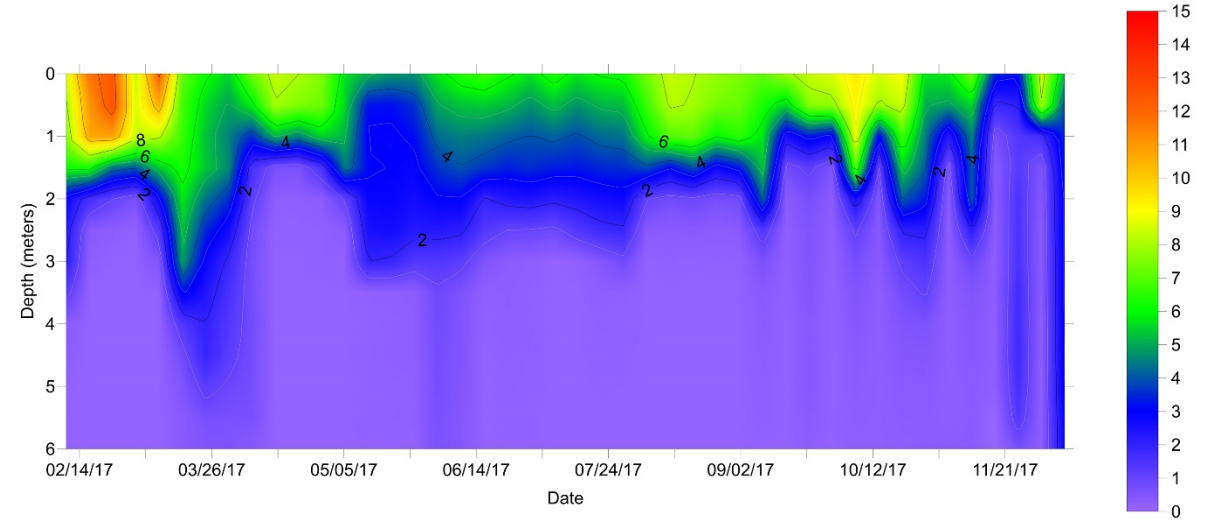

Figure 10. Dissolved oxygen concentration (mg · L-1) in Lake Louise from February – November 2017. Available oxygen mostly restricted to the surface waters, though turnover in November restores a minimum, uniform concentration throughout the column until thermal stratification. During peak stratification, hypolimnion (3 – 6 meters) register as anoxic.

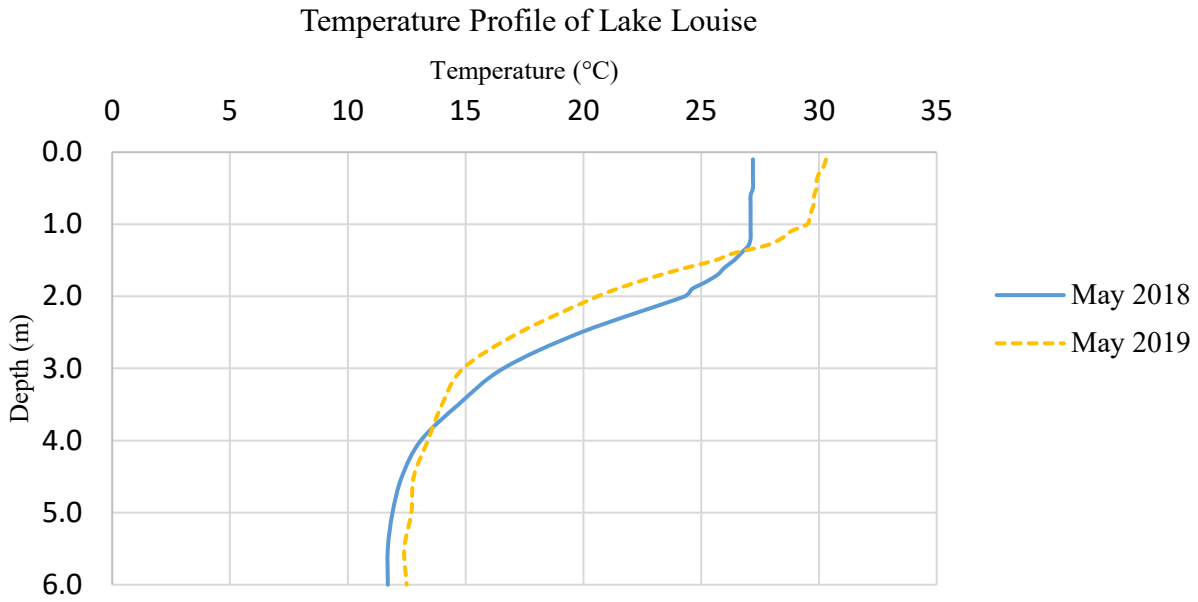

Figure 11. Temperature profile (°C) of Lake Louise during the May 30, 2018 and May 31, 2019 sampling sessions.

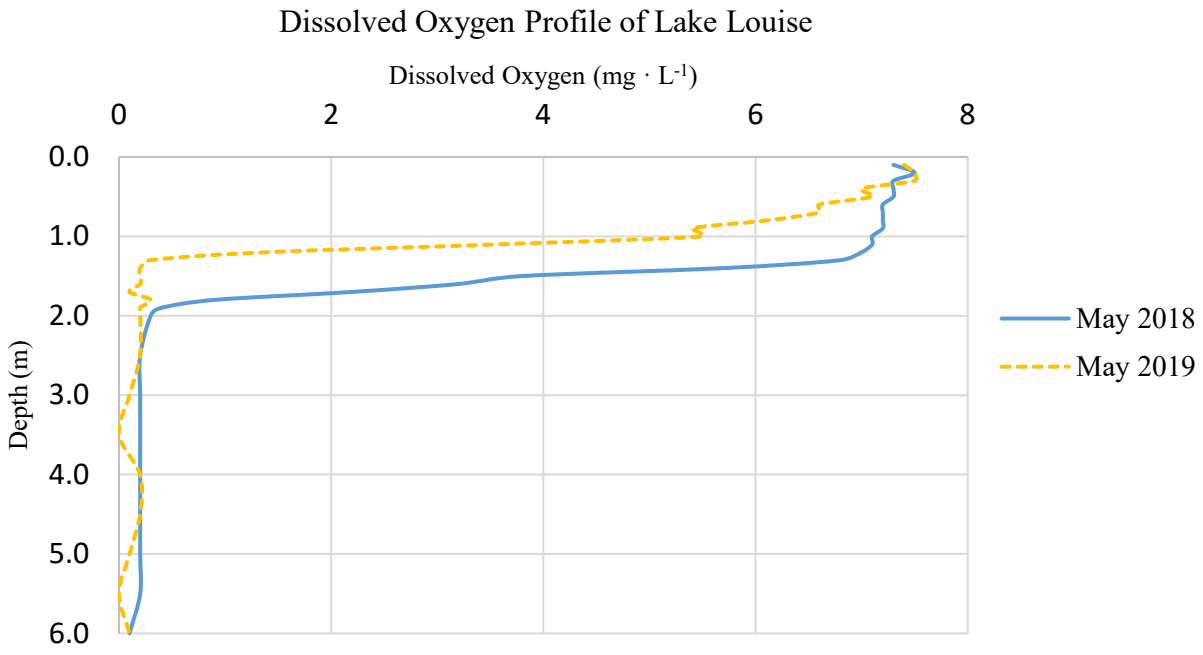

Figure 12. Dissolved oxygen profile (mg · L^{-1}) of Lake Louise during the May 30, 2018 and May 31, 2019 sampling sessions.

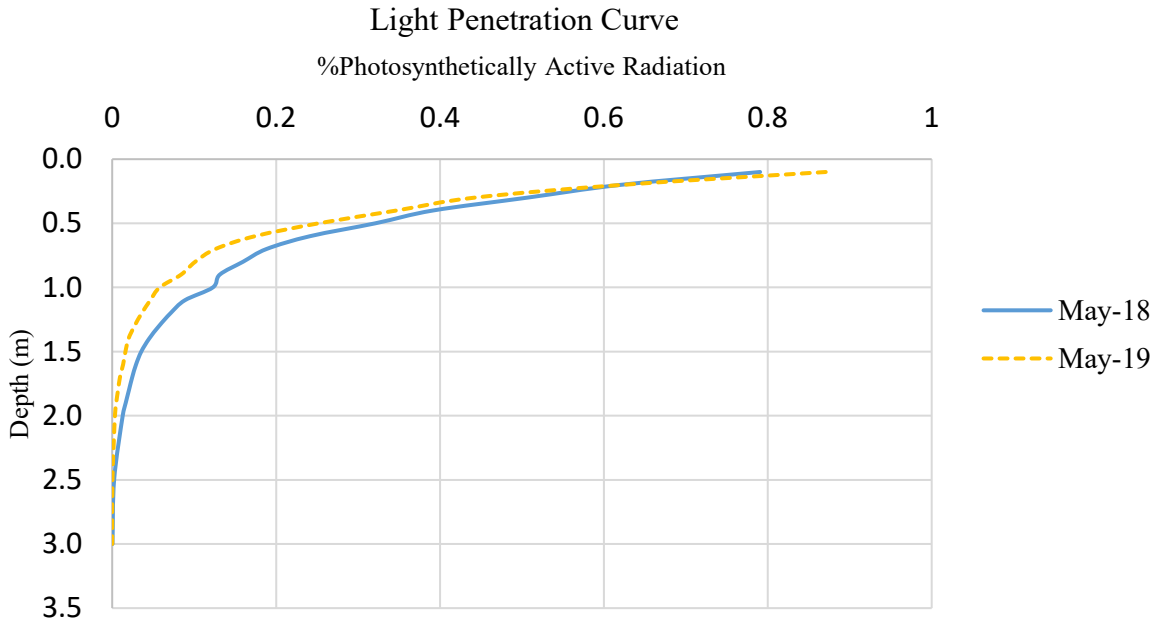

Figure 13. Percent of penetrant PAR in the Lake Louise water column during sampling in 2018 and 2019.

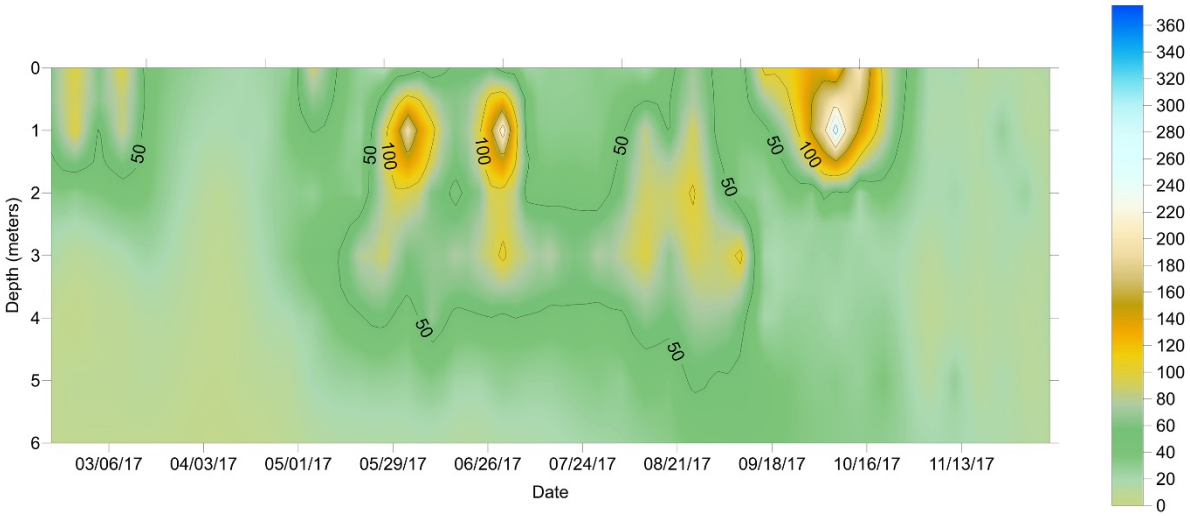

Figure 14. Total chlorophyll *a* pigment (µg · L-1) in samples collected from 0 – 6 meters in Lake Louise during 2017. Highest concentrations observed in epilimnion (0 – 1.5 meters) during thermal stratification with smaller peaks co-occurring within the lower metalimnion (3 meters). Significant reduction in chlorophyll *a* production reported during turnover periods.

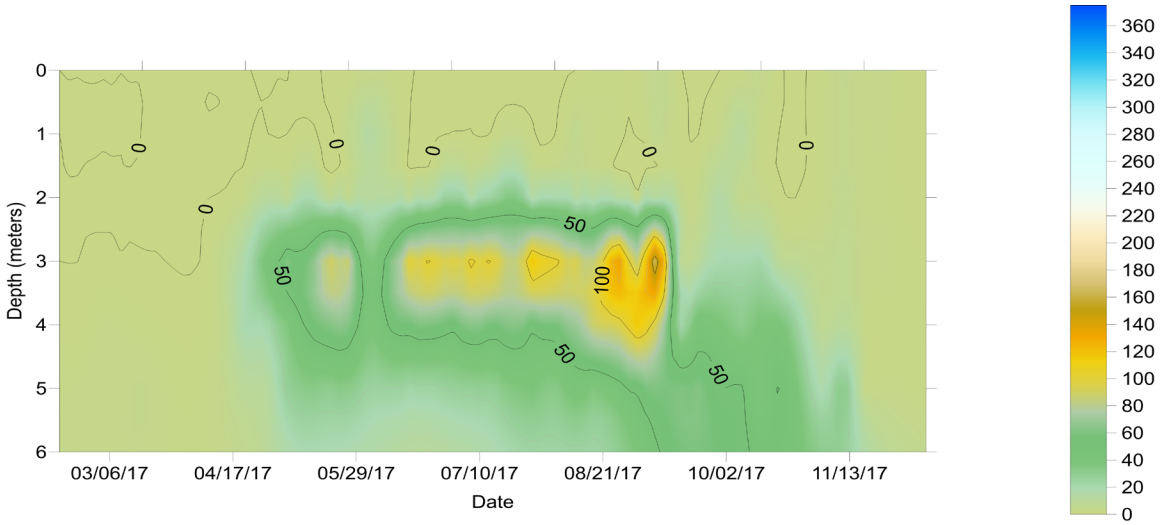

Figure 15. Total chlorophyll *b* pigment (µg · L-1) in samples collected from 0 – 6 meters in Lake Louise during 2017. Exclusively detected in the lower metalimnion and hypolimnion (3 meters – bottom) during thermal stratification from May – September 2017. Strongest peaks occur prior to oxygen restoration from Hurricane Irma.

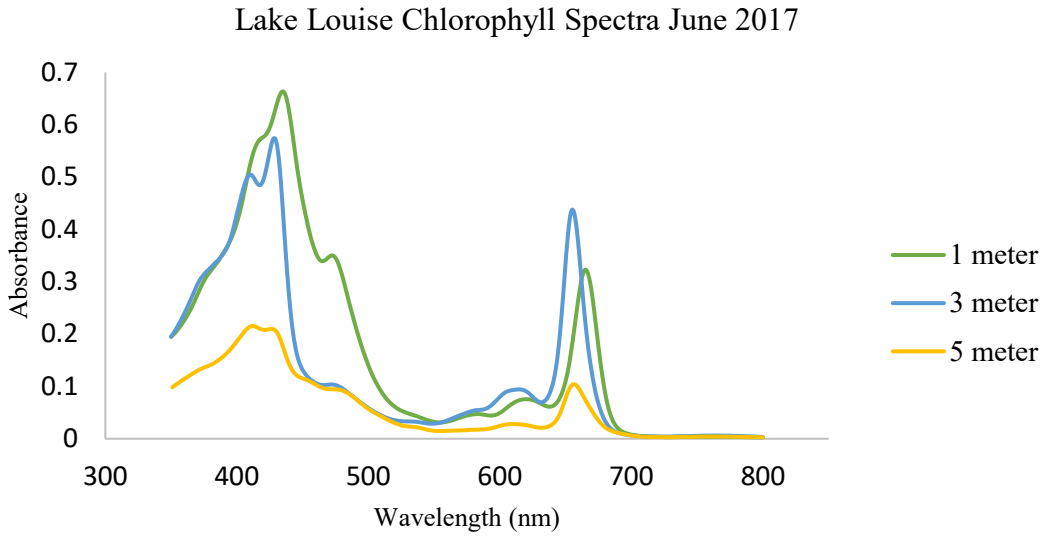

Figure 16. Lake Louise chlorophyll spectra (90% acetone) at depths 1 meter, 3 meters, and 5 meters on June 01, 2017. Peaks plotted for 1 meter and 3 meters demonstrate different absorptive capacities and roughly equal pigment concentration.

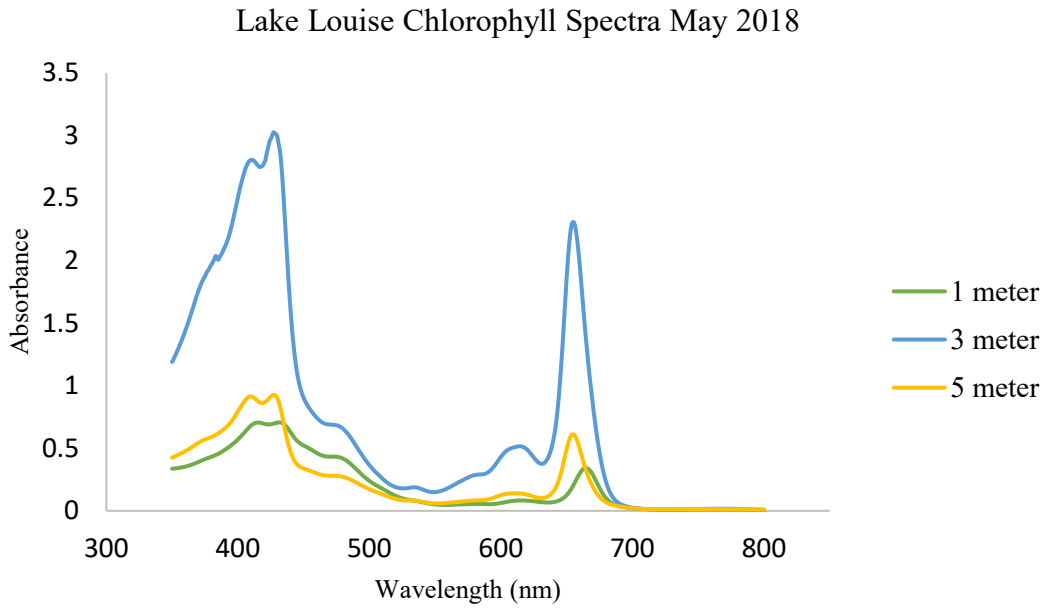

Figure 17. Lake Louise chlorophyll spectra (90% acetone) at depths 1 meter, 3 meters, and 5 meters on May 31, 2018. Pigment concentration was strongest at 3 meters and extracts from all three depths exhibit different absorptive capacities.

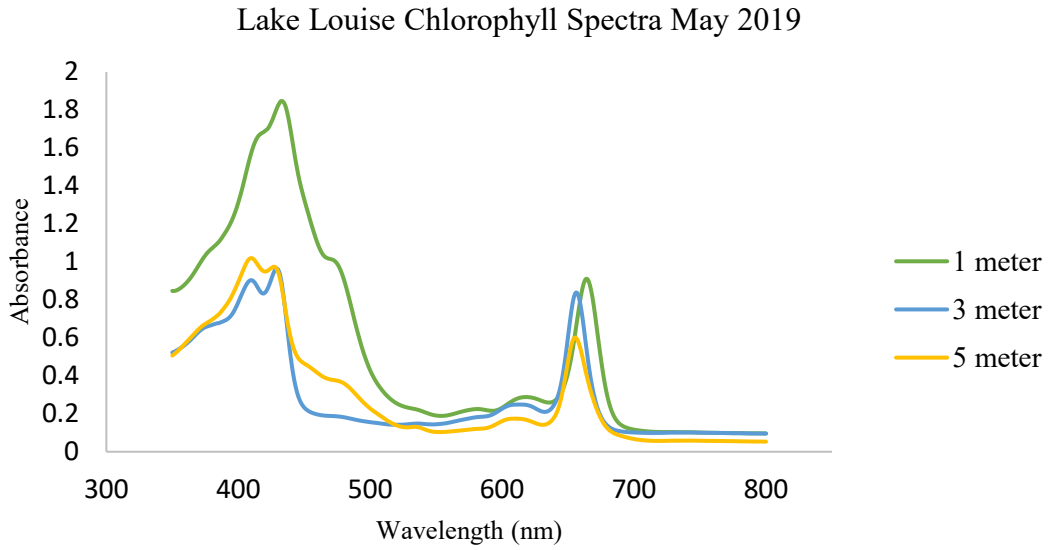

Figure 18. Lake Louise chlorophyll spectra (90% acetone) at depths 1 meter, 3 meters, and 5 meters on May 30, 2019. Pigment concentration was strongest at 1 meter and extracts from all three depths exhibit different absorptive capacities.

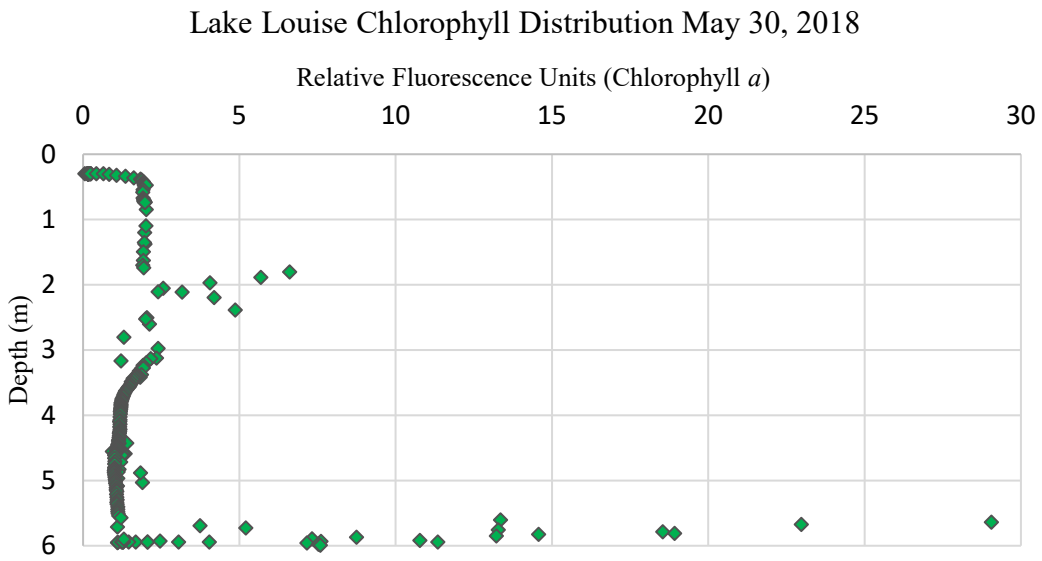

Figure 19. Distribution of chlorophyll *a* in the Lake Louise water column, measured by relative fluorescence.

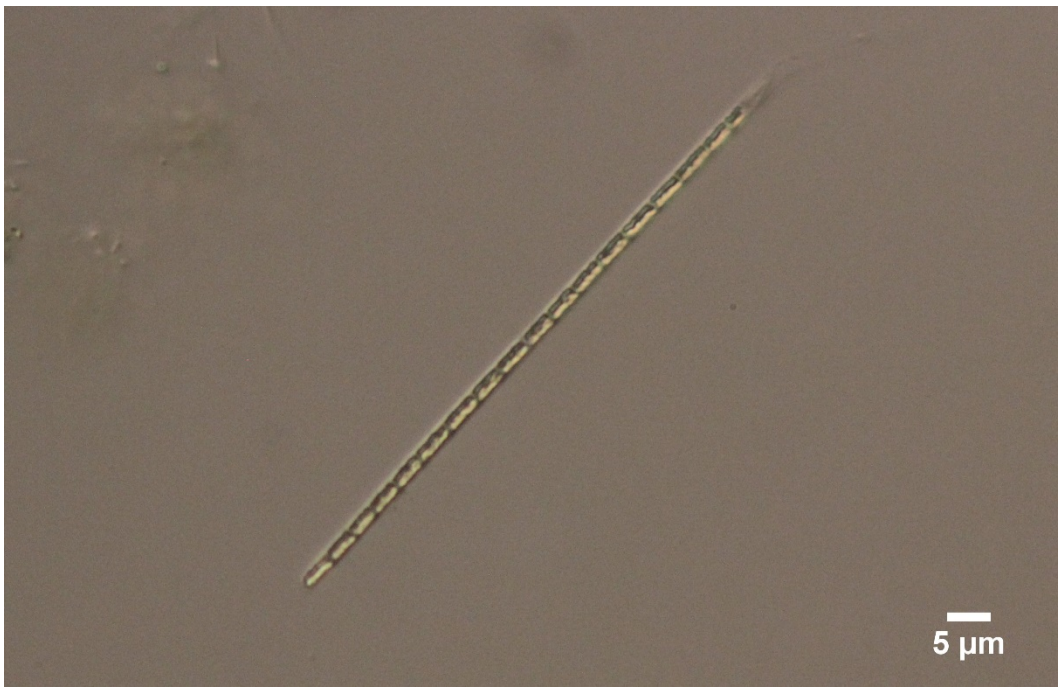

Figure 20. Observed *Chloronema* sp. in Lake Louise, May 30, 2018 at 400x magnification. Cells appear longer than wide, approximately 5 µm long and 1.5 µm wide.

Figure 21. (Top) *Planktolyngbya* sp. at 1,000x magnification; (Bottom) *Planktothrix isothrix* and *Planktolyngbya* sp. at 400x magnification.