

**REVIEW**

# Influence of experimental, environmental, and geographic factors on nutrient-diffusing substrate experiments in running waters

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**Abstract**

1. Freshwater algal growth is often limited by the availability of nitrogen (N), phosphorus (P), or both nutrients (NP). For over 30 years, investigators have conducted nutrient-diffusing substrate (NDS) experiments to quantify algal nutrient limitation or co-limitation in rivers and streams. Previous meta-analyses of NDS have shown that algae are commonly co-limited by N and P and that water column nutrients are weakly predictive of limitation. These analyses have not, however, comprehensively addressed the experimental, environmental, and geographic covariates affecting nutrient limitation results.
2. We surveyed the literature and extracted data for algal biomass effect sizes and a suite of covariates across a total of 649 experiments. We built meta-regression models to identify important controls on NDS results and to gain insights about algal nutrient limitation patterns over space and time. We also reviewed potential mechanisms for the reported result that NDS N and P treatments can inhibit algal growth.
3. Experimental variables including substrate type, chemical concentration, and experimental length significantly affected P and NP effect sizes, while NDS chemical compound influenced N, P, and NP effect sizes. We also found that environmental variables such as in-stream nutrients and riparian canopy cover significantly affected limitation by N, P, and NP. Temperature, stream discharge, and stream velocity only affected limitation by NP. Land use, ecoregion, and season showed clear trends in nutrient limitation for all treatments that could generally be tied to environmental factors like in-stream nutrients and riparian canopy cover.
4. Most experimental and environmental variables that were statistically significant in the meta-regression models produced very low  $R^2$  index values, indicating that the models explained little variation in among-site effect sizes. Spatial factors including stream order, ecoregion, and climate classification had the highest  $R^2$  index values, but these models still produced a large amount of unexplained variance.
5. In light of these findings, we provide recommendations for improving NDS experimental design and pursuing future research avenues using NDS. We also

highlight the need for future experiments to consider algal stressors that may interact with nutrient limitation experiments.

**KEYWORDS**

algae, lotic, meta-analysis, nutrient limitation, nutrient-diffusing substrate

## 1 | INTRODUCTION

Benthic algae serve many critical ecological functions in freshwater stream ecosystems, including primary production, nutrient cycling of both nitrogen (N) and phosphorus (P), and food and habitat provision for macroinvertebrates and fish (Stevenson, 1996). Over the past several decades, N and P concentrations have increased in freshwater systems as a result of human sources such as wastewater treatment plant discharges, agricultural fertiliser runoff, urban stormwater runoff, and atmospheric deposition (Carpenter et al., 1998). Given concerns about stream eutrophication, it is important to understand how algal communities respond to nutrient additions and how a variety of environmental factors can mediate algal responses to increased nutrient loading.

For over 30 years, investigators have used in situ nutrient-diffusing substrates (NDS) to quantify algal nutrient limitation in freshwater streams and rivers (Fairchild, Lowe, & Richardson, 1985). NDS involve filling replicate vessels (e.g. clay pots, plastic vials) with a medium (agar or water), which slowly releases nutrient salts to create locally enriched growth surfaces (Tank, Bernot, & Rosi-Marshall, 2006). These have become a standard method of assessing nutrient limitation in streams and rivers because they have the advantage of being small, replicable and relatively low maintenance. After an in-stream deployment period, control and treatment NDS are compared to quantify the effects of nutrient additions on algal growth and accrual. Structural response variables in NDS studies typically include chlorophyll *a* or ash-free dry mass (AFDM), although some experiments have also measured algal species composition (e.g. Biggs, Kilroy, & Lowe, 1998), algal biovolume or cell density (e.g. Wellnitz, Rader, & Ward, 1996), and fungal biomass (Tank & Dodds, 2003). Functional response variables are measured much more infrequently, but NDS studies have addressed the influence of nutrient inputs on gross primary production (GPP; Reisinger, Tank, & Dee, 2016), respiration (Hoellein, Tank, Kelly, & Rosi-Marshall, 2010), photosynthetic efficiency (Whorley & Francoeur, 2012), and N-fixation (Marcarelli & Wurtsbaugh, 2006).

A previous meta-analysis by Keck and Lepori (2012) reported that in-stream dissolved inorganic nitrogen (DIN) and total phosphorus (TP) concentrations were significantly correlated with decreased algal responses to N and P treatments, respectively. However, the statistical models predicting algal responses from in-stream chemistry contained a large amount of unexplained variance, highlighting the potential importance of other factors in regulating algal production and biomass accrual. Another study (Wold & Hershey, 1999) found

that in-stream  $\text{NO}_3^-$  and soluble reactive phosphorus (SRP) were poor predictors of nutrient limitation.

Many environmental factors can modify algal responses to nutrient additions by either increasing algal accrual (e.g. light, temperature) or decreasing it (e.g. herbivore grazing, scouring flows). For example, one study found that as light became less limiting, secondary N-limitation became apparent (Taulbee, Cooper, & Melack, 2005). For environmental factors to affect NDS results, they must either interact with nutrients in some way or significantly reduce algal biomass across all treatments.

Nutrient-diffusing substrate results are also influenced by the methodological approach used to construct and deploy the substrates. For example, investigators may employ clay pots, plastic vials, or periphytometers as the substrate type (Capps et al., 2011), apply different nutrient chemicals (e.g.  $\text{KH}_2\text{PO}_4$  vs  $\text{NaH}_2\text{PO}_4$ ), utilise nutrient concentrations which yield different N:P molar ratios (Capps et al., 2011), and deploy NDS substrates for differing periods of time, from <2 weeks (Scrimgeour & Kendall, 2002) to >2 months (Gustina & Hoffmann, 2000). Capps et al. (2011) demonstrated that within the same system and over the same time period, the NDS substrate type significantly affected algal nutrient limitation patterns. Moreover, they found potential interactive effects on limitation between the N:P molar ratio of the nutrient addition and the substrate type (Capps et al., 2011).

Limitation of algal growth by N, P, or both nutrients can vary over time within a given system due to shifts in stream physical, chemical, and biological conditions. For instance, a study in New Zealand showed that nutrient limitation was most common in summer and least common in winter. Statistical models showed that these results were due to temperature changes (Francoeur, Biggs, Smith, & Lowe, 1999). Wold and Hershey (1999) completed repeated experiments in Michigan streams and showed that within 2 weeks, a given stream could shift between limitation by N, P, both nutrients, or neither nutrient.

Nutrient limitation clearly varies over space based on regional factors like land use, climate, and nutrient loading. Reisinger et al. (2016) showed regional differences in the nutrient limitation of 15 U.S. streams (spanning the U.S. Midwest, Mountain West, and Arid West) that were associated with in-stream nutrient concentrations and land use. Tank and Dodds (2003) investigated nutrient limitation of autotrophic and heterotrophic biofilms across eight different biomes, finding that nutrient limitation was also associated with in-stream nutrient concentrations. Furthermore, they showed that autotrophic nutrient limitation was linked to photosynthetically active radiation (PAR; Tank & Dodds, 2003).

These highlighted studies reveal that a number of mechanisms may control algal accrual on NDS and that there are context-dependent stream and study characteristics that challenge the replication of results from experiments. Thus, it is not surprising that after 30 years of investigation, no clear consensus has arisen as to how multiple factors (experimental, environmental, and geographic) interact to influence algal responses to nutrients from NDS. To address this shortcoming, meta-analyses can be used as they are powerful ecological tools that involve aggregating effect sizes along with associated variances from multiple experiments (Koricheva, Gurevitch, & Mengersen, 2013). In addition, these analyses can include covariates that may vary across sites, allowing for better insight into their general contributions to algal response variation across systems. Previous meta-analyses have investigated NDS results (Elser et al., 2007; Francoeur, 2001; Keck & Lepori, 2012); however, none of them included a comprehensive suite of covariates to help inform future experimental design and inference. Such an analysis could ultimately assist in management of benthic algal production and biomass in streams and rivers.

In this review, we use meta-analysis techniques to investigate how experimental approach (e.g. substrate construction, experimental length), environmental conditions (e.g. in-stream nutrients, canopy cover, and discharge) and geographic variation (e.g. land use, ecoregion) affect the results of NDS experiments. We also examine the reported result that NDS nutrient treatments can inhibit algal growth (e.g. Bernhardt & Likens, 2004; Sanderson et al., 2009) in light of hypothesised mechanisms such as nutrient toxicity (Fairchild et al., 1985) or  $H_2O_2$  production when agar and  $PO_4^{3-}$  are boiled together in the laboratory (Tanaka et al., 2014). We have four primary objectives: (1) estimate overall effect sizes for N, P and NP; (2) calculate reporting rates for potentially important covariates; (3) develop and analyse univariable and multivariable meta-analysis models; and (4) provide recommendations for future experiments and novel research avenues.

## 2 | METHODS

### 2.1 | Database search

We searched for NDS studies using the online databases Web of Science, Science Direct, and Wiley Online Library. The keyword combinations for database searches are reported in Appendix 1. Studies were defined as journal articles, dissertations, and reports from the time period January 1, 1986 through December 31, 2015. From the search results, we reviewed titles and abstracts from over 3,000 studies. In addition, we applied the Google Scholar “related searches” tool to a subset of references, and we reviewed citations from previous nutrient limitation meta-analyses.

### 2.2 | Criteria

Studies were required to meet several criteria before being used in our meta-analysis. First, they had to include in situ NDS experiments

in freshwater, lotic systems. Studies were excluded if they contained “unnatural” manipulations to the stream environment, such as complete grazer exclusions (e.g. Lourenço-Amorim et al., 2014). We wanted to directly link environmental variability with effect sizes in this meta-analysis, hence studies with pooled results from multiple streams (e.g. Bechtold, Marcarelli, Baxter, & Inouye, 2012) were not considered. We only included studies that measured chlorophyll *a* (biomass per unit area) as the response variable because this metric is representative of the algal fraction of periphyton, and it was commonly recorded in NDS studies. Furthermore, we only included studies that reported replicated NDS controls and at least one replicated nutrient treatment (N, P, or NP) that were deployed in natural flowing water for a discrete experimental period.

For the analysis, if NDS were deployed under unique experimental or environmental conditions within the same stream, they were considered to be separate experiments. For instance, some studies tested the effects of different nutrient ratios (e.g. Capps et al., 2011) or light levels (e.g. Elsaholi, Hannigan, & Kelly-Quinn, 2011) within the same stream, and the results from these manipulations were considered as independent units. Lack of spatial independence of these experiments was then corrected for in the meta-analysis models (see Section 2.6). If multiple experiments were conducted over time at the same site (e.g. Wold & Hershey, 1999), we recorded only one experiment per 30 days to ensure experiments did not overlap.

### 2.3 | Data extraction

For controls and treatments in each experiment, we recorded chlorophyll *a* biomass ( $mg/m^2$ ) mean and variance, and the number of replicates. Some experiments included multiple sampling time points, and in these cases, only the final chlorophyll *a* biomass was recorded. Response variables were extracted from images when necessary, using Web Plot Digitizer version 3.8 (Rohatgi, 2015).

Prior to reviewing the studies, we identified a wide variety of experimental, environmental, and geographic factors which could potentially influence effect sizes across experiments (Table S1 in Appendix 3). We recorded values for these factors and determined which ones were commonly reported so that we could use them as predictor variables in meta-analysis models.

Environmental factors were only recorded when they were relevant to the spatial and temporal scale of the NDS experiments. For instance, average channel water depth did not qualify as experimental depth, and catchment forest cover did not qualify as riparian canopy cover. Annually averaged parameters (e.g. in-stream nutrients, discharge) were not considered to be representative of the experimental period. We computed averages if multiple values were recorded for an environmental variable within the same experimental period. In some cases when environmental data were plotted, the first value from the associated experimental month was extracted using Web Plot Digitizer version 3.8 (Rohatgi, 2015). For canopy cover, we recorded categorical and continuous values separately to capture all available information. We recorded the primary catchment land use category stated by the authors. We determined

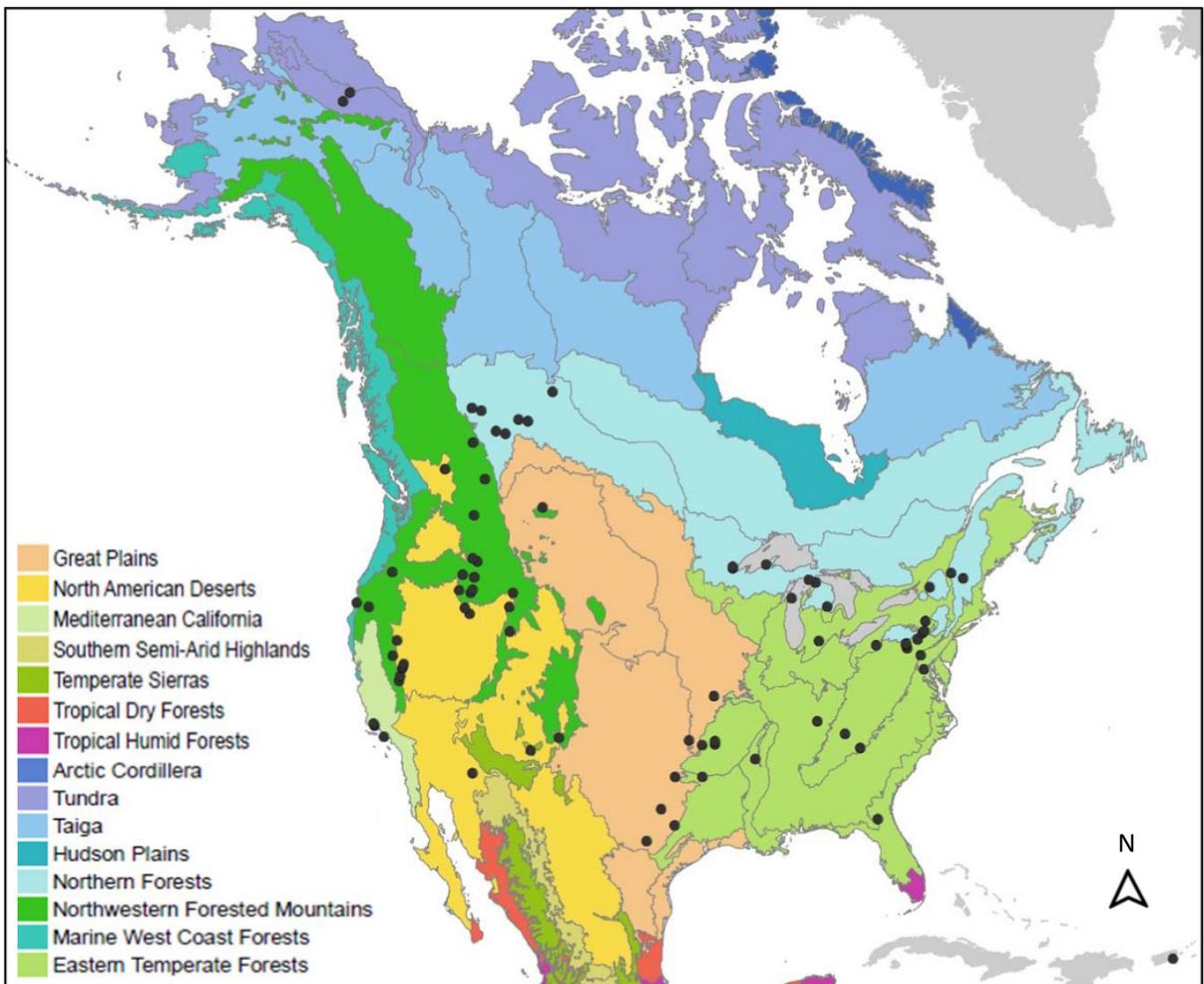
season by comparing solstice and equinox dates with reported experimental dates. If experiments overlapped two different seasons, we recorded the season in which the majority of the experiment took place. All data were assumed to be reliable as reported.

We completed two separate spatial analyses to understand how nutrient effect sizes might change based on ecoregion (North America) and climate classification (global). Ecoregions are areas of similar temperature, precipitation, and vegetation growth potential, and these factors influence background nutrient dynamics and hydrology (Omernik, 1987). However, classifications are only available for North America. Köppen-Geiger climate regions are derived from temperature and precipitation regimes (Peel, Finlayson, & McMahon, 2007), and classifications are available on a global scale. Layers for North American Level 1 Ecoregions (Figure 1; Commission for Environmental Cooperation, 2009) and updated Köppen-Geiger climate classifications (Peel et al., 2007) were downloaded and visualised using Google Earth. We

plotted experimental sites using reported coordinates as well as study site maps and stream names. This allowed us to associate most sites with an ecoregion and/or climate classification.

## 2.4 | Database composition

The final database included 649 experiments from 67 studies (Appendix 2 and Figure S1). Of these experiments, 553 recorded effects of N, 534 recorded effects of P, 591 recorded effects of NP, and 487 recorded effects of all three nutrient treatments. Experimental factors were commonly reported, but certain environmental and geographic factors (e.g. pH and stream slope) were not. If factors were reported in more than 25% of experiments, they were used to develop meta-analysis models. We calculated Pearson's correlation coefficients for commonly reported continuous environmental variables, using pairwise deletion of missing values.



**FIGURE 1** Study sites in North America were mapped based on reported latitudes and longitudes, as well as site maps and stream names. They were classified by Level 1 Ecoregion (Commission for Environmental Cooperation, 2009) to facilitate an analysis of geographic patterns in nutrient limitation

## 2.5 | Effect size and variance metrics

Meta-analysis models require a measure of effect size and associated variance (Koricheva et al., 2013). We used the log response ratio (LRR, Equation 1) as our effect size because of its demonstrated utility in ecological studies (Hedges, Gurevitch, & Curtis, 1999). LRRs are easily interpretable and have been used in previous meta-analyses on nutrient limitation (Elser et al., 2007; Francoeur, 2001; Keck & Lepori, 2012). LRRs were separately calculated in the following way for nitrogen (N-LRR), phosphorus (P-LRR), and nitrogen + phosphorus (NP-LRR) treatments:

$$\text{LRR} = \ln(Y_1/Y_2) = \ln(Y_1) - \ln(Y_2) \quad (1)$$

where  $Y_1$  is the mean chlorophyll *a* biomass from the treatment replicates and  $Y_2$  is the mean chlorophyll *a* biomass from the control replicates in a given experiment (Koricheva et al., 2013). We also calculated the variance of each LRR using the following equation:

$$\text{LRR}_{\text{Var}} = s_1^2/(n_1 Y_1^2) + s_2^2/(n_2 Y_2^2) \quad (2)$$

where  $s_1$  and  $s_2$  are the standard deviations of the treatment and control replicates, and  $n_1$  and  $n_2$  are the number of treatment and control replicates (Koricheva et al., 2013).

## 2.6 | Statistical models

We built meta-analysis models using the metafor package (Viechtbauer, 2010) in R version 3.2.2 (R Core Team, 2015). Meta-analysis models incorporate experimental effect sizes and variances, and thus can account for variability within and among experiments (Viechtbauer, 2010). We chose to use linear mixed models (LMMs) with "site" as a random effect, to account for the correlated effects of experiments within the same stream reach (Gelman & Hill, 2006). LMMs were used to estimate the total effect size of each nutrient, and we also built meta-regression models to determine the effect of experimental, environmental, and geographic predictor variables. Predictors were transformed as necessary to meet LMM assumptions. The "rma.mv" function was used for all models.

Each individual model was created using a different subset of the database, depending on the research question and predictor(s) of interest. As a result, models cannot be compared to one another, and inference can only be made for factors within the same model. For models with multiple predictors, we centred the values to put all coefficients on a scale commensurate with the predictor means and standard deviations (Gelman & Hill, 2006).

We developed models for commonly reported variables (i.e. >25% of experiments, Table S1). Models with NDS chemical concentration as a predictor did not include experiments that used periphytometers because aqueous and agar diffusion rates are likely to be different. Combining multiple predictors reduced the sample size we could consider; therefore, we constructed most models using a single predictor variable.

Investigators use different chemical compounds when constructing NDS. To determine the effect of P substrate chemicals on P-LRR and NP-LRR, we developed univariable models that compared commonly used compounds ( $\text{KH}_2\text{PO}_4$ ,  $\text{K}_2\text{HPO}_4$ ,  $\text{NaH}_2\text{PO}_4$ , and  $\text{Na}_2\text{HPO}_4$ ). We then categorised the phosphates into compounds with sodium ( $\text{NaH}_2\text{PO}_4$  and  $\text{Na}_2\text{HPO}_4$ ) vs potassium ( $\text{KH}_2\text{PO}_4$  and  $\text{K}_2\text{HPO}_4$ ) cations, and compounds with one hydrogen atom ( $\text{K}_2\text{HPO}_4$  and  $\text{Na}_2\text{HPO}_4$ ) vs two hydrogen atoms ( $\text{KH}_2\text{PO}_4$  and  $\text{NaH}_2\text{PO}_4$ ). We used the cation and hydrogen designations as categorical predictors for multivariable models. For N substrate chemicals, we developed univariable models to determine the influence of commonly used compounds on N-LRR and NP-LRR ( $\text{NaNO}_3$ ,  $\text{KNO}_3$ ,  $\text{NH}_4\text{Cl}$ , and  $\text{NH}_4\text{NO}_3$ ).

For each model, we evaluated the sign and magnitude of the coefficients, and whether the predictors explained significant variation in the observed effect sizes. For individual estimates, we determined significance using 95% confidence intervals (CIs) that did not overlap with zero. For categorical factors and multivariable models, we determined significance using an omnibus test of parameters. This method uses a test statistic ( $Q_M$ ) and chi-square distribution to test the null hypothesis:  $B_1 = B_2 = 0$ , where  $B_s$  are model coefficients in the meta-regression models (Viechtbauer, 2010). If the null hypothesis is rejected, we can conclude there is a significant effect of the factor(s) being tested.

We calculated an  $R^2$  index for each meta-regression model based on the equation from Borenstein, Hedges, Higgins, and Rothstein (2009):

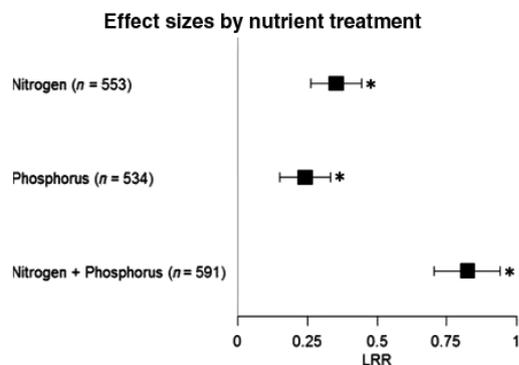
$$R^2 \text{ index} = \tau_{\text{explained}}^2 / \tau_{\text{total}}^2 = 1 - \left( \tau_{\text{unexplained}}^2 / \tau_{\text{total}}^2 \right) \quad (3)$$

where the  $\tau^2$  parameter is estimated as part of the LMM procedure, and represents the true variance between studies in the meta-analysis. We used an intercept-only random effects model to calculate the total variance between sites ( $\tau_{\text{total}}^2$ ), and meta-regression models with moderators to calculate the unexplained variation between sites ( $\tau_{\text{unexplained}}^2$ ). Most index values are expected to range between 0 and 1, but we set any negative values equal to 0 (Borenstein et al., 2009). A high  $R^2$  index indicates the moderator explained additional variation between streams, and a low  $R^2$  index indicates that the moderator did not explain much more variability than the random effects model alone.

We used models with intercepts to determine model parameters, standard errors, and 95% confidence intervals. Statistical significance was also determined using models with intercepts. We used no-intercept model estimates to graphically represent the meta-analysis model effect sizes and 95% confidence intervals.

## 3 | RESULTS

All nutrient effect sizes were positive and significantly different from zero (Figure 2, Table S2). NP had the highest estimate (0.82), followed by N (0.35) and P (0.24).



**FIGURE 2** Log response ratio effect size estimates from the no-intercept models by nutrient treatment, with the centre square denoting the mean, bars denoting upper and lower 95% confidence intervals (CIs), and “\*” indicating a significant effect as determined by CIs that do not overlap with zero in the intercept model (see Table S2)

Many continuous covariates were significantly correlated with other covariates (Table S3). Pairs with high correlation coefficients ( $r > |.5|$ ) included stream order and quantitative canopy cover ( $r = -.654$ ),  $\text{NH}_4^+$  and TN ( $r = -.622$ ),  $\text{NO}_3^-$  and DIN ( $r = .994$ ), DIN and TP ( $r = .886$ ), TN and DIN ( $r = .965$ ), TN and TP ( $r = .712$ ), and SRP and TP ( $r = .989$ ).

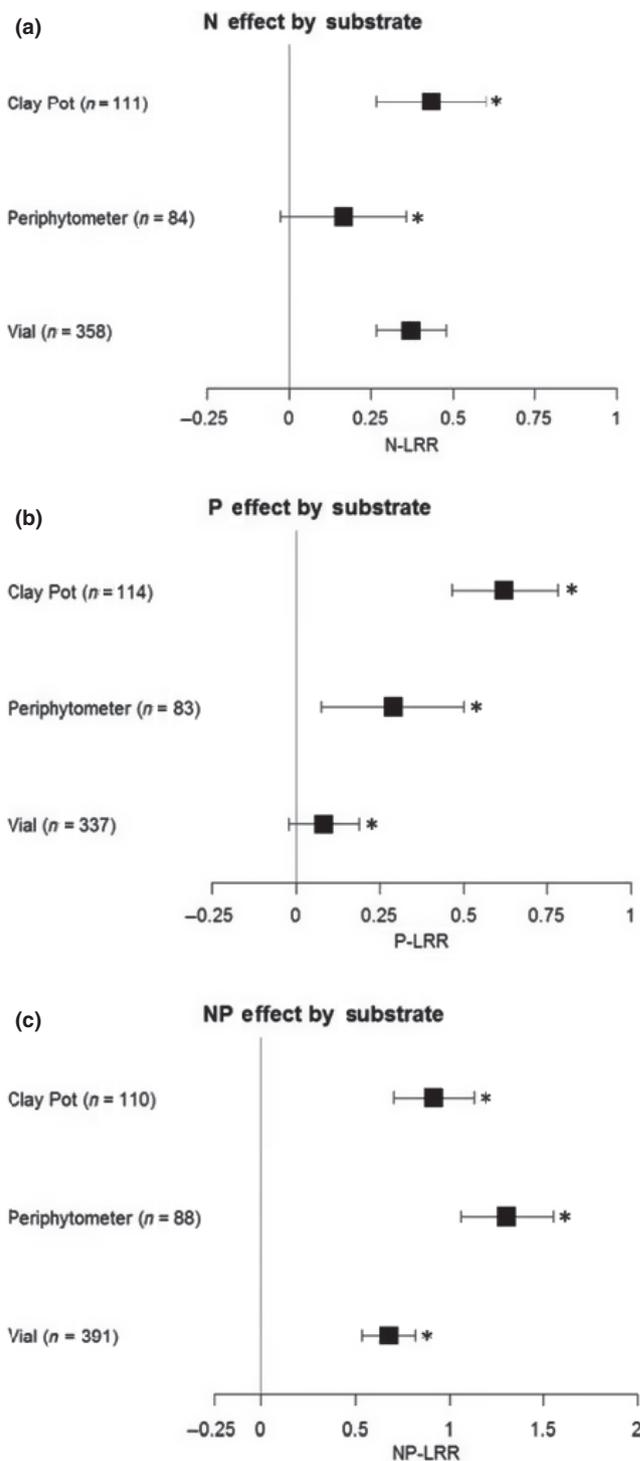
### 3.1 | Experimental approach

Substrate had different effects depending on the LRR being considered. Clay pots had the highest N-LRR estimate, followed by vials and periphytometers (Table S4, Figure 3), but results showed no overall effect of substrate on N-limitation ( $Q_M = 5.31$  and  $p = .07$ ). Clay pots had the highest P-LRR estimate, followed by periphytometers and vials (Table S4, Figure 3). Periphytometers had the highest NP-LRR estimate, followed by clay pots and vials (Table S4, Figure 3). For the P and NP models, substrate type significantly affected nutrient limitation responses (P:  $Q_M = 36.01$  and  $p < .0001$ ; NP:  $Q_M = 20.47$  and  $p < .0001$ ).

$\text{PO}_4^{3-}$  compound significantly affected LRRs for both P ( $Q_M = 16.09$  and  $p = .001$ ) and NP ( $Q_M = 36.14$  and  $p < .0001$ ) models. Compounds with potassium ( $\text{KH}_2\text{PO}_4$  and  $\text{K}_2\text{HPO}_4$ ) produced higher P-LRR and NP-LRR estimates than did  $\text{PO}_4^{3-}$  compounds with sodium ( $\text{NaH}_2\text{PO}_4$  and  $\text{Na}_2\text{HPO}_4$ ; Table S4, Figure 4).  $\text{PO}_4^{3-}$  compounds with one hydrogen atom ( $\text{K}_2\text{HPO}_4$  and  $\text{Na}_2\text{HPO}_4$ ) produced higher P-LRR and NP-LRR estimates than compounds with two hydrogen atoms ( $\text{KH}_2\text{PO}_4$  and  $\text{NaH}_2\text{PO}_4$ ; Table S4, Figure 4). While there were significant main effects of cation and hydrogen in the multivariable models, there was no significant interaction between the two factors (Table S4).

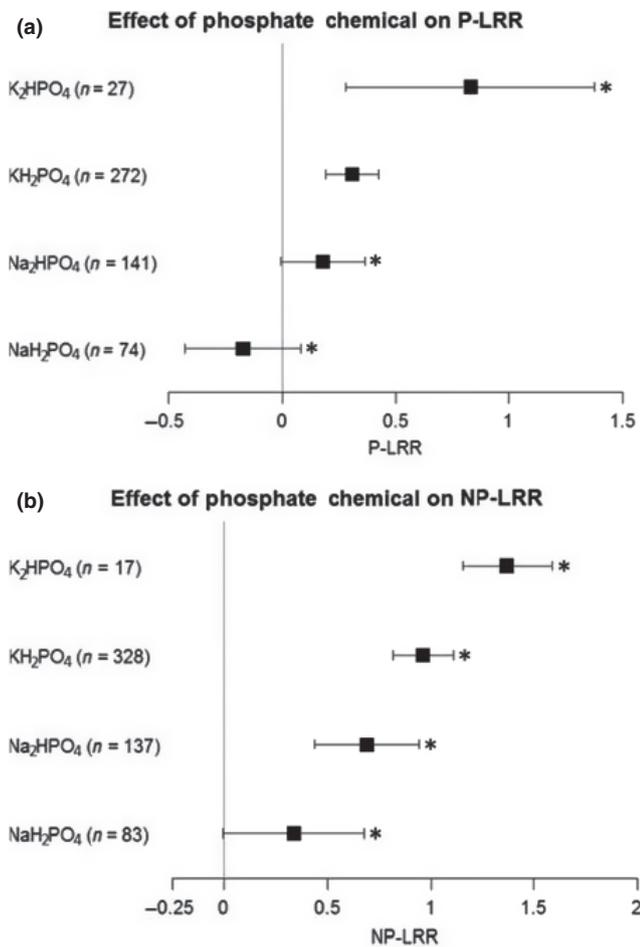
Nitrogen compound significantly affected LRRs for both N ( $Q_M = 201.03$  and  $p < 0.0001$ ) and NP ( $Q_M = 9.07$  and  $p = .028$ ).  $\text{NH}_4\text{NO}_3$  had the highest estimates for both LRRs (Figure 5).

The effect of NDS initial concentration and deployment length was not significant for the N models but was significant for the P and NP models (Table S4). P concentration and number of days led to decreases in P-LRR and NP-LRR, while N concentration also led to decreases in NP-LRR.



**FIGURE 3** Log response ratio effect size estimates from the no-intercept models by experimental substrate for the N (a), P (b), and NP (c) treatments (see Table S4; symbols explained in the Figure 2 caption)

Meta-regression models considering experimental approach generally produced low  $R^2$  index values within the range 0%–2% (Table S4). However, P chemical compound explained 4% of the between-stream variability in P- and NP-LRRs, while substrate type explained over 12% of the between-stream variability in P-LRR.

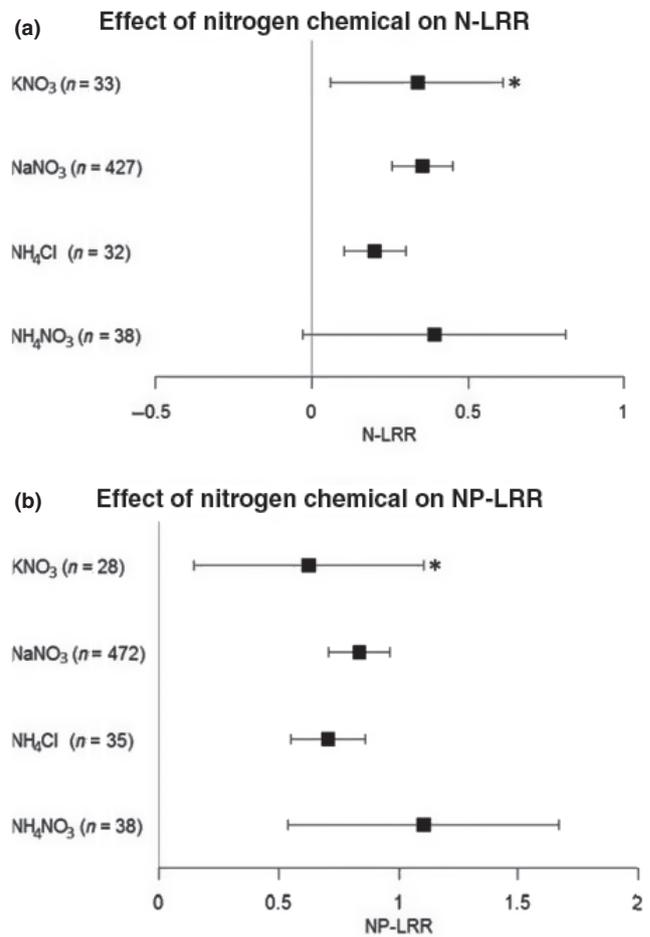


**FIGURE 4** P-LRR (a) and NP-LRR (b) effect size estimates from the no-intercept models by  $PO_4^{3-}$  chemical compound composition (see Table S4; symbols explained in the Figure 2 caption)

### 3.2 | Environmental variables

Environmental variables significantly affecting all three nutrient responses included quantitative canopy cover, SRP, and season (Tables S5–S7, Figure 6). Quantitative canopy cover was associated with increases in N-LRR, but decreases in P-LRR and NP-LRR. SRP was associated with decreases in all nutrient responses. N-LRR and NP-LRR exhibited the same seasonal trends whereby autumn had the highest estimates, followed by summer, spring, and winter (N:  $Q_M = 12.3023$  and  $p = .0064$ ; NP:  $Q_M = 162.7482$  and  $p < .0001$ ). P-LRR had the highest estimate during summer, followed by autumn, spring, and winter ( $Q_M = 97.8791$  and  $p < .0001$ ).

Other environmental variables significantly affecting N-LRR included  $NO_3^-$  and N:P molar ratio (Table S5), which were both associated with decreases in N-limitation. Additional environmental variables significantly affecting NP-LRR included  $NH_4^+$ , qualitative canopy cover, discharge,  $NO_3^-$ , temperature, and velocity (Table S7). Discharge,  $NH_4^+$ ,  $NO_3^-$ , and velocity were all associated with decreases in NP-LRR, while temperature was associated with increases in NP-LRR. Open canopy cover had a higher estimate than closed canopy cover ( $Q_M = 233.9916$  and  $p < .0001$ ).

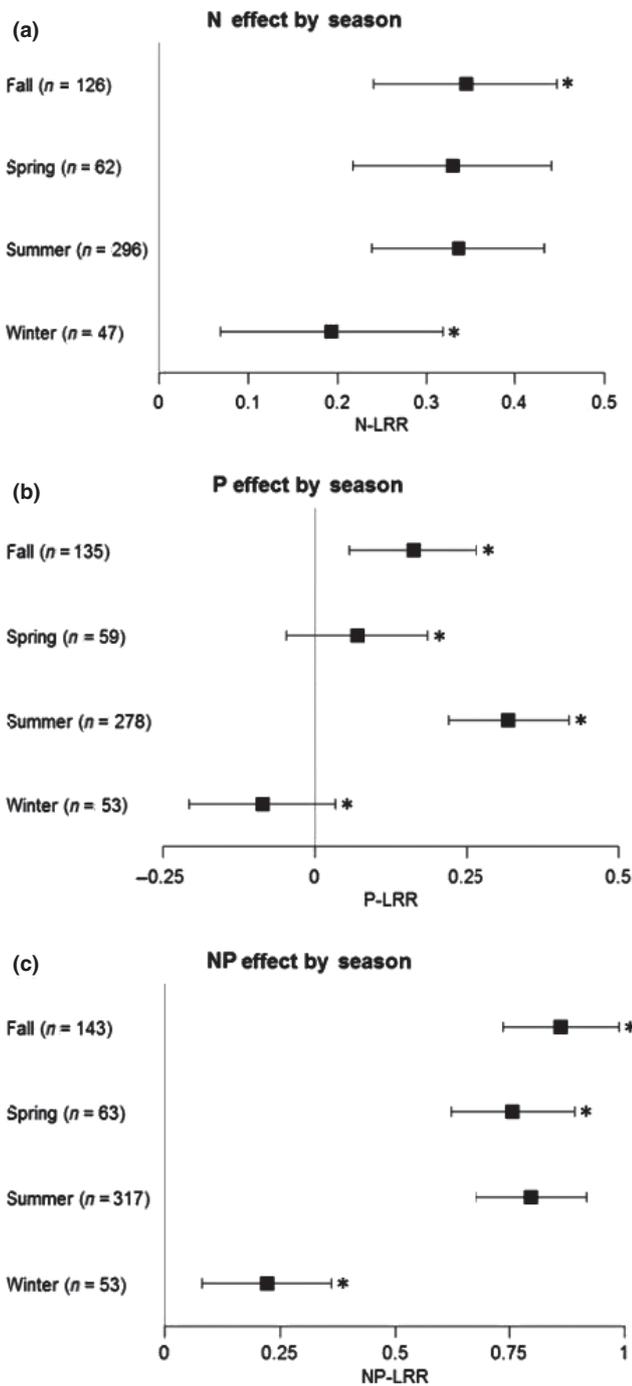


**FIGURE 5** N-LRR (a) and NP-LRR (b) effect sizes estimates from no-intercept models by N chemical compound composition (see Table S4; symbols explained in the Figure 2 caption)

The  $R^2$  indices were also low for the environmental variable models, generally in the range 0%–2% (Tables S5–S7). However, in-stream  $NO_3^-$  explained nearly 8% of the variability in among-site N-LRR and 11% of the variability in NP-LRR. In-stream SRP and quantitative canopy cover explained 9% and 4% of the variability in among-site NP-LRR.

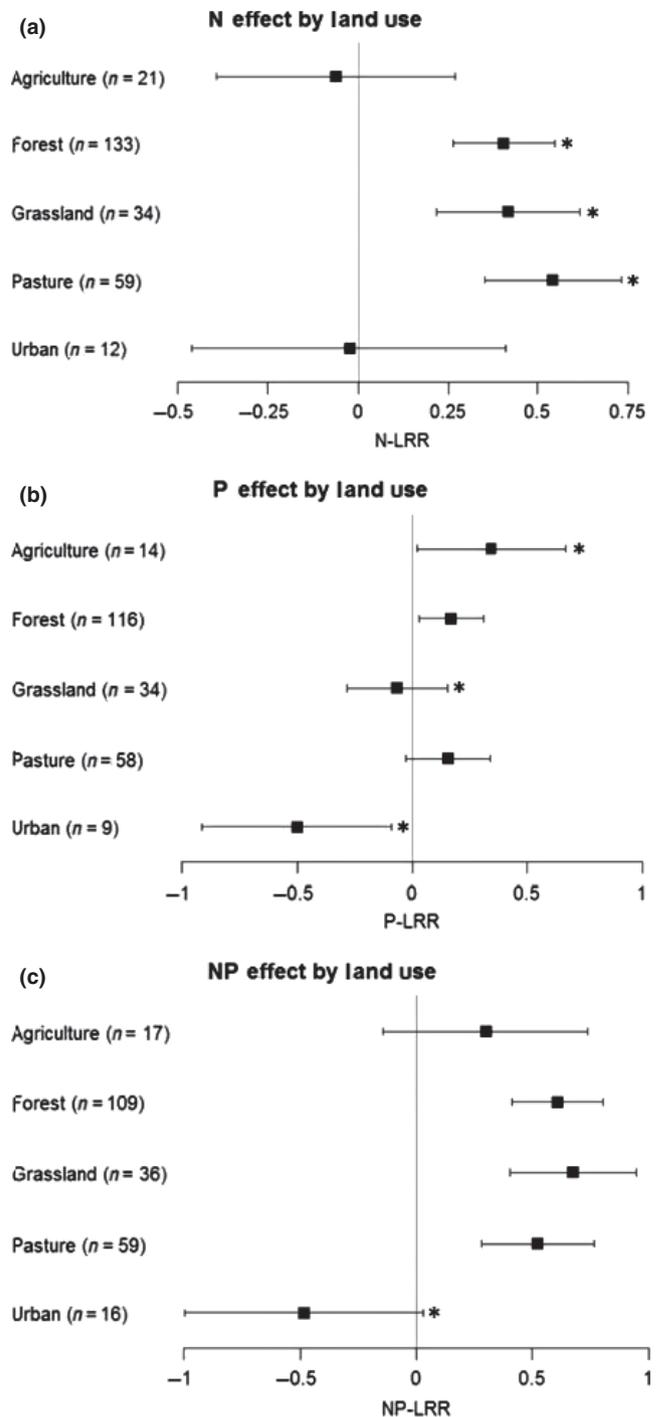
### 3.3 | Geographic factors

Land use was significantly related to all three nutrient responses (Figure 7, Table S8). Pasture had the highest N-LRR estimate, followed by grassland, forest, urban, and agriculture ( $Q_M = 13.0620$  and  $p = .0110$ ). Agriculture had the highest P-LRR estimate, followed by forest, grassland, pasture, and urban ( $Q_M = 15.0838$  and  $p = .0045$ ). Grassland had the highest NP-LRR estimate, followed by forest, pasture, agriculture, and urban ( $Q_M = 17.0689$  and  $p = .0019$ ). The land use  $R^2$  index explained the highest among-stream variability in NP-LRR (12%), followed by P-LRR (10%) and N-LRR (9%). In the P models, stream order was also significantly related to P-LRR, whereby higher stream orders were associated with increases in P-LRR (Table S8). The stream order  $R^2$  index was 5% for N-LRR and 15% for P-LRR but 0% for NP-LRR.



**FIGURE 6** Log response ratio effect size estimates from the no-intercept models by season for the N (a), P (b), and NP (c) treatments (see Tables S5–S7; symbols explained in the Figure 2 caption)

Response ratios differed by North American ecoregion (Table S9, Figure 8), and we found a significant effect of ecoregion in all models (N:  $Q_M = 22.0441$  and  $p = .0025$ ; P:  $Q_M = 27.9145$  and  $p = .0002$ ; NP:  $Q_M = 49.1990$  and  $p < .0001$ ). The Marine Western Forest and Temperate Sierra ecoregions had significantly positive N-LRR estimates. The Marine Western Forests, Northern Forests, and Northwest Forested Mountains had significantly positive P-LRR



**FIGURE 7** Log response ratio effect size estimates from the no-intercept models by land use for the N (a), P (b), and NP (c) treatments (see Table S8; symbols explained in the Figure 2 caption)

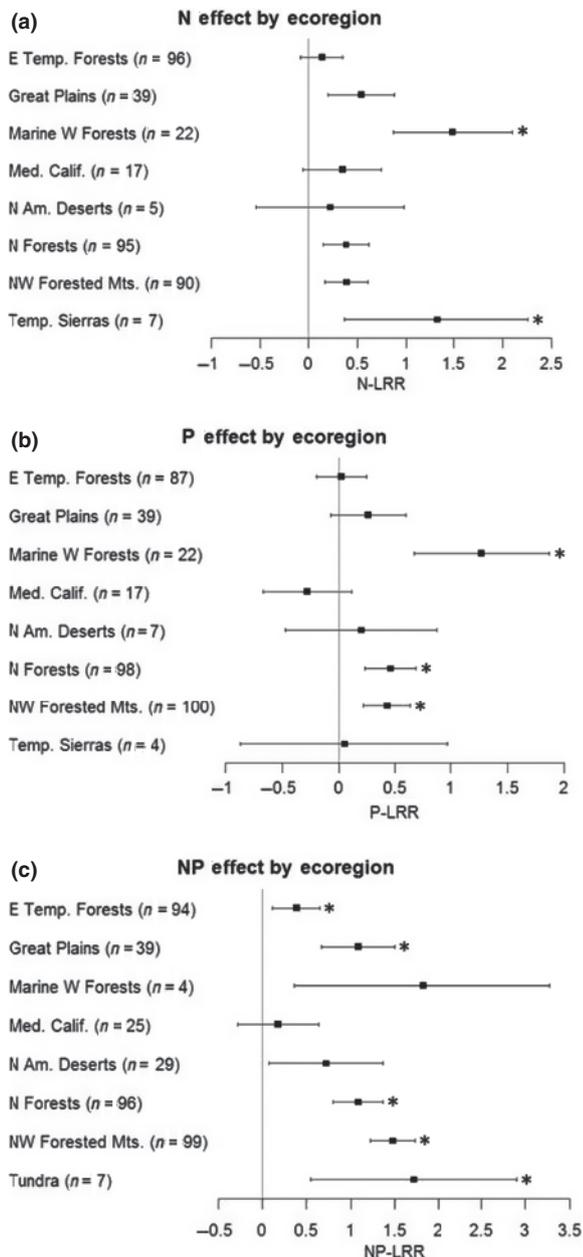
estimates. Finally, the Eastern Temperate Forests, Great Plains, Northern Forests, Northwest Forested Mountains, and Tundra had significantly positive NP-LRR estimates. The ecoregion  $R^2$  index was 8% for N-LRR and P-LRR, and 16% for NP-LRR.

No individual Köppen-Geiger climate classification was significantly related to N- or P-LRR (Table S10), but we did find an overall effect of climate classification in those models (N:

$Q_M = 21.5962$  and  $p = .0173$ ;  $P: Q_M = 23.7271$  and  $p = .0048$ ). The Cfa, Dfc, and Dsb classifications were all significantly related to NP-LRR (Table S10), and we found a significant effect of climate classification on NP-limitation ( $Q_M = 59.3328$  and  $p < .0001$ ). Climate classification explained 4%, 6%, and 18% of the among-stream variability in N-LRR, P-LRR, and NP-LRR, respectively.

## 4 | DISCUSSION

By including experimental, environmental, and geographic covariates, our meta-analysis has produced new insights into what drives



**FIGURE 8** Log response ratio effect size estimates from the no-intercept models by North American Level 1 Ecoregion for the N (a), P (b), and NP (c) treatments (see Table S9; symbols explained in Figure 2 caption)

nutrient limitation in running waters over space and time. Our findings show that algal production on NDS P treatments depends on the experimental approach, including the substrate type, nutrient concentrations,  $PO_4^{3-}$  compound chemical composition, and experimental duration. In fact, NDS substrate type explained more variability in P-LRR than any other experimental or environmental variable. This suggests a need to standardise NDS approaches in future experiments, which we discuss below. We also found that spatiotemporal factors (land use, ecoregion, and season) significantly influenced NDS response ratios, with spatial factors having the highest power to explain variation in LRRs. Environmental variables that promote algal growth rates have been well-reported and studied, and our results reaffirm the importance of in-stream nutrients but also identify other factors that may be influencing nutrient limitation. We found that variables that decrease algal growth and accumulation (e.g. turbidity, grazing) are understudied, but NDS may be a useful method to understand how these factors interact with nutrients to regulate algae.

### 4.1 | Overall effect sizes

Our study supports previous meta-analyses of nutrient amendment experiments that demonstrated high effect sizes of NP treatments in lotic ecosystems (Elser et al., 2007; Francoeur, 2001). Furthermore, in agreement with Allgeier, Rosemond, and Layman (2011), we found synergistic, non-additive effects of NP additions (i.e. the NP-LRR estimate was higher than the sum of the N-LRR and P-LRR estimates). We found a higher N than P effect size, which is in contrast to the slightly higher P effect size reported by Elser et al. (2007) in freshwater streams. However, we restricted our analysis to NDS experiments, while Elser et al. (2007) included a broader class of nutrient enrichment experiments.

### 4.2 | Effects of experimental approach on nutrient limitation

#### 4.2.1 | NDS and nutrient inhibition

We tested the effect of multiple experimental factors on NDS study outcomes, with a particular focus on why some studies have found an inhibitory effect of P additions on algal biomass as compared to control treatments (e.g. Bernhardt & Likens, 2004; Sanderson et al., 2009). These inhibitory effects introduce questions about whether researchers are actually using appropriate experimental methods to test for P-limitation. Unexpectedly, NDS studies have reported P-inhibition by P treatments but rarely by NP treatments (but see Reisinger et al., 2016), despite the fact that the two treatments generally include the same P concentrations and chemicals. Although we found that P-inhibition was more common than NP-inhibition, we identified a set of experimental factors that led to significant declines in both P-LRR and NP-LRR.

We found that increased NDS P concentrations and longer experimental periods led to decreased P-LRR and NP-LRR (Table S4).

The experimental lengths ranged from 11 to 67 days, but depending on how they are constructed, NDS may diffuse nutrients for only 18–20 days (Tank et al., 2006). Higher NDS concentrations lead to more sustained diffusion (Rugenski, Marcarelli, Bechtold, & Inouye, 2008), but we advise against deploying experiments for more than 3 weeks unless diffusion rates have been measured. In addition, benthic algae colonising bare substrates have been shown to exhibit an accrual phase followed by a loss phase due to autogenic sloughing, grazing, or the drag of current velocity (Biggs, 1996). Researchers are generally most interested in measuring chlorophyll *a* around the time of peak algal biomass, but lengthy experiments might lead to chlorophyll *a* measurements during a biomass loss phase. Algal accrual observations on tiles or scrubbed rocks could be employed to achieve a stream-specific estimate of when peak biomass occurs.

Higher P concentrations may have had a negative effect on P and NP treatment responses by creating an unfavourable N:P molar ratio for algal growth. Many studies use the Redfield ratio (N:P = 16:1; Redfield, 1934) to relate stream water chemistry to algal growth, and P additions could induce a ratio below 16:1 in certain streams. However, an unfavourable N:P ratio would not necessarily inhibit algal biomass on P treatments relative to controls. Inhibition could result from direct effects of P toxicity, but this phenomenon has rarely been reported in the literature. P toxicity has sometimes been inferred in NDS experiments when algal biomass on controls exceeds algal biomass on P treatments (Fairchild et al., 1985), but alternative hypotheses should also be considered.

We found higher algal biomass stimulation when experiments used potassium phosphates ( $\text{KH}_2\text{PO}_4$  and  $\text{K}_2\text{HPO}_4$ ) as compared to sodium phosphates ( $\text{NaH}_2\text{PO}_4$  and  $\text{Na}_2\text{HPO}_4$ ). Possible explanatory mechanisms could include algal potassium limitation or sodium inhibition (Sudhir & Murthy, 2004). On the other hand, we found similar effect sizes when experiments used  $\text{NaNO}_3$  and  $\text{KNO}_3$ , showing that the cation was not important for N-LRR and NP-LRR. This could have been because of the small sample size for  $\text{KNO}_3$  ( $n = 33$  for N-LRR and  $n = 28$  for NP-LRR), but could also indicate that the cations differentially interact with  $\text{NO}_3^-$  and  $\text{PO}_4^{3-}$  uptake or assimilation.

The number of hydrogen atoms in the  $\text{PO}_4^{3-}$  compound may have had an effect on P-LRR and NP-LRR by changing the pH conditions of the growth surface.  $\text{KH}_2\text{PO}_4$  and  $\text{NaH}_2\text{PO}_4$  can lower the pH of the surrounding water, while  $\text{K}_2\text{HPO}_4$  and  $\text{Na}_2\text{HPO}_4$  can raise the pH of the surrounding water (W. Beck, unpublished data). In our analysis, chemicals that lower pH had significantly lower P- and NP-LRRs. Stream pH can affect algal biomass and community composition (Planas, 1996) and may be a mechanism by which P chemicals influence LRRs. However, additional field and laboratory experiments are required to explore this mechanism.

Two other ideas about P-inhibition could not be tested using the dataset compiled here. First, Tanaka et al. (2014) reported that autoclaving  $\text{PO}_4^{3-}$  with agar produces  $\text{H}_2\text{O}_2$  that inhibits microbial growth in a laboratory setting, and it is plausible that  $\text{H}_2\text{O}_2$  could inhibit in-stream algal growth as well. However, it remains unclear whether autoclaving is necessary to produce  $\text{H}_2\text{O}_2$ , or whether simply boiling the agar solution and  $\text{PO}_4^{3-}$  can yield the same results. In any case,

studies do not commonly report these laboratory methods. In the *Methods in Stream Ecology* second edition, Tank et al. (2006) recommend mixing  $\text{PO}_4^{3-}$  and agar during the heating process, leading us to speculate that most experiments have used that protocol. If harmful levels of  $\text{H}_2\text{O}_2$  are produced by this method, we might expect P-inhibition of algae to be more common than is currently reported (Reisinger et al., 2016).

Second, it has been proposed that heterotrophic bacteria and fungi could utilise P additions and outcompete algae in epilithic biofilms (Bernhardt & Likens, 2004). However, few studies provide enough data to adequately test this idea. Most studies only report broad structural responses to nutrient additions including chlorophyll *a* biomass and (less commonly) AFDM. Bechtold et al. (2012) measured both chlorophyll *a* and AFDM in NDS experiments, finding apparent competitive suppression of chlorophyll *a* when dissolved organic carbon was added as an NDS treatment. It is possible that an analysis of studies reporting both chlorophyll *a* and AFDM would show the same type of competitive suppression with P additions, but such an analysis was beyond the scope of this study. A few studies have tested functional responses to nutrient additions such as GPP and ER (e.g. Marcarelli, Bechtold, Rugenski, & Inouye, 2009; Reisinger et al., 2016) and this approach may provide information on the relative importance of autotrophic vs heterotrophic growth in NDS experiments with different types of  $\text{PO}_4^{3-}$ .

Finally, we did find a significant effect of N chemical compound, but the compound type did not consistently influence N-LRR and NP-LRR. Different algal species may preferentially take up  $\text{NO}_3^-$  or  $\text{NH}_4^+$  (Dortch, 1990), and we did see that adding both chemicals together elicited the greatest N-LRR and NP-LRR. However, we found that  $\text{NH}_4\text{Cl}$  had the lowest N-LRR while  $\text{KNO}_3$  had the lowest NP-LRR. Background water chemistry and algal community composition may influence how algae respond when N is added in these different forms.

#### 4.2.2 | NDS substrate type

Our substrate model results contrast a previous experiment that tested the effect of substrate on nutrient limitation. In our analysis, clay pots had the highest reported N-LRR and P-LRR, whereas Capps et al. (2011) found plastic vials to have the highest N-LRR and periphytometers the highest P-LRR. For NP-LRR, however, our findings agree with Capps et al. (2011) that periphytometers have the highest estimate. The difference in these results could be driven by the Capps et al. (2011) experiment being completed in one stream during one season, emphasising the importance of considering spatial and temporal context when interpreting NDS experimental outcomes.

NDS substrate may affect experimental results via chemical interactions, and if this is the case, in-stream solutes would likely make these interactions very stream dependent. Brown, Maher, Norris, and Mathieu (2001) concluded that clay pots do not consistently diffuse nutrients because agar clogs the substrate pores, and pot types vary widely in pore size and diffusion rates. Furthermore, clay pots tend to bind P because of high aluminium and iron oxide

content (Brown et al., 2001). In our analysis clay pots had the high coefficient of variation (0.46–0.55) across all treatments, which does support Brown et al.'s (2001) conclusions about clay pot porosity and chemistry producing variable results. However, our results showed that rather than completely binding the chemical additions, clay pots had the highest N-LRR and P-LRR estimates. We hypothesise that the unique chemistry of clay pots could negate any inhibitory effects of nutrient additions, such as cation or nutrient toxicity (Fairchild et al., 1985) or  $\text{H}_2\text{O}_2$  toxicity (Tanaka et al., 2014).

Surface texture is another mechanism by which NDS substrate could affect algal accumulation. Rough, heterogeneous surfaces (e.g. clay pots or fritted glass discs in plastic vials) could support higher LRRs by promoting strong algal attachments that are resistant to loss by shear stress (Dudley & D'Antonio, 1991). Clay pots supported the highest control, P, and NP chlorophyll *a* biomass means of all the treatments, although the control means were comparable to vials. Periphytometer surfaces generally consisted of smooth glass fibre filters, and these treatments had the lowest control and NP chlorophyll *a* means.

Because NDS substrate type had a higher  $R^2$  index for P-LRR than all other experimental and environmental variables, we recommend standardising this factor across experiments. We caution against using clay pots as they produce highly variable NDS results. We do recognise that in larger rivers clay pots may be easy to anchor and deploy (Scrimgeour & Chambers, 1997), but plastic vials have been successfully deployed in large rivers (Marecchelli et al., 2009; Reisinger et al., 2016). Periphytometers are unique because they often employ smooth glass fibre-filter growth surfaces that are not necessarily representative of natural stream substrates. In addition, they are deployed with growth surfaces perpendicular to water flow (Matlock, Matlock, Storm, Smolen, & Henley, 1998). Benefits of periphytometers include a more constant nutrient diffusion rate and the ability to completely recover chlorophyll *a* from the glass fibre filters (Matlock et al., 1998). However, for comparability across experiments and to best emulate natural stream conditions, we concur with Tank et al.'s (2006) recommendation to use vials covered with a rough substrate such as a fritted glass disc whenever possible. Chlorophyll *a* can be completely recovered from these discs when the whole disc is placed in the extraction medium, and vials are easily deployed so that the growth surface is parallel to water flow (Tank et al., 2006).

### 4.3 | Drivers of spatiotemporal patterns in nutrient limitation

Urban and agriculture landscapes, which generally have higher in-stream nutrient loadings compared to forested or otherwise undisturbed land surfaces, produced lower N- and NP-LRRs than forest, grassland, and pasture land uses. This is consistent with the environmental variable models, where stream nutrient concentrations ( $\text{NH}_4^+$ ,  $\text{NO}_3^-$ , SRP) were always associated with decreases in LRRs. In a recent study across multiple streams spanning three U.S. regions, Reisinger et al. (2016) also showed a negative

relationship between nutrient LRRs and catchment per cent developed lands (urban and agriculture), as well as in-stream  $\text{NO}_3^-$  concentrations.

Contrary to our expectations, P-LRR was highest in agricultural streams. Agriculture can have variable effects on stream N:P molar ratios, but in our dataset, agricultural streams had the highest N:P ratios of any land use ( $\text{NO}_3^-:\text{SRP} = 155.7$ ). It is likely that algae in these streams were P-limited, causing the algae to respond to increased P from the NDS.

In-stream SRP was associated with decreases in N-LRR, which could occur if in-stream SRP and N were positively correlated in streams. For instance, we might expect that agricultural and urban land uses produce higher loads of both N and P (Carpenter et al., 1998), leading to a lower N effect size. In our dataset the correlation between in-stream SRP and  $\text{NO}_3^-$  was weak but highly significant (Table S3).

N:P molar ratios have been shown to be good predictors of N-limitation but not P-limitation (Keck & Lepori, 2012), which is consistent with our findings. However, neither this analysis nor Keck and Lepori (2012) used TN:TP molar ratios. TN and TP may be important predictors of nutrient limitation because they capture additional information about nutrient resources over the long term, while DIN and SRP are representative of instantaneous, bioavailable nutrients. In our dataset, TP was highly correlated with SRP and DIN was highly correlated with TN, but this does not necessarily mean the variables are interchangeable. We recommend that future studies should compare the utility of DIN:SRP ratios (commonly reported) and TN:TP ratios (rarely reported) for predicting algal biomass, algal responses to NDS, and nutrient uptake rates.

Canopy cover was an important variable for predicting algal responses to all nutrient treatments, but was not clearly correlated with land use in our study. Even streams in forested catchments were often described as having "open" riparian canopies. We expected canopy cover to be associated with decreases in all LRRs, as light becomes more limiting than N and P (e.g. Taulbee et al., 2005). However, we found that canopy cover was associated with increases in N-LRR but decreases in P-LRR and NP-LRR. In our dataset, canopy cover was significantly negatively associated with in-stream  $\text{NO}_3^-$  and  $\text{NH}_4^+$  but was not associated with in-stream SRP or TP (Table S3), which may explain the patterns of higher N-limitation under closed canopies.

While canopy cover can be a useful surrogate for available light, few studies reported turbidity and experimental depth, both of which are confounding factors that would affect light penetration to substrate surfaces. Continuous PAR measurements would be an ideal method to compare light differences among experiments. However, at a minimum we recommend reporting quantitative canopy cover, experimental depth, and turbidity to account for light differences.

In concordance with our land use results, ecoregions with intense urbanisation or agriculture (and likely higher nutrient loads) generally had lower responses to all three nutrient treatments, including Mediterranean California, Eastern Temperate Forests, and North American Deserts. Most of the Mediterranean California

streams were located in urban areas (e.g. Busse, Simpson, & Cooper, 2006). Similarly, Eastern Temperate Forests are characterised by high population density and dominance of urban and agricultural industries (Commission for Environmental Cooperation, 1997), and the North American Desert ecoregion is characterised by large-scale irrigated agriculture (Commission for Environmental Cooperation, 1997).

In contrast, ecoregions with lower human population densities and more intact forests generally had higher LRRs, including Marine West Coast Forests, Northern Forests, Northwest Forested Mountains, and the Tundra. In the case of Marine West Coast Forests and Northern Forests, soils are known to be relatively nutrient poor, which could lead to low in-stream nutrients in relatively undisturbed streams (Commission for Environmental Cooperation, 1997).

Ecoregions are described by temperature and precipitation regimes, vegetative land cover, topography, and soil nutrient status (Omernik, 1987) and may explain some natural variation in algal production. However, human activities greatly modify hydrologic processes and nutrient loading characteristics and thus alter algal dynamics in complex ways. Metrics such as catchment population density or quantitative catchment land use may have helped interpret the ecoregion results, but these were rarely reported in the studies we reviewed.

Köppen-Geiger climate classifications did not produce interpretable geographic patterns in LRRs, but in all models we did find a significant effect of climate classification and a relatively high  $R^2$  index as compared to experimental and environmental models. Ultimately the climate classification models did not provide clear insight about particular combinations of temperature and precipitation that led to differences in algal responses to nutrient additions. Additional factors that vary over space (e.g. elevation, stream slope) may have helped interpret spatial patterns in response ratios, but this information was rarely reported. Even exact latitude and longitude coordinates of streams were only reported in 25% of experiments. Future studies should provide more detail on geographic setting to facilitate comparisons across experiments.

Stream order was only a significant predictor of P-limitation and explained the highest among-site variation in P-LRR (15%). Order was not significantly correlated with in-stream P, but was negatively correlated with quantitative canopy cover. It is likely that P-LRR increased with stream order as light became less limiting.

We expected to find temporal differences in nutrient limitation based on season, especially since only nine experiments were conducted in tropical zones with little seasonality. We expected summer to have the highest estimate for all response variables because of high temperature and insolation. We found that autumn had the highest estimates for N-LRR and NP-LRR, while summer had the highest estimate for P-LRR followed by autumn. Francoeur et al. (1999) showed that nutrient limitation in 12 New Zealand streams was most common during summer, and that nutrient limitation was significantly associated with temperature. However, temperatures in our dataset (spring: 14°C, summer: 15.4°C, autumn: 11.9°C, winter:

6.6°C) varied less by season than did temperatures in the New Zealand dataset (spring: 9.2°C, summer: 14.4°C, autumn: 10.5°C, winter: 3.5°C). In our dataset, winter had much lower LRRs than did other seasons, indicating that low temperatures and light levels may have decreased algal growth. Autumn may have had high LRRs because of moderate temperatures but low canopy cover. Ultimately, interpreting seasonal variation in algal production requires considering context-specific factors like temperature, light, grazing activity, and in-stream chemical parameters like C, N, and P availability.

Temperature and precipitation regimes can affect algal patterns among streams (Biggs, 1996). Temperature does not significantly affect diffusion rates from NDS (Rugenski et al., 2008); however, temperature may increase algal growth rates (Biggs, 1996). We did find a significant relationship between temperature and NP-LRR in our dataset. We caution that in NDS studies most temperature measurements are instantaneous, and few experiments reported the full range and variability in temperatures experienced by the algal communities.

## 5 | CONCLUSIONS

NDS experiments can be valuable tools to determine stream nutrient limitation when the scope of inference is applied to an appropriate spatiotemporal scale. Our analysis has shown that NDS produce variable results that are not easily explained by single experimental and environmental factors. Broader spatial factors like ecoregion, climate classification, and stream order explained the most variation in nutrient limitation results, but the  $R^2$  index was still below 20% for all models. Thus, NDS experiments may be most useful for site-specific or regionally specific research questions. Comparing experiments across regions is likely only informative when methods are standardised (e.g. Reisinger et al., 2016; Tank & Dodds, 2003), and such studies should measure environmental gradients that are expected to be important such as light or turbidity.

While many studies have focused on the effects of resources on algal nutrient limitation, there is a clear need for studies that consider how algal stressors interact with nutrients. Instantaneous discharge and water velocity were sometimes reported, but metrics of flow variability or flood occurrence were lacking in the studies we analyzed (but see Biggs et al., 1998; Francoeur et al., 1999). Similarly, studies rarely reported herbivore grazing metrics, even though grazing is just as important as nutrients for controlling algal accrual (Hillebrand, 2002). A few studies (e.g. Biggs et al., 2000; Francoeur et al., 1999) did quantify insect grazers on the NDS growth surfaces at the end of the experimental periods. However, this is an instantaneous measure unlikely to sufficiently quantify grazing activity over the experimental period. A census of grazer densities and complete grazer exclusions would be required to accurately determine the influence of grazers on algal accumulation. Finally, turbidity can affect light penetration into streams as well as

scour algal cells from benthic growth surfaces. Turbidity was measured in some studies and not found to be a significant factor (e.g. Atkinson, Vaughn, Forshay, & Cooper, 2013), but we maintain that turbidity may interact with factors like canopy cover, water velocity, water depth, or vertebrate and invertebrate grazing to influence algal growth on NDS.

Determining drivers of nutrient limitation in streams is challenging because of the wide range of experimental methods employed, as well as a lack of reporting for environmental data. Given what has been reported in the literature, our meta-analysis emphasises the importance of in-stream nutrients, light levels, streamflow, season, and land use, as well as experimental methodologies. We have provided recommendations for standardising methodologies and reporting environmental variables that may drive and help explain NDS results as they relate to basic research and management of benthic algae. However, understanding the relative contributions of environmental and geographic factors requires future experiments on how methodologies may impact P-inhibition of algal growth. Future studies should also deploy NDS across a priori defined gradients of key environmental variables, particularly focusing on algal stressors that are, thus far, understudied.

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## SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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