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Dissolved organic carbon manipulation reveals coupled cycling of carbon, nitrogen, and phosphorus in a nitrogen-rich stream

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Abstract

To investigate the coupling between carbon (C) and phosphorus (P) cycling in a human-altered stream, we conducted a whole-ecosystem manipulation of the labile dissolved organic carbon (DOC) pool in a nitrate (NO_3^-)-rich stream in the midwestern United States. For 6 d, we increased stream DOC by ~ 1 mg L^{-1} through a continuous addition of sodium acetate. On the sixth day of the addition, ammonium (NH_4^+) was increased by ~ 130 μg N L^{-1} to examine the potential for nitrogen (N) to mediate coupled C and P cycling. Of the added DOC, 85% was retained within the treatment reach, which increased ecosystem respiration with respect to the reference reach. Alkaline phosphatase activity (APA) increased from day 1 to day 6; however, water column P uptake only increased on day 6 concurrent with the NH_4^+ addition. Gross primary production decreased during the DOC addition relative to the reference reach, yet seemed to recover on day 6 (NH_4^+ addition). These results suggest that during the DOC addition, heterotrophs out-competed autotrophs for N and that sediment-sorbed P sustained the heterotrophic community while P uptake from the water column was dominated by autotrophs. Because APA and P uptake were stimulated by the simultaneous DOC and NH_4^+ addition, P cycling appeared to be N limited, despite the high ambient NO_3^- concentration; this indicates a strong preferential uptake of NH_4^+ over NO_3^-. In streams, C and P cycling can be intrinsically coupled through biological mechanisms, and this coupling can be mediated by the availability of different forms of inorganic N.

Carbon (C), nitrogen (N), and phosphorus (P) are three of the main components of biomolecules in cells, making them essential elements for life. The structure and function of an organism dictates the ratios of C, N, and P that the organism requires for its survival, growth, and reproduction. In turn, the assimilation of each of these elements is governed by their availability in the environment and the capacity of the organism to acquire them. This elemental balance at the organismal level is often reflected in the stoichiometry of interactions and processes at the ecosystem scale (Sterner and Elser 2002) and represents a main pathway by which the global biogeochemical cycles of C, N, and P are interconnected (Finzi et al. 2011). Microorganisms are particularly important in these connections because of their capability of transforming inorganic material into biomass as well as mineralizing organic matter. Therefore, they are fundamental in the cycling of essential elements between the abiotic environment and higher trophic levels.

Predicting global responses of the C, N, and P pools to current and future environmental changes requires a clear understanding of the links between C, N, and P cycles at the microbial scale (Finzi et al. 2011). A number of anthropogenic activities, including fossil fuel burning (Hejzlar et al. 2003), land-use changes (Wilson and Xenopoulos 2008), and declining sulfur deposition (Monteith et al. 2007), have resulted in both decreases and increases in dissolved organic C (DOC) concentrations in freshwaters (Freeman et al. 2001; Driscoll et al. 2003). Alteration of DOC concentrations in aquatic ecosystems can potentially exert strong responses in P and N dynamics through changes in heterotrophic metabolic rates and consequent shifts in the inorganic nutrient demands for both assimilatory and dissimilatory processes. These changes could indirectly affect ecosystem processes such as competition, energy and nutrient transfer to higher trophic levels, and resource allocation, among others (Sinsabaugh and Foreman 2003).

A recent mesocosm study investigating the interactions between DOC and N cycling in oceans determined that heterotrophic N limitation resulted in DOC accumulation and that the increased availability of labile DOC reduced phytoplankton biomass and activity because of competition with bacteria for N (Thingstad et al. 2008). In streams, studies examining the coupling between DOC and N have found that increased labile DOC concentrations can stimulate assimilatory N demand by heterotrophs (Bernhardt and Likens 2002; Johnson et al. 2012) and consequently decrease nitrification rates as a result of competition for N between heterotrophs and nitrifiers (Bernhardt and Likens 2002). Experimentally, increased labile DOC concentration in a stream demonstrated that cycling of water column C can be decoupled from N processes like denitrification, which occurs mainly in deeper anoxic layers of the sediments (Johnson et al. 2012). Changes in DOC pools could also exert strong biological responses in the P cycle; however, there is a paucity of research that explicitly examines coupled P and DOC cycling in aquatic ecosystems (but *see* Peterson et al. 1993).

Differing from the N cycle, which is dominated by biological transformations, the P cycle is strongly affected by physicochemical processes such as sediment sorption

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(Klotz 1985) and, to a certain extent, precipitation from the water column. Sorption of P to stream sediments is regulated by a variety of factors, including the ionic strength of the stream water, pH, and the chemical composition and crystalline structure of the sediment particles (Froelich 1988). Experimental whole-stream phosphate (PO_4^{-3}) additions and laboratory assays based on sediment sorption isotherms have shown that abiotic factors play a major role in P retention in streams (Meyer 1979; Mulholland et al. 1990). The abiotic component of the P cycle provides another level of complexity to the potential mechanisms by which P cycling in streams can be linked to other elemental cycles.

To examine the extent of coupling between DOC and P cycling in streams and to investigate the mechanisms controlling such interactions, we conducted a whole-ecosystem DOC enrichment over a 6 d period in Sycamore Creek, a third-order stream in Indiana, and monitored responses in C and P cycling. To evaluate the effect of the DOC addition on the C cycle, we measured (1) whole-stream metabolism, (2) water column DOC uptake, and (3) benthic biofilm growth. To determine the effect of the DOC addition on P cycling, we examined the responses in P dynamics of three different processes: (1) direct uptake of P from the water column, (2) demand for sediment-bound P by benthic microorganisms, and (3) the abiotic role of sediments as a sink or a source of P.

According to stoichiometric relationships in ecological interactions, if an increased availability of DOC stimulated heterotrophic activity and growth, this would result in an increase in P demand. However, this response is subject to the availability of other limiting nutrients. For example, increased C availability under N-limiting conditions might not exert any changes in the P biological demand. As a result of agricultural activities in the headwaters, nitrate (NO_3^-) concentrations in Sycamore Creek average 1200 μg N L^{-1} annually. Under these conditions, we expected that the interactions between C and P cycles in this stream were not constrained by N availability. However, preferential uptake of ammonium (NH_4^+) over NO_3^- by heterotrophic and autotrophic microorganisms has been documented in aquatic ecosystems (Axler et al. 1982; Middelburg and Nieuwenhuize 2000) and is generally attributed to the lower energetic requirements of NH_4^+ assimilation. To investigate the potential influence of different forms of inorganic N on the interaction between C and P cycling, we also increased NH_4^+ concentrations in the stream on day 6 of the DOC enrichment and determined the response of the three P-related processes mentioned above.

We expected the DOC addition would stimulate heterotrophic activity in the stream and increase microbial demand for P, which would provide evidence of the biological coupling between the C and P cycles. We hypothesized that if there was no microbial N limitation or preferential NH_4^+ uptake, P uptake would increase concurrently with C uptake and exhibit no additional response to the simultaneous DOC and NH_4^+ enrichment on day 6. The results of this study will help improve the understanding of coupled DOC and P cycling in the face of differing inorganic N availability and broaden our knowledge about nutrient transport and retention in streams.

Methods

Study area and experimental design—This study was conducted in July 2010 in a third-order section of Sycamore Creek located on the Indiana University Research and Teaching Preserve (39°32′N, 86°26′W, Morgan County, Indiana). Land use in the 35.5 km^2 watershed includes row-crop agriculture and pasture (47%) in the headwaters and forest (46%) and low-density urban development (7%) in the lower portion of the basin (Engel unpubl.). The watershed is located over the Mississippian geologic formation, dominated by siltstone (Indiana Geological Survey 2012). At the study site, discharge averaged 150 L s^{-1} from June 2010 to November 2010, and during that period, the maximum discharge was 1078 L s^{-1}. Throughout 2010, NH_4^+–N concentrations were ≤ 10 μg L^{-1} for most of the year. In the case of NO_3^-–N, concentrations varied from 0.3 to 1.9 mg L^{-1}, whereas DOC averaged 2.4 mg L^{-1} and peaked at 4.2 mg L^{-1} in November.

For the whole-stream manipulation, we delineated a treatment (455 m) and a reference (225 m) reach based on 1 h travel times; 10 sampling stations were distributed within each reach. The downstream end of the reference reach was located $\sim$ 20 m upstream from the beginning of the treatment reach. Travel time was measured preceding the enrichment as the time-to-peak conductivity of a slug addition of a saturated sodium chloride (NaCl) solution (36% mass : volume). In our study, we quantified direct uptake of water column PO_4^{-3} by conducting short-term P releases (Ensign and Doyle 2006) on the basis of the nutrient spiraling model (Stream Solute Workshop 1990). P uptake derived from this model reflects a combination of biological P uptake and physicochemical adsorption. To examine biological P demand specifically, we measured alkaline phosphatase activity (APA) in the sediments. Phosphatases are ecto- and extracellular enzymes secreted by bacteria, algae, and fungi to hydrolyze PO_4^{-3} groups from organic matter and minerals in the sediments, and their production is induced under conditions of P limitation (Chróst 1991). The potential effects of the DOC addition on the physicochemical P sorption by sediments were quantified through adsorption isotherms. This technique determines the equilibrium P concentration at zero release or retention (EPC_0), which is an indicator of the buffering capacity of the sediments with respect to the concentration of P in solution (Froelich 1988).

Carbon and ammonium enrichment—A solution of sodium acetate trihydrate ($NaCH_3CO_2 \cdot 3H_2O$) and sodium bromide (NaBr) was delivered to the stream at the beginning of the treatment reach continuously for 6 d (14–19 July 2010). We used a Masterflex E/S portable pump (Cole-Parmer) to add the solution at a constant rate (150 mL min^{-1}) to elevate stream DOC concentrations $\sim$ 1 mg L^{-1} above ambient concentrations. During the

sixth day of the addition (19 July 2010), we also added NH_4^+ simultaneously with C for a period of 8 h (10:00 h to 18:00 h). For this purpose, ammonium chloride (NH_4Cl) was added to the acetate solution to elevate NH_4^+–N concentration in the stream to $\sim$ 200 μg L^{-1}. Water samples for DOC, acetate-C, NH_4^+–N, NO_3^-–N, soluble reactive phosphorus (SRP), and bromide (Br^-) concentrations were collected before (day −1, 13 July 2010), during (days 1–6), and after (day 14, 27 July 2010) the DOC enrichment. Samples were collected from the thalweg at each station of the treatment reach and the end of the reference reach (*see* the Analytical chemistry and quality control section below for a detailed description of sample collection and processing). Adding Br^- as a hydrologic tracer in conjunction with the acetate allowed us to calculate daily acetate-C uptake in the treatment reach.

Whole-stream metabolism and biofilm growth—To examine the influence of the manipulation on ecosystem metabolism, we measured whole-reach ecosystem respiration (ER) and gross primary production (GPP) using the one-station open channel method (Owens 1974). Dissolved oxygen (DO) concentrations and stream temperature were measured at 10 min intervals with DO optical probes (Yellow Springs Instruments 600OMS-O) located at the downstream end of the reference and treatment reaches (475 m apart). Because of logistic constraints, the probes were not deployed until day 2 (15 July 2010). Before deployment the probes were calibrated in the field using the one-point saturated air procedure and kept together in the stream for 1 h to confirm that readings were consistent between the two meters. Probes were withdrawn from the stream on 23 July 2010. Photosynthetically active radiation sensors (Odyssey) were deployed at the same time and locations as the DO probes. Dissolved oxygen fluxes were corrected for atmospheric exchange with a reaeration coefficient (k_r) calculated using the steady state tracer gas method with sulfur hexafluoride (SF_6) as the tracer gas (Wanninkhof et al. 1990; Marzolf et al. 1994). The SF_6 injections were conducted in the treatment reach on day 3 and day 14 of the study period. The O_2 reaeration coefficient, kO_2 was calculated from kSF_6 using the ratio of their Schmidt numbers (1.345): $kO_2 = kSF_6 \times 1.345$ (Wanninkhof et al. 1990). We transformed respiration rates from O_2 to C on the basis of the stoichiometric ratios of the photosynthesis reaction and multiplied by a respiratory quotient of 0.85 (the number of moles of CO_2 released per mole of O_2 consumed). To examine the effect of the treatment on metabolism, we calculated the difference in GPP and ER between the treatment and the reference reach, relative to the reference reach (ΔGPP and ΔER, respectively).

We monitored benthic biofilm growth by measuring ash-free dry weight (AF dry wt) accumulation on artificial substrata placed on the streambed at the beginning of the manipulation. We deployed three groups of three ceramic tiles along two cross sections of the stream located $\sim$ 100 m upstream and $\sim$ 50 m downstream of the addition point. All ceramic tiles were collected on day 5, and biofilm was carefully scrubbed and transferred into opaque jars for organic content analysis. In the laboratory, known volumes of the slurry were filtered using preweighed filters (0.7 μm nominal pore size), dried at 60°C for quantification of dry mass and oxidized in a muffle furnace at 550°C to calculate ash-free dry weight.

Phosphorus and acetate-C uptake—Phosphorus uptake was determined using short-term PO_4^{-3} releases (Stream Solute Workshop 1990) in both the treatment and reference reach, on days 1, 3, 5, and 6 of the DOC addition as well as before (day −4) and after (day 14) the DOC addition. Concentrated solutions of sodium phosphate (Na_3PO_4) were delivered to the stream using a QB2 pump (Fluid Metering Inc.) at a rate of 50 mL min^{-1}. NaBr was used as a hydrologic tracer. We targeted a concentration of SRP of 20 μg L^{-1} in the stream (background concentrations averaged 5 μg L^{-1}) and either 10 or 40 μg L^{-1} of Br^- for each release. Once steady state was reached ($\sim$ 1 h), samples were collected from the thalweg at each station in the reach. Water samples were also collected at each station of the treatment and reference reaches before each short-term release to determine ambient concentrations of SRP and Br^-. The PO_4^{-3} releases were conducted at approximately the same time each day, and in the case of day 6, $>$ 2 h after starting the NH_4^+ enrichment.

To calculate PO_4^{-3} uptake, concentrations of SRP above ambient were normalized by Br^- concentrations to correct for dilution and dispersion. Corrected SRP concentrations were natural log–transformed and regressed as a function of distance from the injection point. The slope of this regression corresponds to k_s, the first-order rate constant. Only slopes significantly different from zero (simple linear regression, $p < 0.05$) were considered to have measurable PO_4^{-3} uptake. When k_s was significant, we calculated uptake length (S_w), uptake velocity (v_f), and areal uptake rates (U) according to the Stream Solute Workshop (1990). Acetate-C uptake parameters were also calculated following the model described above. In this case, acetate-C concentrations used to determine S_w were obtained from the 10 longitudinal samples collected daily and corrected for dilution and dispersion using Br^- concentrations in the same samples. Added acetate-C areal uptake rate (U_{add}) was calculated as the product of acetate-C v_f and the geometric mean of background-corrected acetate-C concentrations of longitudinal samples collected across the treatment reach.

Alkaline phosphatase activity and P adsorption measurements—On days −1, 1, 3, 5, 6, and 14 of the experiment, three sediments cores (3 cm diameter and 1 cm deep) were collected from three points across the stream at Sta. 4 of the treatment reach and Sta. 5 of the reference reach. These stations were located 125 m downstream, and 170 m upstream of the addition point, respectively. Cores were sieved (2 mm) and composited to give three analytical replicates per station. The sediments were immediately transported to the laboratory on ice and subsampled for adsorption isotherms, ash-free dry weight, and APA. The subsamples for APA were stored at −80°C until laboratory analysis (approximately 2 wk after collection), and the

subsamples for ash-free dry weight and EPC_0 were processed immediately. The sediment collection was carried out at approximately the same time each day and always before the PO_4^{-3} short-term releases.

Alkaline phosphatase activity was determined following standard techniques (Sayler et al. 1979). Briefly, *p*-nitrophenyl phosphate (*p*-NPP) was exposed to phosphatases in the sediments, which hydrolyze the PO_4^{-3} group. The products of the hydrolysis are PO_4^{-3} and *p*-nitrophenol (*p*-NP), a colored compound at an alkaline pH that can be quantified spectrophotometrically. For the laboratory assay, known amounts of sediment were added to a buffering solution (Tris, pH 8.6) containing *p*-NPP and mixed with a vortex mixer for 10 s. Samples were incubated at 37°C for 1 h. The hydrolysis reaction was stopped by raising the pH above 11, and the samples were mixed and centrifuged. Concentration of *p*-NP in the supernatant was determined as a function of absorbance.

We measured EPC_0 through sediment SRP adsorption isotherms. A series of five potassium dihydrogen phosphate (KH_2PO_4) equilibrium solutions (0–1000 μg P L^{-1}) were prepared using fresh stream water. For each sediment sample, 40 mL of equilibrium solution was added to 5 mL of wet sediment in a 50 mL centrifuge tube. Samples were shaken for 14 h and centrifuged for 10 min at 500 × *g*. The supernatant solution was filtered through a 0.45 μm membrane and analyzed for SRP. EPC_0 was calculated following McDaniel et al. (2009). In brief, SRP in solution after 14 h of equilibration (normalized to sediment mass) was regressed against the initial concentration of SRP in solution (normalized to sediment mass). The regression was fitted to a logarithmic function and the *x*-axis intercept of this function represents the SRP concentration at which there is negligible P adsorption or release from the sediments to the solution (EPC_0). Values of $EPC_0 >$ SRP concentration in the stream water indicate that the sediments are a source of P, whereas values of $EPC_0 <$ SRP indicate that the sediments act as a P sink (Froelich 1988). Sediments were not sterilized; however, studies have shown biotic uptake in adsorption isotherms is negligible (Meyer 1979), especially in sediments of 1 mm particle size or less (Lottig and Stanley 2007).

Analytical chemistry and quality control—All water samples were filtered on site into 60 mL high-density polyethylene Nalgene bottles using Whatman glass fiber filters (0.7 μm nominal pore size) for DOC, and Millipore membrane filters (0.45 μm nominal pore size) for acetate-C, NH_4^+–N, NO_3^-–N, Br^-, and SRP. Samples were kept on ice and transported to the laboratory, where they were frozen until analysis. A Lachat QuikChem 8500 Flow Injection Analysis system (Hach Company) was used to measure SRP, NO_3^-–N, and NH_4^+–N using QuickChem methods 10-115-01-1-Q (molybdate blue complex, method detection limit [MDL] = 5 μg P L^{-1}), 10-107-04-1-A (cadmium reduction and ethylenediaminetetraacetic acid red complex, MDL = 2.5 μg N L^{-1}), and 10-107-06-1-J (indophenol blue complex, MDL = 10 μg N L^{-1}), respectively. DOC was determined via high-temperature oxidation using a Shimadzu total organic carbon-CPN analyzer (MDL = 150 μg C L^{-1}). Acetate–C and Br^- concentrations were measured on a Dionex ICS-2000 Ion Chromatograph with an AS11-HC analytical column (MDL = 10 μg C L^{-1} and 8 μg Br^- L^{-1}). Absorbance of *p*-NP was measured using a Shimadzu UV-2101PC spectrophotometer. SF_6 was measured on a 450GC-Varian gas chromatograph using an electron capture detector. For all chemical analyses, commercial standards of known concentration were measured every 10 samples to ensure the accuracy of the standard curves and correct for instrument drift. For all analytes, we collected field duplicates and ran analytical replicates to confirm reproducibility of the sampling and analysis methodology.

Statistical analyses—Statistical significance in our study was determined at $\alpha = 0.05$, and data normality was tested using the Shapiro–Wilk test; statistical analyses were conducted using Sigma Plot 11.0 (Systat Software). To identify significant effects of the DOC addition on APA and EPC_0, we used two-way analysis of variance (ANOVA) with reach (treatment vs. reference) and day as the main factors followed by Tukey's multiple comparisons test. We used a *t*-test to examine differences in the organic matter content on artificial tiles located in the reference and treatment reach. Differences in the slope of the uptake regressions (k_s) in the treatment reach among days were examined through analysis of covariance (ANCOVA) followed by a Tukey's multiple comparisons test of the slopes (Zar, 1999).

Results

General physicochemical parameters—During DOC enrichment, discharge varied from 88 to 329 L s^{-1} (Fig. 1A), averaging 164 L s^{-1}. Nighttime rain events during days 2 and 3 caused large but brief increases in stream flow; these changes were minor compared with the annual variation in the hydrograph, and they did not result in flood events. We did not observe any changes in stream width or depth during the daytime. Throughout the study period, reference concentrations of NH_4^+–N and SRP were low (mean = 15, 5 μg L^{-1}, respectively; Table 1), and mean reference NO_3^-–N and DOC concentrations were 1.1 and 2.3 mg L^{-1}, respectively (Table 1). Average DO concentrations were lower in the treatment reach than in the reference reach on days 4, 5, and 6, whereas on days 2, 3, and 14, DO concentrations in the treatment reach were similar to the reference reach (Table 1). In the treatment and the reference reach, pH averaged 8.0 across days −1 to 6, and there was little variation among days or reaches. SF_6 reaeration coefficients (kSF_6) were 0.0011 m^{-1} and 0.0009 m^{-1} on day 3 and day 14, respectively. We calculated metabolism parameters for all days of the experiment using the value obtained on day 3 because discharge on day 3 was most similar to discharge on days 2–10 (Table 1).

Alteration of the C cycle—On average, we increased DOC concentration by 72% over ambient concentrations throughout the enrichment. Concentration of DOC in the

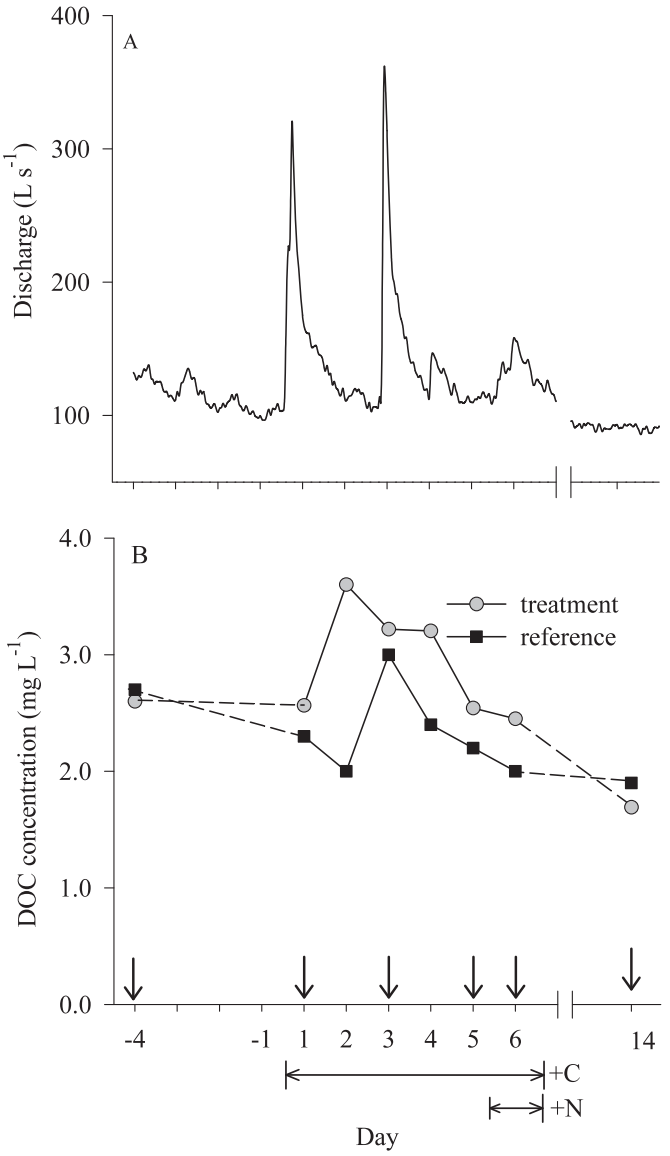

Fig. 1. (A) Discharge in Sycamore Creek and (B) DOC concentration in the treatment and reference reach during the experiment. In (B), reference reach DOC corresponds to samples collected at the most downstream station, and treatment reach DOC represents the average concentration of the 10 stations in the reach. Arrows indicate the days when PO_4^{-3} uptake, APA, and EPC_0 were measured.

stream immediately before the DOC addition was 2.6 mg L^{-1} and was increased to an average of 2.4–3.6 mg L^{-1} in the treatment reach during the DOC addition (Fig. 1B). Each day of the DOC addition, acetate-C concentrations decreased longitudinally from the addition point; however, on day 3, acetate-C k_s was not statistically significant (Table 2). Acetate–C v_f on day 1 was 0.4 mm min^{-1} and increased throughout the experiment to 3.8 mm min^{-1} on day 6 during the simultaneous C and NH_4^+ addition (Fig. 2). Similar to v_f, acetate-C U_{add}, which represents the areal uptake of the added acetate-C, showed a general increase throughout the experiment, but it decreased slightly on day 6 (Table 2). The fast uptake

Table 1. Daily average discharge, temperature and NO_3^-–N, SRP, NH_4^+–N, and DOC concentrations measured in the treatment and the reference reach during the study period. Temperature and chemical parameters represent the average of the 10 sampling stations throughout the treatment reach and the furthest downstream station of the reference reach. (bd, below detection; nd, not determined).

			Reference						Treatment					
Day	Solute added	Average discharge (L s^{-1})	DO (mg L^{-1})	T (°C)	NO_3^-–N (mg L^{-1})	SRP (μg L^{-1})	NH_4^+–N (μg L^{-1})	DOC (mg L^{-1})	DO (mg L^{-1})	T (°C)	NO_3^-–N (mg L^{-1})	SRP (μg L^{-1})	NH_4^+–N (μg L^{-1})	DOC (mg L^{-1})
−1	None	154	nd	21.5	1.18	4	32	nd	nd	21.5	1.18	4	18	nd
1	+C	138	nd	26.1	0.99	6	42	2.3	nd	25.5	1.02	6	13	3.6
2	+C	138	8.9	22.7	1.09	nd	21	2.0	8.8	22.5	1.03	bd	12	3.2
3	+C	165	7.1	23.1	1.00	7	bd	3.0	7.1	23.0	1.01	6	5	3.9
4	+C	123	6.4	23.4	1.02	2	bd	2.4	5.6	23.0	0.95	bd	10	3.2
5	+C	122	6.4	21.6	1.01	5	bd	2.2	5.5	21.6	0.96	4	12	2.5
6	+C+NH_4^+	131	6.8	22.0	1.18	4	bd	2.0	5.9	22.0	1.16	4	146	2.5
14	None	91	7.9	22.7	1.28	7	bd	1.9	7.9	22.5	1.30	11	25	1.7

Table 2. Photosynthetically active radiation (PAR), whole-stream metabolism, and added acetate-C uptake rates (U_{add}) measured throughout the experiment. ΔGPP and ΔER were calculated as the arithmetic difference between the treatment and the reference reach relative to the reference reach. (nd, not determined; ns, k_s was not significant at the 95% confidence level).

			Metabolism (g O_2 m^{-2} d^{-1})						Acetate-C uptake
			Reference reach		Treatment reach				
Day	Manipulation	PAR (mol m^{-2} d^{-1})	GPP	ER	GPP	ER	ΔER (%)	ΔGPP (%)	U_{add} (mg m^{-2} d^{-1})
1	+C	nd	nd	nd	nd	nd	nd	nd	765
2	+C	3.1	5.2	−6.9	5.2	−7.2	4	0	2415
3	+C	5.5	5.9	−8.3	4.1	−8.7	5	−31	ns
4	+C	3.4	3.1	−4.5	2.3	−5.3	18	−26	3369
5	+C	1.9	3.1	−4.7	2.4	−5.8	23	−23	3650
6	+C+NH_4^+	4.4	2.2	−3.6	2.0	−4.6	28	−9	3001
7	None	2.9	3.7	−5.8	3.4	−5.5	−5	−8	
8	None	5.9	3.5	−5.2	3.3	−5.0	−4	−6	
9	None	4.5	3.8	−5.7	3.6	−5.5	−4	−5	
10	None	6.3	2.9	−4.5	2.9	−4.5	0	0	

rates of acetate-C on day 6 resulted in low average ambient acetate-C concentrations and, in turn, lower values of U_{add}.

On day 2, GPP in the treatment reach was equal to the reference reach (Table 2); therefore, ΔGPP was 0%. During DOC enrichment, ΔGPP decreased, averaging −27% (days 3–5) and increased to −9% on day 6 (Table 2). This indicates that DOC enrichment decreased GPP in the treatment reach, but it partially recovered during the simultaneous DOC–NH_4^+ addition on day 6. ΔGPP continued to increase after DOC addition was terminated and was 0% on day 10 (Table 2). ER was always higher in the treatment reach during DOC addition; thus, ΔER was positive and increased gradually from day 2 to day 6. After DOC addition ended, ER in the treatment reach was lower than or equal to that in the reference reach; therefore, on the days after the manipulation, ΔER was negative or 0%

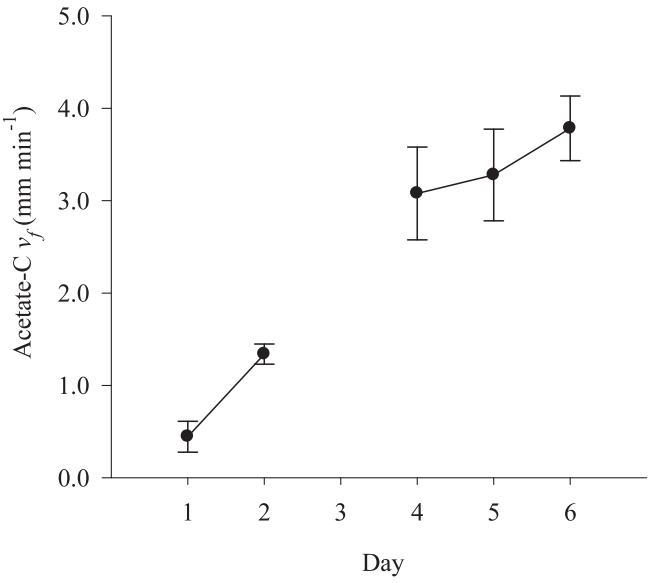

Fig. 2. Acetate-C uptake velocity (v_f) in the treatment reach. No significant acetate-C uptake was measured on day 3. Error bars represent standard error (SE) of the uptake regression model propagated through the calculation of v_f.

(Table 2). Organic matter on ceramic tiles deployed to measure new biofilm growth in the treatment reach was significantly higher (39 μg AF dry wt m^{-2}) than the reference reach (16 μg AF dry wt m^{-2}; *t*-test, $t = 6.3$, df = 10, $p < 0.05$). Furthermore, growth of the bacterium *Sphaerotilus* was observed in the treatment reach after day 4 of the DOC addition. On the basis of a semi-quantitative evaluation of streambed cover carried out on day 6, *Sphaerotilus* filaments occupied 20% of the treatment reach area and were not observed in the reference reach.

Responses in the P cycle to the modification of the C and N pool—On days −4, 1, and 3, APA averaged 16 μmol P (g AF dry wt)$^{-1}$ h^{-1} in both the reference and the treatment reach, respectively, with no significant differences among reaches. In the treatment reach, APA increased consistently throughout the DOC enrichment, with the highest activity on day 6 (53 μmol P [g AF dry wt]$^{-1}$ h^{-1}; Fig. 3A). On days 5 and 6, APA was significantly higher in the treatment reach than in the reference reach (two-way ANOVA, $F_{1,33} = 13.8$; followed by Tukey test; $p < 0.05$).

On days 3, 5, and 6, P uptake for the reference reach was not quantifiable because added PO_4^{-3} did not show significant longitudinal declines (k_s was not significantly different from zero; Table 3). On the remaining days, PO_4^{-3} v_f in the reference reach ranged from 1.9 mm min^{-1} on day 14 to 4.5 mm min^{-1} on day −4 (Fig. 3B). In the treatment reach, PO_4^{-3} v_f was higher on days 1 and 3 (4.5 and 3.8 mm min^{-1}, respectively) than before (day −4) and after (day 14) the DOC enrichment (3.1 and 2.1 mm min^{-1}, respectively; Fig. 3B), and it decreased to 2.1 mm min^{-1} on day 5. Additionally, PO_4^{-3} v_f was highest on day 6 (10.2 mm min^{-1}), the day of the simultaneous DOC-NH_4^+ enrichment. This uptake was more than two times faster than the results observed for any other day, in either the treatment or reference reach (Fig. 3B). The corresponding uptake length was 70 m (Table 3) and was significantly shorter than all other days in the treatment or reference reach (ANCOVA, $F_{5,33} = 8.9$, $p < 0.05$).

EPC_0 in the treatment reach was not significantly different from the reference reach and did not change with

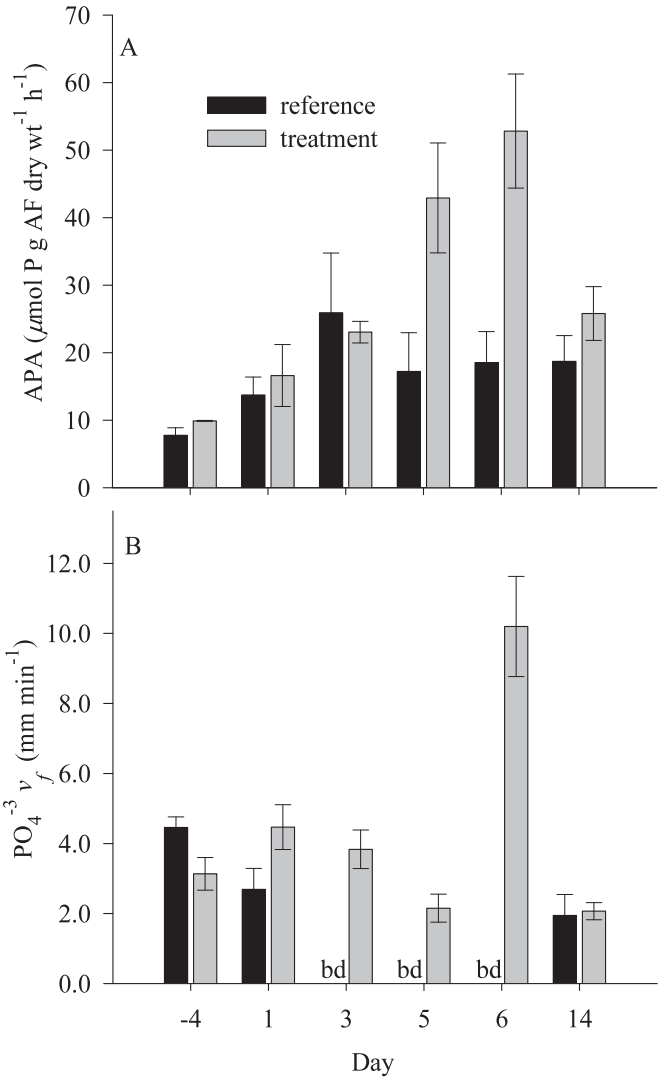

Fig. 3. (A) Average alkaline phosphatase activity (APA) in the treatment and the reference reach. Error bars represent SE ($n = 3$). (B) Phosphate uptake velocity (v_f) in the treatment and reference reach. Error bars represent SE of the uptake regression model propagated through the calculation of v_f (bd, below detection; nonsignificant PO_4^{-3} k_s at the 95% confidence level).

the DOC or NH_4^+ enrichments (two-way ANOVA, $F_{1,33} = 0.14$, $p > 0.05$). Across both reaches, EPC_0 ranged from 2 to 6 μg P L^{-1} (Fig. 4). Sediments in both the treatment and the reference reach were primarily a sink for P ($EPC_0 <$ SRP; Fig. 4). In some cases EPC_0 was very similar to the water column SRP concentration, indicating equilibrium conditions in which sediments presented no net adsorption or desorption of P.

Discussion

Alteration of the C cycle—The DOC quantity in Sycamore Creek was successfully modified by adding a total of 89 kg of C (1006 kg of $NaCH_3CO_2 \cdot 3H_2O$) over a 6 d period. Of the added C, 85% (74 kg) was taken up in the treatment reach, with the remainder exported downstream, and based on whole stream metabolism, ~ 22% (20 kg) was aerobically respired. Using the arithmetic difference between the C that was taken up and the C that was respired, we estimate that 63% of the added C was directed into assimilatory uptake and potentially anaerobic respiration. The level of C enrichment varied slightly among days because of changes in discharge during the study period, particularly the nighttime of days 1 and 2 (Fig. 1). The lower acetate-C concentrations on day 3 possibly hindered the quantification of acetate-C uptake parameters for that day (Fig. 2).

Because reaeration depends largely on discharge, we evaluated the effect of the increased flow on metabolism measurements. For this, we calculated instantaneous kO_2 on the basis of the surface renewal method (Owens 1974) and compared the results with those obtained using the kO_2 derived from the SF_6 injections. We determined that although absolute values of GPP and ER differed moderately among methods during the nights of days 2 and 3, the trends observed in ΔGPP and ΔER were not different from those calculated using a fixed value of kSF_6. However, the effective reach integrated by the DO sensors increased during the night of day 3. This means that the sensor in the treatment reach integrated DO dynamics over a larger stream segment, including a portion of the reference reach. This might explain why ΔER on days 2 and 3 was not as high as it was observed for days 4–6 and, similarly, why ΔGPP on day 2 was not as low as it was on days 4 and 5.

Table 3. Spiraling metrics for PO_4^{-3} uptake in the treatment and the reference reach during the experiment. The superscripts in S_w for the treatment reach indicate the significant differences identified in Tukey's multiple comparison test (ns, not significant at the 95% confidence level). U on days 3, 5, and 6 cannot be calculated because the corresponding S_w are not significant.

		PO_4^{-3} uptake			
		Treatment		Reference	
Day	Manipulation	S_w (m)	U (mg m^{-2} d^{-1})	S_w (m)	U (mg m^{-2} d^{-1})
−4	None	227^{bcd}	24	160	39
1	+C	175^{b}	40	293	19
3	+C	330^{bc}	32	ns	
5	+C	301^{d}	12	ns	
6	+C+NH_4^+	70^{a}	61	ns	
14	None	248^{cd}	33	264	23

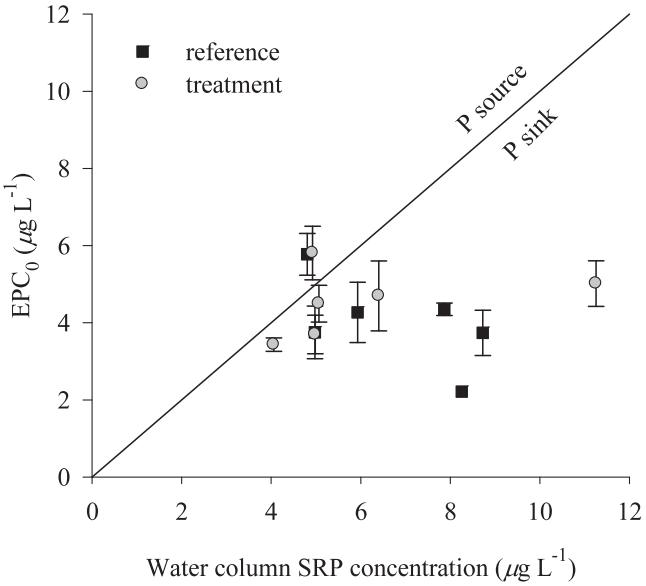

Fig. 4. Equilibrium phosphorus concentrations at zero release or retention (EPC_0) as a function of water column SRP concentration. The 1 : 1 line represents no net release or retention of P by the stream sediments. Error bars represent SE ($n = 3$).

We observed extensive mats of *Sphaerotilus* on the streambed of the treatment reach after day 4 and a significant increase in biofilm ash-free dry weight on the artificial substrata. Our results are consistent with other studies that have altered DOC availability in streams. Bernhardt and Likens (2002) and Johnson et al. (2012) found that acetate-C enrichments resulted in increased bacterial growth and assimilatory demand for N. A consistent response among these studies is the growth of *Sphaerotilus* in the enriched sites. This microorganism is a filamentous sheath-forming bacterium frequently found in organically enriched water, likely because of its ability to degrade a great variety of organic compounds (Madigan 2000). It is clear that the 6 d modification of the C pool stimulated the growth and activity of the benthic microbial community, and at the time of the experiment, the heterotrophic community appeared C limited, suggesting that an increase in biologic P demand should be expected in the treatment reach.

On the basis of an average molar C : N : P ratio for stream benthic bacterial biomass of 75 : 12 : 1 (Cross et al. 2005), the concentrations of P and inorganic N in the stream water necessary to satisfy P and N demand during DOC enrichment ranged from 18 to 33 μg P L^{-1} and from 52 to 93 μg N L^{-1}. To calculate these values we assumed negligible anaerobic respiration of the added carbon and used daily rates of assimilatory uptake and the daily average concentration of acetate-C in the treatment reach. These values confirm that the high NO_3^-–N concentration in the stream (Table 1) should have satisfied the inorganic N demand caused by the DOC enrichment. On the other hand, the concentration of SRP during the experiment averaged only 5 μg L^{-1}, and the increased P demand resulting from the DOC enrichment could not have been satisfied by the P available in the water column.

Coupled C and P cycling—With the exception of days 5 and 6 in the treatment reach, results of APA during our experiment were comparable to those reported by Tank et al. (1998), which ranged from 7 to 23 μmol P (g AF dry wt)$^{-1}$ h^{-1}; these values correspond to phosphatase activity measured in stream wood biofilms using *p*-NPP as substrate (pH 5). Higher values of APA, ranging from 140 to 350 μmol P (g AF dry wt)$^{-1}$ h^{-1}, were reported by Bonin et al. (2000) for stream fine benthic organic matter. These comparisons need to be taken with caution because the studies used slightly different protocols. Particularly important are pH conditions, which can affect enzymatic activity significantly (Chróst, 1991). For the same reason, in our study, differences in pH between the stream water (pH 8.0) and the buffer used in the APA bioassays (pH 8.6) suggest that measured APA does not necessarily reflect in situ APA in stream sediments.

As we predicted, the increase in both acetate-C v_f and APA in the treatment reach suggests that higher C assimilation enhanced biological P demand through a potential shift in the limiting factor from C to P, which stimulated the production of APA. Changes in allocation of resources by microorganisms to acquire the limiting element are common in aquatic and terrestrial ecosystems (Sinsabaugh and Follstad Shah 2012). Specifically for phosphatases, laboratory studies have reported increased phosphatase activity in freshwater amended with C (Stewart et al. 1982), and simultaneous C and N amendments to soil (Allison and Vitousek 2005) and seawater (Nausch and Nausch 2004). In streams, in situ manipulations of DOC pools have been conducted previously (Bernhardt and Likens 2002; Johnson et al. 2012), but to our knowledge, this is the first study of this type to document responses in phosphatase activity.

Although APA in the treatment reach showed a continuous increase through the C addition, it was only significantly higher than the reference reach on days 4 and 5. It is not clear whether the response in APA was delayed or simply undetectable on days 1 and 3. In bioassays, seawater amended with glucose and NH_4^+ did not show an increase in APA until the third day of incubation (Nausch and Nausch 2004). In contrast, the effect of acetate-C additions on aminopeptidase activity (N-acquiring extracellular enzymes) in lake water bioassays was observable within 20 h of incubation (Chróst, 1991). Because our study consisted of a whole-ecosystem manipulation, it is difficult to compare our results with those reported in studies using bioassays. Nonetheless, the significant increase in APA on days 4 and 5 provides evidence for coupling between the C and P cycles in streams through mechanisms that involve utilization of particulate organic P or mineral-sorbed P.

With the exception of the remarkably high PO_4^{-3} v_f value observed on day 6 in the treatment reach, values of PO_4^{-3} v_f measured throughout the experiment fall within the interquartile range (0.02–0.12 mm s^{-1}) reported for 21 third-order streams by Ensign and Doyle (2006). A number of reasons could have caused undetectable uptake of P from the water column in the reference reach on days 3, 5, or 6. For example, the reference reach had a braided channel, and we might not have sampled from the thalweg

at all stations. Despite the increased C uptake in the treatment reach, we did not observe an increase in water column P uptake on days 1–5, indicating that the DOC addition did not stimulate demand for P from the stream water. Thus, the P uptake measured over days 1–5 was likely the result of processes that were not directly affected by the increased DOC, for example abiotic P adsorption, P demand by autotrophs, or heterotrophic organisms unable to use acetate as a source of C.

Studies employing the nutrient spiraling model have found that biological uptake of P can be influenced by the relative importance of heterotrophic vs. autotrophic demand (Bernot et al. 2006; Hoellein et al. 2007). It is possible that the DOC addition actually decreased autotrophic P demand via increased competition with heterotrophs for N, resulting in the observed decrease in ΔGPP after day 2. In support of reduced autotrophic activity, an apparent decrease in PO_4^{-3} v_f was observed in the treatment reach from day 1 to 5. However, the magnitude of the decrease in ΔGPP was rather large and was observed since day 2 (Table 2), whereas changes in PO_4^{-3} v_f appeared to be gradual (Fig. 1B). On day 6, when the DOC enrichment was combined with an enrichment in NH_4^+, ΔGPP increased, and GPP in the treatment reach was similar to the reference reach. From this we conclude that the increased availability of NH_4^+ alleviated possible N limitation and released the autotrophs from competition with heterotrophs for inorganic N. Additionally, uptake of P from the water column was much higher on day 6 than any other day of the experiment, clearly a result of biological demand, because abiotic processes are unlikely to be affected by a short-term increase in NH_4^+ concentration.

Despite the recovery in GPP on day 6, it is not clear whether the increase in uptake of water column P on day 6 was entirely due to photoautotrophic production because GPP in the treatment reach did not exceed that of the reference reach (Table 2). The NH_4^+ addition could have stimulated chemoautotrophic communities such as nitrifying bacteria that would contribute to increasing water column P uptake but do not perform photosynthesis. Furthermore, a variety of studies in streams have documented luxury P uptake by autotrophs (Grimm and Fisher 1988), which would not be strictly reflected in primary productivity. It is also unclear whether the NH_4^+ enrichment affected APA directly or if the continuous increase in APA through day 6 was simply a further response to the DOC addition. On days 1–5, autotrophic P demand seemed to be supported by water column SRP, while the organic P pool in the sediments supported the heterotrophic P demand triggered by the increased DOC availability. These results suggest that SRP concentrations do not necessarily reflect P limitation of the benthos, which agrees with Klotz (1985) and Hill et al. (2010), who found that SRP was not a major factor controlling APA.

EPC_0 measured throughout the experiment, in both the treatment and the reference reach, was generally low (2–6 μg P L^{-1}) compared with values reported in the literature for agricultural midwestern stream sediments (median, minimum–maximum: 83, 1–1180 μg P L^{-1}), possibly as a result of lower organic matter content in the sediments relative to the sites studied by McDaniel et al. (2009). At the time of the experiment, Sycamore Creek sediments were primarily a sink for P, which means that sediments had not reached saturation. Thus, as mentioned before, it is feasible that a fraction of the P uptake measured through PO_4^{-3} releases was due to abiotic adsorption, as has been documented in other studies in streams (Mulholland et al. 1990; Meals et al. 1999). The whole-stream manipulation of the C and N pools did not have a significant effect on EPC_0, indicating that changes in the P cycling in the treatment reach as a response to the C and N additions can be attributed entirely to biological processes.

Response to different forms of inorganic N—The highest PO_4^{-3} U and APA were documented during the NH_4^+ addition on day 6. These results indicate that a preferential uptake of NH_4^+ over NO_3^- occurred in Sycamore Creek and that, despite the particularly high concentrations of NO_3^-, the study reach experienced N limitation. This limitation was at least partly relieved during the NH_4^+ addition, leading to the strongest responses in biological P demand. Because the oxidation state of N in NH_4^+ (−3) is the same as that of N in biomolecules, only 5 mol of adenosine triphosphate (ATP) are needed to incorporate 1 mol of NH_4^+ into biomass. In contrast, the assimilation of NO_3^- requires the reduction of N (+4) to N (−3), which represents an energetic investment of 15 mol of ATP per mole of assimilated N (Gutschick 1981). Greater demand for NH_4^+ relative to NO_3^- under high concentrations of NO_3^- has been documented in streams (Bernot et al. 2006; Arango et al. 2008). However, preferential uptake is more complex than just a direct energetic requirement. Studies focused on marine phytoplankton found that NO_3^- uptake was possibly stimulated by NH_4^+ during certain seasons (Glibert et al. 1982). A review of field studies showed that the reduction of NO_3^- uptake by marine phytoplankton in the presence of NH_4^+ is often minimal and that it is a highly variable phenomenon that depends strongly on other factors, such as light availability and taxonomic group (Dortch 1990).

Implications of coupled C, P, and N cycling for nutrient management—It is now increasingly recognized that a detailed understanding of coupled nutrient cycles can help formulate solutions to current and future environmental problems (Finzi et al. 2011). We are aware that an increase in DOC concentration of the magnitude reached in our experiment does not represent a realistic scenario in nature, particularly in terms of the lability of C; nevertheless, this study shows that biological processing of P in streams can be linked to C dynamics in multiple ways and suggests that natural or anthropogenic alterations of the DOC pool might indeed affect P cycling in streams. This underlines the need to incorporate DOC as a major criterion in stream restoration and management activities (Stanley et al. 2012). Our results also suggest that the water column and sediment P pools can respond differently to changes in DOC and that the link between the C and P cycles is influenced not only by the concentration of N, but by its

chemical form. Human activities have largely modified nutrient loads to waterbodies, generating concentrations that exceed, sometimes by orders of magnitude, the biological demand and have led to eutrophication of streams, lakes, and coastal ecosystems. As has been stressed before (Dodds 2006), effective nutrient regulations in waterbodies must consider both the biochemical aspects of the nutrient and its potential connections to other nutrient cycles.

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