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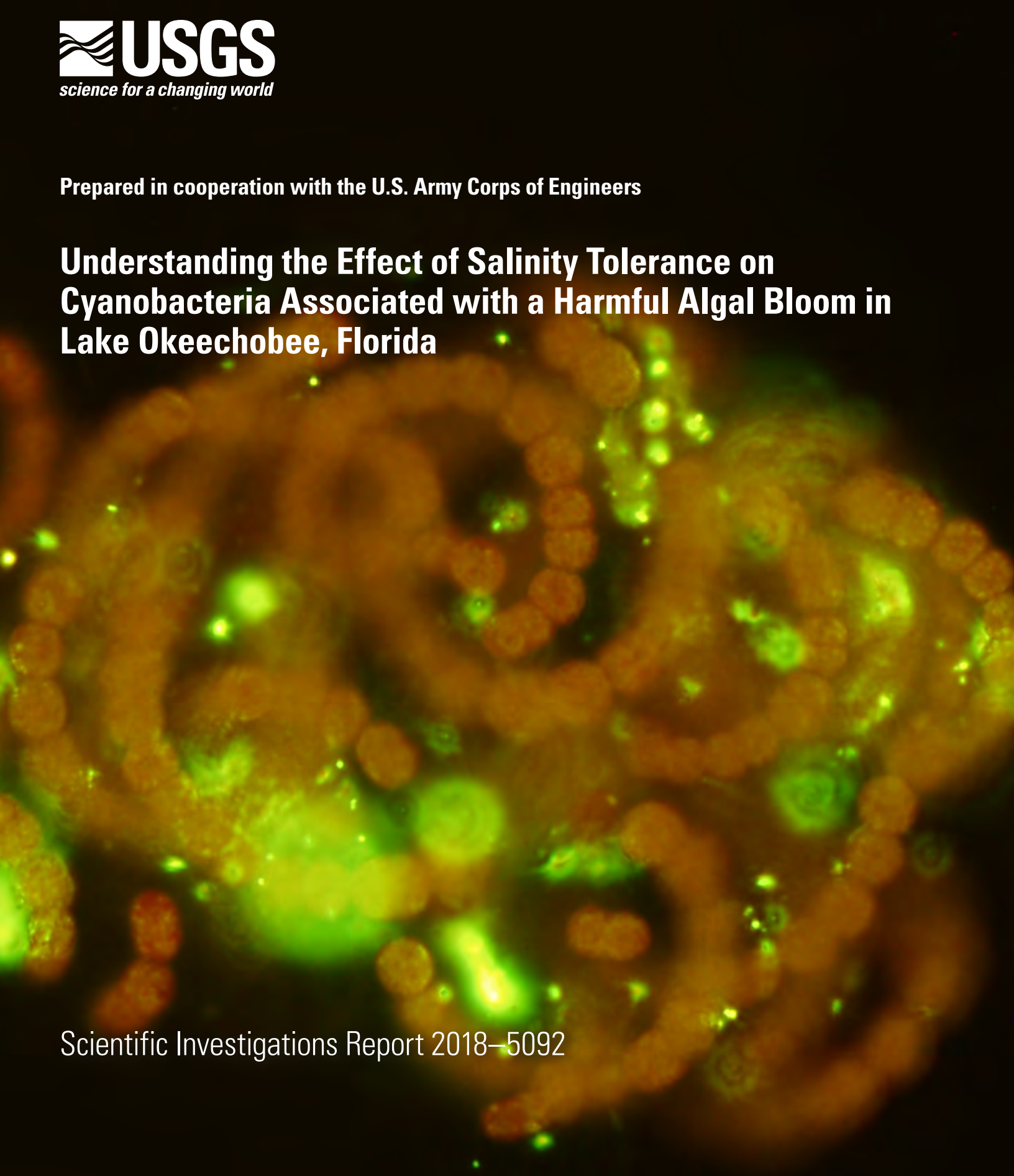
Understanding the Effect of Salinity Tolerance on Cyanobacteria Associated with a Harmful Algal Bloom in Lake Okeechobee, Florida



Scientific Investigations Report 2018–5092

U.S. Department of the Interior
U.S. Geological Survey

Cover. *Dolichospermum circinale* stained with SYTOX® Green. Photograph by Barry H. Rosen, U.S. Geological Survey, July 17, 2015, using an epifluorescence microscope.

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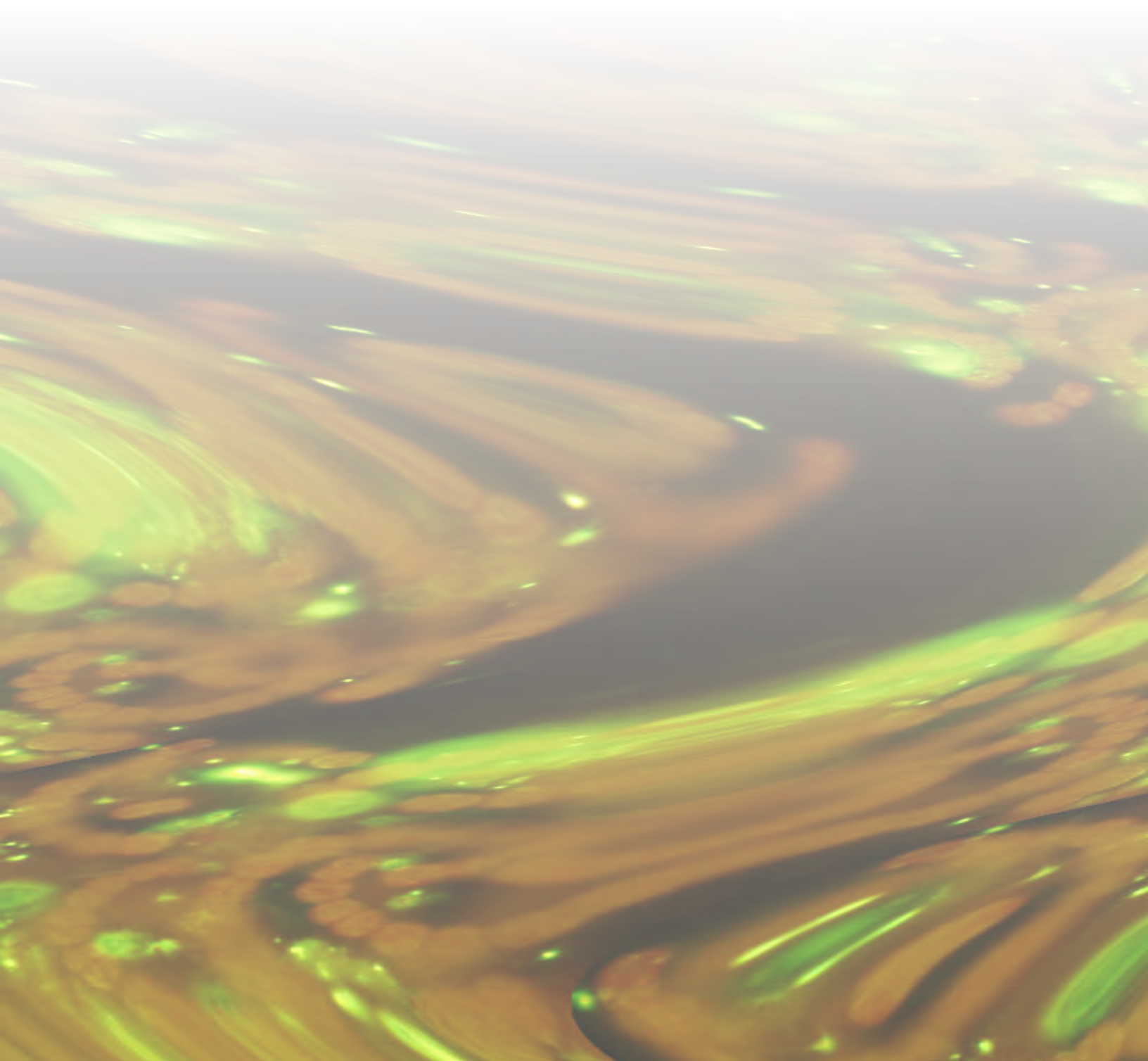

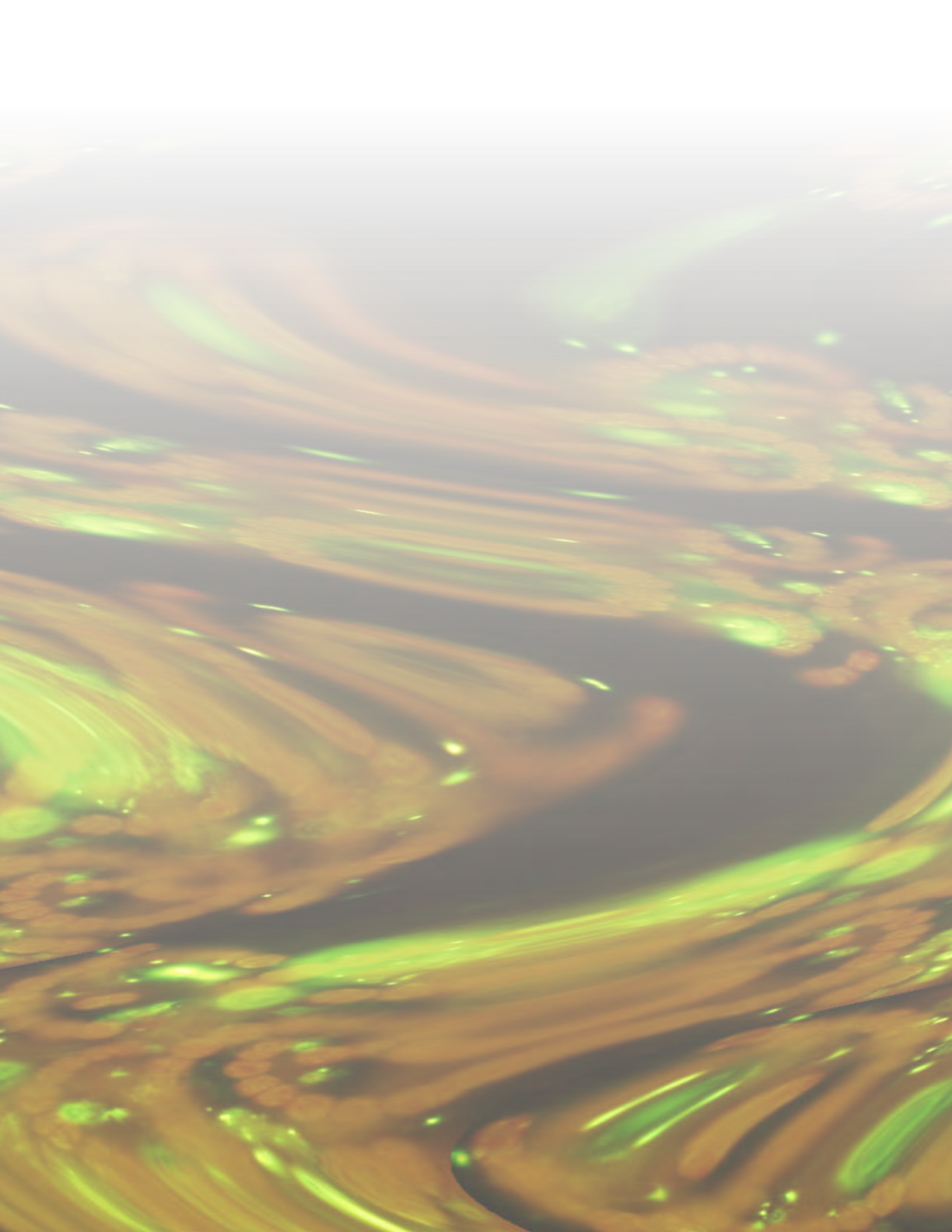

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Conversion Factors

International System of Units to U.S. customary units

Multiply	By	To obtain
	Length	
centimeter (cm)	0.3937	inch (in.)
millimeter (mm)	0.03937	inch (in.)
meter (m)	3.281	foot (ft)
kilometer (km)	0.6214	mile (mi)
kilometer (km)	0.5400	mile, nautical (nmi)
meter (m)	1.094	yard (yd)
	Area	
square kilometer (km^2)	247.1	acre
square kilometer (km^2)	0.3861	square mile (mi^2)
	Volume	
liter (L)	33.81402	ounce, fluid (fl. oz)
liter (L)	2.113	pint (pt)
liter (L)	1.057	quart (qt)
liter (L)	0.2642	gallon (gal)
liter (L)	61.02	cubic inch (in^3)
	Flow rate	
meter per second (m/s)	3.281	foot per second (ft/s)
	Mass	
gram (g)	0.03527	ounce, avoirdupois (oz)

U.S. customary units to International System of Units

Multiply	By	To obtain
	Flow rate	
foot per second (ft/s)	0.3048	meter per second (m/s)

Temperature in degrees Celsius (°C) may be converted to degrees Fahrenheit (°F) as

$$°F = (1.8 \times °C) + 32$$

Supplemental Information

Specific conductance is given in microsiemens per centimeter at 25 degrees Celsius (µS/cm at 25 °C).

Concentrations of chemical constituents in water are given in either milligrams per liter (mg/L) or micrograms per liter (µg/L).

Abbreviations

cells/mL	cells per milliliter
FDEP	Florida Department of Environmental Protection
LM	light microscopy
μWm^{-2}	microwatts per square meter
MCLR	microcystin-LR
mL	milliliters
µL	microliter
µg	microgram
µm	micrometer
NaCl	sodium chloride
PRSD	percent relative standard deviation
psu	practical salinity units
RFU	relative fluorescent units
SFWMD	South Florida Water Management District
USGS	U.S. Geological Survey
UV	ultraviolet
WB	wide blue

Understanding the Effect of Salinity Tolerance on Cyanobacteria Associated with a Harmful Algal Bloom in Lake Okeechobee, Florida

By Barry H. Rosen,[1] Keith A. Loftin,[1] Jennifer L. Graham,[1] Katherine N. Stahlhut,[1] James M. Riley,[2] Brett D. Johnston,[1] and Sarena Senegal[1]

Abstract

In an effort to simulate the survival of cyanobacteria as they are transported from Lake Okeechobee to the estuarine habitats that receive waters from the lake, a bioassay encompassing a range of salinities was performed. An overall decline in cyanobacteria health in salinity treatments greater than 18 practical salinity units (psu) was indicated by loss of cell membrane integrity based on SYTOX® Green staining, but this loss varied by the kind of cyanobacteria present. *Microcystis aeruginosa* was tolerant of salinities up to 18 psu; however, higher salinities caused leaking of microcystin from the cells. *Dolichospermum circinale*, another common bloom-former in this system, did not tolerate salinities greater than 7.5 psu. Stimulation of mucilage production was observed and is likely a mechanism used by both species to protect organism viability. At 7.5 psu, microcystin increased relative to chlorophyll-*a*, providing some evidence of biosynthesis when *M. aeruginosa* is exposed to this salinity. This study indicates that as freshwater cyanobacteria are transported to brackish and marine waters, there will be a loss of membrane integrity which will lead to the release of cellular microcystin into the surrounding waterbody. Additional research would be needed to determine the exact effect of salinity on this relationship.

Introduction

Lake Okeechobee (located at 27º north latitude and 81º west longitude) is a large shallow lake (1,900 square kilometers [km^2]) that receives inflow from the Kissimmee River and other smaller tributaries (fig. 1). The bloom material for this study came from Eagle Bay on the north side of the lake. An earthen dike that surrounds the lake was constructed to control adjacent flooding. When lake stage threatens the integrity of the dike, water is released to the Atlantic Ocean through the St. Lucie Canal and to the Gulf of Mexico through the Caloosahatchee River. Releases follow the Lake Okeechobee Regulation Schedule, developed and implemented by the U.S. Army Corps of Engineers and the South Florida Water Management District (SFWMD), to ensure the lake is at a low enough stage to accommodate summer season runoff and rainfall (U.S. Army Corps of Engineers, 2008).

Lake Okeechobee is classified as a moderately eutrophic (Brezonik and Engstrom, 1998) shallow waterbody that has undergone ecological changes because of external nutrient loading from agriculture (Havens and others, 1996) and, more recently, by internal loading of phosphorus from lakebed sediments (Pollman and James, 2011). Ample phosphorus and other nutrients create the ideal conditions for cyanobacterial blooms and have been documented in the lake since 1970 by the SFWMD (1989). The first large-scale bloom occurred in 1986, was dominated by *Dolichospermum circinale* (formerly *Anabaena circinalis*), and covered approximately 310 km^2.

Some of the conditions that lead to cyanobacterial blooms are warm temperatures, sunlight, water-column stability, and sufficiently high concentrations of nitrogen and (or) phosphorus. While all cyanobacteria need ample phosphorus to thrive, inorganic nitrogen limitation allows a subset of cyanobacteria, those capable of using atmospheric nitrogen, to be more successful. *Dolichospermum* (formerly *Anabaena*) has the ability to fix nitrogen (Rosen and others, 2017), compared to *Microcystis,* a nonnitrogen fixing genus, that needs to acquire nitrogen from inorganic sources.

During the summer of 2016, regulatory discharges from Lake Okeechobee into the Caloosahatchee River and the St. Lucie Canal and Estuary occurred during an extensive cyanobacterial bloom (National Aeronautics and Space Administration, 2016). The Florida Department of Environmental Protection (FDEP), in conjunction with the Florida Fish and Wildlife Commission, reports cyanobacteria species composition and microcystin concentrations weekly in Lake Okeechobee and attending waterways (FDEP, 2018). In late June 2016, a large cyanobacterial accumulation occurred

[1]U.S. Geological Survey.

[2]U.S. Army Corps of Engineers.

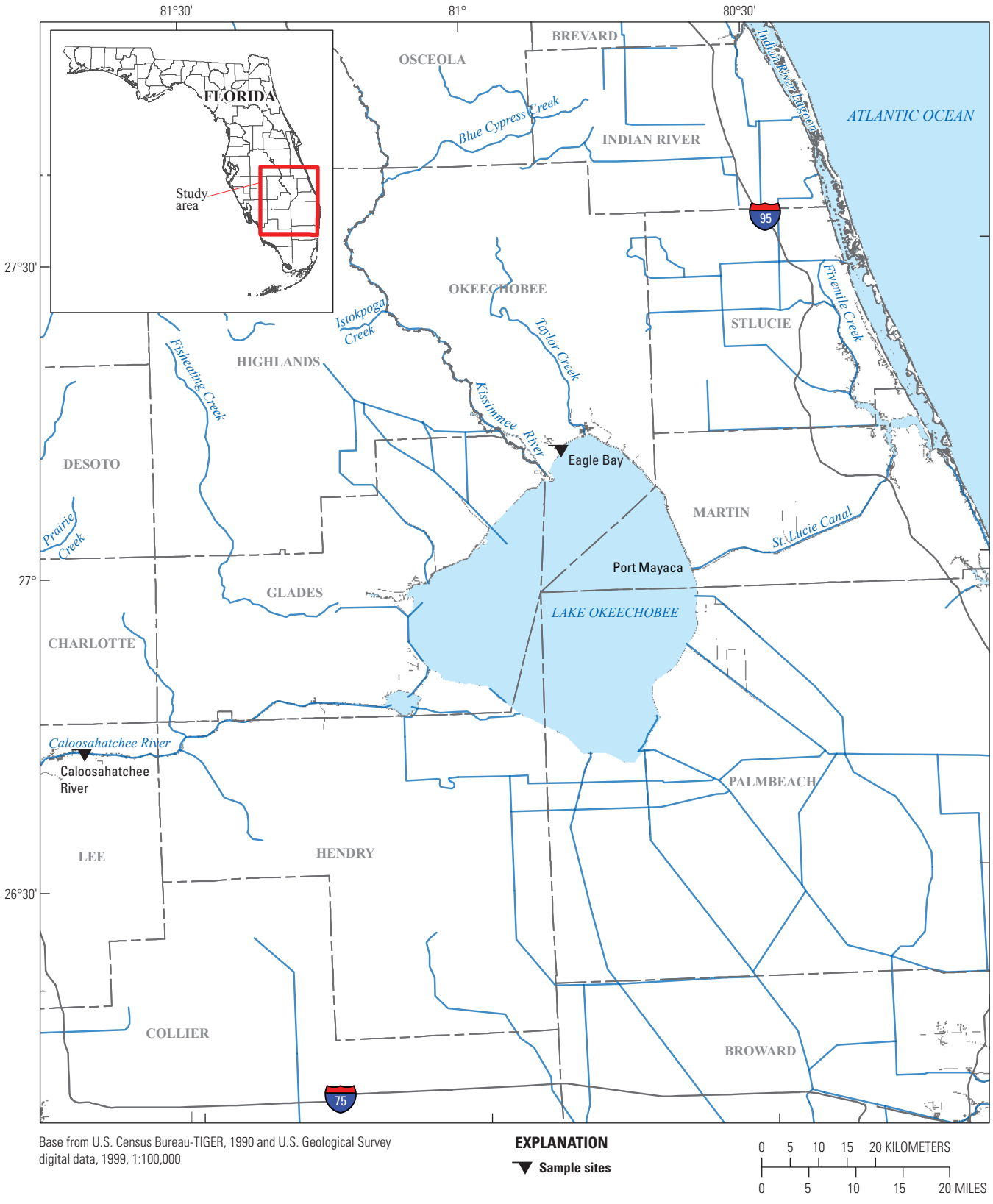


Figure 1. The location of Lake Okeechobee, the tributary system in and out of the lake, and Eagle Bay, where samples were taken for this study, July 7, 2017.

in the St. Lucie Estuary, prompting the Governor of Florida to issue a state of emergency for three affected counties. The FDEP data indicated that Lake Okeechobee and (or) the canal system had existing populations of cyanobacteria (FDEP, 2017) that were likely transported downstream when water was discharged from Lake Okeechobee. Local and coastal basin nutrient runoff, with cyanobacterial proliferation in the backwater areas, also contributed organisms to estuarine and marine habitats, mainly in the St. Lucie Estuary and Indian River Lagoon. In 2005, a similar event occurred with a large cyanobacteria population in the St. Lucie Estuary, which occurred during regulatory discharges from Lake Okeechobee (Phlips and others, 2012).

Salinity tolerance determines how long cyanobacteria originating from freshwater habitats will survive at the salinities typical of estuarine and marine environments (Batterton and Van Baalen, 1971; Orr and others, 2004; Tonk and others, 2007), and collectively, these studies indicate that many freshwater cyanobacteria experience mortality at salinities between 15 and 21 practical salinity units (psu). In the Dutch delta and its associated impoundments, such as Lake Volkerak in the Netherlands, water management strategies have been developed for *Microcystis* blooms (Verspagen and others, 2016) based on salinity tolerance in their systems.

Using cyanobacteria isolated from Lake Okeechobee, the U.S. Geological Survey (USGS), in cooperation with the U.S. Army Corps of Engineers, conducted a 4-day laboratory bioassay with four different salinity treatments and a control. Response variables included morphological changes to cyanobacterial filaments and colonies, changes in growth rate, physiological indicators, intracellular and extracellular (released) microcystin, and an approximation of cell membrane integrity. Collectively, these data provide converging lines of evidence about how Lake Okeechobee cyanobacteria might respond to increased salinity under natural conditions as water mixes in downstream estuaries. Additional data related to this study can be found in King and others (2018a, b).

Methods

The organisms used for the study were collected from Lake Okeechobee and brought into the laboratory in Orlando, Fla., for this study.

Initial Bloom Material

Water and floating bloom material were collected from the upper 10 cm of the water surface at noon on July 7, 2017, from Eagle Bay, Lake Okeechobee, Fla. (latitude 27°11'42.19"N, longitude 80°49'46.24"W). Approximately 35 liters (L) of water and bloom material (fig. 2) were collected in two large carboys and immediately transported to the laboratory, which was approximately 3 hours away, without being cooled or held in the dark. All water and bloom materials were blended and mixed thoroughly in the laboratory and distributed to eighteen 1-L glass graduated cylinders (fig. 3). The 1-L graduated cylinders were used to simulate a water column approximately 35 centimeters (cm) in depth to allow the diurnal migration typically exhibited by planktonic cyanobacteria. Cylinders were incubated in a west-northwest window, and they were treated as batch reactors. Daily temperature and solar irradiance was recorded by using a Li-Cor LI-200/R pyranometer. Average temperature ranged from 23 to 26 degrees Celsius (ºC), and light readings yielded 8.9 microwatts per square meter (μWm^{-2}; plus or minus one standard deviation).

Approximately 20 hours after collection, triplicate cylinders were dosed with sodium chloride (NaCl) to increase salinity by 7.5, 10, 15, or 18 g L^{-1} to yield 7.5, 10, 15, or 18 psu; NaCl was not added to three control cylinders. The time zero samples were taken just prior to dosing the cylinders with the NaCl. Time one began after 24 hours of exposure, at approximately 9:00 every morning. The water in the cylinders was thoroughly mixed by completely inverting the cylinders 5 times, and the volume needed for all the analytical procedures was quickly poured from the cylinder. During the time period of the experiment, no attached algae grew on the cylinder walls. Although not part of the original planned bioassay, some of the original sample was placed into water with higher salinities (20, 25, 30, and 35 psu) in graduated cylinders 2 days after the main bioassay was started. The assessment of these treatments was limited to microscopic observations and physiological assessment.

Biomass Indicator—Chlorophyll-*a*

Chlorophyll provided an overall indication of algal biomass. Ten-milliliter (mL) subsamples collected from each cylinder daily were filtered as described in Hambrook Berkman and Canova (2007), stored (frozen with desiccant), and quantified by using a modification of U.S. Environmental Protection Agency Method 445.0 (Arar and Collins, 1997). Instead of acetone extraction, samples were extracted in heated ethanol (Sartory and Grobbelar, 1986), and the fluorometer was modified with a flow-through cell (Knowlton, 1984). Samples were analyzed in duplicate, and the results were reported as an average.

Biomass Indicator—Cell Numbers

A 10-milliliter (mL) sample from each of the 18 cylinders was collected by 10-mL graduated pipette dispensed into a 15-mL screw-cap plastic centrifuge tube, preserved with 100 microliters (µL) of Lugol's iodine, and refrigerated at 4 ºC until it was processed. For counting, the samples were homogenized by vigorous shaking to disperse the colonies, an aliquot was withdrawn, and the precise weight was determined (x 0.0001 gram [g]) with an Ohaus Explorer EX224 Analytical Electronic Balance. The weight was considered equivalent to the volume (1 mL = 1 g) and evenly distributed under a

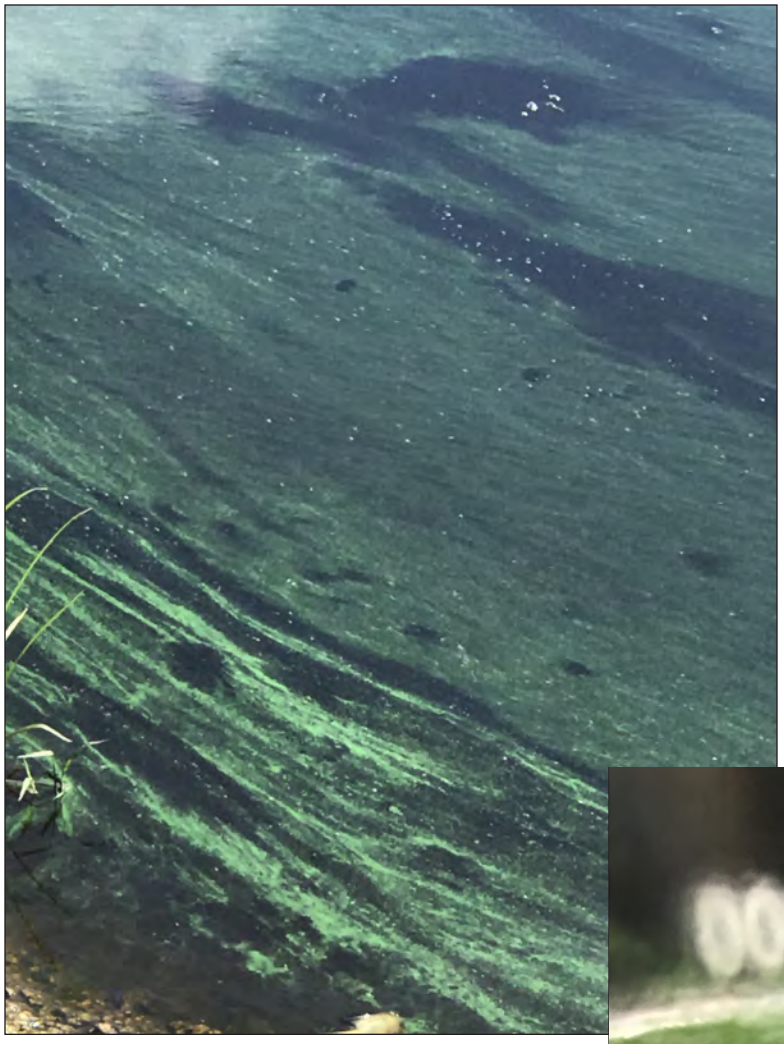


Figure 2. Area where water and bloom materials were collected for bloom bioassay, 2017 Eagle Bay, Lake Okeechobee, Florida. (Photograph by Barry H. Rosen, U.S. Geological Survey.)

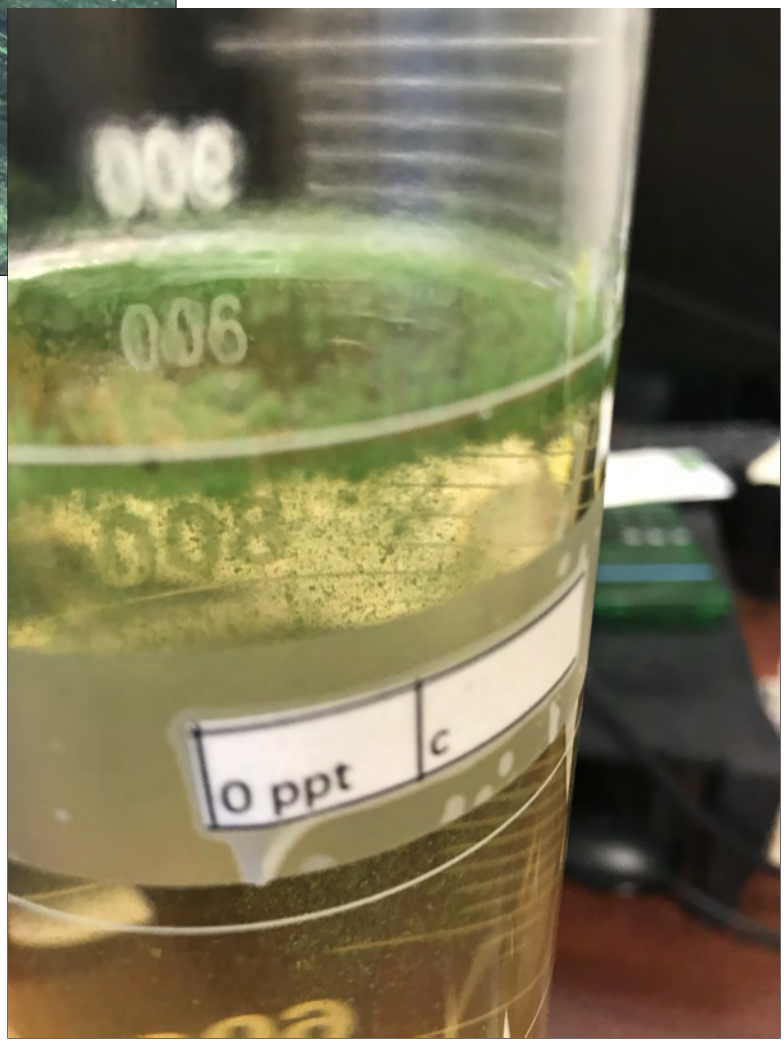


Figure 3. A 1-liter graduated cylinder with initial bloom material. (Photograph by Barry H. Rosen, U.S. Geological Survey.)

22-square-millimeter (mm^2) glass coverslip. Counting was conducted microscopically at 400x by enumerating the cyanobacteria observed in a linear strip along the full length of the coverslip. The diameter of the strip was measured with a stage micrometer. Using the volume distributed under the coverslip per unit area, the number of organisms per unit volume was calculated. For *Microcystis*, its dense, colonial form does not allow an exact direct cell count. We were able to enumerate the number of colonies and optically image a subset of colonies to obtain an average number of cells per colony. This average number of cells per colony was multiplied by the number of colonies found in each sample to get estimated cell count.

Live Organism Physiological Assessment

Approximately 0.020 mL of live material taken from each salinity treatment was examined daily to assess the health of cells by following the protocol of Rosen and others (2010). Microscopic observations were made with a BX51 Olympus microscope with differential interference contrast and epifluorescence using ultraviolet and wide blue excitation sources. SYTOX® Green (Invitrogen, Carlsbad, Calif.), a deoxyribonucleic acid (DNA) stain excluded from live cells, was added (Rosen and others, 2010) to assess if cells lost cellular membrane integrity.

Microcystin Analysis by Enzyme-Linked Immunosorbent Assay

For analysis of total microcystin, a 10-mL subsample was taken daily from each cylinder and frozen. For analysis of dissolved microcystin, the filtrate (Millipore Type TSTP 3.0-micrometer [µm], 25-millimeter [mm] diameter) from a 10-mL subsample was collected daily from each cylinder and frozen. These samples were processed and analyzed as described in Loftin and others (2016), except that an Abraxis streptavidin amplified enhanced sensitivity (SAES) enzyme-linked immunosorbent assay kit was used with a 5-psu sodium chloride microcystin-LR (MCLR) calibration curve custom made by Abraxis, Inc. (Warminster, Pa.). A four-parameter curve-fit was used for calibration. Measurements of kit diluent and laboratory reagent water were below the kit minimum reporting level (0.10 microgram per liter [µg/L]), 0.75-µg/L MCLR kit controls were analyzed every 10th sample, and 28 percent of samples were laboratory replicates. Mean kit control recovery was 106 percent, mean percent relative standard deviation (PRSD) was 9.6 percent, and laboratory replicate PRSD was 20 percent. Particulate microcystin was calculated by subtracting dissolved concentration from total concentration.

Additional Studies

The influence of circulation was evaluated in unamended water and bloom material. Three 4-L glass beakers were filled with 2.5 L of water and bloom material, set atop magnetic stir plates, and separately circulated at velocities of 0.00 foot per second (ft/s; "nonstirred"), 0.27 ft/s, and 0.95 ft/s. The beakers were sealed with plastic to prevent evaporation. Velocity was measured with a USGS pygmy current meter by using the 0.6-depth method (Turnipseed and Sauer, 2010). High-frequency sensor data were collected at 5-minute intervals. Data were collected by using a multiparameter sonde (Xylem/YSI EXO2), calibrated in accordance with manufacturer protocols (YSI Incorporated, 2017) to measure water temperature, specific conductance, dissolved oxygen, pH, chlorophyll-*a* fluorescence, and phycocyanin fluorescence. The high-resolution visualization resulting from these data provides insight into the biogeochemical processes in relation to the results from less frequently collected discrete analyses (Downing and others, 2017).

Results

The majority of the results center on the salinity bioassay conducted; however, ancillary data were collected from the same water and bloom material to provide some understanding of how stirred conditions affect physio-chemical parameters.

Initial Bloom Material

The bloom material collected from Lake Okeechobee on July 7, 2017, contained three species of *Microcystis*: *M. aeruginosa*, *M. flosaquae*, and *M. wesenbergii*. *Microcystis aeruginosa* was the most frequently observed species of this genus. Also present were *Dolichospermum circinale*, *Planktolyngbya contorta, Planktolyngbya limnetica,* and *Cuspidothrix tropicalis* (fig. 4). Microcystin concentration in the surface scum collected directly from Lake Okeechobee was 560 µg/L, indicating the presence of microcystin-producing species.

Although rare in the initial community of organisms, two additional cyanobacteria genera grew during the experimental treatments. *Planktothrix* was found on day eight of the experiment in water with salinity of 15 psu, and *Cuspidothrix tropicalis* was found on day four in water with salinity up to 18 psu.

Biomass Indicators

Chlorophyll-*a* concentrations and cyanobacterial abundance were used to quantify changes in algal biomass during the bioassay (figs. 5–7). For chlorophyll-*a*, all concentrations declined from day zero to day four, with the

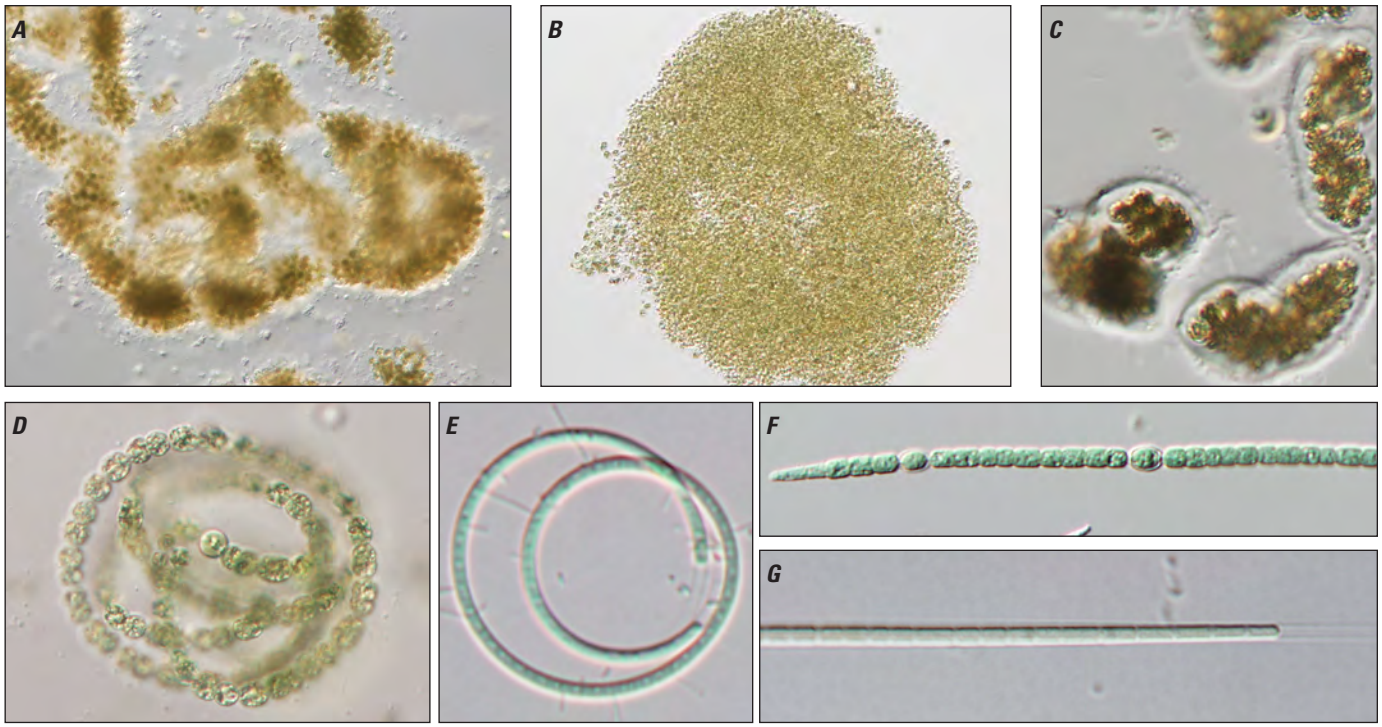


Figure 4. The initial cyanobacterial community in the water sample collected July 7, 2017, from Eagle Bay, Lake Okeechobee, Florida. *A, Microcystis aeruginosa* (most abundant); *B, M. flosaquae*; *C, M. wesenbergii*; *D, Dolichospermum circinale*; *E, Planktolyngbya contorta*; *F, Cuspidothrix tropicalis*; *G, Planktolyngbya limnetica*.

greatest declines in the control (0 psu) and 7.5-psu treatments (fig. 5). The 10, 15, and 18 psu treatments showed greater amounts of chlorophyll-*a* than the lower salinity treatments.

Microcystis aeruginosa and *Dolichospermum circinale* were the dominant cyanobacteria in the initial community used for the bioassay. For *Microcystis*, all of the treatments showed an initial increase in the number of cells during the first time interval from time zero through the first 24 hours, and then the number of cells decreased during the next 24-hour time period (fig. 6), including the control treatment with no added salt. Because the bloom collected from Eagle Bay had been thriving in full sunlight, this adaptation to a laboratory setting was an anticipated response.

Dolichospermum circinale abundance in the control and all of the treatments substantially decreased during the first 24 hours of the bioassay (fig. 7).

Microcystin

Microcystin was reported as a total concentration in whole water and as a dissolved phase in the water after the cells were removed by filtration. Total concentration (fig. 8*A*) decreased to less than 20 µg/L in the control. The 7.5- and 10-psu salinity treatments initially declined but increased to more than 40 µg/L by the end of the 4-day bioassay.

The dissolved phase (fig. 8*B*) is a combination of microcystin leaked from live or unhealthy cells and from those cells that died and released microcystin. In the control, the amount of dissolved-phase microcystin remained less than 2 µg/L, with the largest amounts in the 15- and 18-psu treatments (12–13 µg/L) and in the 7.5- and 10-psu treatments (8–10 µg/L). At all the salinities greater than the control, the amount of dissolved microcystin increased over time (fig. 8*B*), indicating that some of the cells in the treatments were leaking cellular microcystin and cell lysis was occurring. The partitioning of microcystin between the amount retained in cells (calculated particulate) and the dissolved phase (fig. 8*C*) has a similar pattern as the total microcystin (fig. 8*A*), with more retained or produced in the 7.5- and 10-psu treatments.

The calculated particulate microcystin to chlorophyll-*a* ratio was examined (fig. 8*D*) to normalize the toxin concentration to a cell abundance surrogate (chlorophyll-*a*). Cell abundance was not used for this calculation because chlorophyll-*a* was a more precise measurement then cell abundance, given the difficulty and potential variability in estimating cell numbers. In addition, particulate microcystin and particulate chlorophyll-*a* are both intracellular constituents. Using this calculation, by day four, the 7.5- and 10-psu treatments showed an increase in this ratio that is approximately double the ratios in the 15- and 18-psu treatments.

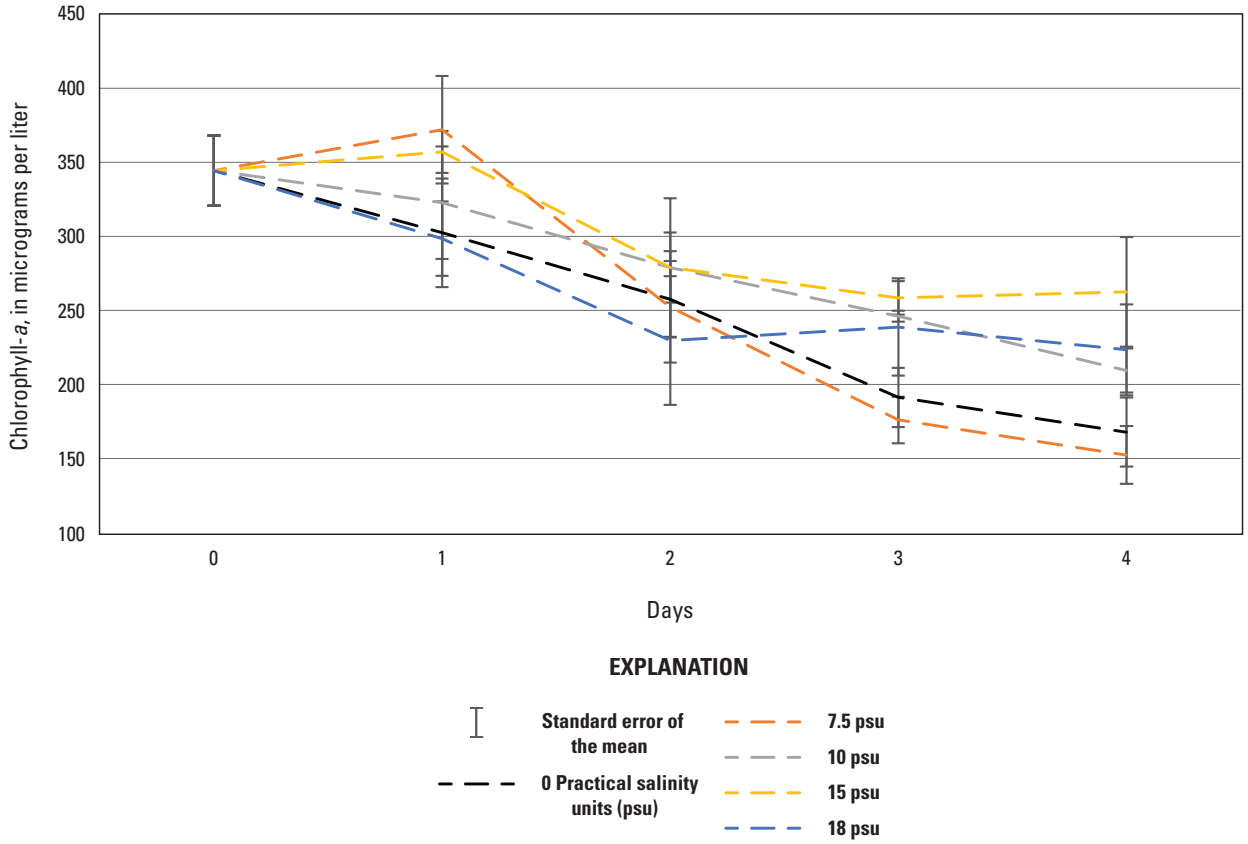


Figure 5. Chlorophyll-*a* concentrations during the bioassay.

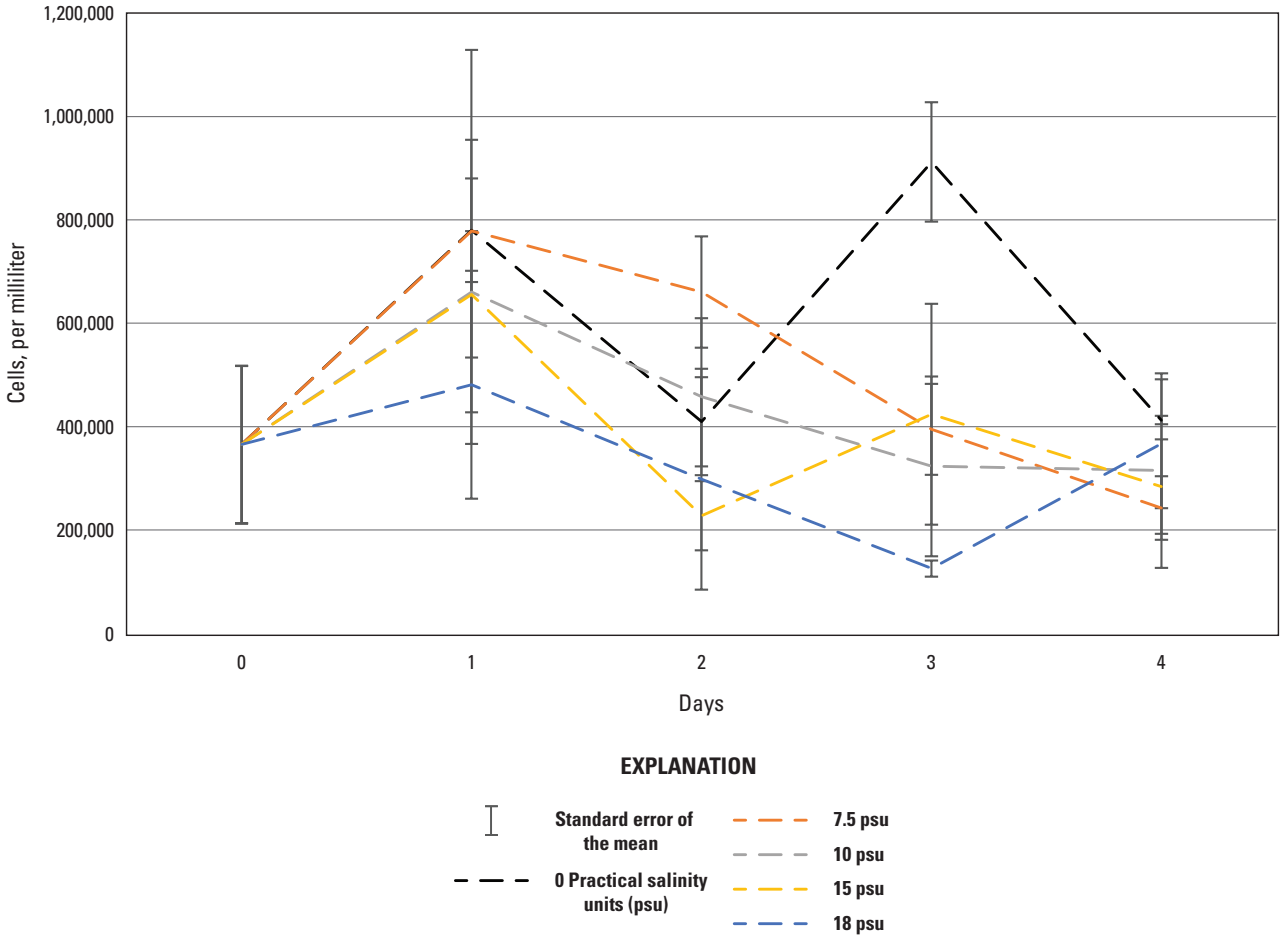


Figure 6. *Microcystis aeruginosa* abundance in response to salinity. The error bars are standard error of the mean.

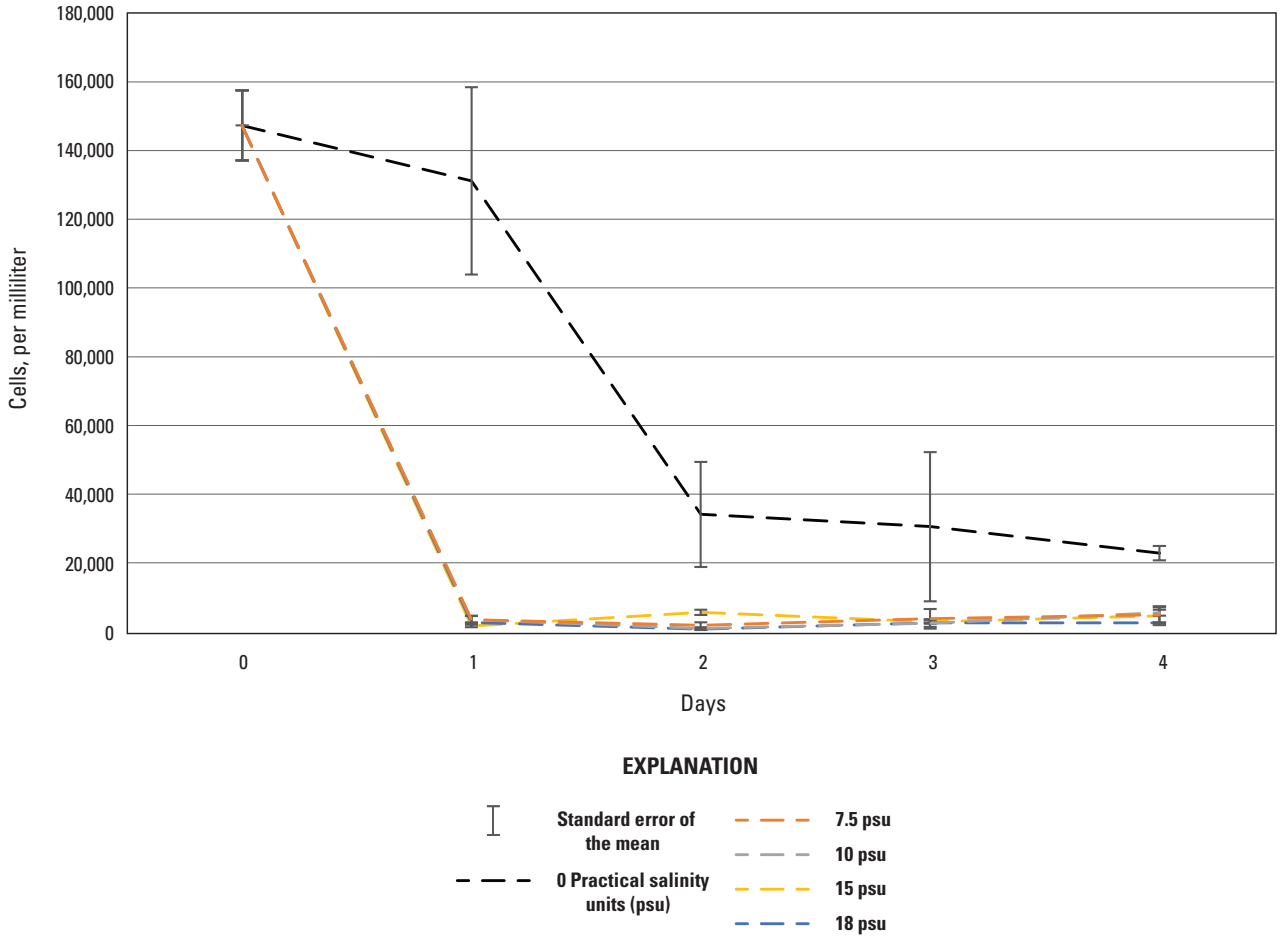


Figure 7. *Dolichospermum circinale* abundance in response to salinity. The error bars are standard error of the mean.

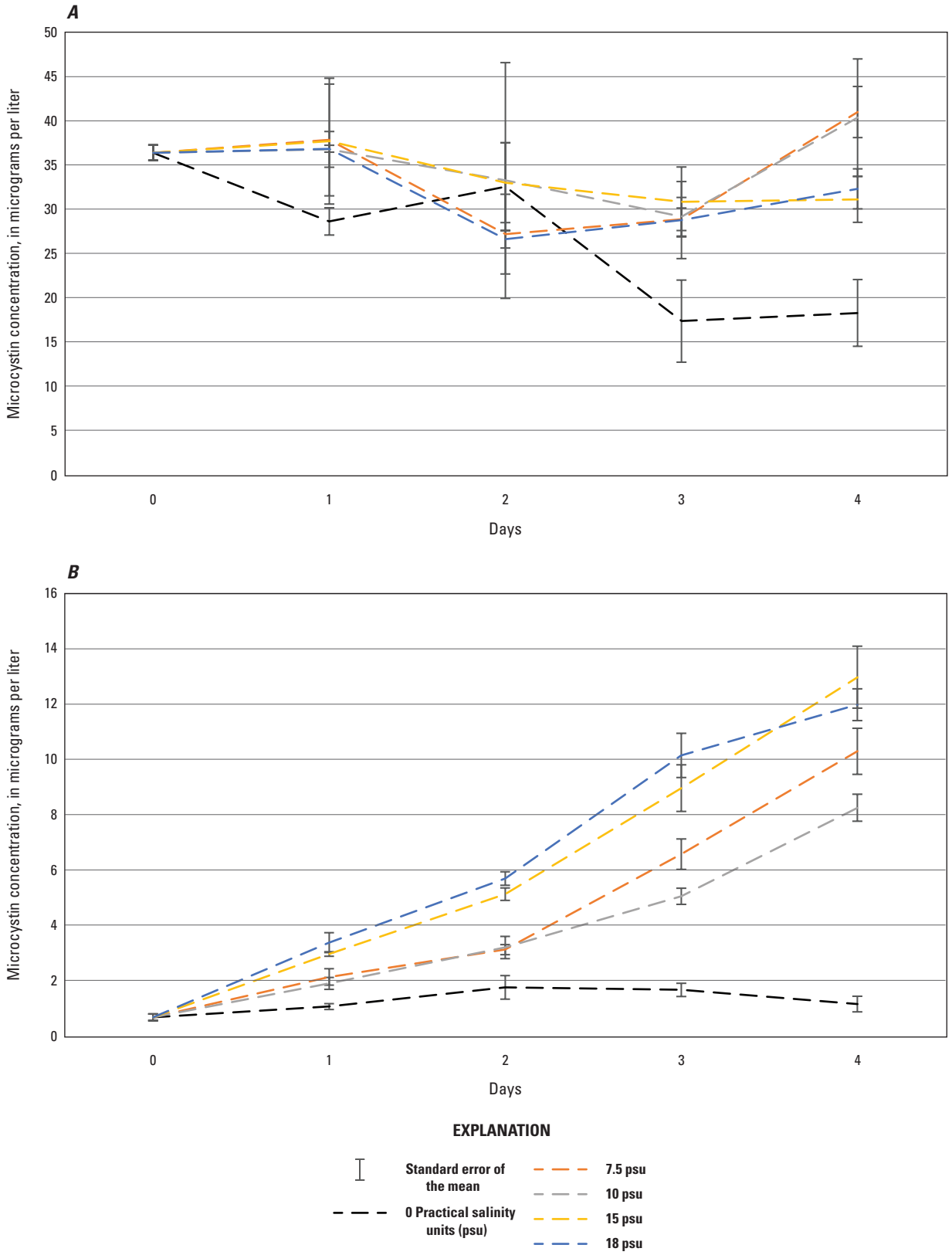


Figure 8. Microcystin response to salinity: *A*, total microcystin; *B*, dissolved phase; *C*, calculated particulate; *D*, calculated particulate microcystin:chlorophyll-*a* ratio. The error bars are standard error of the mean.

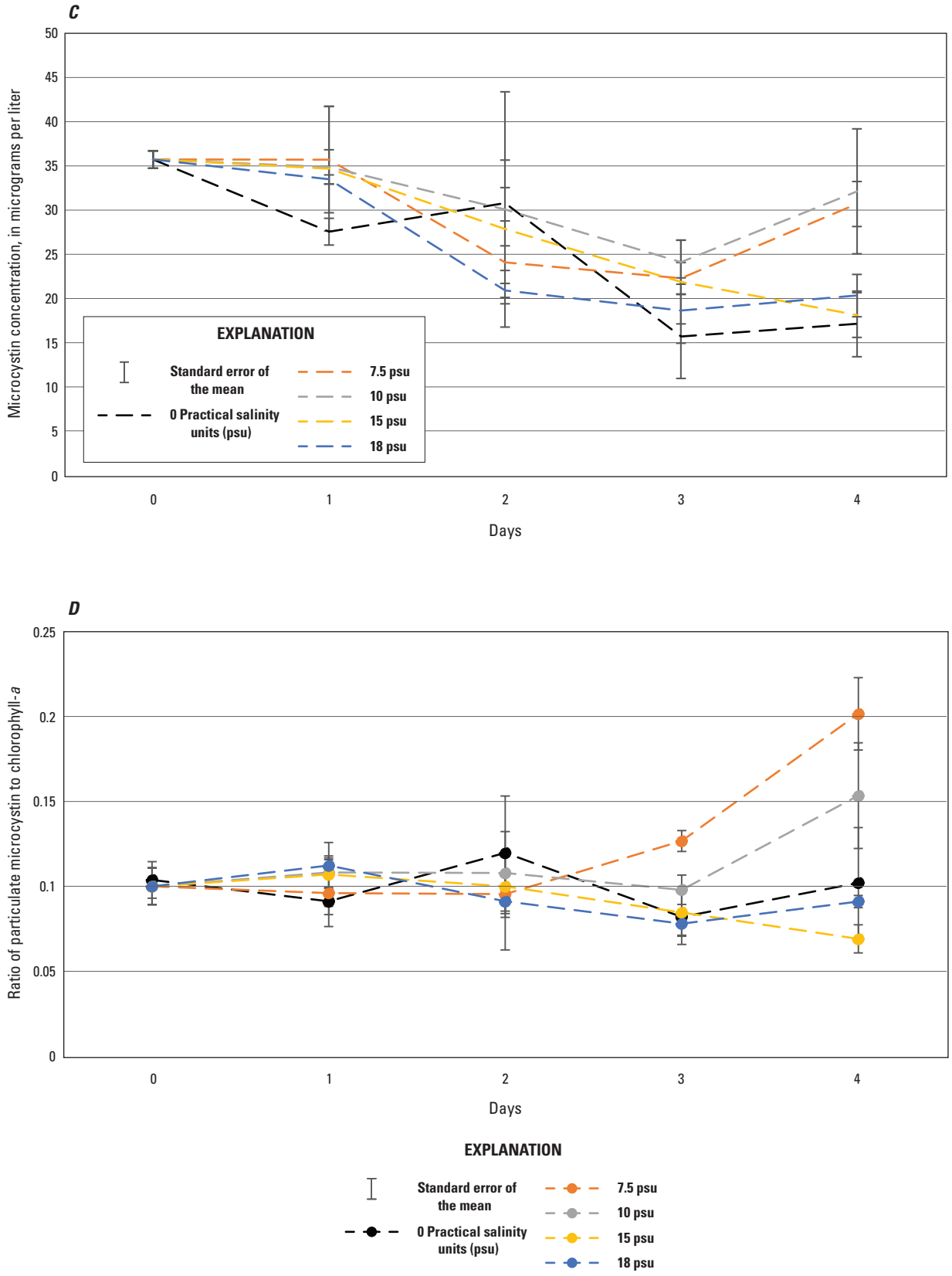


Figure 8. Microcystin response to salinity: *A*, total microcystin; *B*, dissolved phase; *C*, calculated particulate; *D*, calculated particulate microcystin:chlorophyll-*a* ratio. The error bars are standard error of the mean.—Continued

Physiological Response to Salinity

Overall, the *Microcystis aeruginosa* colonies maintained integrity during the first 24 hours of exposure at salinities up to 25 psu (fig. 9; 30 and 35 psu were not photographed at this time interval). After 4 days, complete disintegration of all *Microcystis aeruginosa* colonies did not occur, even up to 35 psu (fig. 10). Although most colonies remained intact, a few disintegrated to small clusters and individual cells (fig. 11).

Dolichospermum circinale was much less abundant, leaving a less complete picture of salinity effects. Intact filaments were present after 1 day; however, the cells seem to be separating in the 20- and 25-psu treatments (fig. 12). After 2 days of exposure, *Dolichospermum* has additional separation of cells at higher salinity (fig. 13). *Dolichospermum circinale* was tolerant of 7.5 psu and was in good condition after 8 days (fig. 14), but was not found at any salinity greater than 7.5 psu by the end of the bioassay.

In addition to overall morphological changes in colonies and filaments of these two genera of cyanobacteria, evidence of adaptation was observed microscopically. The production of mucilage was observed enveloping the filaments and the colonies (fig. 15).

Physiological Response as Determined by SYTOX® Green Visualization

Cell health was analyzed by using epifluorescence microscopy in conjunction with SYTOX® Green, a DNA stain that emits green light in the presence of DNA. If the cellular membrane of an organism is intact, SYTOX® Green does not penetrate, providing an indication of cell health. In figures 16–22, the denotation is (1) LM—organisms illuminated with differential interference light microscopy for overall cell and colony structure; (2) UV—organisms illuminated with ultraviolet light, with variations in pigment color an indicator of cell health; (3) WB—organisms illuminated with "wide blue" as the baseline color before adding SYTOX® Green; (4) SYTOX® Green—organisms stained with this dye are bright green when DNA is present (Rosen and others, 2010). The photographs are representative of the overall condition of the colonies or filaments in the treatments.

After 1 day of exposure, the SYTOX® Green is only seen outside of the *Microcystis* colony, with no penetration of the stain into the cells in all treatments (fig. 16). After 2 days of exposure, the SYTOX® Green penetrated the *Microcystis* cells in the 18-, 20-, and 25-psu treatments as seen in figure 17 where the cells are green compared to the 15-psu treatment. After 3 days of exposure, the SYTOX® Green is seen only external to the *Microcystis* colonies, with no penetration of the stain into the cells in all treatments, with the exception of the 15-psu treatment (fig. 18). In the 15-psu treatment, the SYTOX® Green penetration and the pigment shift to yellow under UV indicate that this colony was no longer viable. After 4 days of exposure, with one of two *Microcystis* colonies in the 15-psu treatment and all of the colonies in treatments of 20 psu or greater salinity, the SYTOX® Green penetration and the pigment shift to yellow under UV indicate that these colonies were no longer viable (fig. 19). After 8 days of exposure, the 18-psu treatments had some *Microcystis* colonies that were still viable and some that were not, while all lower salinity treatments had viable colonies (fig. 20).

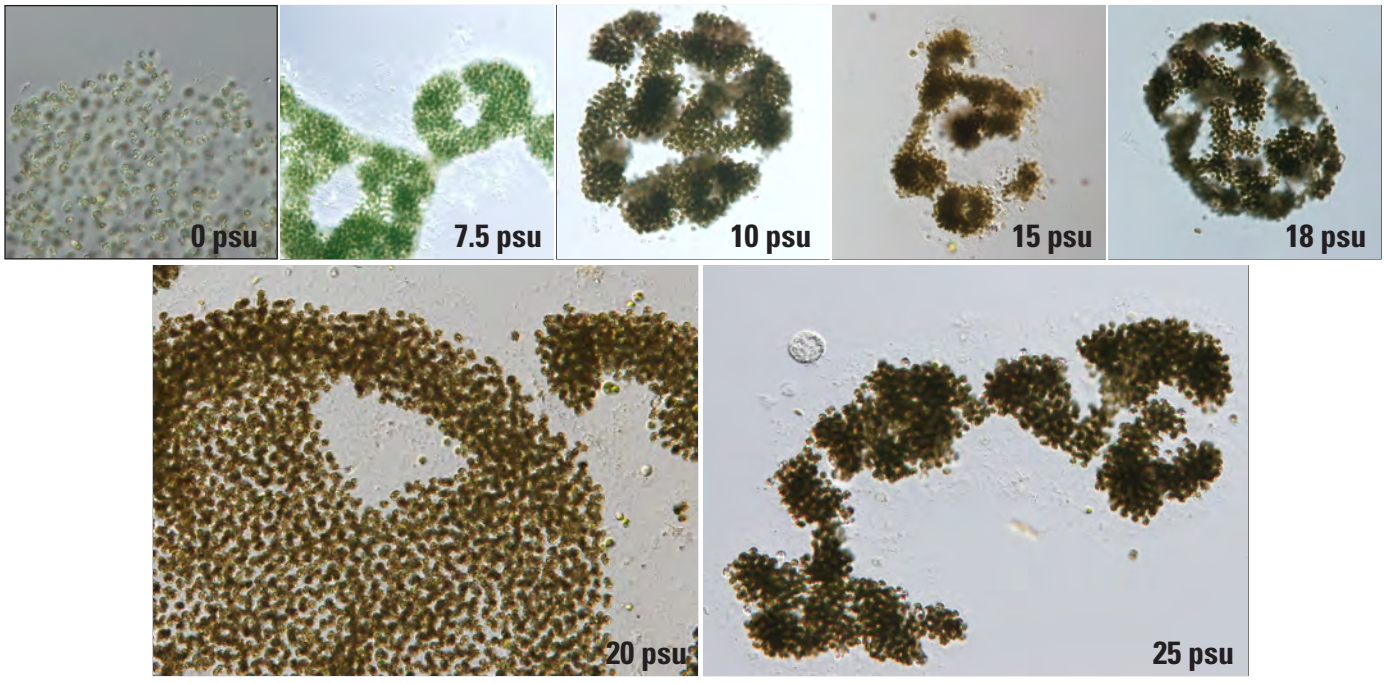


Figure 9. Colony morphology of *Microcystis aeruginosa* in various salinity treatments after 1 day of exposure.

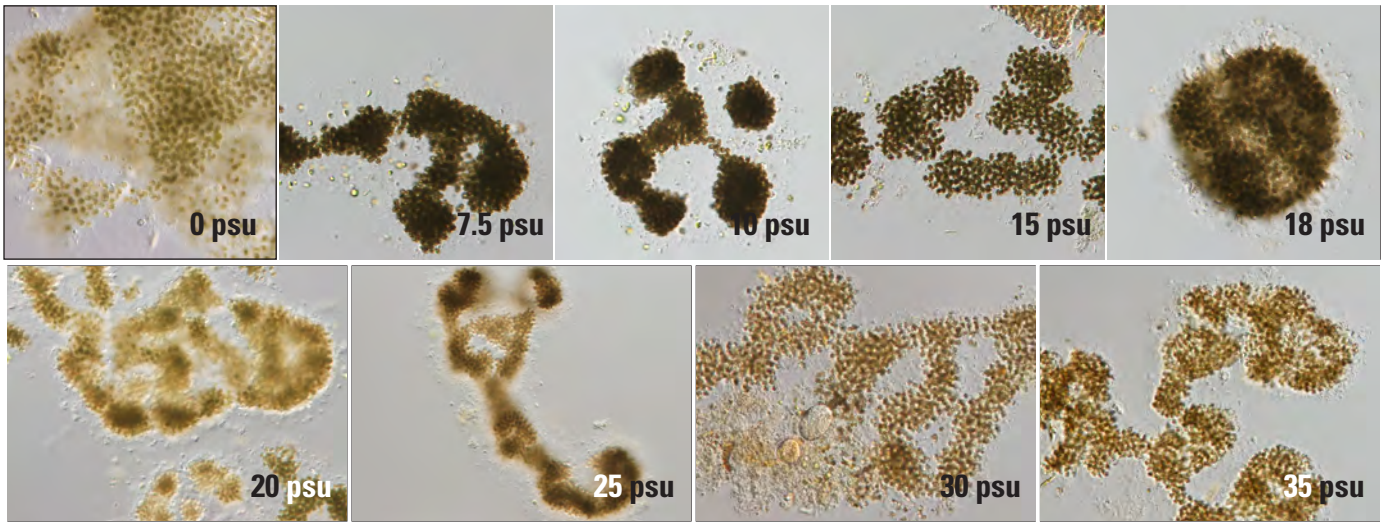


Figure 10. Colony morphology of *Microcystis aeruginosa* in various salinity treatments after 4 days of exposure.

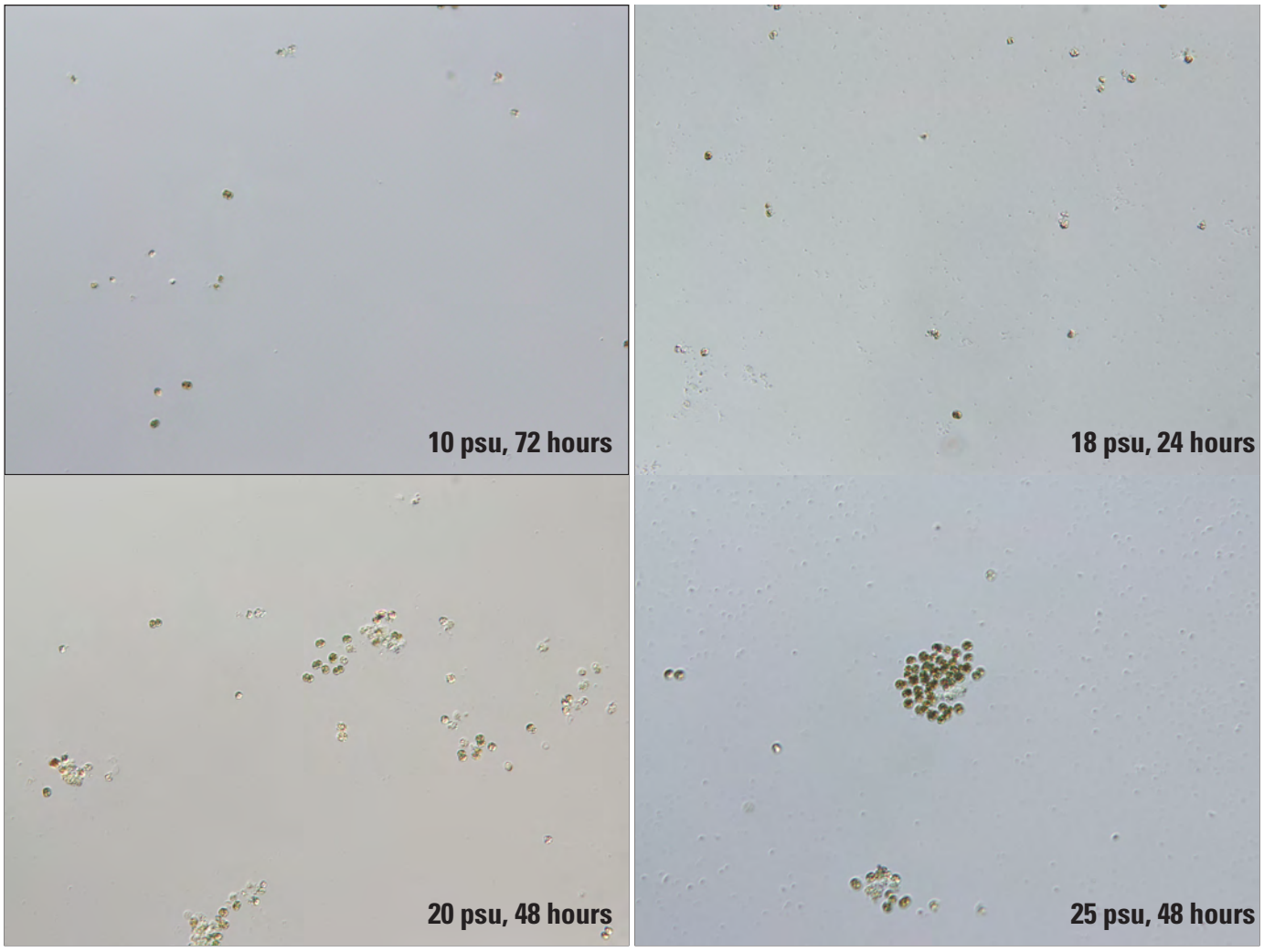


Figure 11. Disintegration of some of the *Microcystis aeruginosa* colonies in salinity treatments greater than 10 psu after 1 or more days of exposure.

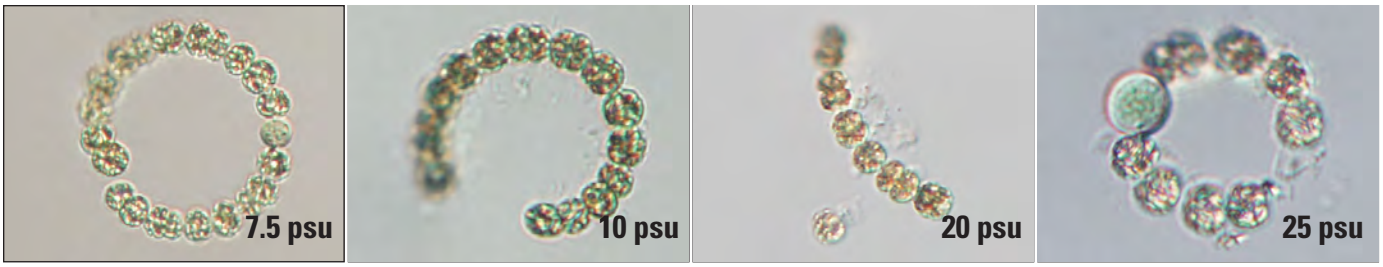


Figure 12. Response of *Dolichospermum circinale* filament morphology to salinity after 1 day of exposure.

Figure 13. Response of *Dolichospermum circinale* filament morphology to salinity after 2 days of exposure.

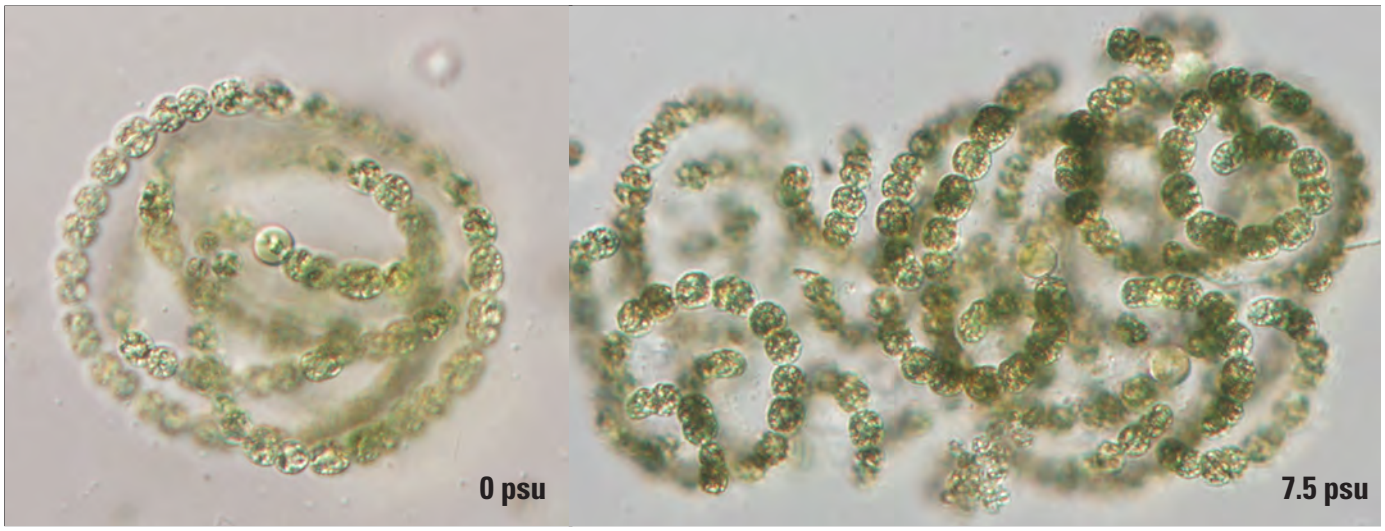


Figure 14. Response of *Dolichospermum circinale* filament morphology to salinity after 8 days of exposure.

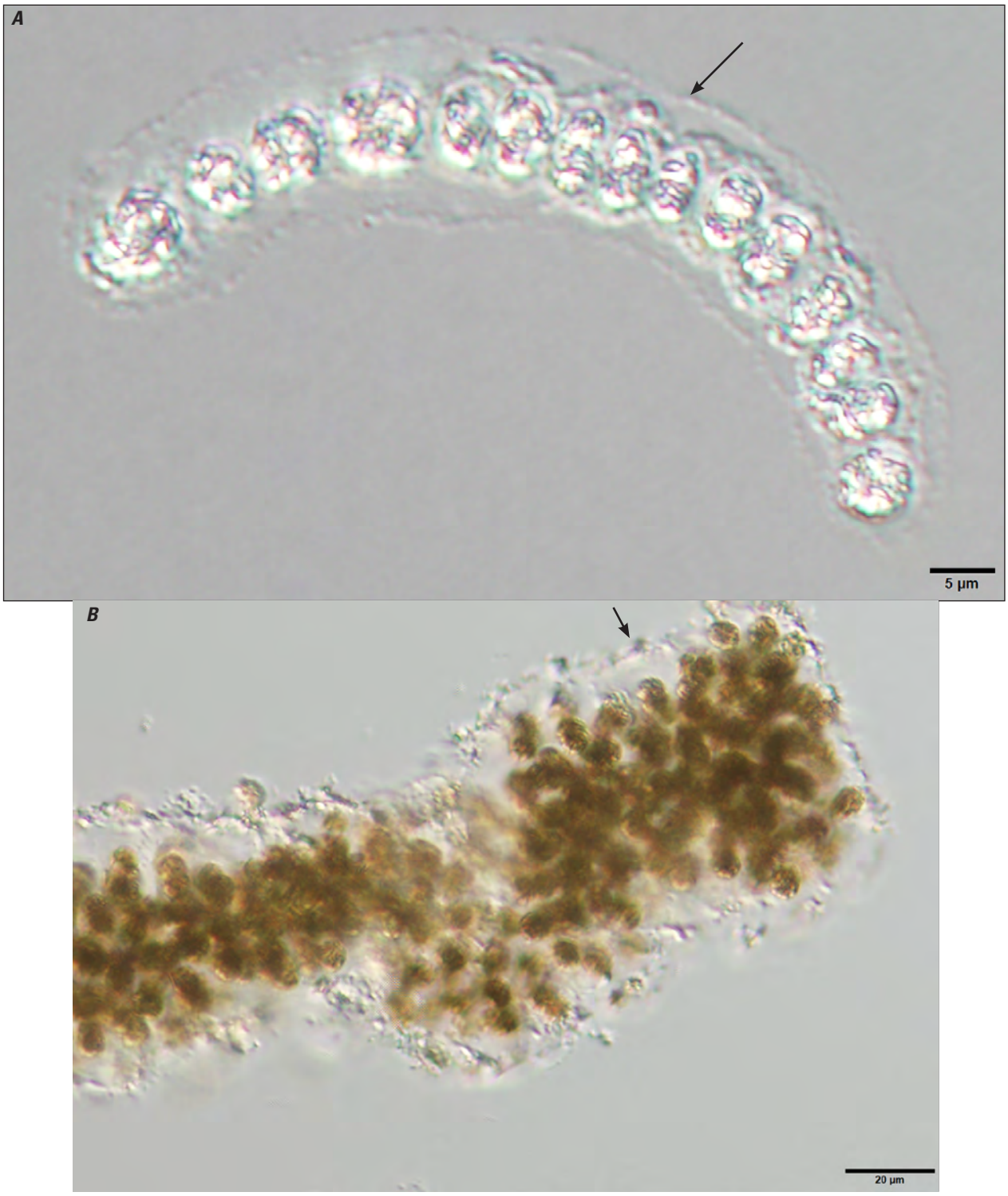


Figure 15. Extracellular mucilage, at arrows, in *A, Dolichospermum circinale*, day four at 25 psu; *B, Microcystis aeruginosa*, day three at 15 psu.

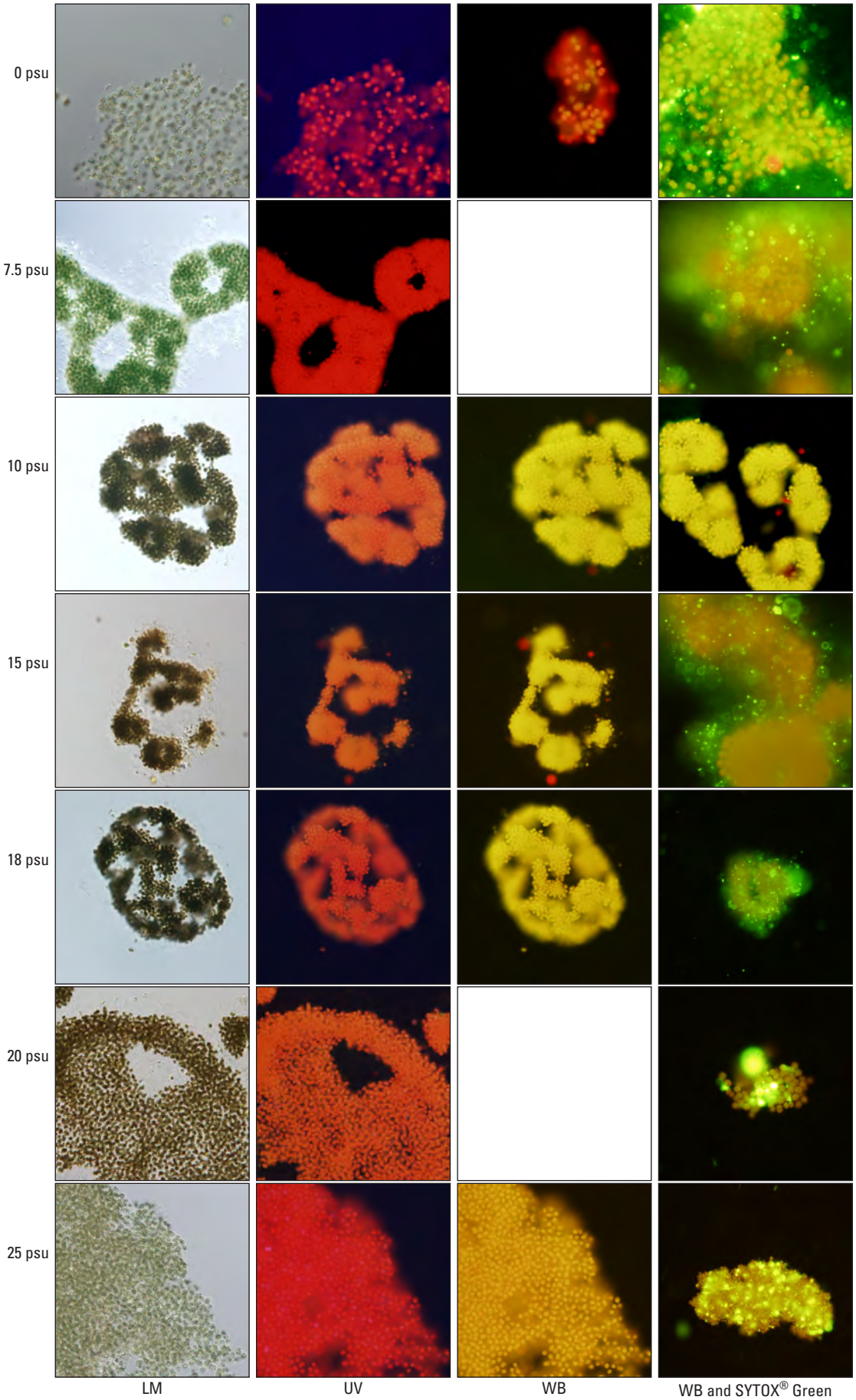


Figure 16. Response of *Microcystis aeruginosa* after 1 day of exposure to treatments with different salinities (psu, practical salinity units; LM, differential interference microscopy; UV, epifluorescence microscopy; WB, wide-blue epifluorescence microscopy; WB and SYTOX® Green, wide-blue microscopy with the DNA stain SYTOX® Green added. Blank boxes indicate that data were not available).

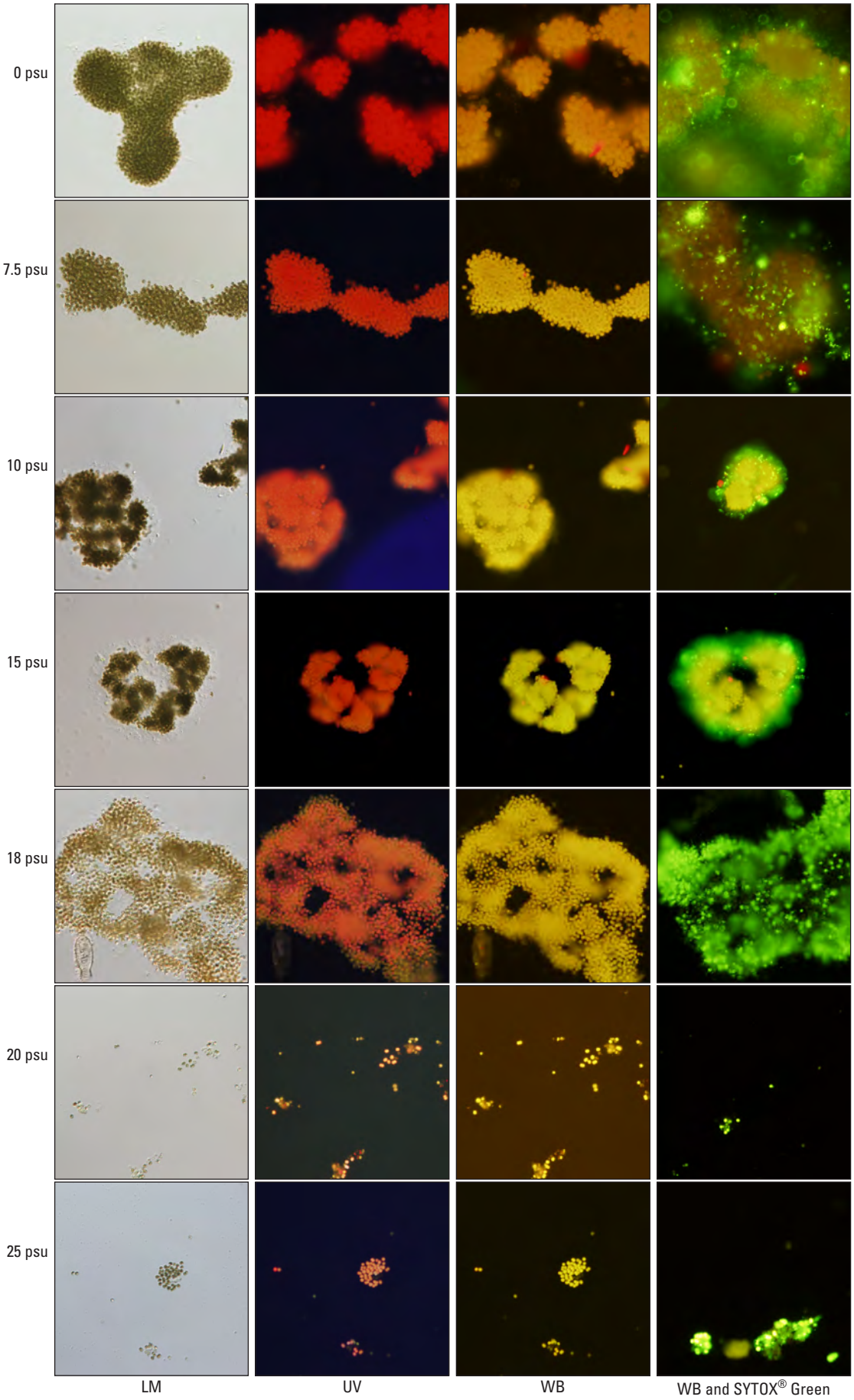


Figure 17. Response of *Microcystis aeruginosa* after 2 days of exposure to treatments with different salinities (psu, practical salinity units; LM, differential interference microscopy; UV, epifluorescence microscopy; WB, wide-blue epifluorescence microscopy; WB and SYTOX® Green, wide-blue microscopy with the DNA stain SYTOX® Green added).

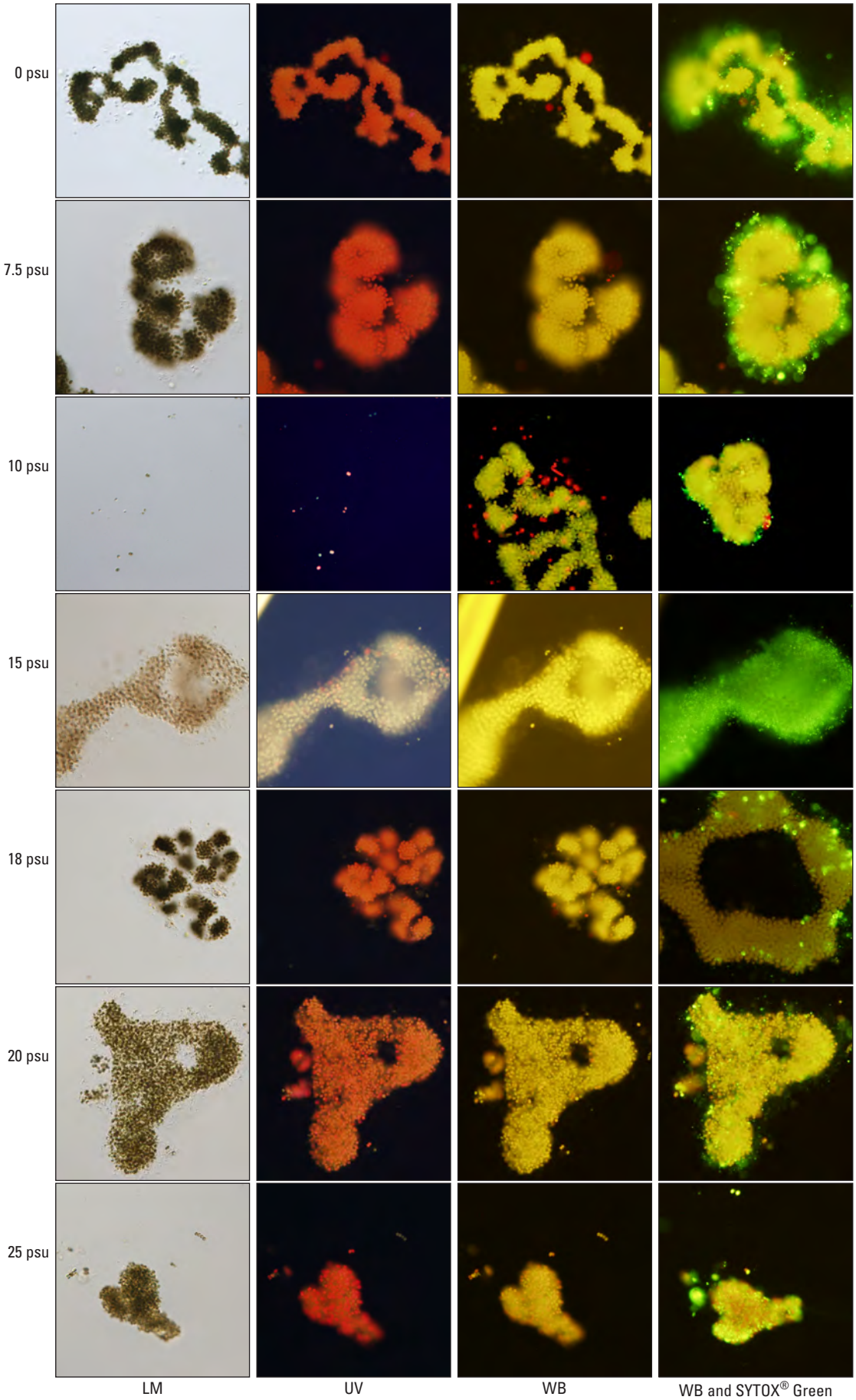


Figure 18. Response of *Microcystis aeruginosa* after 3 days of exposure to treatments with different salinities (psu, practical salinity units; LM, differential interference microscopy; UV, epifluorescence microscopy; WB, wide-blue epifluorescence microscopy; WB and SYTOX® Green, wide-blue microscopy with the DNA stain SYTOX® Green added).

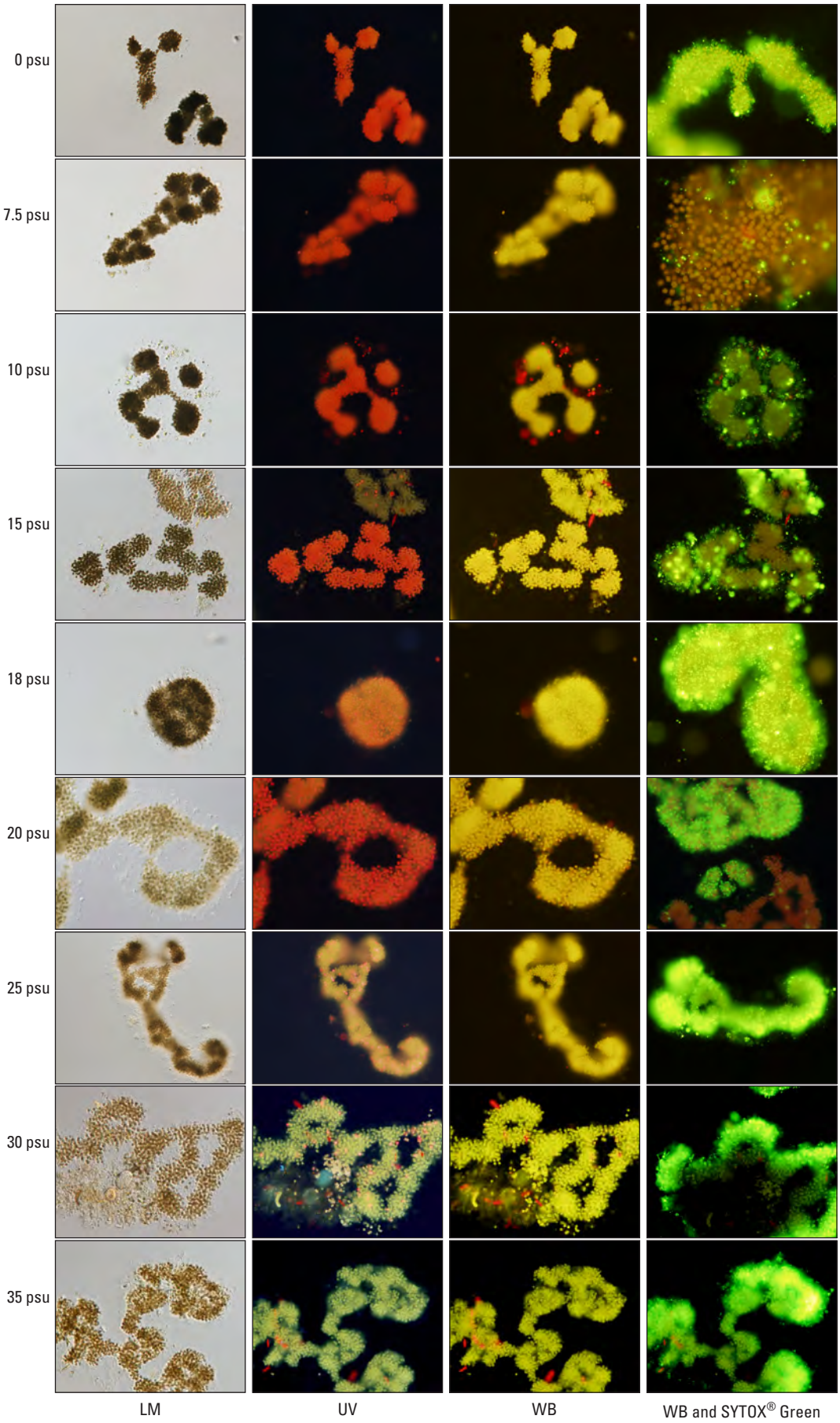


Figure 19. Response of *Microcystis aeruginosa* after 4 days of exposure to treatments with different salinities (psu, practical salinity units; LM, differential interference microscopy; UV, epifluorescence microscopy; WB, wide-blue epifluorescence microscopy; WB and SYTOX® Green, wide-blue microscopy with the DNA stain SYTOX® Green added).

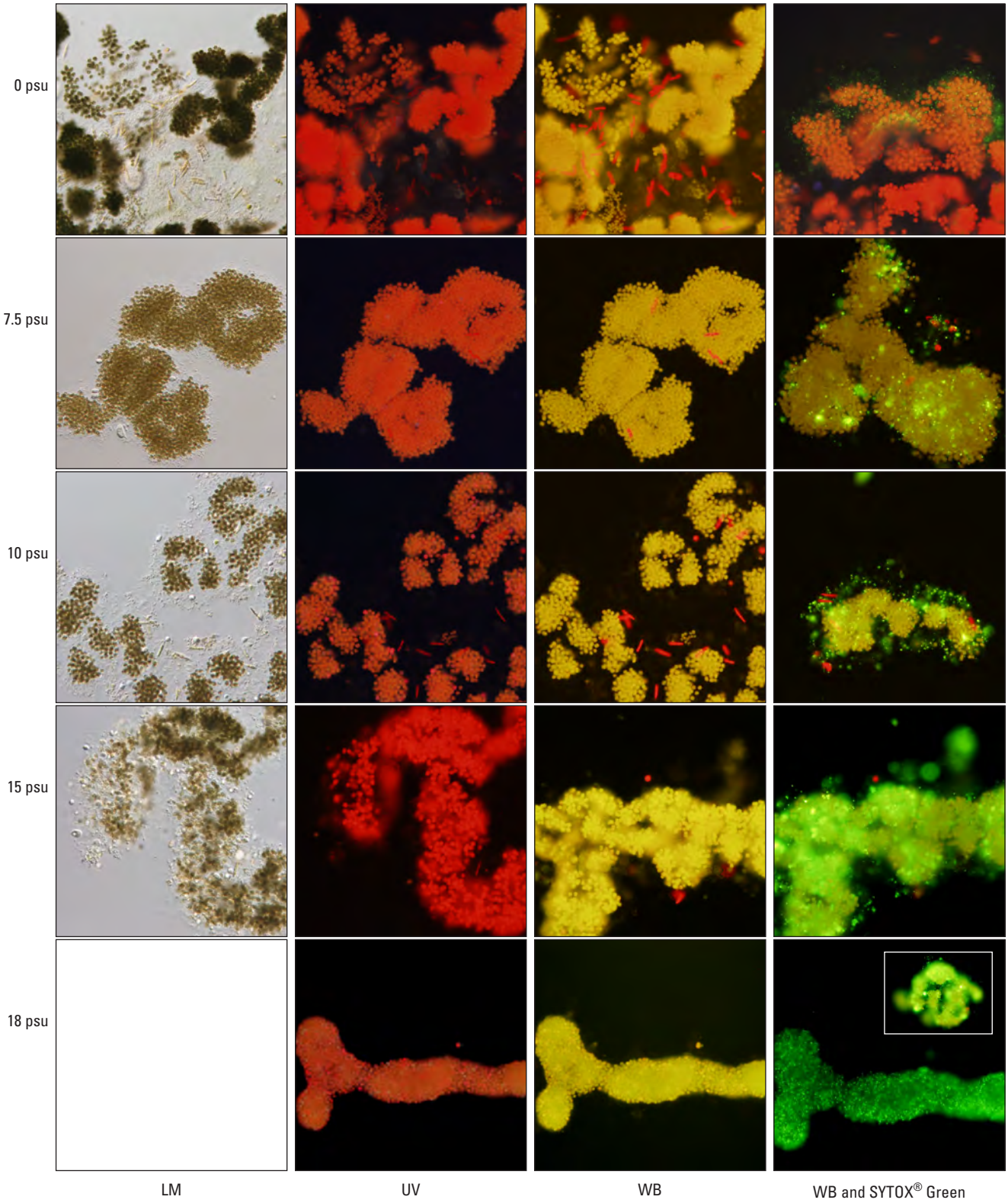


Figure 20. Response of *Microcystis aeruginosa* after 8 days of exposure to treatments with different salinities (psu, practical salinity units; LM, differential interference microscopy; UV, epifluorescence microscopy; WB, wide-blue epifluorescence microscopy; WB and SYTOX® Green, wide-blue microscopy with the DNA stain SYTOX® Green added. Blank boxes indicate that data were not available).

After 1 day of exposure, *Dolichospermum* was much less abundant in the treatments, hence the sparse observations (fig. 21). The Green was observed to have penetrated one of two filaments observed after 1 day of exposure at 20 psu.

Dolichospermum filaments were seen in the control and in the 7.5-psu treatment, but were absent in salinity greater than 7.5 psu after day one. After 8 days, the only treatments with any *Dolichospermum* were the control and the 7.5-psu treatment, and *Dolichospermum* were rare in both samples (fig. 22). The few filaments observed in both of these treatments were viable and appeared healthy.

Physio-Chemical Conditions

Ancillary to the bioassay itself, a continuously recording sonde was used to collect physical and water-chemistry data from water associated with the initial cyanobacteria sample collection and a simulation of water flow without any salinity treatments. Material was placed in 4-L beakers that were either stirred or not stirred. The nonstirred treatment is the most similar to the actual bioassay in the sense that the graduated cylinders used for the bioassay were not stirred. Dissolved oxygen initially showed a small increase to 4 milligrams per liter (mg/L), then decreased and maintained a fairly constant level of 3 mg/L in the nonstirred treatment. A slight diurnal pattern was present that dissipated after day two (fig. 23). Both stirred treatments had higher dissolved oxygen than the nonstirred treatment, with the highest concentration of oxygen at the fastest rate of stirring, 0.95 ft/s, reaching 8 mg/L.

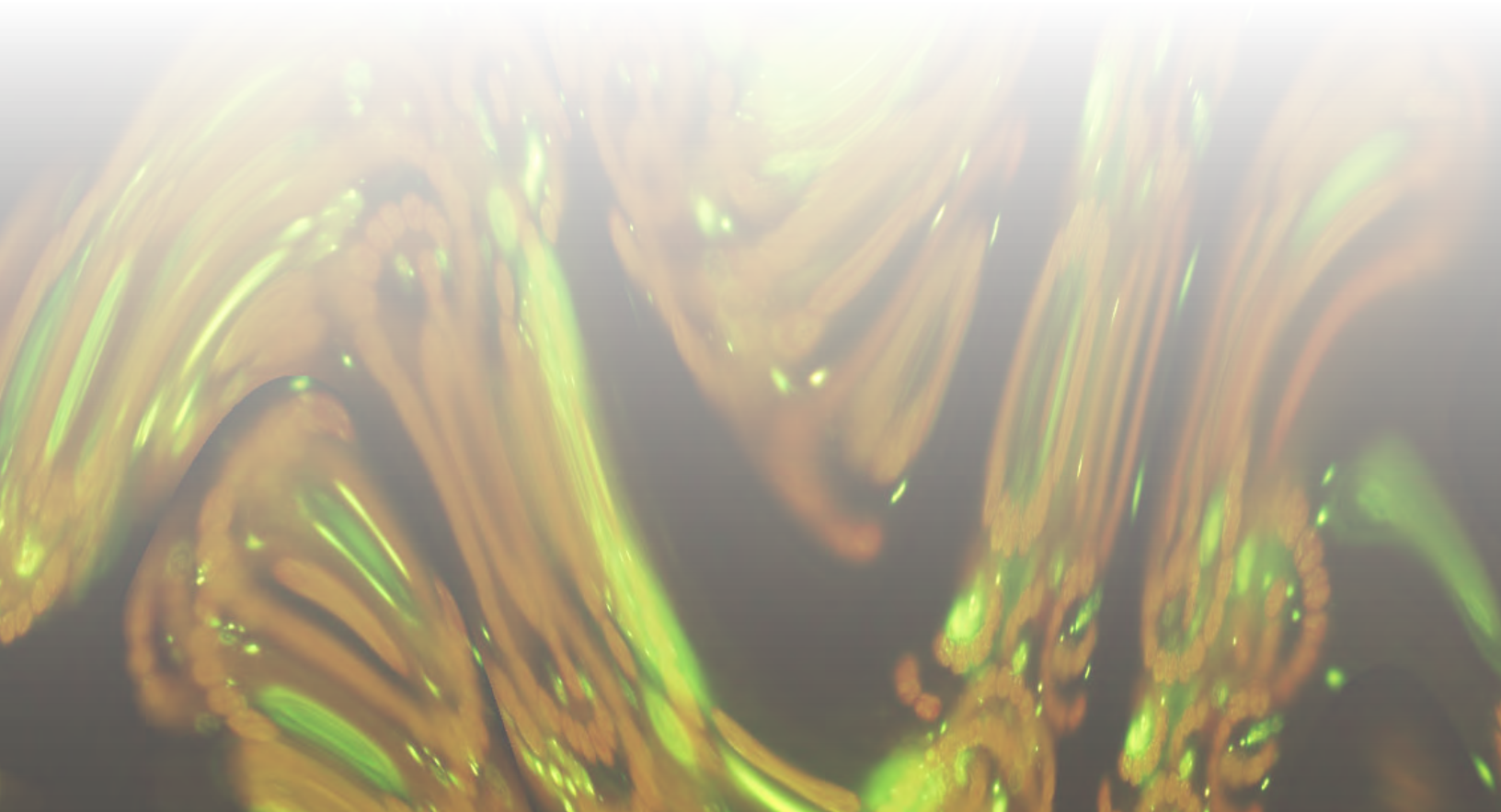
Temperature varied diurnally from a low of 23 ºC to a high slightly greater than 27 ºC in the nonstirred treatment (fig. 24). Water in both stirred beakers (on a magnetic plate with a stir bar) had elevated temperatures that were 2 to 4 ºC higher than temperatures in the nonstirred treatment.

In the nonstirred treatment, specific conductance was 307 microsiemens per centimeter at 25 degrees Celsius (µS/cm at 25 °C) on the first day of the test; specific conductance increased to 322 µS/cm by the end of the test (fig. 25). The stirred treatments had the same initial specific conductance as that of the nonstirred treatment; however, specific conductance was approximately 30 µS/cm greater in the stirred treatments than in the nonstirred treatment by the end of the test.

The pH peaked at just over 7.3 in the nonstirred treatment (fig. 26). In the stirred treatments, pH values were higher on average. In the treatment that was stirred at a rate of 0.27 ft/s, the maximum pH was 7.6. In the treatment that was stirred at a rate of 0.95 ft/s, the maximum pH was 7.9.

For all treatments, chlorophyll-*a* fluorescence exhibited the greatest decrease during the first day as the bloom was moved from Lake Okeechobee to the laboratory setting (fig. 27). The decrease of chlorophyll-*a* fluorescence in the nonstirred treatment leveled off for the duration of the experiment; however, fluorescence in both stirred treatments was higher than in the nonstirred treatment, and chlorophyll-*a* fluorescence increased in the stirred treatments after day three.

Phycocyanin fluorescence decreased substantially across all treatments over the course of the experiment, though fluorescence did remain higher in the treatment that was stirred at the fastest rate (0.95 ft/s; fig. 28).



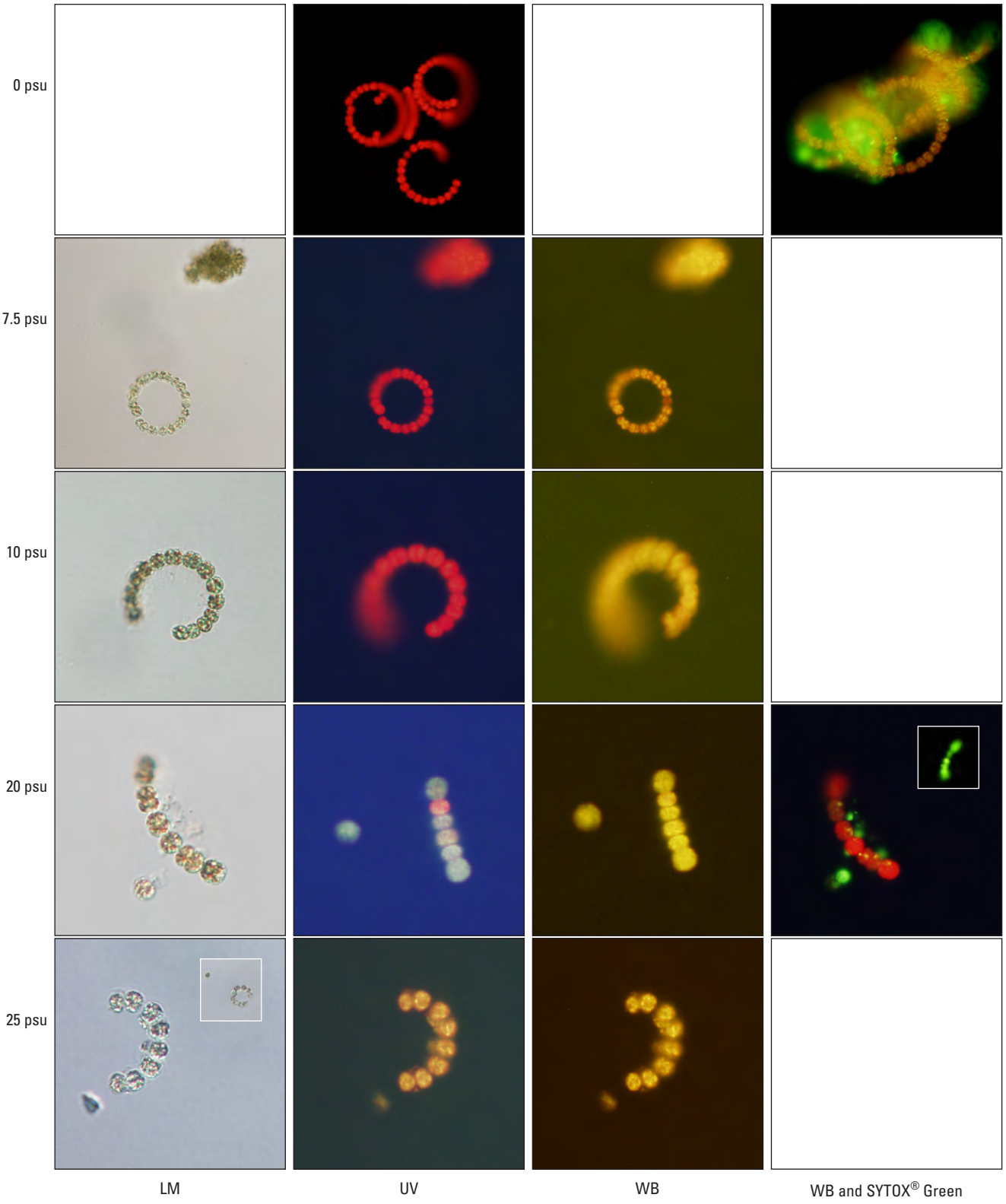


Figure 21. Response of *Dolichospermum circinale* after 1 day of exposure to treatments with different salinities (LM, differential interference microscopy; UV, epifluorescence microscopy; WB, wide-blue epifluorescence microscopy; WB and SYTOX® Green, wide-blue microscopy with the DNA stain SYTOX® Green added. Blank boxes indicate that data were not available).

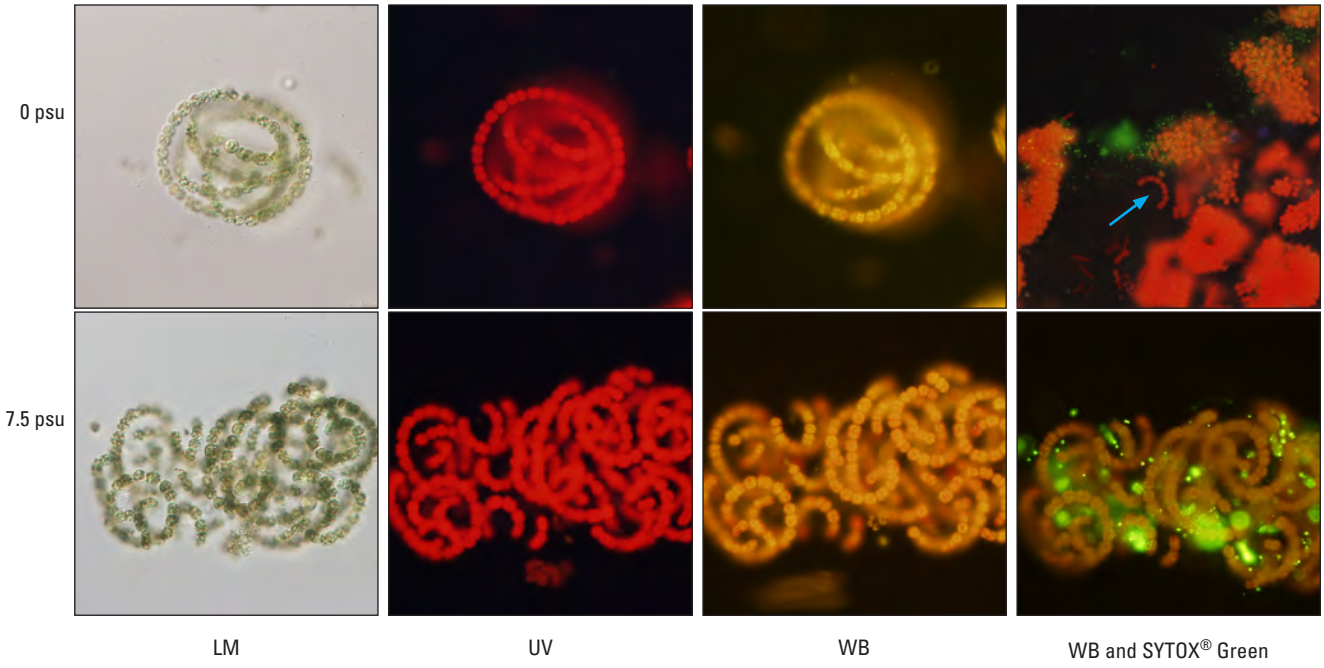


Figure 22. Response of *Dolichospermum circinale* after 8 days of exposure to treatments with different salinities (filament at blue arrow for image at upper right, among the *Microcystis* colonies; psu, practical salinity units; LM, differential interference microscopy; UV, epifluorescence microscopy; WB, wide-blue epifluorescence microscopy; WB and SYTOX® Green, wide-blue microscopy with the DNA stain SYTOX® Green added).

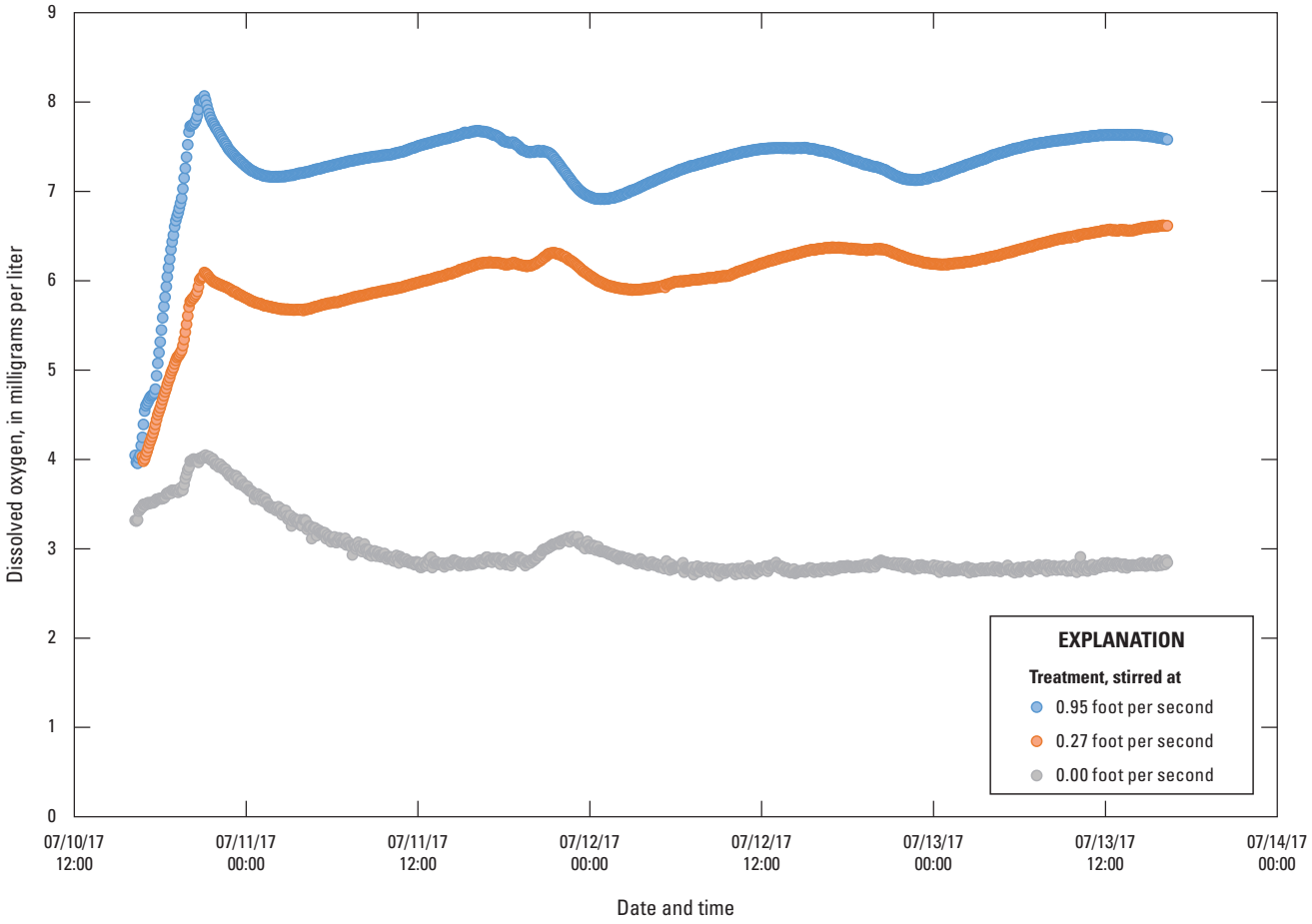


Figure 23. High frequency, optical sensor data for dissolved oxygen concentrations in three treatments: one not stirred, one stirred at 0.27 foot per second (ft/s), and one stirred at 0.95 ft/s.

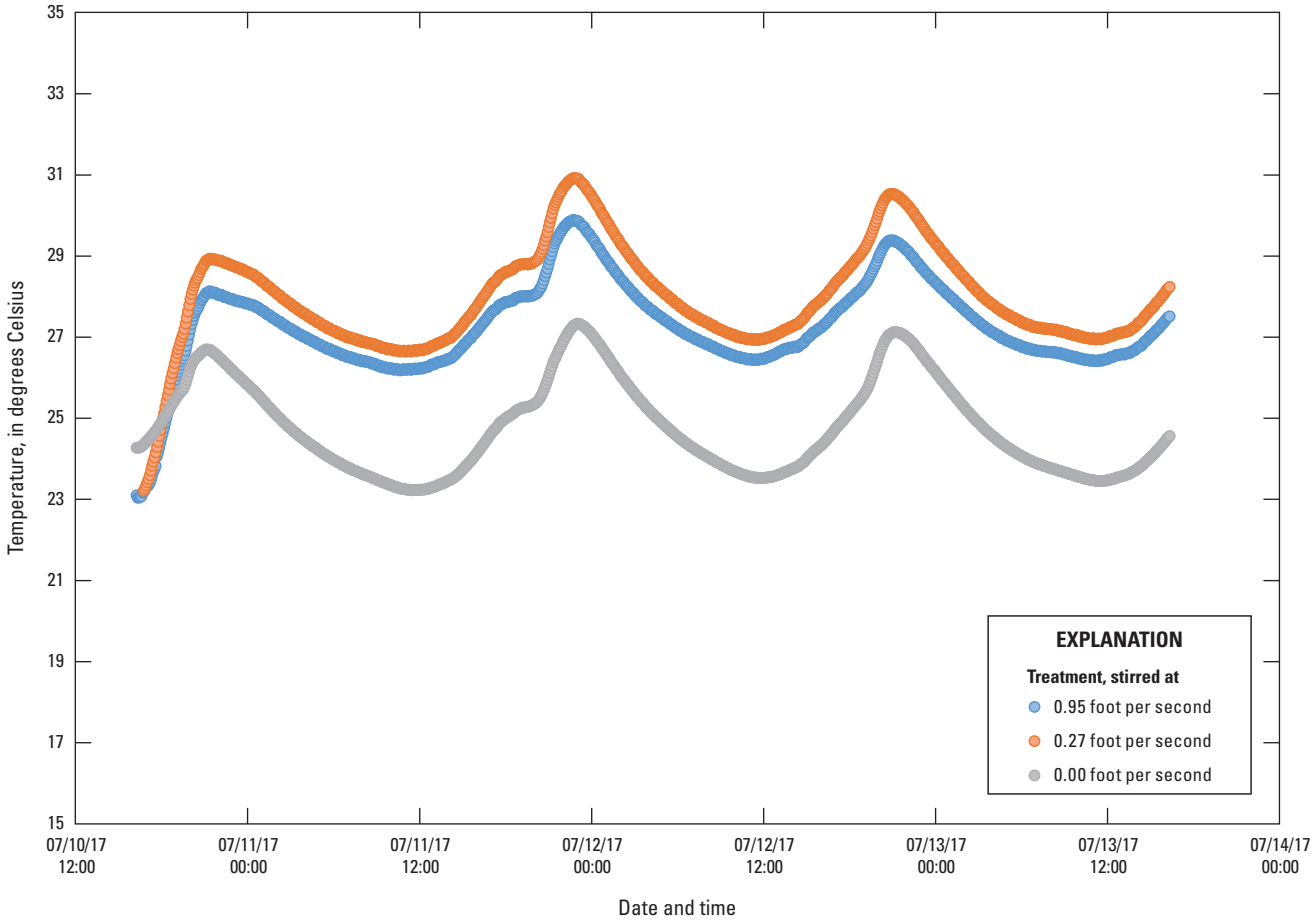


Figure 24. High frequency sensor data for temperature.

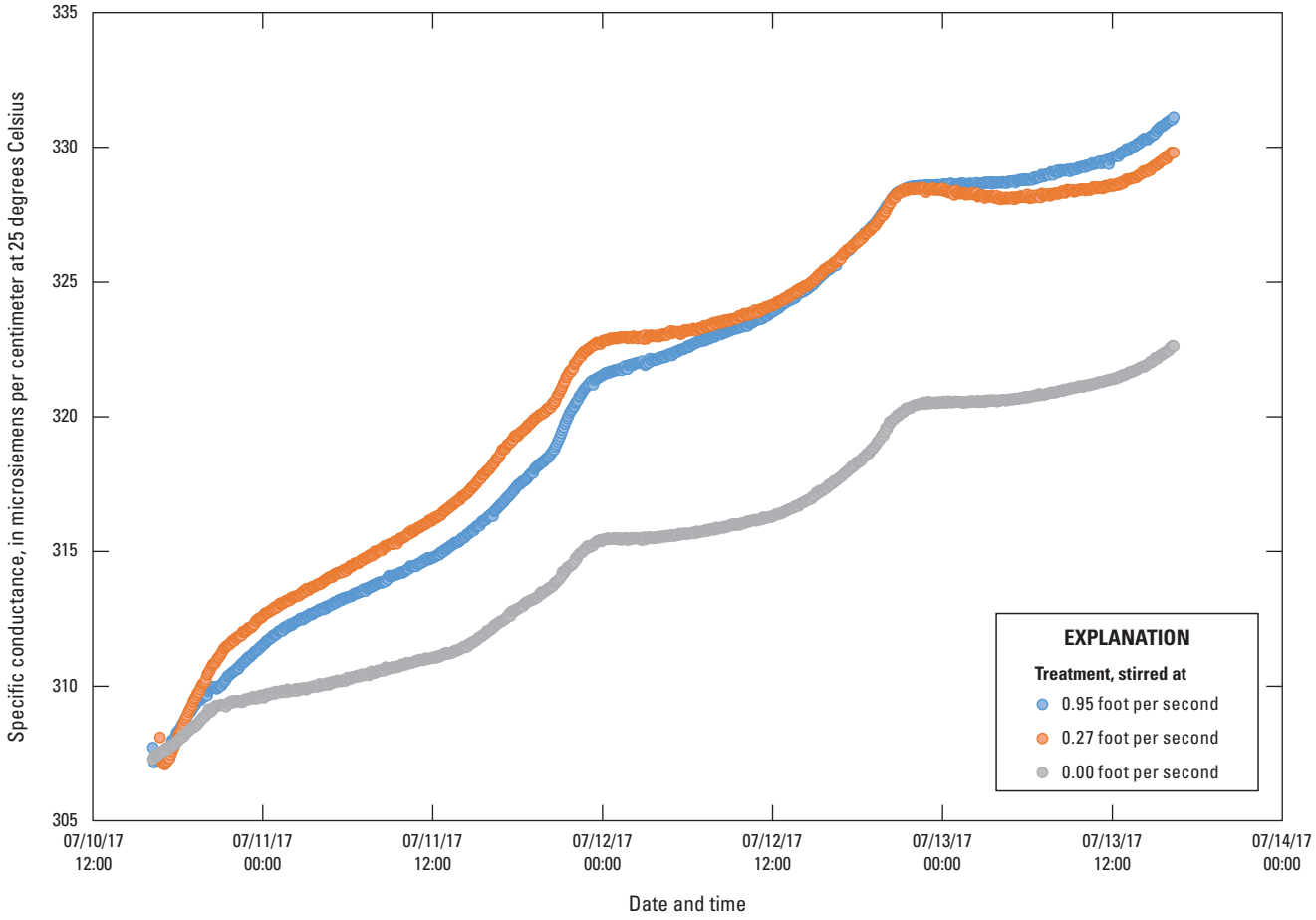


Figure 25. High frequency sensor data for specific conductance.

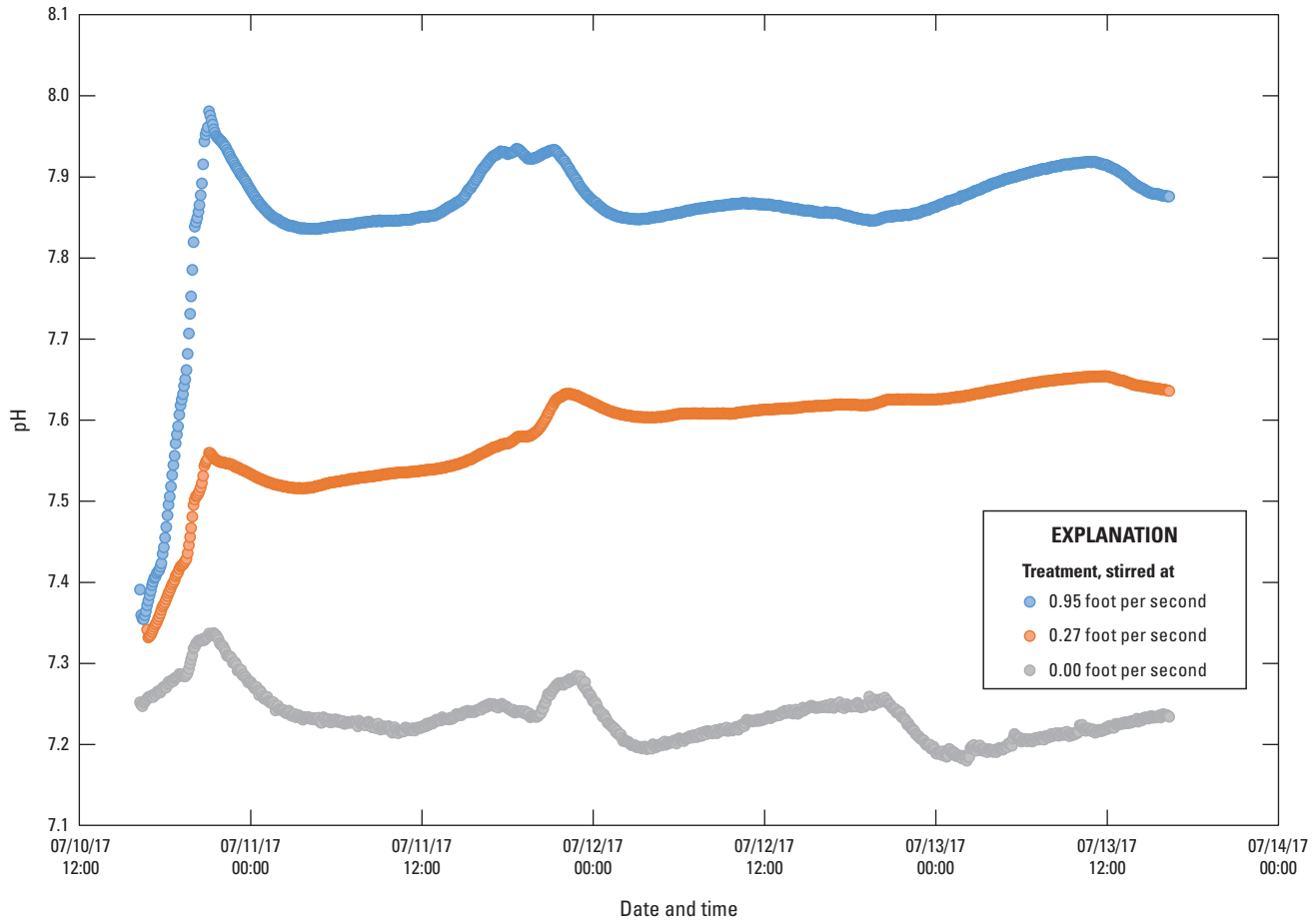


Figure 26. High frequency sensor data for pH.

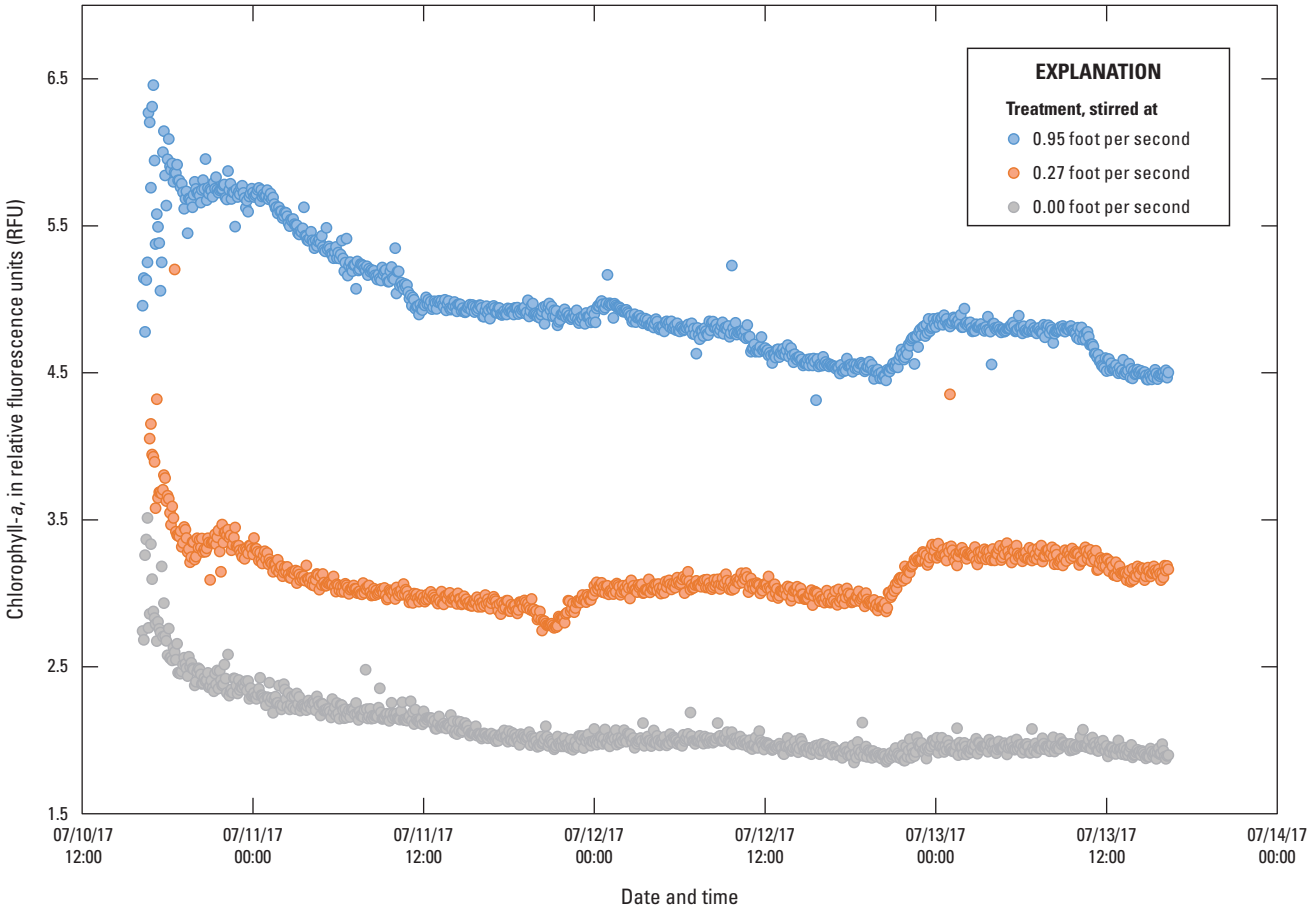


Figure 27. High frequency, optical sensor data for chlorophyll-*a* fluorescence.

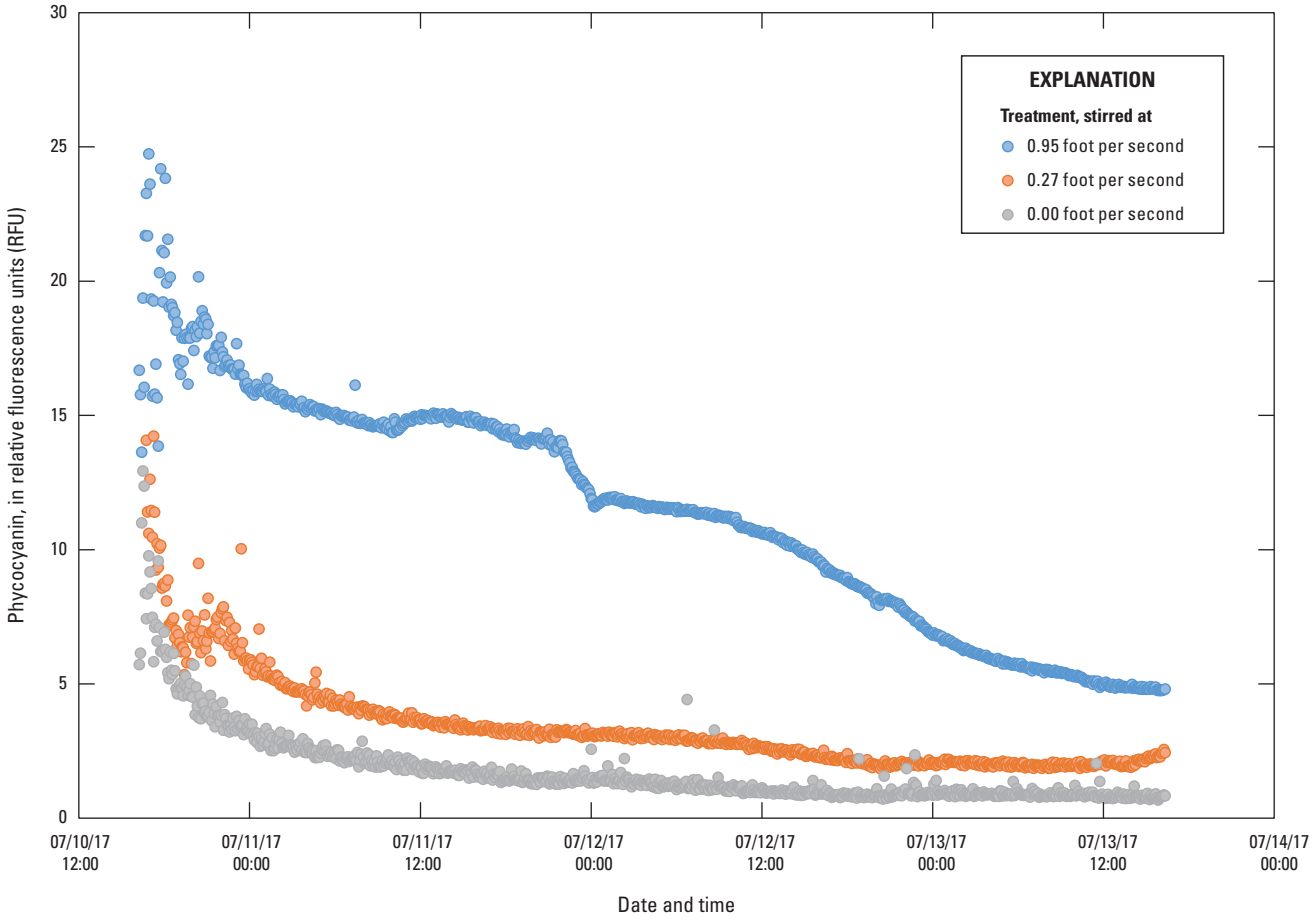


Figure 28. High frequency, optical sensor data for phycocyanin fluorescence.

Discussion

It is frequently assumed that when cyanobacteria from freshwater lakes, reservoirs, and rivers reach saline estuaries, they will die off after some period of time. The hydrologic dynamics in these transition areas influence the salinity conditions that these organisms experience. In a study that included a period of augmented flow from Lake Okeechobee in 2005, Phlips and others (2012) found that *Microcystis aeruginosa* persisted in the St. Lucie Estuary. The Phlips study reported that the South Fork of the St. Lucie Estuary had dense surface accumulations of cyanobacteria, with chlorophyll-*a* reaching 2,863 µg/L^{-1} at the surface, and the cyanobacteria seem to have come from the St. Lucie Canal. Only a few studies have attempted to define the effects of salinity on cell growth, cell death, toxicity, pigmentation, and cell morphology (Batterton and Van Baalen, 1971; Orr and others, 2004). Tonk (Tonk and others, 2007) found the salinity tolerance of *Microcystis* did not exceed 20 psu.

The bloom used for this bioassay was collected from Eagle Bay, Lake Okeechobee, on July 7, 2017, and brought into a laboratory setting. The bloom was moved from an open-water habitat where it experienced wind and wave activity and nutrient and light variability associated with an open waterbody. Moving the community from its natural environment into the laboratory and into batch growth conditions (the graduated cylinders) altered many parameters that would equate to physiological stress on the organisms; however, the approach used in this study ensured physiological variables were similar across the treatments, leaving salinity changes as the only variable. The responses of dissolved oxygen and pH indicate the loss of a typical diurnal pattern that likely existed in the lake (figs. 23 and 26). The increase in specific conductance (fig. 25) may be explained by greater evaporation in the stirred treatments, which were also warmer than the nonstirred treatment (fig. 24) because of the heat produced by the stir plates. On a cellular level, if the cells were being disrupted and leaking cellular contents mineralized by bacteria, it could also explain the increase in specific conductance, as well as being a possible factor for the observed decline in fluorescence.

Overall, the control and treatments showed a downward trend in chlorophyll-*a* and phycocyanin fluorescence over time (figs. 27 and 28), and numerous possibilities exist for this phenomenon, including moving organisms from a high-light environment to a laboratory setting and changing nutrient dynamics that affect pigment fluorescence (Rosen and Lowe, 1984).

The water collected contained the targeted organism, *Microcystis aeruginosa,* with a density of approximately 400,000 cells per milliliter (cells/mL). This original sample contained ample microcystin (560 µg/L), which allowed us to monitor the presence of microcystin in cells and the amount released to the water as a result of salinity stress. Another major bloom-forming organism from the lake (Rosen and others, 2017), *Dolichospermum circinale*, was present at an initial density that was more than 140,000 cells/mL. The response of these two organisms provided information on the colonial and filamentous forms of cyanobacteria, *Microcystis* and *Dolichospermum*, respectively.

Generally, cyanobacteria health declined in salinity treatments greater than 18 psu as indicated by a loss of cell membrane integrity. In any given waterbody, the cyanobacteria colonies and filaments are not identical; rather, they are in various physiological states. Some colonies and filaments contain healthy cells that are likely better able to withstand stress, such as salinity changes, while others are unhealthy and more easily disrupted. Mucilage production, a mechanism commonly observed in response to desiccation in cyanobacteria (Pereira and others, 2009; Rosen and Mareš, 2016) and salinity (Ozturk and Aslim, 2010), was also an indicator of response to salinity in *Microcystis* and *Dolichospermum* (fig. 15). For mucilage production to occur, the filaments/colonies have to be alive and able to synthesize compounds that are excreted from the cells. The extracellular polymeric substance is mainly composed of complex polysaccharides that serve as a protective boundary between the cells and the water that surrounds them (Kehr and Dittmann, 2015). Nitrogen limitation has also been found to increase polysaccharide production in *Microcystis* cells, and light intensity seems to play an important role (Yang and others, 2012).

Initially, individual *Microcystis* colonies contained hundreds of cells (fig. 4*A*), and the effect of salinity on colony integrity did not show a single pattern. Some colonies maintain their overall shape with several hundreds of cells, even after 4 days of exposure in treatments up to 35 psu (fig. 10), while other colonies broke up into individual cells at 10 psu or greater (fig. 11). Although some cells of *Microcystis* lost integrity after 2 days of exposure to salinity greater than 18 psu, as indicated by being stained green with SYTOX® Green (fig. 17), other cells and colonies were left intact even after 4 days of exposure to salinity up to 20 psu. Cell mortality occurred after 4 days of exposure to salinity of 25 psu or greater (fig. 19), even if the colonies appeared to be intact. This range in colony breakdown in response to salinity is likely because of the natural variability in the health of each individual colony in a bloom, similar to the concept of the "paradox of the plankton" (Hutchinson, 1961).

Dolichospermum circinale cells decreased substantially in all treatments during the first 24 hours, indicating this species is not tolerant of the salinities in the ranges tested (fig. 7). Filaments were initially coiled (fig. 4*D*), but became shortened fragments within 1 day of salinity exposure (fig. 12). Separation of the cells in a filament was observed and likely the cause of fragmentation. *Dolichospermum*

tolerated the lowest salinity treatment, 7.5 psu (fig. 14*B*), for 8 days, but filaments were not observed in the higher salinities in this timeframe. From these observations, this other common Lake Okeechobee bloom-forming organism is much less tolerant of salinity compared to *Microcystis*. Although some of the fragments were stained with SYTOX® Green after 2 days of exposure to the 7.5-psu treatment, this salinity does not appear to cause complete mortality, as indicated by filamentous colonies that were still viable after 8 days at 7.5 psu. *Dolichospermum circinale* did not tolerate conditions greater than 7.5 psu.

Total, dissolved, or calculated microcystin concentrations, as total, dissolved, or calculated particulate, increased over time and were greater in the salinity treatments compared to the control. The amount of microcystin was greatest in the dissolved phase at the two highest salinities (fig. 8*B*), indicating that either a greater amount leaked from the cells in general or that cellular contents were released by dead cells. The particulate microcystin and chlorophyll-*a* give us information about the cellular content of organisms, hence we gain a more direct understanding of the cellular microcystin response as a function of salinity. The 7.5- and 10-psu treatments contained a greater amount of microcystin per unit of chlorophyll, indicating toxin stimulation at these concentrations. In contrast, in the 15- and 18-psu salinity treatments, the microcystin to chlorophyll ratios were similar to or lower than in the control, suggesting the inhibition of microcystin at these salinities. Additional experiments are needed to determine the mechanisms by which salinity stimulates or inhibits microcystin production.

Summary and Conclusions

This study documents an overall decline in cyanobacteria health in salinity treatments greater than 18 psu. A dominant bloom in this system, *Microcystis aeruginosa,* was tolerant of salinities up to 18 psu; however, higher salinities caused leaking of microcystin from the cells. *Dolichospermum circinale*, another common bloom-former in this system, did not tolerate salinities greater than 7.5 psu. Both organisms produced extracellular mucilage as a protective response to salinity, which is a common ecological strategy in cyanobacteria undergoing desiccation. At 7.5 psu, microcystin increased relative to chlorophyll-*a*, providing some evidence of biosynthesis when *M. aeruginosa* is stressed at these salinities. This study indicates that as freshwater cyanobacteria are transported to brackish and marine waters, there will be a loss of membrane integrity which will lead to the release of cellular microcystin into the surrounding waterbody. Additional research would be needed to determine the exact effect of salinity on this relation.

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