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Experimental nutrient enrichment of forest streams reduces ecosystem nitrogen and phosphorus storage

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Abstract

Streams store nutrients in standing stocks of organic matter (OM) and associated biologically sequestered elements. Unlike standing stocks of autotrophs, detritus is depleted by nutrient enrichment, potentially reducing areal storage of detritus-associated nutrients. To test effects of nutrient-loading on storage of nitrogen (N) and phosphorus (P) by autotrophic and detrital-pool compartments, we quantified the effects of 2 yr of continuous experimental N and P additions on fine benthic organic matter (FBOM), leaves, wood, and biofilms in five forest streams. Our design tested the relative strength of N vs. P on OM nutrient content, areal OM storage, and areal nutrient storage in OM types. Enrichment increased nutrient content of all OM types; %P increased more than %N in leaves, wood, and biofilms, but not FBOM. Biofilm %P and %N increased more than in all detrital types. Areal FBOM and leaf storage declined with nutrient enrichment. Biofilm standing stocks were generally higher with enrichment but were not related to the streamwater N and P gradients. Despite increased OM nutrient content, total areal nutrient storage in leaves and wood decreased due to reduced OM storage. Although annual nutrient storage was stabilized by FBOM, seasonal variation in nutrient storage increased with enrichment. Leaf-associated nutrient storage was reduced in most seasons, whereas FBOM and biofilm nutrient storage increased in winter and spring, respectively, relative to pretreatment. Overall, the combined responses of all OM types to enrichment resulted in reduced storage and altered seasonal availability of carbon and nutrients, which has implications for consumers and downstream processes.

Nutrient loading to streams can alter ecosystem-scale carbon dynamics in opposing ways. Nutrients increase carbon fixation and production of organic matter (OM) by stimulating autotrophic productivity, while they also decrease OM standing stocks by increasing heterotrophic respiration rates (Elser et al. 2007; Ferreira et al. 2015). The net changes to stream OM budgets can have important implications for the associated available energy and nutrient resources that support food-web production and other ecosystem functions, including the processing and fluxes of nitrogen (N) and phosphorus (P) within the "freshwater pipe" (Maranger et al. 2018). However, the effects of increased streamwater nutrient concentrations on N and P content of stored OM are uncertain. Storage of organic N and P in streams is largely in the form of algae and allochthonous detritus (Mulholland et al. 1985; Grimm 1987). In streams subject to low human impacts, mass-balance studies demonstrate that inputs of nutrients are balanced by export on an annual basis over average hydrologic conditions, indicating no net nutrient loss or retention (Meyer and Likens 1979; Grimm 1987). Excess nutrient loading may change the capacity of ecosystem nutrient storage, as nutrients can alter OM standing stocks, as well as OM nutrient content, and thus affect OM-associated storage of N and P regardless of discharge regime (Maranger et al. 2018).

Nutrients stored in OM (hereafter, nutrient storage) are primarily controlled by the amount and composition of OM

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types, which can vary geographically (Farrell et al. 2018; Tank et al. 2018), seasonally, with flow regime (Junker et al. 2021), and with light availability (Tank et al. 2018). The identity of dominant OM types—autotrophic or detrital—drives potentially different responses to nutrient enrichment through changes in OM standing stocks and their physiological storage capacity for N or P. Stream OM types consist of autotrophs (e.g., algae, macrophytes) and detritus in the form of coarse (e.g., leaves and wood) and fine particulate organic matter (CPOM and FPOM, respectively) colonized by heterotrophic decomposers. Both autotrophic and heterotrophic microorganisms can increase nutrient uptake as streamwater concentrations increase, enhancing N and P immobilization and thus changing the mass-specific nutrient content of these OM compartments (Small et al. 2011; Cheever et al. 2012; Scott et al. 2013; Taylor et al. 2014; Godwin and Cotner 2015; Gulis et al. 2017; Evans-White et al. 2020).

Effects of nutrient loading on nutrient standing stocks are predicted by the relative standing stocks of autotrophs (like algae and macrophytes) and detritus and their distinct responses to nutrients. Autotroph biomass generally increases as a function of limiting nutrients, depending on light and disturbance (Elser et al. 2007; Johnson et al. 2009). In contrast to autotrophs, detrital standing stocks can be reduced by nutrient enrichment, as nutrients increase fungal and bacterial activity associated with dead OM, leading to faster microbial decomposition and increased detritivore feeding (Cross et al. 2007; Ferreira et al. 2015; Manning et al. 2015; Rosemond et al. 2015). Thus, autotroph nutrient storage pools can increase because of both increased autotroph biomass and nutrient content, whereas detrital nutrient storage pools may depend on the relative strength of increases in their nutrient content vs. reductions in detrital OM storage. Although autotrophs play a disproportionate role in nutrient uptake and retention relative to their biomass even in shaded streams (Newbold et al. 1983; Tank et al. 2018; Tomczyk et al. 2022), ecosystem dominance by detrital vs. autotroph OM types may drive either reduced or increased areal nutrient storage with nutrient enrichment.

Here, we tested the influence of dissolved N and P availability on areal nutrient storage in multiple OM types, including fine benthic organic matter (FBOM), leaves, wood, and algae-containing biofilms. We defined N and P storage as analogous to the frequently used metric of OM storage (i.e., a mass of material expressed per unit area; *see* e.g., Jones 1997; Tank et al. 2010). We used enrichments of N and P at different supply ratios in five forested headwater streams. We tested the relative importance of streamwater N and P concentration for OM %N and %P content, and for OM standing stocks. We then combined these responses to quantify the total areal storage pools of N and P in the four OM types and assessed responses of annual and seasonal particulate N and P storage to N and P enrichment. We tested two questions: (1) Does N and P enrichment modify the amount of these elements that are stored within different OM compartments? and (2) do the effects of enhanced nutrient immobilization by specific OM compartments outweigh the effects of reduced standing stocks of detrital OM? Our current study complements others that are part of a larger experiment examining relative effects of N and P in driving detrital carbon dynamics, food-web pathways of carbon flow, production of invertebrates, and growth rates and food-web dynamics of higher-order consumers (Bumpers et al. 2015, 2017; Rosemond et al. 2015; Demi et al. 2018, 2020). These previous studies revealed that detrital loss rates and consumer production were driven by effects of nutrients through both algal and detrital pathways (Manning et al. 2015, 2016; Bumpers et al. 2017; Demi et al. 2018). We predicted that N and P enrichment would increase mass-specific nutrient content of both autotrophic and detrital OM types. Furthermore, we predicted that, despite increases in OM nutrient content, losses of detrital OM standing stocks would outweigh potential gains from autotrophic biomass, and result in reduced total areal nutrient storage.

Methods

Study sites and experimental enrichment

Our experiment was conducted in temperate forest headwater streams at the U.S. Department of Agriculture Forest Service Research Station, Coweeta Hydrologic Laboratory (hereafter Coweeta) in Macon County, North Carolina. We selected streams with similar elevation, aspect, and physiochemical attributes prior to experimental enrichment. One year of pretreatment data collected in the same 70-m reach as the treatments served as a paired reference to the treatment data. Beginning on 11 July 2011, each stream was continuously enriched with N (21% ammonium nitrate) and P (85% phosphoric acid) at distinct target N : P ratios (2, 8, 16, 32, 128) for 2 yr. Nutrients were added such that low soluble reactive phosphorus (SRP) concentrations (target range = 11–90 μg L^{-1}) were paired with high dissolved inorganic nitrogen (DIN; 80–650 μg L^{-1}) concentrations and vice versa. Thus, the lowest N : P enrichments consisted primarily of P (90 μg L^{-1}) with low N concentrations (81 μg L^{-1}) and the highest N : P enrichment consisted primarily of N (650 μg L^{-1}) with low P concentrations (11 μg L^{-1}). Nutrients were added to the streams at discharge-proportional concentrations using solar-powered metering pumps linked to a pressure transducer that recorded stage height. Stream-specific nutrient solutions were injected into a gravity-fed irrigation line where it mixed with ambient stream water and dripped into the stream at approximately 5-m intervals via irrigation spouts throughout the treatment reach. The crossed-gradient design enabled us to assess the relative effects of N vs. P concentration on OM nutrient content and areal storage because concentrations of the two nutrients did not increase together. Our experimental design and analyses assume that neither element was added at inhibitory concentrations for organisms. Consequently, any

negative responses of OM nutrient content or storage to higher dissolved N concentrations (or N : P) were expected to be driven by the inverse (i.e., decreasing) P gradient and vice versa. See Manning et al. (2015) and Rosemond et al. (2015) for detailed site characteristics and a further summary of the experimental nutrient enrichment treatments.

Dissolved streamwater nutrient concentrations were measured every 2 weeks at one location ($n = 1$) upstream of the treatment reach and at three locations ($n = 3$) along each treatment reach. Water was filtered in the field (0.45-μm nitrocellulose membrane filter: Millipore), frozen within 24 h of collection, and analyzed for DIN (NH_4-N + NO_3-N) and SRP concentrations within 28 d (DIN: Alpkem Rapid Flow Analyzer 300; SRP: spectrophotometric method with UV-1700 spectrophotometer, Shimadzu). Measured streamwater nutrient concentrations reflected the effects of uptake. Therefore, we calculated the amounts of DIN and SRP that were actually added to the treatment streams. These "added concentrations" were determined using detailed records of the nutrient solutions added to each nutrient-dosing system, discharge records, and the measured ambient concentration upstream of the dosing system (*see* Manning et al. 2015). The added concentrations (hereafter, "streamwater nutrient concentrations") were used for subsequent analyses because they reflected our nutrient treatments better than measured concentrations.

OM standing stocks

We quantified standing stocks of leaf litter, FBOM, and biofilm monthly from July 2010–July 2013. Details of the leaf litter sampling are outlined in Rosemond et al. (2015) and Kominoski et al. (2018). Briefly, litter was collected every month from the entire wetted width of eight randomly selected 0.15-m wide transects in each stream. Litter was transported back to the laboratory and processed to quantify ash-free dry mass (AFDM). Fine benthic OM was collected using a stovepipe corer following Lugthart and Wallace (1992). Four benthic cores were collected at random locations in each stream. All coarse materials were removed from the core and transferred into a 4-liter plastic jar. The top ~ 10 cm of remaining sediment slurry were then stirred and a subsample retained for further processing. In the laboratory, contents of each core were passed through nested 1-mm and 250-μm sieves over a bucket. The material collected on the 250-μm sieve was subsampled and filtered on to a preashed, preweighed glass fiber filter (0.7-μm nominal pore size; Pall Life Sciences). Filters were then dried, weighed, combusted at 500°C, and reweighed to determine AFDM (mg m^{-2}). The material that passed through the 250-μm sieve was also subsampled, filtered, and processed for AFDM. The subsample of water taken from the core was processed in the same fashion. Thus, FBOM includes all benthic material smaller than 1 mm (*see* Demi et al. 2018 for additional details).

Biofilm was sampled monthly, following 2 months of colonization, using standardized unglazed slate tiles (14.5 × 14.5 cm). Two-month colonization times have been shown to be adequate for detecting effects of variation in light and nutrients on algal assemblages in the Coweeta basin (Lowe et al. 1986). Two tiles were randomly placed at four transects along each study reach and collected 2 months later, with collected tiles replaced with uncolonized tiles each sampling time ($n = 8$ tiles per month per stream). Both tiles from a transect were scrubbed and rinsed together in a pan ($n = 4$ samples per month per stream, except for occasional losses of tiles due to high discharge events). The resulting slurry was then subsampled and filtered onto a preweighed, preashed glass fiber filter, and AFDM was determined as described above, scaled to the total tile area, and expressed on a mass per unit area basis (g AFDM m^{-2}). We note that this method likely overestimated biofilm standing stocks as the stream bed was not exclusively hard substrata in any of the experimental reaches. Thus, estimates of areal nutrient storage in this OM pool (*see* "Calculating nutrient storage" section) may also be overestimates. Nevertheless, while many areas in these streams are depositional, rock substrates are common and have specific surface area greater than 1. Scaling tile substrates (that were subject to being covered by leaves and FBOM) to an areal basis is our best approximation of this standing stock.

We estimated wood standing stocks once during the study period using the line-transect method (Wallace and Benke 1984; Wallace et al. 2000). All wood that intersected a nylon string was measured with calipers at $n = 15$ transects in each stream. At each transect, the wetted width was recorded. Standing stocks of wood in each 70-m reach were then estimated following the equations in Wallace and Benke (1984). All estimates of particulate standing stocks are presented on an areal basis (i.e., g AFDM m^{-2}).

OM nutrient content

Nutrient content of wood (small sticks < 2 cm in diameter) and FBOM were determined quarterly in each of the five streams during all 3 years of the study. Each sampling period, five wood pieces were collected from four randomly selected transects within each 70-m study reach. We collected FBOM from obvious depositional areas within the same transect as the wood. All samples were transported back to the laboratory, dried, homogenized, and weighed. Samples from the same transect were aggregated; thus, each sampling event resulted in four estimates of nutrient content for wood and FBOM ($n = 4$ per stream per event). Leaves and biofilm nutrient content were quantified monthly using the standing stock samples described previously. To compare nutrient content of leaves and biofilm to wood and FBOM, we used only the samples collected during the same month as the FBOM and wood samples. This resulted in one aggregate sample per transect in most months (biofilm, $n = 4$ per stream per event; leaves,

$n = 8$ per stream per event), with the exception of occasional months in which either not all tiles were recovered or there was sample loss.

A subsample of 2–4 mg of the dried material was used to determine %C and %N with an elemental analyzer (1500 CHN analyzer Carlo Erba). Phosphorus content was determined using dry ash/acid-extraction method (leaf litter, wood, biofilms; Allen 1974) or acid-persulfate digestion (FBOM) followed by spectrophotometric analysis (Shimadzu UV-1700) of the extracted solution using the ascorbic acid method (APHA 1998). All nutrient content data are reported as a percentage of dry mass, unless otherwise noted.

Calculating nutrient storage

We calculated the areal mass of particulate N and P stored in each OM type by multiplying the percent N or P by the areal standing stock dry mass (Meyer and Likens 1979; Cross et al. 2005). Nutrient storage pools were determined using seasonal means of OM areal standing stocks (as DM m^{-2}), %N, and %P (both % of dry mass). We compared particulate nutrient storage in all four OM types using the annual average calculated from seasonal means for each year. Because we only had one measurement of wood standing stocks, we used the mean breakdown rate under enriched conditions from Gulis et al. (2004) to estimate the depletion of the measured standing stocks (wood < 2 cm in diameter) over a 2-yr period using the exponential-decay model. The effect of nutrients was compared using the same model for all five streams; thus, we did not account for potential differences due to the streamwater N : P gradient. We assumed steady state of inputs and export of wood. The estimated change in wood standing stocks and thus nutrient storage in wood is a coarse estimate and was done for comparative purposes. We omitted wood nutrient standing stocks from analyses and only consider FBOM, leaves, and biofilm. We also calculated seasonal nutrient storage pools for FBOM, leaves, and biofilm using the seasonal means of dry mass and nutrient content.

Data analyses

Effects of N and P enrichment on OM nutrient content

We assessed the effect of experimental enrichment on OM nutrient content and stoichiometry (C : N, C : P, and N : P molar ratios) using the quarterly data described previously. To compare across OM types, we used the biofilm and leaf litter nutrient data from the same collection month as wood and FBOM. All analyses were conducted in R (R Core Team 2021). To determine the relationship between OM nutrient content and streamwater nutrients, we used linear mixed-effects regression (function "lmer" in R package "lme4"; Bates et al. 2015). We expected each OM type to differ in its nutrient content, so we analyzed models separately for each OM type. We present results for %N, %P, and N : P of OM in the main text and provide results for OM C : N and C : P stoichiometry in the Supporting Information. We tested for relationships between seasonal mean streamwater N and P concentrations (based on added concentrations described previously) and OM %N or % P, and between mean streamwater dissolved N : P and OM N : P. We included stream as a random effect in all models to account for spatial and temporal nonindependence. Due to the seasonality of OM dynamics in the study streams, we expected that season might be an important factor affecting OM nutrient content. We used a drop-in-deviance test to assess if a model that included season as an interaction (model form: [N] × season + [P] × season; N : P × season) vs. an additive (model form: [N] + [P] + season; N : P + season) factor was the more parsimonious for each nutrient response variable. For this analysis, season was coded as follows: autumn as samples from October, winter as samples from January, spring as samples from April, and summer as samples from June or July. We report the results for the best model according to the deviance test. Streamwater N and P concentrations and N : P ratio were $\log_e$-transformed and standardized using *z*-scores for all models. We calculated marginal and conditional R^2 values as a metric of goodness-of-fit of each model. Marginal R^2 describes the variance explained by the fixed effects and conditional R^2 includes the fixed and random effects (Bartoń 2020).

Effects of N and P enrichment on OM standing stocks

We also tested for relationships between our nutrient treatments and the magnitude response of %N, %P, and OM standing stock (AFDM, excluding wood). We calculated response ratios of the effects of nutrient enrichment by stream by dividing the annual average of each treatment year (YR1, YR2) by the annual average during the pretreatment year (PRE). Each ratio was then $\log_e$-transformed before further analyses. We then tested for a relationship between the mean response ratio and $\log_e$-transformed streamwater N : P ratio using mixed-effects models as above.

Effects of N and P enrichment on annual OM-associated N and P storage

We used ANOVA to test for the effects of enrichment on annual nutrient storage pools. We used an effect of "year" as a proxy for our nutrient treatment. We tested each OM type separately. If the effect of year was significant, we performed a Tukey honestly significant difference (HSD) post hoc test to determine which years were different. In the comparison of annual means of all pools, we used a grand mean across all five streams for each year to assess the overall effect of enrichment on areal nutrient storage pools. The effect of year (proxy for treatment) was the only factor included in the model to test for differences in N or P storage. To compare the seasonal nutrient standing stock data, we calculated the response ratio as described above using seasonal means for each OM type and nutrient pool. We first analyzed the seasonal response ratios using mixed-effects models to evaluate the relationship of the magnitude change to the streamwater N : P gradient and season. However, all slopes for N : P gradient had high

uncertainty. Therefore, we tested for differences in means among seasons using ANOVA. Models only included a fixed effect of season and were analyzed separately for each OM type. If the main effect of season was significant, we performed a Tukey HSD post hoc test.

Results

Effects of stream enrichment on OM standing stocks

OM standing stocks were dominated by FBOM and leaves followed by wood and biofilm (Table 1). Standing stocks (as AFDM) of both leaves and FBOM were reduced under enrichment; leaf AFDM was reduced by an average of 52%, while FBOM AFDM was reduced by 24%, relative to pretreatment conditions (Table 1). The magnitude changes in standing stocks of FBOM and leaves were positively related to the dissolved streamwater N : P gradient, indicating greater standing stock reductions at lower N : P (Fig. 1). Mean annual biofilm standing stocks generally increased with enrichment by an average of 20% but were weakly related to the N : P gradient (Fig. 1).

Effects of stream enrichment on annual OM N and P content

All substrates increased in %P and %N when evaluated based on average annual values in enrichment YR1 and YR2 relative to the pretreatment year (Table 1). OM %P increased more than %N for all OM types except FBOM (Fig. 1; Table 1). The magnitude changes in %N and %P varied among substrates: biofilm had the greatest change in both %N and %P compared to pretreatment, while leaf and wood %N and FBOM %P changed the least (Fig. 1; Table 1). The magnitude change in %N was not strongly related to the dissolved N : P gradient for any OM type, indicating similar responses across

Table 1. Annual means (± SE) of OM standing stocks as AFDM, particulate nitrogen (N) and phosphorus (P), and nutrient content as percent P, N, and C across the five study streams. Nutrient standing stocks were determined by multiplying mean percent nutrient concentration by areal dry mass standing stocks (g DM m^{-2}). OM standing stocks were determined from replicate collections that were randomly chosen along each study reach. We report AFDM of OM standing stocks for comparison with previous studies, but dry mass was used in nutrient standing stock calculations. Total represents the summed areal standing stock from each separate OM type. Letters in parentheses indicate significant differences as determined by Tukey's HSD ($p < 0.05$) among treatment years within a given resource. Mean percent change is the average change for each variable during enriched conditions relative to pretreatment conditions.

Year	AFDM (g m^{-2})	g P m^{-2}	g N m^{-2}	%P	%N	%C
Total						
PRE	1023.13 ± 151.91 (a)	2.38 ± 0.27 (a)	25.29 ± 4.48 (a)			
YR1	662.63 ± 69.83 (b)	2.2 ± 0.16 (a)	23.47 ± 1.45 (a)			
YR2	623.01 ± 69.85 (b)	2.16 ± 0.39 (a)	21.97 ± 4.00 (a)			
Mean % change	−37	−8	−10			
FBOM						
PRE	543.25 ± 96.14 (a)	2.183 ± 0.256 (a)	20.9 ± 4.04 (a)	0.050 ± 0.006	0.462 ± 0.055	8.08 ± 1.04
YR1	413.18 ± 46.36 (a)	2.08 ± 0.147 (a)	21.1 ± 1.32 (a)	0.055 ± 0.004	0.567 ± 0.064	10.74 ± 1.27
YR2	412.46 ± 66.88 (a)	2.026 ± 0.381 (a)	19.81 ± 4.01 (a)	0.062 ± 0.003	0.603 ± 0.073	10.96 ± 1.26
Mean % change	−24	−6	−2	17	26	34
Leaves						
PRE	348.3 ± 44.49 (a)	0.166 ± 0.022 (a)	3.41 ± 0.46 (a)	0.039 ± 0.001	0.813 ± 0.025	42.87 ± 0.42
YR1	170.11 ± 19.39 (b)	0.092 ± 0.011 (b)	1.69 ± 0.19 (b)	0.053 ± 0.002	0.879 ± 0.024	41.91 ± 0.63
YR2	162.58 ± 3.31 (b)	0.112 ± 0.004 (b)	1.77 ± 0.07 (b)	0.053 ± 0.002	0.867 ± 0.040	42.27 ± 0.61
Mean % change	−52	−38	−49	37	7	−2
Wood						
PRE	131.46 ± 12.90 (a)	0.033 ± 0.004 (a)	0.98 ± 0.09 (a)	0.013 ± 0.001	0.378 ± 0.008	47.93 ± 0.22
YR1	79.2 ± 7.83 (b)	0.027 ± 0.003 (a)	0.66 ± 0.06 (b)	0.017 ± 0.001	0.424 ± 0.015	47.69 ± 0.15
YR2	47.8 ± 4.69 (c)	0.019 ± 0.003 (b)	0.38 ± 0.04 (a)	0.02 ± 0.002	0.402 ± 0.010	46.91 ± 0.10
Mean % change	−52	−29	−47	48	9	−1
Biofilm						
PRE	0.13 ± 0.004 (a)	0.0003 ± 0.00004 (a)	0.0047 ± 0.0003 (a)	0.036 ± 0.003	0.536 ± 0.031	5.91 ± 0.99
YR1	0.15 ± 0.017 (b)	0.0007 ± 0.00004 (b)	0.01101 ± 0.0009 (b)	0.071 ± 0.005	0.956 ± 0.070	6.42 ± 0.43
YR2	0.16 ± 0.029 (b)	0 0.0007 ± 0.00007 (b)	0.0081 ± 0.0004 (c)	0.071 ± 0.006	0.772 ± 0.009	5.49 ± 0.21
Mean % change	20	127	105	104	75	27

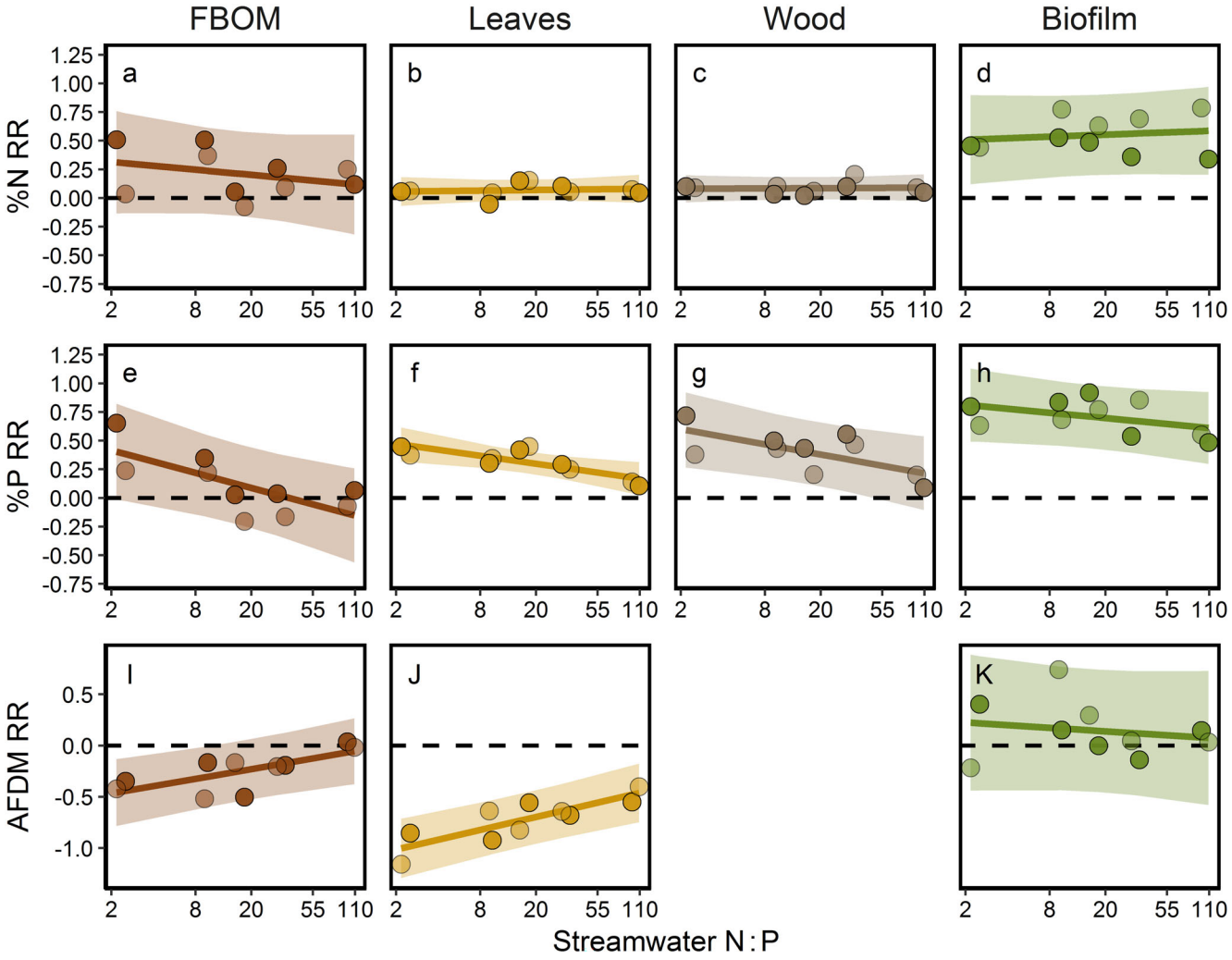


Fig. 1. The response ratio of annual standing stocks of OM (as AFDM) and nutrient content (as %N and %P) in enrichment years 1 and 2 compared to the pretreatment year related to streamwater N : P. Log_e response ratios for percent nitrogen (%N; **a**–**d**), phosphorus (%P; **e**–**h**), and OM standing stocks (AFDM; **i**–**k**). Values above zero indicate a net increase and values below zero indicate a net decrease compared to pretreatment conditions. Regression lines are derived from the mixed-effects models. Shading represents the 95% confidence interval around the slope of the regression. Light points = YR1 of enrichment, dark points = YR2 of enrichment. Regressions were analyzed using log_e streamwater N : P but are presented untransformed to preserve ratio values. Regression equations: (**a**) $y = 0.35–0.05x$, $R^2_{Marginal} = 0.06$; (**b**) $y = 0.05 + 0.006x$, $R^2_{Marginal} = 0.18$; (**c**) $y = 0.08 + 0.002x$, $R^2_{Marginal} = -0.01$; (**d**) $y = 0.50 + 0.02x$, $R^2_{Marginal} = 0.03$; (**e**) $y = 0.51–0.14x$, $R^2_{Marginal} = 0.050$; (**f**) $y = 0.52–0.08x$, $R^2_{Marginal} = 0.78$; (**g**) $y = 0.66–0.10x$, $R^2_{Marginal} = 0.42$; (**h**) $y = 0.85–0.05$; $R^2_{Marginal} = 0.18$; (**i**) $y = -0.54 + 0.10x$, $R^2_{Marginal} = 0.51$; (**j**) $y = -1.1 + 0.14x$, $R^2_{Marginal} = 0.70$; (**k**) $y = 0.25–0.04x$, $R^2_{Marginal} = 0.03$. $R^2_{Marginal}$ = variance explained by the fixed effects.

the nutrient gradient (Fig. 1). Change in OM %P was negatively related to dissolved N : P ratio for all OM types, indicating a greater change in %P at lower N : P and that %P responded more than %N for these OM types on an annual basis with nutrient enrichment (Fig. 1). The absolute slope of the relationship between %P and N : P was greatest for FBOM and weakest for biofilm (Fig. 1).

OM C : N, C : P, and N : P was reduced with nutrient enrichment in leaves, wood, and biofilm, but all these ratios increased for FBOM (Table S1). C : P changed the most (24–46% relative to pretreatment) followed by N : P (14–22%) and C : N (4–20%) for all OM types (Supporting Information Table S1). Biofilm C : P and C : N exhibited the greatest change relative to other OM types.

Effects of streamwater N and P concentrations on OM nutrient content

The magnitude and effect of streamwater N and P on OM nutrient content varied among OM types (Supporting Information Table S2). FBOM %N was negatively related to streamwater N concentration, but the parameter estimates had high uncertainty. Leaf and biofilm %N increased with increasing streamwater N concentration (Fig. 2). FBOM %N was positively related to streamwater P concentration (with a

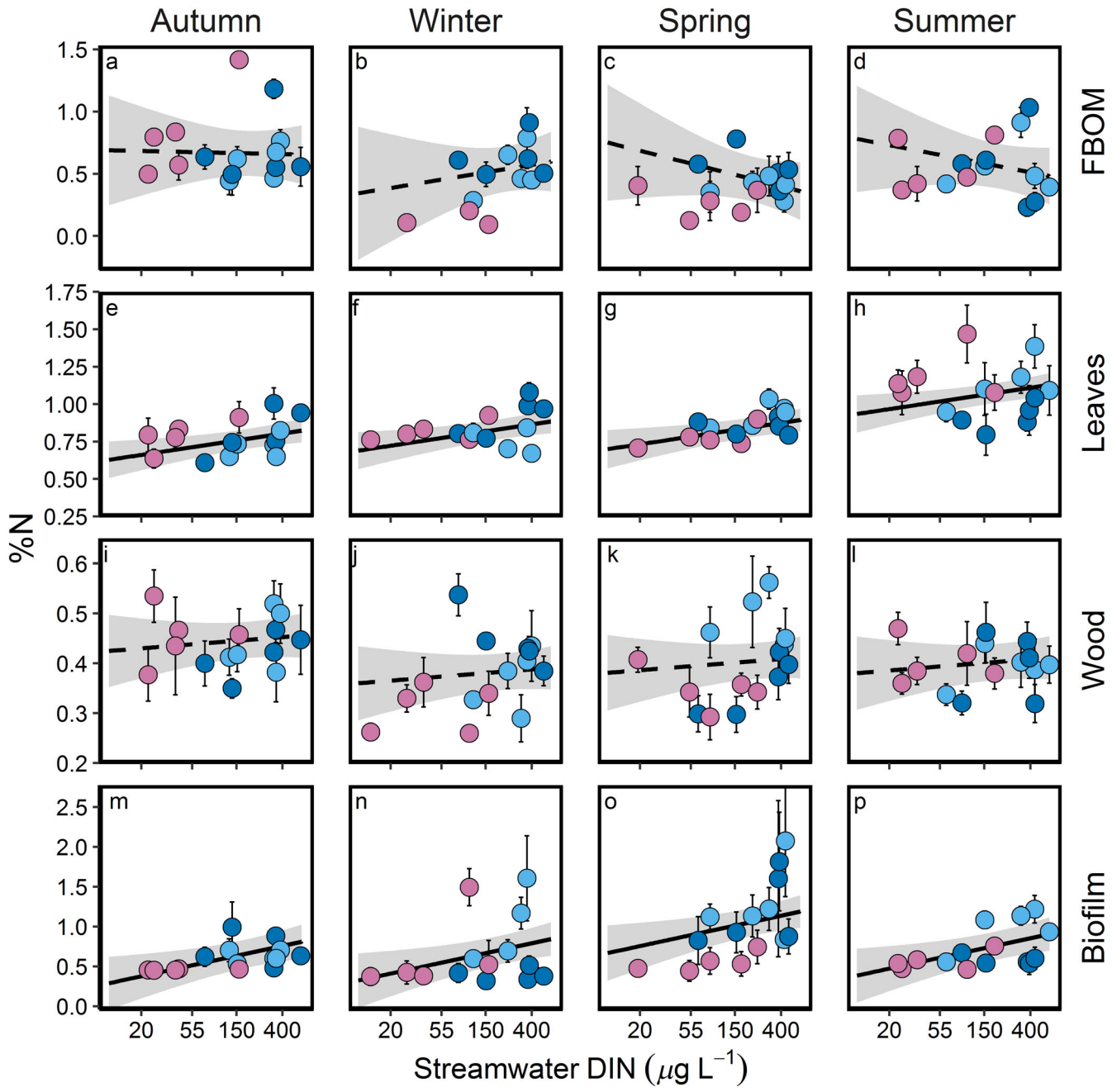


Fig. 2. Percent nitrogen concentration of naturally occurring FBOM (**a**–**d**), leaves (**e**–**h**), wood (**i**–**l**), and biofilm (**m**–**p**) related to streamwater DIN during PRE (pink), YR1 (light blue), and YR2 (blue) of the study. Streamwater DIN represents the ambient plus added (in the case of enrichment years) concentration entering the treatment reaches (*see* "Methods" section for more details). Note the $\log_e$ scale in the *x*-axes. Individual regression lines are derived from the mixed-effects models, which included streamwater N and P concentration and are plotted to show the effect of DIN at the mean concentration of streamwater P. Year was not included as a factor in regression models but is denoted here for illustrative purposes. If the *t*-value of the parameter estimate for DIN is < 2, the regression line is dashed. Points represent the seasonal mean for an individual stream with standard error. Note that *y*-axis range differs among panel rows. See Supporting Information Table S2 for regression statistics.

significant interaction with season; Supporting Information Table S2). Leaf %N declined with streamwater P concentration. Wood %N was weakly related to streamwater N and P concentrations (Fig. 2; Supporting Information Table S2). FBOM was the only OM type for which the effect of streamwater N and P on %N varied among seasons (significant interaction; Supporting Information Table S2). Mean %N was different in at least one season for each OM type.

Phosphorus content was positively related to streamwater P concentration for all OM types (Fig. 3; Supporting

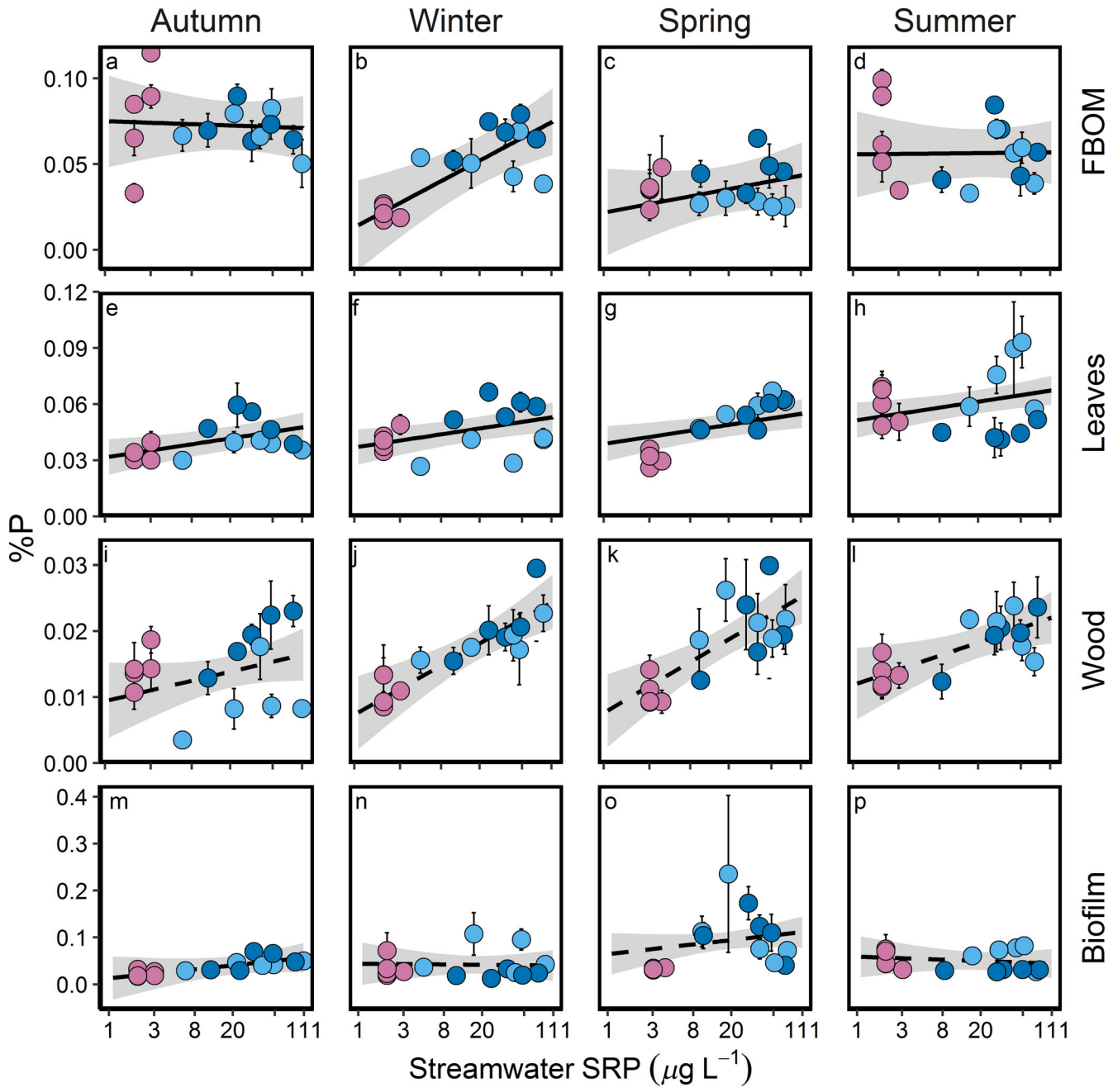


Fig. 3. Percent phosphorus concentration of naturally occurring FBOM (**a**–**d**), leaves (**e**–**h**), wood (**i**–**l**), and biofilm (**m**–**p**) related to $\log_e$ streamwater SRP during PRE (pink), YR1 (light blue), and YR2 (blue) of the study. Streamwater SRP represents the ambient plus added (in the case of enrichment years) concentration entering the treatment reaches (*see* "Methods" section for more details). Note the $\log_e$ scale in the *x*-axes. Individual regression lines are derived from the mixed-effects models, which included streamwater N and P concentration and are plotted to show the effect of SRP at the mean concentration of streamwater DIN. Year was not included as a factor in regression models but is denoted here for illustrative purposes. If the *t*-value of the parameter estimate for SRP is < 2, the regression line is dashed. Points represent the seasonal mean for an individual stream with standard error. Note that *y*-axis range differs among panel rows. *See* Supporting Information Table S2 for regression statistics.

Information Table S2). FBOM and wood %P both exhibited strong relationships with streamwater P concentration and weak or no relationships with streamwater N concentration. Though the season interaction model for wood was significantly better than the additive model, the season × nutrient concentration parameter estimates had relatively large uncertainties that encompassed zero (Supporting Information Table S2). Leaf %P was strongly related to streamwater P but not streamwater N. The effect of streamwater P varied among seasons for FBOM, wood, and biofilm %P. Biofilm %P was not

strongly related to streamwater P concentration but there was a strong streamwater [N] × spring effect (Supporting Information Table S2). Mean %P differed in at least one season for all OM types. Mean FBOM %P differed in all seasons and was greatest in autumn. Consistent with leaf %N, leaf %P was highest in summer. Wood %P was also different in each season and was slightly greater in spring. Like biofilm %N, biofilm %P was highest in spring. The effects of streamwater N and P concentration on OM C : N and C : P were similar to those of %N and %P (*see* Supporting Information Figs. S1, S2; Tables S3, S4). Within leaf and FBOM compartments, the C : N response to streamwater N varied seasonally while the response of C : P to streamwater P was consistent among seasons (Supporting Information Figs. S1, S2; Tables S3, S4).

Streamwater dissolved N : P had a strong effect on OM N : P except for FBOM N : P (Fig. 4; Supporting Information Table S5). OM N : P increased along the streamwater N : P gradient for leaves, wood, and biofilm, but FBOM N : P declined with increasing streamwater N : P (Fig. 4). The effect of streamwater N : P varied among seasons for biofilm but not leaves, FBOM, or wood (Supporting Information Table S5). Mean leaf N : P and FBOM N : P did not vary among seasons but did vary for wood and biofilm. Biofilm N : P was lowest during the spring.

Effects of stream enrichment on N and P areal storage

The mass of N and P storage pools varied among OM types and specifically changed due to enrichment in leaves, wood, and biofilm, but not in FBOM (Table 1). FBOM made up the largest type of OM standing stocks (as AFDM), as well as N and P storage pools, followed by leaves, wood, and biofilm (Table 1). FBOM nutrient storage pools were relatively consistent across years (Supporting Information Figs. S3, S4), although FBOM AFDM decreased by 24% under enrichment. Enrichment reduced nutrient storage associated with leaves and wood, and increased biofilm storage (Fig. 5; Table 1; Supporting Information S3, S4). The net effects of decreased standing stocks of leaves and wood led to slightly reduced total annual storage of N and P across all OM types, despite increased detrital %N and %P and an increase in biofilm standing stocks (Table 1; Supporting Information Figs. S3, S4). The absolute increase in biofilm N and P storage was very small compared to the loss of N and P associated with leaves (Table 1). The magnitude changes in total (sum of all OM types) N and P storage pools relative to pretreatment were generally negative and did not vary consistently across the streamwater N : P gradient (both regression p values > 0.1; Supporting Information Fig. S5).

We observed dramatic changes in the seasonal patterns of N and P storage associated with FBOM, leaves, and biofilm compared to the pretreatment year in our experimental streams. The direction and magnitude of the change differed by OM type; these effects were generally consistent across the experimental gradient, as the magnitude change was not strongly related to the streamwater N : P for any substrate (Fig. 5). Within some seasons there were trends between the N : P gradient and magnitude change (e.g., primarily for biofilm in spring), but the slopes had high uncertainty in all cases (data not shown). Thus, we tested for differences in mean response ratios across seasons. FBOM-associated N and P storage changed little compared to pretreatment patterns, except for relatively large increases during winter (Fig. 5). Leaf-associated N and P storage either had no change or a slight increase during autumn and dramatic reductions in winter, spring, and summer for both nutrients (Fig. 5; Supporting Information Figs. S3, S4). Biofilm-associated N and P storage generally increased, with the greatest and consistent increases in both N and P storage in spring (Fig. 5; Supporting Information Figs. S3, S4). Reductions in leaf-associated N and P storage in the spring were as high as ∼ 85% compared to pretreatment. Increases in biofilm-associated N and P storage were as high as ∼ 600% in spring; however, mass of N and P associated with leaves was much greater than for biofilm (160 × higher for P and 300 × higher for N; Table 1).

Discussion

Our results demonstrate that nutrient enrichment increased nutrient content of all OM types, but this effect did not result in greater areal N or P storage. Rather, storage of N and P was greatly reduced in leaves and wood and elevated in FBOM and biofilm and varied seasonally. Although we observed large relative increases in nutrient storage associated with biofilm, light-limitation in our heavily shaded study streams likely constrained its absolute response, which did not offset losses of nutrient storage in leaves. Leaf litter contributed 25–35% of OM standing stocks but only 5–7% of particulate nutrient storage pools. Small wood comprised 7–13% of OM stocks and 0.8–2% of particulate nutrient stocks. In contrast, FBOM represented 91–94% of particulate nutrient storage pools. FBOM had modest increases in nutrient content and moderate declines in standing stocks. Consequently, there was no significant net effect of nutrient enrichment on FBOM-associated nutrient storage. Although FBOM provided a stabilizing nutrient pool in our treatment streams, dramatic declines in leaf litter and wood standing stocks resulted in declines in nutrient storage within these biologically active compartments, driving decreases in areal N and P storage pools summed across OM types.

The degree to which leaves, wood, or FBOM drive nutrient storage likely differs across stream ecosystem types (Tank et al. 2018). The OM-associated nutrient standing stocks we measured were comparable to other eastern U.S. deciduous streams in which benthic N storage was dominated by FBOM followed by leaves, and wood (Tank et al. 2018). Open canopy streams contain much higher benthic N in biofilms, yet overall benthic storage can still be dominated by FBOM across open and closed-canopy streams (Tank et al. 2018). Nitrogen

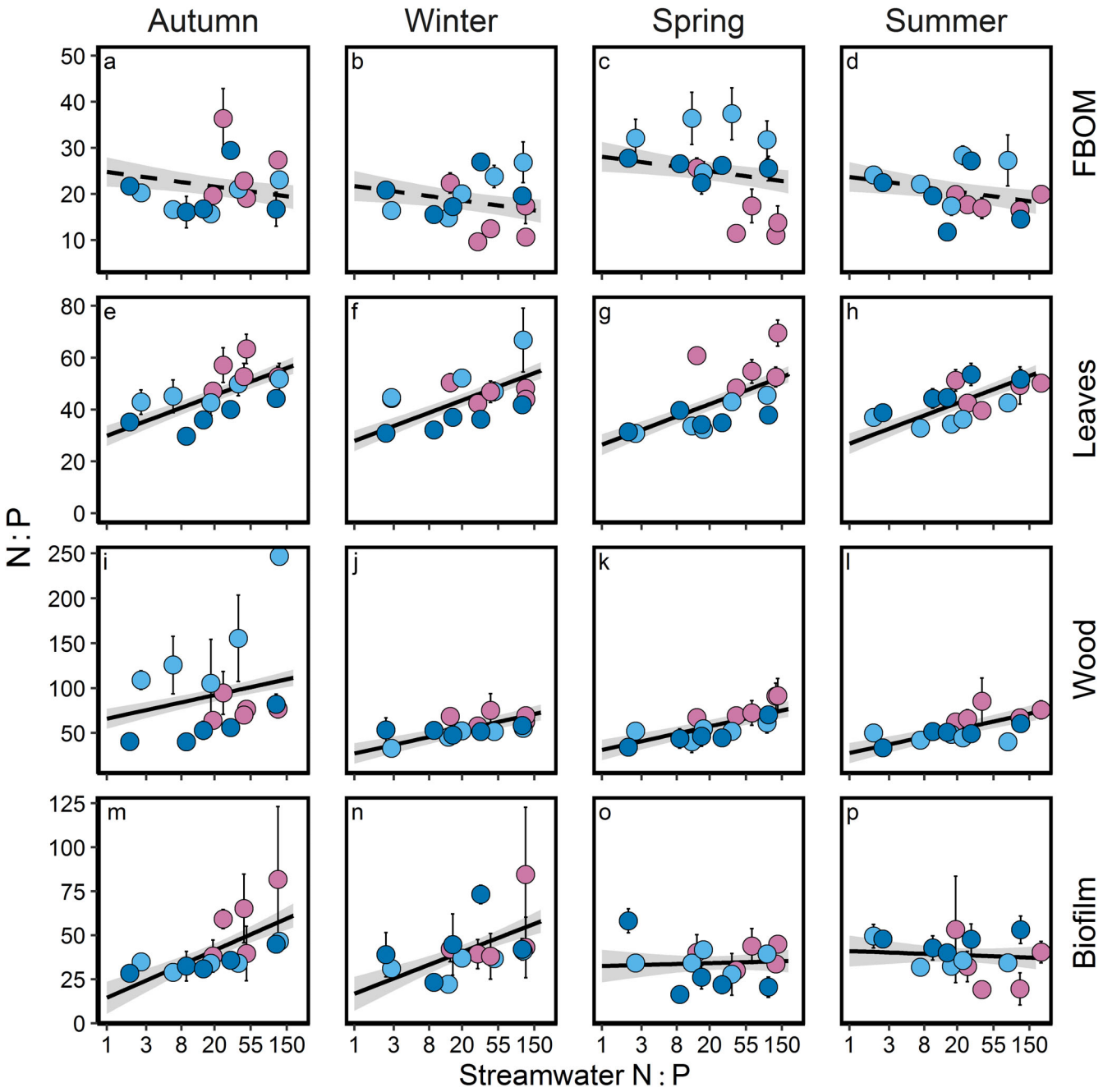
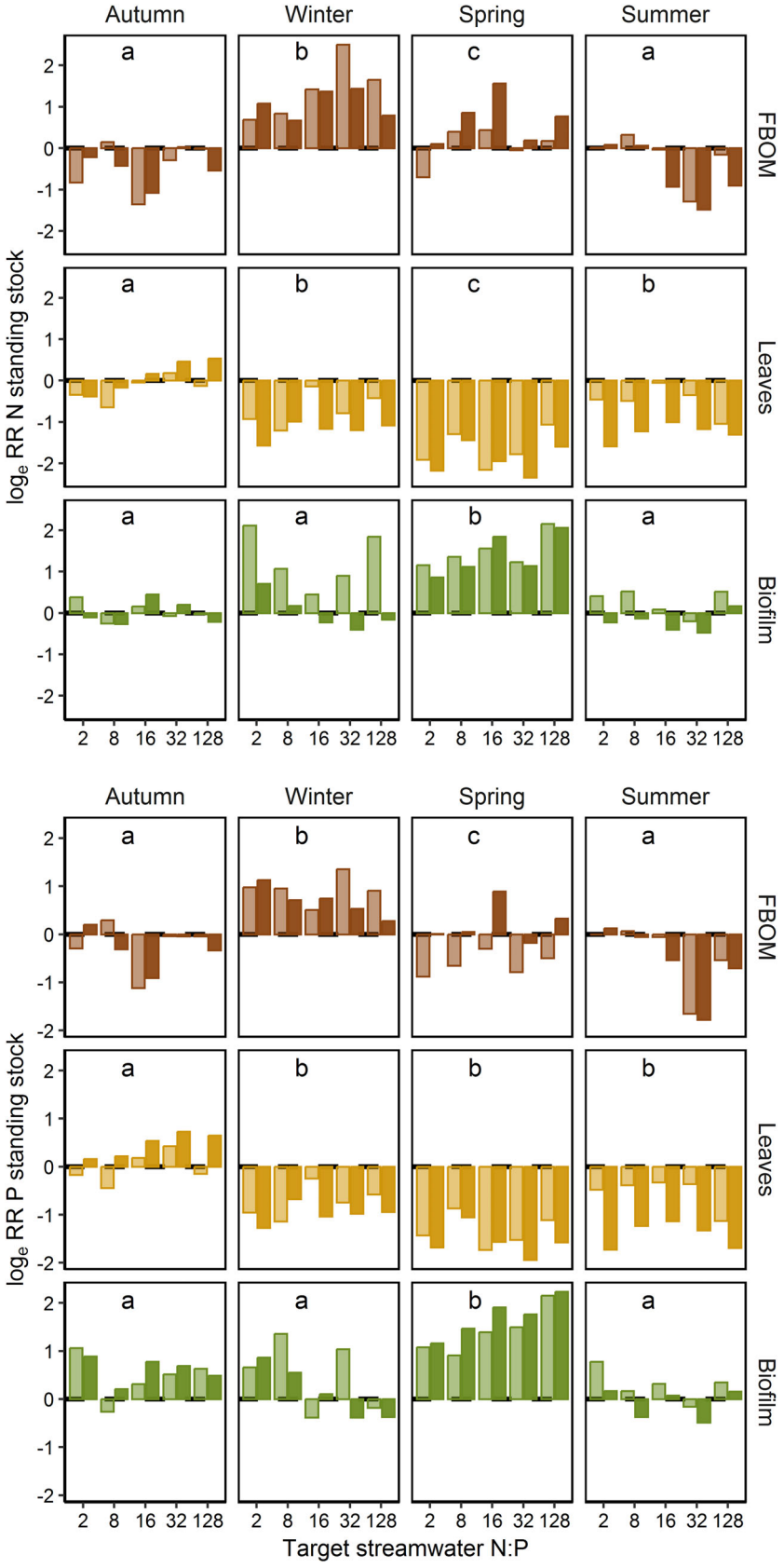


Fig. 4. N : P ratio of naturally occurring FBOM (**a–d**), leaves (**e–h**) wood (**i–l**), and biofilm (**m–p**) related to streamwater N : P during PRE (pink), YR1 (light blue), and YR2 (blue) of the study. Streamwater N : P represents the ambient plus added (in the case of enrichment years) dissolved nutrients entering the treatment reaches (*see* "Methods" for more details). Note the $\log_e$ scale in the *x*-axes. Individual regression lines are derived from the mixed-effects models. Year was not included as a factor in regression models but is denoted here for illustrative purposes. If the *t*-value of the parameter estimate for streamwater N : P is < 2, the regression line is dashed. Points represent the seasonal mean for an individual stream with standard error. Note that *y*-axis range differs among panel rows. *See* Supporting Information Table S5 for regression statistics.

storage was also found to be higher in FBOM than in leaves or wood at three sites across a large latitudinal gradient along the Appalachian Mountains (Sanzone et al. 2001). However, FBOM is less biologically active, as it has been shown to remove N from the water column at lower rates than leaves or wood (Mulholland et al. 1985; Sanzone et al. 2001). Our experimental enrichments lasted 2 yr, so a longer-term response of FBOM may have eventually resulted in declines in FBOM-associated N and P storage if FBOM standing stocks continued to decline with further enrichment. A previous



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study at Coweeta found that FBOM standing stocks declined during 5 years of N + P enrichment (Benstead et al. 2009; Rosemond et al. 2015). During this time, FBOM export and respiration exceeded total annual OM inputs, implying that long-term storage of buried OM was being reduced (Benstead et al. 2009; Benstead et al. 2021). Therefore, chronic enrichment of detritus-based streams may potentially reduce storage even of FBOM over multiyear time periods.

Effects of enrichment on stream OM nutrient content

Although nutrient content increased on all OM types with enrichment, there were greater magnitude changes in %P than in %N. Greater change in P vs. N content of substrates in our study is consistent with others conducted at Coweeta (Rosemond et al. 2008; Tant et al. 2013; Kominoski et al. 2015) and across nutrient gradients elsewhere in the United States (O'Brien and Wehr 2010; Taylor et al. 2014; García et al. 2017; Usher et al. 2020). Greater effects on P than on N occurred in both algal and detrital substrates, likely due to greater potential for sequestration of P than N when both were available (Gulis et al. 2017; Danger 2020). Biofilm exhibited greater changes in substrate N and P content than detrital substrates, which likely occurred due to higher microbial biomass and activity as a proportion of AFDM on biofilms relative to leaves, wood, and FBOM (Tank et al. 2018; Tomczyk et al. 2022). The streamwater N : P gradient was negatively associated with the magnitude change in %P for most OM types (though it was only significant for leaves and FBOM); magnitude changes in OM standing stocks of leaves and FBOM were also negatively related to the N : P gradient.

These patterns suggest that streamwater P had relatively greater effects on P content, as well as OM standing stocks. Furthermore, it is unlikely that negative relationships with the streamwater N : P gradient would be caused by declining dissolved N concentrations. In contrast, the magnitude change in %N was similar across the streamwater N : P gradient. Our concurrent studies showed that invertebrate production was stimulated by reduced leaf litter C : P (Demi et al. 2018) and that larval salamander growth increased with increasing P concentration (Bumpers et al. 2015). These studies demonstrate at least the short-term effects of greater mass-specific P and N content of food resources, even if OM standing stocks declined and became more seasonally variable.

Nutrient content varied seasonally in all OM types. Biofilm responses to the N : P gradient were likely driven by the seasonal dynamics of algae in these streams, which are light-limited except during spring before the deciduous forest canopy closes (Greenwood and Rosemond 2005). Leaf inputs peak in autumn, so we expected that the effect of N or P enrichment on leaf litter nutrient content might vary seasonally. However, we found models that included season as an additive effect to be more parsimonious for all measures of nutrient content, suggesting the effect of streamwater nutrients on leaf litter nutrient concentrations was similar across seasons (i.e., mean leaf litter nutrient content varied by season). The leaf litter and wood we collected reflected the tree species composition of the riparian forests, which naturally vary in nutrient content, leaching rate, and microbial colonization (Marks 2019; Robbins et al. 2019; Danger 2020). In addition, although most leaf litter enters these streams during autumn, litter inputs can occur throughout the year (Wallace et al. 1995) and wood inputs also vary in space and time.

In streams where litter decomposition rates increased the most (as previously observed in the lowest N : P streams; Rosemond et al. 2015), the dominant litter substrates available for sampling were sometimes those that had recently entered the stream, and likely had less time for nutrient immobilization to occur (Cheever et al. 2012). Shorter stream incubation could have skewed our bulk samples toward nutrient-poor substrates, despite strongest nutrient effects on OM standing stocks. Thus, our treatment effects on leaf and wood nutrient content are likely conservative.

Enrichment effects on stream ecosystem nutrient storage

We hypothesized that microbe-driven increases in OM nutrient concentration might increase total areal nutrient storage pools at least during portions of the year. In contrast, we found nutrient storage pools of leaf litter were reduced even during the early part of the "OM year" (e.g., at maximum litter-fall and OM standing stocks in deciduous forest streams). Monthly variation in leaf-associated nutrient storage was reduced, as shown by rapid reductions in N and P after the fall peak (particularly in YR2; Supporting Information Figs. S3, S4). In contrast, seasonal variation in biofilm storage increased compared to pretreatment, with large-magnitude increases in the spring (Supporting Information Figs. S3, S4). Consequently, during any given season organisms were experiencing higher or lower nutrient availability in different OM nutrient pools compared to pretreatment conditions (Fig. 5). On an annual basis, the absolute increase in algal storage pools (which we may have overestimated with our methods) was too low to mitigate the greater loss of nutrient storage in leaf litter (Supporting Information Figs. S4, S5). In streams with greater light availability, it is likely that seasonal

(Figure legend continued from previous page.)

Fig. 5. Seasonal $\log_e$ response ratios of nitrogen and phosphorus particulate standing stocks in each of the five streams (as target streamwater N : P) in enrichment years 1 and 2 compared to pre-treatment for FBOM (brown), leaves (yellow), and biofilm (green). Light points = YR1 of enrichment, dark points = YR2. Values above zero indicate a net increase in standing stocks compared to pretreatment conditions and values below zero indicate a net decrease in standing stocks from the study reaches compared to pretreatment conditions. Letters in each panel above the bars indicate significant differences ($p < 0.05$) among seasons determined by Tukey's HSD. There were no significant differences among streams within a given season.

increases in autotrophic nutrient storage could offset nutrient-induced losses of detrital nutrients.

Total areal storage of OM-associated N or P was only weakly related to the N : P gradient. This was the result of opposing responses of AFDM and %N or %P. For instance, %P of leaves increased with increasing streamwater P, whereas the AFDM of leaves was negatively related to streamwater P. This pattern was similar for each OM type, resulting in no relationship between the magnitude change in total N or P storage and streamwater N : P. This result is likely a function of many factors, including co-limitation of N and P of many of the processes we quantified in this study on an areal basis, for which we have previously determined mechanisms and pathways, particularly for leaf litter loss rates and nutrient sequestration (Manning et al. 2015, 2016; Rosemond et al. 2015). In addition, the nutrient concentrations added to our study streams may have been sufficient to alleviate limitation of both N and P across our experimental N : P gradient (Kominoski et al. 2015).

Lower mass of nutrients retained in OM likely represent long-term effects of enrichment on the benthic storage of nutrients in the study streams. In a test of the effects of this experimental enrichment on N and P uptake from the water column, we found that nutrient uptake was generally balanced with remineralization across the gradient of N : P in the enriched streams (Tomczyk et al. 2022). However, when net uptake occurred, it was related to the experimental N : P gradient (Tomczyk et al. 2022). These results, together, suggest that there were subtle differences in benthic OM nutrient storage and uptake dynamics across an N : P gradient based on relatively low concentrations. To assess nutrient pollution effects on stream nutrient processing more widely, in the future these processes should be explored across larger concentration gradients in N and P and with different stream characteristics.

Implications of altered nutrient storage for stream ecosystem function

Our study provides a comprehensive assessment of temporal variation in areal nutrient storage in response to streamwater N and P availability. The estimates of OM standing stocks allowed us to detect reductions in nutrient storage despite enhanced substrate nutrient content. The reductions in nutrient storage pools occurred at low to moderate N and P concentrations relative to those found in our study region, as well as globally (Scott et al. 2002; Woodward et al. 2012; Manning et al. 2020). Overall, the effects of the streamwater N : P gradient we document here suggest that particulate nutrient storage and transport dynamics are likely altered in many headwater streams affected by current and historical nutrient enrichment, thus, potentially changing the processing of N and P within the freshwater pipe (Maranger et al. 2018).

Changes in the temporal dynamics of nutrient storage also likely affect the feeding ecology and life-history characteristics of macroinvertebrate consumers. Macroinvertebrate production increased in our treatment streams, an effect that was most strongly associated with increases in leaf litter %P (Demi et al. 2018). The effects of enrichment were observed primarily for shredder and scraper feeding groups, with more subdued effects for predators. Taxon-specific effects of enrichment showed that enrichment effects played out positively for taxa that exploited higher-nutrient resources in autumn and winter, but that other taxa responded negatively to enrichment. Negative effects of enrichment in the autumn-winter were seen in taxa with longer larval lifespans (Demi et al. 2019), suggesting that a temporally consistent supply of resources might be particularly important for long-lived taxa (Siders et al. 2018). Furthermore, the enrichment period of our study ran for only 2 yr; a longer enrichment experiment increased production of primary consumers dramatically, while decreasing production of predators, especially that of longer-lived taxa (Davis et al. 2010, 2011). Thus, the reduced capacity to store both C and nutrients may have negative long-term consequences for organisms in these detritus-based ecosystems.

Enrichment-driven reductions in nutrient storage pools are likely most important in shaded streams like ours, which are dependent on seasonal pulses of leaf litter. However, detritus-based streams make up a significant portion of many river networks. Our results may be relevant for up to fourth-order rivers within basins such as the Little Tennessee River in North Carolina, where our study was conducted (*see* Rosemond et al. 2015). Moreover, headwater streams play important roles in the retention and transformation of nutrients (Bernhardt et al. 2003; Alexander et al. 2007). Thus, changes in their nutrient storage capacity could affect the timing and form (organic vs. inorganic) of nutrient transport to downstream (and ultimately marine) ecosystems (Bernhardt et al. 2003; Webster et al. 2016; Maranger et al. 2018).

Data availability statement

Data presented in this paper are available through Zenodo and open access at: https://doi.org/10.5281/zenodo.7915947

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Conflict of Interest

None declared.

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