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# Correlations between *in situ* denitrification activity and *nir*-gene abundances in pristine and impacted prairie streams

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*Denitrification efficiency best correlated to nirS and nirK gene abundances.*

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ABSTRACT

Denitrification is a process that reduces nitrogen levels in headwaters and other streams. We compared *nirS* and *nirK* abundances with the absolute rate of denitrification, the longitudinal coefficient of denitrification (i.e., $K_{den}$, which represents optimal denitrification rates at given environmental conditions), and water quality in seven prairie streams to determine if *nir*-gene abundances explain denitrification activity. Previous work showed that absolute rates of denitrification correlate with nitrate levels; however, no correlation has been found for denitrification efficiency, which we hypothesise might be related to gene abundances. Water-column nitrate and soluble-reactive phosphorus levels significantly correlated with absolute rates of denitrification, but *nir*-gene abundances did not. However, *nirS* and *nirK* abundances significantly correlated with $K_{den}$, as well as phosphorus, although no correlation was found between $K_{den}$ and nitrate. These data confirm that absolute denitrification rates are controlled by nitrate load, but intrinsic denitrification efficiency is linked to *nirS* and *nirK* gene abundances.



## 1. Introduction

Urbanization and human land uses such as agriculture have resulted in increased levels of bio-available nitrogen (N) in streams and rivers (Seitzinger et al., 2006). Increasing N alters the biotic integrity of streams (Dodds and Welch, 2000) in addition to causing significant eutrophication in downstream N-limited waters, such as larger rivers, lakes and marine receiving waters (Rabalais, 2002). Despite increasing N loads, counterbalancing in-stream N-suppression mechanisms exist, including denitrification, which is the microbiologically-mediated anoxic reduction of nitrate ($NO_3^-$) to nitrous oxide ($N_2O$) and nitrogen gas ($N_2$) (Galloway et al., 2003). Unfortunately, what controls rates of *in situ* denitrification and efficiency is only moderately understood because the abundance and function of denitrifying organisms are difficult to determine, largely because of the wide array of microorganisms capable of this function (Philippot and Hallin, 2005; Davidson and Seitzinger, 2006; Wallenstein et al., 2006; Seitzinger, 2008), and environmental conditions that influence rates are not well defined (Wallenstein et al., 2006). Further, direct measurement of *in situ* denitrification is not trivial because background dissolved $N_2$ levels are often very high relative to denitrification rates, which makes denitrification-specific signal detection difficult. As such, limited data exist relating responsible microorganisms, habitat conditions, and ecosystem-level denitrification rates and efficiencies, which are all critical to develop strategies that promote or retain denitrification in streams.

To address this broad question, Mulholland et al. (2008, 2009) performed an extensive evaluation of N-cycle reactions in streams, including denitrification, which spanned 8 regions, 72 headwater streams, and pristine, agricultural, and urban watersheds across North America (Lotic Intersite Nitrogen eXperiment; LINX II). Overall, these studies showed that absolute stream denitrification rates increased with increasing ($NO_3^-$) levels. However, relative nitrate-removal rates are often lower in streams with high ($NO_3^-$) loadings,

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implying N-polluted streams tend to remove proportionally less ($NO_3^-$) than pristine streams (i.e., they were less efficient; Mulholland et al., 2009; O'Brien et al., 2007). Thus, although high rates of denitrification occur in the streams with high N levels, N-removal tends not to be efficient and consequential downstream N-transport still occurs. Unfortunately, previous work on these streams only provided generic descriptions of microbial function (e.g., community respiration, gross primary production, the extent of periphyton) and no detailed quantification of *in situ* organisms or genes responsible for denitrification was performed (Mulholland et al., 2008, 2009). As such, we undertook a complimentary study with LINX II to determine whether relevant gene abundances and denitrification potential might better explain denitrification activity, especially intrinsic efficiency, in streams with different N-loadings (Mulholland et al., 2009; O'Brien et al., 2007). Specifically, genes were quantified in seven prairie streams, which were associated with the non-heme-containing (*nirS*) and copper-containing (*nirK*) nitrite-reductases that encode the key enzyme classes responsible for the conversion of $NO_2^-$ to NO within the denitrification pathway (Philippot, 2002; Tavares et al., 2006). Targeting such genes has been used previously to determine denitrifier community composition (Braker et al., 1998; Philippot and Hallin, 2005; Henry et al., 2006; Wallenstein et al., 2006; Knapp et al., 2009) and also explain related N-cycle phenomena.

Quantitative real-time PCR (qPCR) was used for gene detection because it is rapid, detects genes in both culturable and non-culturable species, and it is quantitative, which allows statistical analysis between gene levels and ecosystem function. Our ultimate goal here was to quantify gene abundances of the two key *nir* genes as surrogates for denitrifier population numbers, and use these gene abundances to determine whether they explain ecosystem-level denitrification rates and efficiencies in the streams. Streams were investigated with broadly differing denitrification activities for comparison, including highly pristine streams to those heavily impacted by agricultural or urban activity.

## 2. Materials and methods

### 2.1. Denitrification determinations

This current study examined seven streams, including: two pristine streams (Kings-N4D and Shane Creeks) at Konza Prairie Biological Station (Kansas, USA); two agricultural streams (Ag-North and Swine Creeks); and three urban streams ("The Ditch", Campus, and Little Kitten Creeks). Details about the streams and methods used to quantify *in situ* denitrification are provided elsewhere (O'Brien et al., 2007). In summary, the size of the streams varied with widths between 0.8 and 3.3 m, average depths from 2 to 14 cm, and reaches from 125 to 1000 m in length. Flow velocities varied between 0.9 and 6.7 m/min.

Denitrification rates were quantified by monitoring isotopic $^{15}NO_3^-$, $^{15}N_2O$, and $^{15}N_2$ levels in each stream after enrichment with $K^{15}NO_3$ ($\delta$ enrichment of 20,000‰ of stream $NO_3^-$ levels) (O'Brien et al., 2007). Samples for water-column $^{15}NO_3^-$ were collected at 6 stations along the stream reach (+12 h and +23 h during release). Samples were filtered (Whatman GF/F) and analysed using an adaptation of the ammonia diffusion method following nitrate reduction (Sigman et al., 1997) and mass spectrometry on a ThermoFinnigan Delta Plus instrument. Gaseous N samples were also collected using plastic syringes and were allowed to equilibrate with 20 mL ultra-pure helium for 5 min; the gases were then collected in 12-mL exetainer vials, and $^{15}N_2$ and $^{15}N_2O$ concentrations were determined by mass spectrometry (Hamilton and Ostrom, 2007).

Actual rates of denitrification were calculated using the longitudinal flux model based on $^{15}N_2$ measurements as previously described (Mulholland et al., 2004). The two measures of denitrification used here were total denitrification, which is the sum of the mass rate of formation of $^{15}N_2O$, and $^{15}N_2$ per unit stream area (mg-N $m^{-2}$ $day^{-1}$), and the coefficient of denitrification ($K_{den}$; $m^{-1}$), which describes the intrinsic longitudinal conversion of $NO_3^-$ to $N_2$ and $N_2O$ along a stream reach and provides an optimal denitrification rate coefficient for given environmental conditions (O'Brien et al., 2007). These two measures differ in that total denitrification is the absolute mass rate of denitrification in a stream, which is biased by both the ambient $NO_3^-$ level and stream width, whereas $K_{den}$ reflects the intrinsic rate of $NO_3^-$ loss per unit distance along the stream independent of N level or stream size. This parameter is calculated from longitudinal flux of $^{15}N_2$ over the stream reach (O'Brien et al., 2007; Mulholland et al., 2008) and is inversely related to the percent removal of N from the stream along the reach according to $\%N - \text{Removal} = (1 - e^{k_{den} \cdot 1000}) \cdot 100$ (O'Brien et al., 2007).

### 2.2. Water-quality determinations

Water-column samples were collected and analysed for water chemistry. $NO_3^-$, total ammonia ($NH_4^+$) and soluble-reactive phosphorus (SRP) were determined using a Technicon auto-analyzer (APHA et al., 1995). Dissolved organic carbon (DOC) concentrations were determined with a Shimadzu TOC5000 high temperature catalysis method. Measurements of ecosystem metabolism, gross primary production (GPP) and community respiration (CR), were calculated from diurnal changes in dissolved oxygen and water temperature values measured by YSI data sondes at a single station (Bott, 1996).

### 2.3. Sample collection for gene analysis

Samples for the quantification of *nirS* and *nirK* genes were collected at least in triplicate from a minimum of four sections along each stream approximately 23 h after $^{15}N$ tracer addition. Specifically, water-column samples were collected aseptically in bottles and kept on ice. At the laboratory, samples were homogenized by inverting the bottles at least 3 times, and 10 mL were centrifuged for 10 min (10,000×*g*); the pellets were retained for DNA extraction.

Sediment cores were collected from 3 streams (King's N4D, Campus and Ag-North) by inserting a liquid-nitrogen filled copper pipe (pinched at the bottom) into the stream sediment; basically, sediment and water were frozen surrounding the pipe in real-time and then were withdrawn intact from the stream bed. Sediments were immediately sectioned in 1-cm intervals (0–3 cm depth) and stored frozen in the field. When coring was not possible (due to substrate conditions or sampler availability), a 15-cm diameter PVC tube sampler was inserted 3-cm into surface sediments. The resulting core was re-suspended into local surface water and handled similar to the water-column samples (except 2 mL were centrifuged and pelleted) (Knapp et al., 2009). Sediment gene abundance values were corrected for water-column values (which were often negligible).

Water-column samples (50–100 mL) were filtered onto pre-weighed glass fiber filters, dried to constant weight at 103 °C and re-weighed to determine total suspended solids (TSS). As with the sediment core samples, approximately 0.5 g (wet-weight) were dried and re-weighed for dry-weight determinations (APHA et al., 1995).

### 2.4. DNA extraction and analysis

DNA samples were extracted using the MoBio UltraClean Soil DNA kit (Carlsbad, CA, USA) according to manufacturer's instructions. Before PCR analysis, DNA extracts were diluted (either 1:10 or 1:100) with molecular-grade water to minimize the presence of PCR inhibitors. Portions of *nirS* and *nirK* genes were quantified using primers nirS1F/nirS6R and nirK1F/nirK5R (Braker et al., 1998). Reactions were performed on either a Roche (Burgess Hill, UK) Light Cycler or a BioRad iCycler (Hercules, CA) qPCR system (Knapp et al., 2009). Each 25 μL reaction mixture combined 2 μL of template DNA, primers (700 nM) and PCR reagent (either Roche Faststartplus SYBR Green or BioRad iQ solution). Reaction conditions involved initial DNA denaturation (10 min, 95 °C), then 40 cycles of denaturation (30 s, 94 °C), primer annealing (40 s, 49 °C) and elongation (40 s, 72 °C) and fluorescence detection. Cloned *nirS* and *nirK* gene fragments (TOPO-TA; Invitrogen, Carlsbad, CA) were used to prepare DNA standards with known quantities of target DNA ($10^3$–$10^8$ copies $\mu L^{-1}$). Quality control included post-analytical melt curves and spiking $10^6$ *nirS* $\mu L^{-1}$ into UV-irradiated environmental DNA extracts.

### 2.5. Data analysis

Data analysis was conducted using SPSS (Chicago, IL; v. 11.0). Gene abundances were normalized to g-dry sediment values. Cumulative distributions of all variables were compared against a normal-distribution function using the Kolmogorov–Smirnov test; as a result, gene abundances and $K_{den}$ values required log transformation to better distribute the data. Denitrification rates were calculated from N-values obtained over the entire sample reach; as such, pooled sediment gene-abundance values (per sample location) were used to calculate length-weighted geometric mean abundances of *nirS* and *nirK*, which were used in bivariate correlation analysis and principal component analysis (PCA), along with whole-stream averages of GPP, CR, DOC, $NO_3^-$, $NH_4^+$, and SRP. PCA was based on a correlation matrix with Varimax rotation and Kaiser normalisation to maximize differences among loadings.

## 3. Results

### 3.1. Distribution of nirS and nirK

Absolute rates of denitrification, $K_{den}$, and stream water chemistry conditions are summarised in Table 1. A close relationship is

**Table 1**
Chemical, physical, and biological parameters of the seven streams in this study.

| Stream Name | Location | Total Denitrification (mg-N $m^{-2}$ $day^{-1}$) | $K_{den}$($m^{-1}$) | DOC (mg $L^{-1}$) | $NO_3^-$ – N (μg $L^{-1}$) | $NH_4^+$ – N (μg $L^{-1}$) | SRP (μg-P $L^{-1}$) | GPP (g-$O_2$ $m^{-2}$ $day^{-1}$) | CR (g-$O_2$ $m^{-2}$ $day^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|
| Shane Creek | Pristine | 0.0 | 0.0 | N/A | 1.2 | 4.7 | 1.0 | 5.4 | −4.5 |
| Kings Creek -N4D | Pristine | 0.2 | $5.13 \times 10^{-5}$ | 3.84 | 8.6 | 0.0 | 0.5 | 6.2 | −3.9 |
| Ag-North | Agricultural | 0.004 | $5.13 \times 10^{-6}$ | 7.36 | 35 | 31.7 | 0.2 | 3.2 | −2.7 |
| Little Kitten Creek | Urban | 12.8 | $1.39 \times 10^{-4}$ | N/A | 168 | 24.2 | 7.2 | 4.3 | −4.2 |
| "The Ditch" | Urban | 43.6 | $2.41 \times 10^{-3}$ | 3.81 | 277 | 28.3 | 35.4 | 12.5 | −7.0 |
| Campus Creek | Urban | 8.8 | $3.05 \times 10^{-5}$ | 10.3 | 2900 | 7.8 | 4.0 | 1.0 | −1.0 |
| Swine Creek | Agricultural | 221 | $3.05 \times 10^{-5}$ | N/A | 21000 | 3.3 | 15.5 | 2.7 | −4.4 |

apparent between ambient $NO_3^-$ level and the absolute rates of denitrification, which is consistent with results elsewhere (O'Brien et al., 2007; Mulholland et al., 2008). However, we also measured *nirS* and *nirK* gene abundances in the water-column and sediment samples to assess how abundances of these genes related to the two measures of denitrification. Before examining relationships between the genes and denitrification activity, a preliminary assessment was performed on the spatial distribution of genes in a sub-set of the studied streams.

Fig. 1 presents observed *nirS* and *nirK* abundances in the water-column and sediments for the three streams, which shows that gene abundances did vary significantly in the top 3 cm of the sediment zone in King Creek, Campus Creek, and Ag-North (three very different streams). Water-column gene concentrations were consistently 1–3 orders of magnitude lower than in the sediments, which is expected and reflects the amount of suspended solids in the water-column. Further, *nirK* abundances were generally higher in the water-column (Campus Creek: $t_{11} = 1.79$, $p = 0.11$; all others: $t > 3.64$, $p < 0.01$) than *nirS*, which is consistent with past observations that *nirK* is often prevails in conditionally $O_2$-exposed environments (Desnues et al., 2007; Knapp et al., 2009).

Given that *nir* genes were predominantly found in the sediments and that gene levels did not vary significantly in near-surface sediments with depth (*ANOVA*; $F_{3,10} < 1.062$; $p > 0.41$), all sediment *nirS* and *nirK* gene data were pooled for each sample-site, and then interpolated for all sample and sites along each stream to develop an estimate of the "total" *nir*-gene levels in each steam. These estimates then were statistically compared with absolute rates of denitrification, $K_{den}$, nutrient levels, and other biological activity data to determine trends (Table 1; O'Brien et al., 2007).

### 3.2. Comparisons between denitrification rates and gene abundances and environmental conditions

Bivariate analysis indicated that $K_{den}$ significantly correlated with the *nirS* ($r^2 = 0.80$, $p = 0.006$), *nirK* ($r^2 = 0.61$, $p = 0.037$), nirSK (the sum of *nirS* and *nirK*; $r^2 = 0.78$, $p = 0.009$), and SRP ($r^2 = 0.57$, $p = 0.049$) levels, whereas absolute denitrification rate only correlated with SRP ($r^2 = 0.80$, $p = 0.006$) and $NO_3^-$ ($r^2 = 0.57$, $p = 0.049$). Fig. 2 shows a clear and similar relationships exist between the two *nir* genes and $K_{den}$ (i.e., note the correlations roughly parallel each other), which suggests that both genes, within detection limits and gene probes employed here, provide a consistent marker of denitrification gene levels in the streams. Interestingly, no significant correlation was found between $K_{den}$ and $NO_3^-$, which differs from significant relationships between absolute denitrification rate and $NO_3^-$.

Table 2 summarises a PCA performed to further examine relationships suggested by the bivariate correlation analyses by grouping parameters that display similar patterns into discrete components. Specifically, Table 2 shows that $NO_3^-$, DOC, and SRP associate with absolute denitrification rate, which have been shown previously to

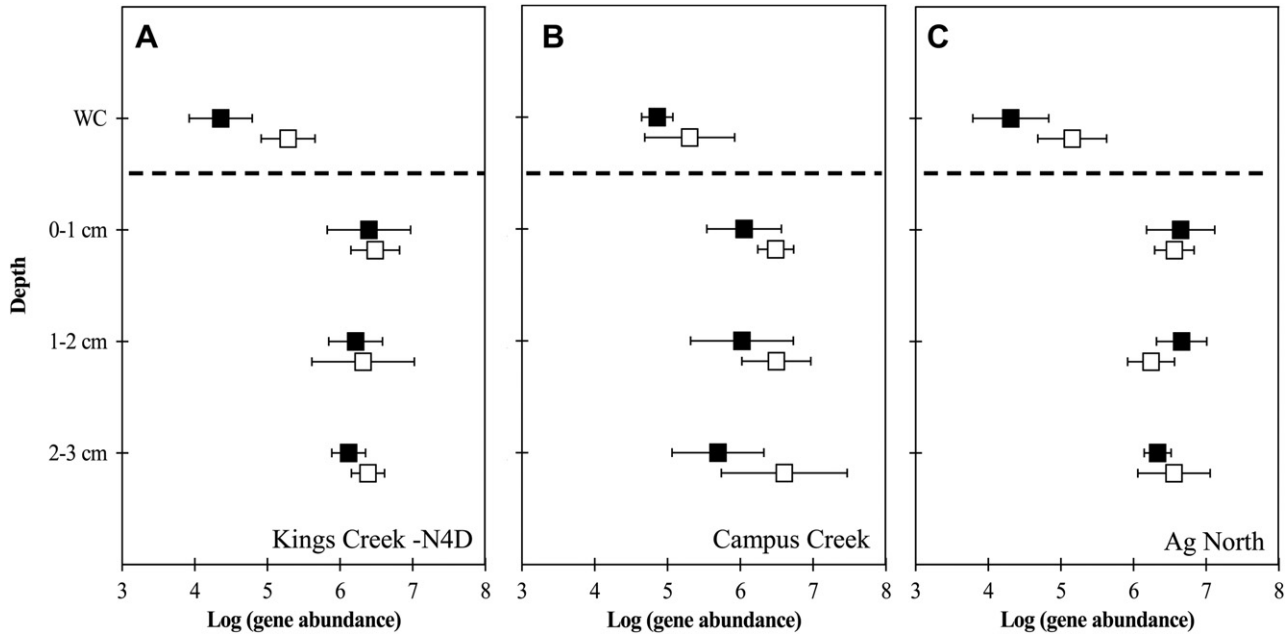


**Fig. 1.** Log-transformed *nirS* (■) and *nirK* (□) abundances in water-column (per mL) and sediment core (per g-dry sediment) samples taken from Kings, Campus and Ag-North Creeks.

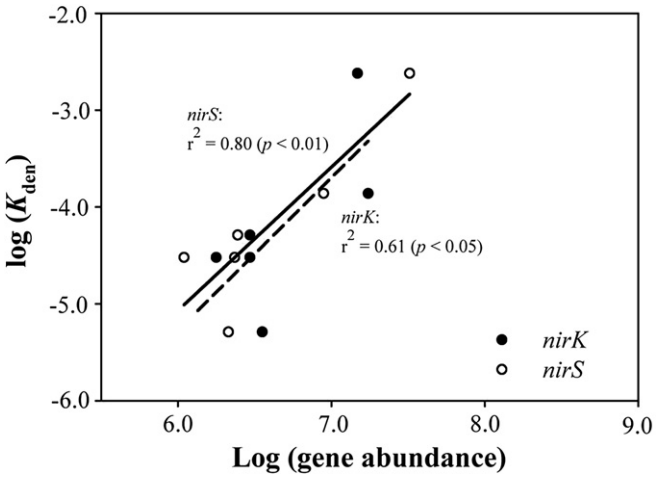


**Fig. 2.** Relationship between log-transformed *nirS*- (○) and *nirK*- (●) gene abundances and log-transformed denitrification coefficient ($K_{den}$). Note: Values for Shane Creek were excluded from the graph due to the inability to log-transform the $K_{den}$ value ($K_{den}$ = .0 $m^{-1}$). *nirS* and *nirK* were $10^{6.2}$ and $10^{6.1}$ genes per g-dry sediment for this stream.

affect denitrification rates in similar streams (Tank and Dodds, 2003; Niyogi et al., 2004; O'Brien et al., 2007). However, the PCA also specifically clusters $K_{den}$ with SRP; it also clusters gross primary production (GPP) and community respiration (CR) with each other; and it separates $NH_4^+$ as a signal component. It is noteworthy that PCA groups SRP with absolute denitrification rate and $K_{den}$, implying it contributes to variability in both measures of denitrification activity.

## 4. Discussion

Many environmental factors influence denitrification rates in streams, including dissolved oxygen (DO), DOC, SRP, and $NO_3^-$ levels (Wallenstein et al., 2006), although how each factor affects *in situ* rates is not clearly understood. However, only limited biological characterisation of organisms presumed responsible for this reaction has been performed in these experiments, which has limited our ability to fully explain and predict rate data. Therefore, we quantified here *nirK* and *nirS* genes in seven streams with differing water-quality conditions and denitrification rates (O'Brien et al., 2007; Knapp et al., 2009) to determine possible relationships. As background, Mulholland et al. (2008) had shown that absolute denitrification rates significantly correlate with $NO_3^-$ levels in streams across many biomes; however, they provide no explanation for why $K_{den}$, the intrinsic denitrification rate, does not also correlate with $NO_3^-$ levels. This is a significant omission because $K_{den}$ is an estimate of the optimal rate of $NO_3^-$ loss per unit distance along a stream, independent of N level or stream size, which is a potentially more valuable measure of denitrification activity for understanding actual factors that affect *in situ* rates.

Our data show through bivariate correlation analysis and a PCA that both *nir* genes significantly correlate/cluster with $K_{den}$, but not with absolute rates of denitrification. Further, $K_{den}$ significantly correlates SRP, but not with $NO_3^-$ level (consistent with Mulholland et al., 2008). Therefore, we provide first evidence of a biological explanation for observed *in situ* $K_{den}$ levels. Although such gene abundances do not exactly define the number of responsible denitrifying organisms present (Philippot and Hallin, 2005), they do provide an indirect useful new measure of intrinsic enzymatic capacity for denitrification, which we contend should be used in future studies. The practical question is "why does $K_{den}$ correlate with gene abundances", which can be answered when one considers what $K_{den}$ actually represents for a stream.

$K_{den}$ is the rate of $NO_3^-$ loss per unit distance along the stream, and is an intrinsic measure of denitrification independent of N level, stream size, or other explicit habitat factors. As such, $K_{den}$ simply clumps all factors that influence denitrification into a single parameter and, in fact, should be most influenced by the limiting factor to denitrification within the system. By analogy, $K_{den}$ is the specific substrate utilization rate coefficient from Monod kinetics for the stream. Similar to Monod for biological activity and growth (Monod, 1949), the actual limiting factor in many systems is not always clearly specified, but this factor must be a reflection or controller of the abundance and—or activity of the responsible organisms. Therefore, if *nir*-gene levels reflect the potential for denitrification in a stream and gene levels are finite, one would expect gene levels to correlate with $K_{den}$, the intrinsic denitrification. We suspect the reason why $K_{den}$ and $NO_3^-$ level do not correlate in the most streams is because $NO_3^-$ is not always the limiting factor to *in situ* denitrification, especially in the streams with high $NO_3^-$ levels. Conversely, *nirS* or *nirK* gene abundances correlate with $K_{den}$ because they best approximate the potential for denitrification, which is a product of present and past habitat and nutrient conditions that have influenced microbial selection. Based on the PCA, one might assume the limiting factor is SRP, or maybe GPP or CP, but this is not certain because other data imply that multiple factors might not be influencing rates (O'Brien et al., 2007).

Regardless, this new work provides a valuable starting point for new investigation on *in situ* denitrification activity. We contend a comprehensive explanation of denitrification rates and efficiencies only will be possible once molecular and classical limnological approaches are combined. Here we showed reach-scale denitrification efficiency correlates with associated ambient gene abundances. Therefore, future studies should employ a similar approach to finally define dominant factors that are most important to enhancing N-removal from streams, which has both local and global significance to understanding the impact of human activity of the N-cycle.

**Table 2**
Principal component eigenvalues and factor loadings for parameters measured in the seven streams.

| | Component | | | |
|---|---|---|---|---|
| | 1 | 2 | 3 | 4 |
| % of Variance | 34.5 | 28.7 | 23.6 | 9.2 |
| Total denitrification | .578 | **.800** | −.038 | −.147 |
| $K_{den}$ | **.949** | .151 | .194 | −.175 |
| *nirK* | **.913** | −.114 | .027 | .260 |
| *nirS* | **.893** | −.021 | .410 | .149 |
| nirSK | **.942** | −.056 | .220 | .211 |
| GPP | .350 | −.409 | **.831** | −.072 |
| CR | .168 | −.086 | **.978** | .061 |
| GPP:CR Ratio | .501 | **−.790** | .083 | −.284 |
| DOC | −.157 | **.786** | −.387 | .311 |
| $NO_3^-$ | .259 | **.870** | −.360 | .047 |
| $NH_4^+$ | .265 | .139 | −.126 | **.912** |
| SRP | **.630** | **.684** | .197 | .055 |

Note: Parameters with significant loadings within each component are highlighted in bold text.

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