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Stimulation of Phytoplankton Production by Anthropogenic Dissolved Organic Nitrogen in a Coastal Plain Estuary

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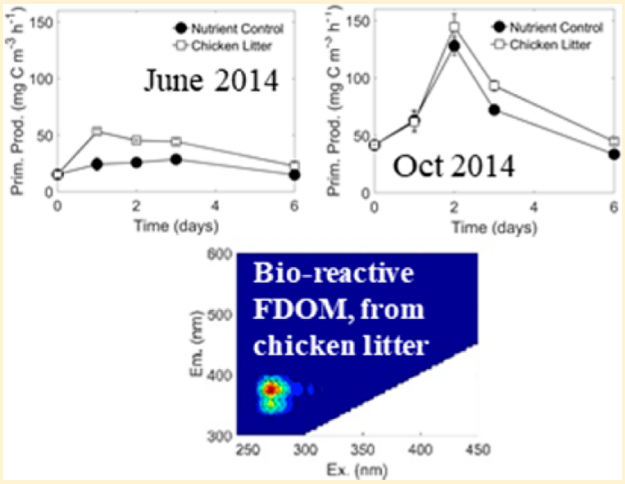
Supporting Information

ABSTRACT: There is increased focus on nitrogen (N)-containing dissolved organic matter (DOM) as a nutrient source supporting eutrophication in N-sensitive estuarine ecosystems. This is particularly relevant in watersheds undergoing urban and agricultural development, leading to increased dissolved organic N (DON) loading. To understand how this shift in N-loading influences estuarine phytoplankton production, nutrient addition bioassays were conducted in the N-limited Neuse River Estuary, North Carolina from 2014 to 2015. Additions included N-rich DOM sources characteristic of urban and agricultural development, including chicken and turkey litter leachate, wastewater treatment facility effluent, and concentrated river DOM (used as a reference). Each DOM addition was coupled with an inorganic nutrient treatment to account for inorganic nutrient concentrations ($NO_{2/3}$, NH_4, PO_4) in each respective DOM addition. Repeated measures analysis of variance (RM-ANOVA) showed that chicken litter leachate stimulated phytoplankton growth greater than its coupled inorganic nutrient treatment. Wastewater treatment facility effluent, turkey litter leachate, and concentrated river DOM did not stimulate phytoplankton growth greater than their respective inorganic nutrient controls. DOM fluorescence (EEM-PARAFAC) indicated the chicken litter contained a biologically reactive fluorescent DOM component, identified as the nonhumic, biologically labile, "N-peak", which may be responsible for stimulating the observed phytoplankton growth in the chicken litter leachate treatments.



INTRODUCTION

Globally, coastal systems are experiencing increasing pressures on ecosystem function and health as a result of rapidly expanding urban, industrial, and agricultural activities in their water- and airsheds.[1−3] These changes in land-uses and activities have resulted in changes in the form of nutrient loading, specifically as nitrogen (N) to downstream coastal systems, as a shift from a combination of inorganic and organic N (ON) to a larger proportion of ON loading.[2,4] Since the mid-1990s, efforts have been enacted to reduce total N loading to N-sensitive, eutrophying systems, e.g., the introduction of total maximum daily loads (TMDLs) that mandated reductions in total N-loading to these systems.[5,6] While efforts to reduce sources of inorganic N to many impaired estuaries have been successful, there has been a simultaneous increase in dissolved organic N (DON) loading, resulting in a shift in the proportion of inorganic N to DON loading.[1,2,4] Primary production in most receiving coastal systems and estuaries is N-limited,[7−9] and N loads continue to exacerbate eutrophication and its associated negative impacts (i.e., nuisance and harmful algal blooms, hypoxia/anoxia, fish kills), despite management efforts.[4,10]

It has been hypothesized that changing watershed land uses and resultant changes in N-loading to estuarine systems play a key role in the continued eutrophication of these N-limited ecosystems.[2,11] We tested this hypothesis by conducting a series of dissolved organic matter (DOM) nutrient addition bioassays on natural phytoplankton and microbial communities from 2014 to 2015 in the eutrophic Neuse River Estuary (NRE), located in eastern North Carolina, USA. Treatments included various N-rich DOM additions that reflected watershed urban and agricultural activities as well as "natural" watershed sources such as forests and wetlands. Using this approach, the study addressed the following research question: Does the DOM found in specific watershed sources (chicken litter, turkey litter, wastewater treatment facility effluent, river DOM) stimulate

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Table 1. Nutrient Additions (DOM and Inorganic Nutrients) for June 2014, October 2014, and July 2015 Bioassays[a]

Date	Treatments			
June 2014	Chicken litter addition	Coupled inorganic nutrient addition (chicken litter)	Effluent addition	Coupled inorganic nutrient addition (effluent)
	2.8 μg L^{-1} NO_x	2.8 μg L^{-1} NO_x	93.8 μg L^{-1} NO_x	93.8 μg L^{-1} NO_x
	10.5 μg L^{-1} NH_4	10.5 μg L^{-1} NH_4	13.6 μg L^{-1} NH_4	13.6 μg L^{-1} NH_4
	31.6 μg L^{-1} PO_4	31.6 μg L^{-1} PO_4	425.6 μg L^{-1} PO_4	425.6 μg L^{-1} PO_4
	140.1 μg L^{-1} DON		140.1 μg L^{-1} DON	
Date	Treatments			
October 2014	Chicken litter addition	Coupled inorganic nutrient addition (chicken litter)	Turkey litter addition	Coupled inorganic nutrient addition (turkey litter)
	44.8 μg L^{-1} NO_x	44.8 μg L^{-1} NO_x	3.8 μg L^{-1} NO_x	3.8 μg L^{-1} NO_x
	154.5 μg L^{-1} NH_4	154.5 μg L^{-1} NH_4	171.3 μg L^{-1} NH_4	171.3 μg L^{-1} NH_4
	89.2 μg L^{-1} PO_4	89.2 μg L^{-1} PO_4	92.8 μg L^{-1} PO_4	92.8 μg L^{-1} PO_4
	140.1 μg L^{-1} DON		140.1 μg L^{-1} DON	
Date	Treatments			
July 2015	River DOM addition		River DOM control	
	31.0 μg L^{-1} PO_4		no addition	
	140.1 μg L^{-1} DON			

[a]Nutrient concentrations for the DOM additions reflect the concentrations inherent to the respective DOM source.

phytoplankton standing stock and primary production in excess of growth stimulated by the dissolved inorganic nutrients also contained in these DOM sources.

Results from this study have important implications for focusing on specific anthropogenic OM and ON sources (chicken litter, wastewater treatment facility effluent, turkey litter) that may require management in order to protect N-impaired systems experiencing continued eutrophication despite ongoing efforts to reduce inorganic N inputs.

MATERIALS AND METHODS

Site Description. The NRE is a river-dominated, microtidal estuary located in the coastal plain of North Carolina, USA (Figure S1). The Neuse River flows through the increasingly urbanized Raleigh-Durham area and several growing, downstream municipalities (Goldsboro, Kinston, and New Bern, NC) before entering the estuary where land use is characterized by agriculture (concentrated animal feeding, mainly as poultry; row crop operations), wetlands, and forested watersheds.[3,11] Due to the mixed land use in the watershed, a variety of nutrient and DOM sources exist in both the river and estuary.[2,11,12] The estuary drains into the USA's second largest estuarine complex, the Albemarle-Pamlico Sound, a semi-lagoonal system which has restricted exchange with the Atlantic Ocean, leading to a freshwater flushing time of about 5 to 8 weeks in the NRE.[13,14] This provides ample time for phytoplankton and associated microbial assemblages to utilize both inorganic and organic nutrients flushed into the system.[13,15] The NRE is mainly N-limited and can exhibit large phytoplankton blooms in the summer and fall months, which exacerbate bottom water hypoxia and fish kills.[5]

Experimental Design. Five nutrient addition bioassays were conducted using natural phytoplankton and bacterial communities collected from the NRE (June 2014, October 2014, July 2015) where phytoplankton blooms are common (Figure S1).[16] Bioassays received different DOM additions representative of N-rich, DOM sources to the NRE, including chicken litter leachate, wastewater treatment facility effluent (effluent), turkey litter leachate, and concentrated river DOM. The volume of DOM source additions was added such that the total DON concentration in each DOM addition equaled 140 μg DON L^{-1}, which is representative of DON concentrations measured in the lower NRE.[17,18] Each DOM addition was paired with an inorganic nutrient addition treatment made up of nitrate/nitrite, ammonium, and phosphate (NO_x^-, NH_4^+, PO_4^{3-}) added to concentrations matching those measured in each respective DOM addition (Table 1). By coupling each DOM addition treatment with an inorganic nutrient addition, the impact of DOM specific sources to phytoplankton productivity could be isolated. For this study, we assume growth responses to dissolved inorganic N and DON are additive, and additional growth stimulated in the DOM treatments compared to the coupled inorganic nutrient treatment is due to the DOM pool contained in the respective DOM source addition. Previous research in the NRE showed that P-limitation is not commonly observed,[16,19] but to ensure that P-limitation did not occur during the bioassay, phosphate was added to each treatment. The coupled nutrient control for the concentrated river DOM treatment did not contain added inorganic nutrients, since none were detectable in the addition. Iron and trace metal levels in the NRE are sufficient for supporting macronutrient (N and P)-stimulated growth[19] and were not added to the bioassays.

Incubation water, which contained natural phytoplankton and bacterial assemblages, was collected 0.5 m below the surface and pumped through 202 μm mesh into precleaned, acid-rinsed polyethylene carboys. Initial characteristics of the incubation water were measured (Table S1). Temperature and salinity were measured *in situ* using a YSI 6600 multiparameter water quality sonde.[20] Riverine discharge was measured at the USGS gauging station #02091814 located on the Neuse River near Fort Barnwell, NC approximately 26 km from the head of the NRE.[15] Nutrient and chlorophyll *a* (Chl *a*) concentrations were measured as described below. Incubation water was transported (<4 h) to the University of North Carolina-Chapel Hill, Institute of Marine Sciences in Morehead City, NC and distributed into pre-aged 4-L transparent polyethylene Cubitainers for nutrient additions and incubation. Pre-aging of Cubitainers reduces possible leaching of optically active compounds.[21] Cubitainers have been shown to transmit ~95% of light in the 400−700 nm (PAR) range[16] and ~20−35% of light in the 300−400 nm range (Peierls, unpublished results). Treatments were incubated for at least 6 days under ambient light and temperature conditions. Quadruplicate (June 2014;

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October 2014) or triplicate (July 2015) treatments were subsampled for nutrient, OM, and biological analyses on day 0, 1, 2, 3, and 6 of the bioassay incubations.

DOM source additions were obtained from watershed sources that are nutrient rich and high in DON. DOM source additions used during June 2014 were selected to reflect both urban (effluent) and agricultural (chicken litter) DOM sources in the NRE watershed. In October 2014, DOM source additions were selected to examine the difference between poultry operations (chicken vs turkey litter). River DOM was used in July 2015 as a contrast to anthropogenic sources of DOM and to reflect more natural DOM sources.

Litter treatments were derived from water-soluble extracts of turkey and broiler chicken litter from poultry operations in the NRE watershed (24 h extraction at room temperature, followed by filtration) which were obtained, blind, from the NC Department of Agriculture laboratory.[22] The chicken litter additions were collected from two different farms and may represent variability in nutrient and DOM concentrations between chicken operations.[23,24] The June 2014 chicken litter was dried and homogenized prior to extraction. The October 2014 litter samples (chicken and turkey) were not manipulated prior to extraction. Effluent was obtained from a wastewater treatment facility located in Raleigh, NC and is representative of effluent discharged into the Neuse River and NRE.[25] Concentrated river DOM originated from Contentnea Creek, an agriculturally dominated tributary of the NRE[26] (Figure S1), and was concentrated via tangential flow filtration using a cellulose filter with 1 kDa cutoff.[25] DOM source additions and volumes are listed in Table S2.

Optical Analyses. Samples for optical analyses were filtered through a combusted (450 °C; 4 h) 0.7 μm porosity, GF/F glass fiber filter, and the filtrate measured for absorbance (colored DOM = CDOM) and fluorescence (fluorescent DOM = FDOM). Absorbance spectra of CDOM filtrate were collected from 800 to 200 nm on a Shimadzu UV-1700 Pharma-Spec spectrophotometer and corrected using a Nanopure water blank measured on the same day as analysis. Fluorescence spectra were measured on a Varian Cary Eclipse spectrofluorometer. Excitation wavelengths were measured from 240 to 450 nm every 5 nm. Emission wavelengths were measured from 300 to 600 at 2 nm intervals. Instrument excitation and emission corrections were applied to each sample in addition to corrections for inner-filtering effects, calibrated against the Raman signal of Nanopure water, and standardized to quinine sulfate units (Q.S.U.).[17,27] Emission scans for each sample were concatenated into 151 × 43 excitation−emission matrices (EEMs).

Parallel Factor Analysis (PARAFAC) is a multiway, statistical decomposition technique that can be applied to a collection of EEMs to identify and track broad classes of FDOM, represented as linearly independent components having excitation and emission properties common to organic fluorophores.[28] Similar to principal components analysis (PCA), but without the constraint of orthogonality, PARAFAC identifies a set of components that explains the underlying fluorescent variability of collected EEMs. Unlike traditional PCA, PARAFAC can be applied to three-way data arrays (i.e., multiple three-dimensional EEMs) by developing a trilinear model.[29] A PARAFAC model was fitted to a total of 225 EEMs collected from all bioassays using the DOMFluor toolbox in Matlab.[27] EEMs were normalized to their total fluorescence prior to PARAFAC modeling.[17] A 5-component PARAFAC model was fitted to the DOM bioassay samples. The model was split-half validated, and all 5 components matched (>95% similarity) with previously identified PARAFAC components on the online database, OpenFluor[30] (Figure S2; Table S3).

Fluorescence was also measured on the DOM source additions (chicken and turkey litter, effluent, river DOM). The sources were not included in the PARAFAC modeling; however, the PARAFAC model generated using the bioassay samples was applied to the five DOM sources. Additionally, a previously developed PARAFAC-based mixing model, FluorMod, was applied to the five DOM sources and the starting NRE incubation water. FluorMod is based on sources of FDOM to the Neuse River watershed.[22] By applying FluorMod to the source and incubation water used during the bioassay, the proportion of watershed FDOM sources in the initial bioassay samples could be assessed.

A second PARAFAC model was developed on residuals (as the difference between raw EEMs and PARAFAC modeled EEMs) for samples collected from the two chicken litter DOM treatments (n = 50) (June 2014; October 2014). By analyzing and modeling the residuals, a better understanding of the FDOM composition of the chicken litter treatment could be inferred.[26,31]

Phytoplankton Biomass, Primary Productivity, and Bacterial Productivity. Phytoplankton biomass was measured as Chl a according to the modified version of EPA method 445.0.[32] Briefly, 50 mL of sample was gently filtered through 25 mm GF/F glass fiber filters. Filters were collected and stored at −20 °C until analysis. Filters were extracted overnight in 90% acetone following processing in a tissue grinder. Extract was analyzed unacidified on a Turner Designs TD-700 fluorometer with narrow bandpass filters. Primary productivity in samples was measured using the ^{14}C method.[33] Bacterial productivity was measured using the tritiated (^{3}H) leucine uptake method.[34]

Nutrient Analysis. Total dissolved N (TDN), nitrate + nitrite (NO_3^- + NO_2^-, reported as NO_x), ammonium (NH_4^+), and phosphate (PO_4^{-3}) were determined colorimetrically.[35] DON was calculated by subtracting dissolved inorganic N species (NO_x^- + NH_4^+) from TDN. DOC was measured on a Shimadzu TOC-5000 analyzer via high temperature catalytic oxidation.[35]

Statistical Analysis. Phytoplankton growth responses (Chl a, primary productivity) to nutrient additions, EEM-PARAFAC FDOM components, and bulk DOC and DON measurements were compared between each coupled treatment (i.e., inorganic nutrient addition compared to the respective DOM addition) with repeated measures analysis of variance (RM-ANOVA) using the statistical program, JASP 0.8.0.0.[36] Spearman's correlation coefficients (ρ) were calculated for correlations between DON and the bioassay PARAFAC components and residual model component in Matlab R2016b.

RESULTS AND DISCUSSION

Phytoplankton Growth Response. Phytoplankton growth response, measured as both phytoplankton standing stock (Chl a) and primary production, was stimulated by the two chicken litter treatments (June 2014; October 2014) above their respective coupled inorganic nutrient treatments based on RM-ANOVA results (Figure 1; Table 2). This indicated there was a specific stimulant in the chicken litter, either as a DOM component or other stimulatory compound, that allowed for greater phytoplankton growth and primary production as compared to the addition of inorganic nutrients (NH_4^+, NO_x,

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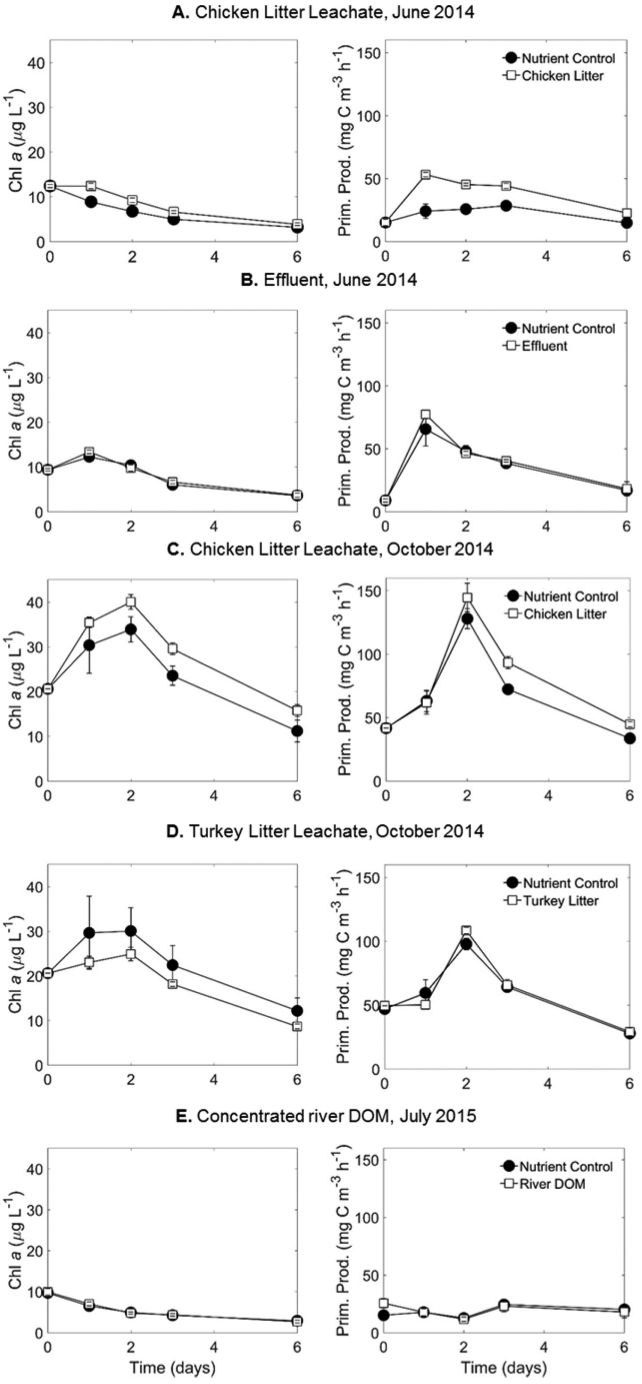

Figure 1. Chl *a* (left) and primary productivity (Prim. Prod.) (right) plotted for each coupled DOM addition treatment. Black circles represent the respective inorganic nutrient addition treatments; white squares indicate the coupled DOM addition. RM-ANOVA was used to determine statistically significant differences between the coupled treatments (indicated by p-values for Chl *a* and primary productivity, respectively). A. chicken litter leachate, June 2014 ($p < 0.001$, $p = 0.001$); B. effluent, June 2014; C. chicken litter leachate, October 2014 ($p = 0.02$; $p = 0.022$); D. turkey litter leachate, October 2014; and E. concentrated river DOM, July 2015.

PO_4^{-3}) alone. When present, inorganic-N sources (NO_x, NH_4^+) were rapidly depleted between Day 0 and 1, resulting in N-limited conditions by Day 1 (Figure S3). Previous work in the NRE concluded micronutrients were not limiting and would not likely have a stimulatory effect on growth,[19] demonstrating the DOM compounds inherent to the chicken litter may be stimulating phytoplankton production greater than the addition of inorganic nutrients alone.

For the effluent, turkey litter, and river DOM treatments, the respective DOM addition treatment did not yield greater phytoplankton production compared to the respective inorganic nutrient addition treatments, indicating the DOM pool in these treatments did not lead to greater phytoplankton production. Primary productivity rates measured for the turkey litter leachate treatment were lower than those measured in its coupled inorganic nutrient treatment ($p < 0.001$) despite equal concentrations of inorganic nutrients in both treatments. This indicates there may have been a constituent in the turkey litter that inhibited phytoplankton growth. The exact mechanism of this inhibition is beyond the scope of this study but could be a result of pharmaceuticals, heavy/trace metal, pathogens, or pesticides contained within the turkey litter that negatively impact phytoplankton productivity.[23,24] Further investigation is necessary to determine a specific mechanism.

DOM Characteristics: EEM-PARAFAC FDOM Components, DOC, and DON. All five identified EEM-PARAFAC components were plotted for each coupled treatment (inorganic treatment + DOM addition treatment) through time (Figure S4; Figure S5; Table S4). All five components exhibited similar patterns during the bioassay, regardless of treatment, which indicates all five components, while identified as mathematically distinct by the PARAFAC model, exhibited similar reactivity irrespective of source. It is assumed the bioassay PARAFAC model is capturing the FDOM pool inherent to the estuarine water used for incubation and is not able to capture FDOM in the sources. Because all five components decreased from day 0 to day 6 for all treatments, regardless of DOM source, primary productivity, or bacterial productivity responses (Figure S6), we conclude the decrease in fluorescence intensity for all five components is likely a function of photobleaching and not consumption by phytoplankton. However, we cannot rule out decreases in FDOM intensity due to removal by baseline bacterial degradation, perhaps facilitated by prior photodegradation.[37] Previous photodegradation studies have demonstrated FDOM components (terrestrial, humic-like; microbial, humic-like; proteins as tyrosine and tryptophan) decrease in fluorescent intensity in response to sunlight exposure, as observed during this study.[38−40] The difference in phytoplankton primary production in the two chicken litter treatments is not a function of any of the five identified bioassay PARAFAC modeled FDOM components.

Bulk DOC and DON concentrations were also measured for each bioassay treatment (Figure S7; Table S5). DON concentrations were correlated (Spearman's ρ) with each bioassay and residual PARAFAC component (Table S6). All components were positively correlated with DON, indicating components can be considered a proxy for DON. DOC and DON concentrations were generally higher in the DOM additions compared to the coupled inorganic nutrient treatments. The June 2014 chicken litter was the only treatment statistically different from its coupled inorganic nutrient addition in terms of DON concentration (Table S5) and which also stimulated phytoplankton production greater than its coupled inorganic nutrient addition. These results indicate the DON portion of the DOM pool in the chicken litter treatment may be stimulating the observed phytoplankton growth. The October 2014 chicken litter treatment also

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Table 2. Results from the RM-ANOVA Conducted on the Coupled DOM Addition and Inorganic Nutrient Addition Treatments for Chl *a* and Primary Productivity (Prim. Prod.)[a]

	June 2014 Chicken Litter			June 2014 Effluent		
	Time	Treatment	Time*Treatment	Time	Treatment	Time*Treatment
Chl *a*	<0.001	<0.001	<0.001	<0.001	0.183	0.024
Prim. Prod.	0.001	0.001	0.031	<0.001	0.695	0.033
	October 2014 Chicken Litter			October 2014 Turkey Litter		
	Time	Treatment	Time*Treatment	Time	Treatment	Time*Treatment
Chl *a*	<0.001	0.02	0.759	<0.001	0.107	0.33
Prim. Prod.	<0.001	0.022	0.072	<0.001	0.511	<0.001
	July 2015 River DOM					
	Time	Treatment	Time*Treatment			
Chl *a*	<0.001	0.488	0.333			
Prim. Prod.	<0.001	0.625	<0.001			

[a]Statistically significant p-values ($p < 0.05$) are highlighted in gray. The time column corresponds to differences through time, the treatment column corresponds to differences between the coupled treatments, and the time*treatment column corresponds to differences between the two coupled treatments through time.

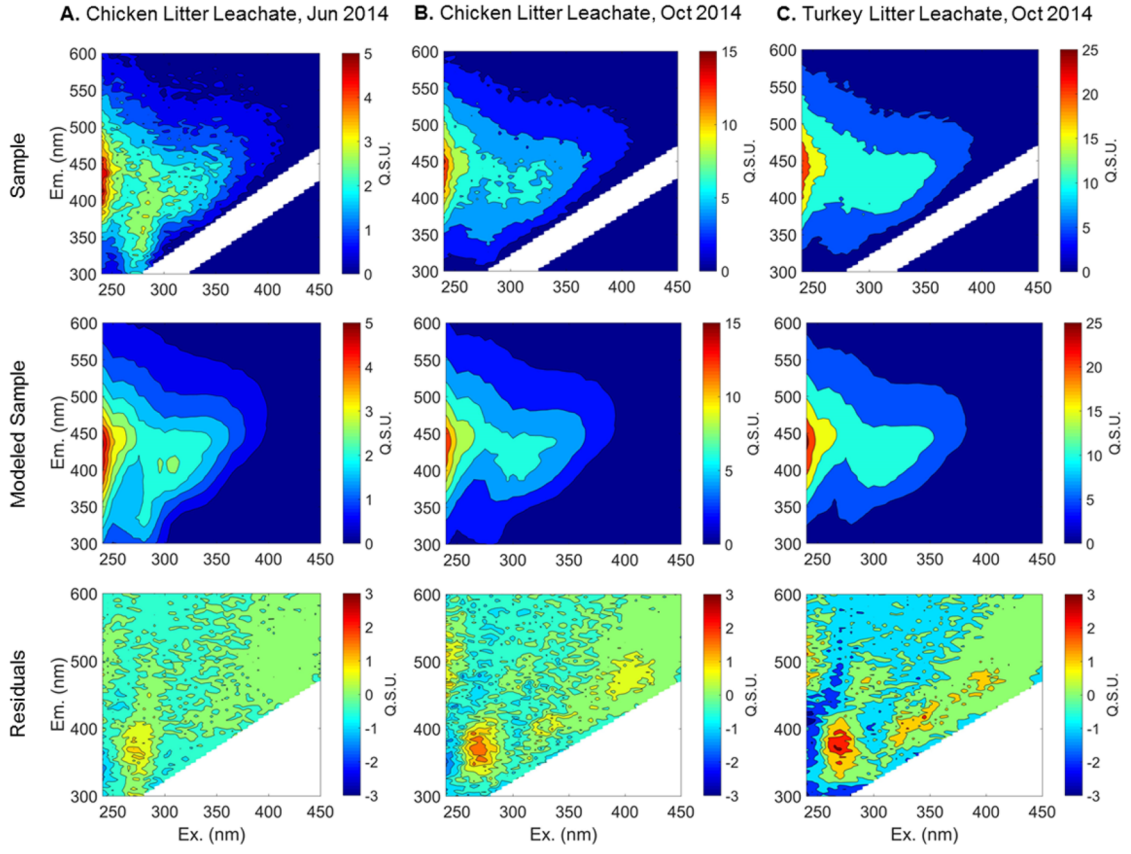

Figure 2. Sample EEM (top), PARAFAC modeled EEM (middle), and residual EEM (bottom) for DOM addition sources: A. chicken litter leachate, June 2014; B. chicken litter leachate, October 2014; and C. turkey litter leachate, October 2014. Fluorescence is plotted as Quinine Sulfate Units (Q.S.U.).

stimulated phytoplankton production; however, the DON concentrations between this DOM addition and its coupled inorganic nutrient treatment were not statistically different, mainly due to the poor replication among quadruplicates. Additional bioassay experiments should be conducted to confirm the linkages between DON, DOM sources, and the stimulation of phytoplankton production to chicken litter, but these preliminary results suggest there is a link. The variability in response to chicken litter (June 2014, October 2014) could either be a function of varying composition and nutrient/DOM

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quality of different chicken litters[23,24] or could be based on seasonal phytoplankton growth and community composition.

Source EEMs. DOM source samples (chicken litter, effluent, turkey litter, concentrated river DOM) were undersampled relative to the DOM treatments in the bioassay experiment and thus were not included in the bioassay PARAFAC model, as these samples would heavily skew modeling results.[30] The bioassay PARAFAC model was applied to the source samples during postmodeling data analysis. The residuals calculated after applying the bioassay PARAFAC model to the source samples were used to identify signals in the source samples that were different from the DOM pool in the NRE water used for incubations.[31] Results indicated that the poultry litter sources (chicken, turkey) contained fluorescence in the protein-like, biologically labile region of the EEMs that was not captured by the bioassay PARAFAC model (Figure 2). This type of fluorescence is considered biologically reactive and has been shown to decrease in fluorescence intensity during laboratory incubation studies, indicating potential uptake by phytoplankton and/or microbial assemblages.[41,42] We argue that the residual fluorescence in the chicken and turkey litter sources is responsible for stimulating primary productivity, but utilization of this DOM component may be inhibited in the turkey litter source due to the presence of contaminants (heavy/trace metals, antibiotics, pesticides, pathogens) which may inhibit phytoplankton growth as explained previously.[23,24]

The bioassay PARAFAC model was also applied to the effluent and river DOM sources (Figure S8). Residual fluorescence in the effluent sample was similar to the residual signal used in FluorMod to identify effluent DOM in the Neuse River basin[22] but distinct from the biologically active, protein region of the uncaptured fluorescence in the three litter sources (chicken litter, June 2014, October 2014; turkey litter, October 2014). For the river DOM source, the bioassay PARAFAC model captured virtually all fluorescence variability in the original sample, indicating that the Neuse River FDOM pool dominates the estuarine water used during the bioassay experiments and confirms the assertion that the bioassay PARAFAC model is dominated by estuarine FDOM signals.

FluorMod was also applied to the five source samples and the initial NRE incubation water (Figure S9). By applying FluorMod to the source samples, it is possible to calculate the relative proportion of eight previously characterized FDOM sources in the Neuse River basin (reference, effluent, wastewater treatment facility influent, poultry, swine, septic, street, soil) contained within each source and incubation water sample.[18,22] The incubation water used for all three time points was largely dominated by the reference signal, which is characteristic of background stream DOM, followed by DOM leached from soil (and possibly from riparian wetlands). Both of these signals are terrestrially derived, high in fluorescent intensity in collected river and estuarine samples, and considered conservative and refractory.[18,22] The incubation water samples also contained a small proportion of poultry litter. All three poultry litter source samples were largely dominated by the reference and poultry signal followed by smaller proportions of the effluent and soil signals. Poultry litter often contains mixtures of soil and bedding material such as wood and straw, which likely explains the presence of other source signals as modeled by FluorMod.[22] For the June 2014 chicken litter source, the sample was dominated (>50%) by the poultry litter signal. This source also represented the greatest stimulation of phytoplankton production compared to its coupled inorganic nutrient treatment.

Residual PARAFAC Model. Because the chicken litter leachates were the only DOM sources which promoted phytoplankton growth beyond the coupled inorganic nutrient treatment, a second PARAFAC model was developed based on the chicken litter treatment residuals (residual model), which were not captured by the bioassay PARAFAC model.[43] A single component was identified (Figure 3). The component was not split-half validated and did not match with any previously identified components in the OpenFluor database;[30] however, this component does appear to represent a separate FDOM class that is inherent to the chicken litter samples and is not accurately captured by the bioassay PARAFAC model. The identified component did match, visually, to the fluorescent N-peak which has been characterized as biologically labile FDOM[44,45] but is not included in the OpenFluor database. Regardless, we have isolated a fluorescence signal specific to poultry litter sources of DOM in the NRE's watershed.

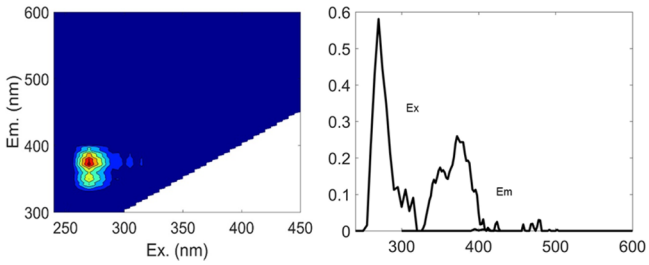

Figure 3. PARAFAC component unique to the chicken litter leachate (June 2014, October 2014) treatment samples (left) and the Ex and Em spectra (right). The component was not split-half validated and was identified as the nonhumic, biologically labile N-peak.[44,45] Excitation maximum at 270 nm; emission maximum at 372 nm.

The residual PARAFAC model was applied to the sample residuals for all bioassay treatments (chicken litter, effluent, turkey litter, river DOM, and the coupled inorganic nutrient treatments) as a tracer for the behavior of the chicken-specific DOM during the experiments (Figure 4; Table S7). The residual component was present in most inorganic nutrient addition treatments (June 2014; October 2014); we interpret this to indicate the general presence of biologically labile, poultry-derived DOM in the NRE, as previously identified by FluorMod. This signal decreased rapidly during the bioassay for both chicken litter treatments (June 2014, October 2014) each of which stimulated greater phytoplankton production than their respective inorganic nutrient treatments. The presence of the residual component in the effluent treatment indicated this signal may not be removed during wastewater treatment. Neither phytoplankton standing stock nor primary productivity was greater in the effluent treatment than its coupled inorganic nutrient treatment, which may be explained by the high inorganic N concentration in the effluent source addition and coupled inorganic nutrient treatment. It is hypothesized these high inorganic-N concentrations stimulated phytoplankton production in both the effluent and its coupled inorganic nutrient treatment greater than potential growth the residual FDOM component could stimulate (Table 1; Figure S3). These results suggest that phytoplankton and microbial assemblages may preferentially use inorganic forms of N, when present, over ON forms for growth.[46]

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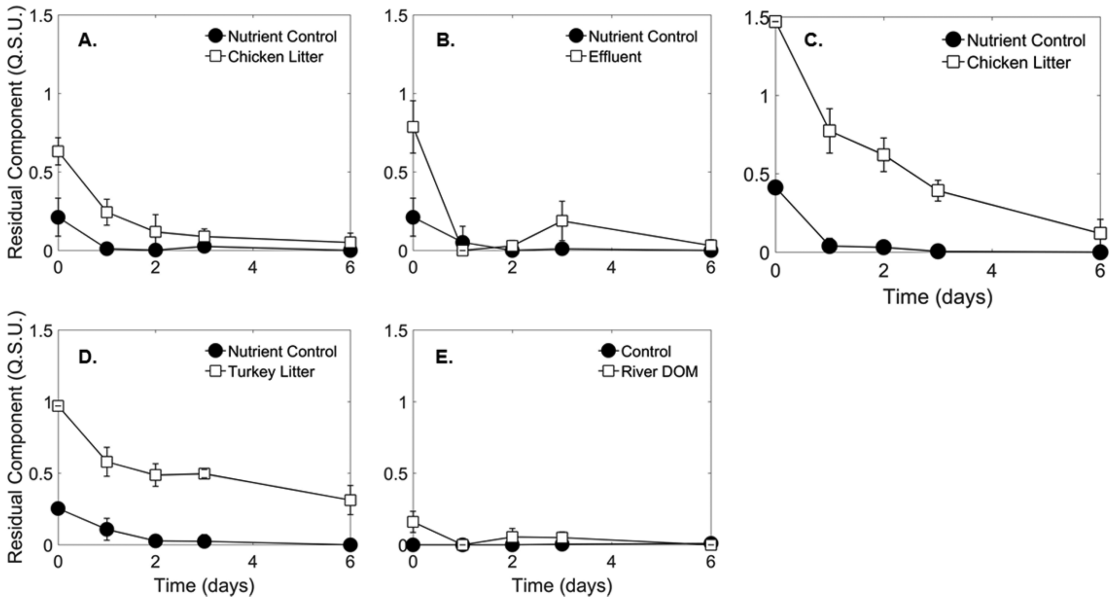

Figure 4. Residual PARAFAC component applied to bioassay fluorescence samples for A. chicken litter leachate, June 2014; B. effluent, June 2014; C. chicken litter leachate, October 2014; D. turkey litter leachate, October 2014, and E. concentrated river DOM, July 2015 plotted through the bioassay for both the respective inorganic nutrient addition (black circles) and DOM addition (white squares).

The turkey litter treatment also contained a high initial intensity of this residual fluorescence but was not completely removed during the bioassay incubation as compared to the chicken litter treatments (Figure 4). As described previously, phytoplankton growth in the turkey litter treatment appears to be inhibited by an unknown compound allowing this reactive, residual FDOM component to persist during the bioassay. The composition of poultry (chicken, turkey) litter has been shown to be variable among poultry operations and can contain a range of nutrient, trace metal, pesticide, and pathogen concentrations, making it difficult to predict a consistent impact of different poultry litters on phytoplankton production.[23,24] Despite this, the consistent phytoplankton growth responses and FDOM composition observed for two different chicken litters indicates this DOM source can be assumed to stimulate phytoplankton growth in this estuarine environment.

Implications for Estuarine Management. Bioassay results suggest DOM from chicken litter leachates stimulates phytoplankton production and standing stock greater than inorganic N (NO_x^-, NH_4^+) alone. This stimulation of growth attributed to a fluorescent component identified with PARAFAC shared spectral similarity to the N-peak region of fluorescence that has been previously identified as non-humic and biologically labile.[44,45] Chicken litter showed the greatest stimulation; the turkey litter DOM treatment appeared to have an inhibitory effect on primary production, not related to the DOM pool, while the effluent and river DOM treatments had no impact on phytoplankton production compared to their respective inorganic nutrient addition treatments.

Additional chemical analysis is warranted to link specific DOM compounds to the labile peak identified[47] to understand how and why this specific FDOM component stimulates primary production. The inhibitory effect of the turkey litter treatment also requires further study to understand the mechanism with which this treatment inhibits primary production and whether this inhibitory effect has ramifications for higher trophic levels. These results and future studies are particularly important to coastal ecosystems globally that, like the NRE, are experiencing rapid growth of poultry operations within their watersheds.[48,49] Results from this study demonstrate the need for comprehensive nutrient management plans to ensure waste products from poultry operations are properly contained and treated prior to entering a hydrologic system. Untreated waste from animal and other sources that are prevalent in the NRE[22] and other coastal watersheds is exacerbated during extreme events such as tropical storms[17,33] and has important implications for estuarine water quality. This study points to the need to account for and manage both inorganic and organic N sources to N-sensitive estuarine and coastal systems in an effort to reduce pervasive eutrophication and its negative impacts.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.7b03538.

Map of the Neuse River Estuary, NC (Figure S1), initial conditions of incubation water (Table S1), DOM source additions (Table S2), component EEMs generated during PARAFAC modeling (Figure S2), EEM component identification (Table S3), plots of inorganic nutrients (NO_x, NH_4, PO_4) plotted through each bioassay (Figure S3), plots of the five PARAFAC identified DOM components plotted through each bioassay (Figures S4, S5), RM-ANOVA results for the five identified DOM components (Table S4), plots of bacterial productivity through each bioassay (Figure S6), plots of bulk DON and DOC concentrations plotted through each bioassay (Figure S7), RM-ANOVA results for DON and DOC (Table S5), Spearman rank correlations between DON and the bioassay and residual PARAFAC components (Table S6), source residual EEM plots for the June 2014 effluent and July 2015 River DOM additions (Figure S8), results of FluorMod applied to initial incubation water and the five DOM sources used during bioassay experiments (Figure S9), and RM-

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ANOVA results for the residual EEM-PARAFAC component (Table S7) (PDF)

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Notes

The authors declare no competing financial interest.

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