The influence of land use on stream biofilm nutrient limitation across eight North American ecoregions

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Abstract: Nutrient diffusing substrata were used to determine the influence of inorganic nitrogen (N) and phosphorus (P) availability on community respiration (CR), gross primary production (GPP), and chlorophyll *a* (chl *a*) on inorganic and organic substrata. We incubated substrata in nine streams each in a total of eight ecoregions (n = 72 streams) located in a range of native vegetation, agriculture, and urban land-use types. On organic substrata, CR was nutrient-limited in 94% of reference streams but showed significant nutrient limitation in only 60% and 65% of agricultural and urban streams, respectively. The relative magnitude of nutrient limitation for CR on organic substrata decreased with increasing percent modified land use in the basin (agriculture + urban). On inorganic and organic substrata, GPP and chl *a* were rarely nutrient-limited across all ecoregions and land-use types, although the magnitude of nutrient limitation increased with increasing light availability. The effect of human land use on nutrient limitation of biofilm CR, GPP, and chl *a* was influenced by ecoregion, yet heterotrophic biofilms were consistently most sensitive to nutrient enrichment across ecoregions. Both heterotrophic and autotrophic biofilm constituents should be considered to fully understand stream ecosystem responses to nutrient enrichment.

Résumé : Nous avons utilisé des substrats diffuseurs de nutriments pour déterminer l'influence de la disponibilité de l'azote (N) et du phosphore (P) inorganiques sur la respiration de la communauté (CR), la production primaire brute (GPP) et la chlorophylle *a* (chl *a*) sur des substrats inorganiques et organiques. Nous avons incubé des substrats dans neufs cours d'eau dans chacune d'un ensemble de huit écorégions (n = 72 cours d'eau) situés dans une gamme de végétations indigènes, de types d'agriculture et d'utilisatiosn urbaines des terres. Sur les substrats organiques, CR est limitée par les nutriments dans 94 % des cours d'eau témoins, mais elle ne montre de limitation significative par les nutriments que dans respectivement 60 % et 65 % des cours d'eau agricoles et urbains. L'importance relative de la limitation de CR par les nutriments sur les substrats organiques diminue en fonction de l'augmentation du pourcentage d'utilisation de terres modifiées (agriculture + urbanisation) dans le bassin versant. Sur les substrats inorganiques et organiques, GPP et chl *a* sont rarement limitées par les nutriments sur l'ensemble des écorégions et dans les différents types d'utilisation des terres, bien que l'importance de la limitation par les nutriments augmente en fonction de la disponibilité de la lumière. L'effet de l'utilisation des terres par les humains sur la limitation par les nutriments de CR, GPP et chl *a* de biofilms varie selon l'écorégion; néanmoins, les biofilms hétérotrophes sont régulièrement plus sensibles à un enrichissement en nutriments dans toutes les écorégions. Il faut tenir des constituants hétérotrophes et autotrophes des biofilms si l'on veut comprendre entièrement la réaction des écosystèmes d'eau courante à l'enrichissement en nutriments.

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Introduction

Benthic biofilms in streams influence ecosystem function via their role in primary production, heterotrophic production, nutrient spiraling, and organic matter decomposition (Cummins 1974). Autotrophic constituents of biofilms fix carbon (C) from the atmosphere, whereas heterotrophs use organic carbon for biosynthesis and growth (Schlesinger 1997). Although biofilm autotrophs and heterotrophs play unique roles in streams, their growth is often regulated by similar factors, including susceptibility to scour from flooding (Biggs and Close 1989; Ryder et al. 2006), temperature (Suberkropp and Chauvet 1995; Francoeur et al. 1999), and nutrient availability (Borchardt 1996; Gulis et al. 2004). Autotrophs are also influenced by light availability (Hill 1996) and heterotrophs can be regulated by substrate (i.e., carbon) quality (Peterson et al. 1993, Gessner and Chauvet 1994).

Nitrogen (N) or phosphorus (P) availability may limit growth of both heterotrophs and autotrophs (Pringle et al. 1986; Tank and Webster 1998). Biofilm autotrophs can be

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N-limited, P-limited, co-limited by N and P, or not limited by nutrients at all (Tank and Webster 1998; Francoeur 2001; Tank and Dodds 2003), and heterotrophs have an equally wide range of responses to nutrient amendment (Hepinstall and Fuller 1994; Tank and Webster 1998; Tank and Dodds 2003). Most importantly, autotrophs and heterotrophs often have different nutrient limitation status when directly compared with each other in the same system (Tank and Dodds 2003), which may reflect physiological differences in nutrient demand. Therefore, both autotrophic and heterotrophic biofilms are potentially sensitive to nutrient availability, and conditions that affect nutrient availability could have a strong and differential effect on nutrient limitation of autotrophic versus heterotrophic biofilms.

Human land use often leads to elevated nutrient concentrations (Carpenter et al. 1998; Kemp and Dodds 2001; Walsh et al. 2005). Agriculture increases nitrate (NO_3^-) concentrations as a result of excess fertilizer application (Howarth et al. 1996; Royer et al. 2004). Urban land use tends to increase ammonium (NH_4^+) and dissolved P concentrations from sewage and septic inputs and lawn fertilizer applications (Paul and Meyer 2001; Walsh et al. 2005). The type and intensity of land use within broad categories (e.g., agriculture, urbanization) is variable and therefore so is its effect on stream ecosystems (Allan 2004). For example, row-crop agriculture can result in extensive channel modifications and nutrient enrichment of stream ecosystems, whereas low-intensity agricultural livestock production in pastures can have a much less pronounced effect (Strayer et al. 2003; Allan 2004). Nutrient input to urbanized streams is generally correlated with population density and impervious surface area (Paul and Meyer 2001; Meyer et al. 2005; Walsh et al. 2005), which are both factors that vary dramatically across the landscape. We expect that autotrophic and heterotrophic nutrient limitation will also vary based on the intensity of human land use.

Variation in nutrient availability can also be driven by regional differences in climate, physiography, and vegetative cover (i.e., ecoregions; Bailey 1976; Omernik 1977), potentially translating into variation in nutrient limitation (Smith et al. 2003). For example, southwestern US streams frequently have low inorganic N concentrations and are subsequently N-limited (Grimm and Fisher 1986). Furthermore, Tank and Dodds (2003) found that biofilm nutrient limitation varied across ecoregions. Regional differences in background nutrient concentrations influence stream ecosystem management (Dodds and Welch 2000; Smith et al. 2003; Suplee et al. 2007). However, we are not aware of studies that have analyzed ecoregions and human land use effects on nutrient limitation across a broad geographic scale.

Our goal was to compare nutrient limitation of heterotrophic and autotrophic stream biofilms among a wide range and intensity of human land use, as well as among ecoregions of North America. Using nutrient diffusing substrata, we quantified N and P limitation across eight ecoregions in nine streams per ecoregion, three from one of each adjacent land-use type: native vegetation (or reference), agriculture, and urban (n = 72 streams total). We compared nutrient limitation of autotrophic and heterotrophic biofilms on two different substratum types (inorganic vs. organic), which served as surrogates for equivalent stream substrata such as cobble (inorganic) and leaves and wood (organic). We predicted that biofilm nutrient limitation in reference streams would differ based on ecoregion, substratum type, and biofilm type, and across all ecoregions, the intensity of human land use would influence autotrophic and heterotrophic biofilm nutrient limitation. These data from 72 streams greatly expand our understanding of how land use may influence biofilm nutrient use and, by extension, factors controlling stream ecosystem function across different ecoregions.

Materials and methods

Sites

Nutrient limitation was assessed via nutrient diffusing substrata (NDS) in 72 streams as part of the Lotic Intersite Nitrogen eXperiment II (LINXII), a large collaborative project examining the influence of land use on stream NO_3^- cycling. Nine headwater streams were selected in eight ecoregions across the US and Puerto Rico encompassing a wide range of climatic conditions (Table 1; for site map, also see Mulholland et al. 2008). For this study, ecoregions were abbreviated in the results using two-letter state postal codes except for the southwest (SW), which included streams in Arizona and New Mexico.

In each ecoregion, three streams were selected to span a range of three land-use types: native vegetation (or reference), agriculture, and urban. Land use was broadly categorized and initially based on visual observations of the riparian land use immediately adjacent to the study reaches (Table 1). Reference streams were bordered by native vegetation of the specific ecoregion including forest, prairie, and desert shrub land, with a coverage in the basin generally >85% (except two streams with 50% and 65%). Agricultural land use in stream basins was highly variable, ranging from row-crop agriculture with associated high fertilizer application to pastures with low-intensity cattle grazing, with a range in basin coverage from 4% to 100%. Urban land use was also variable, consisting of highly urbanized streams with cemented channels, residential–suburban streams, parkland, and even recreational (e.g., golf course) land use, with a range in coverage from ~1% to 100%. After site selection, the percent land cover of each land-use type for each basin was determined using the US Geological Survey (USGS) National Elevation Data Set and the 2001 USGS National Land Cover Datasets and for PR using the 1991–1992 Landsat TM imagery as derived by Helmer et al. (2002). Streams within each land-use category in each region were not considered replicates, but instead span a typical range representative of that land-use type in the ecoregion. Additionally, selection of agricultural and urban streams was not necessarily intended to represent dominance of that land-use type within the basin, and many basins consisted of mixed land uses. As part of the larger LINXII study, three streams (one from each land-use type) were typically studied in each of the eight ecoregions per year (n = 27 per year) for a total of three years (2003–2005, n = 72 streams in total) during base-flow conditions.

Nutrient diffusing substrata

NDS were constructed using 30 mL plastic cups filled with a 2% agar solution amended with 0.5 mol·L^{-1} $NaNO_3$ (N treatment), 0.5 mol·L^{-1} KH_2PO_4 (P treatment), both (N + P treatment), or no amendment as a control (C treatment; Tank et al. 2006). NDS were topped with either inorganic fritted glass disks or organic cellulose sponge cloth totaling five replicates for each treatment for each substratum type. Inorganic and organic substrata were chosen to examine nutrient limitation of a typical range in types of biofilms found in natural stream ecosystems. NDS were placed in riffles that were located in reaches representative of ambient light and flow conditions in each stream (as reported in Table 1). For example, if a stream was mostly shaded, NDS were placed in a shaded location, if a stream was open, then open conditions were sought for NDS placement. NDS were deployed in each stream for 17 days to ensure that nutrients continued to diffuse from the NDS throughout the entire incubation period (Tank et al. 2006). Prior work demonstrated that 17 days was ample time for development of a significant biofilm response in reference streams. NDS were then retrieved from streams, and substrata were removed from plastic cups and immediately placed in 50 mL centrifuge tubes filled with stream water. After transport to the laboratory, substrata were analyzed for autotrophic activity by measuring gross primary production (GPP) via dissolved oxygen (DO) production, autotrophic biomass using chlorophyll *a* (chl *a*) extraction, and heterotrophic community respiration (CR) via dissolved oxygen (DO) consumption (see methods described below).

In 2003, we quantified chl *a* on inorganic substrata and CR on organic substrata. We expanded the methods in 2004 and 2005 to include GPP and measured all three metrics (GPP, chl *a*, and CR) on both substrata, allowing us to confirm selection of autotrophic and heterotrophic biofilms by inorganic and organic substrata, respectively. We measured GPP along with chl *a* as indicators of autotrophic nutrient limitation. GPP may indicate more directly the limitation of ecosystem function, because it is a measure of autotrophic activity and state rather than solely autotrophic biomass (chl *a*), which can vary based on parameters other than nutrients (e.g., grazing, light availability).

GPP and CR were measured using a modified light–dark bottle method in which net community metabolism (NCM) was measured in the light, CR was measured in the dark, and GPP was calculated by adding NCM and CR (American Public Health Association (APHA) 1995; Hill et al. 2002). Centrifuge tubes containing substrata retrieved from NDS were refilled with fresh ambient water from each stream taking care to exclude all air bubbles from tubes. First, NCM was measured as the difference in DO after a 3 h incubation on a shaker table under a wide-spectrum fluorescent grow lamp (1900 lm, photosynthetically active radiation (PAR) = 35 μmol·m^{-2}·s^{-1}; model GE F40PL/AQ, The General Electric Company, Fairfield, Connecticut). We measured NCM with a grow lamp to maintain similar and consistent light conditions across all samples to compare nutrient limitation across biofilms from a variety of streams. Given potential differences in the response of GPP to the constant PAR conditions used during the incubations, we also calculated quantum yield (mol O_2·mol quanta^{-1}) by dividing GPP by incubation PAR to more accurately examine relationships with in situ light availability. Because the experimental PAR level was unlikely to be saturating (Dodds et al. 1999), this strategy allowed for comparison of photosynthetic efficiency independent of variation of in situ light across measurements.

Then, CR was measured using the consumption of DO after a 2 h incubation in the dark with gentle continuous mixing on a shaker table. The same substratum was used for both NCM and CR measurements, but fresh stream water was used for each analysis to maintain ambient nutrient availability and a starting point close to saturating DO concentrations. Following metabolism estimates, chl *a* was extracted from the same substratum using the hot ethanol method (Sartory and Grobbelaar 1984). Substrata were extracted in 95% ethanol, heated to 79 °C for 5 min, and then cooled for 24 h at 4 °C. Following centrifugation, chl *a* in the extract was measured using the nonacidification method on a fluorometer set to avoid interference from phaeophytin (Welschmeyer 1994).

Numerous descriptor variables were quantified in each stream as part of the larger LINXII experimental protocol, which occurred at or near the time of NDS placement, confirming that streams both within and across ecoregions were diverse (Table 1; Mulholland et al. 2008). NO_3^- concentrations were measured using ion chromatography or colorimetry, NH_4^+ concentrations were measured using indophenol colorimetry or fluorometry, and soluble reactive phosphorus (SRP) concentrations were measured using molybdate-blue colorimetry (APHA 1995; Holmes et al. 1999; Taylor et al. 2007). Light availability as percent channel shade was measured at each stream using a canopy densiometer, and PAR was measured every minute for three representative days of the experiment.

Calculations and statistical analyses

Nutrient limitation status (a categorical designation) for each response metric (GPP, CR, or chl *a*) on each substratum type (inorganic or organic) was determined using a two-way analysis of variance (ANOVA) based on the presence or absence of N and (or) P in the agar. For this ANOVA, we used N and P as the main factors, and significant interaction terms determined instances of co-limitation or secondary limitation by N and P (interpreted as in Tank and Dodds 2003). In addition to determining if there was significant nutrient limitation by N or P, we calculated the nutrient response ratio (NRR) as the ratio of the N + P treatment over the control treatment for CR on organic substrata and GPP and chl *a* on inorganic substrata (Francoeur 2001; Tank and Dodds 2003). Thus, a NRR >1 would indicate a positive response of either GPP or CR to added nutrients (N + P treatment > control). Using NRR in addition to nutrient limitation status allowed us to compare the magnitude of nutrient limitation on a relative scale across streams and ecoregions and relate this to other predictor variables used as ecoregion and stream descriptors (see Table 1; Mulholland et al. 2008).

We used a one-way analysis of covariance (ANCOVA) to examine the effect of ecoregion on stream characteristics and NRR for all response metrics with percent modified land use (percent agriculture + urban) in the catchment as the covariate followed by Tukey's post-hoc comparisons to

Table 1. Study stream locations, land-use classifications, and physical and chemical characteristics.

Site	Ecoregion (Omernik 1987)	Stream name	Land-use classification	Catchment area (km^2)	Discharge ($L \cdot s^{-1}$)	NO_3^- ($\mu g\ N \cdot L^{-1}$)	NH_4^+ ($\mu g\ N \cdot L^{-1}$)	SRP ($\mu g\ P \cdot L^{-1}$)	PAR (mol quanta·$m^{-2} \cdot day^{-1}$)	Percent channel shade
MA	Eastern temperate forest: Northeastern Coastal Zone	Cart Creek	REF	3.9	4.8	15.3	293.2	1.5	1.2	76
		Boxford	REF	1.6	12.2	52.8	13.3	9.2	0.5	86
		Gravelly Brook	REF	5.6	2.0	112.2	435.4	80.3	0.7	7
		Runaway Brook	AGR	0.4	0.7	1 164.2	80.2	7.8	19	0
		Long Meadow Brook	AGR	1.0	2.4	989.3	63.1	10.8	4.5	26
		Black Brook	AGR	6.1	120.1	50.3	30.5	33.5	19.9	20
		IS_104	URB	1.2	2.1	1 336.2	121.2	2.1	1.2	91
		Sawmill Brook	URB	4.2	4.9	1 024.7	39.2	11.5	0.2	85
		IS_118	URB	2.1	11.3	512.8	253.8	11.8	9.7	65
NC	Eastern temperate forest: Blue Ridge Mountains	Hugh White Creek	REF	0.3	19.4	7.3	3.2	2.8	0.2	93
		Big Hurricane Branch	REF	0.6	12.2	240.7	5.6	2.5	1.7	93
		Cunningham Creek	REF	1.1	49.3	10.1	2.7	1.9	2.9	94
		Hoglot Branch	AGR	2.7	52.7	154.5	17.1	2.8	25.8	41
		Jerry Branch	AGR	2.6	26.5	405.7	108.2	18.2	6.9	41
		Blacks Branch	AGR	3.6	189.4	172.6	8.5	6.7	29.6	ND
		Crawford Branch	URB	3.0	45.0	102.5	15.4	4.3	2.3	90
		Mud Creek	URB	4.8	51.8	139.5	6.0	2.1	39.4	57
		Sugarloaf Creek	URB	3.7	79.8	54.2	2.6	2.9	ND	96
MI	Eastern temperate forest: Southern Michigan – Northern Indiana Till Plains	Sand Creek	REF	1.1	4.9	282.6	54.8	14.9	1.5	81
		Bullet	REF	3.6	6.5	384.5	11.0	2.3	9.2	42
		Honeysuckle	REF	5.1	99.4	4.2	21.1	3.7	30.3	91
		Steinke Drain	AGR	3.0	1.7	4 158.3	29.4	68.1	21.5	ND
		Buskirk	AGR	2.8	6.0	81.5	20.7	11.3	37.6	65
		Bellingham	AGR	2.9	22.9	1452.9	27.9	1.7	20.9	8
		Dorr	URB	1.9	35.0	1 100.2	127.7	9.2	11.1	ND
		Wayland	URB	2.7	11.7	694.6	74.3	5.4	ND	52
		Arcadia	URB	32.6	110.1	273.5	32.0	10.7	51	0
KS	Great plains: Flint Hills	Kings Creek N4D	REF	1.6	13.4	8.6	0.3	0.5	32.1	39
		K2A	REF	3.3	26.3	0.9	6.7	1.9	22.7	37
		Shane Creek	REF	4.4	4.4	1.2	4.7	1.0	51.7	57
		Agnorth	AGR	5.0	0.2	34.8	31.7	0.2	56	15
		Natalie Creek	AGR	0.9	1.3	6.0	3.1	2.4	29.5	44
		Swine	AGR	10.1	5.4	21 162.3	3.4	16.2	58.8	11
		Campus Creek	URB	1.7	2.9	2 942.0	7.8	4.0	49.8	71
		Walmart Ditch	URB	7.2	1.6	277.4	28.3	35.4	56.1	0
		Little Kitten	URB	5.6	20.1	167.6	24.2	7.2	52.4	76

Table 1 (*concluded*).

Site	Ecoregion (Omernik 1987)	Stream name	Land-use classification	Catchment area (km^2)	Discharge ($L \cdot s^{-1}$)	NO_3^- ($\mu g\ N \cdot L^{-1}$)	NH_4^+ ($\mu g\ N \cdot L^{-1}$)	SRP ($\mu g\ P \cdot L^{-1}$)	PAR (mol quanta·$m^{-2} \cdot day^{-1}$)	Percent channel shade
WY	North American deserts: Middle Rockies or Wyoming Basin	Ditch	REF	74.0	55.7	0.1	1.7	2.4	ND	19
		Two Oceans	REF	29.7	64.5	18.9	3.8	10.0	ND	3
		Spread	REF	260.4	267.8	2.8	2.2	ND	56.7	17
		Giltner	AGR	1.2	158.5	49.7	3.0	2.8	ND	0
		Headquarters	AGR	1.3	131.1	0.7	2.9	15.3	69.9	1
		Kimball	AGR	103.0	153.8	27.9	1.1	4.4	55.9	0
		Golf	URB	1 605.7	110.0	0.8	1.0	2.1	ND	39
		Teton Pines	URB	0.03	9.5	152.1	1.0	3.2	25.3	39
		Fish	URB	8.8	102.9	234.7	4.3	5.9	60.9	0
SW	North American deserts: Arizona – New Mexico Plateau	Agua Fria	REF	645.5	11.9	0.4	1.5	55.9	69.9	13
		Rio Salado	REF	3 410.8	5.8	3.8	3.6	2.5	56	0
		Sycamore Ck	REF	230.8	21.3	57.5	2.0	31.8	57.8	ND
		Bernalillo drain	AGR	0.8	23.5	1.5	1.8	36.7	69.2	0
		Rio Puerco	AGR	16 019.7	2.5	3.6	3.9	13.6	47.6	ND
		San Pedro	AGR	92.5	4.0	297.2	4.4	18.9	65.2	0
		Rio Rancho	URB	2.8	17.8	12.5	3.4	50.2	61.7	1
		Indian Bend Wash	URB	453.8	28.4	99.2	65.2	20.9	2.9	ND
		Tempe Town lake	URB	889.4	18.0	4.3	9.8	25.2	52.2	0
OR	Northwestern forested mountains: Cascades or Willamette Valley	Oak	REF	6.2	7.5	70.5	1.3	34.7	1.4	92
		Mack	REF	5.3	30.7	62.7	5.9	13.0	2	56
		Potts	REF	3.5	19.0	69.3	4.0	24.5	4	88
		Oak	AGR	30.5	5.5	96.0	8.4	47.8	2.7	75
		Camp	AGR	26.8	113.4	54.2	6.1	5.3	21.3	69
		Courtney	AGR	41.7	34.7	96.6	10.6	5.0	26.4	27
		Oak	URB	32.2	5.6	162.7	19.3	45.3	2.6	82
		Amazon	URB	21.8	25.0	2.4	4.8	17.8	39.4	49
		Periwinkle	URB	10.3	2.7	7.7	4.4	208.5	37.6	1
PR	Tropical wet forest	Bisley	REF	0.8	12.5	170.7	2.6	21.5	0.2	88
		RIT	REF	0.3	20.0	130.8	7.2	0.2	0.5	17
		Q. Pared	REF	0.8	5.2	104.7	2.5	7.3	1.3	57
		Grande	AGR	0.7	12.3	275.8	11.1	13.2	6.4	24
		Maizales	AGR	2.6	25.0	205.6	7.1	11.5	12.4	10
		Q. Vaca	AGR	1.7	111.9	445.7	2.6	8.7	1.3	57
		Petunia	URB	1.4	4.7	997.1	15.1	26.3	0.03	89
		Mtrib	URB	1.3	23.2	174.3	2204.3	310.5	0.7	65
		Q. Ceiba	URB	5.2	49.5	511.7	50.3	21.5	1.6	50

Note: Physical characteristics and photosynthetically active radiation (PAR) were calculated during the 24 h ^{15}N tracer experiment, and chemical characteristics were the mean from sampling dates up to a week after the experiment. SRP, soluble reactive phosphorus; ND, no data were available.

examine specific pairwise differences in ecoregion. We also used simple linear regression (SLR) to identify controls on NRR. Paired *t* tests were used to examine differences in CR, GPP, and chl *a* between inorganic and organic substrata. Statistical analyses were performed using SYSTAT 11 (Systat Software, Richmond, California), and statistical significance was determined at the $\alpha = 0.05$ level. To meet the assumptions of parametric statistics, we tested the data using a KS–Lilliefors test for normality ($p > 0.05$) and then transformed non-normal data using logarithmic (CR, all NRR, QY, and NH_4^+ concentrations), arcsine square root (percent channel shading, percent modified land use), or power (GPP, chl *a*, PAR) transformations.

Results

Site characteristics

Across ecoregions and land use, our streams encompassed a wide range in water chemistry characteristics (NO_3^-, NH_4^+, and SRP concentrations) and light availability (PAR and percent channel shade; Table 1). NO_3^- concentrations ranged from 0.1 to 21 162 μg $N \cdot L^{-1}$, with significantly higher concentrations in PR compared with the SW and WY ecoregions (one-way ANCOVA, $p < 0.001$). Additionally, NO_3^- concentrations significantly increased with increasing percent modified land use in the stream basin (one-way ANCOVA, $p < 0.001$), but the strength of this relationship depended on ecoregion (one-way ANCOVA, interaction $p < 0.001$). Similarly, NH_4^+ (range 0.3–2204 μg $N \cdot L^{-1}$) and SRP (range 0.2–311 μg $P \cdot L^{-1}$) concentrations increased with greater percent modified land use (one-way ANCOVA, NH_4^+ $p = 0.003$, SRP $p = 0.007$), but there were no significant differences by ecoregion (one-way ANCOVA, $p > 0.05$). Channel shade and PAR also differed by ecoregion (one-way ANCOVA, $p < 0.001$), with higher PAR in KS, SW, and WY compared with MA and PR and lower percent channel shade in SW and WY compared with OR, PR, and NC. Additionally, percent channel shade decreased with increasing percent modified land use (one-way ANCOVA, $p = 0.02$), but we found no relationship between PAR and percent modified land use (one-way ANCOVA, $p > 0.05$).

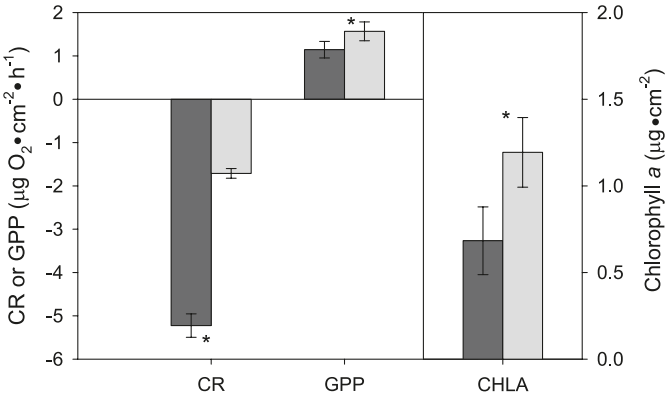
Selection of autotrophs and heterotrophs by substratum type

When combining NDS data across all nutrient treatments for each of our three response metrics (CR, GPP, and chl *a*), we found differences in biofilm function by substratum type (Fig. 1). CR was significantly higher on organic substrata compared with inorganic substrata (paired *t* test, $p < 0.001$), and the absolute value of CR was greater than GPP. In contrast, on inorganic substrata, GPP and chl *a* (autotrophic biomass) were significantly higher than on organic substrata (paired *t* test, GPP $p = 0.028$, chl *a* $p < 0.001$), and the absolute value of CR was approximately equal to GPP. Based on our response metrics (GPP and CR), organic substrata tended to be more heterotrophic than inorganic substrata, which tended to be more autotrophic.

Nutrient limitation of community respiration

Heterotrophic activity (CR) on organic substrata across all land-use types was frequently nutrient-limited, but there was no prevalence of N versus P limitation (Fig. 2). Considering reference streams alone, CR was most frequently N-limited, and when combined with P limitation and co-limitation by N + P, CR was nutrient-limited in all but one stream. In contrast to reference streams, CR was not nutrient-limited in a majority of agricultural and urban streams. Interestingly, the urban streams that were nutrient-limited showed predominantly P limitation, and more urban streams exhibited P limitation than reference streams.

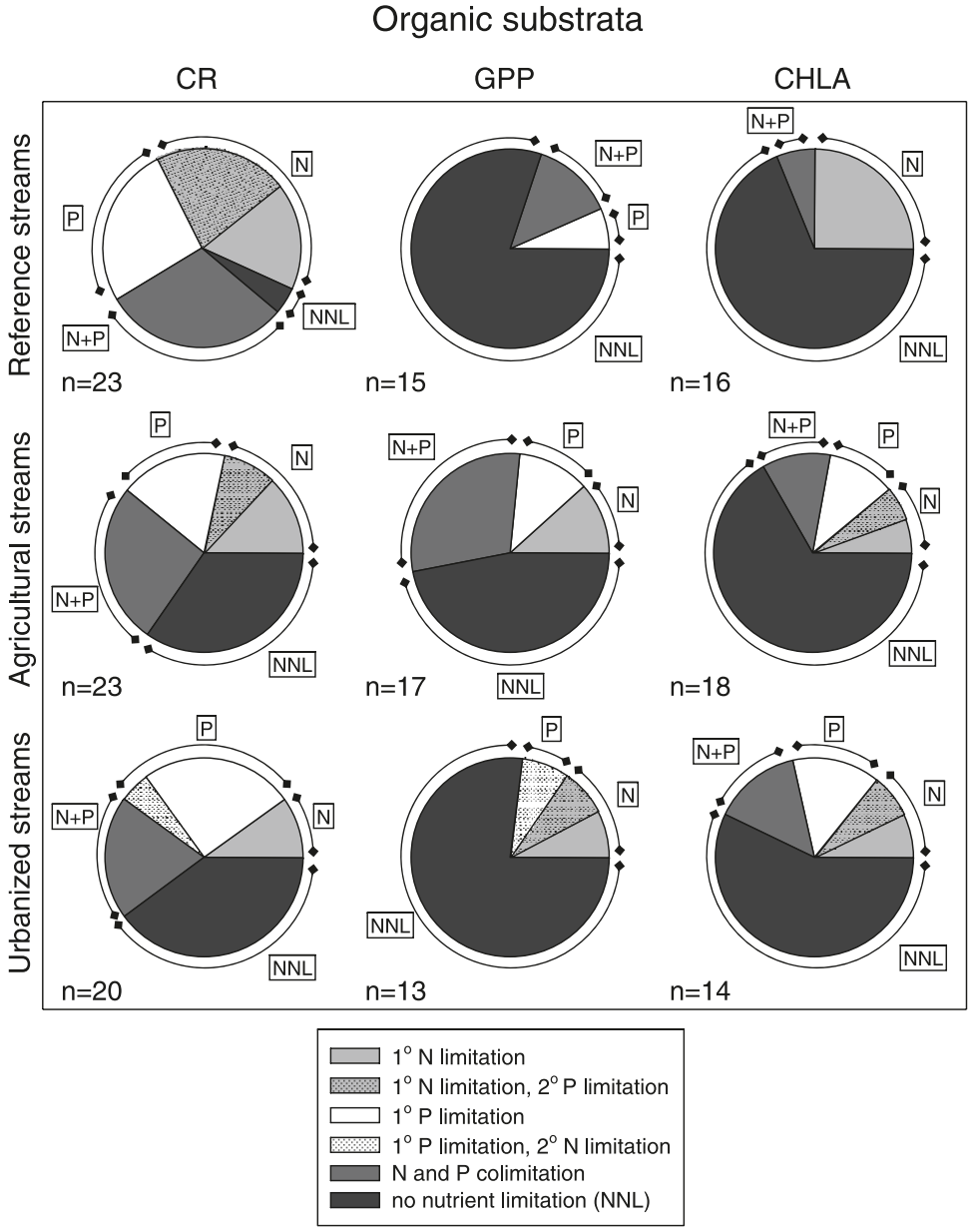
Fig. 1. The effect of organic (bars with dark shading) and inorganic (bars with light shading) substrata on community respiration (CR), gross primary production (GPP), and chlorophyll *a* (CHLA). Means ± standard error (SE) are reported. Asterisks (*) above bars represent significant differences ($p < 0.05$) between inorganic and organic substrata as determined by paired *t* test on transformed data. *P* values, data transformations, and *n* for each response were as follows: CR, $p < 0.001$, $\log(x + 1)$, $n_{organic} = 68$, $n_{inorganic} = 50$; GPP, $p = 0.028$, $x^{1/3}$, $n_{organic} = 46$, $n_{inorganic} = 50$; CHLA, $p < 0.001$, $x^{1/5}$, $n_{organic} = 49$, $n_{inorganic} = 72$.



Because CR was significantly higher on organic substrata than on inorganic substrata (Fig. 1), we examined factors that influenced the nutrient response ratio (NRR) for CR on organic substrata. Within ecoregions and pooling data across land-use types, the largest response to N + P addition for CR on organic substrata was in WY, the lowest was in KS and MA, and the remaining ecoregions had similar NRRs (Fig. 3*a*; one-way ANCOVA, $p = 0.001$). By pooling data across ecoregions, we found that NRR decreased as the percent modified land use (percent agriculture + percent urban) in the catchment increased; however, little variation in NRR was explained by percent modified land use ($r^2 = 0.143$; Table 2). Upon further investigation, we found differential effects of land use depending on ecoregion (one-way ANCOVA, $p = 0.011$). For example, there was a strong effect of land use on NRR (>45% decline in NRR from reference to agriculture or urban) in KS and NC, but only a moderate effect (10%–45% NRR decline) in MI and WY and only a weak effect (<10% NRR decline; Table 3) in MA and PR. Finally, for CR on organic substrata, we analyzed drivers of NRR and found that CR was relatively less nutrient-limited as stream water ammonium (NH_4^+) availability increased (Table 2).

CR was much less frequently nutrient-limited on inorganic substrata than on organic and was not nutrient-limited in 83% of all streams (Fig. 4). CR may have been limited by carbon availability across all streams because inorganic sub-

Fig. 2. Nutrient limitation status of community respiration (CR), gross primary production (GPP), and chlorophyll *a* (CHLA) on organic substrata in reference (native vegetation), agricultural, and urbanized streams. Primary (1°) and secondary (2°) nutrient limitation status, as defined in the key, was determined by a significant response to nutrient treatment: N alone, P alone, and N and P added together (analysis of variance, ANOVA, $p < 0.05$).

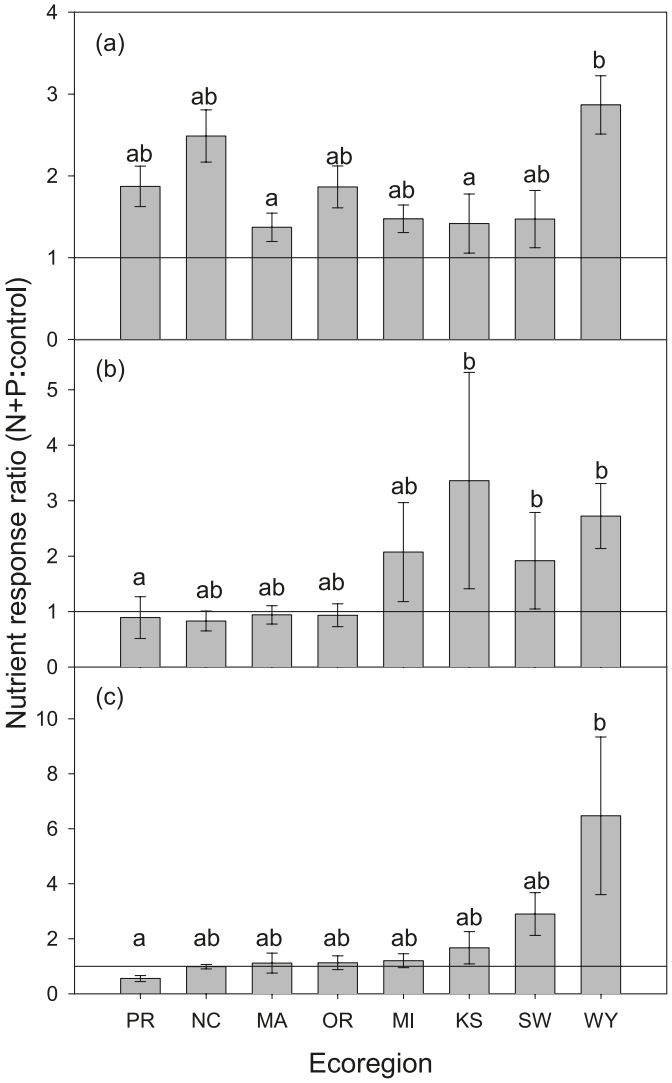


strata did not provide an outside source of carbon to heterotrophs. In the infrequent cases of nutrient limitation, reference streams were never P-limited and urban streams were never N-limited, whereas agricultural streams had cases of N limitation, P limitation, and co-limitation of N + P. Overall, even with land use, CR on inorganic substrata was rarely limited by N or P.

Nutrient limitation of gross primary production

In general, across all land-use types, we found that GPP on organic substrata was less frequently limited by N or P compared with CR and was not nutrient-limited in 67% of all streams (Fig. 2). In reference streams, we found that GPP was never N-limited, and significant nutrient limitation of GPP by N or P was only found in the SW and NC sites. In contrast to what we saw for CR, agricultural land use increased rather than decreased the proportion of streams with nutrient limitation of GPP. Agricultural land use often resulted in reduced channel shading (Table 1), which likely alleviated light limitation, resulting in higher primary production and nutrient demand, increasing the likelihood of significant nutrient limitation of autotrophs. Further, we found that quantum yield (QY, GPP normalized to PAR levels used during assay incubations) on organic substrata increased with in situ light availability as both increasing PAR (data not shown; simple linear regression, $r^2 = 0.121$, $p = 0.02$) and decreasing percent channel shading (data not shown; simple linear regression, $r^2 = 0.20$, $p = 0.003$). Un-

Fig. 3. The nutrient response ratio across ecoregions for (*a*) community respiration (CR) on organic substrata, (*b*) gross primary production (GPP) on inorganic substrata, and (*c*) chlorophyll *a* on inorganic substrata calculated as the ratio of N + P treatments to control treatments, where a ratio of one indicates no nutrient limitation. Means ± standard error (SE) are reported. Bars with different letters represent significant differences among ecoregions as determined by analysis of covariance (ANCOVA) with percent modified as the covariate followed by Tukey's post-hoc comparisons test on log-transformed data ($p < 0.05$).



like agricultural land use, urbanization did not increase the frequency of nutrient limitation, and the proportion of urban streams with no nutrient limitation of GPP by N or P was approximately the same as reference streams.

Similar to organic substrata, GPP on inorganic substrata was not nutrient limited by N or P in 75% of all streams pooled across land-use types (Fig. 4). In reference streams, significant nutrient limitation was slightly more frequent than when pooled across land-use types, and most of the reference streams with nutrient limitation of GPP were located in ecoregions with open canopies in native vegetation (i.e., WY, KS, and SW). Human land use reduced the frequency of nutrient limitation of GPP. In agricultural streams, GPP was never P-limited or co-limited by N + P; whereas in urban streams, GPP was never N-limited. Across land-use types, QY significantly increased with PAR and decreased with percent channel shading (data not shown; simple linear regression: PAR, $r^2 = 0.197$, $p = 0.002$; percent channel shading, $r^2 = 0.204$, $p = 0.002$), indicating that light availability influenced GPP, confounding the potential influence of nutrient limitation on biofilm autotrophs.

Because inorganic substrata had significantly higher GPP and chl *a* compared with organic substrata (Fig. 1), we examined the NRR for GPP on inorganic substrata to determine the magnitude of nutrient limitation across ecoregions and land-use types. Across ecoregions, the NRR for GPP was highest in open canopy systems in KS, SW, and WY and lowest in PR (Fig. 3*b*; one-way ANCOVA, $p = 0.002$), but the effect of percent modified land use on NRR was not significant (one-way ANCOVA, $p > 0.05$). As light availability appeared to influence nutrient limitation status, we examined the influence of PAR on the NRR of GPP and found that the magnitude of nutrient limitation increased as light became more available (Table 2).

Nutrient limitation of chlorophyll *a*

When pooled across land-use types, chl *a* on organic substrata was not nutrient-limited in 65% of all streams, similar to results for GPP on organic substrata (Fig. 2). In reference streams, we found that N limitation was most frequent, there was no significant P limitation of chl *a* on organic substrata, and reference streams where chl *a* was nutrient-limited were again located in ecoregions with open-canopied streams. In both agricultural and urban land use, chl *a* was rarely nutrient-limited, with an equivalent frequency of N limitation, P limitation, and N + P co-limitation. However, in contrast to reference streams, nutrient-limited agricultural and urban streams did not correspond to ecoregions with open canopies. Across land-use types, we found that chl *a* on organic substrata significantly increased with PAR and decreased with percent channel shading (data not shown; simple linear regression: PAR, $r^2 = 0.47$, $p < 0.001$; percent channel shading, $r^2 = 0.33$, $p < 0.001$). Therefore, light availability was a strong driver of algal biomass, potentially obscuring the effects of land use on nutrient limitation.

On inorganic substrata, when data from all land uses were pooled, chl *a* was not nutrient-limited by N or P in 75% of all streams (Fig. 4). In reference streams, chl *a* was never P-limited and was only nutrient-limited in ecoregions with open canopy streams. In comparison with reference streams, human land use slightly relieved nutrient limitation of chl *a*, and both agricultural and urban land use showed similar patterns of nutrient limitation. Similar to results for chl *a* on organic substrata, land use likely has a mixed effect on nutrient limitation (high light and nutrients), making it difficult to identify causal factors for the patterns in nutrient limitation of chl *a* biomass. Across land-use types, chl *a* significantly increased with PAR and decreased with percent channel shading (data not show; simple linear regression: PAR, $r^2 = 0.31$, $p < 0.001$; percent channel shading, $r^2 = 0.297$, $p < 0.001$), indicating again that light availability had a strong effect on chl *a* biomass on inorganic substrata.

We also compared NRR for chl *a* on inorganic substrata across sites and found that like GPP, NRR was highest in

Table 2. Significant simple linear regressions with the nutrient response ratio (NRR) of community respiration (CR) on organic substrata, gross primary production (GPP) on inorganic substrata, and chlorophyll *a* (chl *a*) on inorganic substrata.

Dependent variable	Independent variable	r^2	*p* value	*n*
CR on organic substrata (log-transformed)	NH_4^+ concentration (log-transformed)	0.177 (–)	0.001	62
CR on organic substrata (log-transformed)	% modified land use (% agriculture + urban, arcsin $x^{1/5}$-transformed)	0.143 (–)	0.003	62
GPP on inorganic substrata (log-transformed)	PAR ($x^{1/4}$-transformed)	0.105 (+)	0.035	41
Chl *a* on inorganic substrata (log-transformed)	PAR ($x^{1/4}$-transformed)	0.240 (+)	<0.001	65

Note: All data were transformed as indicated in parenthesis. The type of relationship, either positive or negative, is indicated in parenthesis by the r^2.

Table 3. Mean nutrient response ratio of community respiration (CR) on organic substrata for reference, agriculture, and urban streams from each site (±1 standard error, SE).

Site	Effect of land use	Reference	Agriculture	Urban
KS	Strong	2.43 (0.88)	1.02 (0.10)	0.80 (0.05)
MA	Weak	0.96 (0.29)	1.46 (0.21)	1.70 (0.21)
MI	Moderate	1.64 (0.27)	1.28 (0.12)	1.51 (0.47)
NC	Strong	3.62 (0.47)	1.94 (0.07)	1.91 (0.20)
OR	Strong–moderate	2.29 (0.08)	1.27 (–)	1.61 (–)
PR	Weak	1.80 (0.25)	1.68 (0.06)	2.13 (0.78)
SW	Moderate–weak	1.55 (0.06)	1.11 (0.12)	3.62 (–)
WY	Moderate	3.24 (0.75)	2.71 (0.42)	2.65 (0.82)

Note: Sites are classified by the effect of land use: strong indicates a >45% decrease in the NRR with land use, moderate indicates a 10%–45% decrease, and weak indicates a <10% decrease.

WY, lowest in PR, and approximately the same across all other sites (Fig. 3*c*; one-way ANCOVA, $p = 0.006$), but the effect of percent modified land use on NRR was not significant (one-way ANCOVA, $p > 0.05$). Similar to GPP, chl *a* NRR significantly increased with PAR (Table 2), and variation in NRR also increased with PAR, requiring log transformation to increase homoscedasticity. Therefore, the response of chl *a* to added nutrients on inorganic substrata was greatest when light availability was high, but increased variation during high light availability probably indicates a mixed effect of anthropogenic land use.

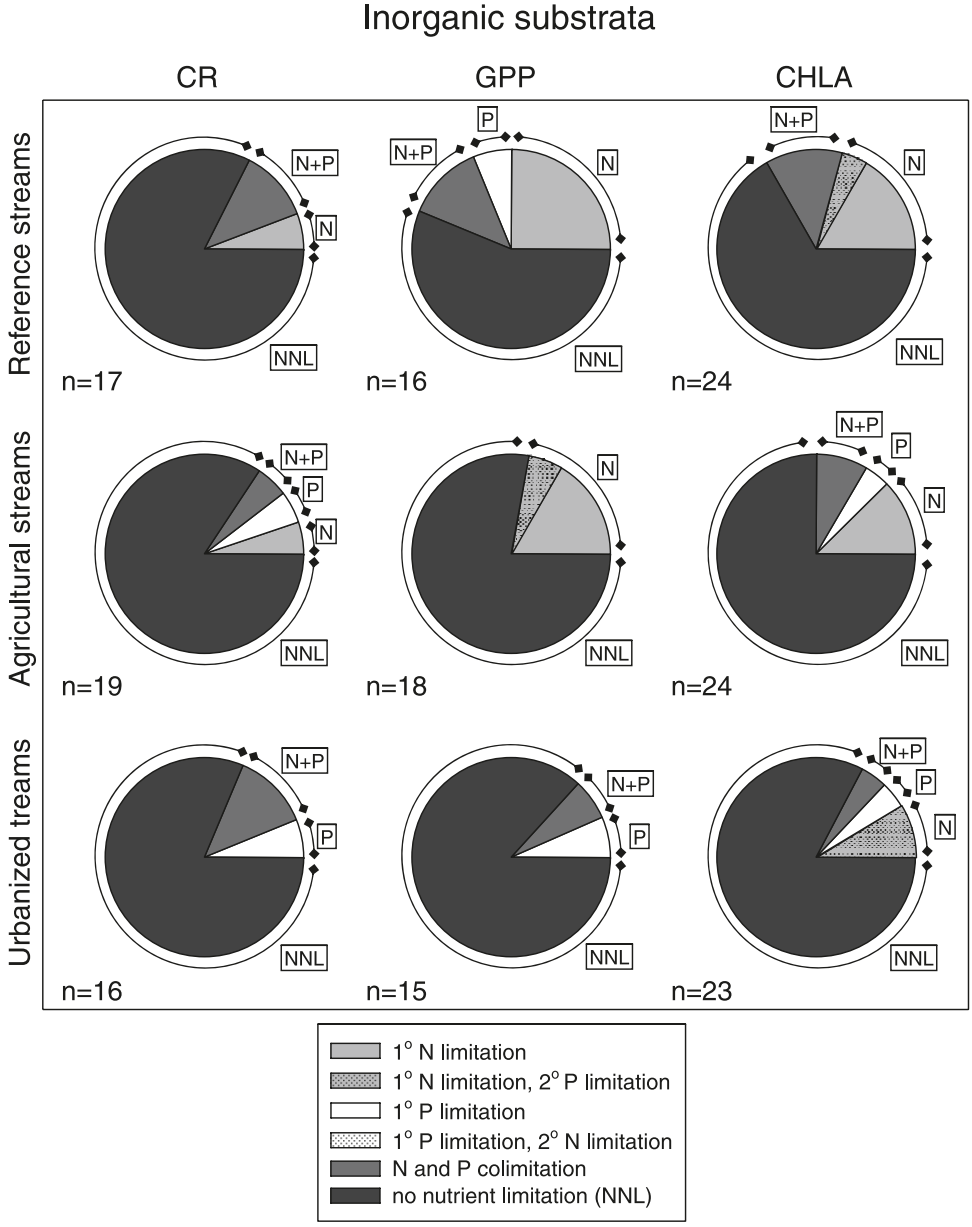
Discussion

Heterotrophic biofilms

Nutrient limitation of heterotrophic biofilms on organic substrata, as indicated by CR, was much more prevalent in reference streams than in agricultural and urban streams. We found significant nutrient limitation of heterotrophic biofilms in 23 out of 24 reference streams, which was a greater proportion than reported by Tank and Dodds (2003), who found that fungal biomass on wood veneers was nutrient-limited in six out of 10 relatively pristine streams across North America. There were two major differences between our results and those of Tank and Dodds (2003): (*i*) our process-related response metric (CR) was a more general heterotrophic response than their structural-based metric (ergosterol as an indicator of fungal biomass), and (*ii*) we used cellulose substrates, which were a more labile carbon source than their wood veneers. Several studies examining natural stream substrates have shown that leaf and wood breakdown (Meyer and Johnson 1983; Greenwood et al. 2007), leaf respiration (Stelzer et al. 2003), and fungal biomass (Grattan and Suberkropp 2001; Gulis et al. 2004) all increase with elevated nutrient concentrations in relatively pristine streams. Overall, these studies indicate that biomass and metabolism of heterotrophic biofilms are sensitive to water column nutrient concentrations and can potentially be influenced by changes in nutrient concentrations from natural or anthropogenic sources.

The frequency of heterotrophic biofilm nutrient limitation on organic substrata declined with agricultural and urban land use, yet the effect of land use was not consistent across all ecoregions. Although it is well documented that human land use influences stream ecosystems (Karr and Schlosser 1978; Paul and Meyer 2001), these effects often vary with land-use intensity (Allan 2004; Meyer et al. 2005). Across our ecoregions, there was high variation not only in the percentage of anthropogenic land use within stream basins, but also in the types of anthropogenic land use, with a net result of high variation in land-use intensity leading to variable stream nutrient concentrations. Typical background nutrient concentrations can change based on ecoregion because of differences in soils and climate (Omernik 1977; Smith et al. 2003), as exemplified by patterns in NO_3^- concentrations in our study, and these regional differences can affect the over-

Fig. 4. Nutrient limitation status of community respiration (CR), gross primary production (GPP), and chlorophyll *a* (CHLA) on inorganic substrata in reference (native vegetation), agricultural, and urbanized streams. Primary (1°) and secondary (2°) nutrient limitation status, as defined in the key, was determined by a significant response to nutrient treatment: N alone, P alone, and N and P added together (analysis of variance, ANOVA, $p < 0.05$).



all impact of agriculture and urban land use on streams. For example, we found that KS was highly impacted by human land use despite the low percent agricultural land use in each catchment, probably arising from a combination of typically low nutrient reference conditions and extensive fertilizer application associated with agricultural land use specific to that ecoregion. However, PR had high percent agriculture and urban land use in the impacted catchments, yet heterotrophic biofilms remained nutrient-limited by N and (or) P across all land-use types. Thus, across ecoregions, anthropogenic land use had a variable effect on streams, yet within ecoregions land use appeared to have a consistent effect, suggesting ecoregional differences in human land use (Omernik 1987).

On inorganic substrata, CR rarely showed significant nutrient limitation; heterotrophic colonization was likely suppressed by low carbon availability. Additionally, CR across all nutrient treatments was lower on inorganic substrata than on organic substrata, further suggesting carbon limitation. Heterotrophs on the inorganic substrata were likely dependent on an organic carbon source from the water column or from release by biofilm autotrophs (Olapade and Leff 2005). Autotrophic constituents of biofilms can support bacterial growth via algal exudates and cell death (Romani and Sabater 1999) and sometimes can be a source of inorganic nutrients (Geesey et al. 1978; Hepinstall and Fuller 1994). Additionally, previous studies suggest that heterotrophic biofilm activity is related to substrate carbon quality (Peterson et

al. 1993; Gessner and Chauvet 1994). Overall, heterotrophic nutrient limitation by N or P will be mediated by substrate carbon quality on which heterotrophic colonization occurs.

Measurement of CR includes both heterotrophic and autotrophic respiration. On organic substrata, CR appeared to be dominated by heterotrophic respiration because CR was much greater on organic substrata compared with inorganic substrata. In contrast, GPP showed the opposite pattern and was greater on inorganic substrata. Thus, large differences between substrata suggest that the high respiration on organic substrata is heterotrophic. The CR on inorganic substrata showed consistent results with GPP and chl *a*, suggesting that we captured a large fraction of autotrophic respiration in these particular measurements. We may have captured a response of autotrophs to nutrients via CR on inorganic substrata that was not significant with GPP or chl *a*.

Autotrophic biofilms

Light availability (as indicated by percent channel shade or PAR) appeared to be an important determining factor of the magnitude of autotrophic nutrient limitation across all land-use types and on both substrata. Autotrophic biofilms on inorganic substrata from reference streams were only nutrient-limited in ecoregions with naturally open canopies, and PAR and canopy cover were drivers of substratum GPP and chl *a* concentrations. Light often controls stream periphyton growth (Hill 1996; Mulholland et al. 2001; Bernhardt and Likens 2004) and nutrient response (Hepinstall and Fuller 1994; Larned and Santos 2000; Mosisch et al. 2001). Furthermore, human land use often influences both light and nutrient availability, obscuring the relationships between autotrophic biofilms and land-use changes (Karr and Schlosser 1978; Greenwood and Rosemond 2005; von Schiller et al. 2007). Our data support this possibility, as increasing percent modified land use led to increased NO_3^-, NH_4^+, and SRP concentrations and decreased percent channel shading, and we found an increase in chl *a* variability with high light availability.

However, a lack of autotrophic response to added nutrients could be associated with other factors besides light availability, especially the influence of invertebrate grazing and (or) substratum scouring as a result of high flows (Steinman 1996; Biggs and Close 1989). Direct observations of grazing by snails and amphipods on the organic substrata in particular occurred in a few streams, yet the proportion of streams that reported obvious herbivory was much less (9%) than those with no autotrophic nutrient limitation (65%). In most of the streams, nutrient diffusing substrata were deployed during base-flow conditions, resulting in a low potential for scouring of developing biofilms; nevertheless, the opportunity for small spates was possible. Finally, we may have been unable to detect a response of GPP to added nutrients if assay incubation PAR was significantly lower than ambient stream PAR. Yet, in 75% of the streams, neither chl *a* nor GPP on inorganic substrata showed a significant response to nutrients. Light limitation is the most parsimonious general explanation for these results.

The contribution of our data to the understanding of stream nutrient limitation

Our study provided substantially more data than previously published meta-analyses of chl *a* based NDS results, particularly by including human-dominated streams. Francoeur (2001) summarized 237 NDS experiments using chl *a* as a response metric and found that a majority (43% of the experiments) showed no N or P limitation. Tank and Dodds (2003) also included a literature survey in their discussion summarizing 172 NDS experiments with chl *a* and showed that N and P co-limitation was most prevalent (41% of the experiments). Our study showed that no limitation of chl *a* was most common (75% of all streams), which was a greater proportion compared with the two previous meta-analyses, probably reflecting the incorporation of anthropogenic land use and a wide range in light availability in our project design. Individuals researching periphytic algal nutrient limitation could be less likely to perform experiments in low-light streams and to publish negative results. Our study adds a new dimension to previous research focusing on autotrophic biofilm nutrient limitation by investigating a wider range of stream types via incorporation of anthropogenic impacts on the landscape.

Management implications

Our results indicated differences in the heterotrophic and autotrophic response to nutrient amendment, which has implications for the management of streams to prevent local and downstream eutrophication. Our data support Dodds' (2006) suggestion that autotrophs and heterotrophs should be assessed separately to determine overall trophic state (eutrophic or oligotrophic) of a stream. For example, a stream receiving nutrient inputs could be eutrophic based on heterotrophic indicators (high CR) but oligotrophic based on autotrophic indicators (low GPP) because of low light availability. The relative importance of the autotrophic and heterotrophic contribution to stream ecosystem function varies across ecoregions and land-use types and by season (which we did not examine here), so the predicted response of stream ecosystems to nutrient enrichment should include these differences when considering potential nutrient assimilation pathways.

Our data support previous research that found that light availability often was a primary control of autotrophs (Hill 1996; Mulholland et al. 2001; Bernhardt and Likens 2004), which can influence the autotrophic state of streams (Dodds 2006), particularly in terms of GPP (McTammany et al. 2007). We also suggest that carbon may be limiting for heterotrophs and thus changes in carbon supply (either dissolved or particulate) could have a large effect on heterotrophic nutrient status. Dodds (2006) proposed that even reduced riparian cover, which may decrease allochthonous organic matter inputs to streams, could have little effect on carbon availability in a stream because autotrophic activity would increase, thus adding new carbon (via carbon fixation) to the system. Similarly, Bunn et al. (1999) found that as GPP increased in response to reduced riparian cover associated with human land use in Australian streams, so did CR. Although their increase in CR could be due to algal respiration, it could also be from heterotrophs that were stimulated by increased algal carbon availability (Kaplan and Bott 1989). Thus, it is imperative to gauge the effect of land use on carbon availability, as well as nutrient availability (i.e., use a stoichiometric approach; Dodds 2007; Dodds and Cole 2007), to fully understand how heterotrophic nutrient status may be mitigated by other controlling factors.

Finally, our results support recent studies that suggest the importance of using ecosystem metrics to investigate stream health (Bunn et al. 1999; Fellows et al. 2006). Although our study does not measure reach-scale ecosystem processes such as whole-stream metabolism, our results imply that ecosystems processes, particularly CR on organic substrata, could be sensitive to the effect of land use. The NDS method has the benefit of separating substrate-specific functional responses to land use, complementing whole-stream metrics of function. Whole-stream GPP and CR can respond to increased agriculture (Bunn et al. 1999; Fellows et al. 2006); however, this response may be largely a result of changes in canopy cover as seen in other agricultural systems (McTammany et al. 2007). Meyer et al. (2005) found that although both NH_4^+ and P demand declined with increased urbanization in a catchment, GPP and CR were not related to indicators of urbanization. Therefore, the effect of human land use on stream ecosystem health may lead to a variety of responses, and future studies should combine metrics of nutrient uptake, whole-stream metabolism, and potential indicators of nutrient limitation such as NDS, thereby covering a range of response scales as an assessment tool.

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