1 **Fluvial seeding of cyanobacterial blooms in oligotrophic Lake**
2 **Superior**

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20

21 **Abstract**

22 Lake Superior has recently begun experiencing
23 cyanobacterial blooms comprised of *Dolichospermum*
24 *lemmermannii* near the Apostle Islands and along the southern
25 shore of the western arm. Little is known about the origin of these
26 blooms. Experiments were conducted during the summers of 2017
27 and 2018 to identify sources of propagules and characteristics of
28 sites that were potential sources. The 2017 experiments were
29 conducting using a factorial design with three source zones
30 (Harbor, River, and Lake), two nutrient conditions (high and low
31 N:P), and three temperatures (15, 20, and 25 °C). At the end of the
32 experiment, cyanobacteria were most abundant from the ‘River’
33 and ‘Harbor’ zones at low N:P and 20 and 25 °C, with *D.*
34 *lemmermannii* most abundant at 20 °C. Subsequently in 2018 we
35 evaluated 26 specific inland locations from three waterbody types
36 (Coastal, Lake/Pond, and River) and explored similarities among
37 those sites that produced cyanobacteria in high abundance when
38 samples were incubated under optimal conditions (low N:P and 25
39 °C). Under these growing conditions, we found high cyanobacteria
40 abundance developed in samples from river sites with low ambient
41 temperatures and high conductivity. Field monitoring showed that
42 Lake Superior nearshore temperatures were higher than rivers.
43 These observations suggest that blooms of *D. lemmermannii* in
44 Lake Superior are initiated by fluvial seeding of propagules and

45 highlight the importance of warmer temperatures and favorable
46 nutrient and light conditions for subsequent extensive
47 cyanobacterial growth. We argue that the watershed is an
48 important source of biological loading of *D. lemmermannii* to Lake
49 Superior, and that when those cells reach the nearshore where there
50 is warmer water temperatures and increased light, they can grow in
51 abundance to produce blooms.

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55 **1. Introduction**

56 Cyanobacterial blooms are a major threat to the beneficial
57 use of marine and freshwater ecosystems globally. Along with the
58 rise in the spatial and temporal extent of nuisance cyanobacterial
59 blooms (Huisman et al., 2018; Taranu et al., 2015), there has also
60 been a rise in the number of blooms that are toxic (Anderson,
61 1989; Sukenik et al., 2015), posing a significant threat to public
62 health. It is widely recognized that cyanobacterial blooms occur in
63 four of the five Laurentian Great Lakes (LGL); however, less well
64 known is that localized surface blooms of *Dolichospermum*
65 *lemmermannii* (formerly known as *Anabaena lemmermannii*
66 (Wacklin et al., 2009) have been observed along the southern shore
67 of the western arm in oligotrophic Lake Superior. In 2012 and
68 2016-2018, cyanobacterial blooms were detected in the nearshore
69 with varying size and duration, most of which were very localized
70 and dissipated quickly; however, in 2018 the bloom persisted for
71 approximately one week in early August and extended
72 approximately 100 km alongshore from Duluth to the Apostle
73 Islands region and 3 km offshore with the highest densities being
74 observed at the shoreline (Sterner et al., submitted for review) .

75 Blooms of cyanobacteria are often associated with
76 increased loading of nutrients, particularly phosphorus (Lürling et

77 al., 2018; Schindler, 1975; Steinberg and Hartman, 1988);
78 however, Lake Superior is a cold oligotrophic system that is low in
79 phosphorus (Sternier, 2010). The watershed in the region where
80 algal blooms have been observed in Lake Superior is primarily
81 forested, but even small differences in land cover and position
82 influence nearshore water quality (Yurista et al., 2011). The
83 western arm has become increasingly subject to extreme
84 precipitation events delivering large amounts of sediment and
85 nutrients to the nearshore (Cooney et al., 2018). Two 500-1000
86 year rainfall events that occurred on the south shore of Lake
87 Superior in 2012 and 2018 (Cooney et al., 2018; Roache et al.,
88 2020) were followed by cyanobacterial blooms varying in size and
89 duration with a lag of 25 days in 2012 and 53 days in 2018;
90 however, small blooms have also occurred in 2016 and 2017
91 without such major rain events. Extreme storms provide large
92 fluxes of nutrients and organic material to the lake (Cooney et al.,
93 2018; Kling et al., 2000; Minor et al., 2014). Cyanobacterial
94 blooms are also generally associated with higher water
95 temperatures (Paerl and Huisman, 2008), and Lake Superior has
96 been warming at the fastest rate of the LGL by maximum summer
97 surface temperature (Austin and Colman, 2007), which may be
98 contributing to the emergence of cyanobacterial blooms (Sternier et
99 al., submitted for review). As summer epilimnetic temperatures

100 increase, conditions can favor cyanobacteria and result in
101 subsequent blooms (Konopka and Brock, 1978; Kosten et al.,
102 2012; Paerl and Huisman, 2009; Robarts and Zohary, 1987).

103 Coastal regions of both marine and freshwater systems can
104 be hotbeds for cyanobacterial blooms (Cloern, 1996; Cloern and
105 Jassby, 2010, 2008). Coastal regions are transitional areas that are
106 significantly influenced by watershed inputs as well as exchange
107 with offshore waters (Cloern, 1996; Howell et al., 2012). Thus,
108 consideration of the surrounding landscape is critical to
109 understanding bloom development in coastal areas (Kratz et al.
110 1997).

111 Rivers are often recognized as sources of nutrients for algal
112 growth in lakes, but seldom are inflowing waters also considered
113 as sources of the living cells that initiate blooms. The riverine input
114 of propagules to receiving lakes is referred to as “fluvial seeding”
115 and the importance of such seeding is referred to as the algal
116 loading hypothesis (ALH) (Conroy, 2007; Loftin et al., 2016).
117 Discussion of the ALH so far is a relatively unexplored question
118 (Conroy et al., 2008), and nearly all of the work done regarding
119 this hypothesis has been in Lake Erie and its watershed, despite
120 any evidence that this process is be unique to that system
121 (Bridgeman et al., 2012; Conroy et al., 2014; Davis et al., 2014;
122 Kutovaya et al., 2012). Conroy et al. (2014) conducted a study

123 evaluating major tributary inputs as sources of seed populations of
 124 cyanobacteria propagules to the Lake Erie blooms and found
 125 elevated concentrations of *Microcystis* in the rivers and observed
 126 visible riverine blooms, concluding that the rivers were playing a
 127 key role in the delivery of seed populations and bloom material to
 128 Lake Erie. In contrast, studies using DNA fingerprinting
 129 techniques have found that the cyanobacterial bloom communities
 130 found in the lake are not very similar to those sampled in the
 131 tributaries (Chaffin et al., 2014; Kutovaya et al., 2012). The
 132 disagreement in findings on the role of fluvial seeding in Lake Erie
 133 demonstrates the lack of knowledge regarding these processes and
 134 illustrates the need for further research in this area. Answers to
 135 these questions are critical in effectively addressing current and
 136 future threats of cyanobacterial blooms in coastal waters.

137 Despite being episodic and relatively small in spatial scale,
 138 algal blooms in Lake Superior have generated public concern due
 139 to their unprecedented nature and their potential impacts on public
 140 health, aesthetics, and the local tourism economy. In this work we
 141 explore how land-lake interactions relate to the emergence of
 142 cyanobacterial blooms in Lake Superior and provide insights to
 143 mechanisms responsible for their appearance. Specifically, we
 144 investigate the question - What are the sources of propagules

145 leading to cyanobacterial blooms in Lake Superior and are there
146 similarities among locations that are potential sources?

147 **2. Material and methods**

148 Laboratory experiments were conducted in summer of 2017
149 and 2018 to identify potential sources of cyanobacterial blooms in
150 western Lake Superior and to identify common characteristics of
151 potential upstream sources. The 2017 experiment aimed to identify
152 the major habitat types that are sources of cyanobacteria and
153 determine whether Lake Superior blooms likely originate in the
154 lake or were initiated in waterbodies further inland (Figure 1). The
155 2018 experiment was informed by results from the 2017
156 experiments and examined 26 specific inland locations as potential
157 sources of blooms. The 2018 experiments also identified some
158 characteristics of sites with higher potential for cyanobacterial
159 growth and thus likelihood of acting as a source for blooms in the
160 large lake (Figure 1).

161 Experiments were designed to assess the propensity of a
162 biological community from a given location to develop into a
163 cyanobacterial bloom over ecologically realistic time scales. Thus,
164 inocula were brought into the lab and incubated in a wide range of
165 nutrient and temperature conditions capable of supporting
166 cyanobacteria. The abundance of major algal groups was recorded
167 over time. This experimental approach was chosen because it

168 ensured that if a viable cyanobacterial population was present, we
169 would observe growth during the experimental time frame. We
170 concluded that inocula that did not develop blooms under bloom-
171 promoting conditions within the experimental period were unlikely
172 to be the source of bloom propagules *in situ*.

173 *2.1 Sample Collection and pre-processing*

174 We defined three “zones” or habitat types during the 2017
175 experiments: 1) Lake Superior near the Apostle Islands (‘Lake’), 2)
176 inflowing rivers (‘River’), and 3) the Duluth-Superior harbor
177 (‘Harbor’). Each of these three zones was comprised of two nearby
178 locations: ‘Lake’ included two nearshore (max depth = 5-10m)
179 locations in Lake Superior, ‘River’ included the Bois Brule and
180 Siskiwit Rivers, and ‘Harbor’ included Wisconsin Point and
181 Superior Bay (Figure 1). Water samples that were collected in each
182 zone during the 2017 experiments were composited to increase
183 chances of collecting viable cyanobacterial propagules. Chemical
184 analyses were conducted on the composited water samples. Habitat
185 types were defined differently in 2018. The 26 sites studied in
186 2018 included two coastal waters (characterized by influence from
187 both inland rivers/streams and Lake Superior) (‘Coastal’), 11
188 upland lakes/ponds (‘Lake/Pond’), and 13 rivers (‘River’), and
189 were selected for their accessibility and connectivity to Lake
190 Superior.

191 Water, net tows, and sediment were collected from each
192 location to be used as inoculant in the laboratory experiments.
193 Whole surface water was collected using a clean, acid washed
194 carboy and filtered through a 150 µm mesh to remove large debris.
195 Horizontal surface net tows were collected with an 80-µm mesh
196 plankton net, and surface sediment was collected via grab sample
197 using a 50 ml centrifuge tube or first collected using a PONAR at
198 deeper sites and then transferred to the centrifuge tube. Net tows
199 were used to provide an additional concentrated inoculant.
200 Supernatant from sediment samples that had been allowed to settle
201 for 1 minute after agitation was added to capture vegetative cells or
202 akinetes that were in the sediment. Filtered water samples, net
203 tows, and supernatant pipetted from sediment samples from each
204 zone were composited with their respective sample and used as
205 inoculant. The amount of each sample type in the inoculant was
206 500 ml water, 17 ml net tows, and 2 ml sediment supernatant in
207 2017, and 200 ml of water, 30 ml of net tow, and 10 ml of
208 sediment supernatant in 2018. Temperature and conductivity were
209 also measured from all sampling sites in July and August in 2018.
210 Electrical conductivity (EC) was measured using an UBANTE™
211 total dissolved solids (TDS) meter in parts per million (ppm). The
212 TDS data was converted to EC in mS/cm and then normalized to

213 25°C (EC₂₅) to account for temperature effects using Eq (1)
 214 (Atkins and de Paula, 2006), where T is temperature in °C .

215 Eq (1) $EC_{25} = EC / (0.889 \cdot 10^{A/B})$, where

216
$$A = 1.37023 (T - 20) + 8.36 \cdot 10^{-4} (T - 20)^2$$

217
$$B = 109 + T$$

218 2.2 Chemical Analyses

219 Water chemistry for 2017 was analyzed for composited
 220 water samples from each zone (3 zones) and 2018 samples were
 221 analyzed for individual sites (26 sites). Particulate fractions of
 222 carbon (POC) and nitrogen (PON) were collected on 25mm pre-
 223 ashed GF/F filters and particulate phosphorus (PP) was collected
 224 on 25mm pre-ashed, acid-rinsed GF/F filters. POC and PON filters
 225 were then dried at 60°C until analysis on a Costech Elemental
 226 Analyzer. Chlorophyll-a (chl-a) samples were filtered using 25mm
 227 0.2 µm cellulose nitrate filters, and frozen in the dark until
 228 analyzed according to Welschmeyer (1994) on a Turner Design
 229 10-AU fluorometer after extraction in 90% acetone for 20-22 hours
 230 in the dark at 4 °C. Total dissolved phosphorus (TDP), soluble
 231 reactive phosphorus (SRP), dissolved organic carbon (DOC),
 232 ammonia (NH₃), and soluble reactive silica (Si) samples were
 233 filtered through 0.22-µm filters, and frozen until analyzed, with the
 234 exception of DOC which was acidified to pH of 2. Total

phosphorus (TP) and nitrate (NO_3^-), were collected as whole-water samples and then frozen for preservation. PP, TP, and TDP samples were digested using potassium persulfate (modification of EPA method 365.1) and then analyzed on a SEAL AQ400. SRP samples were analyzed on a SEAL AQ400 on undigested sample (EPA method 365.1, and Murphy and Riley (1962)). NO_3^- and Si were analyzed according to EPA method 353.2 and Standard Methods 4500-SiO₂, respectively, and also processed on a SEAL AQ400. DOC and TDN were analyzed using a Shimadzu TOC-Vcsh and a TNM-1 module. NH_3 was analyzed according to (Taylor et al., 2007), modified from (Holmes et al., 1999), with the exception of samples that exceeded the upper limit for those methods, in which case they were analyzed on the SEAL AQ400 using EPA method 350.1. Total nitrogen (TN) and was calculated as the sum of TDN and PON, and total organic carbon (TOC) was calculated as the sum of DOC and POC.

2.3 Laboratory Conditions

In 2017, cultures were exposed to high and low nitrogen:phosphorus (TN:TP) nutrient conditions (high: 50:1, low:1.5:1) (all ratios are molar), and three temperatures (15, 20, and 25°C). This broad set of conditions was chosen to insure we would observe cyanobacterial growth, as well as to take a coarse look at ideal conditions, because the species of cyanobacteria

258 observed in Lake Superior blooms has been observed in both cold,
 259 oligotrophic and warm, eutrophic waters. Cultures were run in
 260 triplicate for a total of 54 samples (3 locations x 2 N:P x 3
 261 temperatures x 3 replicates). Cultures were incubated in 1-L
 262 polycarbonate bottles (previously acid washed), continuously
 263 bubbled slowly with room air, and exposed to approximately 250
 264 $\mu\text{mol photons/m}^2/\text{s}$ for 24 hours per day for 21 days. However, the
 265 15°C incubator failed 3 days prior to the scheduled end of the
 266 experiment, and the last growth measurement was taken 3 days
 267 prior to that; therefore, no data were used for the 15°C treatment in
 268 the final 6 days of the experiment, including samples for
 269 taxonomic identification. The experiment began with 100%
 270 environmental sample (500 ml water, 17 ml net tows, and 2 ml
 271 sediment supernatant) and then was run as a semi-continuous
 272 culture, with 150 ml of the sample volume being exchanged for
 273 new sterile media every other day, after thoroughly swirling the
 274 flasks. Culture flasks were exchanged to discourage wall growth
 275 when visible. Cultures were preserved using Lugol's fixing
 276 solution (Wetzel and Likens, 2000) and were examined for
 277 phytoplankton identification and counts (cells and colony units) by
 278 the Wisconsin State Lab of Health and Hygiene, Madison, WI.
 279 Filtered and acetone-extracted chl-a concentration was also
 280 measured in each culture at the end of the experiment.

281 In 2018, cultures were incubated under the same conditions
282 as in 2017 with the following modifications. In 2018 only the low
283 N:P and 25°C conditions were used, as they proved to be the best
284 conditions for cyanobacterial growth in the previous year's
285 experiment. Cultures were also run as batches, duplicates were
286 used instead of triplicates due to space limitations, and the
287 experiment was run for 10 days. Each bottle received 700 ml of
288 low N:P medium and was inoculated with 200 ml of water, 30 ml
289 of net tow, and 10 ml of sediment supernatant. In 2018, a subset of
290 14 samples from sites with the highest cyanobacteria biomass, as
291 measured by the PHYTO-PAM (Walz, 2003), were examined for
292 identification and counts due to cost limitations.

293 2.4 Growth Media

294 The medium used in these experiments was originally
295 designed for an experiment aimed at evaluating competition in
296 natural algal communities in western Lake Superior, western Lake
297 Erie, and Lake Huron, but here we only report its use in
298 experimental work for Lake Superior. The Average Laurentian
299 Great Lakes (A-LGL) Medium was developed for this work based
300 on WC (Guillard, 1975), COMBO (Kilham et al., 1998), SuFr
301 (Twiss et al., 2004), and HH-COMBO (Baer and Goulden, 1998)
302 media, as well as major ion concentrations in the Laurentian Great
303 Lakes (LGL) (Chapra et al., 2012). Table 1 shows the total

304 concentration of major ions in several media and the LGLs. Each
305 of the media were compared to LGL major ion concentrations and
306 used to create a new media recipe, optimized for meeting LGL
307 averages.

308 *2.5 Growth Measurements*

309 Due the large number of samples in the growth
310 measurements, a PHYTO-PAM (Walz, GmbH) was used to
311 monitor growth. The PHYTO-PAM was used with the Phyto-Win
312 software (V 1.45) and the emission detector (ED) unit. The
313 PHYTO-PAM employs multi-spectral fluorescence techniques to
314 differentiate between “blue-green”, “green”, and “brown” algal
315 groups which generally correspond to cyanobacteria, chlorophytes,
316 and diatoms, respectively. High frequency pulses of light are
317 emitted and the excitation of chlorophylls at different wavelengths
318 are measured to separate algal groups. This measurement has
319 proven to provide reliable information on abundance of major
320 groups, provided certain precautions are taken and samples are
321 validated (Lüring et al., 2018; Walz, 2003). To measure growth in
322 our samples, each bottle was subsampled and left in the dark for
323 approximately 30 minutes to be dark-adapted, and all PHYTO-
324 PAM measurements were taken in the dark. Each sample was
325 pipetted into a clean cuvette and placed in the ED unit. The gain
326 was adjusted for each sample, and the amount of chlorophyll in

each algal group was measured using chlorophyll measuring frequency (Chl (MF)) mode with a measuring frequency of 32.

The PHYTO-PAM, like virtually all measures of chlorophyll, is imperfect. Measurements rely on proper reference values being supplied to the software for the samples being analyzed, which can prove complicated when dealing with natural samples with an unknown community composition. To combat this uncertainty, we took the following steps. First, we validated measurements on the PHYTO-PAM using pure cultures of *Microcystis*, *Ankistrodesmus*, and *Cyclotella*, to confirm that the PHYTO-PAM was correctly identifying “blue-green”, “green”, and “brown” algal groups, respectively (Figure S1). We also preserved samples at the end of each experiment for identification. Finally, we treated PHYTO-PAM measurements as a relative measure, meaning that we did not interpret absolute values, but instead evaluated relative changes over time (growth). Though the PHYTO-PAM should not be relied on to give precise biomass estimates, it can provide useful information on the abundances of major algal groups (Lüring et al., 2018).

2.6 Data Analysis

A 3-way MANOVA was used to test for differences in the response of cyanobacterial growth in the 2017 experiments. Each treatment was a different factor (temperature, zone, and N:P) and

350 the response variables were growth rates of the blue-green, green,
 351 and brown algal groups, but because the last 15°C treatment
 352 measurements were taken 6 days prior to the end of the
 353 experiment, only the measurements up to day 13 were used to
 354 calculate growth rates for the 15°C treatment. Growth rates were
 355 calculated as the slope of the natural log of PHYTO-PAM
 356 measurements over time. Growth rates were calculated for the full
 357 duration of the experiment for all treatments as well as up to day
 358 13 to determine if there were differences in results under the two
 359 windows of time and none were found. We also tested for
 360 differences among treatments using taxonomic observations of the
 361 relative abundance of *D. lemmermannii* by applying the non-
 362 parametric Kruskal-Wallis and Kruskal-Wallis multiple
 363 comparison (adjusted to avoid type I errors (Siegel and Castellan,
 364 1988) tests using the *stats* and *pgirmess* packages in R (CoreTeam,
 365 2017), respectively.

366 Due to the spatial nature of the 2018 data, we tested for
 367 spatial autocorrelation in site characteristics and experimentally
 368 derived growth rates by calculating Moran's I (Moran, 1950) using
 369 the inverse distance squared method in ArcMAP (V 10.4.1).
 370 Moran's I tests for tendency of units to be similar to its neighbors
 371 by calculating a correlation coefficient ranging from -1 to 1, where
 372 values near 1, 0, and -1, are clustered, random, or dispersed,

373 respectively. We found that the response variable was not spatially
374 autocorrelated, but many of the potential predictors were
375 autocorrelated as clusters. We then conducted a visual assessment
376 of semivariograms with varied correlation structures to assess
377 severity of autocorrelation, and concluded that corrective measures
378 were not required. We applied paired two-sided t-tests to test for
379 differences in site characteristics and growth rate between months,
380 and one-way ANOVAs to test for differences among waterbody
381 types. If differences were found from the ANOVA test, the Tukey
382 HSD test was applied to identify which groups were different.
383 These tests were performed using the *stats* package in R
384 (CoreTeam, 2017). Four parameters (PP, NH₃, N:P, and Chl-a) did
385 not meet ANOVA criteria for equal variance, so the non-
386 parametric Kruskal-Wallis and Kruskal-Wallis multiple
387 comparison tests were applied to those parameters to test for
388 differences among waterbody types.

389 To identify relationships between growth rates and site
390 characteristics we applied a model selection approach using the
391 Akaike Information Criterion (AIC). Simple linear regressions
392 were used initially to determine which parameters should be
393 included in the selection process, and multiple linear regressions
394 were used for model selection. One outlier was removed from the
395 blue-green growth rate data to evaluate regression models, and the

mean growth rate from July and August experiments was used in the analyses. Non-normally distributed parameters were transformed when necessary. Unless otherwise noted, all analyses were conducted in R for Statistical Computing (V 3.5.0) using the *MASS* and *nlme* packages.

3. Results

3.1 Site characteristics

In 2017, water chemistry for composited samples was analyzed for each of the three zones ('River', 'Lake', and 'Harbor') (Table 3). The most notable difference in water chemistry among the zones was in NO_3^- and Si, where the 'River' zone had much lower and higher concentrations than the 'Lake' and 'Harbor' zones, respectively. Also notable is the lower chl-a observed in the 'River' zone compared to the others. In 2018 water chemistry, temperature, and conductivity were measured at each site in July and August. We compared water quality data from each waterbody type ('River', 'Lake/Pond', and 'Coastal') using boxplots to illustrate differences among the three groups (Figure 2) and performed one-way ANOVAs with Tukey's HSD test to determine differences in site characteristics and cyanobacterial growth rates among waterbody types (Table S1). Note that the 'Coastal' waterbody type refers to sites that are influenced by Lake Superior and inland rivers/streams. TP, TDP, and PP were similar

among groups (Figure 2C, I, and F), as were particulate fractions of carbon and nitrogen (Figure 2D, E). TOC, DOC, TN, and TDN were lower in the ‘River’ than in the ‘Coastal’ and ‘Lake/Pond’ waterbody types (Figure 2A, G, B, and H), with statistical differences ($p < 0.05$) between the ‘Lake/Pond’ and ‘River’ for TOC, DOC, and TDN. The N:P ratio was also lower in the ‘River’ group and had a much narrower range (Figure 2M), and NO_3^- was highest in the ‘River’ group (Figure 2K). Chl-a was highest in the ‘Coastal’ waterbody type, following by ‘Lake/Pond’ and then ‘River’, with statistical differences between the ‘Coastal’ and ‘River’, and the ‘River’ and ‘Lake/Pond’ waterbody types. Temperature was lowest in the ‘River’ group and highest in the ‘Lake/Pond’ waterbodies (Figure 2O), with a statistical difference between the ‘Lake/Pond’ and ‘River’ waterbodies. EC_{25} was highest in the ‘Coastal’ waterbodies (Figure 2P), followed by the ‘River’ and then ‘Lake/Pond’. Paired two-sided t-tests were used to identify statistical differences in site characteristics and cyanobacterial growth rates between months. Significant ($p < 0.05$) differences were observed for all parameters except for N:P, POC, PON, PP, chl-a, and growth rate (Table S2).

3.2 Experimental Results

The results of the 2017 experiments aimed at identifying zones that are sources of cyanobacteria showed that cyanobacteria

442 grew fastest and achieved highest abundance in low N:P conditions
 443 at 20 and 25°C from the ‘River’ and ‘Harbor’ zones (Figure 3N, O,
 444 Q, and R, Table 4). Significant differences in main effects and all
 445 two-way interactions were observed for cyanobacterial growth
 446 (Table 4). Temperature alone had a significant effect on the
 447 “green” algal group, and all treatments and interactions had a
 448 significant effect on the “brown” algal group except for the
 449 interactions between N:P and temperature, and location and
 450 temperature.

451 Taxonomic identification showed that cyanobacteria were
 452 present in cultures with high “blue-green” growth rates measured
 453 by the PHYTO-PAM, and that they were in highest abundance in
 454 the Low N:P and the 20°C and 25°C treatments (Figure 4). The
 455 results of the Kruskal Wallis tests (Table S3) agree with those
 456 found in the MANOVA, that there were significant differences in
 457 the relative abundance of *D. lemmermannii* between the high and
 458 low N:P treatments and the 20°C and 25°C temperature treatments;
 459 however, there were not always significant differences among
 460 locations for a given nutrient and temperature treatment
 461 combination. The relative abundance between the ‘River’ and
 462 ‘Harbor’ locations in the 20°C and low N:P treatment conditions
 463 were not different, and locations were not different in both
 464 temperature levels in the high N:P treatment with exception to

465 20°C-High-River and 25°C-High-Lake, where the abundance was
466 negligible. The taxonomic results support those found using the
467 PHYTO-PAM and also provide additional information: the species
468 of cyanobacteria found in the ‘River’ and ‘Harbor’ locations is the
469 same species observed in Lake Superior blooms, *D. lemmermannii*,
470 and while cyanobacterial growth was high in both 20°C and 25°C
471 at low N:P, *D. lemmermannii* specifically grew in the greatest
472 relative abundance in 20°C and low N:P.

473 The results from the 2017 experiments indicated that
474 upland waters contribute to cyanobacterial blooms in the lake, so
475 in 2018 we evaluated 26 specific locations (rivers, lakes/ponds,
476 and coastal waterbodies) as potential sources of cyanobacterial
477 propagules and looked for characteristics shared among sites that
478 could be potential sources. The results from 2018 showed that sites
479 with the highest cyanobacterial growth rates were widely scattered
480 across the study region (Figure 5). There were no spatial
481 autocorrelations in the growth data as represented in Moran’s I.
482 We then looked for similarities in chemical, biological, and
483 physical characteristics that could identify sites with high potential
484 using single and multiple linear regressions.

485 Simple linear regressions were used to compare
486 cyanobacterial growth rates to site characteristics. Out of 17 water
487 quality parameters tested, four were significant predictors for

488 cyanobacterial growth: temperature ($p = 0.006$, $R^2 = 0.26$, slope = -
489 0.026), EC_{25} ($p = 0.0004$, $R^2 = 0.41$, slope = 2.70), $\ln(SRP)$ ($p =$
490 0.002, $R^2 = 0.34$, slope = 0.17), and $\ln(N:P)$ ($p = 0.02$, $R^2 = 0.20$,
491 slope = -0.17). These results mean that cyanobacteria grew fastest
492 under laboratory conditions from sites that had higher conductivity
493 and SRP and lower temperature and N:P. Those parameters were
494 then used with AIC and backward model selection to identify the
495 best model for cyanobacterial growth rate. The optimized model
496 included only *in situ* EC_{25} and temperature (Table 5). Model
497 selection could only be performed using additive models (i.e.
498 without interactions), as the number of comparisons with
499 multiplicative models would be too computationally expensive,
500 therefore we tested the best additive model for interaction terms
501 and found significant interaction between temperature and EC_{25} ,
502 which resulted in a final model with $R^2 = 0.57$ (Table 6). We also
503 tested for the impact of waterbody type (Coastal, River,
504 Lake/Pond) in the model and found that there was no significant
505 effect.

506 To validate measurements using the PHYTO-PAM, we also
507 examined preserved samples for identification. Preserved samples
508 from 14 sites with the highest final “blue-green” biomass
509 (measured using the PHYTO-PAM) were surveyed. This group
510 included one ‘Coastal’ waterbody, four ‘Lake/Pond’, and nine

511 ‘River’ sites. Seven of these samples were found to be comprised
512 of over 75% *D. lemmermannii* and three sites had greater than 50%
513 (Figure 6). Ten out of the total 14 sites were also among sites with
514 the highest experimental growth rates for “blue-green” algae
515 (calculated using PHYTO-PAM data). Three out of the four sites
516 that were not among the highest observed growth rates were
517 ‘Lake/Pond’ waterbody types, and two of those four sites were
518 comprised of less than 25% *D. lemmermannii* (1 ‘River’ and 1
519 ‘Lake/Pond’).

520 **4. Discussion**

521 The appearance of cyanobacterial blooms in Lake Superior
522 is surprising, given that it is a cold, oligotrophic system with much
523 less anthropogenic nutrient inputs relative to other lakes where
524 blooms are often observed. The current “chemostat paradigm” for
525 cyanobacterial blooms in lakes is that the role of rivers is to supply
526 nutrients to algal populations in the receiving lakes (Bridgeman et
527 al., 2012; Carpenter et al., 1998; Conroy, 2007), but this study
528 points to other potentially important roles for inflowing waters.
529 This work is a critical step toward understanding why we have
530 begun observing cyanobacterial blooms in Lake Superior and it
531 indicates a likely important role of upland waterbodies, particularly
532 rivers, in seeding blooms. There is a wealth of evidence in marine
533 and freshwater systems that bloom-forming cyanobacteria can

534 persist in rivers and estuaries (Conroy et al., 2014; Paerl and Otten,
535 2013; Reif, 1939; Schwartz, 2007; Steinberg and Hartman, 1988).
536 What is not as well understood is how the presence of
537 cyanobacteria in rivers may affect downstream lakes or ponds. The
538 lack of research on this topic may be due to the greater likelihood
539 of observing cyanobacterial blooms in lentic environments than in
540 their lotic counterparts or because riverine conditions (including
541 high turbulence, low light, short residence times, and cooler
542 temperatures) are not typically associated with high algal growth
543 rates (Paerl and Otten, 2013). Nevertheless, a recent study of 11
544 major rivers in the US showed that cyanobacteria was present in all
545 rivers and comprised up to 52% of the community composition
546 (Graham et al., 2020). The present study shows that rivers may
547 play a critical role in the promotion of newly occurring
548 cyanobacterial blooms in Lake Superior.

549 Our work is based on the logic that if an environmental
550 sample can generate cyanobacterial growth under reasonable time
551 scales and favorable conditions in a laboratory setting, it has
552 elevated potential to be a source of blooms compared to samples
553 that do not produce high growth in the lab. This approach has the
554 advantage of not relying on quantifying potentially rare
555 populations that initiate blooms, and it avoids the potential that a
556 given algal taxon may be present but not be viable, issues that

557 could arise under a simple sampling regime no matter how
 558 sensitive it may be. The experiments we conducted in 2017 and
 559 2018 provided insight into the origins and the conditions that
 560 promote cyanobacterial blooms along south shore of Lake
 561 Superior. The conclusions we draw from the 2017 experiment are
 562 that bloom-forming cyanobacteria grew abundantly in low N:P and
 563 at moderate to high temperature, and more importantly, only from
 564 ‘River’ and ‘Harbor’ locations and not from the ‘Lake’ samples.
 565 This finding provides evidence that blooms are unlikely to have
 566 been sourced just from the lake, and that upstream sources are
 567 likely important contributors of living cells to generate blooms.
 568 The subsequent 2018 work then began to narrow down
 569 characteristics of upstream environments that are most likely to be
 570 involved in the generation and delivery of algal seed populations
 571 for Lake Superior blooms.

572 Cyanobacteria in the Nostocales order are diazotrophic;
 573 thus, it is not surprising that *D. lemmermannii* grew better than the
 574 other algal groups in these nitrogen-limiting conditions during
 575 laboratory experiments, but growth under lab conditions does not
 576 necessarily indicate the conditions most responsible for high
 577 growth in a natural setting. PHYTO-PAM measurements in both
 578 2017 and 2018 did not show a dominance of cyanobacteria in river
 579 samples at the beginning of the experiments. In fact, the initial

580 total biomass of samples was low and typically dominated by the
581 “brown” algal group while the “blue-green” only comprised
582 approximately 1-5% of the total biomass (Figure 3). This was also
583 true for ‘Lake’ and ‘Harbor’ samples from 2017, which may
584 mean that while similar concentrations of cyanobacteria were
585 initially present from all zones, cells from ‘River’ and ‘Harbor’
586 samples were more likely *D. lemmermannii* and samples from the
587 ‘Lake’ sites were not, given that this species did not grow in high
588 abundance in any treatment combination from this location. In an
589 analysis of Lake Superior nearshore and offshore phytoplankton
590 survey data from 2011 and 2016, (Kovalenko et al., 2019) found
591 that diatoms accounted for over 75% of the total biovolume for all
592 samples areas, with some codominance in 2016. Codominance of
593 *D. lemmermannii* (by biovolume) was observed for part of 2016 at
594 one site in central Lake Superior offshore (depth > 200m),
595 indicating that *D. lemmermannii* is not prominent in the Lake
596 Superior phytoplankton community. ‘Harbor’ samples did grow
597 cyanobacteria in abundance, but a closer look at the taxonomic
598 data showed that the species present from ‘Harbor’ samples was
599 not predominately *D. lemmermannii* with exception to the low N:P
600 and 20 °C treatment. Analysis of 2018 data revealed that ‘River’
601 sites had the lowest total N:P ratio of the three waterbody types
602 (mean of approximately 19:1); therefore, from a N:P standpoint,

603 rivers feeding into nearshore Lake Superior where blooms have
604 been observed may provide conditions to promote diazotrophic
605 cyanobacteria like *D. lemmermannii*. Consequently, even though
606 the relative abundance of cyanobacteria is not greatest in the
607 overall community composition, the conditions in rivers support a
608 community of cyanobacteria that is capable of proliferating under
609 laboratory conditions.

610 In addition to a clear N:P preference, higher water
611 temperatures stimulated growth during the 2017 experiments, and
612 more specifically, taxonomic data showed that the 20°C treatment
613 resulted in higher *D. lemmermannii* abundances than 25°C. We
614 also observed high cyanobacterial growth rates in the 2018
615 experiments under laboratory conditions (25°C and low N:P), but
616 the sites that produced those high growth rates under laboratory
617 conditions, namely rivers, were characterized by low water
618 temperatures *in situ* (12-26°C, mean of approx. 17 °C). These
619 findings indicate that fast-growing cyanobacteria originated in
620 colder waters, but our 2017 experiments showed that 15°C was too
621 cold for extensive growth of *D. lemmermannii*, even at optimal
622 nutrient conditions. Evidence from subalpine lakes has shown the
623 ability of *D. lemmermannii* to persist in cold water temperature
624 systems and then grow extensively as temperatures warm to

625 greater than 15°C (Salmaso et al., 2015), which is consistent with
626 the temperature dependence we observed in our study.

627 Routine water quality monitoring during the 2018 field
628 season (May-October) showed that the lake is typically warmer
629 than inflowing rivers by several degrees (Figure 7). Cooler river
630 temperatures can be attributed to several factors including size,
631 flowrate, canopy cover, hydrologic inputs, and drainage pattern,
632 whereas water in shallow nearshore embayments is exposed to
633 direct light and the surface layer warms throughout the summer,
634 resulting in higher surface water temperatures in the nearshore than
635 in rivers. Temperature has been identified as a primary control on
636 *D. lemmermannii* growth in other cold, oligotrophic lakes (Callieri
637 et al., 2014; Capelli et al., 2017; Salmaso et al., 2015, 2012), and
638 has also been identified as an important driver in Lake Superior
639 (Sternner et al., in review). So, while rivers are more likely to yield
640 viable *D. lemmermannii* cells that could initiate growth in the lake,
641 the warmer temperature conditions in Lake Superior's nearshore
642 are generally more favorable for growing high concentrations of
643 biomass. Light conditions in the nearshore and rivers was not
644 measured during routine monitoring; however, river plumes in
645 Lake Superior have been studied and it has been shown that light is
646 lower in Lake Superior waters impacted by river plumes (Cooney
647 et al., 2018; Minor et al., 2014), indicating that rivers have higher

648 turbidity and thus, lower light compared to the nearshore.
 649 Detenbeck et al. (2004) also document high turbidity in south
 650 shore streams due to forest type and erosion of fine sediment with
 651 mean field turbidity measurements near approximately 100 NTU,
 652 which corresponds to an extinction coefficient of 5 m^{-1} (Brown,
 653 1984), while typical Lake Superior values are less than
 654 approximately 0.4 m^{-1} (Cooney et al., 2018; Sterner, 2010). We
 655 hypothesize that when *D. lemmermannii* propagules from rivers
 656 with low temperature and light conditions reach the nearshore of
 657 Lake Superior, where temperature is seasonally warmer and there
 658 is greater light exposure, they can grow in greater abundance and
 659 ultimately produce blooms. Further work is needed, however, to
 660 understand the relative contributions of temperature, light, and
 661 nutrients, as well as hydrodynamics, in promoting cyanobacterial
 662 blooms once the cells have entered the lake. Additional work is
 663 also needed to determine whether rivers are supporting a
 664 community of *D. lemmermannii* or acting as a transport
 665 mechanism from other upstream areas.

666 The other predictor that helped explain which sites are
 667 likely sources of cyanobacterial propagules to Lake Superior, in
 668 addition to colder water temperatures, was higher conductivity.
 669 Conductivity is related to salinity and pH, both of which can affect
 670 cyanobacteria growth. There is some evidence that salinity can

671 inhibit growth (Apte et al., 1987; Rai, 1990), but the concentrations
672 used in these studies (55 mM – 500 mM NaCl) typically far
673 exceed those we observed in our study sites (approximately 2.2
674 mM NaCl, assuming that all of the conductivity can be attributed
675 to salinity). Similarly, pH conditions in five south shore Lake
676 Superior streams were circumneutral during multiple sampling
677 events in 2016, indicating that pH is not likely a factor influencing
678 algal growth (J. Delvaux, unpublished data). Hence, it is unlikely
679 that the levels of conductivity we observed are high enough to
680 have an inhibitory effect on growth, and the more likely
681 explanation is that conductivity is related to some other
682 unmeasured parameter that is related to sites with high potential.
683 Conductivity may be related to the geological substrates in the
684 region. The predominate soil types in the study area are Alfisols
685 and Spodosols, commonly referred to as ‘red clay’ due to their rich
686 iron content, are highly susceptible to erosion and the main
687 contributor of dissolved ions to Lake Superior (Cooney et al.,
688 2018; Stortz et al., 1976). We considered the high iron content of
689 the soil as a possible linkage between high potential sites and
690 increased conductivity, but the iron in these soils is present as iron
691 oxides and are highly insoluble in water, and so is not likely
692 stimulating algal growth (Tonello et al., 2019).

693 To summarize, evidence from this study shows that rivers
694 play a critical role in initiating cyanobacterial bloom on the south
695 shore of Lake Superior, with some additional evidence that shifting
696 growth conditions between rivers and lakes are also necessary for
697 bloom formation. Our data showed that *D. lemmermannii* grew
698 under laboratory conditions from water samples that were collected
699 from inland locations, particularly rivers, and grew best in 20°C
700 and low N:P conditions. Somewhat unexpectedly, sites that had the
701 greatest cyanobacterial growth under optimal laboratory conditions
702 were characterized by relatively low temperatures and high
703 conductivity. We ascertain that rivers produce cells of *D.*
704 *lemmermannii* capable of initiating extensive growth, but cold
705 water temperatures and low light conditions inhibit overall growth
706 in these habitats. When these cells enter the Lake Superior
707 nearshore, where temperatures are seasonally warmer and light
708 levels are higher, conditions are more favorable for growth.

709 Based on this evidence, we argue that rivers provide a
710 consistent source of viable propagules of *D. lemmermannii* to Lake
711 Superior, and that the appearance of blooms in the lake is
712 dependent on in-lake conditions. Lake Superior has already begun
713 experiencing significant warming (Austin and Colman, 2007) and
714 extreme precipitation events (Cooney et al., 2018), and these trends
715 are expected to continue. If water temperatures and nutrient inputs

716 continue to increase, along with consistent seeding from rivers,
717 cyanobacterial blooms may become a persistent feature of Lake
718 Superior's nearshore.

719 **Author Contribution Statement**

720 All authors have read and approved the final submission.
721 KLR developed experimental design, carried out laboratory and
722 field research, conducted data analysis, and prepared this
723 manuscript. RWS advised on experimental design and data
724 analysis, and provided significant comments on the manuscript.
725 BLM advised on experimental design, assisted with data
726 collection, and commented on data analyses and manuscript. SLB
727 assisted with field and laboratory research, sample analysis, and
728 commented on the manuscript.

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1008 **Tables**

1009 Table 1. Major ion concentrations of selected algal growth media
 1010 and LGLs. A-LGL was used in this study. All concentration
 1011 reported in $\mu\text{mol/L}$. Ca^{2+} estimates include CaCO_3 .

Major Ions	Ca^{2+}	Mg^{2+}	Na^+	K^+	SO_4^{2-}	Cl^-	HCO_3^-	Total Ions
WC	250	150	1373	100	150	5367	150	7540
COMBO	250	522	150	1374	200	150	150	2796
SuFr	335	100	88	26	60	480	13	1102
HH COMBO	750	225	1513	100	225	1522	1500	5835
A-LGL	600	200	214	25	200	1238	150	2627
Superior	758	116	63	13	40	40	834	1864
Michigan	1974	464	272	36	250	340	2140	5476
Huron	1442	307	168	24	165	186	1563	3855
East Erie	1688	366	373	37	237	411	1758	4870
Ontario	1736	354	503	38	266	552	1789	5238
LGL Average	1520	321	276	30	192	306	834	3479

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1013

1014 Table 2. A-LGL medium recipe.

Major Stocks	μmol/L
CaCl ₂ · 2H ₂ O	600
MgSO ₄ · 7H ₂ O	200
NaHCO ₃	150
Na ₂ SiO ₃ · 5H ₂ O	20
H ₃ BO ₃	388
KCl	25
Algal Trace	μmol/L
Na ₂ EDTA	14.92
FeCl ₃ · 6H ₂ O	3.70
CuSO ₄ · 5H ₂ O	0.01
ZnSO ₄ · 7H ₂ O	0.08
CoCl ₂ · 6H ₂ O	0.08
MnCl ₂ · 4H ₂ O	0.91
Na ₂ MoO ₄ · 2H ₂ O	0.09
H ₂ SeO ₃	0.01
Na ₃ VO ₄	0.01
Vitamins	μmol/L
Thiamine · HCl	296.5
Biotin	0.20
B ₁₂	0.037
Nutrients	μmol/L
K ₂ PO ₄ (High N:P)	0.5
K ₂ PO ₄ (Low N:P)	5.33
NH ₄ NO ₃ (High N:P)	25
NH ₄ NO ₃ (Low N:P)	8

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1016

1017 Table 3. Water chemistry means and standard deviations
 1018 (analytical variation across a given sample) for composited water
 1019 samples from each zone in 2017 experiments.

Parameter	Lake		River		Harbor	
	Mean	SD	Mean	SD	Mean	SD
Chl-a (µg/L)	1.92	0.02	0.79	0.01	3.47	0.34
POC (µM)	26.49	1.37	43.30	1.19	41.11	3.17
PON (µM)	3.02	0.14	4.66	0.68	4.95	0.06
NH ₃ (µM)	0.85	0.10	0.21	0.01	1.44	0.11
NO ₃ ⁻ (µM)	25.81	-	0.95	-	30.27	-
Si (µM)	25.42	0.04	121.45	0.71	50.53	0.02
PP (µM)	0.10	0.00	0.27	0.00	0.32	0.03
TDP (µM)	0.12	-	0.01	-	0.34	-
SRP (µM)	0.05	-	0.37	-	0.26	-
TP (µM)	0.16	-	0.52	-	0.76	-

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1021

1022 Table 4. Results of the 3-way MANOVA test for each algal group
 1023 using growth rate as the response variable. P-values marked with
 1024 an asterisk (*) are significant at $\alpha=0.05$.

Treatment	Blue-green		Green		Brown	
	F value	p-value	F value	p-value	F value	p-value
Temp	6.09	0.0079*	5.01	0.016*	21.6	6.5 x 10 ^{-6*}
Location	7.71	0.0029*	2.34	0.12	79.7	8.4 x 10 ^{-11*}
N:P	13.0	0.0015*	0.940	0.34	66.2	4.5 x 10 ^{-8*}
Location x Temp	2.92	0.044*	1.15	0.36	1.89	0.15
N:P x Temp	12.7	0.00022*	0.380	0.69	2.38	0.12
N:P x Location	8.03	0.0024	0.290	0.75	18.1	2.2 x 10 ^{-5*}
N:P x Location x Temp	1.21	0.31	0.0260	0.97	6.10	0.0078*

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1026

1027 Table 5. Results of model selection using AIC.

Model (N=24)	AIC
Ln(SRP) + Ln(NP) + Temp + EC ₂₅	-85.45
Ln(SRP) + Temp + EC ₂₅	-87.41
Temp + EC ₂₅	-88.58
EC ₂₅	-88.84

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1029

1030 Table 6. Summary of interaction model using temperature and
1031 EC₂₅.

Parameter	Slope Estimate	p-value	Overall R ²	Overall p-value
EC ₂₅	15.7	0.00384	0.57	0.00015
Temp	0.0542	0.0384		
Temp x EC ₂₅	0.227	0.00990		

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1034 **Figure Captions**

1035 Figure 1. Map of study sites. Sites from 2017 are shown in red with
1036 shapes denoting the zone, and 2018 sites are shown in black with
1037 shapes denoting the waterbody type. Stream systems associated
1038 with 2018 sites are also shown. A yellow diamond denotes the
1039 location of the Mawikwe Bay (MB) field sampling site.

1041 Figure 2. Boxplots of water quality parameters for each waterbody
1042 type in 2018 experiments. The central box line indicates the
1043 median, the upper and lower box hinges correspond to the 75% and
1044 25% quartiles, respectively, and the whiskers correspond to 1.5
1045 times the interquartile range. Black dots indicate sample points.

1047 Figure 3. The natural log of chlorophyll concentration of the blue-
1048 green, green, and brown algal groups over the duration of the
1049 experiment for each treatment.

1051 Figure 4. Relative abundance of cyanobacterial species by
1052 treatment combinations. The 15°C treatment was not analyzed due
1053 to mechanical failure of the incubator.

1055 Figure 5. Sampling locations of experimental cyanobacterial
1056 growth rate for the months of July and August. Growth rates are
1057 divided by natural breaks.

1059 Figure 6. Relative abundance of *D. lemmermannii* in 14 sites
1060 (ordered from west to east) with greatest cyanobacterial growth in
1061 culture. Colors indicate waterbody type and asterisks (*) denote the
1062 10 sites that also had the highest cyanobacterial growth rates.

1064 Figure 7. Boxplots of July and August surface water temperature
1065 for nearshore Lake Superior (MB) and a subset of rivers included
1066 in 2018 experiments. The central box line indicates the median, the
1067 upper and lower box hinges correspond to the 75% and 25%
1068 quartiles, respectively, and the whiskers correspond to 1.5 times
1069 the interquartile range. Black dots indicate sample points.

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Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7