A large-scale sustained fish kill in the St. Johns River, Florida: A complex consequence of cyanobacteria blooms

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ABSTRACT

In the summer of 2010, a sustained multispecies fish kill, affecting primarily adult red drum (*Sciaenops ocellatus*) and Atlantic stingray (*Dasyatis sabina*), along with various baitfish such as menhaden (*Brevoortia* spp.) and shad (*Dorosoma* spp.), was documented for six weeks along 50 km of the Lower St. Johns River (LSJR), Florida. An *Aphanizomenon flos-aquae* bloom was present in the freshwater reaches before the fish kill. The kill was triggered by a significant reverse-flow event and sudden influx of high-salinity water in late May that contributed to the collapse of the bloom upstream and brought euryhaline fish downstream into the vicinity of the senescing bloom or its by-products. The decomposing bloom led to a sequence of events, including the release of small amounts of cyanotoxins, bacterial lysis of cyanobacterial cells, high organic loading, and changes in the diversity and dominance of the plankton community to include *Microcystis* spp., *Leptolyngbya* sp., *Pseudanabaena* spp., *Planktolyngbya* spp., and low concentrations of *Heterosigma akashiwo.* Dissolved oxygen levels were within normal ranges in the reach of the fish kill, although elevated ammonia concentrations and high pH were detected farther upstream. These conditions resulted in complex pathological changes in fish that were not consistent with acute cyanotoxin exposure or with poor water quality but were attributable to chronic lethal hemolysis. Potential sources of hemolytic activity included *H. akashiwo*, *Microcystis* spp., and *Bacillus cereus*, a hemolytic bacterium. The continued presence of *A. flos-aquae* in the LSJR could have significant environmental repercussions and ideally the causal factors contributing to bloom growth and maintenance should be fully understood and managed.

Keywords: Fish kill, Cyanobacteria, *Aphanizomenon flos-aquae*, Cyanotoxins, St. Johns River, Hemolysis

1. Introduction

Adverse water quality resulting from harmful algal blooms (HABs) can be varied and may include degradation of the underwater light environment (Orth and Moore, 1983; Onuf, 1996; Greening and Janicki, 2006), depletion of dissolved oxygen (DO) (Officer et al., 1984; Diaz, 2001; Mallin et al., 2006), diminished quality of food of primary consumers (Fulton and Paerl, 1987; Soares, 2010), and biosynthesis of compounds that are acutely toxic to gill-breathing aquatic organisms or, when vectored via bioaccumulation, to primary or secondary consumers (Landsberg, 2002; Rohrlack et al., 2005; Miller et al., 2012). Anthropogenic nutrient enrichment and stratification are primary causal factors in the development of estuarine HABs (Paerl 2006), but the prevailing hydrology can have more subtle effects, because it can alter nutrient ratios (Officer and Ryther, 1980; Howarth et al.,1988; Paerl, 1990), residence time, or salinity (Basu and Pick, 1996; Borsuk et al., 2004) and can create ecotones because of the changing interfaces' fixed (depth, water surface area, nearshore habitat and connected tide marsh) and transient pelagic (water chemistry, plankton and motile aquatic organisms) characteristics. This is particularly evident in river estuaries, where pulses of nutrient-laden freshwater, interspersed with long residence times, lead to the formation of HABs of variable density and composition depending on the temporal and spatial intersection of water chemistry and habitat.

Intense cyanobacteria blooms in three of peninsular Florida's river estuaries: the St. Lucie, Caloosahatchee and the St. Johns; have constituted some of the most significant adverse ecological events to occur in the State's waters in the last four decades (Coveney et al., 2012; Phlips et al., 2012; Pinto et al., 2016; Oehrle et al., 2017; Rosen et al. 2017). Unlike Florida's shallow, inland eutrophic lakes, which tend to maintain relative seasonal stability in

phytoplankton biomass and composition (Huber et al., 1982), these river estuaries exhibit cyclic, episodic blooms. For the Caloosahatchee and St. Lucie estuaries, the most severe blooms typically occur from inter-basin diversions for flood protection, displacing the volume of the estuary with nutrient-rich freshwater from Lake Okeechobee, creating conditions conducive to cyanobacteria blooms, often dominated by *Microcystis aeruginosa* (Doering, 1996; Doering and Chamberlain, 1999; Phlips et al., 2012; Graham et al., 2015; Kramer et al. 2018).

The Lower St. Johns River (LSJR), which drains the northeast portion of the Florida peninsula and is not subjected to the inter-basin water diversions of these estuaries, has nonetheless experienced decades of nutrient enrichment from within its natural basin boundaries. This has led to cultural eutrophication, causing organic sedimentation and degradation of the benthos, nuisance algal blooms, reduced abundance and diversity of zooplankton, episodic low-DO, and fish kills (Hendrickson et al., 2002; St. Johns River Water Management District [SJRWMD], 2008; Pinto et al., 2016). Due to the proximity of runoff from a large agricultural area and effluents from industrial and domestic point sources, coupled with a wide morphology and a shallow water column, blooms have historically attained maximum standing stock in the estuary's freshwater reach. During the exponential growth phase, primary production in this reach typically exceeds 5 g carbon m^{-2} day^{-1} (Paerl et al., 2005). Upstream in Lake George, the head of the LSJR estuary, the productivity of diazotrophic cyanobacteria results in an internal load of nitrogen (N) averaging 763 t yr^{-1} (Piehler et al., 2009). To manage nutrient loading and help prevent annual blooms, total maximum daily loads were established for N and phosphorus (P) in the freshwater reach and for N in the marine reach (Magley and Joyner, 2008).

In the LSJR, discharge significantly influences the composition, density, and spatial extent of HABs (Phlips et al., 2007). Precipitation and runoff affect external nutrient supply, residence

time controls cyanobacterial exploitation of that available nutrient supply, and the down-estuary distribution of the nutrients determines the extent to which freshwater cyanobacteria are supported (Coveney et al., 2012). Seasonal and interannual flow pulses exert significant control over the composition of the phytoplankton and the migration of motile aquatic organisms. The LSJR is a long, narrow estuary, and sustained northeast winds associated with passing fronts can augment the high tide such that they are communicated up-estuary as reverse flows, persisting for several days to a week. Following relaxation of wind, the input of nonpoint source runoff lead to an expansion of pre-storm downstream freshwater extent and an increase in nutrient concentrations. The estuary's unique narrow morphology in its downstream reaches facilitates substantial longitudinal migration of the marine salinity transition zone arising from discharge or temporary variations in Atlantic shelf water level. During 1988 to present day monitoring, the maximum annual encroachment of the freshwater–seawater interface (1,500 mg L^{-1} chloride) has ranged from river kilometer marker 52 (RKM52) to as far upstream as RKM100 (SJRWMD, unpublished data).

Summer convective storms and North Atlantic tropical systems are the dominant influences on the hydrology of the LSJR, typically resulting in a discharge peak in late summer. Winter and spring U.S. continental frontal systems usually dissipate before reaching the headwaters of the LSJR, but often are sustained long enough to produce significant precipitation in the watershed adjacent to the downstream, northern LSJR, leading to a bimodal annual discharge pattern (Kelly and Gore, 2008). Sustained winds from the east-northeast typically follow these continental frontal systems, driving continental shelf water into the river mouth, increasing water level and reversing flow, temporarily forcing water upstream and into the adjacent tidal swamps of this low-head river. Upon relaxation of forcing winds, the mixed water mass will migrate

116 downstream (Sucsy and Morris, 2002). Such events can disrupt developing HABs by increasing
117 the mixing depth, reducing the photic zone and net positive production, and transporting the
118 bloom to river reaches in which it may be exposed to unfavorable light or salinity conditions.
119 Phytoplankton growth in the LSJR (between RKM20–120) has been shown to be strongly N-
120 limited (Paerl et al., 2005). The phytoplankton typically undergo a succession from diatom
121 dominance in late winter to eventual dominance in spring and summer by the diazotrophic
122 cyanobacteria *Dolichospermum circinale* and *Cylindrospermopsis raciborskii* (Paerl et al., 2002;
123 Phlips and Cichra, 2002). The succession of diazotrophic cyanobacteria blooms by non-N-fixing
124 *Microcystis* spp. is a common pattern in the LSJR in the reach grading from fresh to oligohaline,
125 particularly in years with moderate to high river discharges which advect freshwater blooms
126 downstream, simultaneously supplying external N and P. Under this bloom-succession scenario,
127 the LSJR has experienced several large *M. aeruginosa* blooms, with significant fish kills in 1999
128 and 2005 (Williams et al., 2007; Burns, 2008). Cyanotoxins were not confirmed as the causative
129 agents in these fish kills, though during the 2005 event, extremely high levels of microcystin
130 (MCYST) were detected in spot sampling of surface scums (cyanotoxin sampling was not
131 performed during the 1999 event). Low dissolved oxygen was the primary causal factor in the
132 1999 mortality, but the causal agent in the 2005 mortality was undetermined (SJRWMD,
133 unpublished data).
134 The broadscale ecosystem effects of many harmful cyanobacteria blooms are manifested as
135 increases in extracellular toxin concentrations in the water due to cell lysis or release during
136 normal bloom progression or senescence (Mackenthum and Herman, 1948; Barica, 1978; Fallon
137 and Brock, 1979; Landsberg, 2002; Hall et al., 2008), and fish mortalities are usually rapid and
138 nonspecific. Most cyanobacteria-caused fish kills are attributed to anoxic or hypoxic conditions

resulting from the high oxygen demand caused by bloom respiration at night or from oxygen depletion during bloom decomposition (Rodger et al., 1994; Bury et al., 1998). Unless blooms collapse, decompose, or senesce naturally, dissolved cyanotoxins are only occasionally present at concentrations that are chronically or even acutely lethal to fish (Chellappa et al., 2008). In this case, fish mortalities may be caused by a combination of poor water quality, suboptimal environmental conditions, and exposure to toxins, and cyanobacteria-associated kills are acute (Rodger et al., 1994; Ernst et al., 2001; Jewel et al., 2003; Skinner McInnes and Quigg, 2010). Fish kills involving MCYST-producing cyanobacteria have been the most commonly documented, primarily in freshwater ponds, lakes and reservoirs in the Americas, Europe, and Asia (Maloney and Carnes, 1966; Rodger et al., 1994; Zimba et al., 2001; Jewel et al., 2003; Chellappa et al., 2008; Ernst 2008). Fish mortalities may also arise from pathological complications associated with other cyanobacterial bioactive compounds such as lipopolysaccharides and fatty acids (Bury et al., 1998; Pietsch et al., 2001), and some cyanobacterial bloom endotoxins may originate from associated bacteria (Rapala et al., 2002). In many cases, mixed blooms with several cyanotoxins and bioactive compounds are present, and it is not easy to separate a primary toxigenic cause from what may have been synergistic effects associated with multiple toxins and secondary metabolites.

From late May until early July 2010, an atypical fish kill affecting only certain species occurred across a 50-km stretch of the LSJR. Preceded by an *Aphanizomenon flos-aquae* cyanobacterial bloom, the sustained kill affected large numbers of euryhaline fish, particularly adult red drum (*Sciaenops ocellatus*), causing public concern and attracting media attention. The collapse of the bloom was followed by an extensive and noticeable foam event. We report here on the environmental factors contributing to this unprecedented bloom and our investigation of

the unusual sustained multispecies fish kill during the summer of 2010. The ramifications of other potential ichthyotoxic properties of cyanobacteria blooms are presented here for the first time.

2. Methods

2.1 Study area

The LSJR is the largest blackwater river in the southeastern United States, draining a 24,780-km^2 watershed in the temperate/subtropical Atlantic Coastal Plain of northeast Florida (Phlips et al., 2007). It is a low-head river, with an average fall of only 2.2 cm km^{-1} (Toth 1993). The LSJR extends 196 km upstream from the mouth, beginning at Lake George (Bricker et al., 2007). The average annual maximum upstream encroachment of salinity from the Atlantic is to RKM74, with the maximum measured upstream extent to RKM100 (Fig. 1). The mean discharge rate at the mouth is 232 m^3 sec^{-1}, and the mean tide range is 1.38 m (Sucsy and Morris 2002).

The estuary can be divided into three major segments: 1) Lake George (RKM180, 29.2867°, −81.5980°, not shown), a shallow freshwater lake at the head of the estuary; 2) a broad, freshwater lacustrine reach of the LSJR that extends from RKM150 to RKM80; and 3) the transitional marine reach of the LSJR (RKM80 to RKM0), which is broad and lacustrine at the upstream oligohaline end, narrowing to a rapidly flushed and vertically well-mixed mesopolyhaline inlet downstream of the reach (Fig. 1) (Sucsy et al., 2011). Land use in the watershed adjacent to the freshwater reach is dominated by agriculture and silviculture, with only 8% of the area in urban development. Urban land use associated with the Jacksonville

185 metropolitan area characterizes 28% of the watershed contributing to the marine reach;
186 agriculture and forested land covers are proportionately smaller (White et al., 2008).
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188 *2.2 Environmental data*
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190 Routine water quality (e.g., temperature, DO, pH, conductivity, salinity, Secchi depth,
191 alkalinity, biological oxygen demand, total dissolved solids, turbidity, total organic carbon,
192 ammonia, nitrite (NO_2), nitrate (NO_3), TP, TN, pheophytin, chl *a*, and phytoplankton data exist
193 as part of a long-term biweekly monitoring program, collected at fixed stations by the SJRWMD
194 (Fig. 1). Routine water quality samples were collected with an integrating tube, inserted
195 vertically to extract a section of the water column from the surface to below surface at a depth of
196 2.5 m. Three to five "cores" were extracted and emptied into a composite churn, from which all
197 samples for chemistry and phytoplankton were drawn. Field measurements corresponding to the
198 vertical composite sample were calculated as the average reading performed at 0.5 and 2.5 m
199 depth.
200 Additional continuous water temperature, salinity, DO, and phycocyanin data for analysis
201 were obtained from three real-time automated water-quality monitoring sites (using YSI/Xylem
202 Inc. multi-parameter water quality sondes) operated by the U.S. Geological Survey (USGS)
203 (http://waterdata.usgs.gov), located in the LSJR at Dancy Point, near Spuds (station
204 294213081345300, RKM113, 29.7036°, -81.5814°), Buckman Bridge, Jacksonville (station
205 301124081395901, RKM53, 30.1900°, -81.6664°), and Dames Point Bridge, Jacksonville
206 (station 302309081333001, RKM17, 30.3858°, -81.5583°) (Fig. 1). Located in the marine-
207 influenced reach of the LSJR, the Buckman and Dames Point stations were configured to record

208 measurements at two depths corresponding approximately to surface and sub-pycnocline water,
209 and positive pulses in salinity at these sites were used to identify significant reverse flows. Water
210 level and discharge information were obtained from the USGS stations at Buffalo Bluff (station
211 02244040, RKM145, 29.5961°, -81.6833°) and Jacksonville Dames Point (station
212 302309081333001, RKM17, 30.3858° -81.5583°).
213 Rainfall data were obtained from the Florida Automated Weather Network (2015,
214 http://fawn. ifas.ufl.edu) of the University of Florida's Institute of Food and Agricultural
215 Sciences for stations nearest to the LSJR at Macclenny (30.2815°, -82.1380°) for the Ortega
216 River, Putnam Hall (29.6970°, -81.9860°) for Black Creek, and Hastings (29.6933°, -81.4448°)
217 for Deep Creek.
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219 *2.3 Remote sensing*
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221 High-resolution MEdium Resolution Imaging Spectrometer (MERIS) data (300-m
222 resolution), from the European Space Agency's (ESA) Envisat-1 satellite were directly downloaded
223 from the Canada Center for Remote Sensing and provided in near real-time by NASA's Ocean
224 Biology Processing Group. The imagery was further processed to a cyanobacteria index (CI) product
225 (Wynne et al., 2008) by the National Oceanic and Atmospheric Administration (NOAA). Reflectance
226 corrected only for Rayleigh radiance and scaled solar irradiance were generated within the U.S.
227 Naval Research Laboratory's Automated Processing System, which used the rho_s option of
228 NASA's standard l2gen software. The imagery was mapped to 300-m Universal Transverse Mercator
229 with nearest-neighbor sampling. The CI algorithms used to provide a synoptic representation of the
230 bloom events are described further Wynne et al. (2008) and use a spectral shape from:
231

$$SS(\lambda) = R(\lambda) - R(\lambda^-) + \{R(\lambda^-) - R(\lambda^+)\} * \frac{(\lambda - \lambda^-)}{(\lambda^+ - \lambda^-)}$$

where R is the rho_s reflectance and λ indicates the spectral band (central wavelength). The CI = −SS(681), in which the spectral wavelengths being used in the equation are: $\lambda = 681$ nm, $\lambda_+ = 709$ nm, and $\lambda_- = 665$ nm.

2.4 Fish kill reports

Fish kill reports were received by the Florida Fish and Wildlife Conservation Commission's (FWC) Fish and Wildlife Research Institute's (FWRI) Fish and Wildlife Health (FWH) fish kill hotline. If a field response was appropriate, a report was investigated on the same working day or arrangements were coordinated to ship any samples to FWRI in St. Petersburg.

2.5. Event response sample collections

Starting at the beginning of June 2010, FWC-FWRI opportunistically collected surface and bottom water samples along the LSJR in parallel with collections of moribund and freshly dead fish and environmental data (Table 1). Multiple samples collected along the LSJR from RKM23 to RKM97, beyond the extent of the fish kill, included water (for phytoplankton and HAB species, bacteriology, cyanotoxins, and hemolytic activity), foam (for phytoplankton and bacteriology), and fish (for diagnostic tests and cyanotoxins) (Table 1). Depth (m) was noted, and basic water quality parameters (DO, pH, salinity, conductivity, and temperature) were

254 measured between the daylight hours of 0800 to 1500 using a YSI water quality field meter
255 (Yellow Springs Instrument Company, Yellow Springs, OH, USA).
256 Water samples were collected by submerging a 250-mL plastic bottle 5 cm below the water
257 surface and allowing the bottle to fill from depth. Water samples for phytoplankton analysis were
258 preserved with 1% Lugol's solution (Throndsen, 1978). Efforts were also made to collect and
259 process fresh, unpreserved water samples to be screened for hemolytic algae and bacteria, which
260 may have been missed in initial event-response sampling or would not be well preserved in
261 Lugol's fixed samples.
262 Parallel water sample bottles (unpreserved or preserved in Lugol's iodine at a 1% final
263 concentration) were wrapped in wet newspaper, placed in a cooler at ambient temperature, and
264 shipped overnight to FWRI, where they were screened and enumerated for phytoplankton and
265 HAB species of interest. Water samples that needed to be examined live were evaluated with
266 light microscopy within hours of collection to confirm the identity of flagellates (e.g.,
267 *Chattonella* spp. and *Heterosigma akashiwo*) that can be altered during preservation. After
268 identification of such taxa, samples were preserved with Lugol's and stored at 4 °C in the dark
269 until enumeration. Foam and water directly beneath the foam were sampled on 5 August and
270 were analyzed for phytoplankton species (Section 2.6) and for bacteriology (Section 2.8).
271 Water samples were collected for hemolysis assays and for microbiology on 30 June. On 30
272 June and 4 August, during the month of the fish kill and one month after the end of the fish kill,
273 respectively, water samples were collected from 8 stations (from RKM31 to RKM97) spanning
274 the extent of the fish kill area and several kilometers beyond to the north and the south (Fig. 1).
275 Water samples for hemolysis testing were collected in small brown plastic bottles (100 mL),
276 wrapped with wet newspaper, placed into a container at ambient temperature, and shipped

277 overnight to the University of North Carolina at Wilmington. Water samples for bacteriology
278 were collected in sterile small brown plastic bottles (100 mL), wrapped with wet newspaper,
279 placed into a container at ambient temperature and transported for bacteriology to FWRI, St.
280 Petersburg.
281 Moribund and freshly dead fish were collected during June 2010 by FWC-Law Enforcement
282 (LE), FWC-FWRI-Fisheries-Independent Monitoring (FIM) Program, or FWH staff (Table 1).
283 Fish were collected by hand net, placed in a cooler on ice and were then either necropsied within
284 4–6 h at the FWC field laboratory in Jacksonville or within 18–24 h after shipping on ice
285 overnight to FWRI St. Petersburg.
286 On the 15–16 June trip, additional apparently healthy fish (redbreast sunfish, *Lepomis auritus*
287 [*n* = 2], Atlantic croaker (*Micropogonias undulatus*) [*n* = 2], black crappie, *Pomoxis*
288 *nigromaculatus* [*n* = 2], and spot, *Leiostomus xanthurus* [*n* = 2] were sampled from Julington
289 Creek (30.1302°, -81.6115°, Fig. 1), a freshwater to low-salinity tributary adjacent to the
290 mainstem LSJR. These fish were processed using the same diagnostic tests as for moribund fish.
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292 *2.6 Phytoplankton analyses*
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294 Phytoplankton species composition was determined microscopically. Individual
295 phytoplankton (cells, filaments, trichomes, or colonies) were enumerated by settling 3 mL of
296 preserved samples for 1 h in coverglass bottom chambers (Nalge-Nunc Lab-Tek®) then counting
297 (400×) until either 400 in 5 grids or a maximum of 40 grids were evaluated (Edler and Elbrächter
298 2010). The whole chamber was scanned at 100× for large or rare taxa. Cell biovolumes were
299 estimated by assigning combinations of geometric shapes to fit the characteristics of individual
300 taxa. Specific phytoplankton dimensions were measured for at least 30 randomly selected cells

for each individual taxon. Volumes were calculated for each cell, from which a mean cell volume was derived (Smayda, 1978, Hillebrand et al., 1999). The total biovolume per sample was calculated as the sum of estimated cell volumes for all species. All taxa were identified to species, if possible, using a Zeiss Axiovert 100S inverted microscope equipped with an Olympus DP72 digital camera.

2.7. Chemical analyses

Chemical analyses were performed on water passed through a 0.45-µm Gelman© Supor membrane filter following Standard Methods (APHA, AWWA and WEF, 2005) or EPA procedures (USEPA, 1983). Methods determined $NO_2^- + NO_3^-$ (reported as NO_3^-) by copper–cadmium reduction and diazotization colorimetry, NH_4^+ by alkaline phenol-colorimetry, total Kjeldahl nitrogen (TKN) by high-temperature sulfuric acid digestion on whole water followed by NH_4^+ analysis, orthophosphate (PO_4^{3-}) by antimony–phospho-molybdate-complex ascorbic acid colorimetry, and TP by high-temperature sulfuric acid digestion on whole water followed by PO_4^{3-} analysis. Nutrient analyses were performed on a Perstorp EnviroFlow FS 3000 AutoAnalyzer (O-I Analytical, College Station, TX, USA). TN was calculated from TKN plus $NO_2^- + NO_3^-$. Total suspended solids were measured gravimetrically, and chl *a* was determined spectrophotometrically after extraction in 90% acetone.

2.8 Bacteriology

Water samples were apportioned for bacterial counts and were also filtered for MCYST analyses. Each water sample was transferred to a 50-mL conical tube and centrifuged at 3200 × g for 15 min. Supernatants were discarded and pellets kept frozen at −80 °C until use. For 30 June and 4 August water samples, once the pellets had thawed, they were resuspended in 1 mL of tryptic soy broth. Aliquots of 100 µl were plated onto tryptic soy agar (TSA) plates augmented with 5% sheep's blood. All plates were incubated overnight at 27 °C. Cell counts of beta hemolytic bacteria were conducted the following day. Bacterial cell counts were calculated from the mean number of bacterial colonies on replicated (n = 2) plates.

From each plate, the dominant hemolytic bacterial colony was reisolated and identified to species (GEN II MicroStation System, BIOLOG, Hayward, CA, USA). If isolates were unidentifiable, 16s rRNA gene sequencing was performed. DNA was extracted with a commercial DNA extraction kit (Ultra Clean® Microbial DNA Isolation Kit, MoBio Laboratories, Carlsbad, CA, USA). A portion of the 16S rRNA gene was amplified with the universal primer set (fD1-5′-AGAGTTTGATCCTGGCTCAG-3′; and rD1-5′-AAGGAGGTGATCCAGCC-3′) on a MBS0.2S system with the following thermocycler conditions: initialization step at 95 °C for 2 min, 30 cycles at 95 °C for 45s, 55 °C for 45s, 72 °C for 2 min, and a final elongation step at 72 °C for 10 min. The PCR products were purified with a commercial kit (UltraClean™ PCR Clean-up DNA Purification Kit, MoBio Laboratories, Carlsbad, USA). Purified DNA was sequenced (Functional Biosciences Inc., Madison, WI, USA), and results confirmed by BLASTn analysis.

346 Due to the lack of interspecies variabilities in 16s rRNA genes, identification of the *Bacillus*
347 *cereus* group was confirmed by PCR targeting the gyrB gene (Park et al., 2007). The primer sets
348 (BCJH-F-5′-TCATGAAGAGCCTGTGTACG-3′, and BCJH-R-5′- CGACGTGTCAATTCA
349 CGCGC-3') for *B. cereus*, and BTJH-1F-5'-GCTTACCAGGGAAATTGGCAG-3', and BTJH-
350 R-5'-ATCAACGTCGGCGTCGG-3' for *B. thuringiensis* were used. Each 25µl amplification
351 reaction included 12.5 µLPCR Master Mix containing Taq DNA polymerase, dNTPs, $MgCl_2$,
352 and reaction buffer (Promega, Madison, WI, USA), 5ng DNA template, and 10 ρmol of each
353 primer. Thermocycling conditions were: initialization step at 94 °C for 5 min, 40 cycles at 94 °C
354 for 30s, various annealing temperatures for 30s, an elongation step at 72 °C for 30s, and a final
355 elongation step at 72 °C for 10 min. The annealing temperature was changed every 10 cycles
356 from 60 °C to 54 °C in 2 °C decrements. The PCR amplicons were vilified with 1.5% agarose
357 gel with ethidium bromide staining on a Thermo EC Classic ™ CSSU911 (Thermo Fisher
358 Scientific, Waltham, Massachusetts, USA). ATCC *B. thuringiensis* (ATCC 10792) and *B. cereus*
359 (ATCC 14579) strains were used as positive controls.
360
361 *2.9 Hemolytic activity and cyanotoxin analyses*
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363 Hemolytic activity of whole-water samples was assessed using a modified erythrocyte lysis
364 assay (ELA) (Eschbach et al., 2001; Ling and Trick 2010). Pelleted samples were incubated with
365 erythrocyte suspensions to allow lysis, and released hemoglobin was quantified. All samples
366 were centrifuged, triple-rinsed to remove debris, sonicated, and stored at 4 °C no longer than
367 48 h before testing. Assays were run in triplicate with positive (sonicated erythrocytes) and
368 negative (erythrocytes in buffer) controls. ELA incubations were conducted for 4, 10, 24, and

48 h at 18–25 °C, and hemolysis was calculated as a percentage relative to the prepared standard.

Water samples collected by the SJRWMD for routine monitoring between RKM103 and RKM47 were analyzed for cyanotoxins. Samples were prepared by two laboratories, with one freeze–thaw cycle and sonication before analysis. ELISAs were performed by the Lake Superior State University Environmental Analysis Laboratory, MI, for cylindrospermopsin (CYN) and saxitoxin (STX) (Abraxis, Warminster, PA) and MCYST (Envirologix, Portland, ME). Additional water samples collected by FWRI for event response between RKM31 and RKM97 were filtered and analyzed for the same cyanotoxins at FWRI by ELISA using similar standard methods according to the manufacturer's instructions. The toxin limits of detection as performed were approximately 0.04 ng mL^{-1} for CYN, 0.16 ng mL^{-1} for MCYST, and 0.02 ng mL^{-1} for STX.

2.10 Fish diagnostics

Fish were anesthetized in tricane methanesulfonate (Tricane S, Western Chemical Inc., Femdale, Washington, USA), weighed (g), and total and standard lengths (cm) measured. A gross external examination was conducted for clinical signs of infectious pathogens, pathologies, or abnormalities. Fish were necropsied, internal organs examined, and livers were excised and weighed. A small piece of kidney tissue was aseptically plated onto TSA augmented with 5% sheep's blood using standard bacteriological procedures. Plates were incubated overnight at 27 °C and bacterial isolates identified as described above. Pieces of liver, gill, anterior and posterior kidney, gastrointestinal tract (stomach, cecae, or anterior and posterior intestine), spleen, heart, brain, and muscle were fixed in 5% buffered paraformaldehyde for histopathology analysis. All

392 tissues from the fish samples collected from the LSJR and kept less than 12 h on ice were
393 dehydrated in a graded ethanol series, embedded in JB-4 glycol methacrylate resin or paraffin,
394 sectioned at 4 µm, and stained routinely with Weigert's hematoxylin and eosin (H&E) or special
395 stains as needed e.g., Perl's Prussian Blue to demonstrate iron deposition indicative of
396 hemosiderin (hemoglobin breakdown from excess hemolysis) (Luna 1968, Quintero-Hunter et
397 al., 1991). MCYST-LR immunohistochemistry (IHC) was used on sectioned fish livers (Fischer
398 et al., 2000).
399 Pieces of liver and gastrointestinal tract from freshly dead or moribund fish of at least 10 g
400 were separately bagged in whirlpacks and frozen at –80 °C pending cyanotoxin analyses (CYN,
401 STX, and MCYSTs). Tissues were extracted with 1% acetic acid in 80% methanol. Two mL of
402 solvent was added to 2 g of homogenized tissue and incubated for 40 min in a hot-water bath at
403 60°C ± 2°C. The homogenate was centrifuged at 3,500 × g for 10 min and the supernatant
404 decanted into a clean polypropylene centrifuge tube. The pellet was disrupted with an additional
405 2 mL of solvent and the incubation and centrifugation steps repeated. The supernatants were
406 combined. For STXs, 1 mL of the extract was diluted in water 1:4 and C-18–cleaned on a
407 conditioned cartridge column. The aqueous effluent was collected. CYN and STX were analyzed
408 using ELISA kits (Abraxis, Warminster, PA). Samples were analyzed for STXs using high-
409 performance liquid chromatography (HPLC) with fluorescence detection (Lawrence et al., 2005).
410 A Shimadzu HPLC equipped with a Phenomenex 150 × 4.6 mm, 5-µm C-18 column was used to
411 separate STXs. MCYST was analyzed using a protein phosphatase–inhibition assay (PPIA)
412 modified from Tubaro et al. (1996). Modifications were the addition of Tween 20 (0.05%) to the
413 buffer solution and the use of MCYST-LR as a standard. The toxin limits of detection as

414 performed were approximately 4 ng g^{-1} for CYN, 0.2 ng g^{-1} for STX ELISA, 20 ng g^{-1} for STX
415 HPLC, and 3 ng g^{-1} for MCYST.
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417 **3. Results**
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419 *3.1 Hydrological and meteorological conditions*
420
421 Prior to the start of the late May fish kill, the winter of 2010 was unusually cold. In January,
422 February, and March the mean daily water temperature at the Dancy Point USGS station was
423 11.9 °C, 13.2 °C, and 16.1 °C, respectively, compared to the long-term monthly means of 14.7
424 °C, 16.6 °C, and 19.6 °C for the same months. Water temperature sharply increased late in
425 March, rose sharply again the first week in May, and remained above normal for the rest of the
426 summer (Fig. 3).
427 The LSJR discharge in 2010 was above average in April and May and below average through
428 late summer and fall (Fig. 2). Storm fronts in late May and early July affected the usual
429 maturation and succession of blooms. Reverse flows led to pronounced temporary increases in
430 salinity in the oligohaline reach of the LSJR (Fig. 2). The reverse flow in May increased salinity
431 at the Buckman Bridge (RKM53) from fresh to 6.5 over the course of one week and had been
432 propagated upstream and was observable in vertically integrated surface samples as a salinity
433 spike in sampling by 8 June at RKM58 (2.5 m depth) and RKM69 (7.6 m depth), but not as far
434 upstream as RKM80 (Shands Bridge). The early July reverse flow raised salinity at RKM53
435 from a vertically mixed 1 to a 6.7 bottom and 4.5 surface-stratified water column in 3 days and
436 was observable upstream on 7 July sampling at RKM58 (Mandarin Point, 2.5 m depth).

437 *3.2 Phytoplankton bloom chronology, nutrient dynamics, and water quality*

438

439 The 2010 spring bloom was dominated in the freshwater areas of the LSJR by
440 *Aphanizomenon flos-aquae*, whereas the estuarine portion was dominated by a mixed assemblage
441 of diatoms with no clear dominant species. *Aphanizomenon flos-aquae* constituted 22% of the
442 total phytoplankton biovolume upstream in Lake George (RKM180) on 19 April and 31% on 6
443 May, but accounted for only 4% and 3%, respectively, of the relative biovolume on the same
444 dates at Racy Point (RKM103). By 12 May, the *A. flos-aquae* bloom had spread from RKM121
445 to RKM97, with patches of extremely dense accumulations. A multistation transect sampling
446 from RKM125 to RKM100 conducted on that date found that 26 of the 58 samples had chl *a*
447 concentrations > 100 µg L^{-1}, with two stations > 300 µg L^{-1}. Before the tributary runoff and
448 subsequent river (receiving water) reverse-flow event in late May, the *A. flos-aquae* bloom was
449 centered in the freshwater reach of upstream LSJR, from Palatka (RKM127) through Racy Point
450 (RKM103). At the latter station, the cell biovolume was 52,573,710 μm^3 mL^{-1} on 19 May with a
451 pheophytin-corrected chlorophyll of 290.4 µg L^{-1}.

452 Following the 17 May rainfall event, tributary inflow and a relaxation of northeasterly winds
453 repositioned the plug of water containing this bloom downstream near RKM80 (Shands Bridge).
454 TN and TP concentrations measured at RKM103 near the center of this bloom on 19 May were
455 3.53 and 0.172 mg L^{-1}, substantially above the monitoring site values immediately upstream and
456 downstream, and values measured at this location two weeks prior (Fig. 4A, E). These elevated
457 observations were likely the result of high nutrient concentration tributary inflows entering this
458 reach during the May 17 runoff event. Samples collected on 4 May from Rice Creek, a large
459 tributary on the western bank (near RKM120), had a TN concentration of 6.8 mg L^{-1} and TP of

460 0.55 mg L^{-1}; and on 17 May had concentrations of 4.1 mg L^{-1} TN and 0.21 mg L^{-1} TP.
461 Automated samplers recording inflow concentrations in Deep Creek, a large, agriculture-
462 dominated watershed on the eastern bank in this reach, reached peak concentrations on 25 April
463 and 18 May of 13.7 and 26.7 mg L^{-1} for TN and 7.4 and 3.8 mg L^{-1} for TP respectively. An
464 adjacent tributary of this same region, Dog Branch (29.6988°, -81.5816°), had peak stormwater
465 runoff concentrations on 18 May of 12.6 and 9.75 mg L^{-1} for TN and TP. Ambient sampling
466 events from these tributaries indicated P over-enrichment, with the mass ratio of NO_3^- and PO_4^{3-}
467 as N and P in three of the four collections ranging from 2.1–0.6. In addition to these stormwater
468 inputs, atmospheric N-fixation by *A. flos-aquae* (Horne 1979) presumably contributed to the
469 river TN concentration. In mid-May, ammonia and pH levels exceeded 0.016 mg L^{-1} and 9.0
470 respectively at RKM104 and farther upstream (Fig. 4C, D), while upstream daytime DO and DO
471 saturation concentration exceeded 9.0 mg L^{-1} and 120 percent respectively (Fig. 4G, H).
472 Following the reverse flow in late May, the attenuated signature of the freshwater-reach TN,
473 TP, and *A. flos-aquae* concentration peaks were discernable downstream in samples collected
474 through June from Shands Bridge (RKM80) to Piney Point (RKM47) (Fig. 4). Phytoplankton
475 samples collected by both FWC-LE and SJRWMD in this reach on 2–10 June still identified
476 high densities of *A. flos-aquae* (>97 × 10^6 cells L^{-1} at Piney Point, Fig.1), as well as lower
477 densities of *Dolichospermum circinale* and *Microcystis* (comprising three morphospecies, *M.*
478 *aeruginosa*, *M. viridis*, and *M. weisenbergii*). *Microcystis* spp. had not been observed in earlier
479 samples collected upstream. At RKM47, the plankton composition in early June was dominated
480 by diatoms (salinity 3), shifting to a mixed community of salinity-tolerant cyanobacteria
481 (*Cyanodictyon,* a mesohaline species), diatoms, dinoflagellates, raphidophytes, and flagellates in

482 mid-June (salinity, 3.5). The chl *a* peak remained in the vicinity of Shands Bridge through June
483 (Fig. 5A).

484 Also in early June, river concentrations of pheophytin (Fig. 5B), NO_3^- and NH_3 (Fig. 4B–C)
485 reached their highest levels, and DO concentrations in surface water (to 0.5 m depth) fell to their
486 seasonal minimum, reaching 5.29 mg L^{-1} (at 0920) at RKM80, 4.67 mg L^{-1} (at 0945) at RKM97,
487 and 4.9 mg L^{-1} (at 1205) at RKM127 (Fig. 4G). Hypoxic or anoxic conditions were not
488 indicated from the continuous diel DO data for the three USGS stations locations operating in the
489 LSJR (Fig. 6). The lowest recorded DO level was 4.3 mg L^{-1} at 0900 on the 1st of July at
490 RKM113 (Dancy Point). pH levels reached 8.5 at RKM69 and farther upstream after mid-June
491 (Fig. 4D). The reach between RKM80 and RKM59 remained oligohaline (salinity < 2.12) for
492 most of June. The maximum salinity achieved at the downstream end of this reach was 6.0 on 8
493 June, while the maximum daily average salinity recorded at the continuous USGS station at
494 RKM53 was 3.7 on 17 June.

495 By the end of June, *A. flos-aquae* had declined to negligible numbers at the downstream end
496 of the reach, and the raphidiophyte *H. akashiwo* was the dominant phytoplankter at several
497 locations, with cell densities $>10^6$ L^{-1} (Fig. 7A). A second run-off and reverse-flow event
498 starting on 2 July increased salinity (up to 6 at RKM47) throughout the reach and moved the
499 centroid of the remaining *A. flos-aquae* biomass peak back to upstream of, and beyond RKM69
500 (Fig. 7A). This possibly led to stress and the ultimate senescence of this bloom. In riverwide
501 sampling conducted on 6–7 July, pheophytin levels from RKM90–69 were elevated (Fig. 5B),
502 suggesting bloom decline, and in sampling performed 22 July, un-ionized ammonia was elevated
503 in this same region (Fig. 4C). At the time of the 22 July sampling, the second reverse-flow event
504 had dissipated, and salinity had dropped to 1.6 (Fig. 2).

505 A second bloom of the season occurred in mid-August and was centered around RKM103
506 and the Dancy Point USGS continuous monitor (RKM113). The chl *a* concentration at the
507 former site was 104 µg L^{-1} on 24 August. At the beginning of August, phytoplankton sample
508 biovolume data indicated that *Pseudanabaena limnetica* was now the dominant cyanobacterium
509 through much of the reach from RKM115 to RKM69, with *Planktolyngbya*, *Microcystis,*
510 *Aphanizomenon, Dolichospermum and Cylindrospermopsis* also present (Fig. 7B).
511 Phytoplankton samples collected on 12 August upstream at the outlet of Lake George indicated a
512 mixed bloom of *Pseudanabaena* spp. and *Cylindrospermopsis raciborskii*.
513 This longitudinal bloom transport pattern is evident in the CI images (Fig. 8), developed from
514 surface reflectance (rho_s) measured by the MERIS (European Space Agency). These
515 intermittent images from 20 April through 20 September show a cyanobacterial bloom
516 (confirmed by plankton sample analysis) developing in the freshwater reach of the LSJR and
517 expanding and extending downstream in the image recorded on 1 June. In the images recorded
518 from 10 June through 20 June, the bloom gradually declines (Fig. 8). The second freshwater
519 bloom dominated by *Pseudanabaena* spp. in mid-August is evident in the 16 August image (Fig.
520 8). This bloom can be seen to extend south through Lake George.
521
522 *3.3 Cyanotoxins, hemolysins, and hemolytic bacteria*
523
524 Cyanotoxin (CYN, STX, and MCYST) concentrations in water samples collected 13 May to
525 14 September from RKM143 to RKM47 were generally low (< 1.18, 0.64, and < 2.0 ng mL^{-1},
526 respectively). The exceptions were bloom scum samples collected near Shands Bridge (RKM80)
527 on 10 and 16 June that were positive for MCYST, at concentrations of 6,000 and 580 ng mL^{-1} ,

528 respectively (Envirologix EP 022 ELISA) (Table 2). Maximum concentrations of CYN and STX
529 were 1.18 and 0.64 ng mL^{-1}. Cyanotoxin concentrations were low in event response samples,
530 with maxima of 3.21 ng mL^{-1} MCYST (n = 8), 0.041 ng mL^{-1} CYN (n = 4), and 0.18 ng mL^{-1}
531 STX (n = 4).

532 Hemolytic activity of unfiltered whole-water samples (including algal and microbial
533 components) collected on 30 June (four weeks into the fish kill) was highest at RKM63 and was
534 present in the region of the active fish-kill zone where fish were observed dying between
535 RKM52 and RKM79 (Fig. 7A). In general, hemolytic activity paralleled the distribution of
536 *Microcystis* but was related by biovolume. There was no apparent relationship to the high
537 biovolume of *Heterosigma* at RKM47 (Fig. 7A), where hemolytic activity was low.

538 Water samples that were frozen and processed from 30 June for hemolytic bacteria showed
539 dominance by *Bacillus cereus* throughout RKM stations from Shands Bridge (RKM80) to
540 Tallyrand (RKM31) at salinities ranging from 0.5 to 10.4. Occurring with *B. cereus*, one other
541 species, *Aeromonas schubertii*, was detected at Mandarin Point (RKM59), Christopher Point
542 (RKM47) and at Tallyrand (RKM31, salinity 10.4). The mean number of hemolytic bacteria
543 colonies was greatest at RKM47, at the downstream edge of the decomposing bloom. On 4
544 August, one month after the fish kill had ended, *B. cereus* was still the dominant species of
545 hemolytic bacteria, but its density was less than in the prior month.

546

547 *3.4 Fish kill*

548

549 The LSJR fish kill lasted approximately six weeks. Over 300 reports to the FWC-FWRI-
550 FWH fish kill hotline were received from 27 May through 5 July, the period defined by the two
551 major reverse-flow events in the LSJR (Fig. 2). First reports of dead fish spanned the stretch

552 from Shands Bridge (RKM80) to downtown Jacksonville (RKM39) and included euryhaline red
553 drum, striped mullet (*Mugil cephalus*), and ladyfish (*Elops saurus*) and a few freshwater bluegill
554 (*Lepomis macrochirus*). Fish primarily affected were adult red drum, Atlantic stingray, striped
555 mullet, menhaden, gar, catfish, shad, and a few Atlantic croaker (Table 3). However, FIM trawls
556 during the height of the kill (early to mid-June, during the afternoon) showed many fish to be
557 apparently healthy (R. Brodie, personal communication).
558 The extent of the fish mortality reports that were verified independently ranged from RKM97
559 to RKM37 (upstream of Shands Bridge to downtown Jacksonville). Locations of dying and
560 moribund fish were more restricted, noted predominantly along the 25-km stretch between
561 Shands Bridge (RKM80) and the Buckman Bridge (RKM53) and were reported from the end of
562 May until mid-June (Fig. 1). Dead fish were minimally reported in Doctors Lake (typically
563 freshwater), where carcasses were likely wash-in from the mainstem LSJR. Dead fish were
564 observed scattered in generally low numbers along the 60-km stretch of river, with higher
565 numbers reported in and around Jacksonville (RKM55 to RKM37) (likely a function of increased
566 observer effect, river topography [sharp bend], and wind- or current-driven accumulation of
567 carcasses). Reports of a few scattered dead freshwater shad (unconfirmed species), bluegill, and
568 black crappie were noted between Racy Point (RKM103) and Rice Creek (RKM121) in early
569 June. During the first week of June, unidentifiable floating and decomposing fish carcasses were
570 reported as far upstream as Dancy Point (RKM113), with more than 100 dead or decomposing
571 red drum and stingrays noted between Shands Bridge (RKM80) and Scratch Ankle (RKM97).
572 Freshly dead fish were reported up until the end of June.
573
574

575 *3.5 Fish diagnostics*

576

577 Affected species manifested pathology indicative of chronic hemolysis and toxicosis. All fish
578 examined (red drum, *n* = 6; Florida gar, *n* = 1; Atlantic stingray, *n* = 2; striped mullet, *n* = 2)
579 (Table 4), particularly those processed as quickly as possible in the field, showed similar gross
580 clinical signs of disturbances of blood flow or histopathological changes in circulation: skin
581 tissue commonly on the ventral side was reddened in some fish (e.g., in Atlantic stingray and
582 striped mullet) (Fig. 9; Table 4); aneurysms in the brain or hemorrhage associated with
583 telangiectasias in the gill lamellae (although grossly, the gills were rather pale) ; hyperemic eyes;
584 thromboses or proteinaceous materials (or fluids) in the lumen of the blood vessels of the heart;
585 congested livers, kidneys, or brains; and indications of hemolysis in the blood vessels of the liver
586 and heart (Fig. 9), as well as deposition of iron pigments (hemosiderin) in the liver parenchyma
587 (not shown). Other histopathological findings included degenerative changes and necrosis of the
588 kidney tubules and urinary ducts in the six red drum examined. The exocrine pancreatic tissue
589 exhibited atrophy in four red drum.

590 Bacteriological examination was conducted on 17 fish (Florida gar [*n* = 1], striped mullet [*n*
591 = 5], menhaden [*n* = 5], red drum [*n* = 5], and Atlantic stingray [*n* = 1]). Several fish had positive
592 growth on media for bacterial colonies, but this was inconsistent. Uniform bacterial growth was
593 observed in the kidney of 5 fish, red drum (*n* = 1), striped mullet (*n* = 3), and menhaden (*n* = 1).
594 Two of the isolates were lost during media transfer, but others were identified as *Vibrio*
595 *alginolyticus*, *Aeromonas hydrophila*, and *A. veronii*. Two red drum had bacilli in the kidney
596 (Fig. 9). Other bacteria isolates were nonspecific (*Micrococcus* sp., *Aeromonas* sp.) and
597 considered opportunistic.

598 By ELISA, fish (menhaden, Florida gar, mullet, and red drum) had very low or undetectable
599 concentrations of MCYSTs, STXs, and CYNs in livers, stomach contents, or viscera (Table 4).
600 By IHC, MCYSTs were detected in red drum livers, but there was no detectable pathology (data
601 not shown), or indication of hepatotoxicity.
602
603 *3.6 Foam*
604
605 Starting mid-July, several weeks after the fish kill had ended, large quantities of foam were
606 reported through mid-August throughout the LSJR from RKM75 to RKM40. Two separate
607 collections of water and its overlying foam were analyzed for dominant cyanobacteria (i.e., this
608 was not a complete plankton analysis). In general, the cyanobacteria cell concentration in a foam
609 sample was less than that of the corresponding water sample but it was of comparable species
610 composition (data not shown).
611 At RKM64, the dominant cyanobacterium in the foam in late July was *Pseudanabaena* cf.
612 *limnetica* with decreasing biomass downstream, with secondary dominance by *Planktolyngbya*
613 sp. Consistent with phytoplankton observations for late July and early August for the LSJR
614 between RKM47 and RKM80, cyanobacteria were dominant and followed the ambient fresh to
615 low-salinity gradient. The co-occurrence of cyanobacteria in the foam is not unexpected given
616 their persistent presence in the water column of the LSJR during the summer.
617
618 **4. Discussion**
619
620 *Aphanizomenon flos-aquae* is often present in the phytoplankton of the LSJR; in 180 monthly
621 collections made since 1994 it has been observed in Lake George 49 times and in the freshwater

622 LSJR 62 times, but before 2010 its greatest relative abundances were 14.8% at Lake George and
623 9.3% at Racy Point in 2006 (SJRWMD, unpublished data). The dominance of *A. flos-aquae* in
624 the May 2010 bloom was unusual, and it was the first time that it had been observed as a bloom
625 dominant. This may have been either due to the unusually cold winter, which reduced the
626 abundance of the recent dominant diazotroph, *C. raciborskii* (Leonard and Paerl, 2005), a
627 northern-migrating tropical species that inhabits warmer waters (Briand et al., 2004), or a
628 response to the sharp increase in water temperature in early May, which could favor a species
629 that arises from akinetes (Wildman et al., 1974; Yamamoto and Nakahara, 2009).
630 *Aphanizomenon flos-aquae* is diazotrophic, and sediment-to-water-column biotranslocation
631 of P by this cyanobacterium has also been reported (Barbiero and Kann, 1994); hence the
632 pronounced N and P peaks may in part reflect the capability of the phytoplankton to import and
633 entrain N and P in the bloom and in the water column. The succession of *A. flos-aquae* by
634 *Microcystis* spp. is a relatively common occurrence in eutrophic systems, attributed to the release
635 of bioavailable N and P, or increase in temperature (Paerl and Otten, 2016). In this instance, the
636 succession appears to have been facilitated by turbulence, currents, and tributary inflows
637 associated with unusual, sustained nor'easter conditions that disrupted further *A. flos-aquae*
638 expansion and transported the bloom to a reach of the estuary hydrodynamically more favorable
639 for *Microcystis*. This event was unusual in its timing during what is normally the annual
640 precipitation minimum.
641 The sequence of events associated with the LSJR 2010 bloom is presented schematically in
642 Fig. 10. May 2010 started with an *A. flos-aquae*–dominated bloom of unprecedented density in
643 the shallow upstream LSJR freshwater reach between RKM127 and RKM103. A late May front
644 mixed, diluted, and relocated this bloom downstream to the reach between RKM80 and RKM47.

645 Elevated levels of un-ionized ammonia (at a maximum of 0.016 mg L^{-1}, considerably less than
646 levels considered toxic to estuarine fish [0.09–3.35 mg L^{-1}] , Eddy 2005) and pheophytin
647 observed in river sampling conducted in early June suggested a substantial amount of attrition in
648 *A. flos-aquae* associated with this downstream relocation, and though it persisted during June, it
649 was no longer the dominant species of the phytoplankton community. The presence of
650 *Microcystis* spp. at the upstream, freshwater end and of *H. akashiwo* at the downstream, saline
651 end represented relatively significant abundance in the phytoplankton during June in this
652 relocated bloom. Extremely high levels of MCYSTs were measured in bloom scum samples
653 collected in early June, and high levels of hemolytic activity were observed at the end of June
654 (hemolytic activity had not been measured earlier); the unusual mortality of mostly adult
655 euryhaline fish occurred during this period. At the end of June, neither *A. flos-aquae*, *Microcystis*
656 spp., nor *H. akashiwo* was dominant, but euryhaline fish continued to die, presumptively affected
657 by the senescing *A. flos-aquae* bloom and its by-products, and hemolytic bacteria were present in
658 the LSJR. A second front and associated reverse flow reinitiated the bloom-senescence sequence,
659 and the subsequent mixing with high-salinity water ended the bloom. A second spike of
660 pheophytin and un-ionized ammonia was observable at this time, as was a widespread oxygen
661 decline (though not at levels acutely lethal to fish). Shortly afterward, in mid-July, widespread
662 unusual accumulations of foam were reported, most likely associated with the intense
663 decomposition of cyanobacteria biomass (Marshall et al., 2005).
664 The fish mortality event was unusual in that not all species known to be present in the LSJR
665 (Miller et al., 2012) were affected; the fish that were killed were primarily adults or larger
666 individuals of susceptible species, particularly red drum and Atlantic stingrays. Occasionally, as
667 noted here, only larger fish are affected during a fish kill, but in those cases the putative cause

668 has been hypoxia (Perkins et al., 2000). Although an estimated several hundred (to likely
669 thousands) of baitfish were also reported dead, we recognize that the counts are underestimates
670 and no effort was made to quantify dead fish or potentially extrapolate quantities from
671 measurable surface area counts as per American Fisheries Society procedures (AFS 1992).
672 However, the baitfish counts still appear to be skewed by comparison to the benthic species
673 affected. Inherently, such numerically dominant schooling fish in the LSJR (MacDonald et al.
674 2009) should die in greater numbers (if all fish species were affected by the same environmental
675 factors) than the lower density benthic red drum, stingrays, catfish, and gar. The release of bi-
676 products and/or associated sub-optimal water quality from the decomposing bloom may have
677 been more significant near the benthos than at the surface and perhaps differentially affected
678 benthic species.

679 Coincident with the reverse flow during late May and early June, dead individuals of
680 euryhaline fish species were reported as far upstream as RKM97 (although in lesser numbers),
681 suggesting that with the higher salinity, fish moved upstream. The appearance of carcasses along
682 the river do not necessarily reflect where the fish died; they may also have been carried by water
683 current and wind during the reverse-flow period of early June. This suggests that euryhaline fish
684 were in the vicinity of the senescing and decomposing *A. flos-aquae* bloom around RKM80 in
685 early June. In fish obtained freshly dead or moribund from the LSJR, pathologies were consistent
686 in multiple species and suggested a complex multifactorial etiology. In contrast to previous fish
687 kills associated with cyanobacterial blooms, fish morbidity was not consistent with acute
688 cyanotoxin exposure or with low dissolved oxygen, but with chronic lethal hemolysis leading to
689 thromboses or to cardiac pathology. To our knowledge, such chronic effects on fish during
690 bloom events have not been previously documented. (It should be noted that experimentally

691 lethal doses of MCYST-LR can induce atypical pulmonary thrombosis in mice [Slatkin et
692 al.,1983], but parallels with the pathology observed here in fish, if any, are unclear).
693 Fish kills attributed to monospecific *A. flos-aquae* blooms or blooms with *A. flos-aquae and*
694 other codominant cyanobacterial species have been reported in North and South America,
695 Europe, and Asia (Prescott, 1933; Mackenthum and Herman, 1948; Schwimmer and
696 Schwimmer, 1968; Barica 1978; English et al., 1994; Kann 1998; Perkins et al., 2000; Jewel et
697 al., 2003). Such events have usually been rapid, and all fish have been affected in the zone of
698 impact. To our knowledge, the type of pathology seen in the LSJR fish kill has not been
699 associated with *A. flos-aquae* blooms or documented as a consequence of fish exposure to
700 cyanotoxins or secondary metabolites.
701 *Aphanizomenon flos-aquae* is reported to produce STXs (Sawyer et al., 1968; Ikawa et al.,
702 1982; Mahmood and Carmichael 1986; Ferreira et al., 2001), anatoxin-a (Rapala et al., 1993;
703 Sivonen and Jones 1999), and CYNs (Preussel et al., 2009), but not MCYSTs (Sivonen and
704 Jones 1999; Lyra et al., 2001; Šulčius et al., 2015; Cirés and Ballot 2016). However, earlier
705 reports (Sawyer et al., 1968; Mahmood and Carmichael 1986) may have misidentified *A. flos-*
706 *aquae* as other species (Li et al., 2000) such as *Cuspidothrix issatschenkoi* or *A. gracile*, which
707 are confirmed to produce STX (Li et al., 2003; Wood et al., 2007; Cirés and Ballot, 2016).
708 By-products of decomposing *A. flos-aquae* blooms considered to contribute to fish kills
709 include hydrogen sulfide and hydroxylamine (Prescott, 1948). Additionally, *A. flos-aquae*
710 produces other potentially harmful secondary metabolites (Papendorf et al., 1997; Underdal et
711 al., 1997; Dembitsky et al., 2000; Murakami et al., 2000; Kaya et al., 2006; Řezanka and
712 Dembitsky, 2006; Lv et al., 2016), but it remains unknown to what extent, if any, by-products of
713 the *A. flos-aquae* bloom played a role in the mortality event.

714 The presence in the LSJR of CYNs, anatoxin-a, and MCYSTs (Paerl et al., 2002; Williams et
715 al., 2007) and of STXs (FWC, Phlips et al., unpublished data) is not unusual, but concentrations
716 depend on which bloom taxa are dominant. During 2010, the presence of these toxins suggested
717 sources from several taxa. While lysing *A. flos-aquae* (and to a lesser extent *C. issatschenkoi*)
718 likely contributed to STXs and CYNs, systematic analysis of toxigenic algae in the LSJR has yet
719 to be completed. *Cylindrospermopsis raciborskii* was present only at low concentrations, and this
720 species is not known to produce CYNs in this system (Yilmaz et al., 2008). During the fish kill,
721 STXs, CYNs, and MCYSTs were found at low concentrations in the water column and in fish
722 tissues. Traces of anatoxin metabolites were also detected (C. Burleson, personal
723 communication). All of these cyanotoxins are considered as potential cofactors in the mortality
724 event.
725 MCYSTs were localized and detected at high concentrations only in and around RKM79 on
726 10 June in parallel with the *Microcystis* bloom that followed the downstream relocation and
727 partial senescence of the original *A. flos-aquae* bloom crash. Although contributing as added
728 stressors to the fish, MCYSTs are not considered the primary etiological agent in the mortality.
729 Overall, except for highly concentrated scum areas, water concentrations of MCYSTs were
730 generally low, becoming diluted or generally declining after several weeks. Degradation of
731 MCYST congeners -LR and -RR can take more than 3 weeks, with concentrations decreasing to
732 30–37% of the original in salinities ranging from 0 to 24 (Jones and Orr 1994; Lahti et al., 1997;
733 Harada and Tsuji 1998; Mazur and Pliński 2001).
734 MCYST concentrations in fish tissues were also low, indicating rapid depuration (Mohamed
735 and Hussein 2006) or toxin dilution through the food web (Ibelings and Havens 2008). In
736 cyanotoxin-related fish mortality events in which MCYSTs have been implicated, tissue

737 concentrations were at least one order of magnitude greater than those detected here (Chellappa
738 et al., 2008), a finding similarly supported by fish exposure studies assessing lethal MCYST
739 doses (Råbergh et al., 1991; Tencalla et al., 1994; Carbis et al., 1997; Ernst 2008). In some cases,
740 the ichthyotoxic mechanism for MCYSTs has been debated with conflicting interpretations of
741 the factors involved during a bloom-associated fish kill (Zimba et al., 2001; Snyder et al., 2002).
742 While species' sensitivities to cyanotoxins may vary with respect to toxicokinetics and
743 environmental variables, and there is increasing evidence that subacute or chronic cyanotoxin
744 exposure causes lethal and sublethal effects on fish (Carmichael et al., 1975; Råbergh et al.,
745 1991; Landsberg, 2002; Malbrouck and Kestemont, 2006; Martins and Vasconcelos, 2009), it
746 was not apparent in the 2010 event that a lethal threshold was reached. But exposure studies are
747 still needed to mimic the LSJR fish kill scenario before this supposition can be excluded. We
748 recognize the strong likelihood of a multifactorial etiology following the crash of the *A. flos-*
749 *aquae* bloom, with the potential influence of chronic exposure to sub-optimal water quality and
750 low-level cyanotoxins operating in combination with putative hemolytic factors.
751 Considering that cyanotoxins likely played only a secondary or synergistic role in the fish kill
752 and recognizing that there were mixed algal and microbial communities in the LSJR following
753 the collapse of the *A. flos-aquae* bloom, we assessed other potential sources of hemolytic
754 activity. In addition to environmental factors, a number of taxa in estuarine assemblages can be
755 hemolytic, with differing mechanisms, modes of physiological exposure (i.e., water column
756 versus systemic) and subsequent organismic effects. Many HAB species produce hemolysins
757 (Landsberg, 2002; Schug et al., 2010) that can cause fish kills in natural and artificial systems
758 (Deeds et al., 2002; Kempton et al., 2002; Landsberg, 2002; Mooney et al., 2010).

759 Differentiating the exact mechanism of a fish kill is often difficult under natural conditions, as
760 some microalgae produce multiple bioactive compounds (Landsberg, 2002).
761 Hemolytic HABs tend to be transient, with acute effects that induce rapid, nonspecific kills
762 within a zone of impact (Landsberg, 2002), the effects manifested after algal biomass reaches a
763 hemolytic threshold. Hemolytic activity usually correlates with the production of high
764 concentrations of specific fatty acids, glycolipids, or reactive oxygen species and, like toxin
765 production, may vary with environmental conditions, bloom stage and strains, and genetic
766 factors (Landsberg, 2002; Dorantes-Aranda et al., 2009; Zhou et al., 2011). Although HAB
767 bioactive compounds can be hemolytic experimentally, determined by their ability to lyse
768 erythrocytes, their ichthyotoxic mechanism is not usually expressed by causing hemolysis *in*
769 *vivo*. Fish pathology associated with hemolytic algae manifests typically as acute effects on the
770 gills, and hemolysis has not been well demonstrated by hematological and pathological
771 diagnostics. Although certain algae are characterized as hemolytic, hemolysis occurs because
772 bioactive products released into the water, or upon the gills upon cell lysis, cause targeted
773 pathological changes in epithelial cell membranes of the gill, not because hemolytic algae are
774 ingested (or are invasive like hemolytic bacterial pathogens), nor are hemolysins absorbed into
775 the bloodstream resulting in hemolysis of erythrocytes. Few studies have investigated or
776 demonstrated the potential of HAB hemolysins to cause systemic pathology (Kim et al., 2010).
777 Waterborne or cell-borne algal hemolysins act by altering the structure of the phospholipid gill
778 membrane in affected species (Arzul et al., 1995; Bodennec et al., 1995), typically inducing the
779 formation of pores in the membrane and targeting ion channels, disrupting the membrane and
780 causing ionic imbalances, resulting in osmotic stress and death by asphyxia (Sola, 1999; Deeds et
781 al., 2006; Van Wagoner et al., 2008; Ma et al., 2011).

782 The 2010 LSJR fish kill did not manifest as an acute die-off like those typically associated
783 with HAB toxins or low DO concentrations, nor was this scenario supported by the water quality
784 data. Overnight readings of DO levels from the USGS continuous monitoring sondes did not
785 indicate hypoxic or anoxic conditions sufficient to initiate or contribute to a fish kill. Further,
786 clinical signs and pathology of moribund fish lacked the physiological response typically
787 associated with hemolytic HABs. Some three weeks after the fish kill started, in mid-June, water
788 samples from the central zone of the fish kill had low concentrations of *H. akashiwo* (known to
789 produce hemolysins) (Ling and Trick, 2010) at a maximum of $>1.2 \times 10^6$ cells L^{-1} in Doctors
790 Lake and at lower concentrations in and around RKM63. By the end of June, while a high
791 biovolume of *H. akashiwo* was present at RKM47, this site was not demonstrating much
792 hemolytic activity compared with farther upstream, where it was absent. While this species is
793 suspected as a primary cause of fish kills, we consider here that it was only a coincidental co-
794 occurring factor.
795 Globally-reported ichthyotoxic densities of *H. akashiwo* are usually an order of magnitude
796 greater (Chang et al., 1990; Landsberg, 2002; Rensel, 2007) than those that were found in the
797 LSJR during the fish kill. Elsewhere, *H. akashiwo*–associated kills are highly acute, are
798 accompanied by obvious gill pathology, are nonspecific (very different from the LSJR event)
799 with respect to fish susceptibility and are not always attributable solely to hemolytic activity
800 (Chang et al., 1990; Carrasquero Verde, 1999; Twiner et al., 2001). Additionally, the highest *H.*
801 *akashiwo* cell concentrations were present in the adjoining freshwater Doctors Lake (30.1364°,
802 -81.7438°), where no active fish kills were observed, and although *H. akashiwo* is broadly
803 halotolerant (Tomas, 1978), its growth is reduced to negligible at brackish to low salinities
804 (Martinez et al., 2010). In the mainstem LSJR, *H. akashiwo* concentrations were low, restricted

805 to the downstream end of the bloom (near RKM70 to RKM50) and were still below ichthyotoxic
806 levels. Last, *H. akashiwo* did not appear in the plankton community until halfway through the
807 fish kill. Even though *H. akashiwo* can have a patchy distribution, we do not believe we missed
808 an active area of a bloom. Interestingly, this species was found at a salinity of 0.9, which is lower
809 than optimal and typically inhibits its growth (Martinez et al., 2010; Frederickson et al., 2011),
810 and could explain its low biomass. The appearance of *H. akashiwo* in mid-June at RKM63 and
811 then in late June at RKM47 may have been a response to the downstream influx of nitrogenous
812 compounds produced by the senescing *A. flos-aquae* bloom. *Heterosigma akashiwo* may initiate
813 or maintain blooms in response to exogenous nitrogenous sources (Tomas, 1979; Wood and
814 Flynn, 1995; Herndon and Cochlan, 2007), so we might hypothesize in the aftermath of the *A.*
815 *flos-aquae* bloom that *H. akashiwo* initially responded to available N sources. But a high
816 biomass with ichthyotoxic potential was not reached.
817 Having ruled out dinoflagellates and raphidophytes as sources of the hemolytic activity in the
818 LSJR, we considered other possible causes. Only a few cyanobacteria (e.g., *Synechococcus* sp.,
819 *Synechocystis* sp., and *Anabaena variabilis*) are hemolytic or known to produce exotoxic
820 hemolysins (Hashimoto et al., 1976; Sakiyama et al., 2006; Wang et al., 2007; Liu et al., 2008)
821 and have been shown only experimentally to be cytotoxic (Mitsui et al., 1989). In aquatic
822 systems, to our knowledge, cyanobacteria have not been documented to be hemolytic at natural
823 levels or associated with fish kills primarily induced by hemolysis. Even though MCYSTs are
824 hemolytic experimentally (Grabow et al., 1982; Sicinska et al., 2006), their role in natural
825 systems is unknown. But a possible role for hemolytic bioactive compounds originating from the
826 decomposing *A. flos-aquae* bloom should be considered as proposed here.

827 The growth, maintenance, termination, and decline of a cyanobacteria bloom can be strongly
828 influenced by microbial communities (Daft et al., 1975; Paerl, 1976; Mitsutani et al., 1987;
829 Rashidan and Bird, 2001; Kolmonen et al., 2004). Such blooms may also vector human bacterial
830 pathogens (Berg et al., 2009), but potential associations with animal pathogens are not
831 commonly investigated. The possibility that cyanobacteria-associated hemolytic bacteria could
832 play a role in animal mortality events has been minimally addressed. Notably, systemic disease
833 outbreaks by hemolytic bacteria can be triggered by marine HABs (Glibert et al., 2002), which
834 can act as stressors and increase susceptibility of fish to infectious pathogens. While associated
835 bacteria have been considered necessary determinants of HAB toxicity (Carrasquero Verde,
836 1999), a direct role for HAB-associated hemolytic bacteria in fish mortalities has not yet, to our
837 knowledge, been demonstrated.
838 Interestingly, predatory and algicidal cyanobacteriolytic *Bacillus* spp., including *B. cereus*
839 (Reim et al., 1974; Nakamura et al., 2002; Shunyu et al., 2006; Gumbo et al., 2010), play a
840 significant role in cyanobacterial decomposition and senescence (Daft et al., 1975; Rashidan and
841 Bird, 2001) and in cyanotoxin degradation (Christoffersen et al., 2002). More specifically, *B.*
842 *cereus* primarily targets *A. flos-aquae* (Nakamura et al., 2003; Shunyu et al., 2006). To our
843 knowledge there have been no reports of associated pathology in fish caused by waterborne
844 bacteriolytic activity during decomposition of a cyanobacteria bloom.
845 In considering possible sources of hemolysins, a dominant microbe might have been
846 significant following the collapse of the *A. flos aquae* bloom. It is undetermined whether lytic
847 bacteria can reach a biomass during bloom decomposition such that their bioactivity affects fish
848 (putatively indicated here in part as extracellular hemolysins) and whether potentially virulent
849 cyanobacteria-associated bacterial pathogens are also contributing factors. Using selective

850 media, we consistently documented the presence of beta-hemolytic *B. cereus* at all stations
851 sampled from RKM80 to RKM31 at salinities of 0.5 to 10.4 during the active fish kill and at
852 lower densities one month later in the same general location at which high hemolytic activity
853 was detected in the water samples. While we neither have control data collected before the fish
854 kill nor are aware of the normal distribution of this bacterium in the LSJR, the co-localization of
855 this hemolytic species with the decomposing *A. flos aquae* bloom is of particular interest, as fish
856 were presumably dying from a hemolytic pathogen or from exposure to unknown sources of
857 hemolysins in the water column. But it is not clear whether the demonstrated hemolysis is solely
858 from cyanobacteriolytic activity or from a combination of hemolytic factors, including bioactive
859 compounds created from decomposition of the bloom. Also, hemolytic activity in the field was
860 assessed from whole, unfiltered water samples, so these results could have included bioactivity
861 from multiple sources.

862 Sustained or chronic fish kills due primarily to bacterial pathogens (Austin and Austin, 2007)
863 sometimes include broadscale mortality following or triggered by HABs (Glibert et al., 2002).
864 Few studies have documented or appear to have investigated the presence of hemolytic bacteria
865 in the water and their possible role during fish kills. Even more specifically, *Bacillus* spp. are not
866 commonly implicated in fish kills (Pasnik et al., 2008); rarely are these potential pathogens
867 reported to be systemic or cause bacteremia in fish (Goodwin et al., 1994; Oladosu et al., 1994;
868 Ferguson et al., 2001). *Bacillus cereus* is found in a range of terrestrial and aquatic habitats and
869 has a broad salinity tolerance, ranging from freshwater to 66 (Kim et al., 1998; Ivanova et al.,
870 1999). Not typically connected or investigated during HAB events, pathogenic *B. cereus* causes
871 food poisoning and disease (Kotiranta et al., 2000; Bottone 2010) by producing beta hemolysins
872 and other exotoxins (Beecher and Wong 1994; Kim et al., 1998). The persistent co-occurrence of

873 hemolytic *B. cereus* in the water during the 2010 LSJR fish kill suggested a causative role for
874 this pathogen. One possibility is that we may have underestimated the presence of other
875 potentially hemolytic bacteria as the samples were frozen overnight prior to processing.
876 Therefore, some more sensitive species might have been missed, if they had been present. Not
877 unexpectedly, low concentrations of cyanotoxins were found in fish tissues, so we cannot rule
878 out a synergistic role for chronic exposure to cyanotoxins and hemolytic compounds. Overall,
879 the widespread distribution of *B. cereus* in the LSJR during the termination of the *A. flos-aquae*
880 bloom and its possible role in the fish kill raised broader questions about bacteria in the LSJR
881 during bloom and nonbloom conditions, their interactions with HABs, and their hemolytic,
882 pathogenic, and ichthyotoxic capacities.

883 Although hemolytic bacteria (e.g., *Vibrio*, *Aeromonas*, and *Streptococcus* species) cause
884 systemic disease in fish (Austin and Austin, 2007), we did not consistently isolate any primary
885 pathogen in moribund individuals. Bacterial isolates were nonspecific and likely secondary
886 opportunistic pathogens, and the clinical and pathological signs of examined fish were not
887 suggestive of an infectious pathogenic process. But for those fish in which rapid pathological
888 analysis was possible, the presence of ghost cells from lysed erythrocytes and other pathologies
889 associated with hemolytic activity was a consistent factor (and not considered to be artefact). We
890 could not confirm systemic infections of *B. cereus* in moribund fish, although large bacilli
891 consistent with *Bacillus* cell morphology were noted in tissues. But Koch's postulates still must
892 be met, since the co-occurrence of a hemolytic bacterium in the water, while strongly
893 circumstantial and hypothesized here to be ichthyotoxic, does not necessarily prove causality
894 without confirmatory experimental exposures.

Cyanobacteria blooms directly or indirectly influence water quality. As well as affecting DO levels and increasing turbidity, the endogenous or extrinsic production of nitrogenous compounds by or associated with cyanobacteria blooms can increase concentrations of NO_2, NO_3, and ammonia to levels that can impair fish health (Lindholm et al., 1989; Havens, 2008). In dense freshwater blooms, elevated temperature and pH lead to reaction kinetics that increase the levels of un-ionized ammonia, concomitantly increasing the potential toxicity to fish. Adverse conditions during *A. flos aquae* blooms can occur with pH exceeding 10 and concentrations of un-ionized ammonia exceeding 1 mg L^{-1} and hypoxic conditions following bloom die-offs (Kann 1998; Perkins et al., 2000). Such bloom effects contribute to poor water quality and increased fish susceptibility to bacterial infections (Morris et al., 2006).

Such a dramatic poor water quality scenario was not demonstrated for the LSJR 2010 fish kill. During the periods of decline and collapse of the *A. flos-aquae* bloom and subsequent plankton community succession, water quality was suboptimal, but DO and un-ionized ammonia did not generally exhibit levels that would be expected to lead to acute fish mortality, and the region of poor water quality was found farther upstream from the fish kill. It is feasible that a combination of sub-optimal water quality factors, along with increased water temperature promoting susceptibility to bacterial infections, and the cyanobacteria bloom sequelae could have contributed to a chronic multifactorial fish kill.

During the upstream *A. flos-aquae* bloom in May, and in general following the bloom crash, freshwater fish were negligibly affected compared to downstream euryhaline species. Gill pathology (showing telangiectasis) of affected euryhaline fish was not indicative of acute exposure to high pH, or high ammonia or NO_2 concentrations (Randall and Tsui, 2002; Lease et al., 2003; Kroupova et al., 2005). There was no evidence for blood methemoglobinemia or

pathology typically associated with chronic NO_2 exposure. NO_2-associated hemolytic anemia has been reported in freshwater fish (Tucker et al., 1989), but it is not a common health problem for euryhaline species, where chloride ions in brackish or low-salinity areas usually buffer any potential effects of NO_2 on fish (Kroupova et al., 2005). Even when marine fish take up NO_2, plasma NO_2 and methemoglobin concentrations typically remain below external levels (Grossel and Jensen, 2000) and pathological effects and hemolysis are induced only at extremely high levels (>700 ppm) (Park et al., 2007) at concentrations that were not environmentally relevant during the LSJR fish kill. Even though euryhaline red drum appear to be more sensitive than other marine species to the effects of NO_2 in low salinities, effects are noted at concentrations greater than 3 mg L^{-1} (Wise and Tomasso, 1989), concentrations more than 10 times those observed during the LSJR kill. While the NO_2 levels in the LSJR were apparently within optimal limits for fish, we cannot rule out chronic effects of these NO_2 levels in synergy with other factors. Compounded with exposure to low-level cyanotoxins, chronic exposure of fish to localized suboptimal water quality conditions may have exacerbated the effects putatively caused by hemolytic compounds (from an as yet unknown source).

The appearance of vast quantities of foam predominantly through RKM75 to RKM40 followed the crash of the mixed cyanobacteria bloom that persisted just above this area through June and following the fish kill. Decomposing cyanobacteria blooms are sometimes associated with foam production (Marshall et al., 2005). Foam is associated with numerous plant and microbial species and is a by-product of, e.g., protein, lipopeptide, or fatty acid surfactants (Schilling and Messner, 2011). In the 2010 LSJR bloom, the production of foam occurred following the end of the fish kill with the demise of the cyanobacteria bloom and is not considered to be a factor in the fish mortality event.

941

942 **5. Conclusion**

943

944 A hypothetical sequence of events and factors that contributed to the LSJR 2010 fish kill and
945 production of foam is proposed. The kill was triggered by a significant salinity spike caused by a
946 reverse-flow event in late May that contributed to the collapse of a dominant freshwater
947 *Aphanizomenon* bloom farther upstream. Euryhaline fish were associated with the senescing
948 bloom or its associated by-products downstream. The decomposing bloom released cyanotoxins,
949 caused bacterial lysis of cyanobacteria cells, and produced a high organic loading that led to
950 changes in the diversity and dominance of the plankton community. DO levels and other water
951 quality parameters were within normal ranges in the reach of the fish kill, although elevated
952 ammonia levels and pH were noted farther upstream in the vicinity of the decomposing bloom.
953 Fish were exposed to a combination of stressors, including hemolysins, low concentrations of
954 cyanotoxins, and poor water quality. These consequences resulted in complex pathological
955 changes on fish that were not consistent alone with acute cyanotoxin exposure or poor water
956 quality but were attributable primarily to chronic lethal hemolysis. Potential sources of hemolytic
957 activity included the dinoflagellate *H. akashiwo*, cyanobacteria *Microcystis* spp., and the
958 potentially pathogenic bacterium, *Bacillus cereus.* While all of these factors coincided, the
959 source of the putative hemolysins, although strongly suspected to be bacterial, remains
960 unconfirmed.

961 Extrapolating cyanobacteria-associated fish mortalities in the field with only partial
962 knowledge of changing environmental conditions and without systematic single and
963 multifactorial experimental exposures can only be interpreted hypothetically and on the weight

964 of evidence presented. The complex of metabolites and toxins as well as dynamic changes in
965 water quality that follow *A. flos-aquae* crashes need to be better understood. It is important to
966 monitor the changes in plankton dominance in relation to nutrient dynamics and water quality,
967 where shifts in HAB dynamics and dominance may change the prevailing balance of toxins,
968 bioactive compounds, microbes, and water quality, and thus create differential effects on
969 susceptible biota.
970 The sequence of events in the LSJR in 2010 provides a compelling reason to better
971 understand the interplay between HAB and bacterial populations, the function of HABs as
972 vectors of pathogens, and the system ecology during the demise of a cyanobacteria bloom. The
973 potential role for hemolytic bacteria involved in decomposing senescing cyanobacteria blooms as
974 etiologic agents of fish mortalities represents a new and uninvestigated pathway in which HABs
975 might affect estuarine food webs and vector pathogens into coastal ecosystems.
976
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978
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Fig. 1 Map of the LSJR showing the mainstem monitoring stations (red dots), weekly locations of reported dead and moribund fish (various color triangles and stars, respectively), river kilometer markers (white plus signs), and USGS stations with continuous water quality monitoring sensors (Dames Point, Buckman Bridge, Dancy Point, and Buffalo Bluff; peach pentagons). (A) estuarine/freshwater reach (RKM0 to RKM80), (B) freshwater reach (RKM80 to RKM150). Water quality monitoring stations are identified by name and nearest kilometer marker upstream from the river mouth.

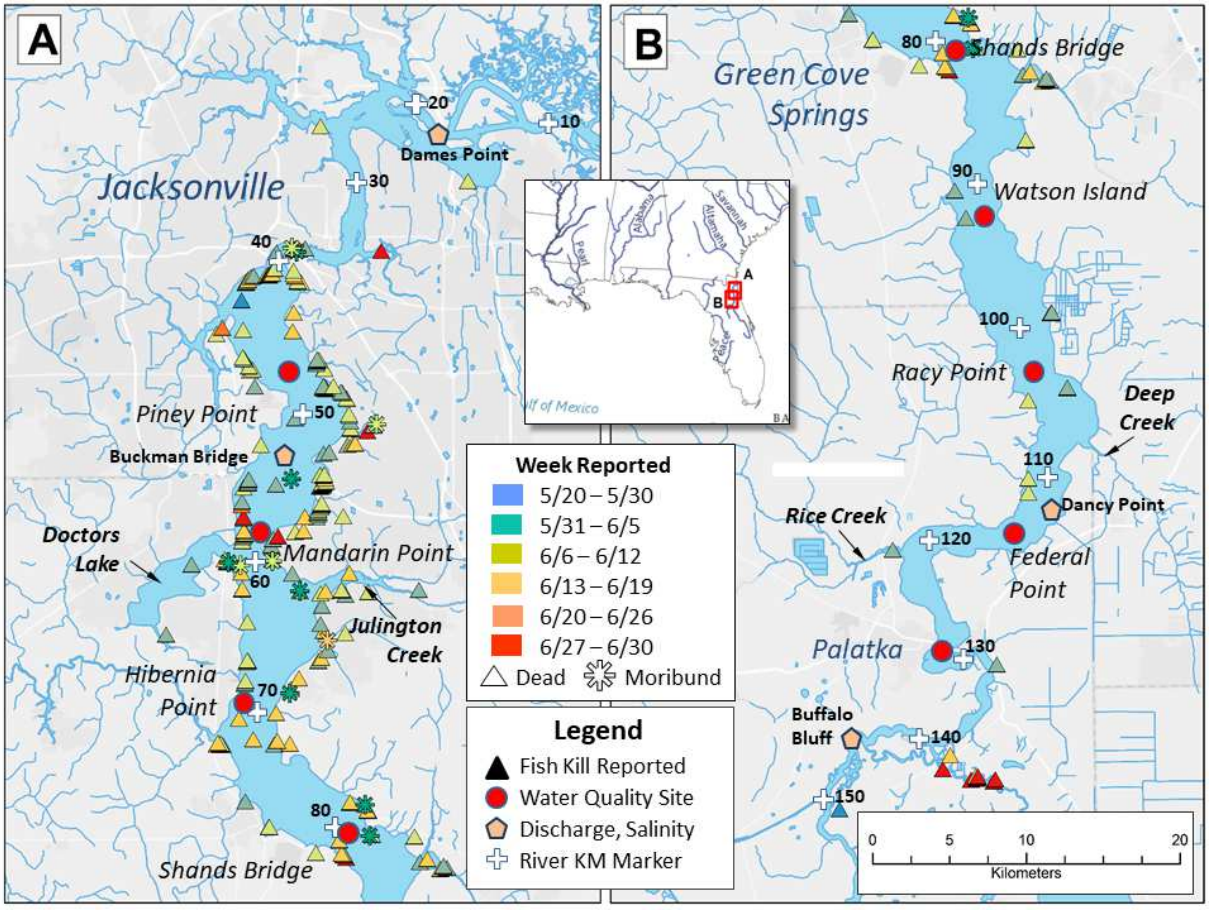

Table 1 Sample types (routine or event response), collection dates, and analytical tests on water (water quality [WQ], nutrients, phytoplankton, microbiology, hemolysis) and fish (diagnostics, toxins) samples. F = foam sample also collected. *Fish diagnostics included gross pathology, histopathology, bacteriology, parasitology, mycology, and hematology.

Date (2010)	Analysis							
	Water						Fish	
	WQ	Nutrients	Phytoplankton	Toxins	Microbiology	Hemolysis	Diagnostics*	Toxins
Routine								
21–22 April	+	+	+	+				
3–5 May	+	+	+	+				
13 May	+	+	+	+				
17–20 May	+	+	+	+				
8–10 June	+	+	+	+				
15–16 June	+	+	+	+				
21 June				+				
23–24 June	+	+	+	+				
6–7 July	+	+	+	+				
21–22 July	+	+	+	+				
29 July	+	+	+	+				
9–10 Aug	+	+	+	+				
24–26 Aug	+	+	+	+				
31 Aug	+	+	+	+				
8–9 Sept	+	+	+	+				
14 Sept	+	+	+	+				
28–29 Sept	+	+	+	+				
Event								
2–3 June	+		+	+			+	+
7–10 June	+		+	+			+	+
15–16 June	+		+	+			+	+
23 June	+		+					
30 June	+		+	+	+	+		
22 July	+		+ F					
4 Aug	+		+		+			
5 Aug	+		+ F		+F			
22 Aug	+		+ F		+F			

1535 **Fig. 2** Discharge and salinity time series during 2010 for the USGS stations in the LSJR at Buffalo Bluff (discharge,
1536 RKM 145) and at Buckman Bridge (salinity, RKM 53).
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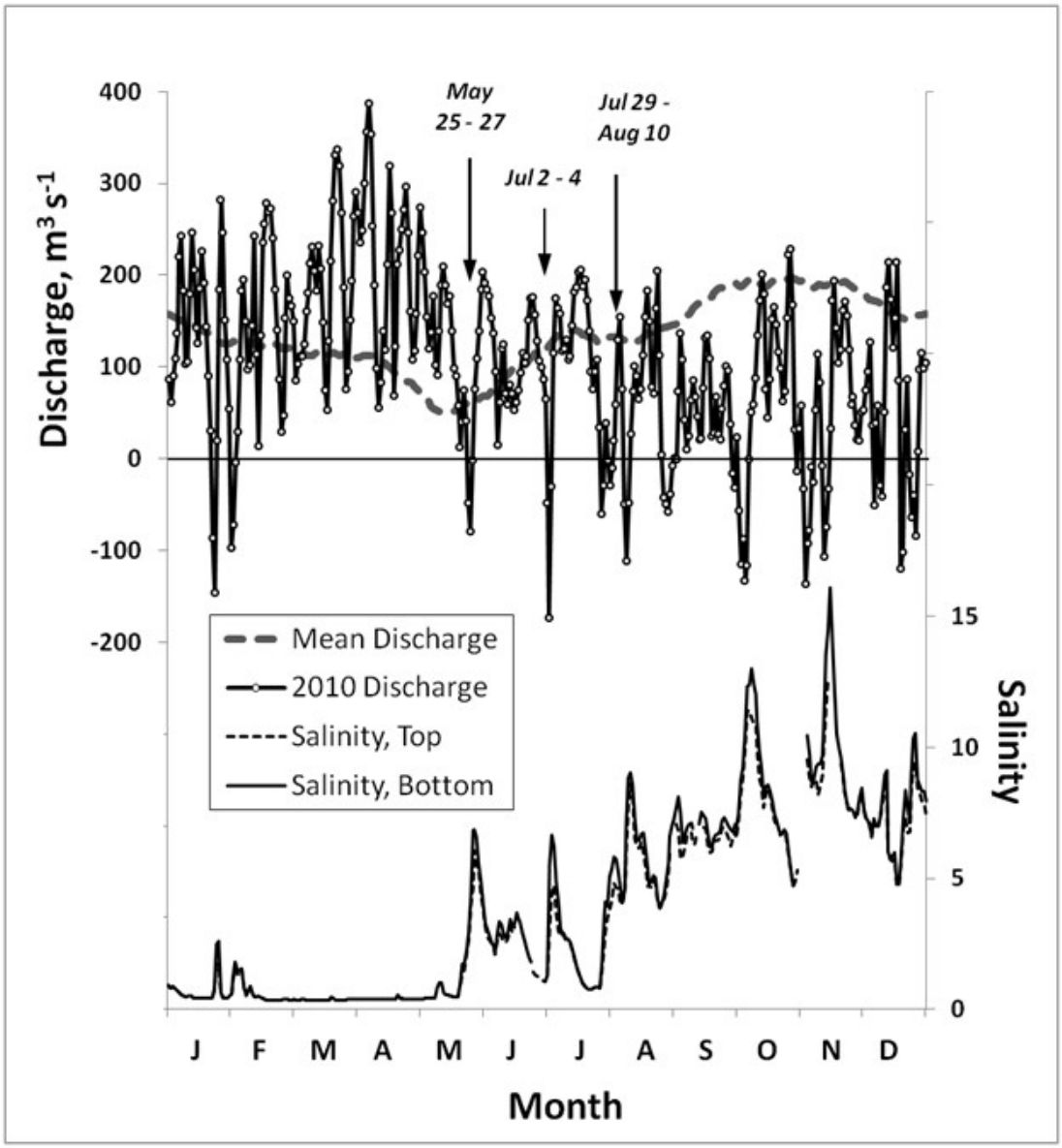

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1543 **Fig. 3** Daily water temperature recorded at Dancy Point (RKM113) during 2010.

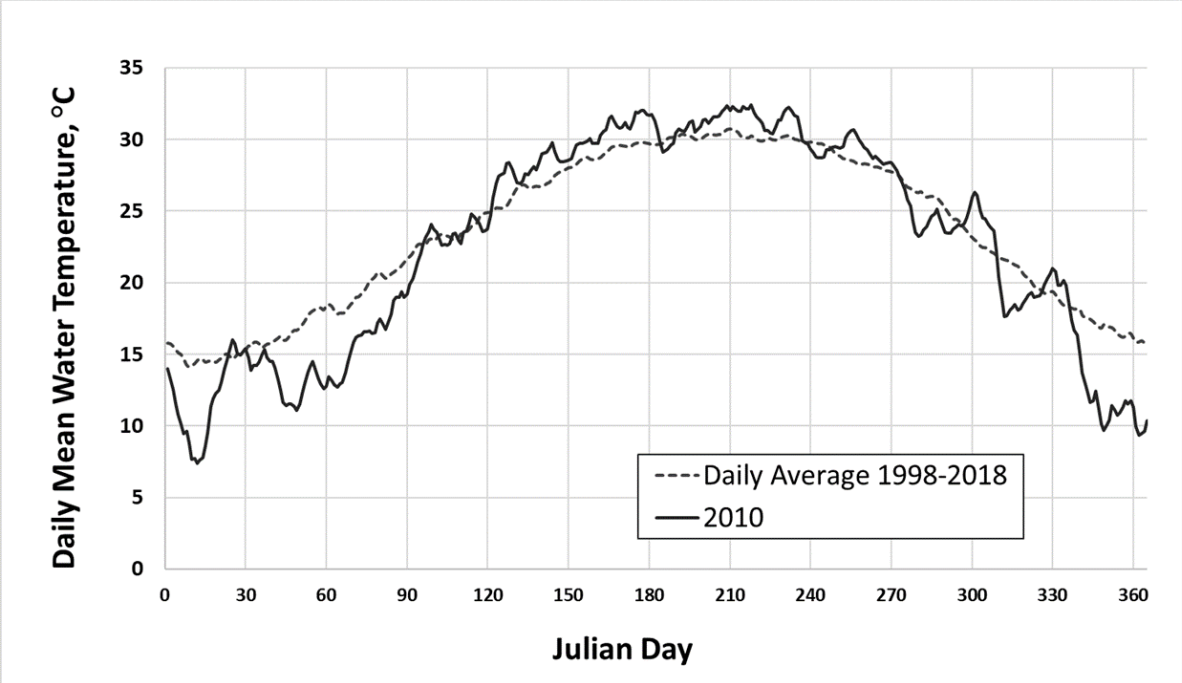

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Fig. 4 Longitudinal transect chemistry data for six LSJR sampling excursions from 4 May through July 22. (A) TN, (B) nitrate + nitrite –N, (C) un-ionized ammonia, (D) pH, (E) TP, (F) PO_4^{3-}, (G) DO, and (H) DO percent saturation (%). Concentrations for (A) to (G) are in units of mg L^{-1} (x-axes). Note that x- axes are not uniform in scale. The y axis is the river kilometer distance from the mouth. Events are numbered in chronological order.

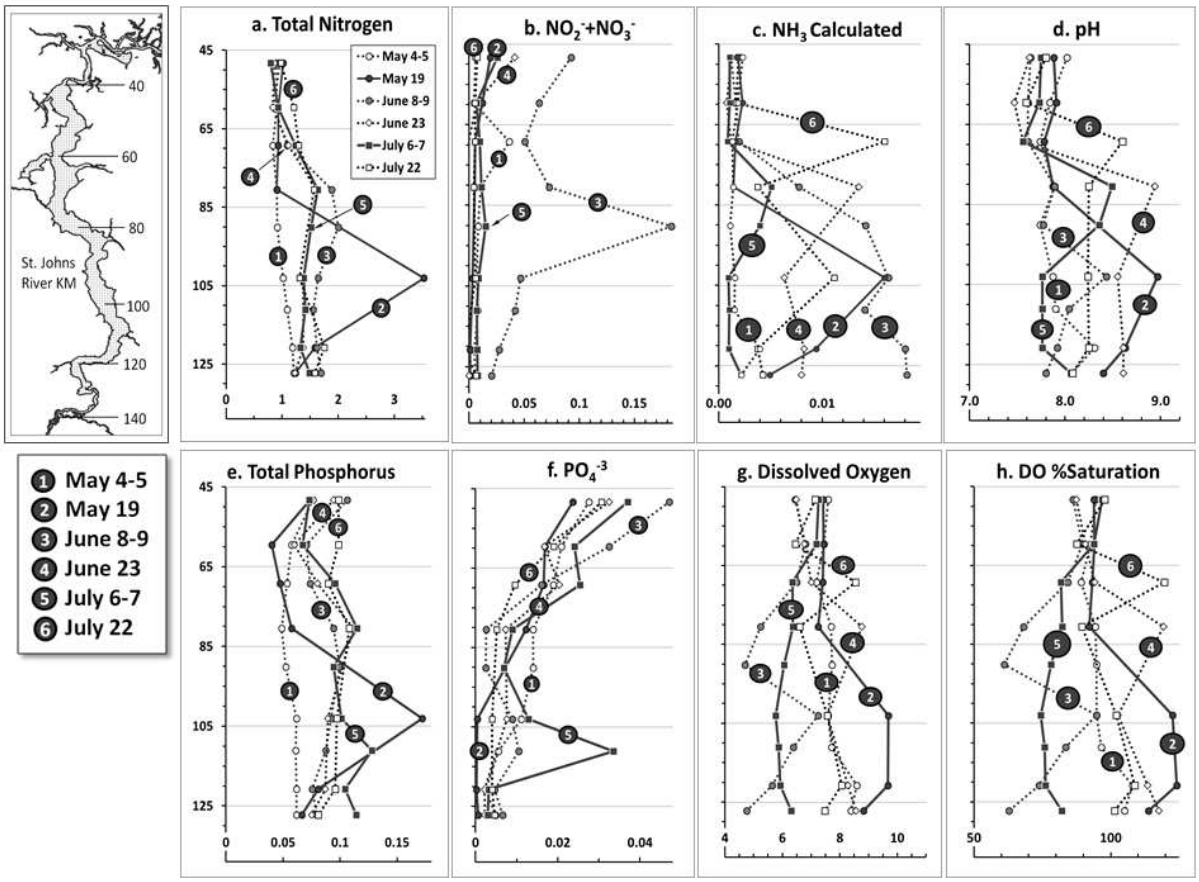
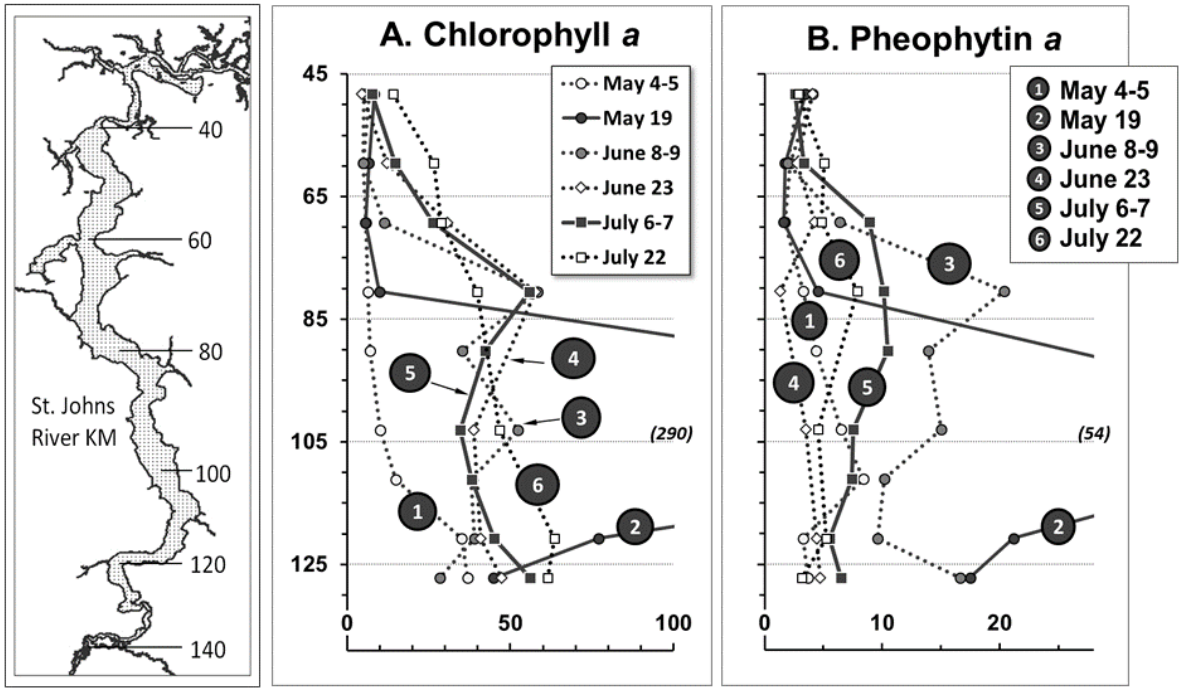


Fig. 5 Longitudinal transect for (A) chl *a* and (B) pheophytin *a* data for six LSJR sampling excursions from 4 May through July 22. Concentrations are in mg m^{-3}. Note that x- axes are not uniform in scale. The y axis is the river kilometer distance from the mouth. Events are numbered in chronological order.



1585 **Fig. 6** Continuous hourly dissolved oxygen concentrations measured in the LSJR, April–September 2010. USGS
1586 continuous water quality sites are Dames Point (RKM 17, blue line), Buckman Bridge (RKM 53, black line), and
1587 Dancy Point (RKM 113, green line).
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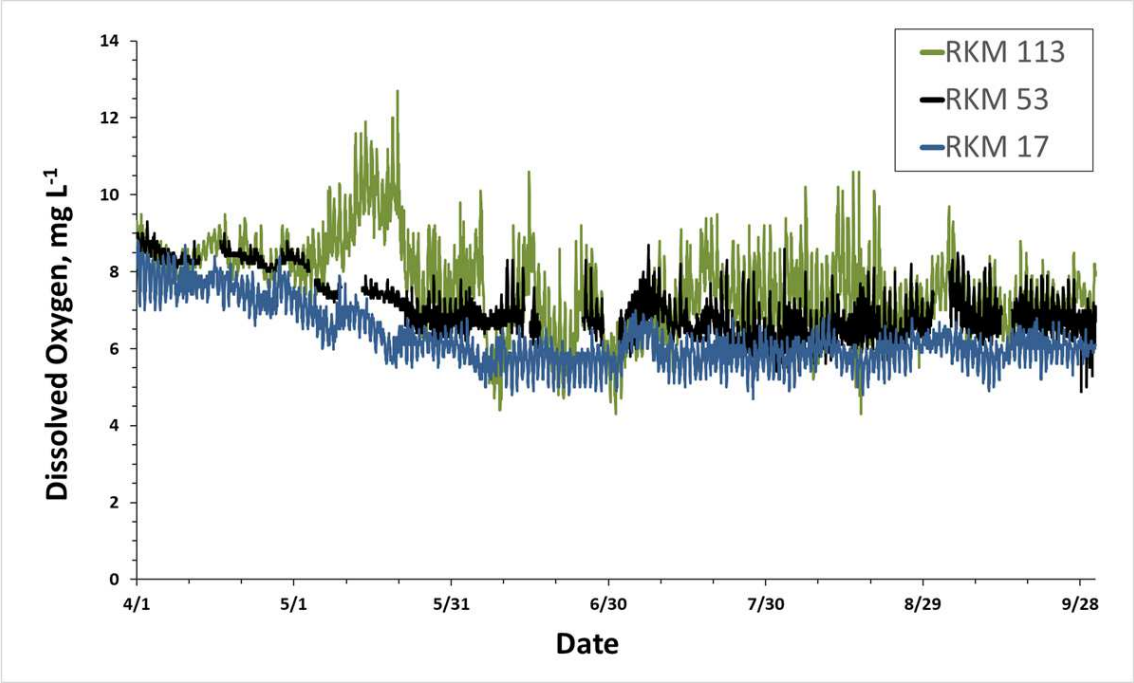

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1601 **Fig. 7** Biovolume of phytoplankton (bars), abundance of hemolytic bacteria (black line, mean number of colonies
1602 per plate, $n = 2$), and hemolytic activity (brown line) of water samples taken along the LSJR upstream (freshwater) –
1603 downstream (estuarine) gradient from Shands Bridge (RKM80) to Tallyrand (RKM31) on (A) 30 June 2010 and (B)
1604 4 August 2010 (minus hemolytic activity).

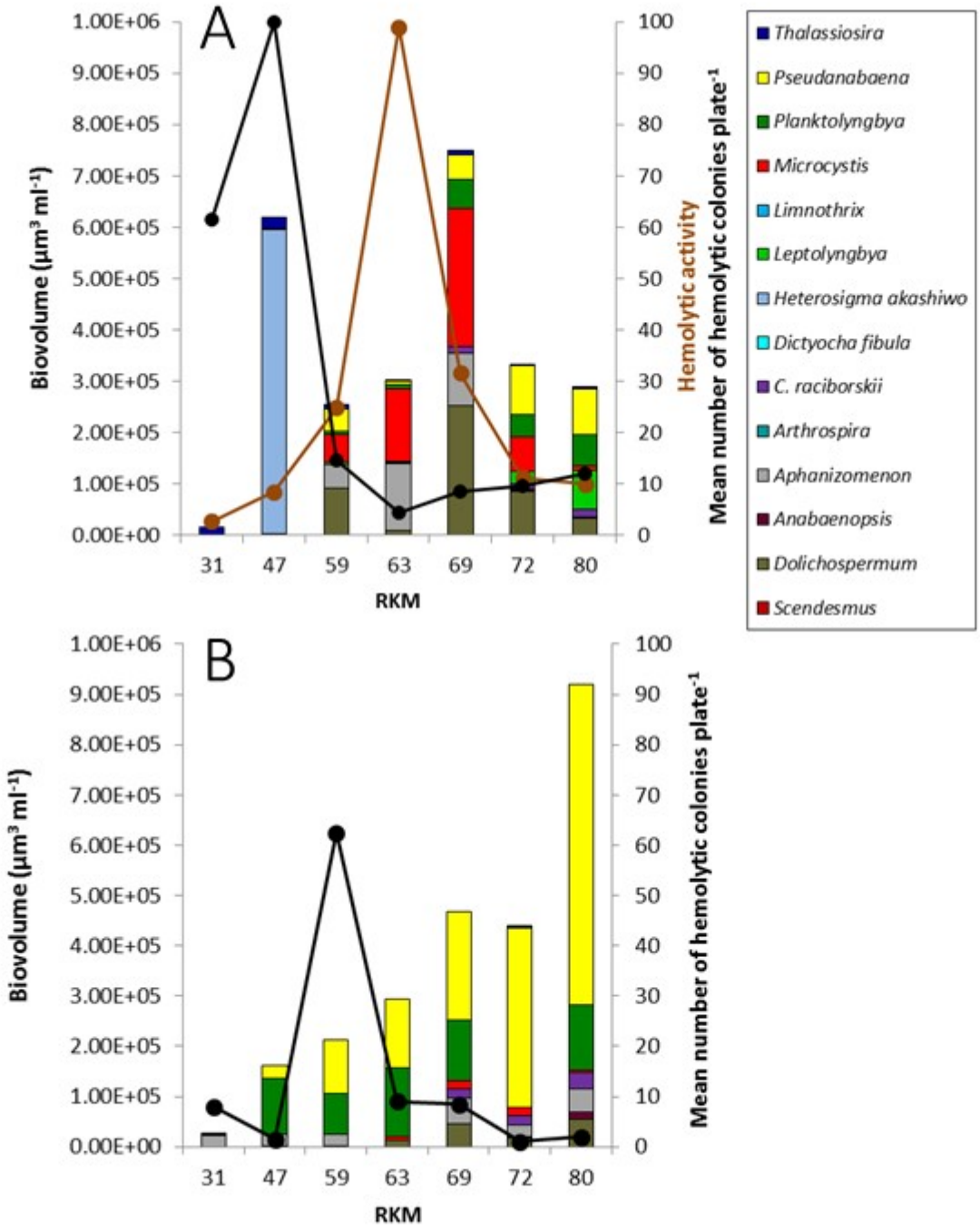

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Fig. 8 Time series from 20 April through 20 September 2010 of the CI for the LSJR. Black indicates pixels with no bloom, gray indicates clouds or missing data, and colored pixels are probable locations of cyanobacterial blooms. Warmer colors (red, orange, and yellow) represent high concentrations of cyanobacteria and cooler colors (blue and purple) low concentrations. Image areas cover the LSJR from Lake George (RKM180) downstream to Buckman Bridge (RKM55).

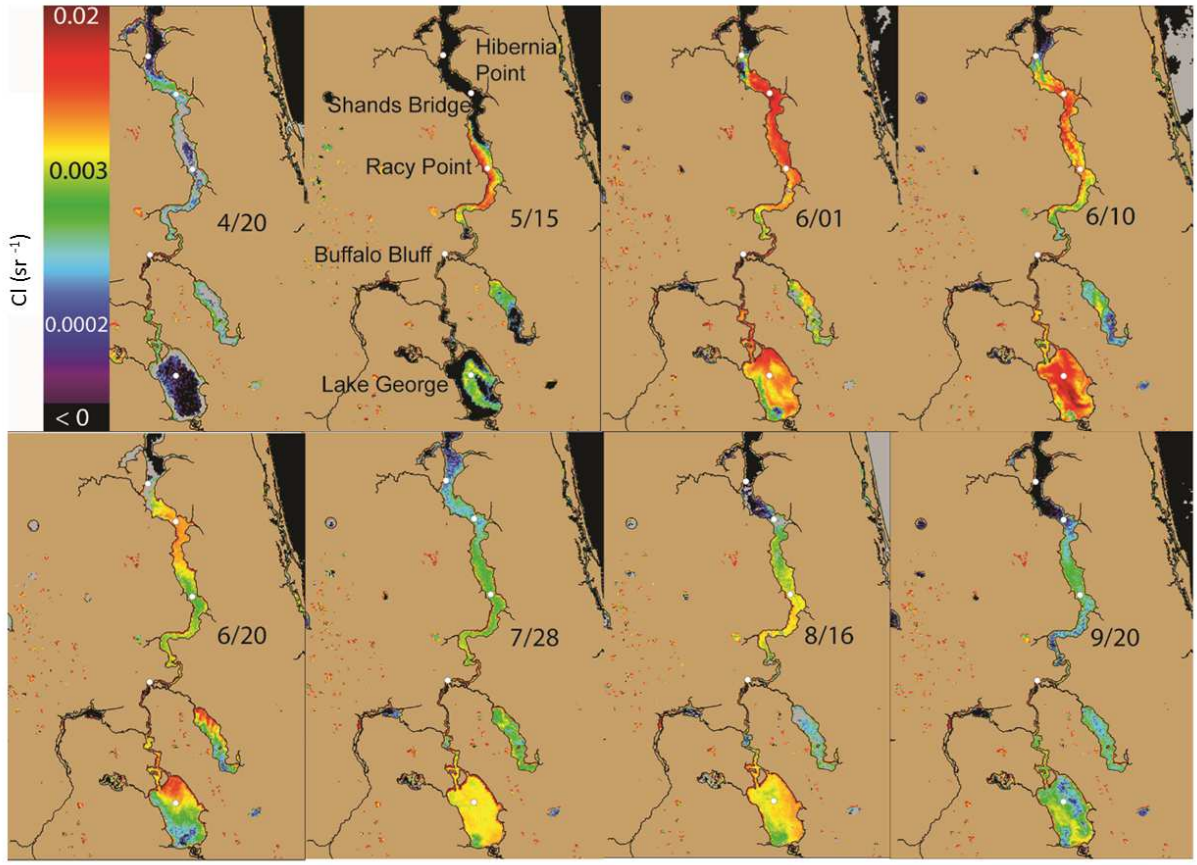


Table 2 Range of cyanotoxin concentrations in routine LSJR water samples collected from 13 May to14 September 2010 from stations at RKM143 to RKM47. RKM = river kilometer (measured from the estuary mouth), *n* = number of samples, <LD = below detection limit, CYN = cylindrospermopsin, STX = saxitoxin, MCYST = microcystin.

RKM	Date (2010)	*n*	CYN ng mL^{-1}	STX ng mL^{-1}	MCYST ng mL^{-1}
143	15 June	1	<LD	0.07	13.86
140	24 June–14 Sept	4	0.06–0.95	<LD–0.29	0.17–2.0
138	26 Aug–14 Sept	2	0.26–0.98	<LD–0.12	<LD–0.29
127	22 July–9 Sept	3	0.28–1.18	<LD–0.3	<LD–0.19
121	13 May	2	<LD	0.07–0.09	0.22–0.25
108	19 May	1	<LD	0.11	0.21
103	22 July–9 Sept	3	0.22–0.70	<LD–0.54	0.16–0.29
100	19 May	2	<LD	0.07–0.24	0.23–0.25
81	26 May–16 June	2	0.07–0.09	0.12	0.18–580
80	22 July–9 Sept	3	0.09–0.3	0.02–0.64	<LD–0.36
79	10 June	2	<LD–0.05	0.07–0.18	3.75–6000
66	21 June	1	0.17	0.14	1.75
58	22 July–8 Sept	3	0.07–0.25	<LD–0.08	<LD–0.75
47	22 July–8 Sept	3	0.07–0.20	<LD–0.06	<LD–0.61

Table 3 Numbers of fish species (and blue crabs) reported dead or moribund. The generic common names for some fish were reported and were not necessarily identified independently to species; these are noted as most likely. Shad is a composite of gizzard shad (*Dorosoma cepedianum*) and threadfin shad (*D. petense*). Catfish includes white catfish (*Ameiurus catus*), hardhead catfish (*Ariopsis felis*), gaftopsail catfish (*Bagre marinus*), and channel catfish (*Ictalurus punctatus*). Gar comprises longnose gar (*Lepisosteus osseus*) and Florida gar (*L. platyrinchus*). Sunfish could include bluegill, redbreast, and redear (*Lepomis microlophus*). Bass is a composite of largemouth bass and striped bass. Shiner could include golden shiner (*Notemigonus chrysoleucas*). Minnow could include sheepshead minnow (*Cyprinodon variegatus*). Baitfish mostly includes menhaden (Atlantic menhaden, *Brevoortia tyrannus*, yellowfin menhaden, *B. smithi*), bay anchovy (*Anchoa mitchilli*), scaled sardine (*Harengula jaguana*), and blueback herring (*Alosa aestivalis*). Note the figures for dead fish are likely to be significantly underestimated as no independent count verification was conducted and reporters' numbers were approximate. UNK = unknown.

Common name	Species	# Reported dead	# Reported moribund
Red drum	*Sciaenops ocellatus*	1674	48
Shad	*Dorosoma* spp.	443	
Menhaden	*Brevoortia* spp.	419	
Baitfish		266	
Stingray	*Dasyatis sabina*	229	
Striped mullet	*Mugil cephalus*	224	1
Catfish		165	1
Gar	*Lepisosteus* spp.	107	5
Sunfish	*Lepomis* spp.	50	
Bass		39	
Ladyfish	*Elops saurus*	31	
Shiner		20	
Minnow		17	
Black crappie	*Pomoxis nigromaculatus*	11	
Atlantic croaker	*Micropogonias undulatus*	10	
Southern flounder	*Paralichthys lethostigma*	8	
Spotted seatrout	*Cynoscion nebulosus*	4	
Black drum	*Pogonias cromis*	3	
Eel	*Anguilla rostrata*	3	
Sheepshead	*Archosargus probatocephalus*	2	
Striped bass	*Morone saxatilis*	UNK	1
Unidentified fish		874	
Blue crab	*Callinectes sapidus*	145	

Fig. 9 Clinical signs and histopathological changes of selected euryhaline fishes affected during the kill. (A) freshly dead Atlantic stingray showing hemorrhage and discoloration (arrows) of the skin, (B) hyperemic eye of red drum, (C) histological section of lumen of red drum cardiac blood vessel showing ghost cells of erythrocytes (circle) and intact erythrocytes; H&E stain, (D) histological section of red drum kidney showing long chains of bacilli (arrows); H&E stain, (E) hematocrit tubes after the blood components have separated in a moribund red drum. Pink plasma (arrow) is indicative of hemolysis. (This fish had a hematocrit value of 63%).

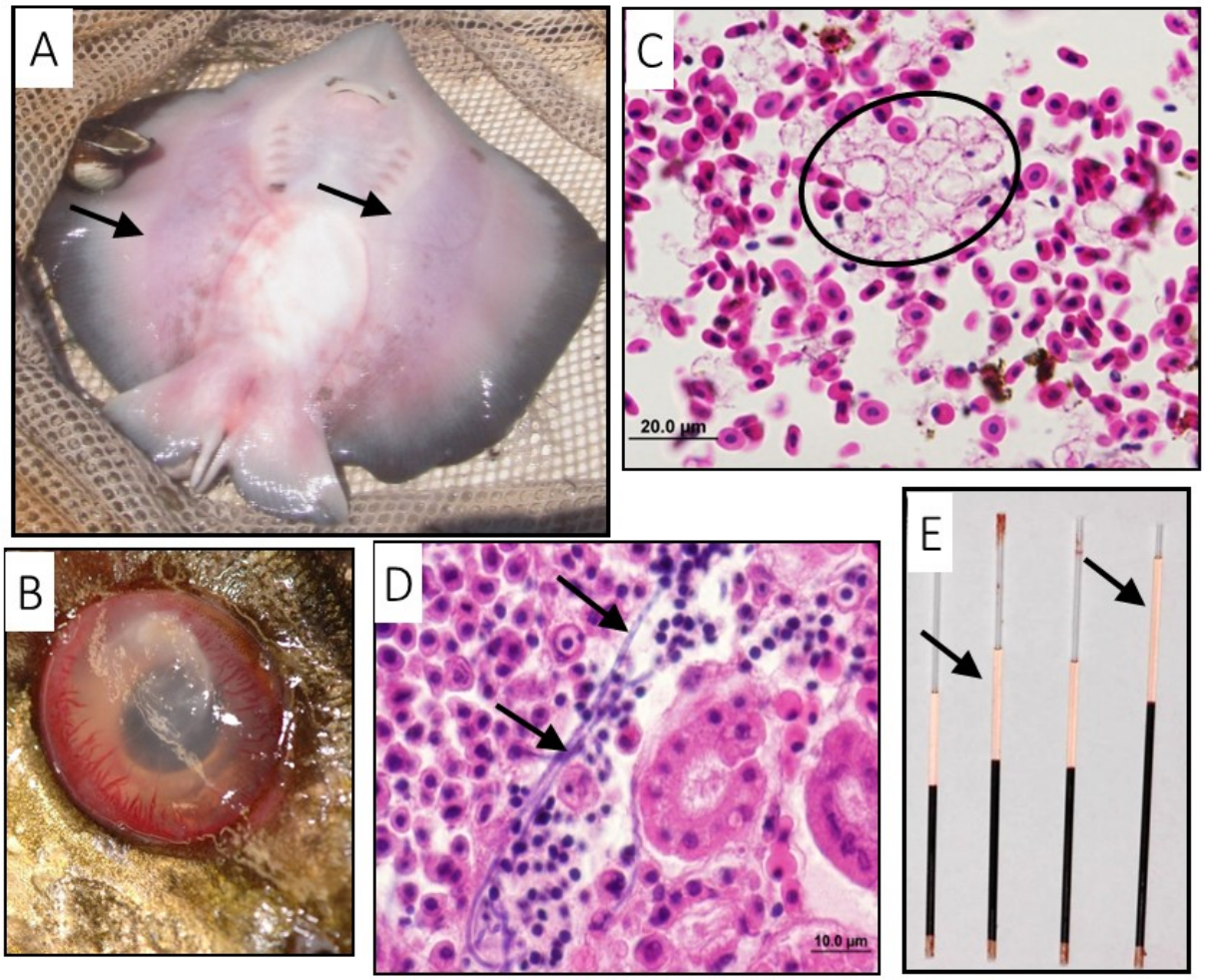
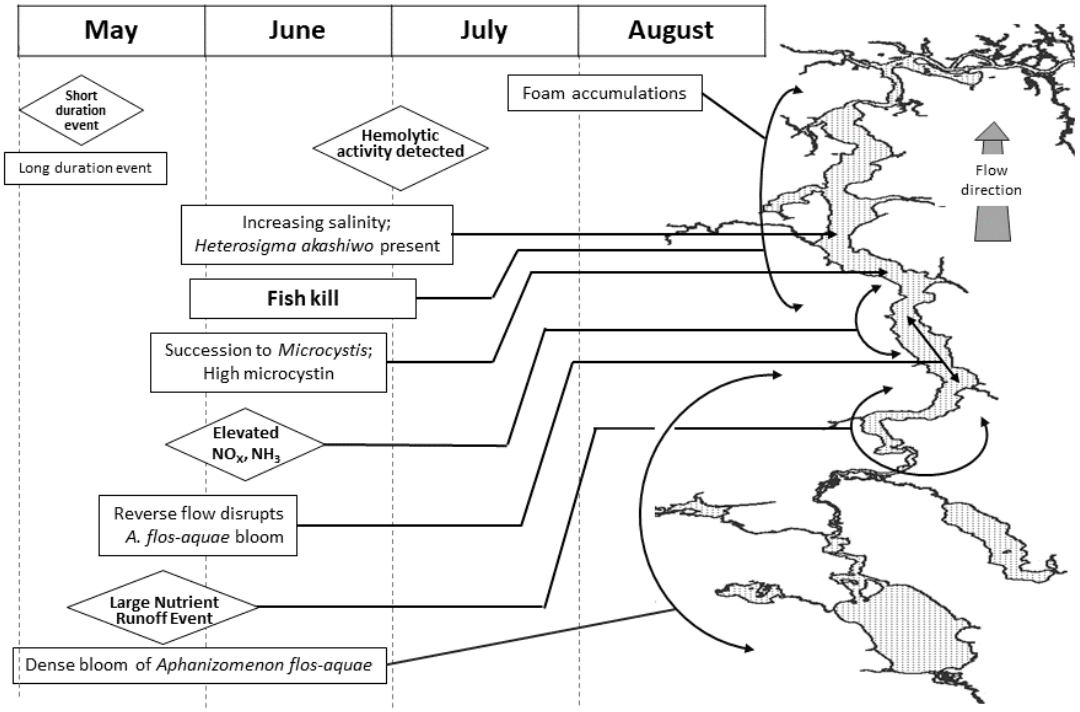


Table 4 Diagnostic findings indicative of hemolysis or disturbance of blood flow (H) and cyanotoxin concentrations in freshly dead or moribund fish collected from RKM61 to RKM22. [a–c] = pooled samples. + = pathological changes. ND = not done. <LD = below detection limit. CYN = cylindrospermopsin, STX = saxitoxin, MCYST = microcystin.

Date	RKM	Species	Condition	Tissue	H	CYN ng g^{-1}	STX ng g^{-1}	MCYST ng g^{-1}
061010	61	menhaden (n = 2)	freshly dead	viscera[a]		-	<LD	<LD
061010	61	menhaden (n = 2)	freshly dead	intestine[b]		<LD	3.08	3.73
061010	61	menhaden (n = 2)	freshly dead	viscera[c]		<LD	<LD	<LD
061610	53	red drum	freshly dead	gills	+	ND	ND	ND
				heart	+	ND	ND	ND
				kidney	+	ND	ND	ND
				liver	+	ND	ND	ND
				spleen	+	ND	ND	ND
				eye	+	ND	ND	ND
				brain	+	ND	ND	ND
060210	47	red drum	moribund	heart	+	ND	ND	ND
				kidney	+	ND	ND	ND
				liver	+	5.10	0.62	<LD
				stomach		<LD	<LD	<LD
				brain	+	ND	ND	ND
060210	47	red drum	moribund	heart	+	ND	ND	ND
				kidney	+	ND	ND	ND
				liver	+	5.78	0.78	<LD
				stomach		<LD	1.00	<LD
				brain	+	ND	ND	ND
060210	47	red drum	moribund	gills	+	ND	ND	ND
				heart	+	ND	ND	ND
				liver	+	6.73	0.54	<LD
				stomach		4.57	<LD	<LD
				brain	+	ND	ND	ND
060210	47	Florida gar	moribund	gills	+	ND	ND	ND
				heart	+	ND	ND	ND
				kidney	+	ND	ND	ND
				liver		<LD	0.68	6.6
				spleen	+	ND	ND	ND
				stomach		<LD	<LD	<LD
				brain	+	ND	ND	ND
060810	47	striped mullet	freshly dead	heart	+	ND	ND	ND
				kidney	+	ND	ND	ND
				liver	+	<LD	15.21	<LD
				stomach		<LD	<LD	<LD
				skin	+	ND	ND	ND
061010	47	red drum	moribund	gills		ND	ND	ND
				heart		ND	ND	ND
				liver	+	8.62	<LD	<LD
				intestine		5.17	<LD	<LD
061010	47	red drum	moribund	gills		ND	ND	ND
				heart	+	ND	ND	ND
				kidney	+	ND	ND	ND
				liver	+	4.69	<LD	<LD
				intestine		6.08	<LD	<LD
				brain	+	ND	ND	ND
061010	47	Atlantic stingray	moribund	liver	+	<LD	<LD	<LD
				intestine		<LD	<LD	<LD
				kidney		ND	ND	ND
				skin	+	ND	ND	ND
060710	22	striped mullet	moribund	kidney	+	ND	ND	ND
				liver	+	<LD	86.78	<LD
				spleen	+	ND	ND	ND
				skin	+	ND	ND	ND

1667 **Fig. 10** Spatial sequence of events associated with the decomposition of the *Aphanizomenon flos-aquae* bloom.



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