Wakulla Spring Dark Water: Causes and Sources Phase II

Final Report

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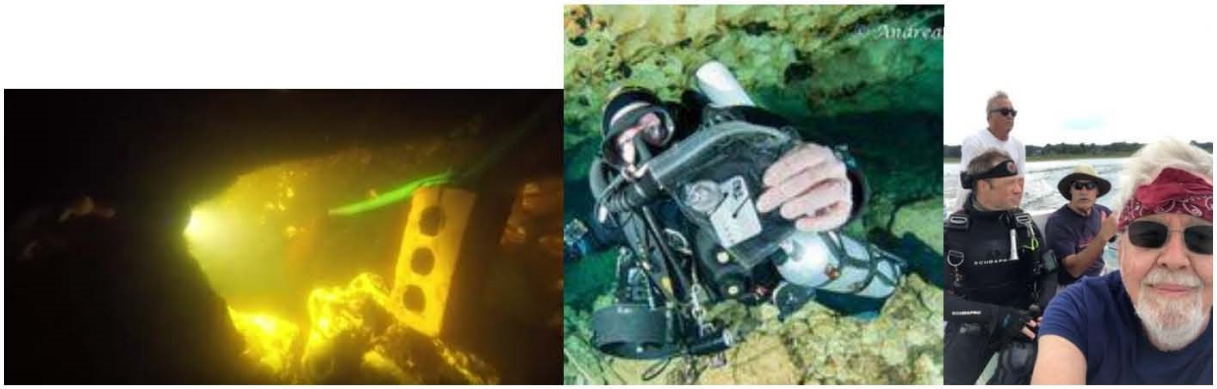

This project was completed for the Wakulla Springs Alliance by McGlynn Laboratories, Inc. with financial assistance provided by the Fish and Wildlife Foundation of Florida, Inc. through the Protect Florida Springs Tag Grant Program, project PFS #1617-08.

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Acknowledgements

This project was completed for the Wakulla Springs Alliance with financial assistance provided by the Fish and Wildlife Foundation of Florida, Inc. through the Protect Florida Springs Tag Grant Program, project PFS #1617-08. We would also like to thank Wakulla Springs State Park staff who collected daily samples from the spring and provided boat access and assistance with weekly light measurements and the following individuals: Katy McGlynn, Julia McGlynn, Sophie Wacongne-Speer, Emily Speer, and Richard Speer who assisted with weekly field work; Cal Susan Jamison who participated in all field work and played a major role in the design and execution of the dye studies, including oversight of deployment of charcoal packs; diver Andreas Hegberg who assisted with injecting dye into Porter Hole Sink and with placing and retrieving charcoal packs at several sites; divers Michael Barnette, Travis Kersting, and Brandon McWilliams who deployed and retrieved other charcoal packs; Sherry Carpenter who assisted with the Porter Hole Sink dye injection; Brendan McGlynn who assisted with injecting dye into Fallschase Sink at Upper Lake Lafayette; and Drs. Richard Long and Thomas Sawicki devoted many hours to designing and implementing the environmental DNA study including analysis and presentation of the data and results. Thanks also to the following property owners for providing access to sinks and springs on or through their properties: Bob Brown, Don Fortner, the Harveys, Norma Skaggs, Mitch and Barbara Spears, Teddy Tollett, and Wakulla Springs State Park.

Executive Summary

This second phase of our Dark Water study of Wakulla Spring was designed to extend prior research funded by the Fish and Wildlife Foundation to determine the causes and sources of "dark water" conditions at Wakulla Springs which have resulted in the nearly complete curtailment of glass bottom boat tours and the loss of most of the highly productive eelgrass and fish community that previously occupied the spring bowl. This Phase II project focused on

documenting the sources of chlorophyll from algae that cause the green "dark water" conditions that occur when the spring has historically cleared after rain events.

Our principal findings include the following:

1. Extension of weekly water quality sampling to include the Wakulla Springs cave system as well as the spring boil has demonstrated that most if not all of the chlorophyll responsible for “green dark water” conditions experienced in the spring boil is entering the spring in the ground water in addition to tannins which have historically caused “brown dark water” conditions.
2. Extension of our weekly sampling of photosynthetically available radiation (PAR) at the spring boil has further confirmed findings from Phase I that dark water conditions are complex phenomena not simply explained by any one of the three major light-adsorbing substances: tannins, chlorophyll a, and its degradation product, phaeophytin.
3. Dye studies we conducted demonstrated for the first time suspected hydrogeologic connections between Wakulla Spring and Lake Jackson (35-day travel time) and Upper Lake Lafayette (30-35 day travel time).
4. Taxonomic analyses of algae samples collected from Upper Lake Lafayette, Lake Jackson, Lake Munson, and the L well which taps the cavern system 430 feet from the Wakulla Spring vent identified three algal taxa at the L well unique to each of the three lakes, indicating that chlorophyll entering the spring could be coming from all three lakes. However, 67% of the taxa identified in the L well were not found in any of the three lakes suggesting that chlorophyll may be coming from one or more other surface water bodies. Lake Iamonia is the most likely other source. We will therefore conduct a dye study of Iamonia during Phase III and include it in two subsequent algal taxonomic studies.
5. Environmental DNA analysis conducted at the same time as the taxonomic studies also identified unique taxa from each of the three lakes found in samples from the spring. As with the algal taxonomic study, the environmental DNA analysis also found algal taxa in the spring not found in one of the three lakes suggesting additional surface water bodies may be contributing chlorophyll to the green dark water conditions in the spring. Two additional DNA studies will be conducted in Phase III along with the taxonomic analyses.

1. Introduction

This project extended research conducted in Wakulla Spring Dark Water: Causes and Sources Phase I which was supported by funding from the Fish and Wildlife Foundation of Florida, Inc. through the Protect Florida Springs Tag Grant Program, project PFS #1516-05. Phase I demonstrated that dark water conditions experienced at Wakulla Spring are caused both by tannins and chlorophyll a including its degradation product, phaeophytin. We extended weekly sampling at the Wakulla Spring boil for an additional 88 weeks (8/11/16 – 3/29/18) for tannins (measured as true color), corrected chlorophyll a, and phaeophytin, plus spectral radiometric measurements of light attenuation with depth, to provide a more robust data set for identifying patterns of dark water conditions and associated water quality. We also designed Phase II to attempt to identify likely sources of the chlorophyll which we hypothesized was entering the spring in the ground water rather than being produced by algae inhabiting the vent and spring bowl. To this end, Phase II expanded weekly sampling of tannins and chlorophyll to seven wells that tap the major caverns flowing into Wakulla Spring (see figure 1.1). During Phase II we also conducted dye studies of two large karst lakes in the Wakulla springshed, Lake Jackson and Upper Lake Lafayette, which we hypothesized may be sources of the chlorophyll entering the spring. Both lakes receive urban storm water inflows from the Tallahassee area and experience extensive algal blooms each year. Previous dye studies had documented the hydrologic connection of a third urban karst lake which also experiences extensive algal blooms, Lake Munson (Kincaid et al., 2007). In an effort to document specific linkages of chlorophyll at Wakulla Spring to one or more of these lakes, we collected samples from all three lakes for taxonomic and environmental DNA analyses in October 2017.

We present our findings in the following chapters. Chapter two compares weekly sampling data from the six wells with measurements from the spring boil. Chapter three extends our analyses of the relationships between the depth limit of photosynthetically available radiation (PAR) and color and chlorophyll (corrected chlorophyll a and phaeophytin). Chapter four presents the results of three dye studies, one for Lake Jackson and two for Upper Lake Lafayette. Chapter five presents findings from the algal taxonomic and environmental DNA analyses of samples from the three lakes and Wakulla Spring. We present a summary of our findings and recommendations for further in chapter six.

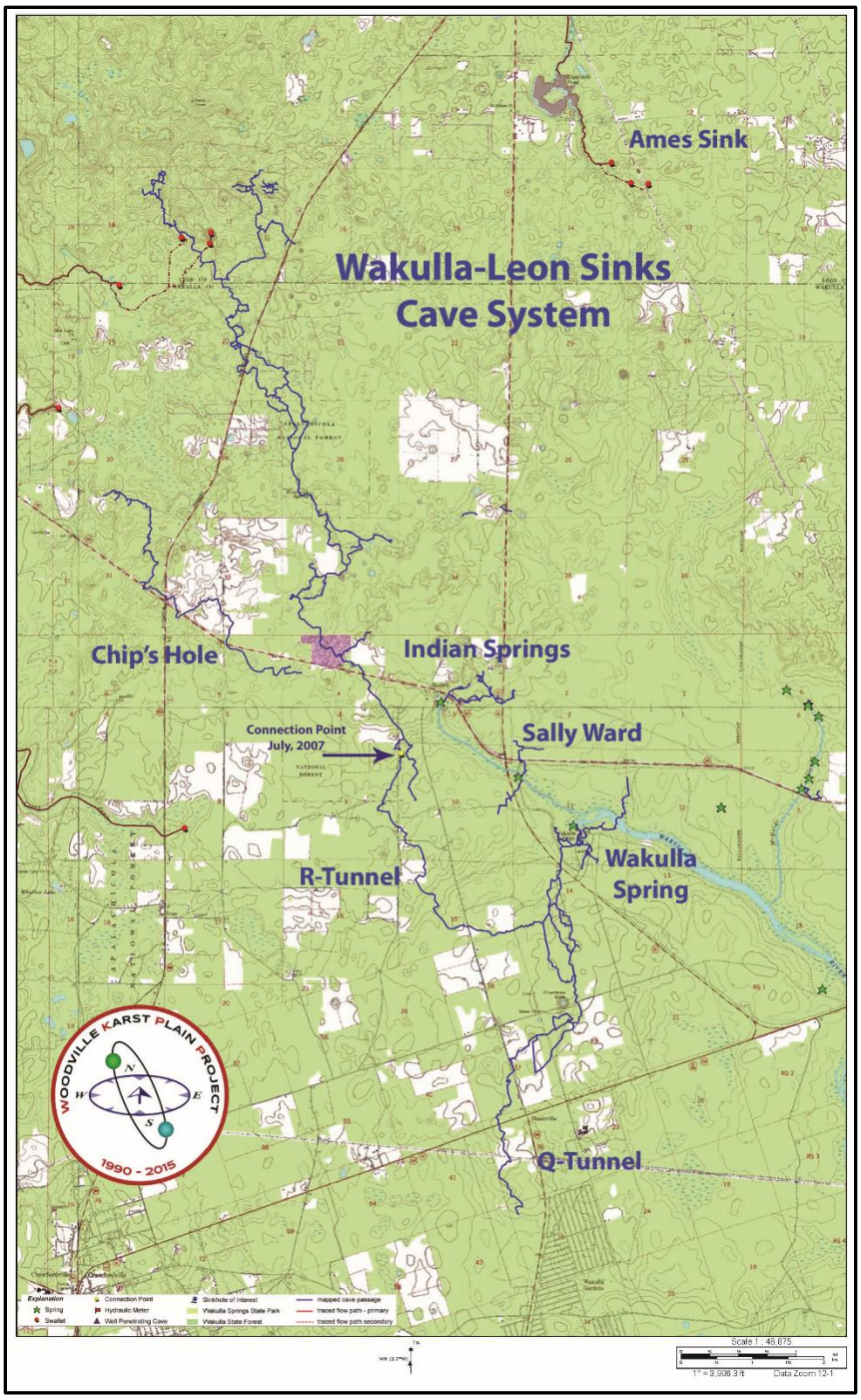

Figure 1.1. Wakulla Spring cave system (Woodville Karst Plain Project, 2015).

2. Water Quality in the Wakulla Caves and Spring

One of the principal objectives of Phase II was to test the hypothesis that most or all of the chlorophyll detected in the Wakulla Spring boil originates in the ground water flowing from the vent rather than from algae growing within the spring bowl. Informal microscopic examination

of algal material filtered from samples taken from the boil during Phase I indicated that algae in the spring outflow are phytoplankton species that typically reside in the water column of lakes rather than benthic or epiphytic species that inhabit bottom sediments, rocks, and the surfaces of submerged aquatic plants. Furthermore, the high rate of flow at the vent, which averaged 875 cubic feet per second during Phase I, would preclude the persistence of community of phytoplankton in the spring boil.

We approached this question by expanding our weekly analysis of water samples from the spring boil to include samples pumped from seven wells that tap into the complex cave system that feeds into Wakulla Spring (figure 2.1) for a one-year period from 8/8/16 through 8/10/17: K, AK, AD, D, C, B, and L. Six of the wells have been used for water quality sampling for a number of years, but sample a complex area of intersecting caves several thousand feet from the spring vent. The L well, however, which was installed in 2015 as part of an upgrade to the lodge air conditioning system, is situated approximately 430 feet from the vent, tapping into the ceiling of the so-called Grand Canyon chamber underneath the lodge (see figure 2.2). We anticipated that the water quality of samples taken from the L well would be nearly the same as those taken from the boil and should, therefore, offer the best measure of the water quality mix reaching the spring from its cave system. We do not know, however, how well water within that chamber is mixed. It is possible that some colder, denser water flows along the base of the chamber.

Figures 2.3-2.5 compare the average weekly levels of specific conductivity, tannins (measured as true color),[1] and chlorophylls[2] from the spring boil (WS), the seven wells, the Sally Ward Spring (SW), and Spring Creek (SC) measured over a one-year period from August 8, 2016, through August 10, 2017.[3]

Figure 2.3 displays average weekly specific conductivity. Specific conductivity serves as a proxy for the concentration of ions in solution by measuring the ability of a solution to conduct electricity. Surface water bodies in North Florida typically have low specific conductivity of 50 or less uS/m, while springs in karst limestone can have values of 300 to 400 uS/m. Salt water, on the other hand, exhibits conductivities as high as 40,000 uS/m. Among the caves tested, weekly average specific conductivity is lowest at the K and AD cave wells, most likely reflecting the influx of sinking stream flows from Black, Fisher, and Jump Creeks in Apalachicola National Forest which discharge into the R cave through swallets (figure 2.6). Lower levels measured at the AD well compared to AK are puzzling since, as reported below, low true color levels at the D well suggest little surface water flow into the D cave. The higher average specific conductivity measured at the Wakulla Spring boil compared to the L well may reflect additional inflows between L and the Wakulla vent from matrix flow through the karst limestone or incomplete mixing within the Grand Canyon chamber. Water with higher dissolved ions may be sufficiently dense to flow along the bottom of the chamber and on to the vent with less dilution.

The one-year average weekly true color measured in the K cave (figure 2.4) mostly reflects tannins from the northern sinking streams carried via the R cave. True color measured in AK derives from combined tannins from K and from the Lost Creek swallet carried north via Q.

[1] Method detection limit (MDL) = 0.41 PtCo.
[2] MDL = 0.21 µg/L.
[3] See McGlynn and Deyle (2016) for sampling and analytic methodology details.

Weekly true color averages for WS, AD, AK, and L are nearly equal suggesting that tannins detected in C and D may be diluted by matrix inflows and that tannins are well mixed within the Grand Canyon chamber.

Figure 2.5 reveals that the one-year weekly average corrected chlorophyll a levels are nearly all below the minimum detection level of 0.21 μg/L. Phaeophytin levels are higher, sometimes double, reflecting the probable degradation of algal cells during transit from their sources through the aquifer. The K and AD well weekly averages exceed those of both the Wakulla Spring boil and the L well, which are almost identical. The intermediate average for AK may reflect dilution of the K cave flow by inflow from the A cave, while the higher weekly average for AD versus AK likely reflects an influx of chlorophyll through the D cave. The incomplete mapping of the cave system offers few clues as to the likely source of chlorophyll detected in the K and D caves. Dye tests conducted by Hazlett-Kincaid, Inc. (Kincaid et al., 2007) documented hydrologic connections between Ames sink, which receives discharge from Lake Munson, and Indian Spring, and between Indian Spring and Wakulla Spring. To date, however, divers have not succeeded in finding the underground connection between Indian Spring and Wakulla. The nearly identical weekly averages for the spring boil and the L well suggest that chlorophylls are well mixed within the Grand Canyon chamber.

Figures 2.7-2.10 present line graphs of weekly measurements of true color, corrected chlorophyll a, phaeophytin, and total chlorophyll a for the Wakulla Spring boil, all of the cave sample wells except L, and Sally Ward Spring for the one-year period August 8, 2016, through August 10, 2017. Separate charts comparing weekly levels of these parameters measured at the L well and the Wakulla Spring boil for a more extended period, March 28, 2016, through March 29, 2018, are presented in figures 2.11-2.14.

Figure 2.7 shows that tannin levels measured as true color at the C and D wells are consistently low, but sometimes exhibit peaks coincident with those of the wells we expect to be most influenced by the influx of sinking stream flows via the R cave connecting with the K cave, i.e. K, AK, and AD (figure 2.1). Color levels measured at the B well, while also low, rarely exhibit a pattern consistent with the wells most affected by sinking streams discharges to the aquifer.

Weekly color levels measured at the spring boil generally mirror those at the L well over the longer time period (figure 2.11). Occasionally color levels measured at the spring boil are greater than those in the L well, suggesting that at times ground water within the Grand Canyon chamber tapped by the L well is not fully mixed. At other times the patterns are not fully synchronized with color peaks at the boil occurring a week before, and occasionally after, those at the L well suggesting that sometimes flow to the vent may temporarily short-circuit the Grand Canyon carrying either high-tannin or low-tannin water to the vent with little mixing in the Grand Canyon. Regression analysis (table 2.1) indicates that true color levels in the L-well explain about 45% of the observed variance in true color levels measured at the boil ($R^2 = 0.453$; significant at the 99.9999 % level).

Many of the weekly corrected chlorophyll a measurements (figure 2.8) from the six cave wells are below the minimum detection limit (MDL) of 0.21 μg/L. As is the case with the one-year

weekly averages, weekly phaeophytin levels tend to be higher (figure 2.9) and exceed the MDL more often. Focusing on total chlorophyll a levels (figure 2.10), reveals that among the six cave wells, each has the highest level in multiple weeks: K(7), D(5), C(5), AD(5), B(4), AK(2). On about a dozen dates, total chlorophyll a levels measured at the spring boil exceed those of any of the caves.

Comparisons of the L well and the spring boil over the longer time interval (March 28, 2016 – March 29, 2018) also reveal a number of occasions when higher levels were measured in the spring boil than the L well for corrected chlorophyll a (figure 2.12), phaeophytin (figure 2.13), and total chlorophyll a (figure 2.14) and some minor out-of-phase patterns. All but two (4/28/16 and 10/6/16) of the spring boil peaks in corrected chlorophyll a and all but one of the phaeophytin peaks (10/6/16) are associated with peaks in the L well suggesting that most if not all of the chlorophyll detected in the boil originates from the cave system but that there may at times be incomplete mixing in the Grand Canyon chamber. No L well data were collected on 4/28/16. The 10/6/16 peaks appear anomalous. However, they are associated with low color (20.8 PtCo) indicative of reduced sinking stream inflow and less dilution of the base groundwater flows that presumably carry the chlorophyll originating from sinking lakes in the springshed. If the base flow is higher density with higher concentrations of carbonates than the inflow from the sinking streams, it may have largely by-passed the Grand Canyon chamber flowing directly to the vent. Simple linear regression models for L well versus spring boil measures of corrected chlorophyll a, phaeophytin, and total chlorophyll a (see table 2.1) are all statistically significant, but their explanatory power is low reflecting both the out-of-phase peaks and the higher peaks detected at the boil. L-well concentrations of corrected chlorophyll a explain only 7% of the observed variance in those measured at the spring boil, 5% for phaeophytin, and 11% for total chlorophyll a.

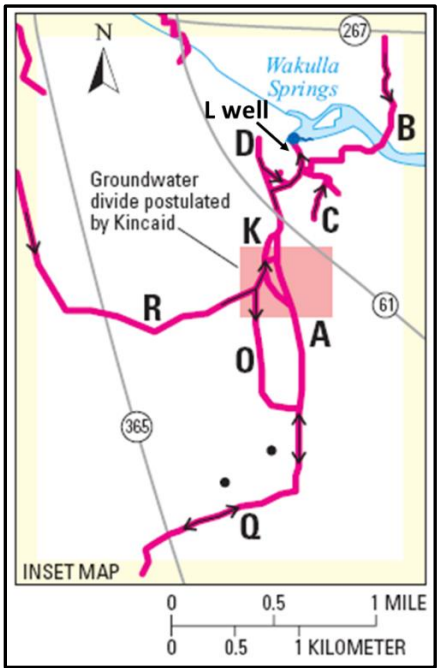

Figure 2.1. Wakulla Spring cave system detail (Davis et al., 2010).

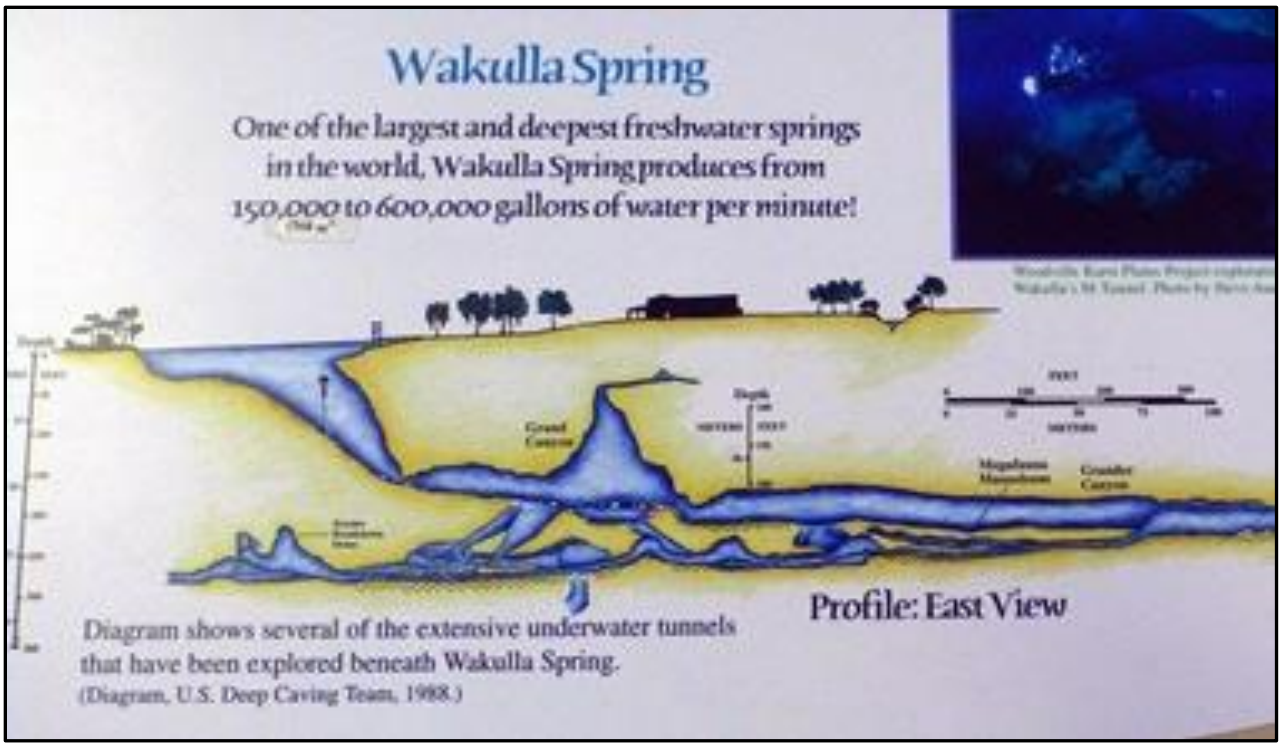

Figure 2.2. Cross section of Wakulla Spring caverns (U.S. Deep Caving Team, 1988).

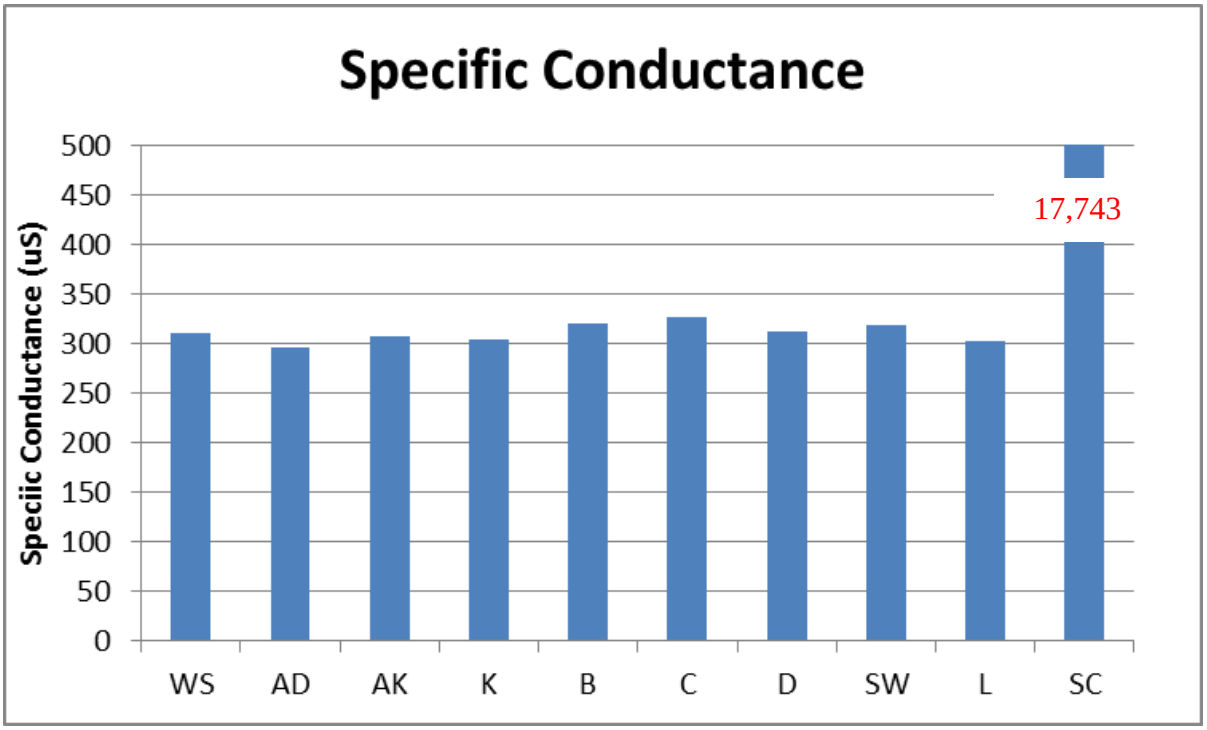

Figure 2.3. Average weekly specific conductivity in micro Siemens per meter at the Wakulla Spring boil (WS), seven cave sample wells (C, D, B, K, AK, AD, L), Sally Ward Spring (SW), and Spring Creek (SC), 8/8/16-8/10/17.

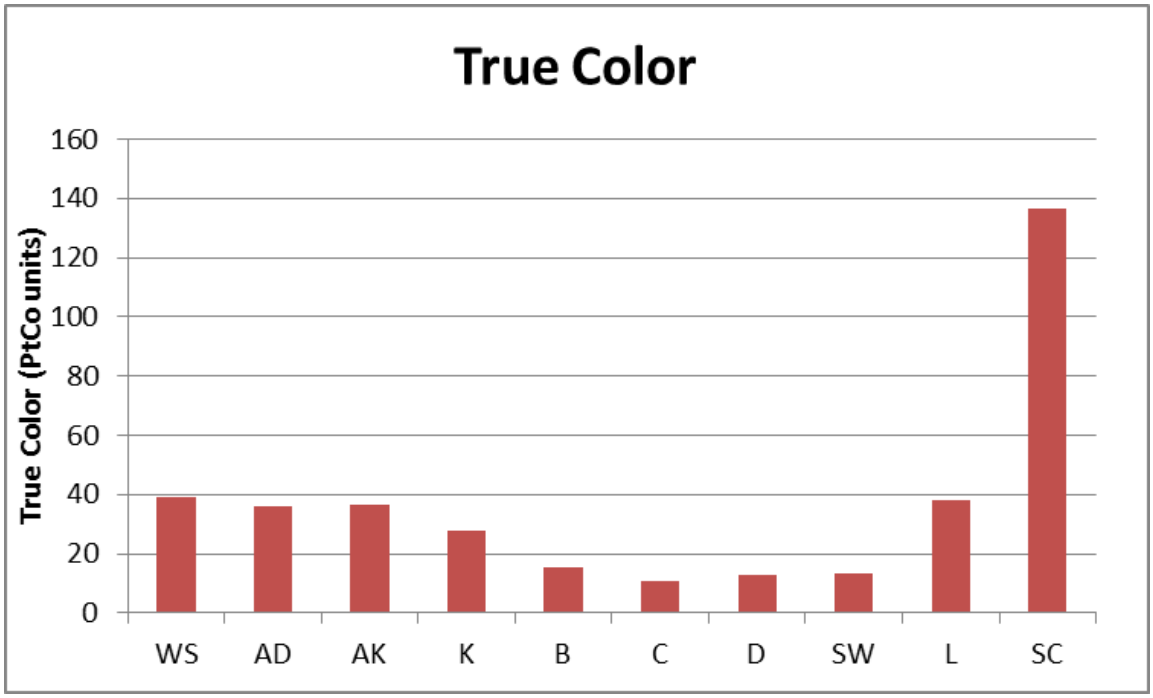

Figure 2.4. Average weekly true color in platinum cobalt units at the Wakulla Spring boil (WS), seven cave sample wells (C, D, B, K, AK, AD, L), Sally Ward Spring (SW), and Spring Creek (SC), 8/8/16-8/10/17.

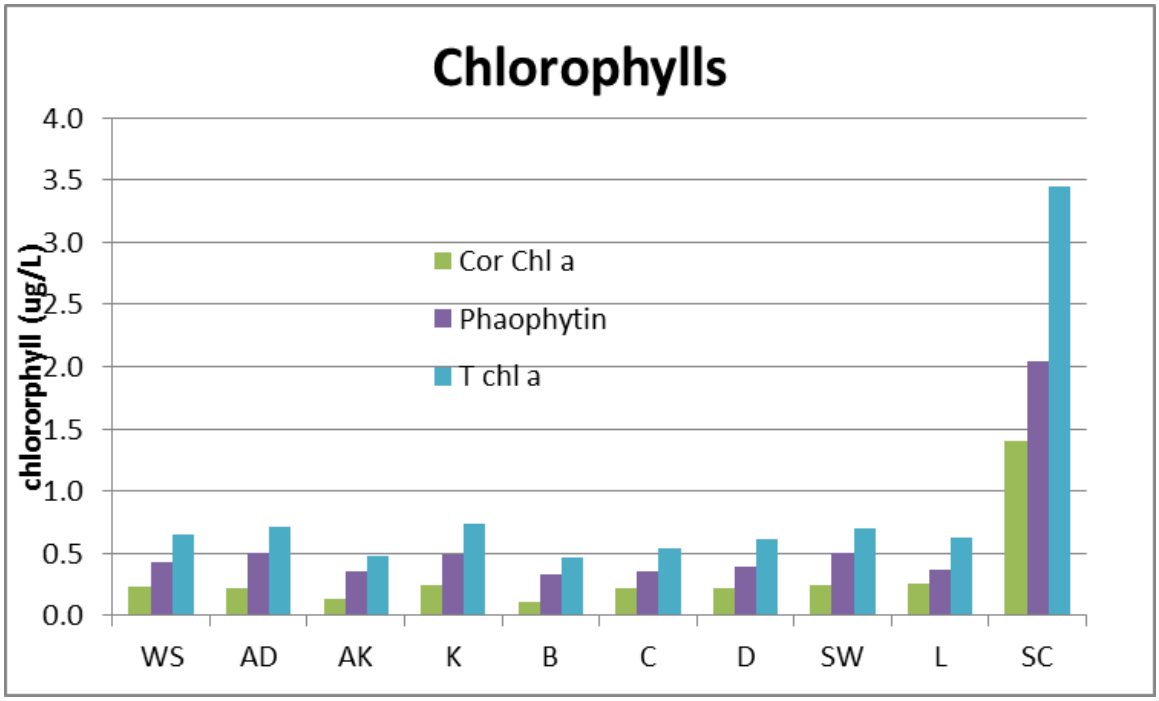

Figure 2.5. Average weekly corrected chlorophyll a, phaeophytin and their sum (total chlorophyll a) in micrograms per liter at the Wakulla Spring boil (WS), seven cave sample wells (C, D, B, K, AK, AD, L), Sally Ward Spring (SW), and Spring Creek (SC), 8/8/16-8/10/17.

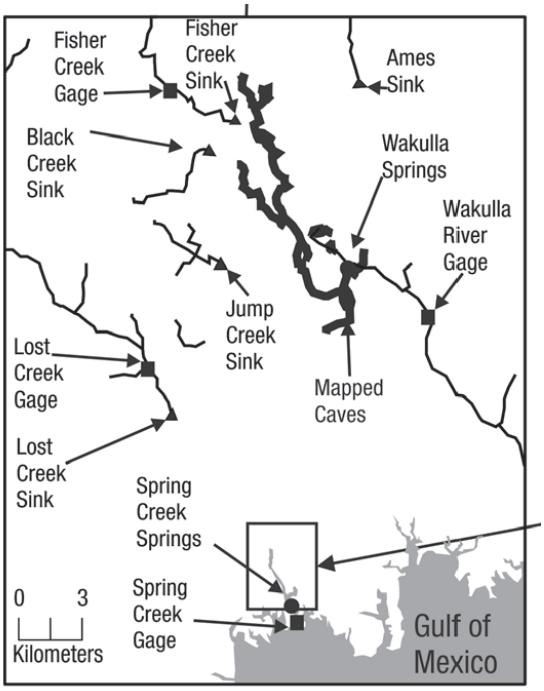

Figure 2.6. Sinking streams and major mapped caves in Wakulla Spring basin (Davis and Verdi, 2014).

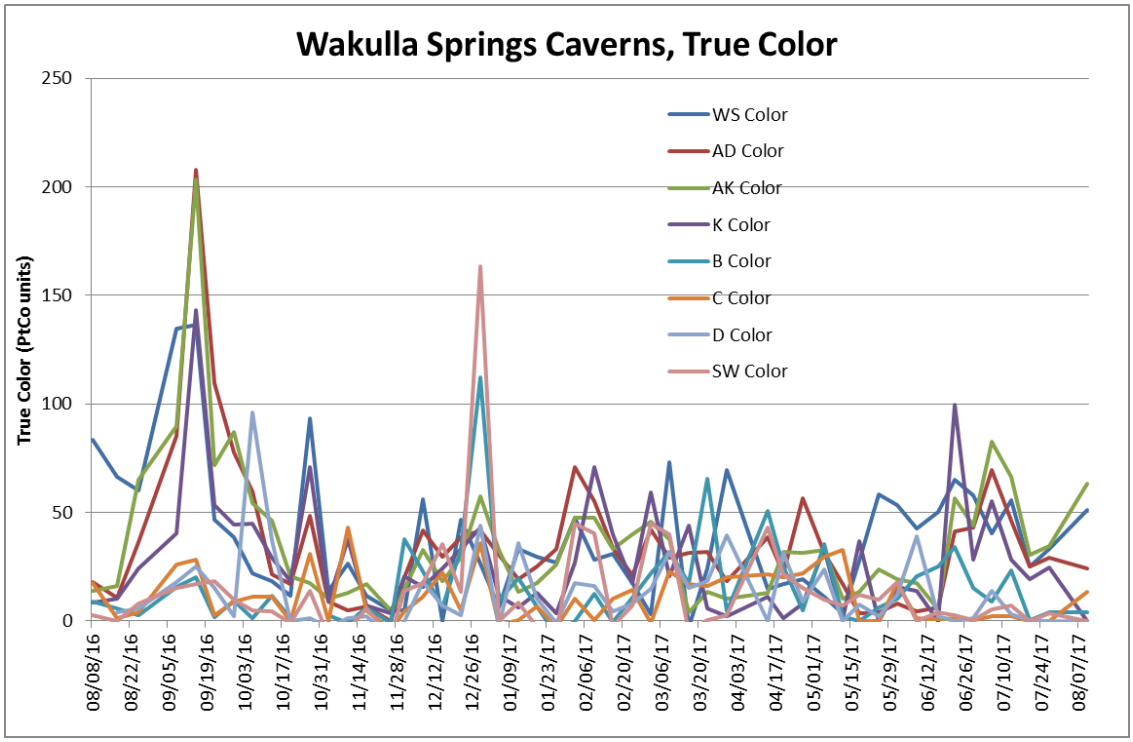

Figure 2.7. Weekly true color in platinum cobalt units at the Wakulla Spring boil (WS), six cave sample wells (C, D, B, K, AK, AD), and Sally Ward Spring (SW), 8/8/16-8/10/17.

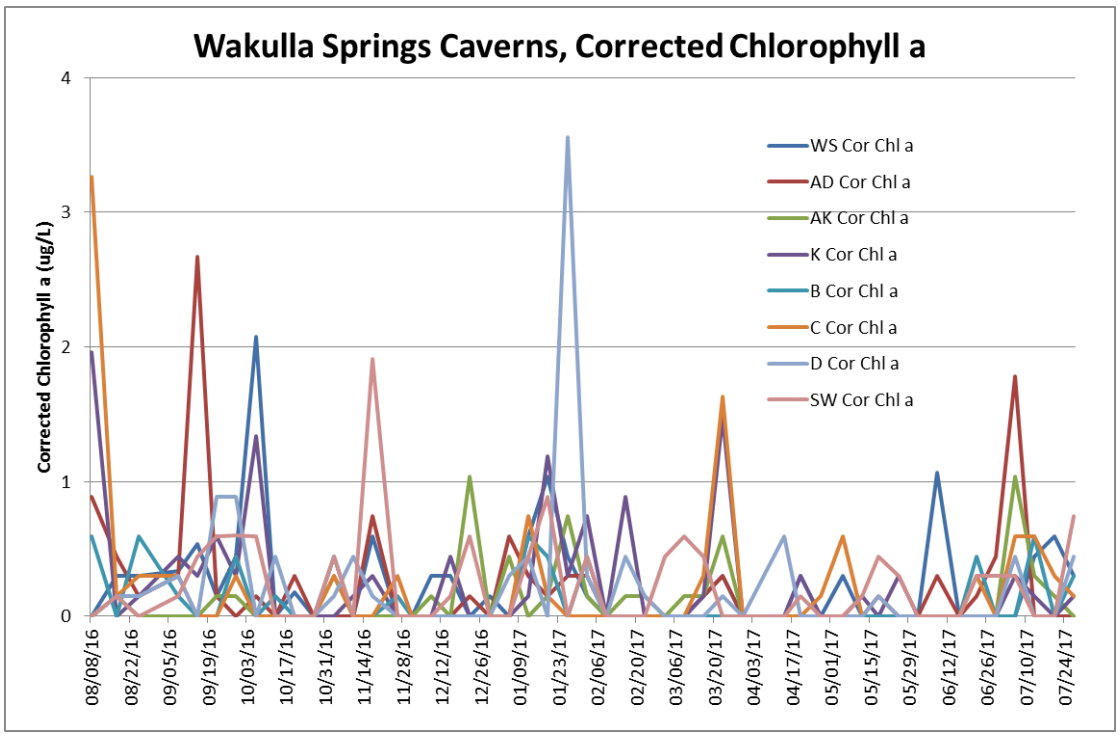

Figure 2.8. Weekly corrected chlorophyll a in micrograms per liter at the Wakulla Spring boil (WS), six cave sample wells (C, D, B, K, AK, AD), and Sally Ward Spring (SW), 8/8/16-8/10/17.

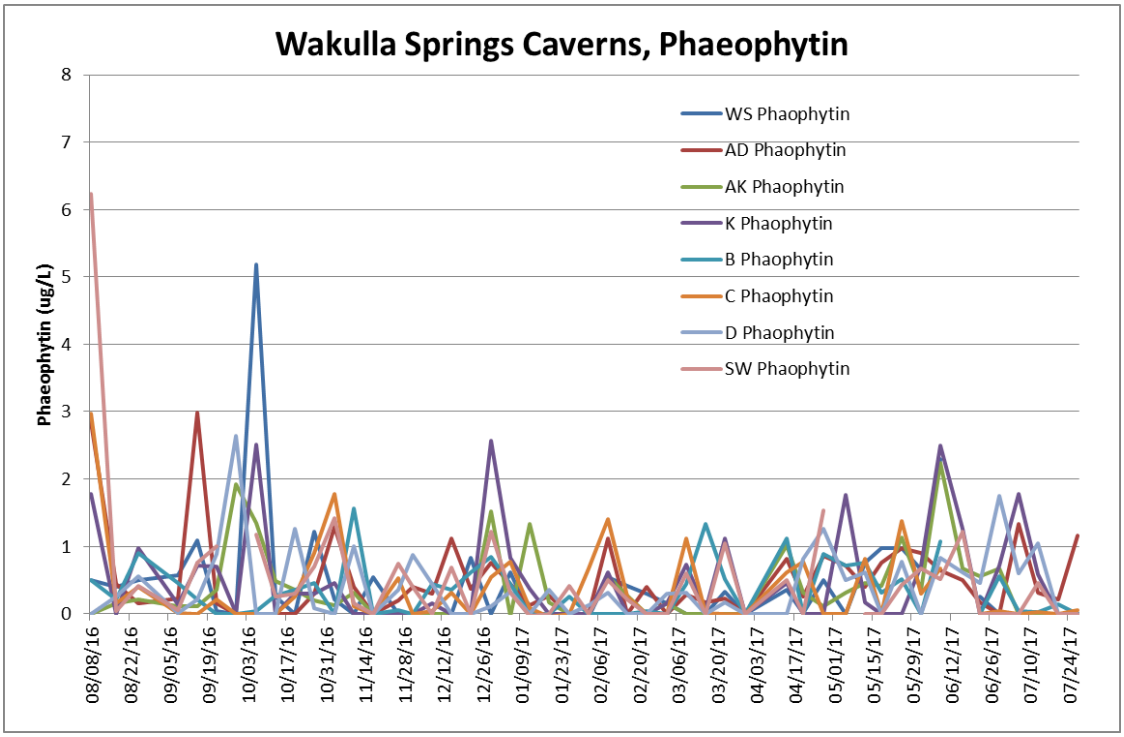

Figure 2.9. Weekly phaeophytin in micrograms per liter at the Wakulla Spring boil (WS), six cave sample wells (C, D, B, K, AK, AD), and Sally Ward Spring (SW), 8/8/16-8/10/17.

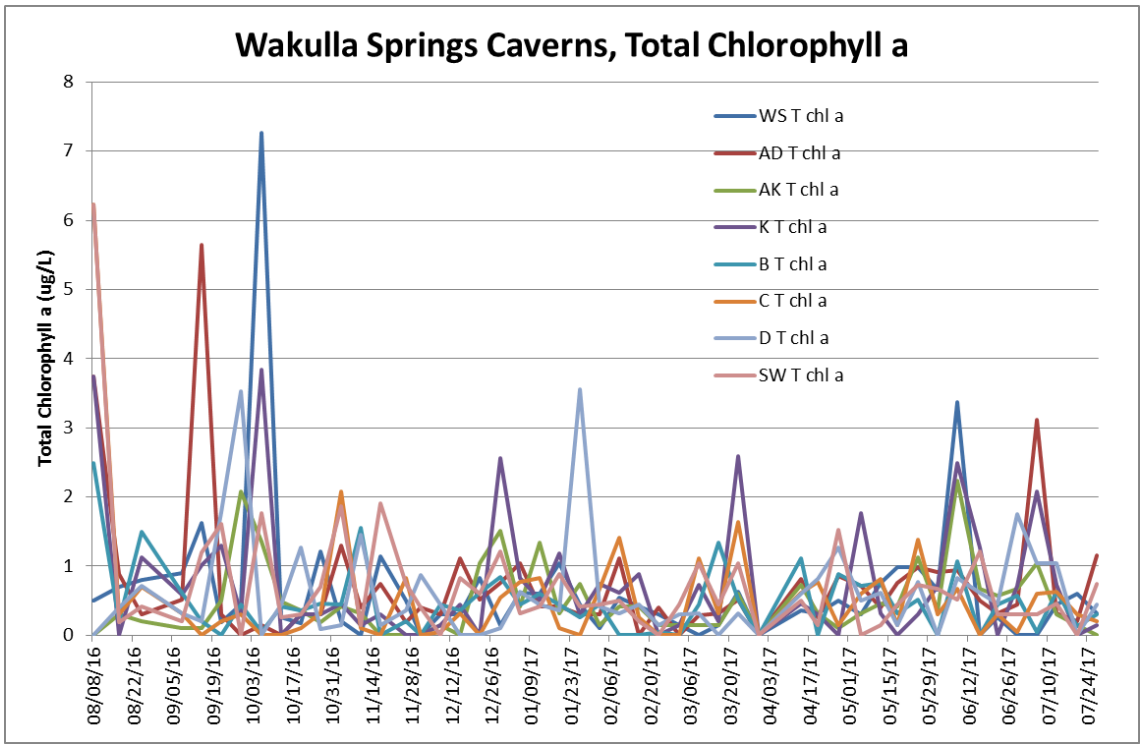

Figure 2.10. Weekly total chlorophyll a (corrected chlorophyll a + phaeophytin) in micrograms per liter at the Wakulla Spring boil (WS), six cave sample wells (C, D, B, K, AK, AD), and Sally Ward Spring (SW), 8/8/16-8/10/17.

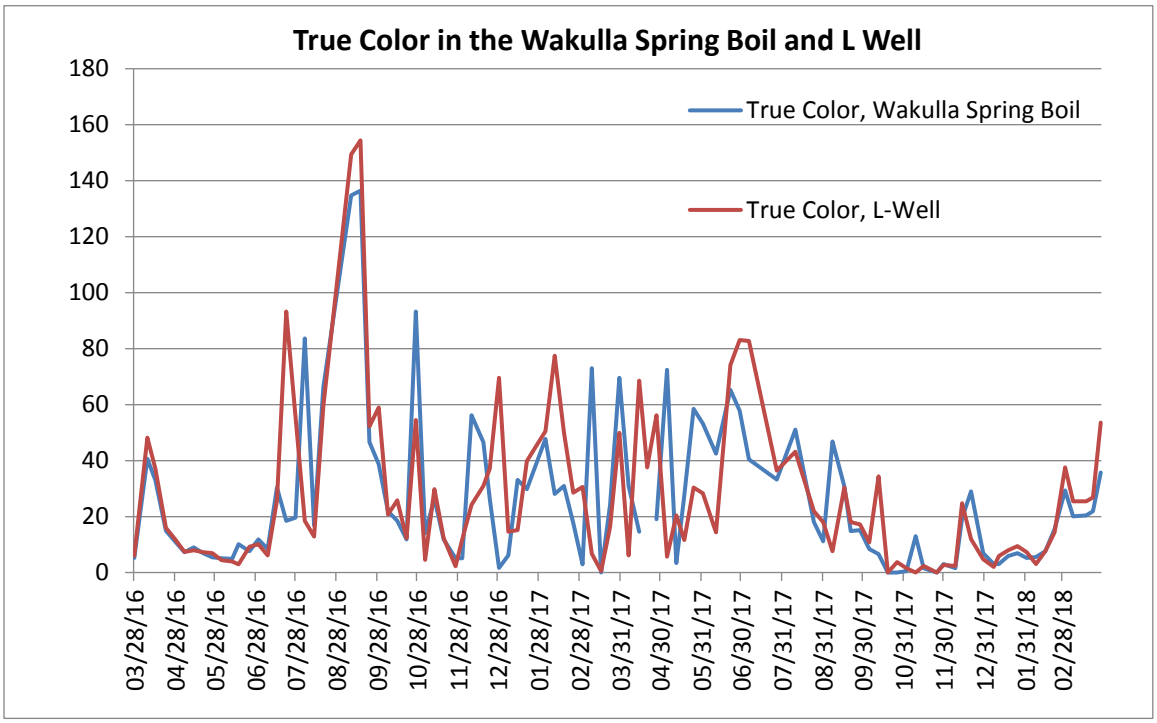

Figure 2.11. Weekly true color in platinum cobalt units at the Wakulla Spring boil (WS) and the L well, 3/28/16-3/29/18

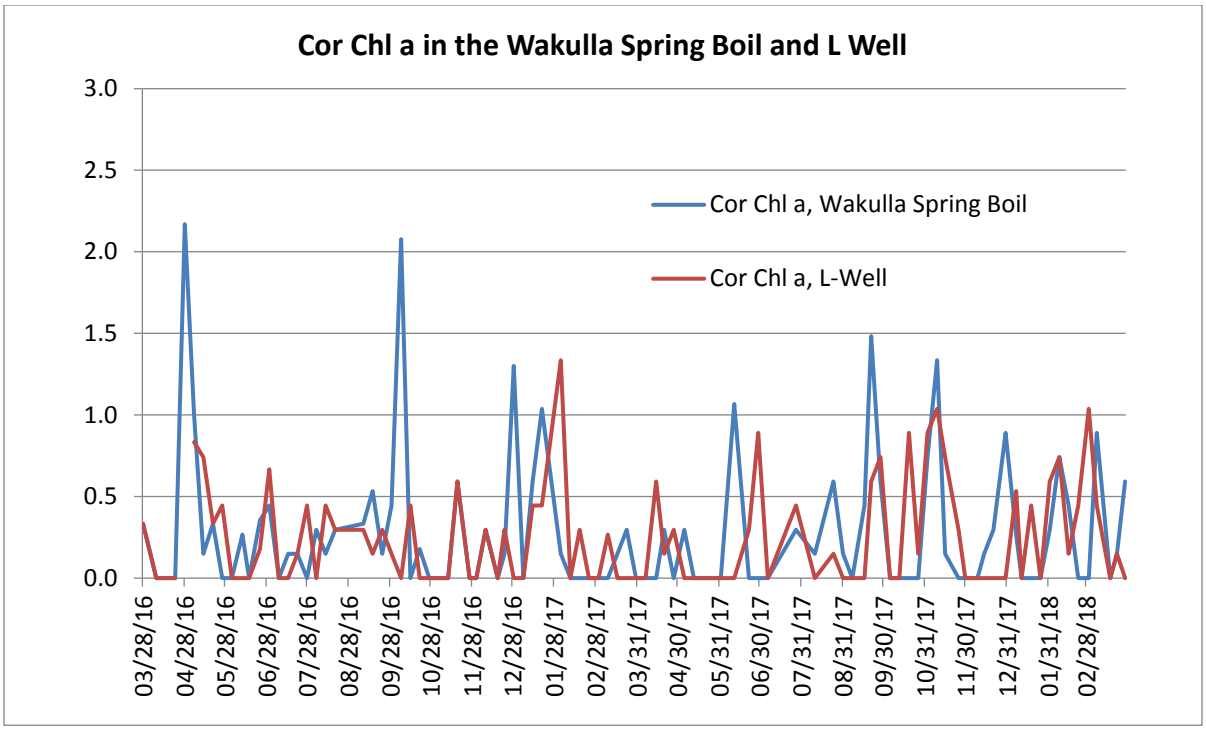

Figure 2.12. Weekly corrected chlorophyll a in micrograms per liter at the Wakulla Spring boil (WS) and the L well, 3/28/16-3/29/18.

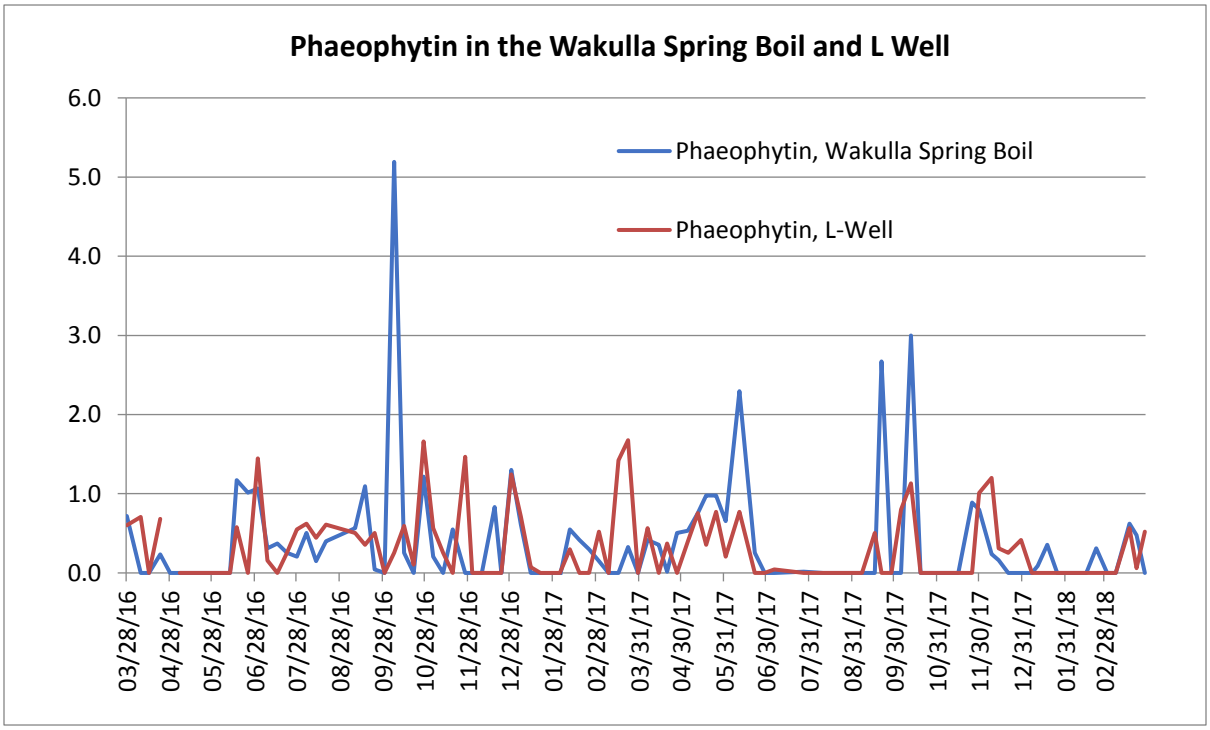

Figure 2.13. Weekly phaeophytin in micrograms per liter at the Wakulla Spring boil (WS) and the L well, 3/28/16-3/29/18.

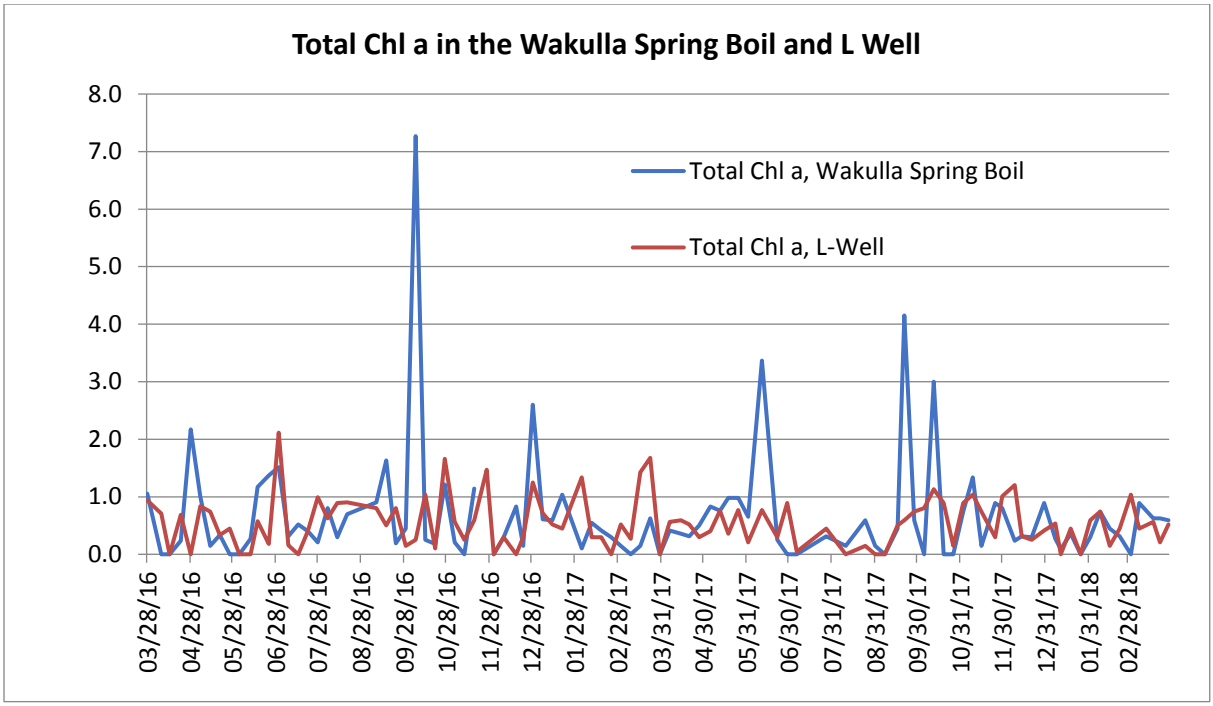

Figure 2.14. Weekly total chlorophyll a (corrected chlorophyll a + phaeophytin) in micrograms per liter at the Wakulla Spring boil (WS) and the L well, 3/28/16-3/29/18.

Table 2.1. Regression analysis of L well correlation with the Wakulla Spring boil for true color and chlorophylls

Parameter	R-squared	F-statistic significance
True color	0.453	99.9999%
Corrected chlorophyll a	0.069	99.0000%
Phaeophytin	0.053	95.0000%
Total chlorophyll a	0.107	99.9000%

3. Factors Influencing PAR Depth Variation

This chapter extends our analyses of the relationships between the depth limit of photosynthetically available radiation (PAR) and color and chlorophyll (corrected chlorophyll a and phaeophytin). Figures 3.1-3.4 present the trend lines for those relationships for the duration of the Phase I and Phase II studies.

In the Phase I study we found that while PAR depth limit was generally greatest when tannins were low, there was not a simple, direct, inverse relationship between PAR depth limit and true color. A simple linear regression model of the effect of color on PAR depth limit, while statistically significant at the 99% level, only explained 10% of the observed variance in PAR depth limit. As shown in figure 3.1, that pattern continued through the Phase II study period

which began in August 2016. A regression model for true color during the entirety of Phases I and II as depicted in figure 3.1 was more robust explaining 15% of the observed variance in PAR depth limit and statistically significant at the 99.9999% level.

Phase I revealed inconsistent relationships between PAR depth limit and chlorophyll a measured as corrected chlorophyll a, phaeophytin, and the sum of the two. Simple linear regression models for each of the three measures of chlorophyll with PAR depth limit based on the Phase I data were not statistically significant. Figure 3.2 suggests a more robust inverse relationship between corrected chlorophyll a and PAR depth limit over the duration of Phase I and Phase II. Results for phaeophytin are less compelling. Figure 3.3 exhibits several large peaks in phaeophytin concentration that are not associated with prominent declines in PAR depth limit. These are reflected in the total chlorophyll a trends shown in Figure 3.4. Nonetheless, individual simple regression models for each of three chlorophyll measures remain statistically insignificant for the extended Phase I plus Phase II data.

A multiple regression analysis of the effects of true color, corrected chlorophyll a, and phaeophytin on PAR depth limit conducted on the Phase I data did yield a statistically significant model at the 99.9 percent level that explained 39% of the observed variance in PAR depth limit. A comparable model for the extended time period was significant at the 99.9999% level but only explained 17% of the observed variance in PAR depth limit. These findings support the hypothesis that the combined effects of tannins and chlorophyll are at work but that tannins may be the primary determinant of dark water conditions. However, explaining PAR depth limit based on water quality is likely to be less precise than with direct measures of light absorbance attributable to the three factors. We are currently exploring alternative analytic approaches for doing so.

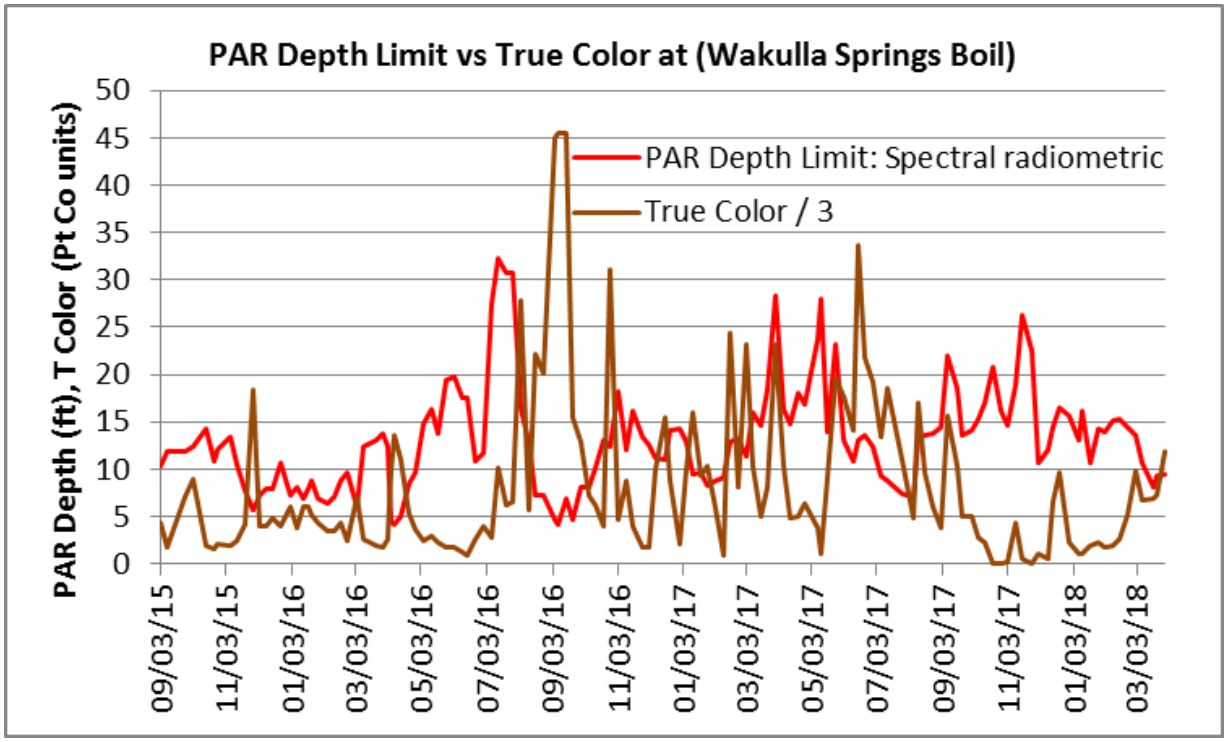

Figure 3.1. Weekly PAR depth limit and true color, 9/3/15 – 3/29/18.

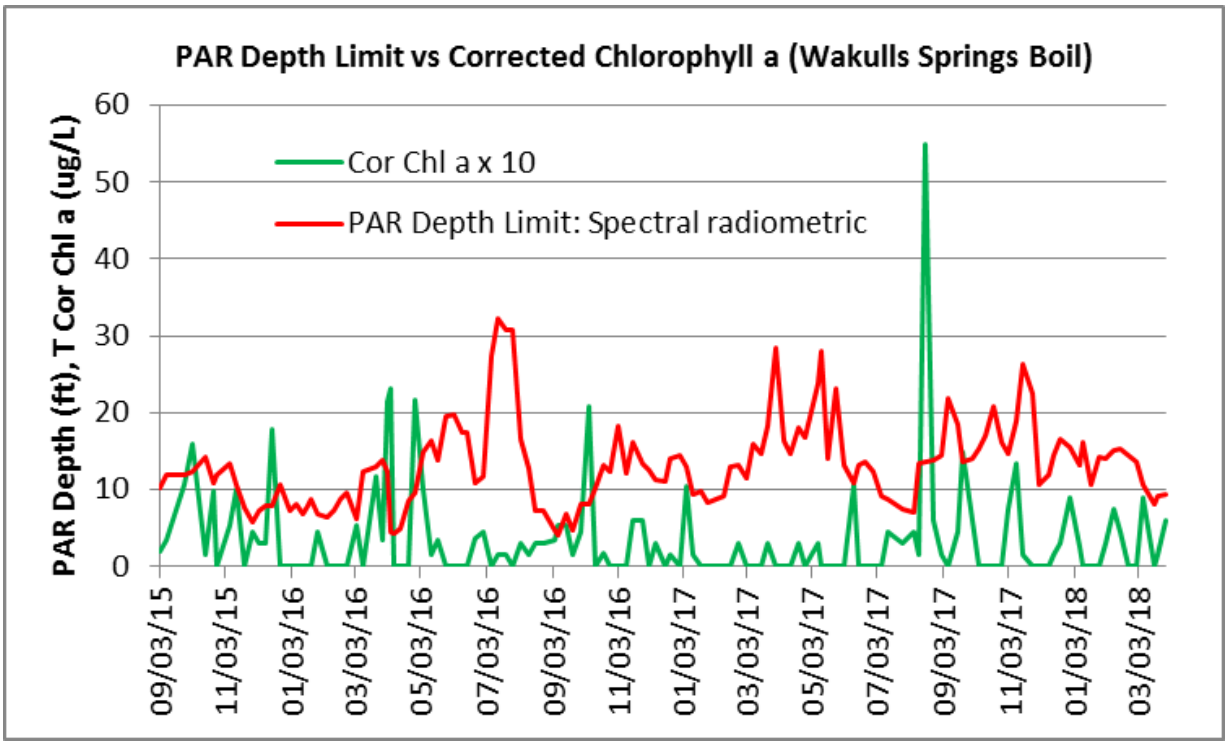

Figure 3.2. Weekly PAR depth limit and corrected chlorophyll a, 9/3/15 – 3/29/18.

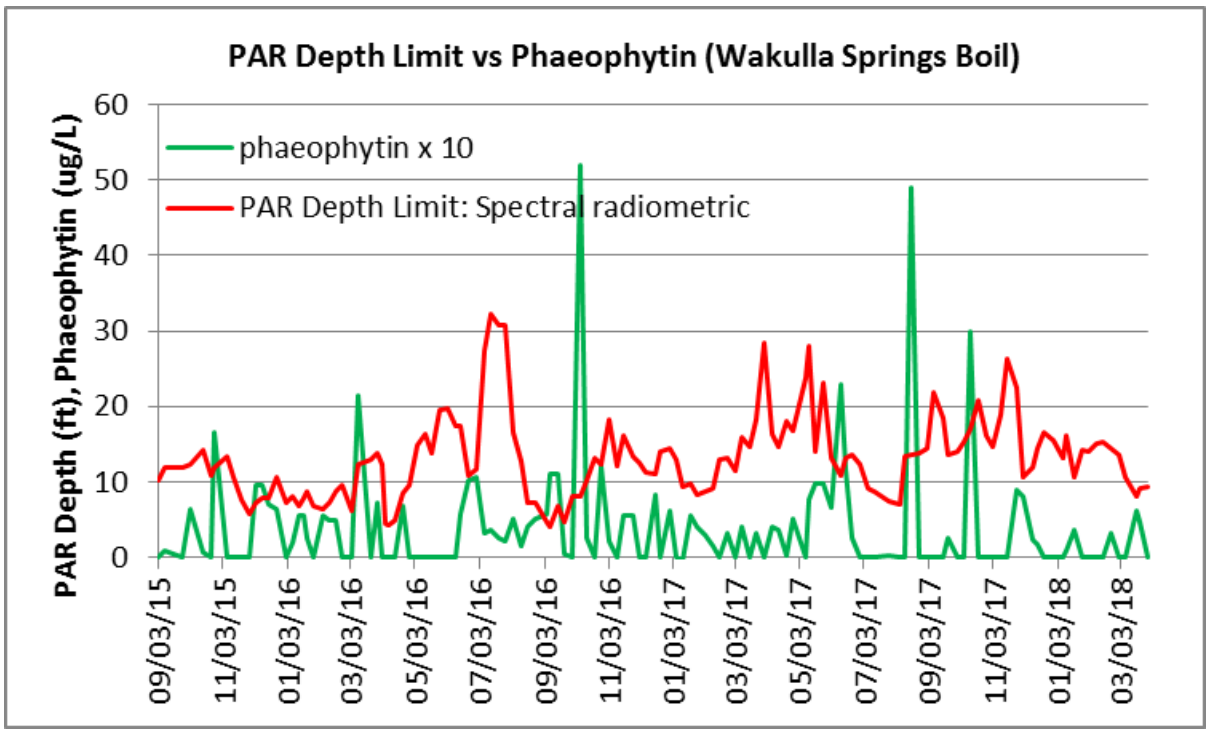

Figure 3.3. Weekly PAR depth limit and phaeophytin, 9/3/15 – 3/29/18.

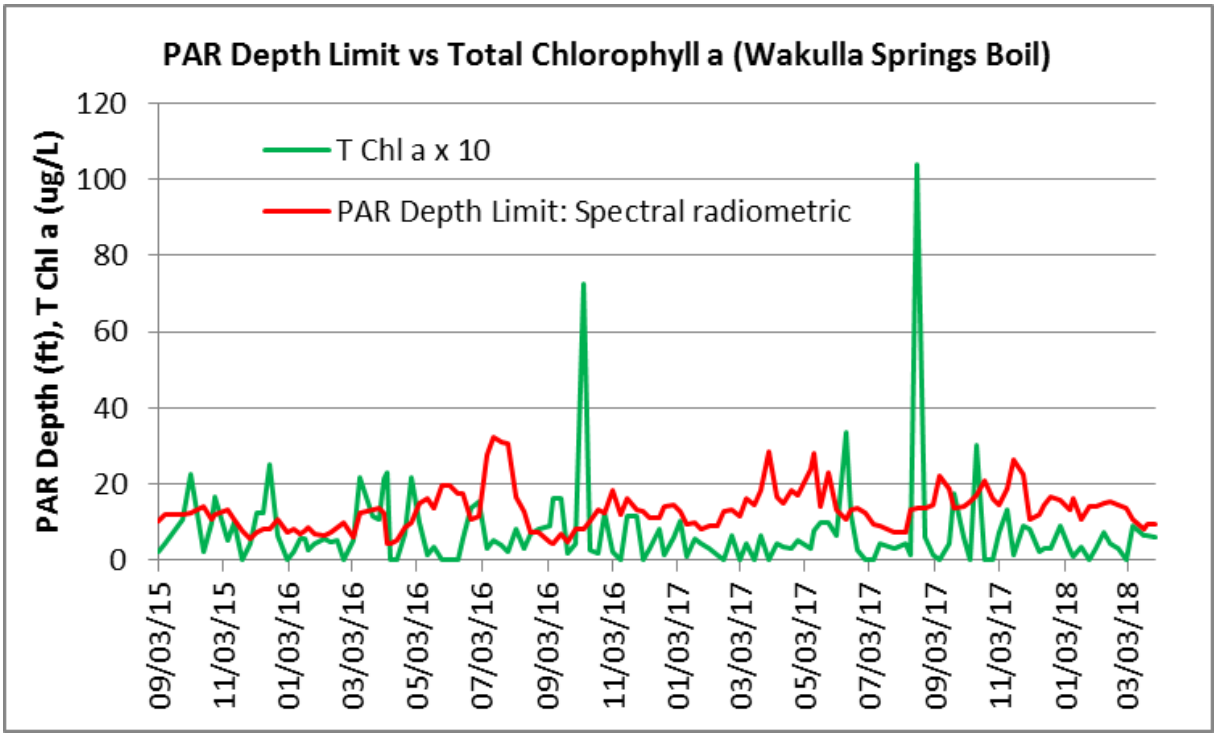

Figure 3.4. Weekly PAR depth limit and total chlorophyll a (corrected chlorophyll a + phaeophytin), 9/3/15 – 3/29/18.

4. Dye Studies of Lake Jackson and Upper Lake Lafayette

Our hypothesis that most or all of the chlorophyll and phaeophytin measured in the outflow of the Wakulla Spring boil originates from one or more karst lakes in the spring basin led us to conduct dye studies of two urban lakes in the Tallahassee area: Lake Jackson and Upper Lake Lafayette (figure 4.1). These two lakes, as well as Lake Munson, receive substantial inflows of urban storm water and experience extensive algae blooms during much of the year.

Previous dye studies completed by Hazlett-Kincaid, Inc. in 2004 and 2005 documented a hydrologic connection between Ames Sink, which receives outflow from Lake Munson via Munson Slough, and Wakulla Spring, with peak travel times of 22 to 23 days over a straight-line surface distance of 5.2 miles (Kincaid et al., 2007). Lake Munson receives untreated storm water from central and southwestern Tallahassee via Munson Slough and until 1984 received treated sewage effluent as well. The Florida Department of Environmental Protection (FDEP) has established total maximum daily loads for the lake for dissolved oxygen, nutrients (trophic state index), and turbidity (Gilbert et al., 2013). The lake has had persistent algal blooms (see figure 4.2) since 2005 (McGlynn and Deyle, 2016). The Leon County Department of Public Works (2018c) reports that the lake exceeded state thresholds for chlorophyll and nutrients several times between 2004 and 2017: corrected chlorophyll a threshold of 20.0 µg/L exceeded four times, most recently in 2015 and 2016; total nitrogen standard of 1.05-1.91 mg/L three times, most

recently in 2015; total phosphorus threshold of 0.03-0.09 mg/L every year since 2005 except 2011 when no sampling was conducted due to drawdown of the lake.

Lake Jackson receives urban storm water from Tallahassee via Meginnis Creek as well as runoff from commercial and residential development along its southern and eastern shores. FDEP (2016) has designated the lake as impaired for dissolved oxygen and nutrients. It experiences frequent algae blooms during summer months (figure 4.3). The Leon County Department of Public Works (2018a) reports that the lake exceeded state thresholds for chlorophyll and nutrients several times between 2004 and 2017: corrected chlorophyll a threshold of 6.0 µg/L exceeded five times, most recently in 2015, 2016, and 2017; total nitrogen standard of 0.51-0.93 mg/L three times, most recently in 2015 and 2017; total phosphorus threshold of 0.01-0.03 mg/L six times, most recently in 2015, 2016, and 2017. Lake Jackson contains several sinkholes and seepage areas which discharge water to the Upper Floridan Aquifer. Porter Hole Sink is the major active sink at this time (McGlynn and Deyle, 2016). We injected dye into Porter Hole Sink on September 19, 2017. It is located along the southwest shore of the lake near Faulk Landing (latitude 30.524662; longitude -84.322059), a straight-line surface distance of approximately 20 miles from Wakulla Spring (figure 4.4).

Upper Lake Lafayette (ULL) receives urban storm water from the Northeast Drainage Ditch and Lafayette Creek. In 2015 the City of Tallahassee constructed the Weems Pond storm water management facility along the Northeast Drainage Ditch. It uses alum to remove dissolved phosphorus. The U.S. Environmental Protection Agency (2012) and FDEP (Wieckowicz et al., 2003) have issued total maximum daily load standards for ULL for nutrients and dissolved oxygen. ULL also experiences regular, extensive algae blooms (figure 4.5). The Leon County Department of Public Works (2018b) reports that ULL exceeded state thresholds for chlorophyll and nutrients several times between 2004 and 2017: corrected chlorophyll a threshold of 20.0 µg/L exceeded six times, most recently in 2015 and 2017 (no data for 2016); total nitrogen standard of 1.05-1.91 mg/L once, in 2017; total phosphorus threshold of 0.03-0.09 mg/L six times, most recently in 2015 and 2017 (no data for 2016). It has a major sinkhole along its north shore, Fallschase Sink (latitude 30.455352; longitude -84.202655), a straight-line surface distance of approximately 16 miles from Wakulla Spring (see figure 4.6). We injected dye into Fallschase Sink on two occasions (January 19, 2017 and April 9, 2018).

4.1 Dye

The fluorescent dyes that have been used in the Wakulla Springs drainage basin have distinctive emission and excitation wavelengths. The fluorescent properties allow for detection at parts per billion (ppb) concentrations measured as µg/L. We chose a dye, rhodamine WT, that is never used by the Florida Geological Survey (FGS) and has only been used by McGlynn Laboratories Inc. in the Wakulla Springshed, most recently in 2004. For each of the dye studies we injected 100 lbs of approximately 20% liquid rhodamine WT dye from Abbey Color Inc.

For the Lake Jackson study, we pumped the dye from a 17-foot Boston Whaler with a 300 GPH, 50 psi 4500 RPM, 12 volt, Pacific Hydrostar utility pump. A professional diver, Andreas Hagberg, swam the tubing down about 22 feet into Porter Hole Sink (figure 4.7). A video of the injection can be viewed on YouTube at: https://www.youtube.com/watch?v=JmNWs_I9-

oU&feature=youtu.be. We followed a similar protocol for the two ULL dye studies injecting the dye directly from the shore into the Fallschase Sink (figures 4.8 and 4.9).

4.2 Sampling and Analysis

In situ sample readings were collected with Hydrolab MS5 multi-parameter water quality sondes equipped with rhodamine WT submersible fluorimeter sensors designed for either profiling or unattended monitoring (figure 4.10.A). The rhodamine WT sensor is a modified Turner Designs Cyclops-7 submersible fluorimeter (figure 4.10.B) with sensitivities as low as 0.01 µg/L (ppb) and capable of detecting concentrations as high as 1,000 µg/L (ppb). Dye concentrations were measured continuously every 10 minutes. The fluorimeters in the sondes were swapped out at weekly intervals with fully charged and calibrated devices and their data downloaded to a laptop computer.

Sensors were placed in the field about a month before the expected arrival of the dye and discrete grab samples were measured for background fluorescence weekly for another two months prior to deploying the sensors in the field. Two-liter grab water samples were collected according to Florida Department of Environmental Protection (FDEP) protocol and analyzed with the rhodamine fluorimeter. The sides of the vials were labeled with the project name, sample ID, sample date, and time with a black permanent felt-tip pen. The vials were placed in the dark and refrigerated immediately after collection and maintained under refrigeration until shipment. All grab sample background levels were below the detection limit for rhodamine WT.

For the initial ULL dye study we deployed a single sonde at the Wakulla Spring boil. We deployed additional sondes at Sally Ward Spring in the Lake Jackson and second ULL dye trace studies and a third sonde at Indian Spring for the second ULL study. We also deployed charcoal samplers (also called activated carbon or charcoal packets) at the Wakulla Spring and Sally Ward Spring boils and several other sites for the Lake Jackson and second ULL dye studies (see figure 4.11 and table 4.1).

We purchased the charcoal samplers from Ozark Underground Laboratory (OUL). They are packets of fiberglass screening about 4 inches long by 2 inches wide partially filled with 4.25 grams of activated 20-mesh size coconut charcoal (figure 4.10.C). We prewashed the charcoal packs and placed them in the field so as to be exposed to as much water as possible. We attached the packs with plastic cable or baling wires in the flow. We rinsed the collected samplers with distilled, demineralized, tracer-free reagent water to remove dirt and accumulated organic material and shook the packs to remove excess water. Next, we placed the packs in plastic Ziploc bags and labeled them using pens with black ink because colored inks may contain fluorescent dyes. The notations included station name or number and the date and time of collection.

We used charcoal packs for the first time during the Lake Jackson dye study, but we did not deploy them fully until the second ULL study. According to Ozark Underground Laboratory, Inc. (Aley and Beeman, 2015, p. 13), “[t]here is generally little or no detectable fluorescence background in or near the general range of . . . rhodamine WT dyes encountered in most groundwater tracing studies.” Nonetheless, a minimum of two charcoal packs ideally should be deployed at each sample site: one prior to the dye injection to detect background levels of dye

from other studies or other substances that may fluoresce similarly to the rhodamine dye and one placed immediately after dye injection so as to catch the pulse from the dye study.

At the laboratory, following the protocol defined by Aley and Beeman (2015), we emptied the charcoal packs into 50 ml centrifuge tubes which we then filled with an eluting solution of 5% aqua ammonia and 95% isopropyl alcohol saturated with sodium hydroxide pellets. The aqua ammonia solution is 29% ammonia. The isopropyl alcohol solution is 70% alcohol and 30% water. The sodium hydroxide is added until a super-saturated layer is visible in the bottom of the container. This eluting solution will elute fluorescein, eosine, rhodamine WT, and sulforhodamine B dyes.

After centrifuging we capped the sample beakers and allowed them to stand for 60 minutes under gentle agitation. Then the liquid was decanted off the charcoal into an appropriately labeled beaker with the laboratory identification number. Samples were kept refrigerated until analyzed with the rhodamine WT submersible fluorimeter from the Hydrolab MS5 multiparameter water quality sonde which functions reproducibly and reliably under different ambient light conditions. The fluorimeter was emersed in the eluant and read in the same manner as in the field.

Charcoal pack concentrations should be interpreted with caution because they reflect the adsorption of dye over time, and they may be exposed for different lengths of time at different stations. If the dye passes as a single pulse over one or two days, that may be less of a factor.

4.3 Quality Assurance/Quality Control

Following guidance from Aley and Beeman (2015), we run 6 standards made from one commercial standard and a second source standard from another standard source. Standard number 3 is used as a continuing calibration standard every 10 samples. The standard curve must have a coefficient of determination greater than 99.5 percent. The recoveries of all standards must be above 90%. The concentrations of the standards must also bracket the range of concentrations of the elution samples. Laboratory blanks are run before and after each sample set. Duplicates are run every 10 samples and matrix spike duplicates are run one per sample set and must have a recovery of +/- 90%. All materials used in sampling and analysis work are routinely analyzed for the presence of any compounds that might create fluorescence peaks in or near the acceptable wavelength ranges for any of the tracer dyes.

Criteria used for quantifying rhodamine WT dye in elutants from charcoal samplers:

1. There must be at least one fluorescence peak in the sample in the range of 565.2 to 571.8 nm.
2. The dye concentration associated with the rhodamine WT peak must be at least 3 times the detection limit. The detection limit in elutant samples is 0.170 μg/L, thus this dye concentration limit equals 0.510 μg/L.
3. The dye concentration must be greater than the lab blank test and at least 10 times greater than any other concentration reflective of background at the sampling station in question.[4]

[4] This is the threshold used by Ozark Underground Labs, Inc. but Aley and Beeman (2015, p. 16) do not justify it.

4. The shape of the fluorescence peak must be typical of rhodamine WT. In addition, there must be no other factors which suggest that the fluorescence peak may not be dye from the groundwater tracing work under investigation.

Criteria used to quantify rhodamine WT dye in the weekly grab water samples:

1. In most cases, the associated charcoal samplers for the station should also contain rhodamine WT dye in accordance with the criteria listed above. These criteria may be waived if no charcoal sampler exists.
2. There must be no factors which suggest that the fluorescence peak may not be rhodamine WT dye from the tracing work under investigation. The fluorescence peak should generally be in the excitation (530 nm) and emission (555 nm).
3. The dye concentration associated with the fluorescence peak must be at least three times the detection limit. Our rhodamine WT detection limit in water samples is 0.015 μg/L, thus this dye concentration limit is 0.045 μg/L.
4. The dye concentration must be at least 10 times greater than any other concentration reflective of background at the sampling station in question.

4.4 Findings

Table 4.1 summarizes the results of the three dye studies; details are presented in table 4.2. Sample sites are ordered more-or-less geographically from north to south with the exception of Chicken Branch and Horn Springs which are located east of the approximate hydrologic boundary between the Wakulla and St. Marks springsheds. We list those two karst features at the ends of the tables with Spring Creek which lies at the extreme southern limit of the combined Wakulla-Spring Creek springshed.

Red cells in table 4.1 indicate that no dye was detected at that site from either an in situ sonde fluorimeter or charcoal sampler packs. Dark green cells indicate that the presence of dye was confirmed by either a sonde fluorimeter reading in the field or from analysis of charcoal samplers for which we met the OUL threshold of the post-injection sampler having a concentration at least 10 times greater than the background sampler. Light green cells indicate sites where the post-injection sampler recorded a greater concentration than background but that concentration was less than ten times background. Yellow cells indicate that charcoal samplers yielded elutant that fluoresced at the rhodamine WT wavelengths, but there was either no background sampler deployed at that site, or the post-injection sampler concentration was less than or equal to that of the background sampler. Orange cells indicate situations where charcoal samplers indicated no dye was present but some element of the sampling protocol makes that conclusion tentative.

As shown in table 4.2, the sonde fluorimeters placed at Wakulla Spring first detected dye after 35 days from both the initial ULL injection and the Lake Jackson injection. The sonde fluorimeter placed at the Sally Ward Spring for the Lake Jackson dye study first detected dye after 31 days. A single peak of 0.24 μg/L was recorded at Wakulla Spring for the first ULL study (figure 4.12). The Lake Jackson dye study also produced a single peak of 6.31 μg/L at Wakulla Spring (figures

4.13 and 4.14) spread over a few hours, while it yielded multiple "hits" at Sally Ward Spring spread over two days (figures 4.15 and 4.16) with a maximum concentration of 3.25 μg/L.

In the second ULL dye study, the Wakulla sonde fluorimeter recorded multiple dye peaks: an initial pulse (0.59 μg/L) appeared on 4/16/18, only 7 days after injection and peaked at about 5.0 μg/L after 11 days on 4/20/18 (figure 4.17). A second larger pulse that peaked at 123.88 μg/L followed on 5/9/18 after 30 days followed by a cluster of intermediate level spikes on 5/28/18-5/31/18. The Sally Ward Spring sonde fluorimeter also recorded an early pulse of 21.64 μg/L on 4/20/18 followed by sporadic much lower hits throughout the rest of April (figure 4.18).

The rapid transit of the first pulse was likely the result of an unusual situation. On April 11, 2018, two days after dye injection at Fallschase Sink, a sinkhole opened in nearby Buck Lake (figure 4.19) and drained about 48 acre-feet of water into the aquifer. Dye that had been injected into the aquifer was pushed back into ULL the next day raising its water level about three feet (figure 4.20). The lake subsequently cleared 12 days later (4/23/18). We hypothesize that the Buck Lake discharge may have quickly pushed a small amount of the dye south toward Wakulla Spring at the same time that it pushed most of the dye back into ULL. The travel time for the second pulse recorded at Wakulla Spring after 30 days is more nearly similar to the 35-day period for the earlier dye study in January 2017.

During the Lake Jackson study we mostly deployed single charcoal packs. As a result we cannot draw definitive conclusions about the presence of dye from the injection as it is possible that other substances that fluoresce similarly to rhodamine WT may have been adsorbed by the charcoal. While this is very unlikely (Ales and Beeman, 2015), we have treated those findings as less certain than those from sites where we had concentrations in the post-injection elutant at least 10 times greater than from the background sampler. During the second ULL study we were able to deploy charcoal packs to measure background conditions at most sample sites. However, we did not anticipate the first pulse arriving so quickly, so some of the background packs may have picked up dye from that initial pulse.

As shown in table 4.1, charcoal packs placed at the Wakulla Spring and Sally Ward Spring boils during the Lake Jackson dye study confirmed the presence of dye detected by the sonde fluorimeters placed at those locations. The single charcoal pack deployed at Wakulla Spring was retrieved three days after the peak detected by the sonde. In the absence of a background measurement, we cannot be absolutely certain that all of what fluoresced in the elutant was dye. However, the sonde data, as well as the background analysis of grab samples prior to injecting the dye, demonstrated that no dye was present prior to deployment of the charcoal pack.

The concentration measured in the elutant from the post-injection sampler at Sally Ward Spring was only 1.3 times greater than that from the background sampler (30.3 versus 23.0 μg/L). However, both charcoal packs deployed at Sally Ward Spring during the Lake Jackson study were exposed during or after the initial peak detected by the sonde placed there, so the dye measurement from the first pack as well as the second probably reflect the arrival of the dye. The sonde was deployed on the same date as the first charcoal pack (10/4/17) thus demonstrating that no dye was present at that time.

We only placed single charcoal packs at the following sinks and springs during the Lake Jackson study: Freedom Sink, Spiral Garden Sink, Whiskey Sink, McBride Slough, and No Name Spring. We placed the charcoal packs at Freedom and Spiral Garden sinks on October 24, 2017, the same day dye was first detected at Wakulla Spring and four days after Sally Ward. These sinks are located well north of Sally Ward and Wakulla (see figure 4.11). Due to the timing and the absence of background samples we cannot be certain that dye was adsorbed by these packs.

We set out packs at Whiskey, Meetinghouse, and McBride before the dye was first detected at Sally Ward Spring or Wakulla Spring, so the fluorescence measured from these sites may have been from the dye injection. However, in the absence of background samples we cannot be certain. Whiskey and Meetinghouse are situated north of Sally Ward and Wakulla. McBride Slough is east-southeast of Wakulla Spring.

We did deploy a background charcoal pack at Emerald Spring during the Lake Jackson study and retrieved it five days later. The calculated dye concentration was slightly higher for the background pack than the second pack (5.9 versus 4.2 μg/L) despite the fact that the second pack was in place for 27 days. Emerald Sink is located northwest of Sally Ward and Wakulla, and the first pack was deployed 21 days after dye injection, so it is possible that the dye detected by both charcoal packs reflects dye that arrived at the spring prior to its arrival at Sally Ward or Wakulla. However, it also is possible that both measurements reflect other substances the fluoresce similarly to the rhodamine WT dye.

Results for the two charcoal packs deployed at Indian Spring during the Lake Jackson study indicate that dye may have traveled there from Lake Jackson. The concentration of the second charcoal sampler (in situ for 28 days from 10/15 – 11/12/17) of 20.8 μg/L is substantially greater than the 4.9 μg/L measured from the first pack during the background period of 5 days (10/10 – 10/15/17). The difference of 15.9 μg/L does not, however, meet OUL's 10 times background threshold for concluding that the dye detected exceeded background.

We deployed background charcoal packs at Harvey's Clear Lake and Harvey's Aphasta Pit located well to the south of Wakulla Spring on October 9 and retrieved them on October 16, four days before dye was detected by the sonde fluorimeter at Sally Ward Spring and eight days before dye was detected at Wakulla Spring. At Harvey's Clear Lake the calculated dye concentration was less than background while at Harvey's Aphasta, it was slightly higher. These findings indicate that it is very likely that all the measured fluorescence was from substances other than the dye we injected for this study.

We set out a single charcoal pack at Spring Creek which discharges into the Gulf of Mexico 11 miles south of Wakulla Spring. While we calculated a concentration of 6.5 μg/L, in the absence of a background sample we cannot ascribe that to the dye we injected with certainty.

As indicated in table 4.1, we deployed background charcoal packs at most sample sites for the second ULL dye study in January and February 2018, well before the April 9 injection date. However, the Buck Lake draining event posed an unforeseen complication: we retrieved some of our background samplers after the initial pulse had arrived, unbeknownst to us, at Wakulla Spring and Sally Ward Spring.

The Sally Ward charcoal sampler results are consistent with the sonde fluorimeter finding that dye injected into ULL did travel to that location. The charcoal sampler results for Wakulla Spring, however, indicate that rhodamine dye or other substances that fluoresce similarly to rhodamine may have been adsorbed to the background charcoal sampler prior to the arrival of dye from this study. The background sampler was retrieved on 4/12/18, the same date that we deployed the sonde and three days after we injected the dye at ULL. While the background charcoal sampler yielded an estimated dye concentration of 48.6 μg/L adsorbed over 75 days between 1/28/18 and 4/12/18, the sonde fluorimeter did not detect dye until 4/16/18. Tests of weekly grab samples from the spring prior to this date did not indicate that any dye was present. The second charcoal sampler yielded an estimated concentration of 83.2 μg/L over 49 days, well below the OUL threshold of 10 times background for concluding that the injected dye was responsible for the observed fluorescence.

As shown in table 4.1, charcoal sampler results indicate that none of the dye injected at ULL travelled to Church Sink, Freedom Sink, Gasoline Sink, Emerald Sink, Indian Springs, Weigers Sink, Harley Davidson Sink, Rhodes Spring, or Spring Creek. However, the charcoal sampler data did indicate that dye from ULL travelled to the following sites: Spiral Garden Sink, Northside Sink, McBride Slough, No Name Spring, and Revell Sinks North and South.

In the case of Meetinghouse Sink, which lies north of Wakulla and Sally Ward Springs, the background sampler was retrieved after the first dye pulses were detected at Wakulla and Sally Ward. Thus, the nearly identical measurements for the two samplers either both reflect the presence of dye or comparable levels of background substances that fluoresce similarly to rhodamine WT. At Stafford Sink, which is located west-northwest of Wakulla Spring, the background sampler was retrieved on 4/18/18, after the initial pulse was detected at Wakulla on 4/16/18. In this case, however, the calculated concentration of the second sampler (48.7 μg/L over 48 days) was lower than that of the background sampler (75.9 μg/L over 65 days) despite being in place when the larger pulse was detected at Wakulla on 5/9/18. The relatively high concentrations suggest the presence of dye in both samplers rather than just other fluorescing substances, but the lower concentration calculated for the second sampler is puzzling.

We deployed charcoal packs at Chicken Branch Spring and Horn Spring in eastern Leon County to ascertain whether or not discharges from ULL into the Upper Floridan Aquifer cross the presumed hydrologic boundary between the Wakulla Spring and St. Marks River basins. Our findings are inconclusive. While both Chicken Branch samplers yielded no dye, the second sampler which was removed on 4/26/18, may have been retrieved too early since the larger pulse was recorded at Wakulla Spring on 5/9/18. We did record apparent dye with a concentration of 14.0 μg/L for the second charcoal sampler deployed at Horn Spring. However, the background sampler was not recovered, so we cannot ascertain whether what fluoresced was dye or some other substances.

In sum, the sonde fluorimeters documented hydrogeologic connections between Lake Jackson and ULL with both Sally Ward Spring and Wakulla Spring. Charcoal pack samplers documented a connection between Lake Jackson and Indian Spring, but a sonde fluorimeter deployed at Indian Spring during the second ULL study found no evidence of a connection.

Charcoal samplers without background controls provided evidence that dye from Lake Jackson may have flowed to several of the sinks located north and west northwest of Wakulla Spring: Freedom, Spiral Garden, Emerald, Whiskey, and Meetinghouse. Of these, only Spiral Garden and Meetinghouse also recorded possible dye from the second ULL study. We did not test Stafford Sink, which is situated west of Indian Spring, for the Lake Jackson study. Charcoal sampler results from the second ULL study indicate that dye may have travelled there from ULL.

Charcoal samplers indicate that dye which appeared at Wakulla and Sally Ward also most likely arrived at Northside Spring, McBride Slough, and No Name spring from both Lake Jackson and Upper Lake Lafayette. These karst features are located close to Wakulla. We also detected dye from the second ULL study at the Revell Sinks which are located south-southwest of Wakulla Spring and from the Lake Jackson study at the Harvey sinks further south in the combined Wakulla-Spring Creek springshed. A single charcoal sampler deployed at Spring Creek during the Lake Jackson study indicated that dye may have reached that location at the terminus of the combined Wakulla Spring-Spring Creek springsheds. A single charcoal sampler retrieved from Horn Spring also indicates that dye may have traveled east from ULL into the St. Marks basin during the second ULL study.

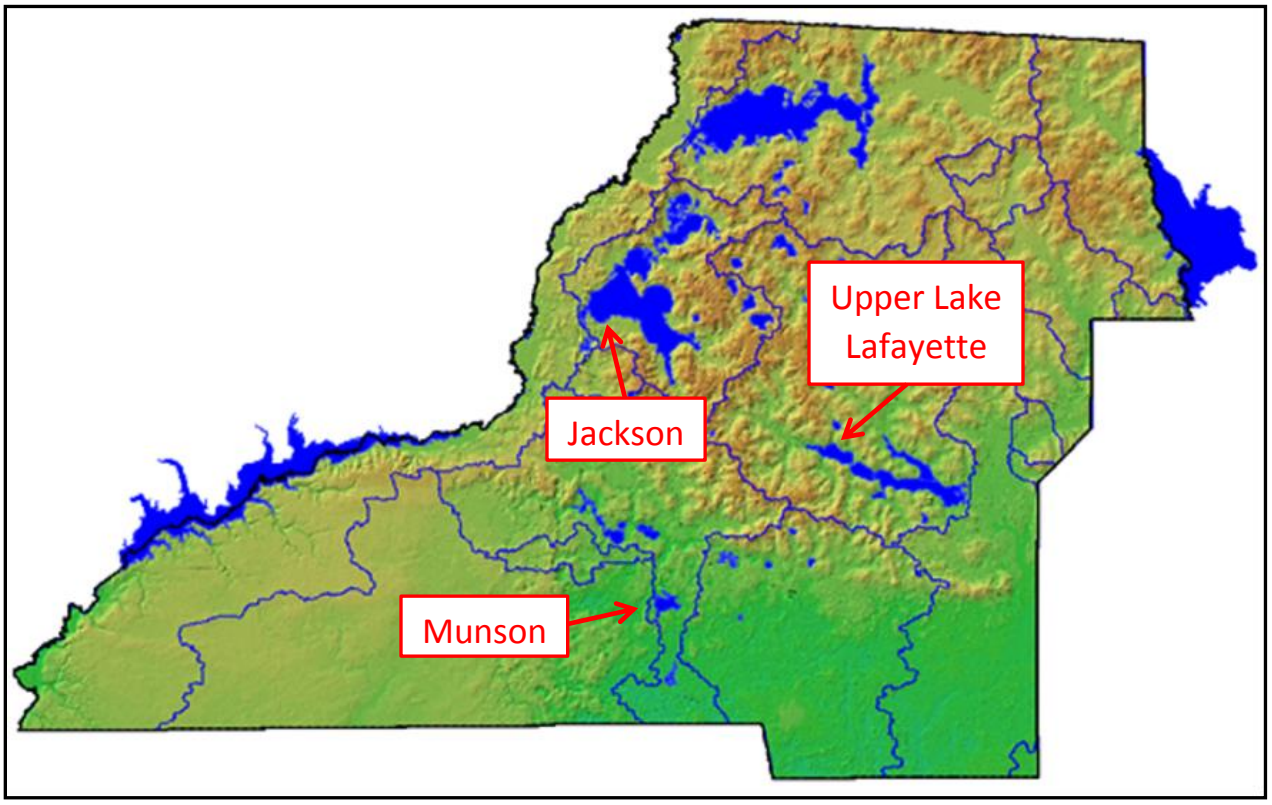

Figure 4.1. Lake location map.

Figure 4.2. Lake Munson algae bloom, April 2003 (Sean McGlynn).

Figure 4.3. Lake Jackson algae bloom, March 2011 (Sean McGlynn).

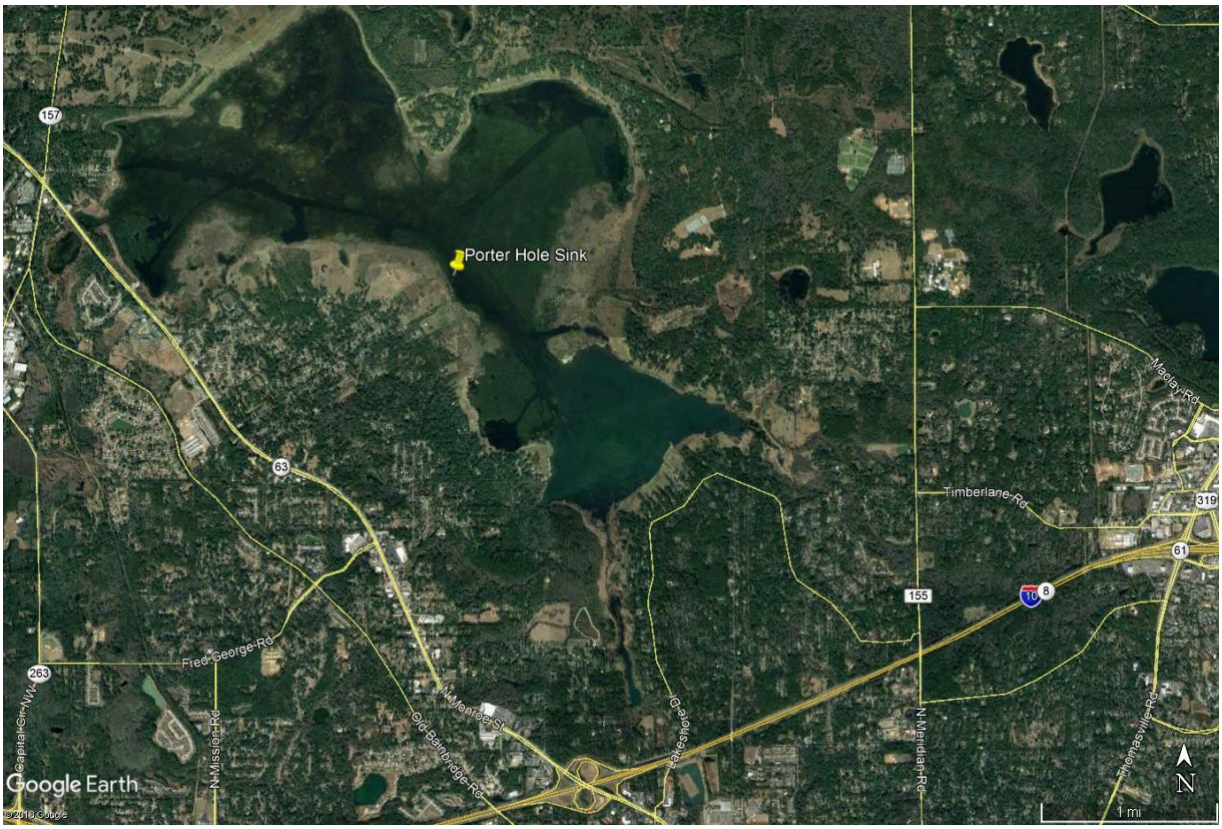

Figure 4.4. Porter Hole Sink, Lake Jackson.


Figure 4.5. Upper Lake Lafayette algae bloom and fish kill, November 2005 (Sean McGlynn).

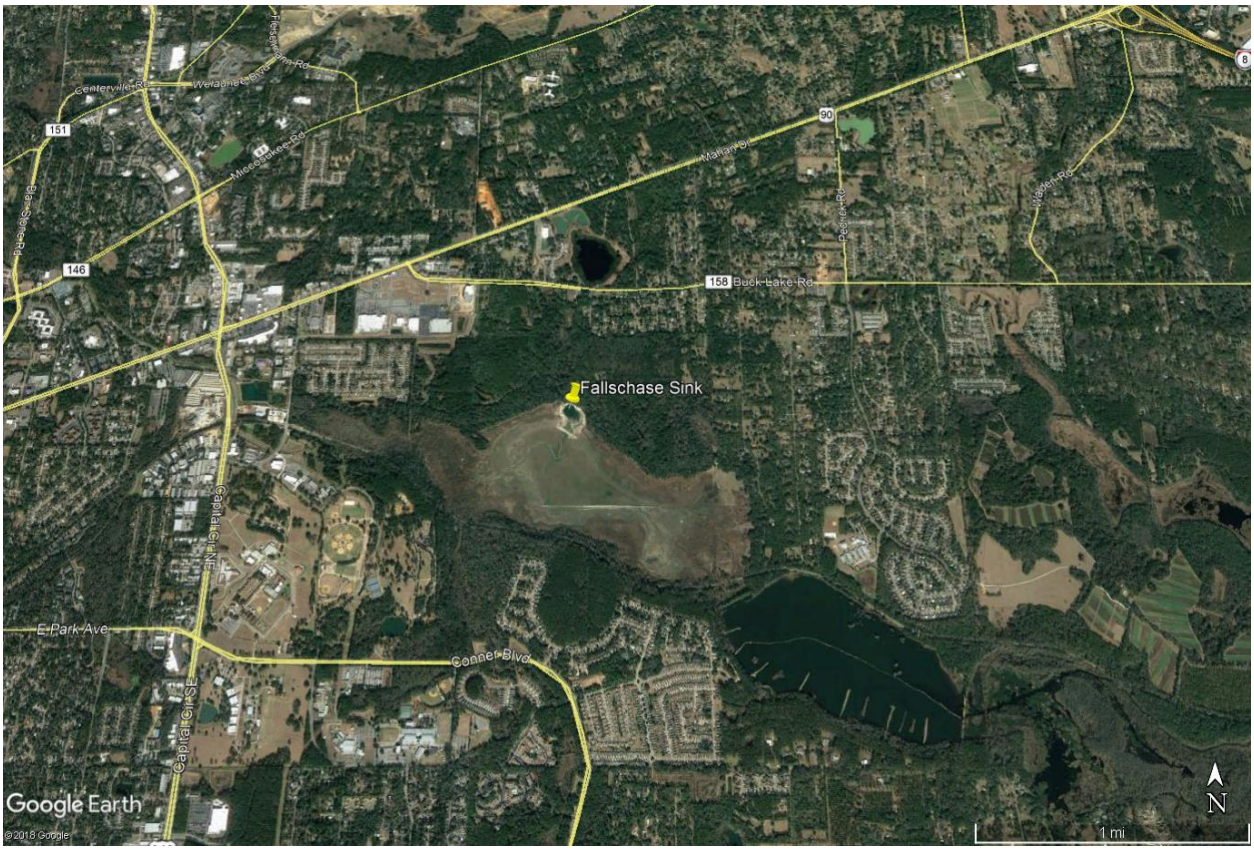

Figure 4.6. Fallschase Sink, Upper Lake Lafayette.

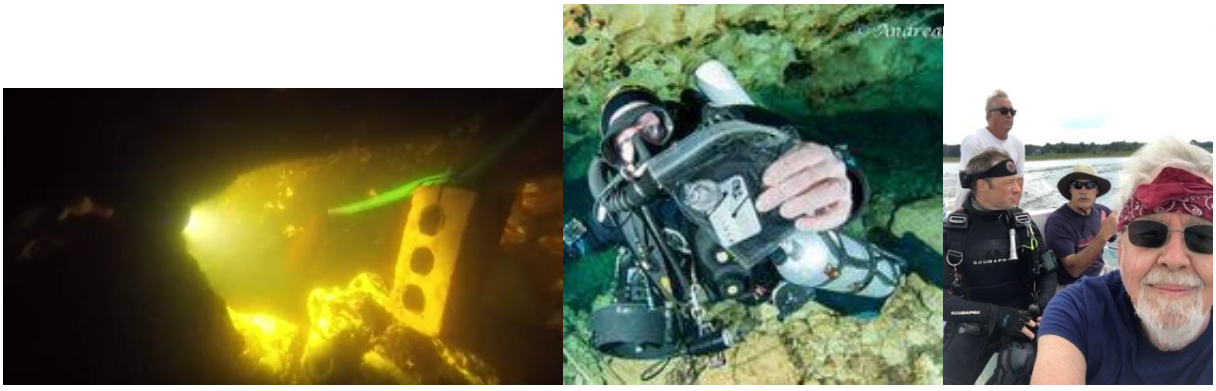
Figure 4.7. Lake Jackson dye injection, 09/19/17.

Figure 4.8. Upper Lake Lafayette dye injection, 01/19/17.

Figure 4.9. Upper Lake Lafayette second dye injection, 4/9/18 (Andreas Hagberg).

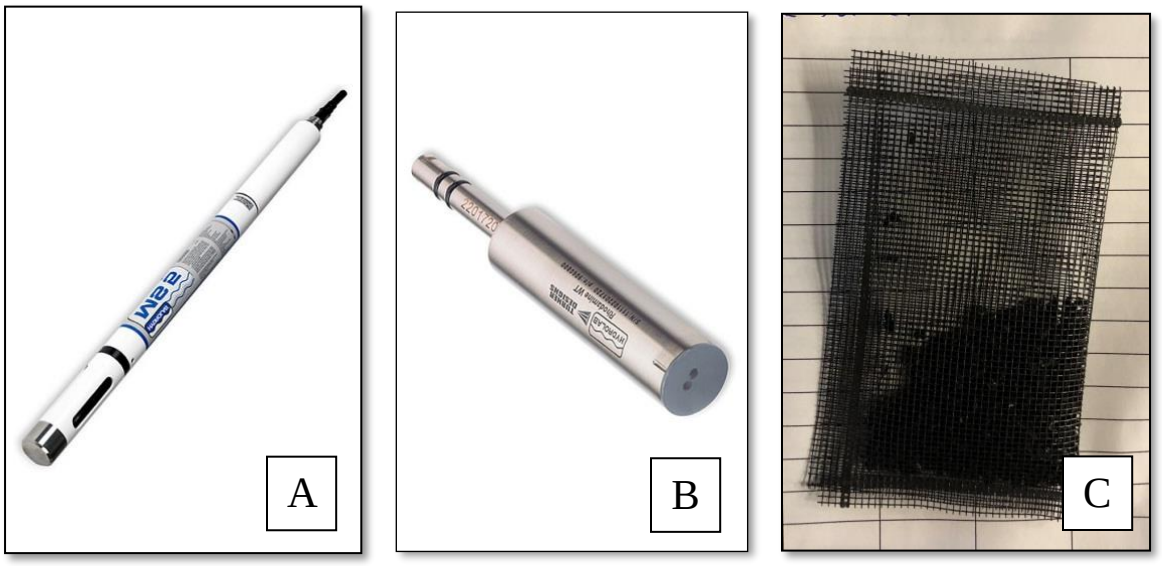

Figure 4.10. Dye sampling devices: A) Hydrolab MS5 multiparameter water quality sonde; B) rhodamine WT modified Turner Designs Cyclops-7 submersible fluorimeter; C) Ozark Underground Laboratory charcoial pack.

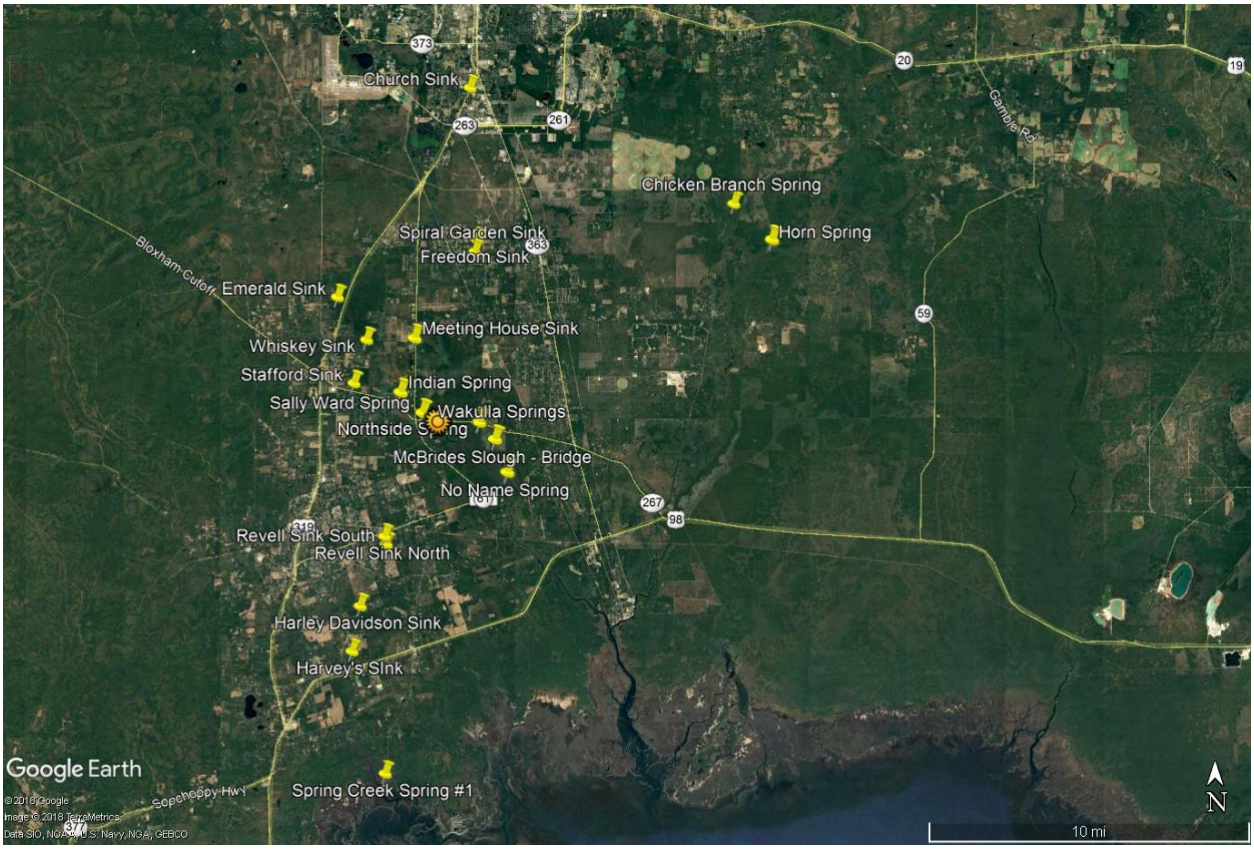

Figure 4.11. Charcoal pack sampling sites.

Table 4.1. Lake Jackson and Upper Lake Lafayette dye trace study summary.

	Lake Jackson	Upper Lake Lafayette 1	Upper Lake Lafayette 2
Church Sink – charcoal packs			N
Freedom Sink – charcoals	nb		N
Spiral Garden Sink – charcoal packs	nb		Y
Gasoline Sink – charcoal packs			N
Emerald Sink – charcoal packs	#2<#1		N
Whiskey Sink – charcoal pack	nb		
Meeting House Sink – charcoal packs	nb		#1~#2
Stafford Sink – charcoal packs			#2<#1
Indian Spring sonde			N
Indian Spring - charcoal packs	Y		N
Sally Ward Spring - sonde	Y		Y
Sally Ward Spring - charcoal packs	<10xb		Y
Wakulla Spring - sonde	Y	Y	Y
Wakulla Spring – charcoal packs	nb		<10xb
Northside Sink – charcoal packs			Y
McBride Slough @ park boundary – charcoal packs	nb		Y
No Name Spring – charcoal packs	nb		Y
Revell Sink North – charcoal packs			Y
Revell Sink South – charcoal packs			Y
Weigers Sink – charcoal packs			N
Harley Davidson – charcoal packs			N
Harvey's Clear Lake – charcoal packs	<b		
Harvey's Aphasta Pit – charcoal packs	< 10xb		
Chicken Branch Spring – charcoal packs			#2 too early?
Rhodes Spring – charcoal packs			N
Horn Spring – charcoal packs			nb
Spring Creek – charcoal packs	nb		N

Y = presence of dye determined from sonde fluorimeter or charcoal pack where post-injection > 10 times background
<10xb = post-injection charcoal pack < 10 times background
nb = detected by charcoal pack with no background control
<b = post-injection charcoal pack < background
#1~#2 = post-injection charcoal pack approximately equal to background
#2<#1 = post-injection charcoal pack < background
#2 too early? = post-injection charcoal pack registered 0 but may have been retrieved too early
N = no dye detected

Table 4.2. Lake Jackson and Upper Lake Lafayette dye trace study result details.

	Lake Jackson	**Upper Lake Lafayette 1**	**Upper Lake Lafayette 2**
Dye Injection Site	Porter Hole sink	Fallschase sink	Fallschase sink
Date of Dye Injection	9/19/17	1/19/17	4/9/18
Church Sink – charcoal pack 1	n/a	n/a	
Date In			1/28/18
Date Out			4/15/18
Concentration (µg/L)			0.0
Church Sink – charcoal pack 2	n/a	n/a	
Date In			4/15/18
Date Out			6/3/18
Concentration (µg/L)			0.0
Freedom Sink – charcoal pack 1		n/a	
Date In	10/24/17		2/12/18
Date Out	11/07/17		4/17/18
Concentration (µg/L)	10.8		0.0
Freedom Sink – charcoal pack 2	n/a	n/a	
Date In			4/17/18
Date Out			5/22/18
Concentration (µg/L)			0.0
Spiral Garden Sink – charcoal pack 1		n/a	
Date In	10/24/17		2/12/18
Date Out	11/07/17		4/17/18
Concentration (µg/L)	7.5		0.0
Spiral Garden Sink – charcoal pack 2	n/a	n/a	
Date In			4/17/18
Date Out			5/22/18
Concentration (µg/L)			22.8
Gasoline Sink – charcoal pack 1	n/a	n/a	
Date In			2/12/18
Date Out			4/19/18
Concentration (µg/L)			0.0
Gasoline Sink – charcoal pack 2	n/a	n/a	
Date In			4/19/18
Date Out			5/22/18
Concentration (µg/L)			0.0
Emerald Sink – charcoal pack 1		n/a	
Date In	10/10/17		1/28/18
Date Out	10/15/17		4/15/18
Concentration (µg/L)	5.9		0.0

	Lake Jackson	Upper Lake Lafayette 1	Upper Lake Lafayette 2
Emerald Sink – charcoal pack 2		n/a	
Date In	10/15/17		4/15/18
Date Out	11/12/17		6/3/18
Concentration (µg/L)	4.2		0.0
Whiskey Sink – charcoal pack		n/a	n/a
Date In	10/11/17		
Date Out	11/07/17		
Concentration (µg/L)	3.2		
Meeting House Sink – charcoal pack 1		n/a	
Date In	10/15/17		1/21/18
Date Out	11/12/17		4/24/18
Concentration (µg/L)	12.4		16.7
Meeting House Sink – charcoal pack 2	n/a	n/a	
Date In			4/24/18
Date Out			6/3/18
Concentration (µg/L)			16.5
Stafford Sink – charcoal pack 1	n/a	n/a	
Date In			2/12/18
Date Out			4/18/18
Concentration (µg/L)			75.9
Stafford Sink – charcoal pack 2	n/a	n/a	
Date In			4/18/18
Date Out			6/5/18
Concentration (µg/L)			48.7
Indian Spring - charcoal pack 1		n/a	
Date In	10/10/17		3/27/18
Date Out	10/15/17		4/24/18
Concentration (µg/L)	4.9		0.0
Indian Spring - charcoal pack 2		n/a	
Date In	10/15/17		4/24/18
Date Out	11/12/17		5/23/18
Concentration (µg/L)	20.8		0.0
Indian Spring - charcoal pack 3	n/a	n/a	
Date In			5/23/18
Date Out			6/3/18
Concentration (µg/L)			0.0
Indian Spring sonde			
Date In			4/24/18
Date Out			5/24/18
Concentration (µg/L)			0.0
Transit Time			n/a

	Lake Jackson	Upper Lake Lafayette 1	Upper Lake Lafayette 2
Sally Ward Spring - sonde		n/a	
Date In	10/04/17		4/20/18
Initial Detection Date	10/20/17		4/20/18
Maximum Concentration (µg/L)	3.25		21.64
Transit Time	31 days		LE 11 days
Sally Ward Spring - charcoal pack 1		n/a	
Date In	10/04/17		1/24/18
Date Out	10/24/17		4/13/18
Concentration (µg/L)	23.0		0.0
Sally Ward Spring - charcoal pack 2		n/a	
Date In	10/24/17		4/13/18
Date Out	11/07/17		6/5/18
Concentration (µg/L)	30.3		23.5
Wakulla Spring - sonde			
Date In	9/21/17	1/19/17	4/12/18
Initial Detection Date	10/24/17	2/23/17	4/16/18
Maximum Concentration (µg/L)	6.31	0.24	5.00
Transit Time	35 days	35 days	7 days
Second pulse	n/a	n/a	5/9/18
Maximum Concentration (µg/L)	n/a	n/a	123.88
Transit Time	n/a	n/a	30 days
Wakulla Spring – charcoal pack 1		n/a	
Date In	10/5/17		1/28/18
Date Out	10/27/17		4/12/18
Concentration (µg/L)	18.0		48.6
Wakulla Spring – charcoal pack 2	n/a	n/a	
Date In			4/12/18
Date Out			5/31/18
Concentration (µg/L)			83.2
Northside Sink – charcoal pack #1	n/a	n/a	
Date In			1/24/18
Date Out			4/13/18
Concentration (µg/L)			0.0
Northside Sink – charcoal pack #2	n/a	n/a	
Date In			4/13/18
Date Out			6/5/18
Concentration (µg/L)			15.2
McBride Slough @ park boundary – charcoal pack #1		n/a	
Date In	10/11/17		1/24/18
Date Out	11/07/17		4/13/18
Concentration (µg/L)	26.9		0.0

	Lake Jackson	Upper Lake Lafayette 1	Upper Lake Lafayette 2
McBride Slough @ park boundary – charcoal pack #2	n/a	n/a	
Date In			4/13/18
Date Out			6/5/18
Concentration (µg/L)			38.4
No Name Spring – charcoal pack 1		n/a	
Date In	10/24/17		1/24/18
Date Out	11/07/17		4/18/18
Concentration (µg/L)	30.2		0.0
No Name Spring – charcoal pack 2	n/a	n/a	
Date In			4/18/18
Date Out			6/5/18
Concentration (µg/L)			29.5
Revell Sink North – charcoal pack 1	n/a	n/a	
Date In			1/28/18
Date Out			4/24/18
Concentration (µg/L)			0.0
Revell Sink North – charcoal pack 2	n/a	n/a	
Date In			4/24/18
Date Out			6/3/18
Concentration (µg/L)			15.5
Revell Sink South – charcoal pack 1	n/a	n/a	
Date In			1/28/18
Date Out			4/29/18
Concentration (µg/L)			0.0
Revell Sink South – charcoal pack 1	n/a	n/a	
Date In			4/29/18
Date Out			6/3/18
Concentration (µg/L)			16.2
Weigers – charcoal pack 1	n/a	n/a	
Date In			2/16/18
Date Out			4/20/18
Concentration (µg/L)			0.0
Weigers – charcoal pack 2	n/a	n/a	
Date In			4/20/18
Date Out			5/30/18
Concentration (µg/L)			0.0
Harley Davidson – charcoal pack 1	n/a	n/a	
Date In			3/27/18
Date Out			4/25/18
Concentration (µg/L)			0.0

	Lake Jackson	Upper Lake Lafayette 1	Upper Lake Lafayette 2
Harvey's Clear Lake – charcoal pack 1		n/a	n/a
Date In	10/09/17		
Date Out	10/16/17		
Concentration (µg/L)	4.8		
Harvey's Clear Lake – charcoal pack 2		n/a	n/a
Date In	10/16/17		
Date Out	11/13/17		
Concentration (µg/L)	3.2		
Harvey's Aphasta Pit – charcoal pack 1		n/a	n/a
Date In	10/09/17		
Date Out	10/16/17		
Concentration (µg/L)	6.1		
Harvey's Aphasta Pit – charcoal pack 2		n/a	n/a
Date In	10/16/17		
Date Out	11/13/17		
Concentration (µg/L)	7.8		
Chicken Branch Spring – charcoal pack 1	n/a	n/a	
Date In			2/15/18
Date Out			3/28/18
Concentration (µg/L)			0.0
Chicken Branch Spring – charcoal pack 2	n/a	n/a	
Date In			3/28/18
Date Out			4/26/18
Concentration (µg/L)			0.0
Rhodes Spring – charcoal pack 1	n/a	n/a	
Date In			4/19/18
Date Out			4/25/18
Concentration (µg/L)			0.0
Horn Spring – charcoal pack 1	n/a	n/a	
Date In			2/5/18
Date Out			4/19/18
Concentration (µg/L)			Not recovered
Horn Spring – charcoal pack 2	n/a	n/a	
Date In			4/19/18
Date Out			5/22/18
Concentration (µg/L)			14.0
Spring Creek @ Spears' dock – charcoal pack 1		n/a	
Date In	10/04/17		2/15/18
Date Out	11/07/17		3/8/18
Concentration (µg/L)	6.5		0.0

	Lake Jackson	Upper Lake Lafayette 1	Upper Lake Lafayette 2
Spring Creek @ Spears' dock – charcoal pack 2	n/a	n/a	
Date In			3/8/18
Date Out			4/26/18
Concentration (µg/L)			0.0
Lab blank - charcoal pack 1			
Concentration (µg/L)	3.5	n/a	0.0

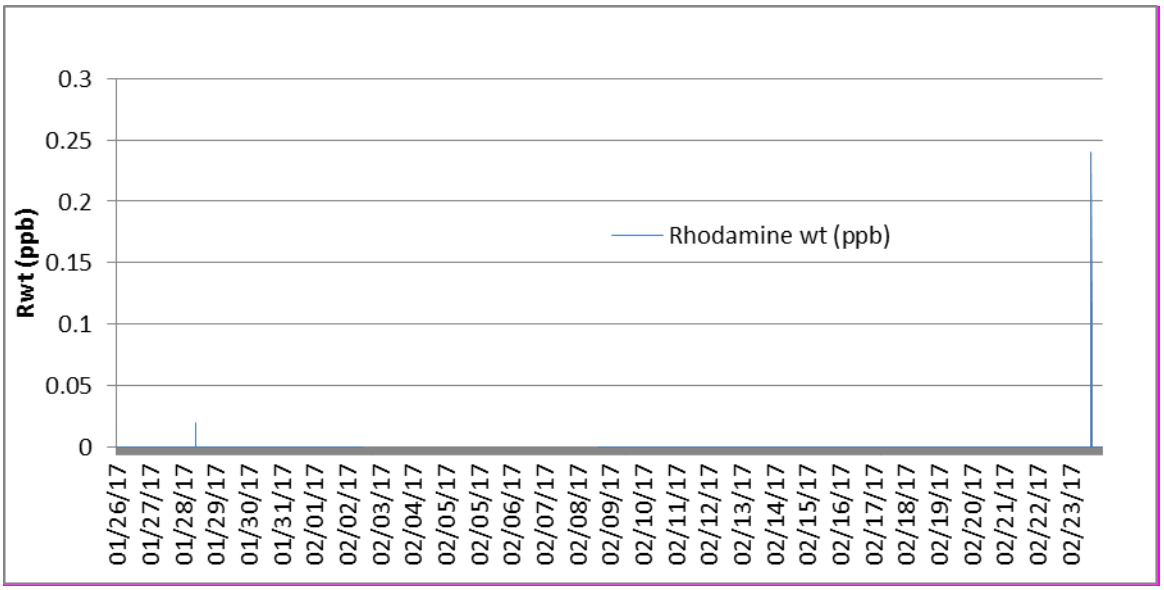

Figure 4.12. Wakulla Spring sonde meter dye detection: first Upper Lake Lafayette dye study.

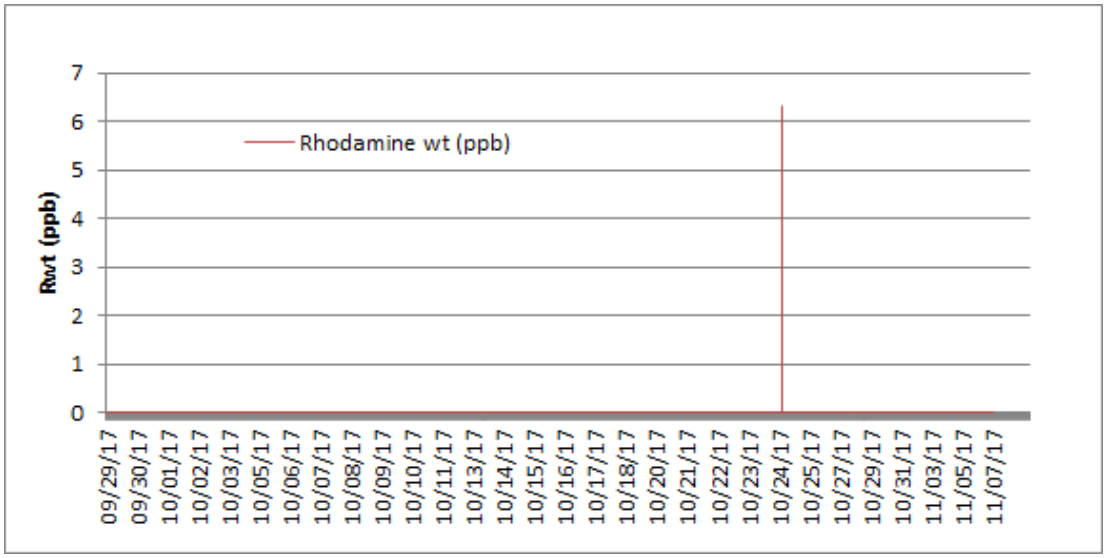

Figure 4.13. Wakulla Spring sonde meter dye detection 9/29/17 - 11/07/17: Lake Jackson dye study.

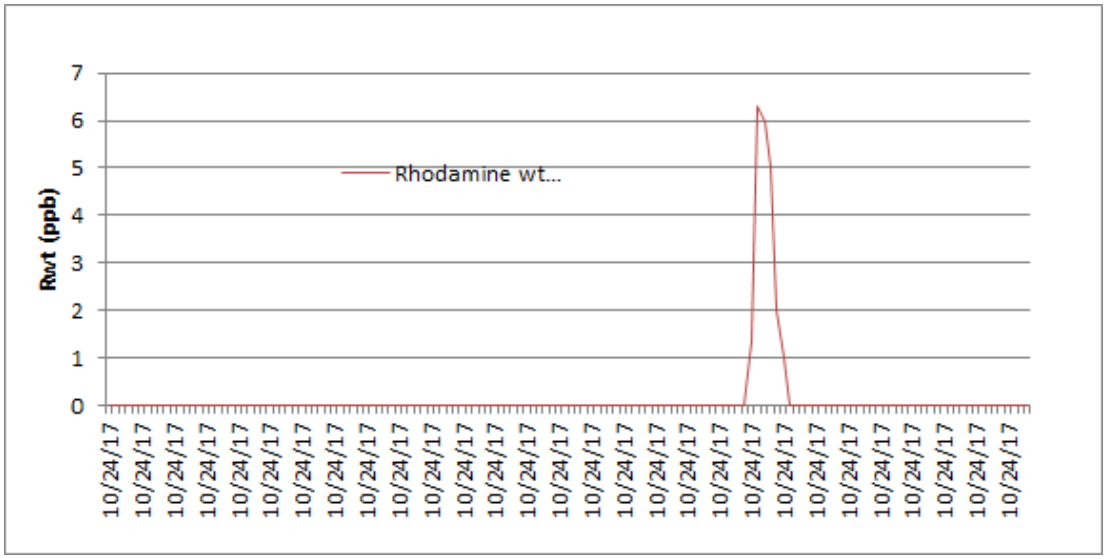

Figure 4.14. Wakulla Spring sonde meter dye detection 10/24/17: Lake Jackson dye study.

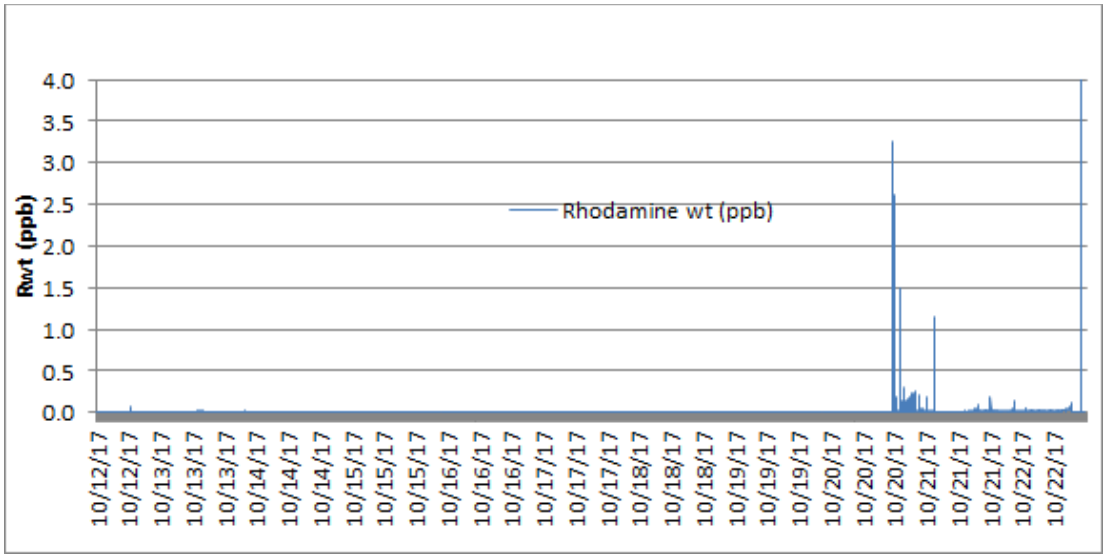

Figure 4.15. Sally Ward Spring sonde meter dye detection 10/12/17 - 10/22/17: Lake Jackson dye study.

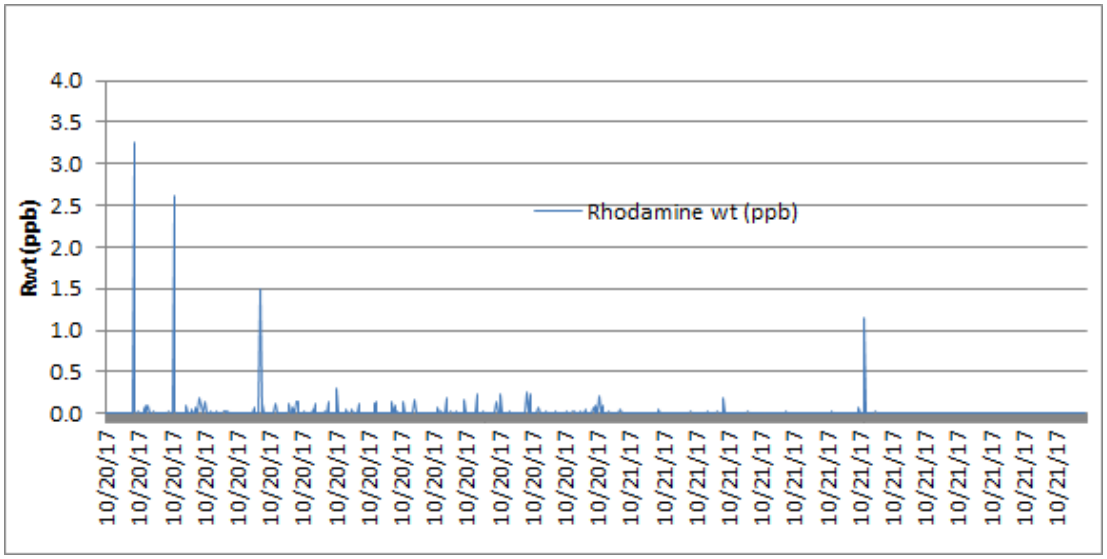

Figure 4.16. Sally Ward Spring sonde meter dye detection 10/20/17 - 10/21/17: Lake Jackson dye study.

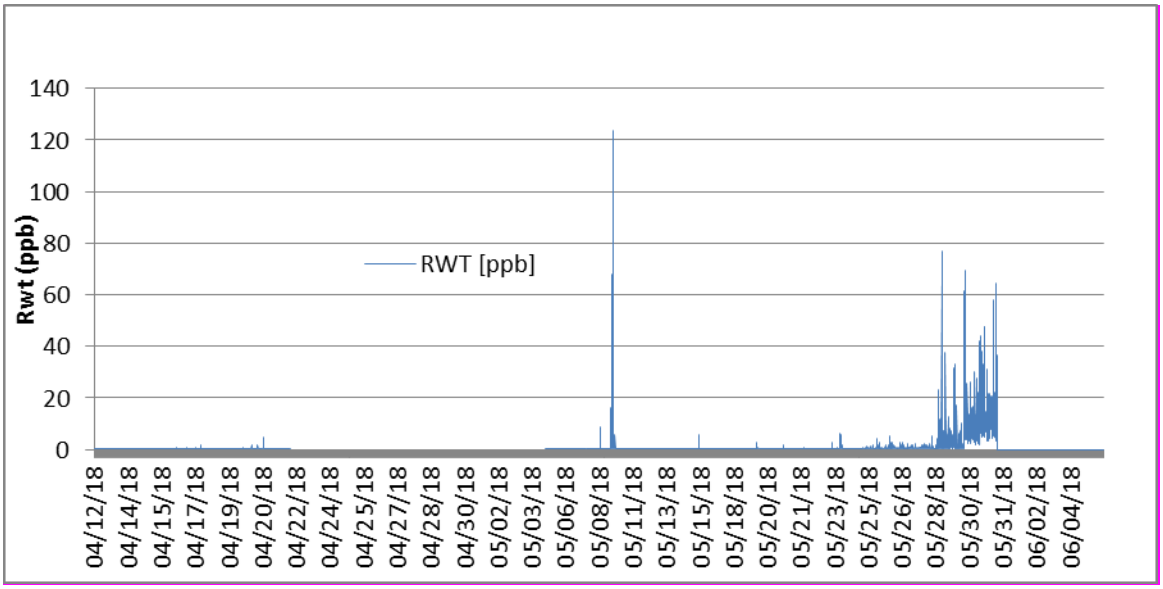

Figure 4.17. Wakulla Spring sonde meter initial dye detection: second Upper Lake Lafayette dye study.

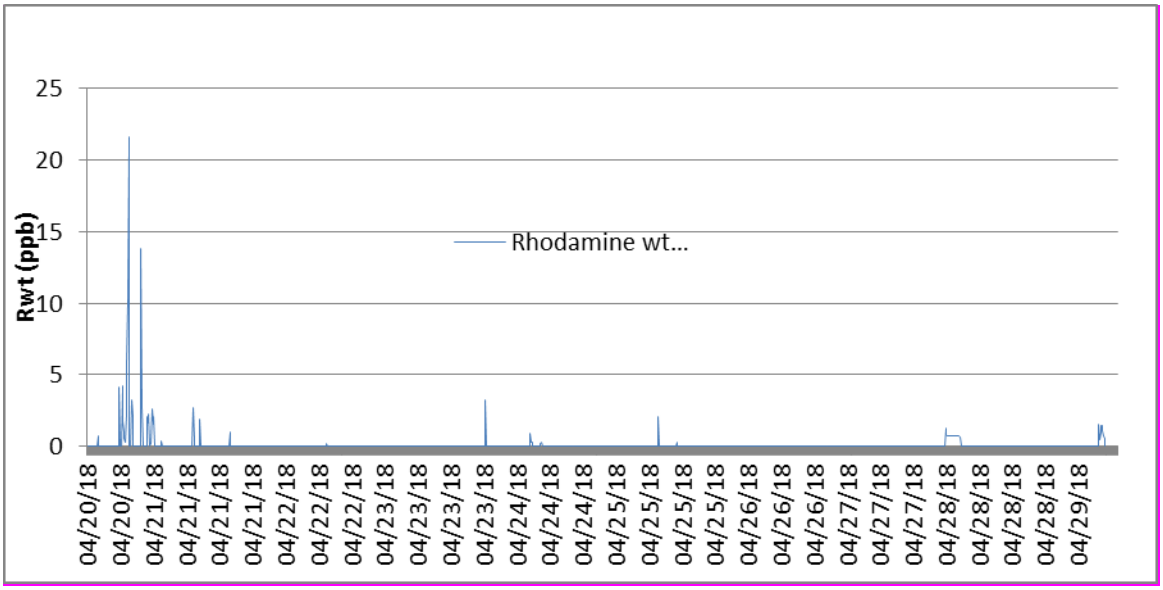

Figure 4.18. Sally sonde meter dye detection: second Upper Lake Lafayette dye study.

Figure 4.19. Locations of Buck Lake (A) and Upper Lake Lafayette (B).

Figure 4.20. Dye backup into Upper Lake Lafayette after draining of Buck Lake, April 17, 2018.

5. Tracking Chlorophyll Sources

The spectralradiometric analyses that we completed during Phase I demonstrated that prolonged dark water conditions at Wakulla Spring are due in part to chlorophyll a and its degradation product, phaeophytin. As reported here in Chapter 3, water quality sampling of the spring boil and L well during Phase II showed that most if not all of the chlorophyll and phaeophytin observed in the spring boil is entering in the ground water flowing through the spring vent. Previous dye studies and those completed for this project have shown that the three large karst lakes in Leon County that receive large volumes of urban storm water and that experience frequent and prolonged algae blooms are connected to the spring: Lake Jackson, Lake Munson, and Upper Lake Lafayette (ULL).

In this chapter we present findings from algal taxonomy and environmental DNA analyses of samples from the three lakes and the L well/spring boil at Wakulla Spring that we undertook in an effort to identify the possible sources of the chlorophyll entering the spring.

5.1 Algal Taxonomic Analysis

Dr. Akshinthala Prasad of the Florida State University Department of Biology performed the algal taxonomic analysis of samples collected by Sean McGlynn. We describe his analytic approach and findings here.

5.1.1 Sampling and Analysis

We collected 1-liter surface water samples from the three lakes on October 6, 2017: Porter Hole Sink in Lake Jackson; the dam in Lake Munson; and at Fallschase Sink in ULL. We preserved the samples with acidified Lugol's solution. Because of the significant dilution that occurs in the ground water, we collected the sample for algae analysis at Wakulla Spring on October 12, 2018, from the L well rather than the boil. Doing so allowed us to pump 160 gallons of water through a 64-micron (μm) plankton net to concentrate the algae. We then rinsed the residue from the net into a container and fixed the sample with Lugol's.

Dr. Prasad allowed the preserved samples to settle for at least 48-72 hours. After settling, samples were concentrated by siphoning off the supernatant water to yield a 50-ml volume for microscopic analyses. Each concentrated sample was mixed thoroughly and divided into two subsamples: one sub-sample was used for microscopic analyses for estimating absolute abundance and the other for the preparation of permanent slides of diatoms.

Concentrated water samples were rinsed in de-ionized water and were mixed with hydrogen peroxide (30%) and sulfuric acid and were boiled for 15-20 minutes. The acidified samples were rinsed free of acid at least ten times and stored in vials in de-ionized water. Permanent slides of diatoms were prepared by placing a small aliquot of acid-cleaned material onto a cover glass and dried; the dried cover glass was inverted and mounted in a Naphrax resin of high refractive index. Each slide was labeled and stored in cool dark cabinets. These permanent resin-mounted-diatom slides and the Lugol's-preserved and acid-cleaned voucher samples are deposited in Dr. Prasad's collections in the Microalgae Collections of the R.K. Godfrey Herbarium, Department of Biological Science, Florida State University.

For microscopic analysis three aliquots of 0.05 ml each were placed onto separate slides and covered with 30 mm x 24 mm cover glasses (#1 thickness). Slides were sealed temporarily with sealant to prevent evaporation. All phytoplankton cells were counted at 400x magnification in each aliquot and were identified to the lowest taxonomic category possible using a Leica DMLB photo microscope and a Nikon Labophot microscope, equipped with phase contrast optics and differential interference contrast objectives. Diatom material for light microscopy was prepared as described in Prasad et al. (1990), Prasad and Fryxell (1991), and Prasad and Livingston (2005).

Absolute abundances for each taxonomic unit were calculated and expressed as number of cells per liter. Relative abundance was estimated as percentage of abundance. No absolute phytoplankton abundances were estimated from the L well sample because the 64-μm mesh size was large enough to allow much smaller cells to escape.

5.1.2 Findings

As shown in table 5.1, the predominant taxonomic classes of algae in the three lakes differed to varying degrees. All three contained substantial proportions of diatoms and green algae. Only diatoms could be identified in the sample collected at the L well. This likely reflects in part the plankton mesh size: diatoms are generally larger than most other freshwater algae (20-200μm).

However, this also may be due to the degradation of most algae cells during the 30-or-more-day journey through the aquifer. Because diatoms have siliceous cell walls, they are structurally more robust and therefore more likely to survive the journey in identifiable condition. Table 5.2 presents the predominant genera and species found in each water body organized by genus or species. At this higher level of taxonomic specificity, the four water bodies diverge even further.

Cyanobacteria (class = Cyanophyceae), sometimes inaccurately referred to as bluegreen algae, are of particular concern because they produce toxins that can harm other organisms including humans. They comprised small proportions of the photosynthetic microorganisms identified in the three lakes: 2.8% in Lake Jackson, 0.4% in ULL, and 4.2% in Lake Munson. Lake Munson had the greatest diversity of genera and species (table 5.2).

Three species/genera of diatoms were found in the L well net sample and only one of the three lakes:

- *Cocconeis placentula* – Lake Jackson
- *Pinnularia spp.* – ULL
- *Staurosira spp.* – Lake Jackson

Although Dr. Prasad (2017) stated that all three taxa are common in freshwater systems in north Florida over the course of the year, the temporal distinctions between the three water bodies at the time of sampling suggest that each of the three lakes may be contributing chlorophyll to Wakulla Spring.

There is, however, a diatom disconnect. Most of the diatoms identified in the L well sample (14 of 21 = 67%) were not found in any of the lakes and an even higher proportion of the diatoms identified in the lakes were not identified in the L well sample (24 of 31 = 77%). Dr. Prasad reports that the L well diatom taxa "are primarily benthic (epipelic, epipsammic or periphytic)[5] but [are] often found displaced in [the] water column with planktonic diatoms in shallow systems" while the "[m]ajority of algal taxa in the lake samples are strictly planktonic with a few exceptions, such as *Cocconeis placentula* [found only in the Lake Jackson sample]." These discrepancies may reflect the fact that we collected our samples from the surface waters of the lakes while in both Lake Jackson and ULL, the principal discharge to ground water is through sinkholes in the bottoms of the lakes. The grab samples would likely have had a higher proportion of planktonic species while the water discharged through the sinkholes may have had higher proportions of benthic species.

5.2 Environmental DNA Analysis

Drs. Richard Long and Thomas Sawicki of the Florida A and M University Department of Biology conducted the environmental DNA sampling and data analyses. DNA sequencing was conducted by Molecular Research LP (MR DNA).

[5] Epipelic = growing on bottom sediments; epipsammic = living on sand grains; periphytic = growing on submerged surfaces including plants, sediments, and rocks.

5.2.1 Sampling and Analysis

Dr. Sawicki collected samples for the environmental DNA analyses at the same time that we collected the samples for algal taxonomic analysis. In addition to the three lakes and the L well, he drew samples from the Wakulla Spring boil and the K, B, D, and C cavern wells (see figure 2.1).

The environmental DNA analysis centered on the DNA that comprises the 16S rRNA ribosome ribonucleic acid gene to identify cyanobacteria and eukaryotic algae (cells with a nucleus) to genus. The 16sRNA gene is the primer[6] that organisms use to produce ribosomal RNA which is essential to all cell function. It is present in all organisms and must be predominantly stable; deleterious mutations lead to cell death. Thus the "conserved" regions of the genomic sequence are very stable and can be targeted in all species. The "variable regions" allow for differentiation among genera and species. As shown in figure 5.1, there are nine hypervariable regions found in the 16s rRNA gene, and each of these regions is flanked by a highly conserved region. The two regions used for this study are targeted for identifying cyanobacteria and eukaryote chloroplasts.

Drs. Long and Sawaicki and their lab assistants prepared samples by filtering them through a 0.2 μm filter. They then extracted and amplified the DNA and sent the DNA samples to MR DNA for sequencing analysis. The FAMU researchers extracted the 16srRNA DNA by smashing the cells, using a molecular filter to remove everything except the DNA, and separating the paired DNA strands into single strands. They then amplified the sequences using polymerase chain reaction (PCR) to produce multiple copies of each DNA sequence in the sample. The resulting DNA sequence frequencies in each sample "data library" are approximately proportional to the number of gene copies in the sample prior to amplification.

MR DNA compared the resulting sequences against the Michigan State Ribosomal Database Project database to identify associated organisms to genus. MR DNA assigned genus-level Operational Taxonomic Units (OTUs) for the entire merged sample based on predefined "bins" of gene sequences with 97% similarity. They reported abundance values for each OTU for each sample site data library. It is important to note that OTUs are not the same as individual organisms; some organisms may contain multiple copies of the same OTU sequence.

Dr. Long conducted 10 Monte Carlo simulations for each sample site data library using the OTU sequence frequencies produced by MR DNA. Each Monte Carlo "community" consists of 10,000 OTUs that are iteratively derived based on the weight of the each individual OTU relative to the entire DNA library for each sample site. This is done to normalize the library sizes across sample sites due to the variation in sequence numbers across sites. Dr. Long used the average values for each OTU from the simulations to create "heat maps" of OTU prevalence. Values of less than 10 OTUs per 10,000 are not considered significant.

[6] "A primer is a short strand of RNA or DNA (generally about 18-22 bases) that serves as a starting point for DNA synthesis. It is required for DNA replication because the enzymes that catalyze this process, DNA polymerases, can only add new nucleotides to an existing strand of DNA."
(https://en.wikipedia.org/wiki/Primer_(molecular_biology))

5.2.2 Findings

In addition to heat maps, Dr. Long calculated Jaccard similarity index coefficients for each of the lakes compared to the spring boil. The index represents the overlap between the gene sequence OTUs. It is defined as the size of the intersection between the sample units divided by the size of the union of the sample sets (figure 5.2). A coefficient of 1 would indicate that the two samples are identical. A coefficient of 0.50 would indicate that half of the combined sequences of the two samples are the same.

Figure 5.3 presents a class-level heat map for the eukaryote algae for the three lakes, the L well, and the spring boil (recall that values less than 10 are considered not significant). It reveals greater phylogenetic diversity in the L well than the taxonomic analysis, offering further evidence that the 64-μm plankton net missed many of the non-diatom algae present in the L well. Class-level OTUs are mostly similar for the L well and spring boil with a few exceptions (the Jaccard coefficient = 1.0). Cryptophytes (Cryptophyta) and euglenoids (Euglenida) are two to three times more prevalent in the boil than the L well, while diatoms (Bacillariophyceae) are eight times more prevalent. This may reflect incomplete mixing in the Grand Canyon.

Table 5.3 presents a Jaccard coefficient matrix for the eukaryote algae genus-level OTUs. The Jaccard coefficients for each of the three lakes with the spring boil (BLA) indicate substantial similarity between each lake and the spring:

- Lake Munson (LMA) - 0.542
- Lake Jackson (LJA) - 0.464
- ULL (FSA) - 0.435

The L well (LWA) coefficient of similarity with the spring boil is one of the highest in the matrix, 0.690. However, the lake Jaccard coefficients with the L well are not as high as those with the boil. This may reflect incomplete mixing in the Grand Canyon as discussed in Chapter 2:

- Lake Munson (LMA) - 0.481
- Lake Jackson (LJA) - 0.419
- ULL (FSA) - 0.385

Table 5.3 also reveals that Lake Munson is more similar to each of the wells and Wakulla Spring than the other two lakes.

The K cavern and B cavern wells (KWA and BWA) are the most similar to the L well aside from the spring boil and the most similar to the boil aside from the L well. They are likely the principal caverns from which the chlorophyll entering the spring originates. The fact that these samples are more similar to the boil than the lakes may reflect cell mortality during transit to the spring as well as inputs of chlorophyll from one or more additional surface water bodies in the springshed.

Figure 5.4 presents a genus-level heat map for the eukaryote algae for the three lakes, the L well, and the spring boil. It reveals that at least one eukaryote algae genus-level OTU defined for the spring boil sample is found in only one of each of the three lakes. Consistent with the algal taxonomic study, three of the four are diatoms (Bacillariophyceae).

- *Coscinodiscus* (Bacillariophyceae) - Jackson
- *Melosira* (Bacillariophyceae) - Munson
- *Phaeodactylum* (Bacillariophyceae) - Munson
- *Skeletonema* (Bacillariophyceae) - ULL
- *Teleaulax* (Cryptophyceae) - Munson

Together, these findings provide further evidence that each of the lakes may be contributing to the chlorophyll being discharged into the spring. Note that three of the five possible indicators are found solely in Lake Munson.

Looking solely at the cyanobacteria genus-level OTUs, Dr. Long found that they are far more prevalent in the lakes than in the samples from the L well and the spring boil (figure 5.5). Furthermore, the cyanobacteria OTUs of the three lakes are more similar to each other than they are to those at the spring. This is not surprising since they are highly susceptible to degradation during the 30+ day journey through the cave system. The four cyanobacteria genus-level OTUs identified from the spring boil and L well are each present in two or more lakes:

- *Cyanobacterium*
- *Leptolyngbya*
- *Prochlorococcus*
- *Synechococcus*

As with the algal taxonomic analysis, the environmental DNA analysis also revealed some disconnects. Among the eukaryote algae, six genus-level OTUs, three of which were diatoms (Bacillariophyceae), were found at the spring but not in any of the three lakes: *Cyanidium* (Cyanidiophyceae), *Cymatopleura* (Bacillariophyceae), *Cymbella* (Bacillariophyceae), *Euglenaformis* (Euglenophyceae), *Neoptilota* (Florideophyceae), *Odontella* (Bacillariophyceae). Four eukaryote genus-level OTUs were identified from one or more of the lakes but not from the spring boil or L well: *Chrysosphaera* (Chrysophyceae), *Cyanophora* (Glaucophyceae), *Pavlova* (Pavlovophyceae), *Phacus* (Euglenophyceae). Two genus-level OTUs were identified from one or more of the lakes and the L well but not the spring boil: *Euglena* (Euglenophyceae) and *Nitzschia* (Bacillariophyceae).

These findings coupled with the diatom disconnect for the algal taxonomic analysis suggest some additional factors may be at work in addition to the distinction between benthic and planktonic diatoms discussed above. It is possible that the algae populations we sampled in the lakes had changed over the approximately 30 to 35 days that the water we sampled at the L well and the spring had travelled through the ground water cave system. It also is possible that the chlorophyll flowing into the spring originates from additional karst water bodies within the springshed. The most likely other source is Lake Iamonia which McGlynn and Deyle (2016)

determined was discharging the highest load of chlorophyll to the aquifer among the major karst lakes in the springshed.

The latter issue is being addressed during Phase III of this project. A dye study will be conducted for Lake Iamonia and two additional rounds of taxonomic and environmental DNA analyses will be conducted that add Iamonia to the sample regime. The Phase III algal taxonomy study will use a finer plankton net mesh (0.25 micron) in an effort to capture other algae classes at the L well beyond the diatoms. The Phase III environmental DNA study will examine longer 16S rRNA DNA sequences to enable sequencing to species-level OTUs which will allow for more precise definition of the OTU communities of the four lakes and the spring. We also may analyze a different hypervariable region targeted more precisely at the eukaryote algae.

Table 5.1. Predominant algae classes

	Water Body			
Class	**Lake Jackson**	**Upper Lake Lafayette**	**Lake Munson**	**L Well at Wakulla Spring**
Golden brown algae –Chrysophyceae	38%	0%	0%	0%
Diatoms – class Bacillariophyceae	19%	11%	71%	100%
Green algae – Chlorophyceae	19%	73%	7%	0%
Cryptophytes –Cryptophyceae	13%	0%	12%	0%
Raphidophytes – class Raphidophyceae	0%	8%	0%	0%

Table 5.2. Predominant algae genera/species

Genus/Species	Class	Water Body			
		Lake Jackson	**Upper Lake Lafayette**	**Lake Munson**	**L Well at Wakulla Spring**
Dinobryon bavaricum	Chrysophyceae	33%	0%	0%	0%
Aulacoseira granulata	Bacillariophyceae	0%	0%	68%	0%
Cocconeis placentula		0%	0%	0%	7%
Cymbella spp.		0%	0%	0%	6%
Naviculas spp.		0%	0%	0%	30%
Nitzschia spp.		0%	7%	0%	16%
Staurosira spp.		7%	0%	0%	0%
Scenedesmus spp.	Chlorophyceae	0%	43%	0%	0%
Chlorogonium tetragamum		0%	14%	0%	0%
Undetermined cryptophytes	Cryptophyceae	11%	0%	11%	0%
Undetermined raphidophyte	Raphidomonadeae	0%	8%	0%	0%
Undetermined dinoflagellates	Dinophyceae	5%	0%	0%	0%
Aphanizomenon flos-aquae	Cyanophyceae	0%	0%	0.9%	0%
Aphanizomenon spp.		0%	0%	0.1%	0%
Anabaena spp.		0%	0.4%	2%	0%
Dactylococcopsis spp.		0%	0%	<0.1%	0%
Nodularis spp.		3%	0%	0%	0%
Nostic spp.		0%	0%	0.1%	0%
Oscillatoria spp.		0%	0%	0.6%	0%
Schizothrix spp.		0%	0%	0.1%	0%
Spirulina spp.		0%	0.1%	0%	0%
Spirulina princeps		0%	0%	0.3%	0%

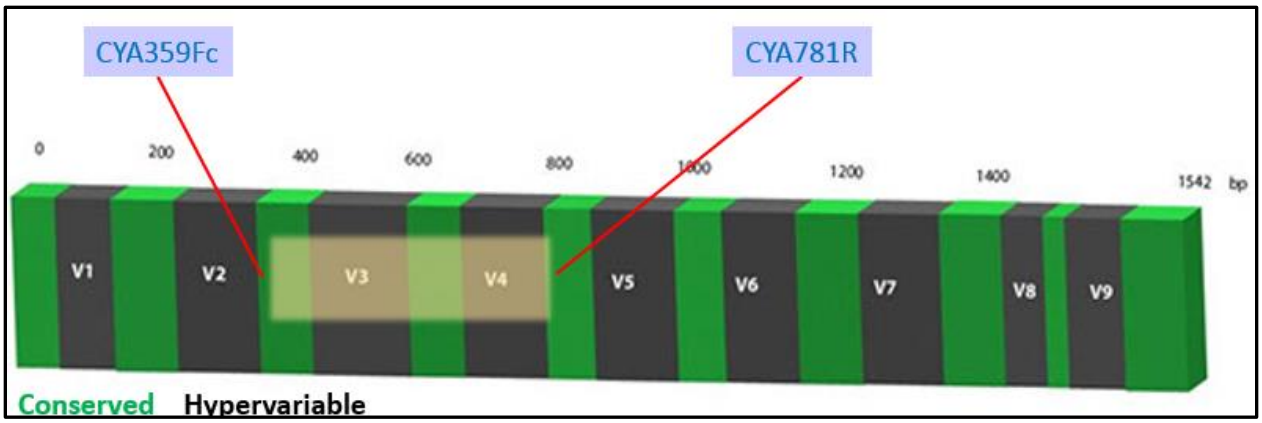

Figure 5.1. 16S rDNA primers for cyanobacteria and eukaryote chloroplasts.

$$J(A,B) = \frac{|A \cap B|}{|A \cup B|} = \frac{|A \cap B|}{|A| + |B| - |A \cap B|}.$$

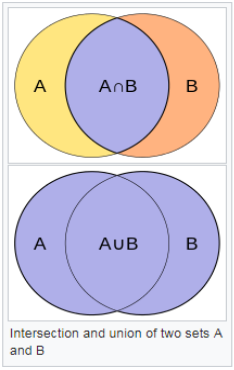

Figure 5.2. Jaccard similarity index (https://en.wikipedia.org/wiki/Jaccard_index).

Table 5.3. Eukaryote algae genus-level OTUs Jaccard coefficient matrix.

Proximity matrix (Jaccard coefficient(1)):

	KWA	BWA	DWA	CWA	LWA	BLA	LJA	LMA	FSA
KWA	1	0.690	0.455	0.576	0.688	0.645	0.394	0.448	0.357
BWA	0.690	1	0.577	0.667	0.679	0.692	0.444	0.591	0.476
DWA	0.455	0.577	1	0.517	0.533	0.483	0.520	0.417	0.500
CWA	0.576	0.667	0.517	1	0.515	0.621	0.400	0.462	0.417
LWA	0.688	0.679	0.533	0.515	1	0.690	0.419	0.481	0.385
BLA	0.645	0.692	0.483	0.621	0.690	1	0.464	0.542	0.435
LJA	0.394	0.444	0.520	0.400	0.419	0.464	1	0.455	0.474
LMA	0.448	0.591	0.417	0.462	0.481	0.542	0.455	1	0.500
FSA	0.357	0.476	0.500	0.417	0.385	0.435	0.474	0.500	1

KWA = K well; BWA B well; DWA D well; CWA C well; LWA L well; BLA Wakulla Spring Boil; LJA = Lake Jackson; LMA = Lake Munson; FSA = Fallschase Sink (Upper Lake Lafayette)

Class	L Well	Spring Boil	Lake Jackson	Lake Munson	ULL
Bacillariophyceae	91.2	720.8	21.4	25.9	9.2
Bangiophyceae	71.3	33.8	0	0.8	3.1
Chrysophyceae	2264.9	2642.2	332.2	13.9	80.2
Coscinodiscophyceae	709.7	845	262.1	5612.1	201.8
Cryptophyta	103.7	323.7	140.6	249.6	153.3
Dinophyceae	38.3	51.6	10.1	165.6	31
Euglenophyceae	114.6	239.4	79.8	180.9	2.5
Eukaryota	0	0	24.1	0	0.3
Eustigmatophyceae	2493.2	1818.8	8515.9	3268.4	321.6
Florideophyceae	105.1	25.4	8	1.6	11.1
Fragilariophyceae	464.5	441.2	51	110.9	1299.6
Glaucocystophyceae	86.5	65.4	37.9	0.1	0.1
Mediophyceae	73.4	55.6	0	1.7	0.7
Pelagophyceae	0	0	2.2	0.8	3.3
Raphidophyceae	3412.2	2747.8	513.6	372	7876.8

Figure 5.3. Eukaryote class-level heat map for the three lakes, L well, ad Wakulla Spring boil.

Genus	L Well	Spring Boil	Lake Jackson	Lake Munson	ULL
Acanthamoeba	0	0	0	0	0
Asterionella	457.3	430	52	109.9	1289.9
Callithamnion	0	0	0	0	0
Chrysosphaera	0	0	113.9	6.1	1.3
Coscinodiscus	177.8	71.2	36.8	5.4	0.4
Cryptomonas	34.9	35.9	60.4	118.3	43.1
Cyanidium	32.3	36.2	0	0.4	0
Cyanophora	0	0	24.3	0	0
Cyanoptyche	83.8	64.4	13.3	0.5	0
Cymatopleura	0	144.4	0	4.2	1.1
Cymbella	0	212.7	0.7	1.7	0
Diatoma	0	4.8	1.7	0.8	7.3
Dinophysis	33.1	50.2	11.5	166	31.3
Euglena	24.4	0	0	13.1	0.4
Euglenaformis	95	208.8	0	0	0.1
Euptilota	11	0	2.9	0.3	0.8
Guillardia	47.5	38.8	52.4	38.5	95.6
Heterosigma	3373.1	2702.5	504.1	356.4	7867.1
Hypnea	0	0	0	0	0.1
Malawimonas	0	0	0	0	0

Melosira	10.2	15.1	1.6	60.8	3.2
Nannochloropsis	2515	1841.9	8501.3	3235.6	331.6
Neoptilota	35.2	19.2	0	0	0
Neosiphonia	44.8	0	0	0.7	0.1
Nitzschia	38.2	0	17.1	1	0
Ochromonas	2264	2645.8	233.5	9.3	86
Odontella	72.1	54.9	0	1.2	0.3
Palmaria	0	0	2	0.1	0.4
Paralemanea	0	4.7	1.7	0.9	8.4
Pavlova	0	0	29.4	0	0.2
Pelagomonas	0	0	1.7	0.6	3.4
Phacus	0	0	36.9	0	0.2
Phaeodactylum	60.3	373.1	0.7	16.1	6.1
Plumaria	13.2	0	0	0	0
Porphyra	35.3	0	0	0.4	0.4
Ptilocladia	0	0	0	0.2	0.1
Rhodomonas	11.7	228	27	60.4	9.5
Sciurothamnion	0	0	0	0	0
Skeletonema	45.6	16.2	0.4	4	44.5
Stephanodiscus	298.3	373.5	46.2	4137.2	26.9
Strombomonas	0	35	22.4	171.4	2.3
Teleaulax	10.6	11.6	9.6	41.9	1.8
Thalassiosira	178.2	381.8	187.2	1450.5	128.3

Figure 5.4. Eukaryote genus-level heat map for the three lakes, L well, and Wakulla Spring boil.

Genus	L Well	Spring Boil	Lake Jackson	Lake Munson	ULL
Acaryochloris	0.2	0.7	0.2	3.2	0.4
Aerosakkonema	0	0.2	0.1	6.9	0.6
Aetokthonos	0	0	0	0	0
Anabaena	4	5.8	3.4	20	9.5
Annamia	0	0	0	0	0.5
Aphanocapsa	0.1	0.4	0	0.2	0.5
Arthronema	0	0	0	0	0
Arthrospira	0.3	0	36.5	18.3	26.7
Blennothrix	0	0.7	0	0	0
Calothrix	0	0	0	0.1	0
Chamaesiphon	0.3	0.3	0.3	5.8	1.1
Chlorogloea	1.4	1.3	0.6	1.6	6.5
Chroococcidiopsis	4	4.7	36.7	57.1	114.9
Chroococcus	0.8	1.7	1.2	1.5	14.7
Coelomoron	0.5	0.7	31.4	1.4	3.2

Coleofasciculus	0	0	0	0	2.1
Cuspidothrix	0	0.2	0.8	16.4	13.8
Cyanobacterium	40.2	101.8	5600.4	3271	3152.1
Cyanobium	0	0	35.6	3.5	16.8
Cyanodictyon	0	0	0	0.5	0.8
Cylindrospermopsis	0.1	0	3.5	3.4	0.9
Cylindrospermum	0	0	0	1.3	1.3
Dactylococcopsis	0	0	2.9	0	0
Dermocarpa	0.1	0	0.4	0	1.3
Dolichospermum	5.3	3.5	17.6	764.8	69.8
Euhalothece	0	0.9	0	0	0
Geitlerinema	0	0	6.8	1	0.4
Geminocystis	0	0	0.6	0	1
Gloeobacter	7.2	10	2.8	3.8	0.8
Gloeocapsa	0	0	0	0	0
Gloeothece	0	0	0	1	0.2
Halomicronema	0	0	0	0	0
Halospirulina	0	0	0	0	2.4
Hydrocoleum	0	0	0	0	3
Jaaginema	1	0.8	0	0	0
Johansenia	0	0	0	0	0.8
Kamptonema	0	0	0	0	0
Komvophoron	0	0	0	0	5.6
Leptolyngbya	17.6	27	6	16	85
Limnothrix	0	2.2	0.8	11.2	0
Loriellopsis	0	0	0	0	0.2
Lyngbya	0	0	0	0.8	0
Merismopedia	0	1	8.4	5	103.2
Microchaete	0	0	0	0	0
Microcystis	2.8	2.4	34.4	146.8	7
Microseira	0	0	0	0	1.8
Myxosarcina	0	0	0	0.8	0
Nodosilinea	0.2	0	0	0	0.2
Nodularia	0	0	0	0	0
Nostoc	0	0	0	1.2	9
Oculatella	0.2	0	0	0	0.8
Oscillatoria	0	0.8	5.4	0	5
Pannus	0	0	3.8	0	0.8
Phormidesmis	1.2	0.8	0	0	0
Phormidium	1	7	35	1.2	22
Planktothricoides	0.4	3.8	0	0	1.2

Planktothrix	0	0.8	0.8	0	2.6
Plectonema	0.8	0	30.2	2.4	5.8
Pleurocapsa	0	0	1.2	3.2	0
Prochlorococcus	573.6	525.8	241.2	2670.4	1477.4
Prochlorothrix	7.2	0	0	0.8	0
Pseudanabaena	0	3	1.4	8.8	3.6
Pseudophormidium	0	0	0	0	0.2
Radiocystis	1	0	0	0	0
Scytonema	0	0	0	0	0.2
Solentia	0	0	0	0	0
Sphaerospermopsis	0	0	0	52	4.6
Spirulina	0	0	0	0	0.8
Stanieria	0	0	0	0	3.4
Stigonema	0	0	1.2	0	1
Synechococcus	45.8	143.4	3469	378	3203.2
Synechocystis	0	0	0.4	0.4	3.2
Thermosynechococcus	0.8	0.8	0	0	0
Tolypothrix	0	0	5	1.6	1.8
Xenococcus	0	0.4	0.4	0	2.4

Figure 5.5. Cyanobacteria genus-level heat map for the three lakes, L well, and Wakulla Spring boil.

6. Summary of Findings and Further Research

Our principal findings include the following:

1. Extension of weekly water quality sampling to include the Wakulla Springs cave system as well as the spring boil has demonstrated that most if not all of the chlorophyll responsible for “green dark water” conditions experienced in the spring boil is entering the spring in the ground water in addition to tannins which have historically caused “brown dark water” conditions.
    a. The L well, which is located 430 feet from the spring vent, ideally should reflect the aggregate conditions of all of the caverns that come together to feed Wakulla Spring. However, the L well taps the top of a huge underground room, the Grand Canyon which may not be thoroughly mixed.
    b. Regression analysis indicates that true color levels in the L-well explain about 45% of the observed variance in true color levels measured at the boil.
    c. Time series data for corrected chlorophyll a and phaeophytin reveal occasions when higher levels were measured in the spring boil than the L well for corrected chlorophyll a and phaeophytin (figure 2.13) as well as some minor out-of-phase patterns when peaks in the spring either lag behind or precede those in the L well.
    d. Nevertheless, all but two of the spring boil peaks in corrected chlorophyll a and all but one of the phaeophytin peaks are associated with peaks in the L well

suggesting that most if not all of the chlorophyll detected in the boil originates from the cave system but that there may at times be incomplete mixing in the Grand Canyon chamber.

e. Simple linear regression models for L well versus spring boil measures of corrected chlorophyll a, phaeophytin, and total chlorophyll a (see table 2.1) are all statistically significant, but their explanatory power is low reflecting both the out-of-phase peaks and the higher peaks detected at the boil. L-well concentrations of corrected chlorophyll a explain only 7% of the observed variance in those measured at the spring boil, 5% for phaeophytin, and 11% for total chlorophyll a.

2. Extension of our weekly sampling of photosynthetically available radiation (PAR) at the spring boil has further confirmed findings from Phase I that dark water conditions are complex phenomena not simply explained by any one of the three major light-adsorbing substances: tannins, chlorophyll a, and its degradation product, phaeophytin.
    a. A regression model for true color during the entirety of Phases I and II explains 15% of the observed variance in PAR depth limit and is statistically significant at the 99.9999% level.
    b. As found in Phase I, individual simple regression models for each of three chlorophyll measures remain statistically insignificant for the extended Phase I plus Phase II data.
    c. A multiple regression analysis of the effects of true color, corrected chlorophyll a, and phaeophytin on PAR depth limit conducted on the Phase I data did yield a statistically significant model at the 99.9 percent level that explained 39% of the observed variance in PAR depth limit. A comparable model for the extended time period of Phase I plus Phase II was significant at the 99.9999% level but only explained 17% of the observed variance in PAR depth limit. These findings support the hypothesis that the combined effects of tannins and chlorophyll are at work but that tannins may be the primary determinant of dark water conditions. However, explaining PAR depth limit based on water quality is likely to be less precise than with direct measures of light absorbance attributable to the three factors. We are currently exploring alternative analytic approaches for doing so.
3. Dye studies we conducted demonstrated for the first time that as has been suspected, direct hydrogeologic connections exist between Wakulla Spring and Lake Jackson (35-day travel time) and Upper Lake Lafayette (30-35 day travel time). We also documented hydrogeologic connections between Lake Jackson and ULL with Sally Ward Spring and between Lake Jackson and Indian Spring.
    a. Charcoal samplers without background controls provided evidence that dye from Lake Jackson may have flowed to several of the sinks located north and west northwest of Wakulla Spring: Freedom, Spiral Garden, Emerald, Whiskey, and Meetinghouse. Of these, only Spiral Garden and Meetinghouse also recorded possible dye from the second ULL study. We did not test Stafford Sink, which is situated west of Indian Spring, for the Lake Jackson study. Charcoal sampler results from the second ULL study indicate that dye may have travelled there from ULL.

b. Charcoal samplers indicate that dye which appeared at Wakulla and Sally Ward also most likely arrived at Northside Spring, McBride Slough, and No Name spring from both Lake Jackson and Upper Lake Lafayette. These karst features are located close to Wakulla. We also detected dye from the second ULL study at the Revell Sinks which are located south-southwest of Wakulla Spring and from the Lake Jackson study at the Harvey sinks further south in the combined Wakulla-Spring Creek springshed. A single charcoal sampler deployed at Spring Creek during the Lake Jackson study indicated that dye may have reached that location at the terminus of the combined Wakulla Spring-Spring Creek springsheds. A single charcoal sampler retrieved from Horn Spring also indicates that dye may have traveled east from ULL into the St. Marks basin during the second ULL study.

4. Taxonomic analyses of algae samples collected from Upper Lake Lafayette, Lake Jackson, Lake Munson, and the L well which taps the cavern system 430 feet from the Wakulla Spring vent identified three algal taxa at the L well unique to each of the three lakes, indicating that chlorophyll entering the spring could be coming from all three lakes.
    a. However, 67% of the taxa identified in the L well were not found in any of the three lakes suggesting that chlorophyll may be coming from one or more other surface water bodies. Lake Iamonia is the most likely other source. We will therefore conduct a dye study of Iamonia during Phase III and include it in two subsequent algal taxonomic studies.
    b. In addition, 77% of the diatoms reportedin the lakes were not identified in the L well. This may be due in part to the mesh size of the plankton net used to sample the L well. We will use a smaller mesh size when we conduct additional sampling during Phase III.
    c. These apparent disconnects also may be due to temporal discontinuities: the algal populations of the lakes may have changed during the 30-35 days that their discharges took to travel to Wakulla Spring. We are addressing this possibility during Phase III by staggering sample times to reflect the travel times revealed by our dye studies.
    d. A third factor may reflect differences in algal populations at the surface versus the bottoms of Lake Jackson and ULL at the mouths of their sinkholes. We will address this by taking future samples from the sinkholes.
5. Environmental DNA analysis conducted at the same time as the taxonomic studies also identified unique taxa from each of the three lakes in samples from the spring. Three of the four are diatoms.
    a. Jaccard similarity coefficients calculated for genus-level Operational Taxonomic Units (OTUs) revealed substantial similarity between each lake and the spring, however, the lake Jaccard coefficients with the L well are not as high as those with the boil. This may reflect incomplete mixing in the Grand Canyon in which the L well is located.

b. The Jaccard similarity matrix also revealed that Lake Munson is more similar to each of the wells and Wakulla Spring than the other two lakes and that the K cavern and B cavern wells are the most similar to the L well aside from the spring boil. They are likely the principal caverns from which the chlorophyll entering the spring originates. The fact that these samples are more similar to the boil than the lakes may reflect cell mortality during transit to the spring as well as inputs of chlorophyll from one or more additional surface water bodies in the springshed.
c. A class-level heat map for the eukaryote algae for the three lakes, the L well, and the spring boil revealed greater phylogenetic diversity in the L well than the taxonomic analysis, offering further evidence that the 64-µm plankton net used to gather the sample for taxonomic analysis missed many of the non-diatom algae present in the L well.
d. As with the algal taxonomic study, the environmental DNA analysis also found algal taxa in the spring not found in one of the three lakes suggesting additional surface water bodies may be contributing chlorophyll to the green dark water conditions in the spring and/or that algae populations changed during the 30-35 day travel time between the lakes and the spring.
e. Two additional DNA studies will be conducted in Phase III along with the taxonomic analyses. The Phase III environmental DNA study will examine longer 16S rRNA DNA sequences to enable sequencing to species-level OTUs which will allow for more precise definition of the OTU communities of the four lakes and the spring. We also may analyze a different hypervariable region targeted more precisely at the eukaryote algae.

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