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EVALUATING BIOLOGICAL NUTRIENT REMOVAL FOR DISSOLVED ORGANIC NITROGEN QUALITY AND ITS IMPACT ON PHYTOPLANKTON BLOOM DYNAMICS IN A EUTROPHIC, FRESHWATER RESERVOIR

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Abstract: There is increasing evidence that dissolved organic nitrogen (DON) can stimulate phytoplankton growth and play an important role in structuring phytoplankton community composition. Specifically, high ratios of DON to dissolved inorganic nitrogen (DIN) may select for potentially toxic harmful algal blooms (HABs) which continue to plague lakes, rivers and freshwater reservoirs. Recent attention has been focused on the composition and quality of DON discharged in waste water treatment plant (WWTP) effluent, particularly as WWTPs switch from conventional activated sludge (CAS) to biological nutrient removal (BNR) processes in response to increasing pressure to reduce total nitrogen (TN) loading in WWTP effluent. BNR systems significantly reduce TN discharged in effluent, but increases the concentration of DON that may be bio-reactive and capable of stimulating phytoplankton growth. The goals of this project were to determine whether BNR processes produce bio-reactive DON and determine whether these compounds stimulate phytoplankton growth, particularly HAB species. A combination of bulk DON analyses, fluorescent spectroscopy, humic, and protein DON analyses were used to characterize the DON in the influent and effluent waters of a representative BNR WWTP to assess whether DON is produced during BNR treatment or is inherent to the influent water. Two experimental nutrient additions of influent and effluent waters were made during summer and fall 2018 to natural phytoplankton and microbial assemblages from a eutrophic reservoir (Jordan Lake, North Carolina) to determine the potential stimulatory role of effluent DON and to assess whether bio-stimulatory DON is produced by the BNR process or is inherent to the received influent water. Influent and effluent characterization revealed that influent waters contain high concentrations of potentially bioreactive protein. However, about 80% of the protein is removed within the WWTP process, and protein contained in the effluent appeared largely refractory. During the summer experiment, effluent additions stimulated total phytoplankton biomass beyond the stimulation that could be attributed to the effluent inorganic nutrient content. Accessory pigment analysis, indicated that diatoms/chrysophytes, chlorophytes, and dinoflagellates were the primary taxa that were stimulated. During the fall experiment, only the diatom/chrysophyte group was stimulated by effluent additions. Potentially harmful cyanobacteria taxa were either slightly inhibited (summer) or showed no response to effluent organic matter additions (fall). Based on canonical phytoplankton stoichiometry and lack of decline in the DON pool, phytoplankton growth during the experiments could be best explained solely by observed uptake of DIN. Therefore, it seems possible that the observed stimulation of phytoplankton growth is due to some other substance, e.g. a vitamin, other co-factor, or micronutrient, rather than direct incorporation of effluent DON. Results from these experiments indicate that although BNR effluent may contain a growth stimulatory substance, it does not contain significant quantities of highly labile DON that could fuel high biomass attainment beyond the carrying capacity set by its DIN concentration. Thus, in nature, BNR effluent would not be expected to strongly stimulate phytoplankton biomass increases in highly N limited reservoirs like Jordan Lake.

Introduction: Since the 1970's, there has been an increasing recognition of and concern for the role nutrient (nitrogen, N and phosphorus, P) over-enrichment (eutrophication) plays in the ecological and economic decline of aquatic ecosystems, specifically the growing threat to usable and safe water supplies posed by increased frequencies and magnitudes of harmful algal blooms (HABs) (Cloern, 2001; Conley et al., 2009; Otten & Paerl, 2015; Paerl, 2014). In order to remedy this growing issue, state and federal legislation was enacted in the mid-1990's to reduce N and P loading to impaired ecosystems by reducing source loading of these nutrients. One approach was to update technology in wastewater treatment plants (WWTPs) to reduce total N and P concentrations in discharged effluent. Many WWTPs switched from conventional activated sludge (CAS) to biological nutrient removal (BNR) systems, which significantly reduced concentrations of total N and P, specifically as nitrate/nitrite and phosphate, from effluent. Recent research indicates that while BNR systems are efficient at reducing total N and inorganic nutrients, the process generates increasing proportions of dissolved organic N (DON) particularly as low molecular weight DON (LMW DON) produced during treatment (Eom et al., 2017). Conventionally, DON has been considered refractory to phytoplankton in receiving waters; however, recent studies have demonstrated that phytoplankton are capable of using DON and LMW DON for growth (Bronk et al., 2007; Eom et al., 2017; Qin et al., 2015). This increase of DON, despite reductions in dissolved inorganic N, could be contributing to eutrophication, and resultant impairment of both freshwater and coastal systems. Additional research has demonstrated DON can preferentially stimulate HABs, many of which are toxic and can significantly complicate and add to the costs of treating drinking and irrigation water (Altman & Paerl, 2012; Bronk et al., 2007).

DON is an emerging issue both in terms of wastewater treatment and in protecting drinking water sources. Motivation for this study was two-fold: 1) to understand the quality of DON released from BNR systems and how the released DON may impact phytoplankton growth and community composition in natural ecosystems, and 2) by determining if and how DON compounds stimulate phytoplankton growth or select for HABs, we can better understand if and how release of DON may impact water quality of freshwater lakes and reservoirs used as drinking water sources. The project represents a first step towards understanding what DON compounds are released by BNR systems and how released compounds may impact phytoplankton production and composition at the base of aquatic food webs. A science-based decision can then be made on the relative importance of these compounds to eutrophication and a targeted approach can be taken to reduce these compounds in effluent. The proposed work addressed the following research objectives:

1. *Characterize DON released from BNR systems.* DON concentration and quality were measured and assessed for a WWTP conducting BNR in its influent and effluent to understand if DON is produced during treatment and how it relates to the characteristics of the influent water.
2. *Conduct nutrient addition bioassays to explore how BNR influent and effluent impact phytoplankton production and community composition and whether BNR effluent selects for HABs in receiving waters.* By comparing the phytoplankton growth response between influent and effluent waters, we were able to determine if bio-reactive DON is mainly due

to characteristics of the influent water or if BNR processes within the WWTP produce bioreactive DON compounds.

Previous studies have demonstrated BNR processes increase the proportion of potentially bioavailable DON in effluent compared to influent water received (Czerwionka et al., 2012; Eom et al., 2017; Sattayatewa et al., 2009). Sattayatewa et al., (2009) determined DON contained in the influent water is largely refractory and derived from drinking water sources and over land run-off. They determined, however, that BNR produced bioavailable DON that was utilized by phytoplankton and bacterial assemblages. This was largely produced as soluble microbial products (SMPs) that are generated by microbes used in BNR (Sattayatewa et al., 2009). Qin et al., (2015) characterized and assessed the bioavailability of the hydrophilic and hydrophobic fractions of DON produced from a WWTP BNR system. The study determined that hydrophobic DON fractions were largely un-reactive while the hydrophilic DON fraction was considered to be bioavailable to phytoplankton for growth in receiving waters. Additionally, Sattayatewa et al., (2009) determined DON produced during BNR was about 28-57% bioavailable to phytoplankton and bacterial assemblages, even in the presence of mixed nitrate and DON species. The study concluded that while phytoplankton and microbial assemblages preferentially use inorganic N species (as nitrate) over DON, a fraction of the DON produced during BNR was still bio-available to phytoplankton and microbial assemblages (Sattayatewa et al., 2009), indicating that DON produced during BNR is an important N-source to phytoplankton and microbial assemblages in receiving waters and may be contributing to eutrophication and impairment of these systems. It is important to note that the Qin et al., (2015) and Sattayatewa et al., (2009) studies used single-species laboratory phytoplankton and microbial cultures that are not necessarily representative of *in situ* phytoplankton and bacterial assemblages. Bronk et al., (2017), demonstrated a stimulatory response of BNR effluent derived DON on natural estuarine microbial communities in the Chesapeake Bay while Eom et al., (2017), found that LMW DON from certain BNR processes may be responsible for stimulating phytoplankton production in estuarine environments.

Our study expanded on these previous studies by examining the effects of BNR effluent on natural mixed phytoplankton and microbial communities in a freshwater reservoir, Jordan Lake, NC, in the presence of environmentally relevant mixed inorganic and organic N sources. Reservoirs like Jordan Lake not only serve as important recreational sites but as drinking water sources for a large portion of the nationwide population. Additionally, we specifically focused on the impacts of released DON on phytoplankton community structure, including the potential selective stimulation of HABs (e.g. toxic cyanobacteria). Our study represents an important first step to begin systematically answering questions relating to whether bio-reactive DON is produced during the BNR process and if yes, assess the production, quality, and bioavailability of produced DON to natural phytoplankton and microbial assemblages in freshwater reservoirs (Figure 1). As outlined in Figure 1, once the initial questions of the study are addressed, it will be possible to assess whether additional research is needed to further constrain the ubiquity of BNR systems to produce DON, the type and quality of DON produced by these systems, and the long-term degradation and bioavailability of this nutrient source to bacterial populations.

Study Site: *Jordan Lake, NC:* Jordan Lake was used as a study site for the proposed project (Figure 2). The reservoir is downstream of several WWTPs that serve the citizens of Greensboro, Burlington, Durham, and Chapel Hill/Carrboro. Additionally, the reservoir is an important drinking water source for > 300,000 residents in the Town of Cary, City of Durham, and Chapel Hill/Carrboro. Jordan Lake is also an important recreational area but has been plagued with eutrophication issues, including the occurrence of HABs (mainly as potentially-toxic cyanobacteria) since its inception in the 1970's and 1980's (NCDEQ). Recent nutrient addition bioassays have concluded the lake is primarily co-limited by N and P (Paerl et al., unpublished results), indicating the importance of N-sources, including DON from WWTPs, for controlling phytoplankton blooms and HABs in the lake. Due to its history of impairment, the number of WWTPs that discharge within its watershed, and its importance as a drinking water source, Jordan Lake is an ideal ecosystem in which to study the potential effects of DON produced by BNR on freshwater phytoplankton communities. Water samples for the bioassay were collected upstream of Farrington Point in the New Hope Creek arm of Jordan Lake. The choice of this location was based on preliminary water quality and nutrient-sensitivity studies currently taking place there (NCDEQ; UNC-CH Collaboratory, 2017) that serve as background limnological data. These data show that this location of Jordan Lake is a site of elevated phytoplankton biomass with chlorophyll a concentrations frequently in violation of NC water quality standards.

Methods: *Characterization of DON:* Research objective #1 was met by conducting seasonal (summer, fall, winter, spring) monitoring at a selected WWTP conducting BNR in order to better understand the formation and composition of DON in both the influent and effluent. A single WWTP was selected, upon consultation with the Urban Water Consortium (UWC), to seasonally monitor DON quantity and quality as well as to test the bio-reactivity of DON contained in the influent versus the effluent (see *Experimental Nutrient Addition Bioassays* below). The selected WWTP conducts BNR treatment processes and is representative of other BNR plants. Operating conditions of the WWTP (i.e., solids retention time, SRT; mixed liquor suspended solids, MLSS; hydraulic retention time, HRT; aeration basin volume; aerated and non-aerated fractions, influent characteristics) were cataloged and recorded along with collected samples. By collecting these types of data, an understanding of how BNR processes impact the composition of the discharged effluent can be drawn and the results expanded to similarly operated WWTPs.

Samples were collected from the influent and effluent, and then analyzed for a suite of dissolved nutrients (total dissolved N, nitrate/nitrite, ammonium, DON – by subtraction, phosphorus). Fluorescence (excitation emission matrices, EEM) were used to characterize the relative composition and quality of the bulk organic matter pool (Osburn et al., 2012). Additionally, collaborator Dr. Chul Park at the University of Massachusetts, Amherst analyzed proteins and humic acids (Frolund et al., 1995) in collected WWTP samples. The goal of these analyses was to assess the total dissolved nutrient and the DON pools in the influent and effluent to characterize the quantity and quality of DON that is contained in the influent water and/or may be produced during BNR.

Nutrient Addition Bioassays: Nutrient addition bioassays have been successfully used in freshwater systems to assess nutrient enrichment on the production and composition of natural

phytoplankton communities (Paerl et al., 2015; Paerl & Peierls, 2014). Surface water was collected from Jordan Lake near Farrington Point (Figure 2) and transported to the UNC-CH Institute of Marine Sciences in Morehead City, NC (< 4 hr) where water was partitioned into triplicate, photosynthetically active radiation (PAR)-transparent Cubitainers® (for each treatment) and incubated under natural temperature and light conditions in outdoor, flowing water ponds for 6 days (Figure 3). Incubations were conducted outdoors under natural light and temperature conditions in order to more accurately mimic the variability of the lake environment. Cubtainers® were covered with neutral density cloth to prevent photo-inhibition during incubation (Figure 3b). Photosynthetically active radiation (PAR) was measured continuously during the incubation period using a LiCOR light sensor-data logger.

The bioassay incubation period was limited to 6 days to focus on the initial impact of discharged DON on phytoplankton production and community composition and to minimize bottle effects (i.e., increased competition, pH changes, nutrient depletion) that can develop and confound results within experiments of greater duration (Giddings et al., 1983). Changes in phytoplankton biomass and class-level changes in community composition were determined as chlorophyll a, and by analysis of class-specific accessory photopigments (Pinckney et al., 1999). Experimental bioassays coupled with analyses for chlorophyll *a* (Chl *a*) and high performance liquid chromatography (HPLC) diagnostic pigment analysis are relevant and useful for measuring phytoplankton growth rate and community shift responses in just a few days because growth (doubling) rates of relevant phytoplankton taxa are generally 0.5 to 1 day, well within the bioassay incubation period (Pinckney et al., 1999).

Bioassay nutrient additions included; 1) control (no nutrient additions), 2) influent inorganic nutrient control, 3) influent treatment, 4) effluent inorganic nutrient control, and 5) effluent treatment (Table 1). Additions were made such that the DON concentration of each addition were about 0.7 mg N L^{-1}, which mimics natural N concentrations measured *in-situ* in Jordan Lake (Paerl et al., unpublished data). Phosphate was added to each addition to ensure P-limitation would not occur. The dissolved inorganic nitrogen (DIN) control contained levels of nitrate and ammonium that mimicked the nitrate and ammonium concentrations in the respective influent and effluent. The influent and effluent treatments consisted of bulk influent and effluent, respectively, added such that the DON concentration was about 0.7 mg N L^{-1}. The DIN control treatment accounted for the inorganic nitrate and ammonium that are inherent to the influent and effluent, respectively. By comparing the DIN control treatment with the respective influent or effluent treatments, we assessed whether DON was capable of stimulating phytoplankton growth greater than the inorganic nutrients in the influent and effluent. Due to the low levels of DON present in the WWTP effluent, large volumes (~2 L) were required to achieve the targeted DON concentration of 0.7 mg N/ L. Inorganic nutrient controls were therefore diluted by a similar amount with a solution containing the major ions of the representative lake water to ensure that initial phytoplankton biomass levels were equal between the effluent addition treatment and the DIN controls. By comparing the growth response of the influent versus effluent, we assessed whether bio-reactive DON is inherent within the influent water or is produced during BNR processes and discharged in the effluent.

Bioassays were conducted during summer and early fall to capture phytoplankton biomass and community composition responses during the productive seasons in Jordan Lake (Paerl et al unpublished data). Phytoplankton growth (Chl *a*) and community structure (phytoplankton

pigments by HPLC) (Pinckney et al., 1999) were analyzed on days 0, 1, 2, 3, and 6 of each bioassay. Analyses for humic acids and proteins were conducted for a single triplicate selected for each treatment on day 0 and day 6. Analyses for bulk inorganic and organic nutrients ($NO_{3/2}$, PO_4, NH_4, DON) as well as dissolved organic matter quality via the EEM technique were conducted for all Cubitainers® (n = 28) on days 0, 1, 2, 3, and 6.

Characterization of Influent and Effluent Nutrients: Inorganic nitrogen forms, nitrate+nitrite and ammonium, and DON were measured by UNC-IMS from influent and effluent water collected for bioassay experiments during summer and fall of 2018, and again during winter and spring of 2019 (Figure 4). Nutrient data collected by the WWTP at weekly to monthly intervals were additionally used to characterize influent and effluent nutrient concentrations and to determine whether concentrations that occurred during the bioassay experiments were representative of typical operating conditions at the WWTP.

From the four seasonal samples measured by UNC-IMS, total dissolved N of influent waters ranged from about 20 to 30 mg/L. Nitrate comprised a negligible fraction of influent nitrogen. Influent nitrogen was dominated (~80%) by DON during summer and winter with the remainder comprised of ammonium. In fall and spring, these ratios switched with influent N dominated (~80%) by ammonium and the remaining ~20% composed of DON. Monthly measurements of influent ammonium concentration made by the WWTP ranged from 26 to 32 mg/L which makes the low ammonium values (~ 4 mg/L) observed during July and January unusual, but is similar to the influent ammonium observed during October and April. Total Kjeldhal N as measured by the WWTP was generally 40-50 mg/L and subtracting off ~30 mg/L gives typical DON concentrations of 10-20 mg/L. This compared well with the range (~4 – 23 mg/L) of DON measured during UNC-IMS's four seasonal measurements. The WWTP measurements also indicated that nitrate and nitrite were minor components, ~1%, of the N pool of the influent waters.

Both the four seasonal measurements by UNC-IMS and more frequent WWTP measurements indicated that effluent N concentration was approximately an order of magnitude lower than influent concentrations, and was comprised nearly evenly by a mix of DON (~0.5-1 mg/L) and oxidized (nitrate + nitrite) inorganic N (0.5-1.5 mg/L). In both the four seasonal measurements by UNC-IMS and the WWTP measurements, ammonium comprised a small fraction, generally less than 10 %, of total effluent N.

Representativeness of WWTP operations during sampling for experiments: To further ascertain whether the operating conditions during sample collection for the experiments were typical for the WWTP, a range of other operational parameters that describe flow through the WWTP (effluent flow, volume and hydraulic residence time (HRT) of the aeration basin, the aerobic HRT, and additional influent and effluent concentrations characteristics including pH, temperature, suspended solids, chemical (COD) and biochemical oxygen demand (BOD), and total P) were measured. Figures 5-7 show time series of these parameters during the study period. Days when influent and effluent were collected for experiments and for the four seasonal measurements by UNC-IMS are highlighted by filled squares. On days when water was sampled for the two experiments, all parameters except for effluent BOD for the October experiment were

well within their normal operating ranges. This indicates that the physical, chemical, and biological characteristics of the influent and effluent waters used for the experiment were typical for this WWTP.

Phytoplankton production stimulation by dissolved organic N from WWTP influent and effluent: During the August 2018 experiment, addition of WWTP effluent significantly stimulated phytoplankton production as measured by chlorophyll *a*. This stimulation cannot be solely attributed to the inorganic nitrogen within the effluent because the DIN control treatment contained 25% higher DIN but exhibited slower growth. The effluent addition and DIN control treatments exhibited similarly weak growth during the first day of the experiment but growth in the effluent treatment increased rapidly and significantly compared to the control between days one and two (Figure 8; Table 2; Table 3). By day three, biomass of the effluent treatment (~80 μg/L) was more than double that of the DIN control (~ 40 μg/L). Between days three and six, chlorophyll *a* declined in the effluent treatment while chlorophyll *a* levels increased in the DIN control. On day 6, chlorophyll *a* in the DIN control (~65 μg/L) was more than double that in the effluent treatment (~30 μg/l). By day 6, DIN had been completely depleted in both the effluent treatment and DIN controls. Despite the apparent stimulation of growth by the organic fraction of the effluent, DON concentration in the effluent fraction remained very close to 600 μg/L throughout the experiment and provided no indication of DON depletion and incorporation into the particulate phytoplankton pool. Further, based on the range of commonly observed chlorophyll *a* to N utilization ratio of 7-28 g N per g chlorophyll *a* (Li et al. 2010; Gowen et al. 1995; Carpenter and Dunham 1985), the approximate 70 μg/L increase in chlorophyll *a* in the effluent treatment can readily be explained solely by the observed 700 μg/L decrease in the DIN pool. Thus, it seems likely that stimulation by WWTP effluent may be a response to a vitamin, co-factor or signaling molecule in the effluent that stimulates growth and uptake of DIN rather than direct stimulation via direct assimilation of a highly bioavailable DON form.

Analysis of the response of accessory pigments to effluent treatments indicated that the positive growth response to effluent DON additions was primarily due to fucoxanthin and chlorophyll *b* containing phytoplankton which include diatoms, chrysophytes, haptophytes, chlorophytes, and euglenophytes (Figure 10; Table 2; Table 3). These groups do not form surface scums or produce toxins and therefore are generally not considered harmful algae. Potentially harmful cyanobacteria taxa, indicated by concentrations of zeaxanthin and myxoxanthophyll, showed no apparent stimulation by the WWTP effluent. Dinoflagellates (indicated by peridinin) were modestly stimulated by WWTP effluent.

In the August 2018 experiment, influent additions resulted in small but statistically significant weaker growth responses compared to the DIN control (Figure 8; Table 2; Table 3). It is unclear what caused the weaker growth response in the influent DON treatment. The initial phosphate concentration was two-fold lower in the influent treatment compared to the DIN control (Figure 8). Phosphate declined rapidly in both the influent treatment and DIN control but due to lower initial concentrations was nearly depleted by day 1 in the influent treatment. Thus, it is possible that P limitation caused the weaker growth response in the influent DON treatment. It is also possible that some component of the influent waters caused a weak inhibitory effect on

phytoplankton growth compared to the DIN control (Figure 8; Table 2). Accessory pigments indicated that myxoxanthophyll containing cyanobacteria, chlorophyll *b* containing chlorophytes and euglena, and alloxanthin containing cryptophytes were the groups most inhibited relative to the DIN control (Figure 10; Table 2; Table 3).

Results from the effluent experiment in October 2018 were qualitatively similar to the effluent experiment in August (Figure 9). However, the degree of stimulation by the effluent addition was weaker and statistically insignificant (Figure 9; Table 2; Table 3). Despite an insignificant response of total phytoplankton biomass, effluent addition did stimulate production of fucoxanthin containing phytoplankton taxa as in the August experiment. It is unclear, however, whether stimulation of fucoxanthin containing phytoplankton was due to a substance produced by the BNR process or a substance that entered through influent waters because influent water additions resulted in a similar degree of growth stimulation (Figure 10; Table 2; Table 3). In October, chlorophyll *b* containing phytoplankton were also significantly stimulated by influent waters and the large positive response of these two groups drove a significant positive response of chlorophyll *a* to influent additions.

As in the August experiment, DON remained nearly constant at ~600 μg/L in the effluent treatment, suggesting little net utilization of effluent DON, and chlorophyll *a* production was accompanied by declines in DIN that match the well-established stoichiometric ratio. As previously mentioned, total phytoplankton biomass was significantly higher for the influent organic addition. However, the DIN control for the influent experiment contained approximately 1000 μg/L less DIN than the influent addition. By day six of the experiment, DIN had been depleted in the DIN control whereas ~200 μg/L DIN still remained in the influent treatment. In the influent treatment, the 2500 μg/L decline in DIN was accompanied by an increase of about 170 μg/L chlorophyll *a*; a mass ratio of N:chl *a* of 15 that agrees well with established stoichiometry. Based on this ratio, the 1000 μg/L excess DIN in the influent treatment compared to the DIN control would be expected to produce about 70 μg/L chlorophyll *a* which was very close to the observed final difference (67 μg/L) in chlorophyll *a* between the two treatments. Thus, the treatment effect likely resulted in the inability to accurately implement a DIN control with the same DIN concentration as in the influent treatment.

Characterization of dissolved organic matter: The dissolved organic matter pool for the collected influent and effluent seasonal samples as well as samples collected during the bioassays was assessed using excitation-emission matrices (EEMs) as well as colorimetric analyses of humic and protein content. The location of fluorescent peaks within EEMs space reveals information about the composition of the fluorescent dissolved organic matter (FDOM) pool. All collected seasonal influent samples exhibited a strong peak in the protein-like region of fluorescence with additional fluorescent signatures in the A and C peak regions of fluorescence (Figure 12a). Protein-like fluorescence is often associated with lower molecular weight FDOM and is thought to be biologically reactive and available as a nutrient source, specifically as an N-source, for microbial and phytoplankton growth (Fellman et al.,2010). Analysis of the protein content of influent waters corroborated the presence of high levels of protein in the influent waters, and the 400% and 100% reductions during the six day bioassays indicate that a significant fraction of the influent protein is labile (Figure 13). Fluorescence in the A peak region

has been associated with highly complex, terrestrially-derived, humic and fulvic-acids (Fellman et al., 2010). Fluorescence in the C peak region has been correlated with anthropogenic sources of FDOM and is often observed in natural aquatic systems that have been heavily impacted by humans (Coble, 2007).

The protein peaks in the effluent samples were not as prominent as in the influent samples, indicating this type of fluorescence was largely removed during the WWTP treatment process (Figure 12b). Protein analysis of influent and effluent waters confirmed this conclusion with effluent protein content (2.3-3.6 mg/L) only about 20% of influent (12.7-16.3 mg/L) concentrations. High fluorescence signals were retained in the A and C peak regions of fluorescence, indicating the types of FDOM associated with these peaks was largely retained during wastewater treatment. Humic concentrations measured colorimetrically, however, did show between a 100 and 200% reduction in humics within the WWTP and significant degradation of humics occurred during the October experiment with influent waters (Figure 13).

In order to visualize how the FDOM pool changed through the bioassays, the fluorescent intensity as measured in certain regions of each EEM were extracted from each EEM sample and plotted through time (i.e., peak picking). The peak identifications and characterizations, as well as locations of extracted EEMs peaks are listed in Table 4 (Coble, 2007; Fellman et al.,2010; Osburn et al., 2012). The fluorescence concentrations between the influent and its associated control are similar for all fluorescence components (Figures 14 and 15), likely because of the small volumes used for the influent addition treatment (a few mL's). For the effluent OM treatment, however, a large volume (1-2 L) was used to obtain the necessary amount of added DON; therefore, there was a much greater difference in the fluorescence characteristics between the effluent OM treatment and its associated DIN control (Figures 14 and 15). Despite this, the initial fluorescent intensities of both the influent and effluent OM treatments were comparable. For most treatments and fluorescence peaks, the intensity appeared to remain relatively constant through the bioassay, however, the microbial peak for the effluent treatment did appear to decrease during the duration of the bioassay, indicating this FDOM pool may have been degraded during the experiment. While it is nearly impossible to determine whether this was due to photochemical or biological degradation, it does indicate this fluorescent component is reactive in the environment. Similar results were obtained for the October 2018 bioassay (Figure 15).

For the humic-like, terrestrial FDOM pool (Peaks A and C), initial fluorescent patterns between the influent and effluent treatments were similar, such that there was much greater fluorescence in the effluent OM treatment than the DIN control but that starting fluorescent intensities between the influent and effluent OM treatments were relatively similar (Figure 16). The exception to this was the 'C peak' which had a much greater intensity in the effluent treatment than the influent treatment. This peak is often associated with anthropogenic sources of FDOM (Coble, 2007; Osburn et al., 2012) and may be produced during WWTP treatment (Murphy et al., 2011), potentially explaining why there would be greater intensity of this peak in the effluent vs. influent OM treatment. For the effluent treatment, both peaks A and C appeared to decrease through the bioassay. Fluorescent peaks A and C are generally thought of as unavailable to microbial and/or primary production due to its complex structure and low nitrogen content (Fellman et al., 2010); however, both peaks are known to be photochemically reactive

(Moran et al., 2000). The decrease in fluorescent intensity for these two peaks was likely a result of photochemical degradation during the bioassay. This was further confirmed by comparing results between the August 2018 and October 2018 bioassays (Figure 17). There was a much greater decrease in fluorescent intensity during the August 2018 bioassay when daylight is longer and the shortwave and longwave radiation stronger as compared to October 2018.

Conclusions: Influent and effluent characterization revealed that influent waters contained high concentrations of bioreactive protein. Study results suggest these compounds are largely removed from the influent during WWTP treatment, and that these compounds were not utilized by phytoplankton as a nutrient source for growth. Fluorescence results from the seasonal influent and effluent sampling and the two bioassays suggest the proteins are characterized as low molecular weight and rich in tryptophan and tyrosine. However, about 80% of the protein was removed within the WWTP process, and effluent protein appeared largely refractory. Results from the OM addition, effluent treatments do suggest there were fluorescent compounds that decreased during the bioassay (Peak M, C, and A), however, we cannot determine whether this decrease in fluorescent intensity was due to biological or photochemical degradation. Additional studies would be needed to control for photochemical and biological degradation to specifically determine FDOM degradation pathways.

During the summer experiment, effluent additions stimulated the growth rate of the total phytoplankton biomass beyond the stimulation that could be attributed to the effluent inorganic nutrient content. Accessory pigment analysis, indicated that diatoms/chrysophytes, chlorophytes, and dinoflagellates were the primary taxa that were stimulated. During the October experiment, only the diatom/chrysophyte group was stimulated by effluent additions and it is possible that the substance that produced the stimulation was contained within the influent waters rather than produced within the WWTP. Potentially harmful cyanobacteria taxa were either slightly inhibited (summer) or showed no response to effluent organic matter additions (fall). Although many marine HABs are dinoflagellates, few freshwater dinoflagellates are HAB species, and none are known to produce toxins. Therefore, while it appears that there may be some stimulatory response of BNR effluent on total phytoplankton biomass, the experiments provide no evidence for selective stimulation of HAB taxa and there was no evidence for selective stimulation of HABs by effluent additions.

Although the growth rate of some phytoplankton groups was stimulated by effluent additions, the final biomass yield was not. Based on canonical phytoplankton stoichiometry and lack of decline in the DON pool, final phytoplankton biomass accumulation during the experiments could be best explained solely by observed uptake of DIN. Therefore, it seems possible that the observed stimulation of phytoplankton growth is due to some other substance, e.g. a vitamin, other co-factor, or micronutrient, rather than direct incorporation of effluent DON. Results from these experiments indicate that although BNR effluent may contain a growth stimulatory substance, it does not contain significant quantities of highly labile DON that could fuel high biomass attainment beyond the carrying capacity set by its DIN concentration. Thus, in nature, BNR effluent would not be expected to strongly stimulate phytoplankton biomass increases in systems like Jordan Lake where DIN is most often near zero and phytoplankton biomass is, therefore, at its carrying capacity. Growth stimulation by WWTP effluent may, however, have significant impacts on biomass development in riverine

systems where phytoplankton biomass rarely reaches its carrying capacity and biomass accumulation is more highly influenced by intrinsic growth rate (Kennedy and Whalen 2008).

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Table 1. Nutrient addition bioassay treatments for summer and fall 2018 WWTP influent and effluent phytoplankton growth stimulation experiments

Treatment Replicates	Treatment Name
1-3	Control (no addition)
4-6	Influent inorganic nutrient control (contains NO_3 and NH_4 in the same concentration as in the WWTP influent addition)
7-9	WWTP influent treatment (bulk influent added to a concentration of 0.7 mg DON L^{-1})
10-12	Effluent inorganic nutrient control (contains NO_3 and NH_4 in the same concentration as in the WWTP effluent addition)
13-15	WWTP effluent treatment (bulk effluent added to a concentration of 0.7 mg DON L^{-1})

Table 2. Summary of response of phytoplankton photopigments to the addition of WWTP influent or effluent in comparison to a control treatment with similar inorganic nutrient content during the August and October 2018 experiments. +/- indicates pigment concentrations in the WWTP addition treatments greater/less than the control. NS indicates that the treatment effect of a repeated measures ANOVA was not statistically significant.

Pigment (associated phytoplankton group)	August		October	
	Influent	Effluent	Influent	Effluent
Chlorophyll *a* (Total phytoplankton biomass)	-	+	+	NS
Zeaxanthin (Cyanobacteria)	NS	+	-	NS
Myxoxanthophyll (Cyanobacteria)	-	-	NS	NS
Peridinin (Dinoflagellates)	NS	+	NS	NS
Fucoxanthin (Dinoflagellates)	NS	+	+	+
Chlorophyll *b* (Chlorophytes and Euglena)	-	+	+	NS
Alloxanthin (Cryptophytes)	-	NS	-	NS

Table 3. Results from a repeated measures ANOVA of phytoplankton photopigments on nutrient treatment, measurement time point, the interaction of nutrient addition treatment and measurement time point, and individual subject effects.

Chlorophyll *a*

Influent August 2018

Source	SS	df	MS	F	Prob>F
Time	8674.3	4	2168.56	147.62	0
Group	150.2	1	150.21	8.74	0.0417
Interaction	6183.3	4	1545.84	105.23	0
Subjects (matching)	68.7	4	17.18	1.17	0.361
Error	235	16	14.69		
Total	15311.6	29			

Effluent August 2018

Source	SS	df	MS	F	Prob>F
Time	40284.2	4	10071.05	103.57	0
Group	1252.9	1	1252.85	22.68	0.0089
Interaction	791.4	4	197.85	2.03	0.1378
Subjects (matching)	221	4	55.25	0.57	0.6894
Error	1555.9	16	97.24		
Total	44105.3	29			

Zeaxanthin

Source	SS	df	MS	F	Prob>F
Time	223.058	4	55.7645	294.09	0
Group	55.681	1	55.6809	82.39	0.0008
Interaction	177.859	4	44.4647	234.5	0
Subjects (matching)	2.703	4	0.6758	3.56	0.0292
Error	3.034	16	0.1896		
Total	462.335	29			

Source	SS	df	MS	F	Prob>F
Time	377.327	4	94.3317	93.39	0
Group	0.043	1	0.0433	0.02	0.8845
Interaction	39.39	4	9.8476	9.75	0.0003
Subjects (matching)	7.233	4	1.8083	1.79	0.1801
Error	16.161	16	1.01		
Total	440.155	29			

Myxo-xanthophyll

Source	SS	df	MS	F	Prob>F
Time	263.389	4	65.8472	146.13	0
Group	66.168	1	66.1676	68.96	0.0011
Interaction	215.946	4	53.9865	119.81	0
Subjects (matching)	3.838	4	0.9595	2.13	0.1243
Error	7.21	16	0.4506		
Total	556.55	29			

Source	SS	df	MS	F	Prob>F
Time	423.483	4	105.871	123.88	0
Group	120.089	1	120.089	155.8	0.0002
Interaction	90.399	4	22.6	26.44	0
Subjects (matching)	3.083	4	0.771	0.9	0.486
Error	13.674	16	0.855		
Total	650.728	29			

Peridinin

Source	SS	df	MS	F	Prob>F
Time	0.11081	4	0.0277	15.27	0
Group	0.04773	1	0.04773	14.19	0.0197
Interaction	0.13109	4	0.03277	18.06	0
Subjects (matching)	0.01345	4	0.00336	1.85	0.168
Error	0.02904	16	0.00181		
Total	0.33212	29			

Source	SS	df	MS	F	Prob>F
Time	0.24593	4	0.06148	13.07	0.0001
Group	0.0002	1	0.0002	0.03	0.8776
Interaction	0.016	4	0.004	0.85	0.514
Subjects (matching)	0.02943	4	0.00736	1.56	0.2317
Error	0.07525	16	0.0047		
Total	0.36681	29			

Fucoxanthin

Source	SS	df	MS	F	Prob>F
Time	121.729	4	30.4323	398.12	0
Group	58.803	1	58.8032	750.83	0
Ineratcion	93.187	4	23.2968	304.77	0
Subjects (matching)	0.313	4	0.0783	1.02	0.4245
Error	1.223	16	0.0764		
Total	275.256	29			

Source	SS	df	MS	F	Prob>F
Time	106.807	4	26.7018	82.86	0
Group	2.303	1	2.3033	3.27	0.1448
Interaction	3.386	4	0.8464	2.63	0.0735
Subjects (matching)	2.817	4	0.7042	2.19	0.1171
Error	5.156	16	0.3223		
Total	120.469	29			

Chlorophyll *b*

Source	SS	df	MS	F	Prob>F
Time	187.931	4	46.9828	275.08	0
Group	29.508	1	29.5082	1055.62	0
Interaction	107.209	4	26.8023	156.92	0
Subjects (matching)	0.112	4	0.028	0.16	0.9537
Error	2.733	16	0.1708		
Total	327.493	29			

Source	SS	df	MS	F	Prob>F
Time	431.166	4	107.791	479.33	0
Group	12.831	1	12.831	29.6	0.0055
Interaction	15.945	4	3.986	17.73	0
Subjects (matching)	1.734	4	0.434	1.93	0.1548
Error	3.598	16	0.225		
Total	465.274	29			

Alloxanthin

Source	SS	df	MS	F	Prob>F
Time	0.67471	4	0.16868	32.65	0
Group	0.00114	1	0.00114	0.07	0.8049
Interaction	0.75947	4	0.18987	36.76	0
Subjects (matching)	0.06571	4	0.01643	3.18	0.0421
Error	0.08265	16	0.00517		
Total	1.58369	29			

Source	SS	df	MS	F	Prob>F
Time	15.6925	4	3.92313	265.66	0
Group	1.1463	1	1.14634	49.39	0.0022
Interaction	1.235	4	0.30874	20.91	0
Subjects (matching)	0.0928	4	0.02321	1.57	0.2298
Error	0.2363	16	0.01477		
Total	18.4029	29			

Table 3. Continued

	Influent October 2018					Effluent October 2018				
Chlorophyll *a*	Source	SS	df	MS	F	Prob>F	Source	SS	df	MS	F	Prob>F
	Time	1160.43	4	290.107	123.33	0	Time	58744.9	4	14686.2	384.21	0
	Group	13.08	1	13.079	8.27	0.0452	Group	658.6	1	658.6	16.22	0.0158
	Interaction	106.36	4	26.589	11.3	0.0002	Interaction	6243.1	4	1560.8	40.83	0
	Subjects (matching)	6.32	4	1.581	0.67	0.6209	Subjects (matching)	162.4	4	40.6	1.06	0.4072
	Error	37.64	16	2.352			Error	611.6	16	38.2		
	Total	1323.82	29				Total	66420.5	29			
Zeaxanthin	Source	SS	df	MS	F	Prob>F	Source	SS	df	MS	F	Prob>F
	Time	1.94485	4	0.48621	138.12	0	Time	93.3669	4	23.3417	382.67	0
	Group	0.01645	1	0.01645	4.13	0.1118	Group	1.4447	1	1.4447	47.64	0.0023
	Interaction	0.25481	4	0.0637	18.1	0	Interaction	2.9795	4	0.7449	12.21	0.0001
	Subjects (matching)	0.01592	4	0.00398	1.13	0.377	Subjects (matching)	0.1213	4	0.0303	0.5	0.7382
	Error	0.05633	16	0.00352			Error	0.976	16	0.061		
	Total	2.28835	29				Total	98.8883	29			
Myxo-xanthophyll	Source	SS	df	MS	F	Prob>F	Source	SS	df	MS	F	Prob>F
	Time	4.52683	4	1.13171	82.91	0	Time	174.01	4	43.5024	94.82	0
	Group	0.00878	1	0.00878	0.59	0.4845	Group	0.423	1	0.4232	0.97	0.3803
	Interaction	1.00394	4	0.25099	18.39	0	Interaction	24.712	4	6.1781	13.47	0.0001
	Subjects (matching)	0.05928	4	0.01482	1.09	0.3965	Subjects (matching)	1.744	4	0.436	0.95	0.4609
	Error	0.21839	16	0.01365			Error	7.341	16	0.4588		
	Total	5.81722	29				Total	208.23	29			
Peridinin	Source	SS	df	MS	F	Prob>F	Source	SS	df	MS	F	Prob>F
	Time	0.01137	4	0.00284	5.44	0.0058	Time	0.0449	4	0.01122	13.77	0
	Group	0.00207	1	0.00207	1.73	0.2592	Group	0.00049	1	0.00049	0.12	0.7438
	Interaction	0.00199	4	0.0005	0.95	0.4597	Interaction	0.00539	4	0.00135	1.65	0.2096
	Subjects (matching)	0.00481	4	0.0012	2.3	0.1035	Subjects (matching)	0.01591	4	0.00398	4.88	0.0092
	Error	0.00836	16	0.00052			Error	0.01304	16	0.00082		
	Total	0.0286	29				Total	0.07972	29			
Fucoxanthin	Source	SS	df	MS	F	Prob>F	Source	SS	df	MS	F	Prob>F
	Time	101.249	4	25.3124	529.64	0	Time	742.503	4	185.626	207.14	0
	Group	28.756	1	28.7559	257.36	0.0001	Group	26.982	1	26.982	428.99	0
	Interaction	39.672	4	9.918	207.53	0	Interaction	40.62	4	10.155	11.33	0.0001
	Subjects (matching)	0.447	4	0.1117	2.34	0.0995	Subjects (matching)	0.252	4	0.063	0.07	0.9901
	Error	0.765	16	0.0478			Error	14.338	16	0.896		
	Total	170.889	29				Total	824.696	29			
Chlorophyll *b*	Source	SS	df	MS	F	Prob>F	Source	SS	df	MS	F	Prob>F
	Time	12.4892	4	3.12231	93.79	0	Time	2707.48	4	676.87	170.22	0
	Group	0.0381	1	0.03814	1.13	0.3479	Group	112.63	1	112.634	19.31	0.0117
	Interaction	1.7231	4	0.43077	12.94	0.0001	Interaction	521.27	4	130.318	32.77	0
	Subjects (matching)	0.1352	4	0.03379	1.01	0.429	Subjects (matching)	23.33	4	5.833	1.47	0.2585
	Error	0.5327	16	0.03329			Error	63.62	16	3.976		
	Total	14.9183	29				Total	3428.34	29			
Alloxanthin	Source	SS	df	MS	F	Prob>F	Source	SS	df	MS	F	Prob>F
	Time	1.76274	4	0.44069	66.45	0	Time	38.292	4	9.5729	116.3	0
	Group	0.00019	1	0.00019	0.02	0.8916	Group	51.049	1	51.0486	345.55	0
	Interaction	0.19106	4	0.04777	7.2	0.0016	Interaction	30.844	4	7.711	93.68	0
	Subjects (matching)	0.03615	4	0.00904	1.36	0.2905	Subjects (matching)	0.591	4	0.1477	1.79	0.1793
	Error	0.10611	16	0.00663			Error	1.317	16	0.0823		
	Total	2.09625	29				Total	122.092	29			

Table 4. Peak designations, locations (as excitation and emission maximum) and organic matter class assignment for each of the 5 fluorescence peaks plotted through time for bioassay samples (Coble, 1996; Coble et al., 2014; Osburn et al., 2012).

Peak Designation	Excitation Maximum (nm)	Emission Maximum (nm)	Organic matter class
A	< 250	400-460	Terrestrial, humic-like; fulvic acid
C	320-360	420-460	Terrestrial, humic-like; anthropogenic sources
M	290-310	370-410	Microbial humic-like
T	275-280	340-344	Protein, tryptophan
B	275	305	Protein, tyrosine

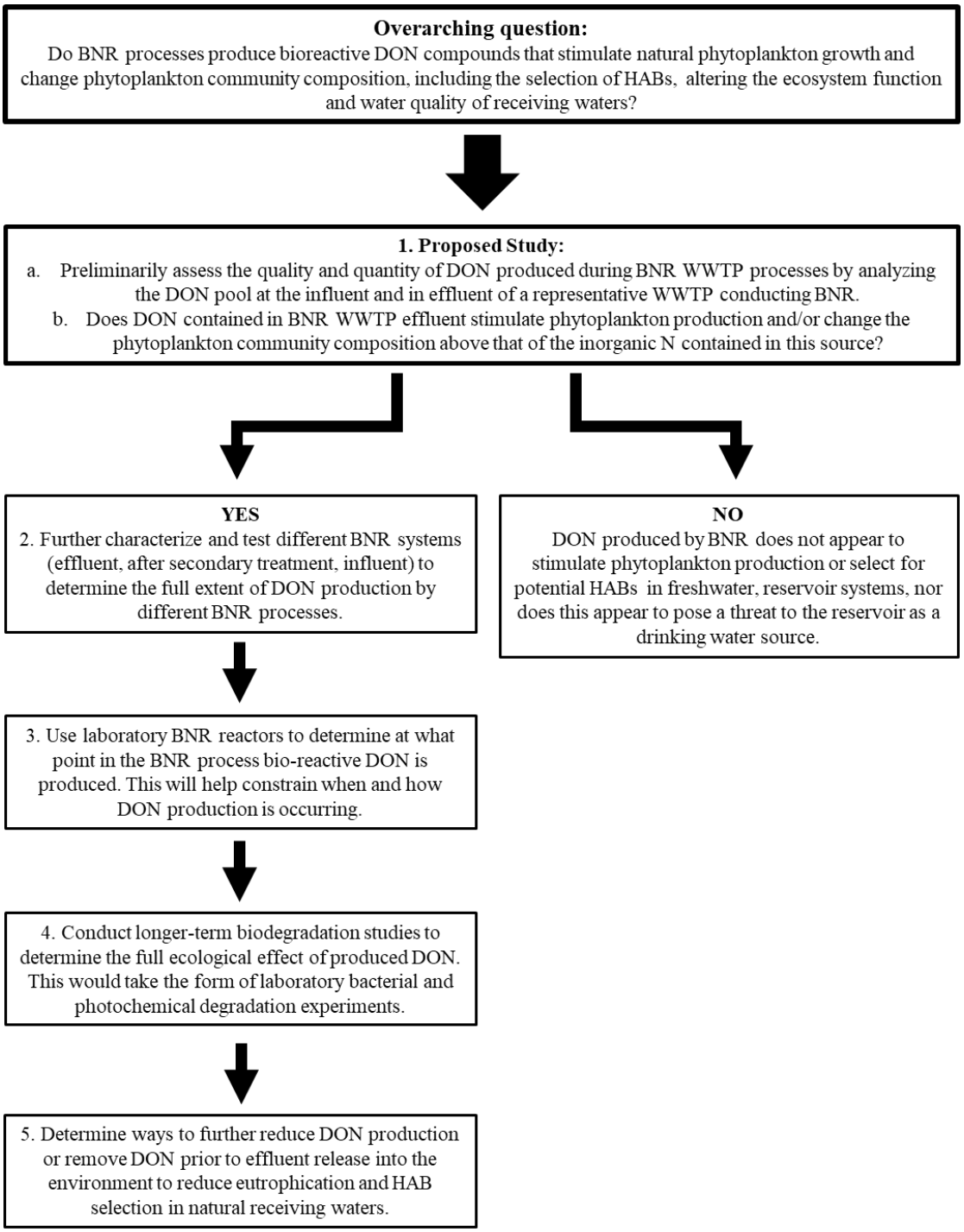

Figure 1. Flow-chart of questions, monitoring work, and laboratory experiments needed to fully assess the production of DON during BNR and whether the DON produced is stimulatory to phytoplankton growth in receiving waters.

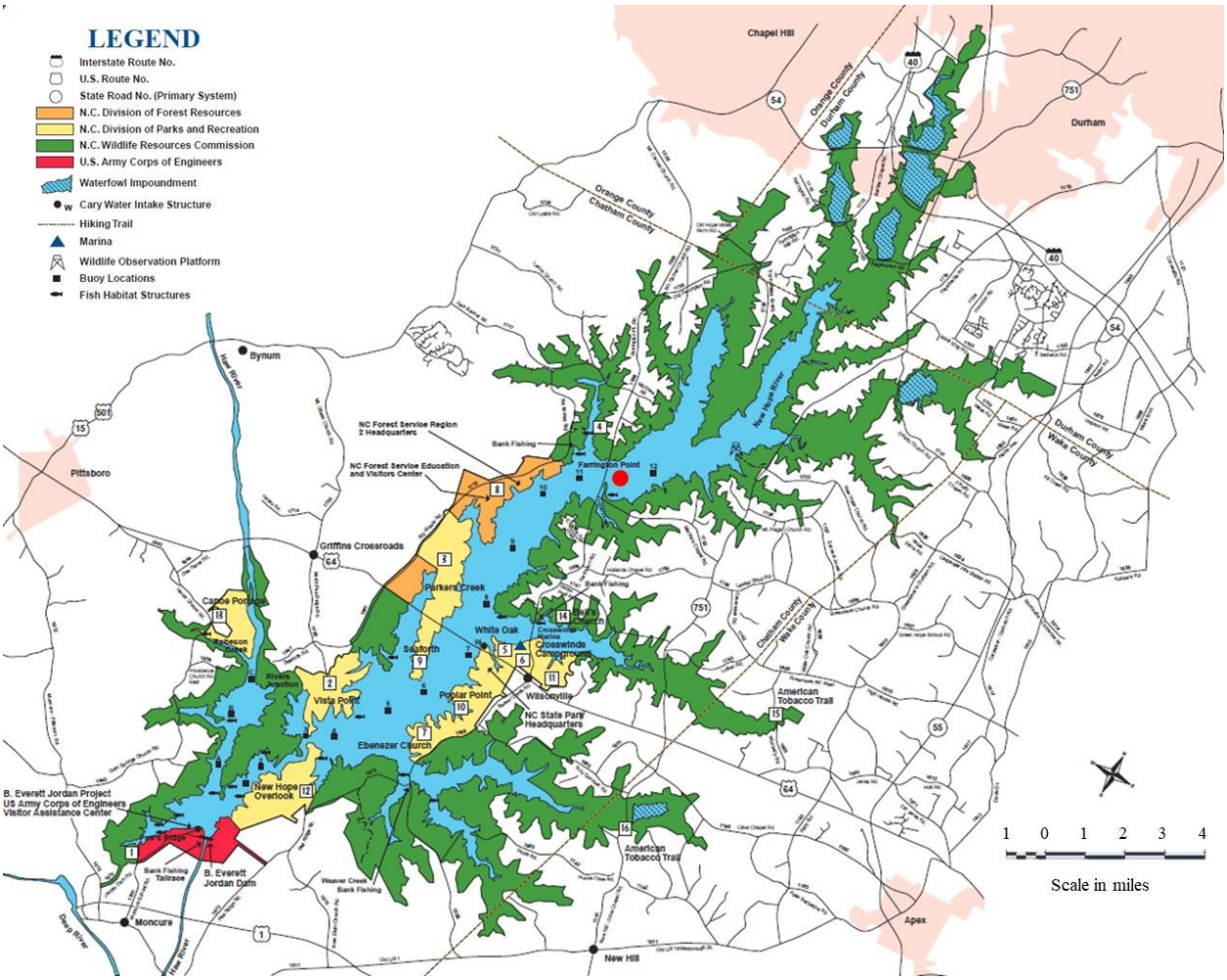

Figure 2. Map of Jordan Lake (modified from the US Army Corps of Engineers). The red dot indicates the proposed location for incubation water collection to be used as natural phytoplankton and bacterial assemblages in the proposed nutrient addition bioassays.

Figure 3. A. Experimental samples in Cubitainers receiving nutrient additions measured and dispensed via pipette. B. Experimental samples incubating in outdoor, flowing water ponds under natural light and temperature conditions.

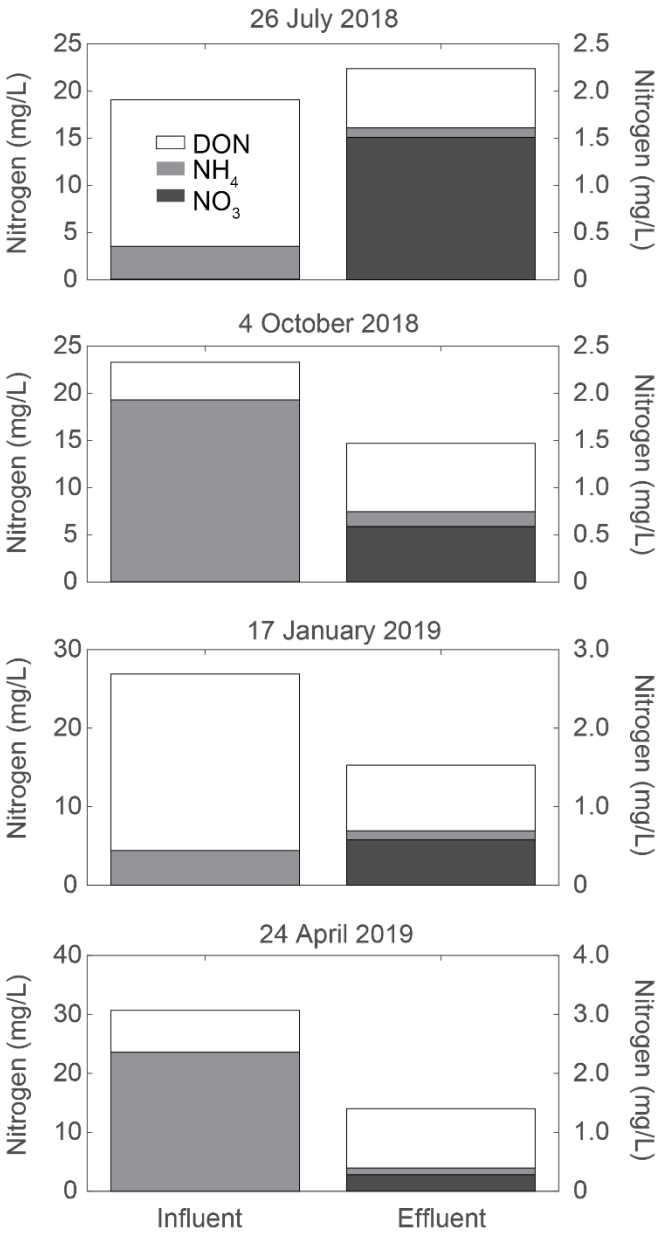

Figure 4. Seasonal measurements of influent and effluent nitrogen forms at the WWTP.

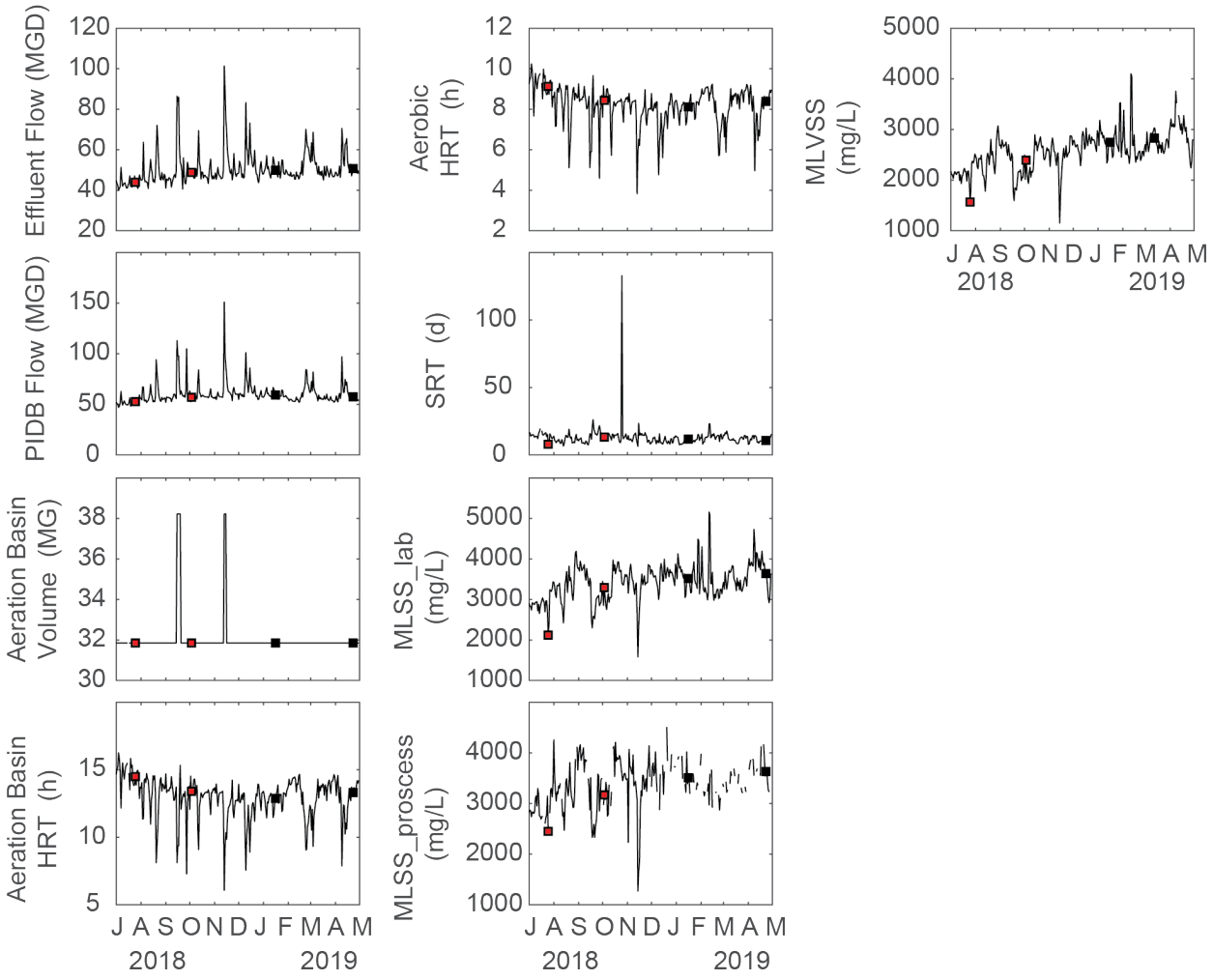

Figure 5. Time series of WWTP operational parameters during the study period. Filled squares indicate timing of sampling by UNC-IMS, and red filled squares indicate timing of influent and effluent collection for bioassay experiments. PIDB = Primary Influent Distribution Box. HRT = hydraulic residence time. SRT = solids retention time, MLSS = mixed liquor suspended solids, MLVSS = mixed liquor volatile suspended solids.

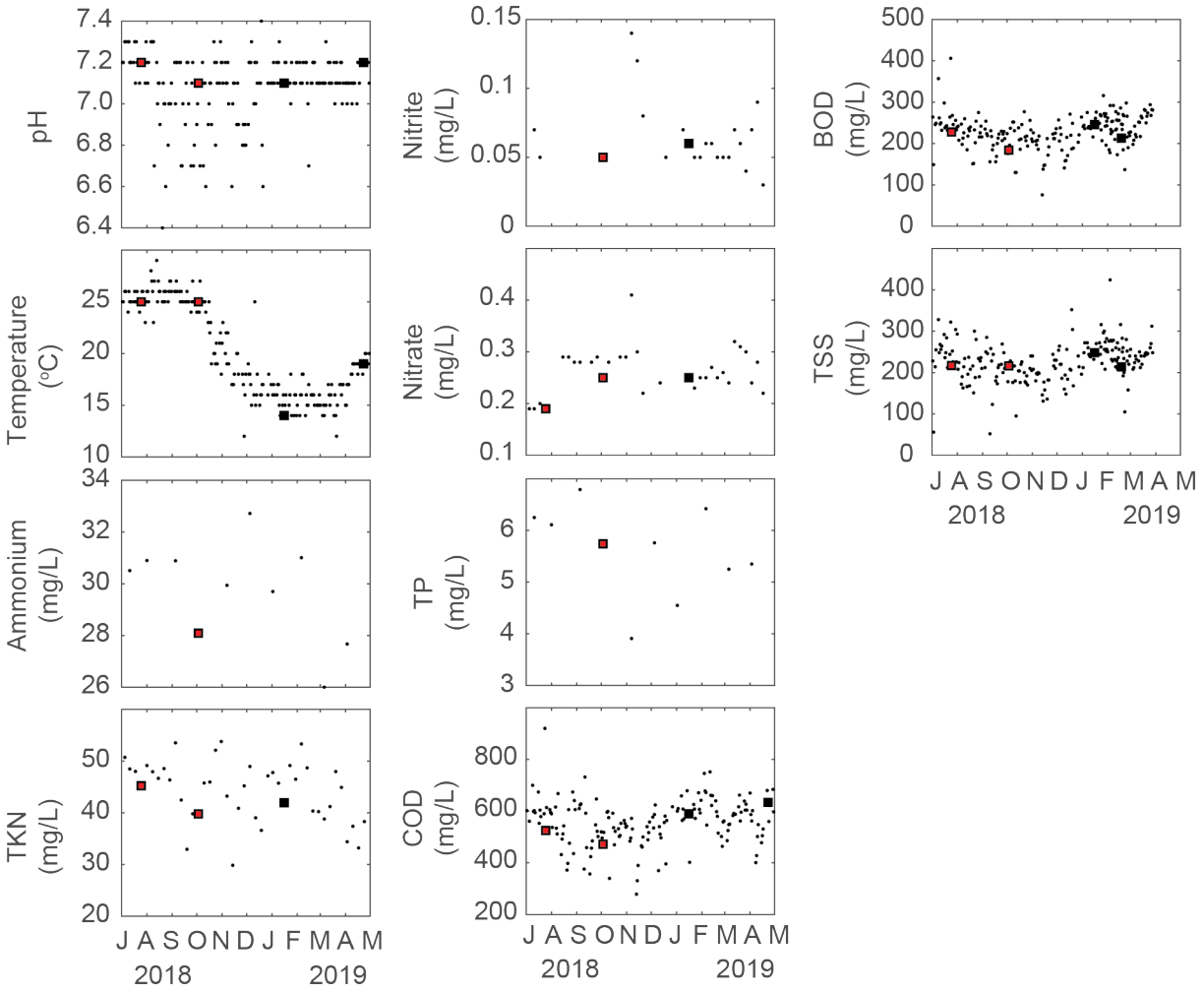

Figure 6. Time series of WWTP influent characteristics during the study period. Filled squares indicate timing of sampling by UNC-IMS, and red filled squares indicate timing of influent and effluent collection for bioassay experiments.

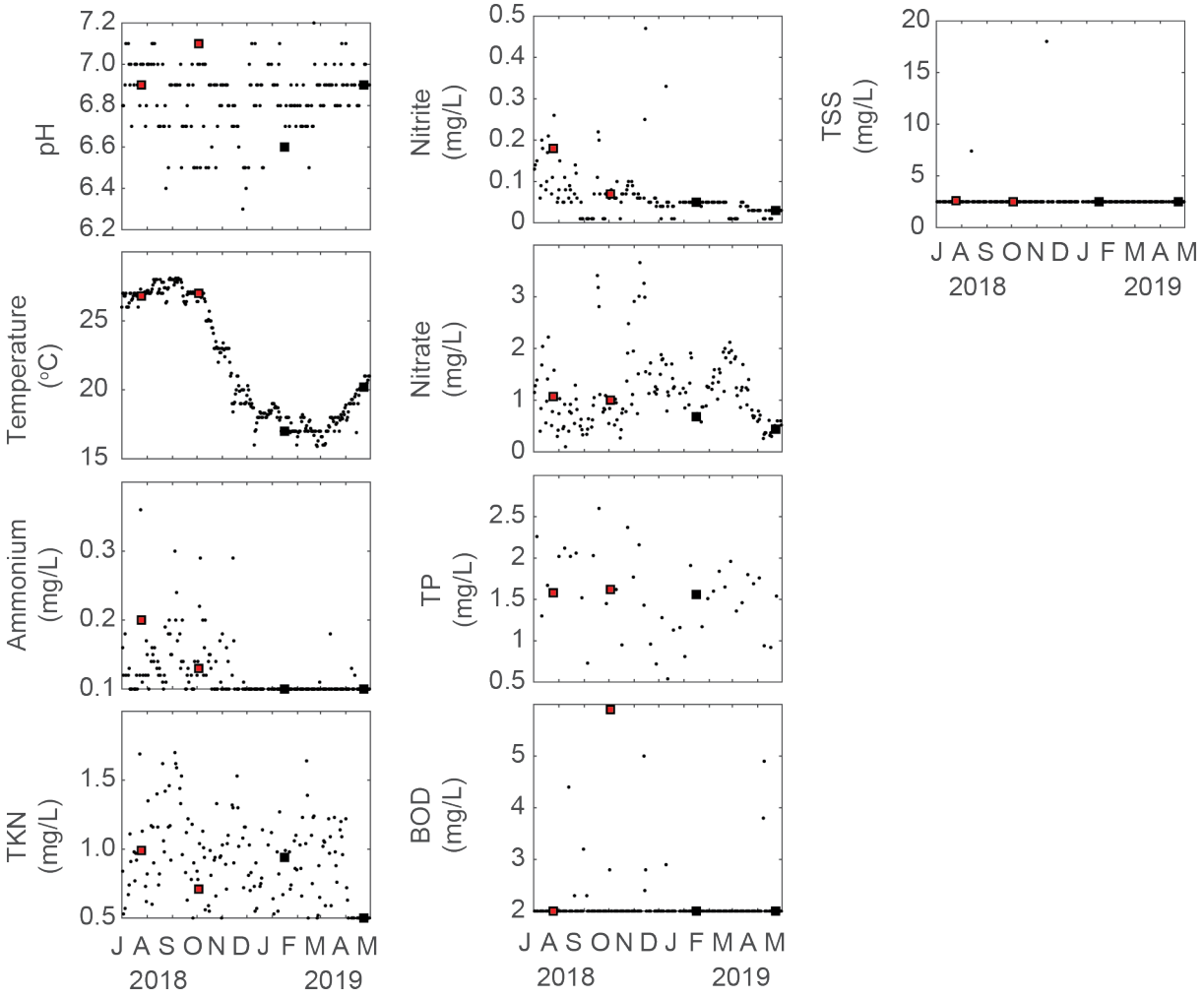

Figure 7. Time series of WWTP effluent characteristics during the study period. Filled squares indicate timing of sampling by UNC-IMS, and red filled squares indicate timing of influent and effluent collection for bioassay experiments.

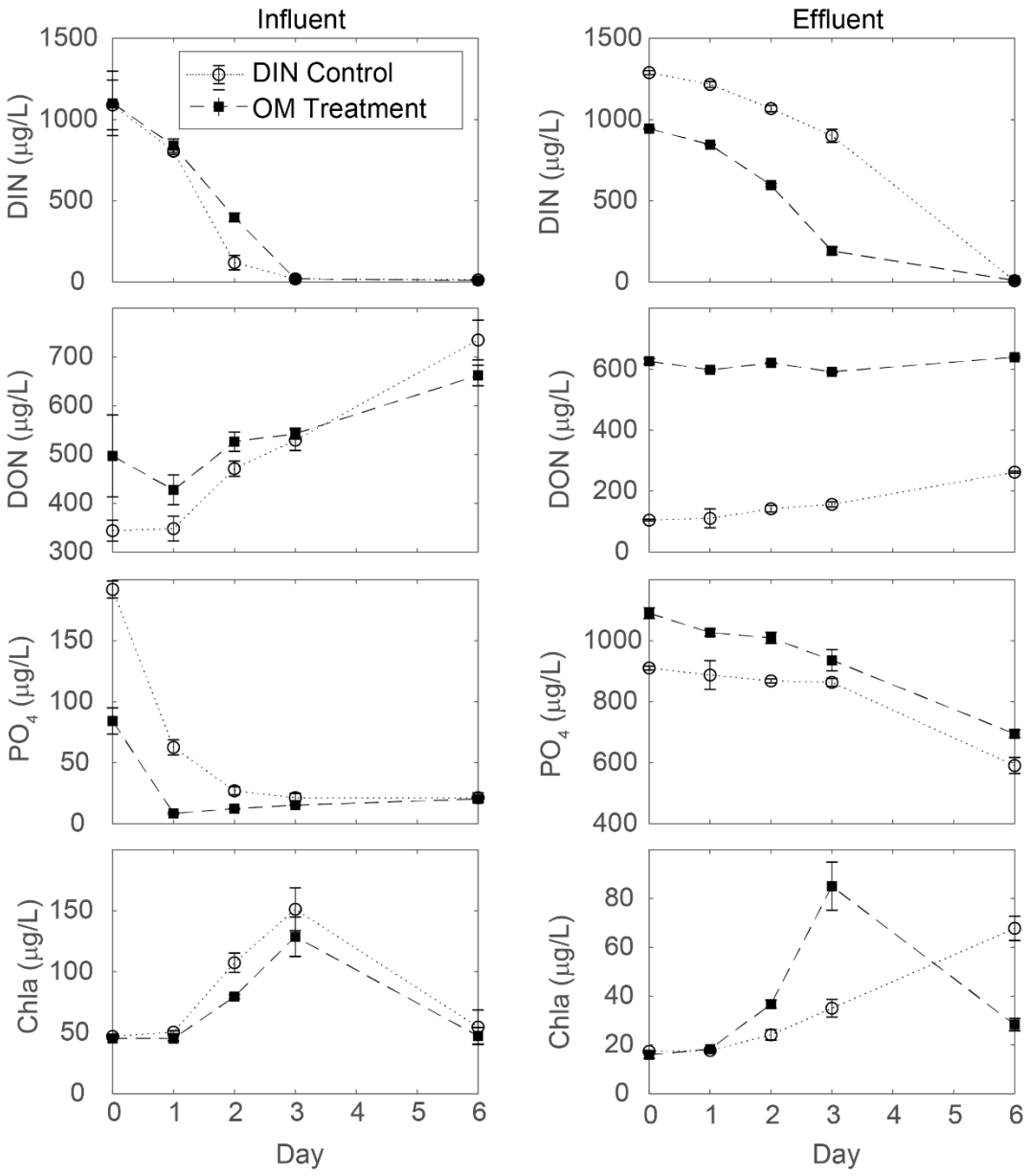

Figure 8. Time series of dissolved N and P forms and phytoplankton biomass as chlorophyll a during WWTP influent and effluent addition experiments to Jordan Lake water in August 2018. OM Treatment consisted of adding influent or effluent water to achieve a final total dissolved organic N concentration of 0.7 mg/L. The DIN control treatment consisted of adding dissolved inorganic N to achieve a final concentration of DIN equivalent to that in the OM Treatment. Data points and error bars are means and standard deviations of triplicated experimental units.

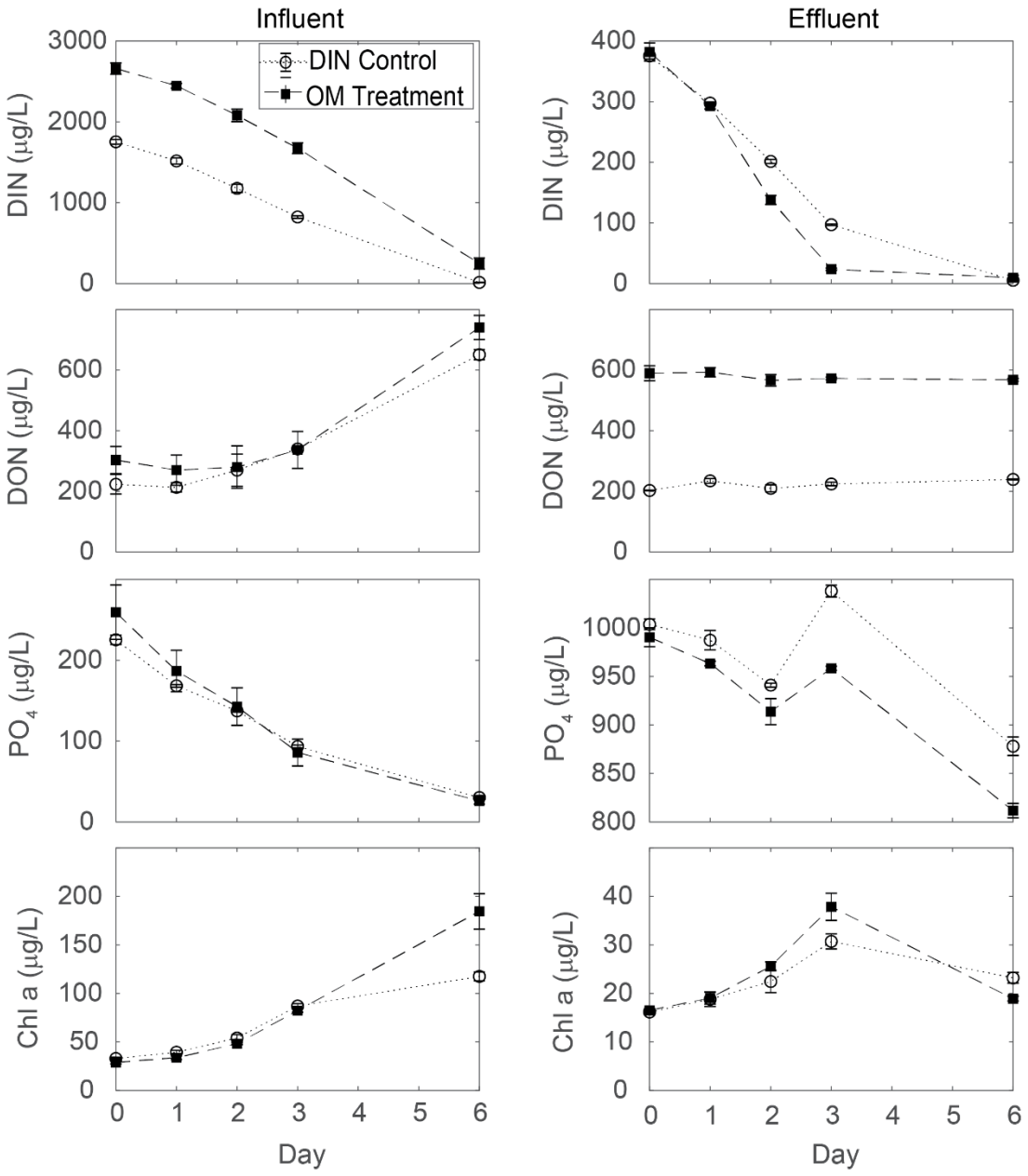

Figure 9 Time series of dissolved N and P forms and phytoplankton biomass as chlorophyll a during WWTP influent and effluent addition experiments to Jordan Lake water in October 2018. Figure configuration is identical to figure 8.

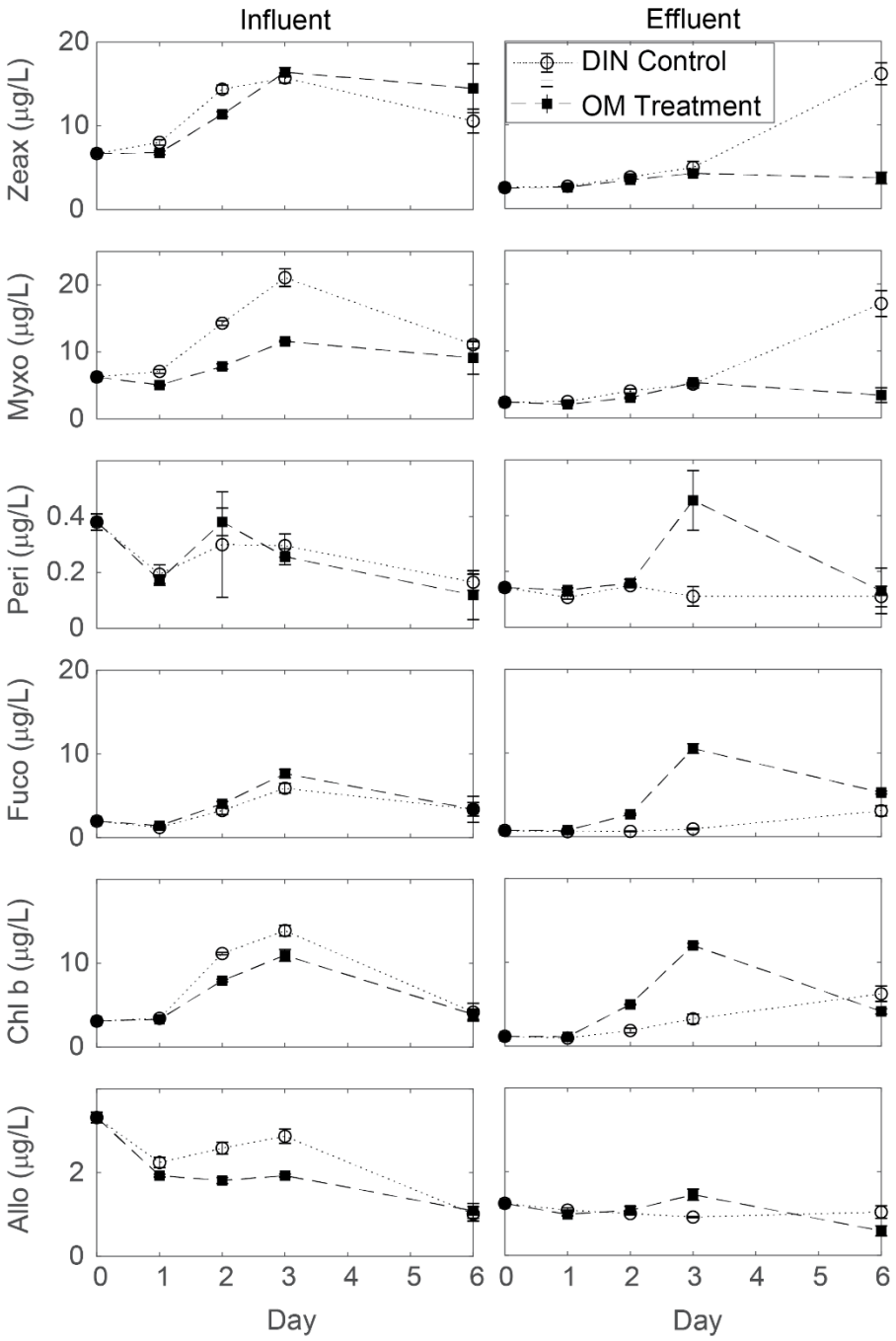

Figure 10. Time series of taxa specific accessory phytoplankton photopigments during WWTP influent and effluent addition experiments to Jordan Lake water in August 2018. Figure configuration is identical to figure 8.

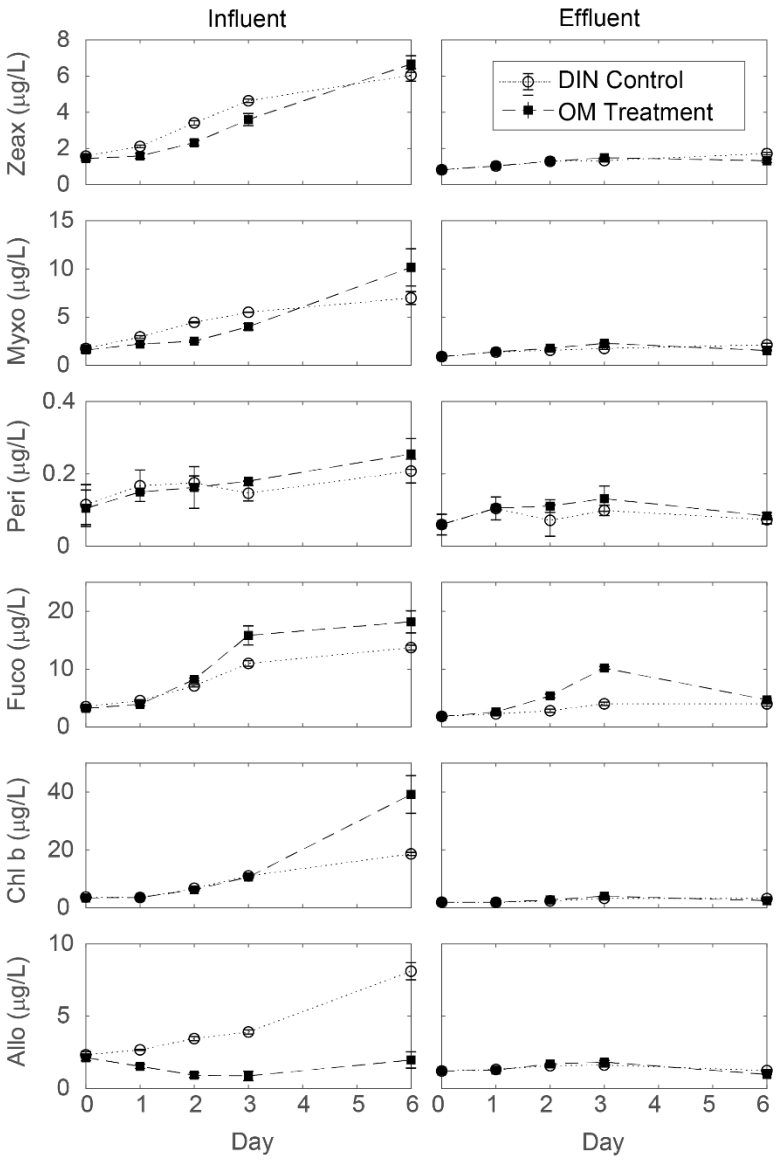

Figure 11. Time series of taxa specific accessory phytoplankton photopigments during WWTP influent and effluent addition experiments to Jordan Lake water in October 2018. Figure configuration is identical to figure 8.

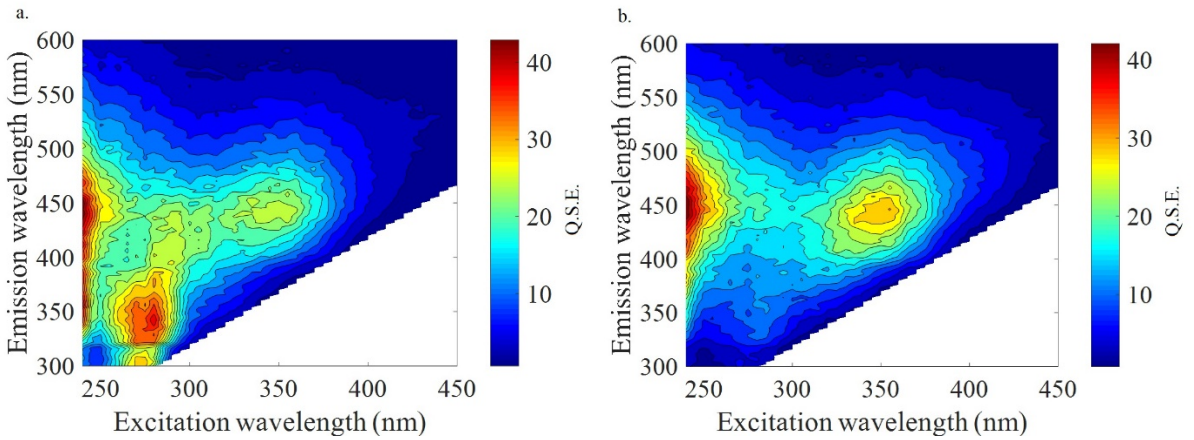

Figure 12. a. Representative EEM obtained from the October 2018 seasonal sampling and b. a representative EEM obtained from the October 2018 seasonal sampling. All seasonal samples exhibited strong fluorescence in the protein-like region of fluorescence for the influent samples. In the effluent samples, fluorescence in this location of the EEMs was largely absent.

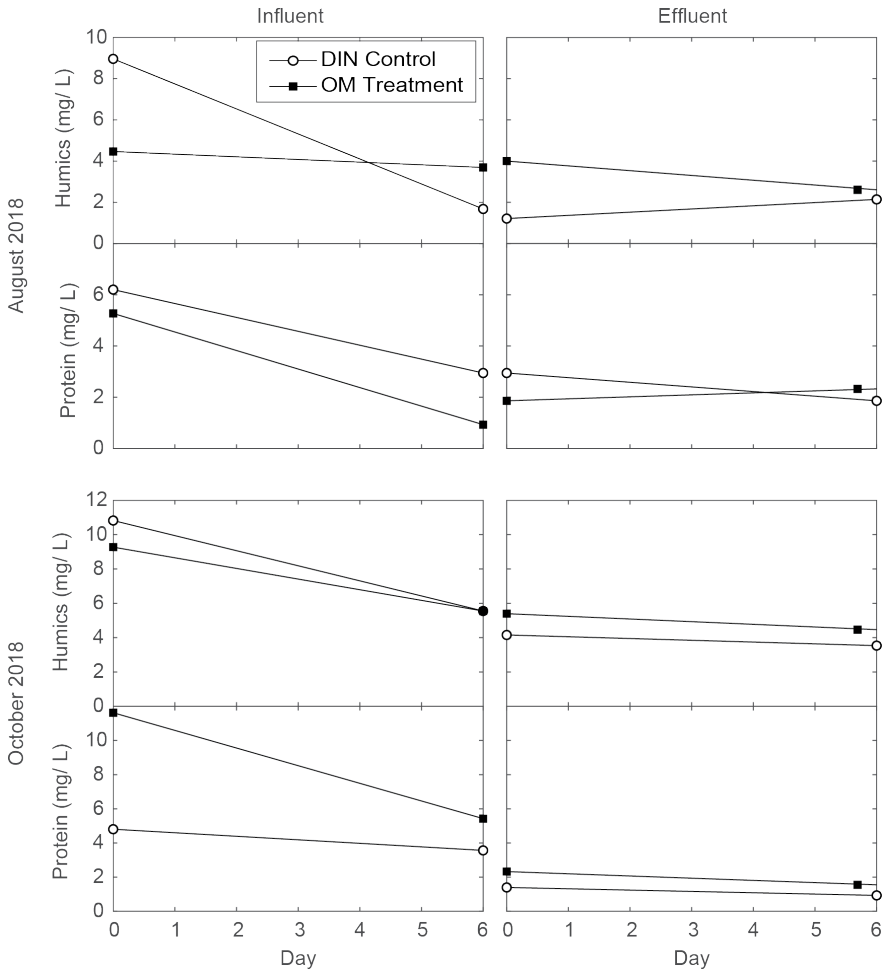

Figure 13. Time series of protein and humic compound concentrations during the August and October 2018 bioassays for the DIN control and respective OM treatment (influent or effluent).

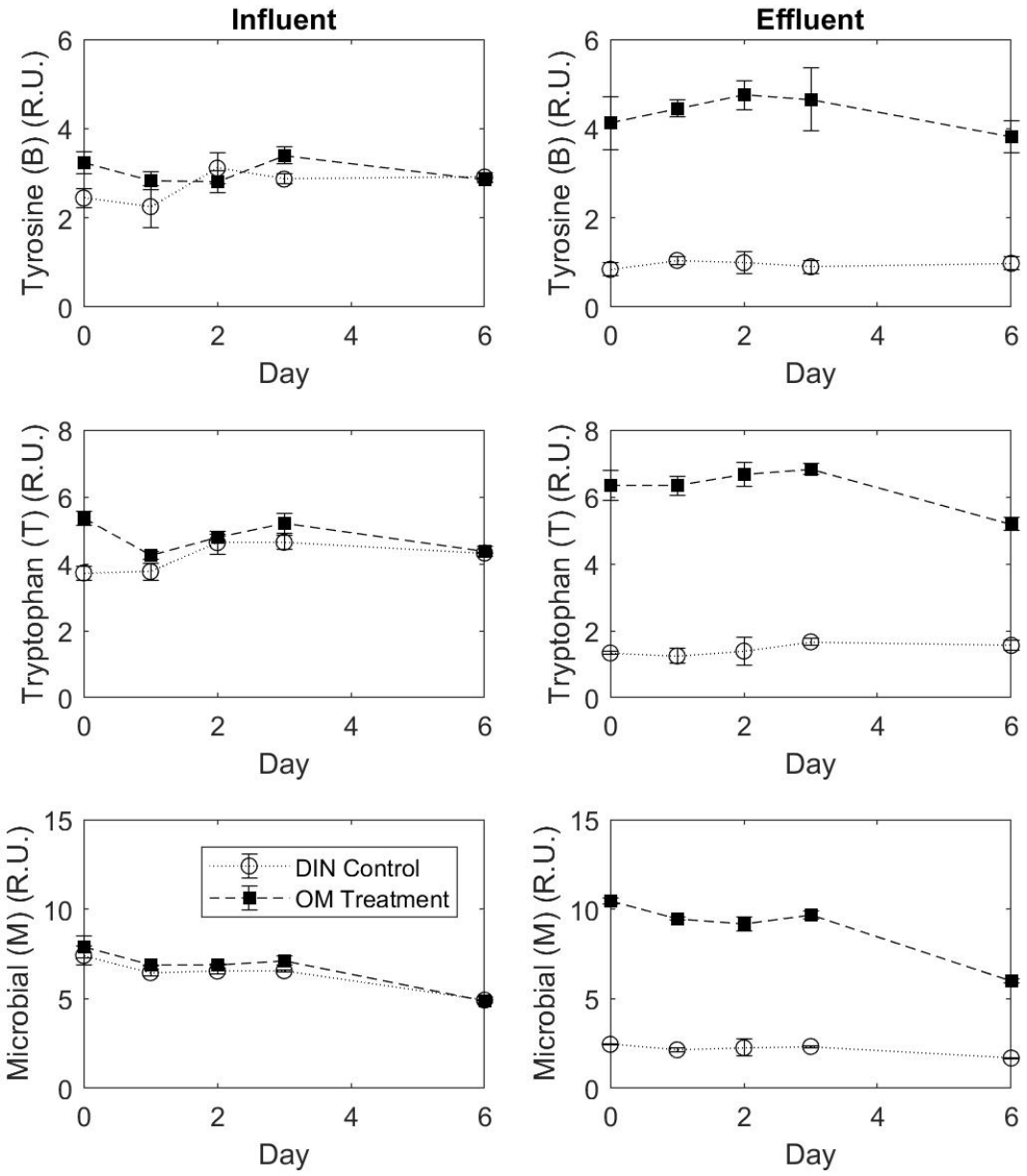

Figure 14. Protein (T and B) and microbial (M) fluorescence peak intensities (as quinine sulfate equivalents) plotted through time for the August 2018 bioassay for the DIN control and respective OM treatment (influent or effluent).

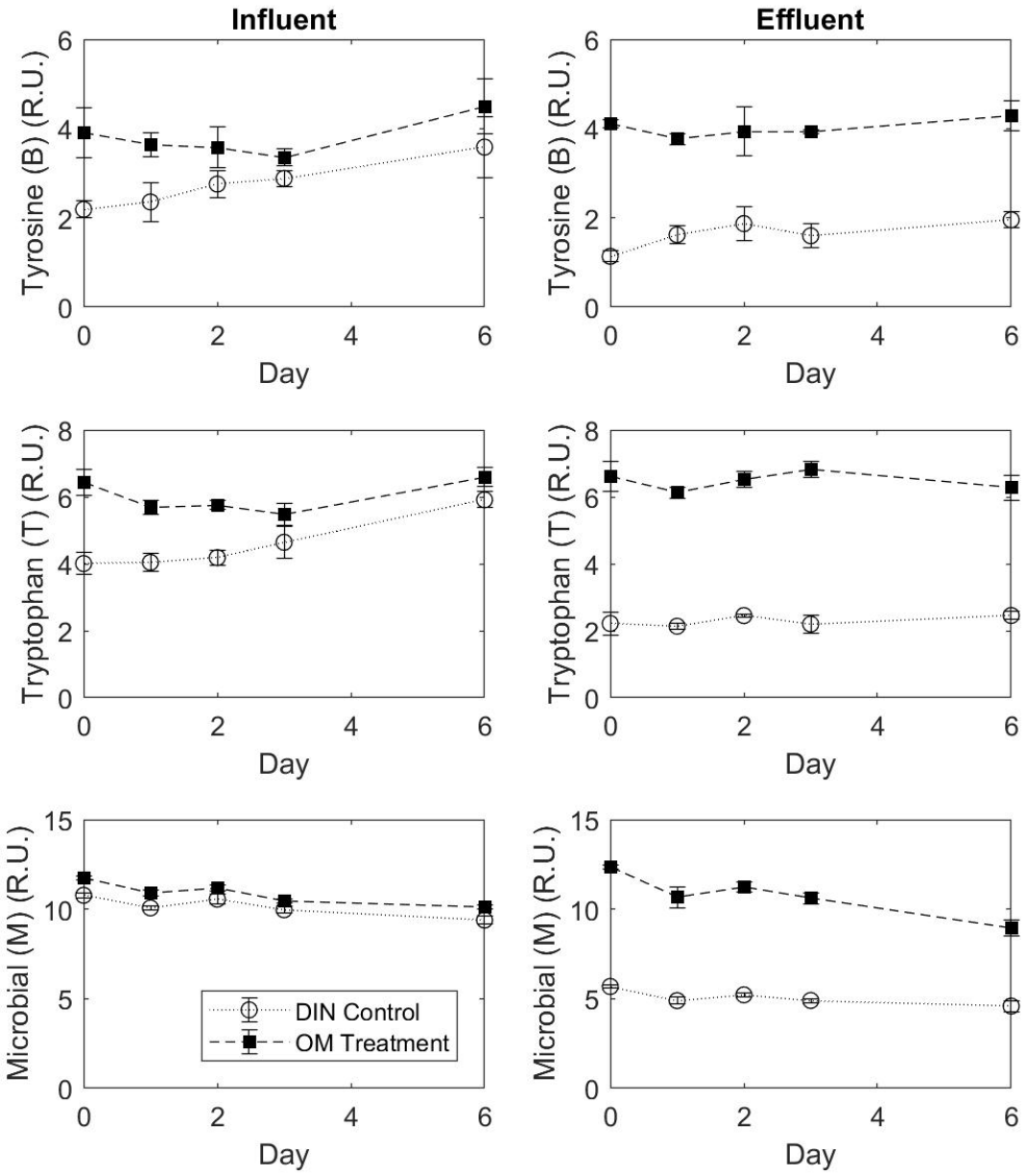

Figure 15. Protein (T and B) and microbial (M) fluorescence peak intensities (as quinine sulfate equivalents) plotted through time for the October 2018 bioassay for the DIN control and respective OM treatment (influent or effluent).

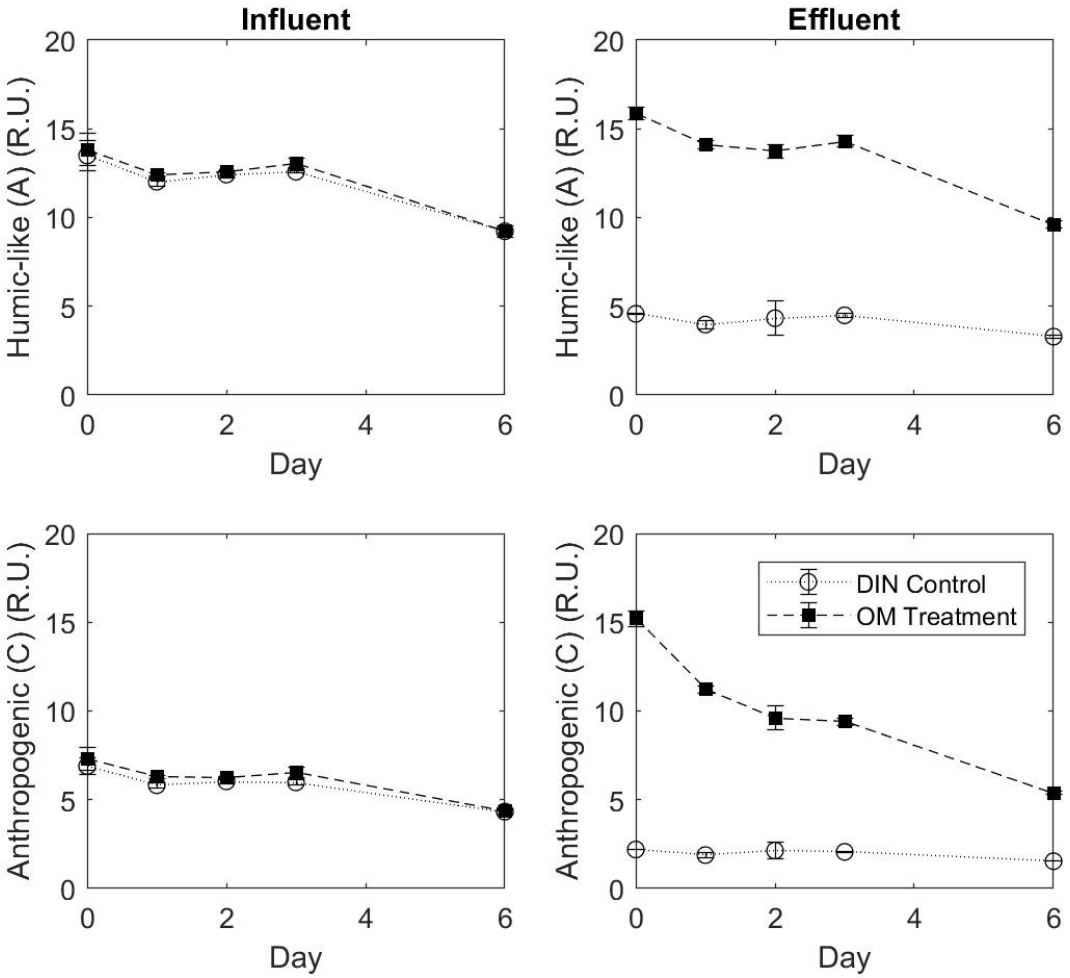

Figure 16. Humic-like (peaks A and C) fluorescence peaks identified in bioassay samples plotted as fluorescence intensities through time. The associated DIN control and OM treatment (as influent and effluent) are plotted for the August 2018 bioassay.

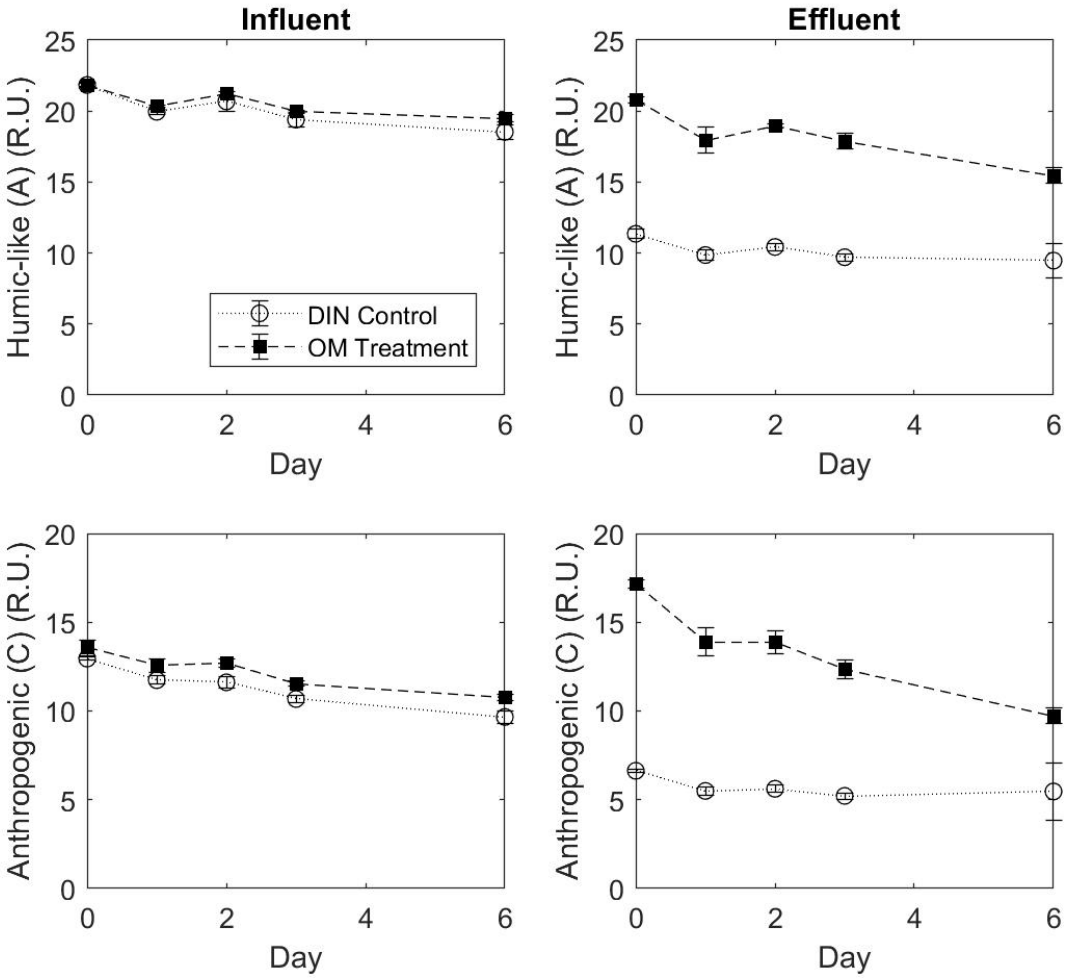

Figure 17. Humic-like (peaks A and C) fluorescence peaks identified in bioassay samples plotted as fluorescence intensities through time. The associated DIN control and OM treatment (as influent and effluent) are plotted for the October 2018 bioassay.