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Abstract

The concentration of dissolved organic nitrogen (DON) is increasing in many northern hemisphere lakes, yet its use by phytoplankton and fate in the environment seldom have been quantified. We conducted 1 week, in situ, microcosm incubations across 25 lakes in northeastern North America to understand how DON, dissolved inorganic nitrogen (DIN), and dissolved inorganic phosphorus (P) affected phytoplankton biomass. In addition, we tested whether lakes were limited by single macronutrients (N or P) or colimited by both. Phytoplankton biomass in 80% of lakes responded similarly to DON and DIN additions. Of the lakes where N form produced differential responses, the majority of phytoplankton communities exhibited greater biomass accumulation with DON than DIN. Colimitation was the most common type of nutrient limitation among the study lakes, followed by P limitation. Limitation type shifted with N form in 40% of the study lakes, but without consistent patterns explaining how shifts occurred. Regardless of N form, lakes with watersheds more dominated by agriculture and higher total dissolved nitrogen (TDN) tended to show P-limited phytoplankton responses, while lakes with less agricultural watersheds and lower TDN tended to show colimited phytoplankton responses. Finally, ambient TDN and total phosphorus (TP) nutrient concentrations were stronger predictors of limitation

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type than ambient TDN : TP ratios. The different contributions of DON and DIN to phytoplankton biomass in some of our study lakes suggest that DON loading from surrounding watersheds may be an overlooked component in predicting phytoplankton productivity and nutrient limitation dynamics in freshwater ecosystems.

Dissolved inorganic nitrogen (DIN) has long been considered the essential form of nitrogen (N) for phytoplankton (Bronk et al. 2007; Bergström 2010). However, considering the net effects of all nitrogen forms (organic and inorganic) could enhance the ability to predict changes in phytoplankton biomass, particularly in low nutrient lakes where dissolved organic nitrogen (DON) is often the dominant N form (Boëchat et al. 2019). Many northern hemisphere, temperate lakes are experiencing shifts in N form, with increasing DON from lake browning, land use change, and organic N fertilizers (Williamson et al. 2015; Swarbrick et al. 2020; Xenopoulos et al. 2021). While decreasing DIN may be associated with decreases in phytoplankton biomass, DON can be a viable N source for phytoplankton under N limitation and may even promote phytoplankton blooms under a range of conditions (Berman 2001; Glibert and Burkholder 2011). Determining the role of DON in regulating phytoplankton biomass in oligotrophic to mesotrophic freshwater lakes will elucidate nutrient limitation dynamics and associated phytoplankton blooms in low nutrient systems.

DON is often assumed to have a negligible effect on phytoplankton growth (Bronk et al. 2007) because it has traditionally been considered refractory and required additional cellular resources and enzymes prior to assimilation. However, many phytoplankton taxa can access portions of the DON pool (Berman and Chava 1999; Bronk et al. 2007; Fiedler et al. 2015; Mackay et al. 2020). Urea, for example, is an N-rich organic compound that has been shown to yield comparable phytoplankton growth to DIN in microcosms (Berman and Chava 1999; Fiedler et al. 2015) and could be a key resource driving phytoplankton blooms in eutrophic lakes, even when DIN is available (Belisle et al. 2016; Swarbrick et al. 2020). Several phytoplankton species, assessed in monospecific culture experiments, can use a range of DON forms for growth, but the overall growth response decreases with increasing molecular complexity (Antia et al. 1975, 1991; Fiedler et al. 2015). This contradiction in the ability of phytoplankton to use organic N forms emphasizes the need to understand the role of bulk DON, which contains both usable and refractory components in controlling freshwater productivity.

Setting clear stoichiometric cut-offs for nutrient limitation of phytoplankton communities has been challenging (Bergström 2010; Ptacnik et al. 2010). The majority of lake ecosystems fall within a range of moderate N : P molar ratios (10–200; Moon et al. 2021). These N : P conditions have been associated with N-limited, P-limited, and colimited phytoplankton communities, depending on the season, lake trophic status, and available nutrients (Bergström 2010; Andersen et al. 2020; Lewis et al. 2020; Moon et al. 2021). To complement N : P

stoichiometric nutrient limitation assignments and help overcome some uncertainty under intermediate N : P conditions, the limiting nutrient can be assessed operationally via measurements of ecosystem responses to experimental additions of N and P (Elser et al. 2007; Andersen et al. 2020). Nutrient limitation assessments via in situ incubation experiments have consistently identified the prevalence of colimitation of phytoplankton growth by both N and P in nutrient-poor lakes (Sterner 2008; Harpole et al. 2011; Lewis et al. 2020).

Despite the potential role of DON in controlling phytoplankton growth, nutrient limitation is almost exclusively determined by addition of inorganic N and P with little consideration of the importance of DON (but see Mackay et al. 2020 and Swarbrick et al. 2020). In addition, TN concentration and TN : TP are used typically to assess N availability, but this coarse measurement masks the relative availabilities and transformations of various N forms and can produce misleading assessments of the macronutrient form that limits phytoplankton biomass (Bergström 2010; Ptacnik et al. 2010). Ratios of N : P based on biologically available forms of N and P tend to provide the best indicators of the controlling nutrients of phytoplankton growth (Ptacnik et al. 2010). In particular, DON may be a broadly available source of N for phytoplankton uptake either directly or indirectly through microbial enzymatic pathways (Berman 2001), especially in oligotrophic and mesotrophic lakes where a substantial portion of DON can turn over within days to weeks (Bronk et al. 2007; Boëchat et al. 2019). Failure to account for DON, some of which may quickly be converted into DIN, in limitation assessments diminishes our ability to determine drivers of phytoplankton growth (Berman 2001). In this study, we consider the overall potential of DON to support phytoplankton community biomass.

To assess the response of phytoplankton communities to DON vs. DIN additions and the impact of N form on limitation type (N or P or colimitation), we performed in situ microcosm incubations in 25 lakes in northeastern North America. We assigned nutrient limitation type into categories (Harpole et al. 2011) based on phytoplankton biomass responses to nutrient additions. Our study aims were to: (1) compare phytoplankton nutrient limitation type relative to N form, (2) determine the major direct and indirect drivers of phytoplankton nutrient limitation type across lakes, (3) compare phytoplankton biomass responses to DON and DIN additions, and (4) predict phytoplankton biomass responses to nutrient additions based on ambient TDN and TP concentrations and TDN : TP stoichiometric ratios. We hypothesized that DON additions would elicit the same limitation type response and similar phytoplankton biomass growth as DIN additions. We also hypothesized that ambient N : P stoichiometry and watershed land use

and land cover type, would affect phytoplankton biomass responses and limitation types (Lewis et al. 2020). In particular, we predicted that the amount of agricultural land within a lake's watershed would be a driver of limitation type.

Methods

Site descriptions

We performed in situ, microcosm incubation experiments in 25 lakes across 5 U.S. states and 1 Canadian province (Table 1). Our study lakes were mostly dimictic lakes whose watersheds were primarily deciduous and coniferous forests with varying degrees of agriculture and development. The lakes range over an order of magnitude in maximum depth (2.4–98 m), over several orders of magnitude in surface area (7–20,670 ha), and varied from oligotrophic to eutrophic, based on nutrient and chlorophyll *a* (Chl *a*) concentrations with most lakes being clear-water, non-dystrophic, and low-conductivity systems (Table 1). For each lake, watersheds were delineated using USGS StreamStats, Version 4 and Ontario Flow Assessment tools (OMNR 2000; Ries et al. 2017). The U.S. National Land Cover Database and Southern Ontario Land Resource Information System were used to extract each lake's land use and cover as a percent of total surrounding watershed area. To make LULC data comparable, individual U.S. and Ontario land use and cover classifications were pooled within related fields (water body, forest, developed, grassland, agricultural, and wetland).

Background water chemistry

At the start of the experiment, pelagic water samples were collected from the epilimnion for analysis of dissolved organic carbon (DOC), TDN, and Chl *a* concentration, with processing for each analyte done in a single lab with standardized methods across all lakes. To determine DOC and TDN concentrations, we filtered lake water through ~0.7-μm glass fiber filters and then acid sparged the sample prior to combustion using a Shimazdu TOC-L analyzer equipped with a TNM-L module at the Rubenstein Ecosystem Science Laboratory, University of Vermont (Badr et al. 2003). Samples were analyzed with the inclusion of a 5-point calibration curve spanning a range of concentrations from 1 to 10 mg C L^{-1} from potassium hydrogen phthalate and 0.5 to 5 mg N L^{-1} from NO$_3$. Nicotinic acid was used as a check standard. Due to uncertainty in sample results from the phosphorus analysis for this study, TP data were compiled from other projects that were conducted at a similar time in all but eight lakes (which were excluded from any analyses using TP or nutrient molar ratios, Table 1). All TP samples were collected within 2 weeks of the initial day of our experiment and processed using standard TP methods with a persulfate digestion followed by single reagent ascorbic acid photometric analysis (Ebina et al. 1983; Pritzlaff 2003). We calculated N : P molar ratios using TDN and TP. Ambient Chl *a* concentrations were used as a proxy for phytoplankton biomass in each lake and were processed

by filtering water samples through 0.7-μm glass fiber filters, followed by extraction using hot ethanol and subsequent spectrophotometric analysis (Lewis et al. 2020) at the State University of New York at New Paltz Biology Department.

Experimental design

We used in situ incubation microcosm (500-mL Bitran™ Fisher Scientific or resealable plastic bags) incubation experiments with a factorial design of DON as glycine, DIN as nitrate and ammonium, and inorganic P as phosphate (building on Williamson et al. 2010; Lewis et al. 2020). Prior to experimental set-up, ~20 L of lake water was collected at each location as a discrete surface sample at ~0.2 m in the pelagic zone. All water was filtered through a mesh sieve (ranging 80–160 μm depending on the lake) to remove large zooplankton grazers. For each lake, we had eight distinct nutrient treatments with four replicates in each treatment (Table 2). The eight treatments tested were control (C), DIN, DON, DIN + DON (DION), P, DIN + P, DON + P, and DION + P. All forms of N additions increased background N concentration by 168 μg N L^{-1}. P additions increased background phosphate concentration by 31 μg P L^{-1}. Nutrient additions at these levels have been shown to elicit a phytoplankton response across the range of ambient nutrient concentrations reported in this study (Lewis et al. 2020). Three replicates for each treatment were randomly positioned in a grid on polyvinyl chloride (PVC) racks, covered with neutral density filters (window screen mesh), and incubated for 1 week at the lake surface. Incubations for all lakes began between 21 June 2018 and 11 July 2018. The in situ microcosm design allowed for standardization of light and temperature conditions across all treatments within a lake. Following the incubation period, each experimental bag was processed for Chl *a* concentration via hot ethanol, acetone, or methanol extraction from 0.7-μm glass fiber filters or measured in vivo. Chl *a* concentration was determined on a spectrophotometer (Arar 1997) or a fluorometer (Welschmeyer 1994; Arar and Collins 1997). To address the analytical variance among the Chl *a* protocols used at various labs, we used standardized Chl *a* effect sizes to compare among lakes as described below.

Nutrient limitation type assignment and analysis

For each study lake, we used 2×2 two-way ANOVAs to assign nutrient limitation type using Chl *a* as the response variable and N and P additions as factors (Fig. 1; Harpole et al. 2011). This analysis was repeated in each lake for each of the three N forms (DIN, DON, DION); therefore, each lake was assigned three nutrient limitation types. In cases of unequal variances (Bartlett test, $p < 0.05$), we log-transformed the Chl *a* concentrations prior to running the ANOVA. We generated Type III fixed effects using the "cars" statistical package in R for all ANOVA analyses (Fox and Weisberg 2019; R Core Team 2020). If there was a significant interaction, we used a Tukey HSD post hoc comparison to compare all interaction terms. Nutrient limitation types (Fig. 1) were assigned based on the categorical system of Harpole et al. (2011). When there

Table 1. Lake characteristics for 25 northeastern U.S. and Canadian lakes, sorted by increasing Chl *a* concentration. Location (describes either the US state or Canadian province, latitude (Lat), longitude (Long), max depth, surface area (SA), DOC, Chl *a* concentration, TP, TDN, molar ratio of TDN : TP, specific conductivity (Cond), and percent agriculture (% Ag) are reported below. TP values for each lake are concentrations reported from each lab that conducted incubations. *Shelburne Bay is located on Lake Champlain, VT.

Lake	Location	Lat (°N)	Long (°W)	Depth (m)	SA (ha)	DOC (mg L⁻¹)	Chl *a* (µg L⁻¹)	TP h (µg L⁻¹)	TDN (mg L⁻¹)	TDN : TP⁻	Cond (µS cm⁻¹)	Ag h (%)
Awosting	NY	41.706	74.290	27.0	39	2.8	0.1	3.1	0.10	32.3	5	0.0
Shelburne Bay*	VT	44.426	73.232	25.0	962	3.7	0.3	13.0	0.35	26.9	154	37.7
Pleasant	ME	44.023	70.520	18.7	540	3.3	0.7	NA	0.16	NA	64	2.4
Willoughby	VT	44.763	72.064	98.0	683	2.6	0.8	13.0	0.22	16.9	106	3.6
Sunapee	NH	43.380	72.053	33.0	1674	3.8	0.9	9.4	0.20	20.7	100	2.6
Panther	ME	43.933	70.474	21.3	570	3.8	1.1	NA	0.17	NA	76	2.2
Minnewaska	NY	41.726	74.235	23.4	14	4.0	1.1	1.1	0.18	159.1	18	0.0
Otsego	NY	42.756	74.896	51.0	1637	3.1	1.2	5.7	0.75	130.7	288	37.6
Auburn	ME	44.139	70.253	35.9	925	3.2	1.5	9.0	0.19	20.6	80	7.2
Great Pond	ME	44.533	69.869	21.0	3453	4.4	1.6	17.9	0.21	11.6	57	3.7
Oneida	NY	43.200	75.900	16.8	20,700	4.2	1.9	8.0	0.41	51.5	353	23.4
Silver	NH	42.757	71.598	7.3	16	4.7	2.1	NA	0.34	NA	NA	7.3
Giles	PA	41.220	75.050	24.0	48	2.7	2.1	15.0	0.17	11.0	72	0.7
Long Pond	ME	44.506	69.914	32.3	1035	3.7	2.4	6.1	0.20	32.8	56	3.7
Lacawac	PA	41.376	75.093	13.0	21	6.8	2.5	15.0	0.33	21.7	22	0.0
Opinicon	ON	44.561	76.323	9.4	790	6.2	2.6	NA	0.36	NA	204	18.1
Warner Lake	ON	44.528	76.381	7.5	9	7.0	2.6	NA	0.39	NA	282	1.6
Elbow Lake	ON	44.477	76.427	9.0	16	9.0	2.7	NA	0.39	NA	79	5.8
Mohonk	NY	41.766	74.158	18.5	7	3.2	2.8	8.4	0.21	25.0	107	0.0
East Pond	ME	44.613	69.780	8.2	695	3.4	3.2	14.7	0.20	13.6	63	1.1
Moe Pond	NY	42.430	74.560	2.4	16	4.7	5.4	37.0	0.39	10.5	43	0.0
Carmi	VT	44.983	72.869	10.0	557	5.8	7.9	24.0	0.30	12.3	127	28.4
Sabattus	ME	44.138	70.102	4.9	790	4.9	10.2	NA	0.25	NA	103	11.0
Waynewood	PA	41.230	75.210	12.5	28	8.0	11.8	13.0	0.40	30.8	94	0.0
Bear	NY	42.347	79.385	10.0	46	7.4	12.3	NA	0.41	NA	135	18.2

Table 2. Full factorial microcosm experimental design.

Treatment	Treatment abbreviation	Nutrients added	Nutrient chemical formula	Background nutrient concentration increased by
Control	C	—	—	—
Inorganic N	DIN	Nitrate	$NaNO_3$	84 µg N L^{-1}
		Ammonium	NH_4Cl	84 µg N L^{-1}
Organic N	DON	Glycine	$C_2H_5NO_2$	168 µg N L^{-1}
Inorganic and organic N	DION	Nitrate	$NaNO_3$	56 µg N L^{-1}
		Ammonium	NH_4Cl	56 µg N L^{-1}
		Glycine	$C_2H_5NO_2$	56 µg N L^{-1}
Inorganic P	P	Phosphorus	NaH_2PO_4	31 µg P L^{-1}
Inorganic N and inorganic P	DIN + P	Nitrate	$NaNO_3$	84 µg N L^{-1}
		Ammonium	NH_4Cl	84 µg N L^{-1}
		Phosphorus	NaH_2PO_4	31 µg P L^{-1}
Organic N and inorganic P	DON + P	Glycine	$C_2H_5NO_2$	168 µg N L^{-1}
		Phosphorus	NaH_2PO_4	31 µg P L^{-1}
Inorganic N, organic N, and inorganic P	DION + P	Nitrate	$NaNO_3$	56 µg N L^{-1}
		Ammonium	NH_4Cl	56 µg N L^{-1}
		Glycine	$C_2H_5NO_2$	56 µg N L^{-1}
		Phosphorus	NaH_2PO_4	31 µg P L^{-1}

was not a significant interaction and there were no main effects, phytoplankton limitation type was assigned as None, indicating that Chl *a* did not significantly change and N and P were not limiting or limitation could not be determined from the experimental design. When there was a significant N main effect only (no significant interaction), limitation type was assigned as N limitation. When there was a significant P main effect only (no significant interaction), limitation type was assigned as P limitation. When there were both significant N and P main effects (no significant interaction), limitation type was assigned as Independent N and P colimitation, indicating that phytoplankton respond to both resources when added independently. When there was a significant N * P interaction with no significant main effects, limitation type was assigned as simultaneous N and P colimitation, indicating that both N and P were needed together to stimulate growth. When there was a significant N * P interaction and a significant N main effect, limitation type was assigned as serial-N limitation, indicating that the degree to which N additions stimulate growth depends on P addition. Alternatively, when there was a significant N * P interaction and a significant P main effect, limitation type was assigned as serial-P limitation. When there was a significant N * P interaction, N main effect, or P main effect, but the magnitude of the interaction response was lower than the other treatments, limitation type was assigned as nutrient inhibition (Fig. 1).

Drivers of limitation type

To test whether molar nutrient ratios (TDN : TP) published in the literature reflected the type of nutrient limitation we assigned based on chlorophyll responses, we examined the distribution of our assigned limitation types on a plot with regions designated as N-limited, P-limited, or colimited based on the analysis in Lewis et al. (2020). The literature-defined limitation regions suggest that N : P molar ratios of >110 (49.7 by mass) indicate P limitation and <44 (19.9 by mass) indicate N limitation; between those ratios indicates colimitation (Elser et al. 2009; Lewis et al. 2020). To determine the drivers of the ANOVA classified limitation types under DIN, DON, and DION treatments, we ran a regression tree analysis using the R package, "partykit" (Hothorn and Zeileis 2015). We grew two trees using the "anova" method, with the first focusing on biogeochemical variables (ambient Chl *a* concentration, TDN, TP, TDN : TP, DOC, pH, conductivity) and the second on land use and cover variables (grassland, wetland, forest, developed, agriculture, shrub as percent of watershed area). Both trees also included N form as a potential predictor variable. To prune the overfit tree, we used the one standard deviation rule (Knoll et al. 2015), where we found the smallest tree whose relative error was less than or equal to one standard deviation above the tree with the smallest relative error.

Biomass response to nutrient additions

To assess whether phytoplankton communities responded similarly to DON and additions, we compared biomass from DIN and DON treatments both across and within lakes and in the presence and absence of added P. To account for differences in Chl *a* analysis methods among labs, we determined the effect size for each treatment by calculating the ratio between the Chl *a* concentration of a given nutrient treatment with the mean Chl *a* concentration of the control for the same lake. Within lake standard error for control treatments ranged from
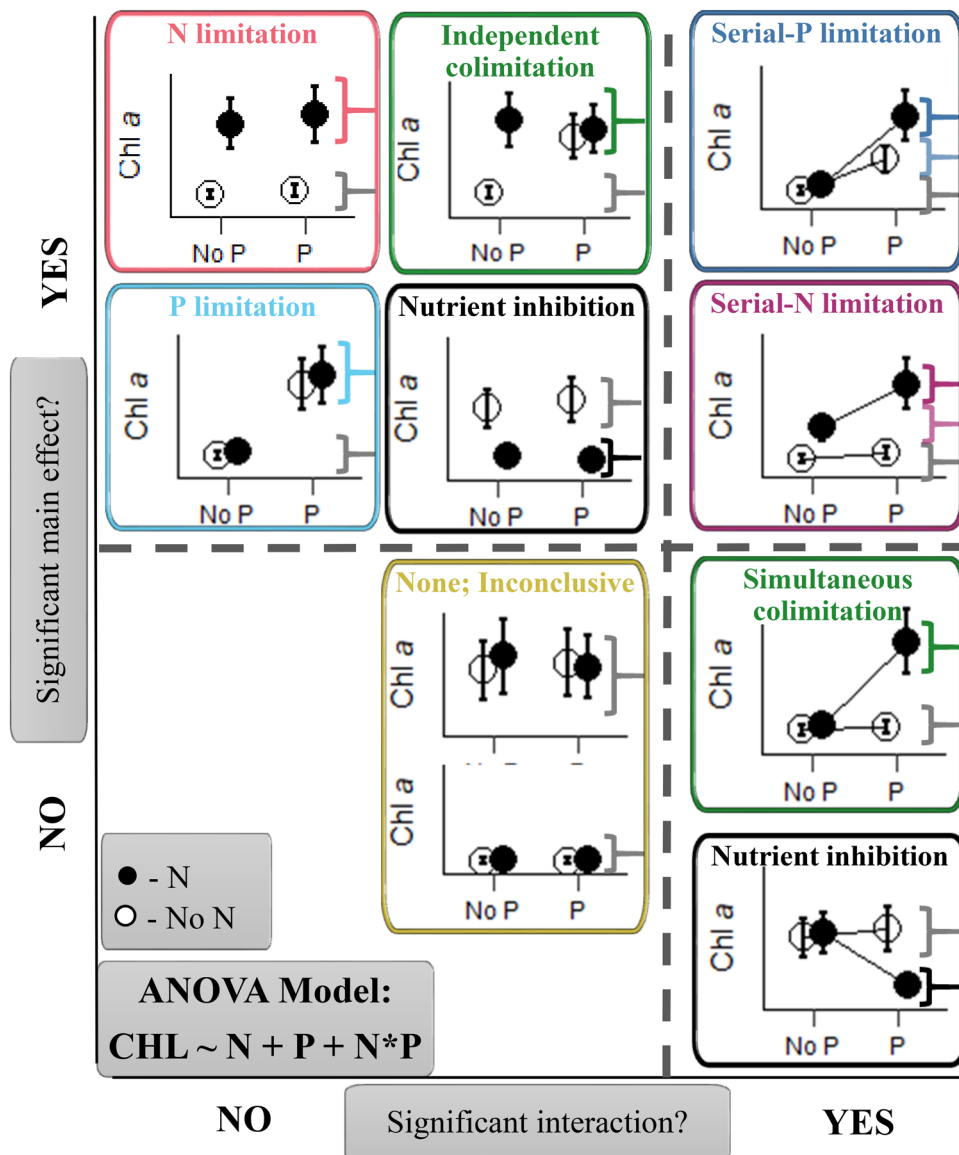
Fig. 1. Theoretical phytoplankton biomass (Chl a) responses to experimental nutrient additions. Nutrient limitation type is determined through a two factor, full model ANOVA. The main effects are nitrogen (N) and phosphorus (P) with levels of nutrient addition or no nutrient addition. The control treatment received neither P nor N. Closed circles indicate N additions, open circles indicate no N additions. Under this design the control treatment is labeled as no P and no N. See "Methods" section for a description of each limitation type.

0.02 to 1.29 µg Chl a L^{-1}. Ratios were then natural-log transformed as in Elser et al. (2007) and averaged across replicates. This produced seven Chl a effect size values (ES) for each lake, one for each nutrient treatment combination (ES$_{DIN}$, ES$_{DON}$, ES$_{DION}$, ES$_P$, ES$_{DIN+P}$, ES$_{DON+P}$, ES$_{DION+P}$).

Within each lake, we used a two-sample t-test to compare ES$_{DIN}$ vs. ES$_{DON}$, using a Tukey correction for the 25 comparisons (family α = 0.05). We used categorical and regression approaches to assess N form effects across all lakes. First, we used an ANOVA to test whether ES$_{DIN}$, ES$_{DON}$, and ES$_{DION}$ differed across all lakes. Then we used a 2 × 2 two-way ANOVA to compare the effect of N form with or without P

additions (interaction factors: ES$_{DIN}$, ES$_{DON}$, ES$_{DIN+P}$, ES$_{DON+P}$). Next, we used linear regression to compare effect sizes of DIN and DON additions with and without P across all 25 lakes (e.g., ES$_{DIN}$ vs. ES$_{DON}$ and ES$_{DIN+P}$ vs. ES$_{DON+P}$). Where the regression was significant, we compared the slope of the regression line to 1.

Finally, we tested the relationship of all dual nutrient effect sizes (ES$_{DIN+P}$, ES$_{DON+P}$, ES$_{DION+P}$) or all N-only effect sizes (ES$_{DIN}$, ES$_{DON}$, ES$_{DION}$) with TDN concentration, TP concentration, and TDN : TP ratios using general additive model (GAM) analysis in the "mgcv" R package (Wood 2011). GAM smoothing parameters were estimated using restricted maximum

likelihood (reml). Three models using univariate smooths (s) or tensor product smooths (te) were explored to explain effect size. The models were: (1) $s(TP) + s(TDN)$, (2) $te(TP, TDN)$, and (3) $s(TDN : TP)$. Akaike information criterion (AIC) and model fit via gam.check() were used for model selection (Wood 2011).

Results

Nutrient limitation types

Phytoplankton communities were mostly colimited, regardless of the added N form (Fig. 2; Supporting Information Fig. S1; Table S1). More than half of the lakes (56%) were colimited when DON was the N form (Fig. 3), 64% were colimited when DIN was the N form (Supporting Information Fig. S2), and 60% were colimited when DION was the N form (Supporting Information Fig. S3). Nearly half (48%) of the lakes were consistently colimited across all three different N treatments. All colimitation was simultaneous colimitation; we did not observe independent colimitation. For the rest of the manuscript, we use "colimitation" to refer to simultaneous colimitation. Overall, most study lakes (80%) experienced dual nutrient limitation (colimitation, serial-N, or serial-P) in at least one of the three N treatments (Supporting Information Table S1; Fig. 2). Only 28% of lakes experienced single nutrient limitation (N-limited, P-limited) at least once across N treatments; 12% of lakes remained consistently P-limited across all N treatments (Supporting Information Table S1).

Depending on the N form added, the remaining 40% of lakes that were not consistently colimited or P-limited changed limitation type at least once with no consistent trend of limitation type under various N forms (Fig. 2; Supporting Information Table S1).

Drivers of limitation type

Looking at all of our nutrient additions across the 25 lakes, limitation type was calculated a total of 75 times. Of these, only 33% of limitation types fell within ranges predicted by stoichiometric ratios of ambient nutrient concentrations (Fig. 4a). From regression tree analyses, both TDN concentration and the percentage of agricultural development in the watershed of a lake were found to be predictors of limitation type, regardless of the N form added. Lakes with a TDN concentration above $0.41 \, \mathrm{mg \, N \, L^{-1}}$ ($n = 3$) were generally characterized as P-limited (Fig. 4b). Similarly, lakes were more likely to be identified as P-limited if they had more than 18% agricultural development in their watershed (Fig. 4b).

Phytoplankton biomass response to nutrient additions

In the absence of P additions, phytoplankton response to DON or DIN addition ranged from positive, negligible, to negative across lakes (median effect size: 0.4, range: −1.4 to 1.2; Fig. 5). However, when DON or DIN additions were combined with P additions, phytoplankton responded positively in all lakes (median effect size: 1.7, range: 0.1–4.3). When phytoplankton communities were provided with either DON or
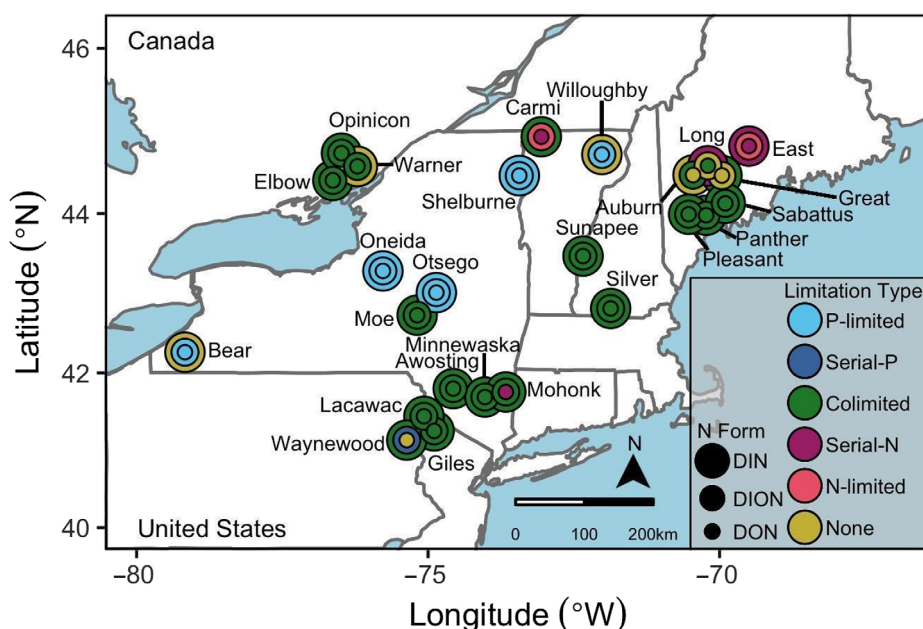

Fig. 2. Map of 25 study lakes across the northeastern United States and Canada including limitation type classification in response to different nitrogen forms. Phytoplankton in each lake were assigned nutrient limitation types depending on Chl *a* response to each nitrogen form: DIN, DON, DION. Colors indicate limitation type for each lake including single (P-limited, N-limited), multiple (Serial-P, Serial-N, and Colimited), and no limitation (None). Circle size depicts each of the three different experimental nitrogen additions (DIN, DION, DON) for each lake. The color in the largest ring is the nutrient limitation type under DIN, middle ring is the limitation type under DION, and the smallest circle is the limitation type under DON.
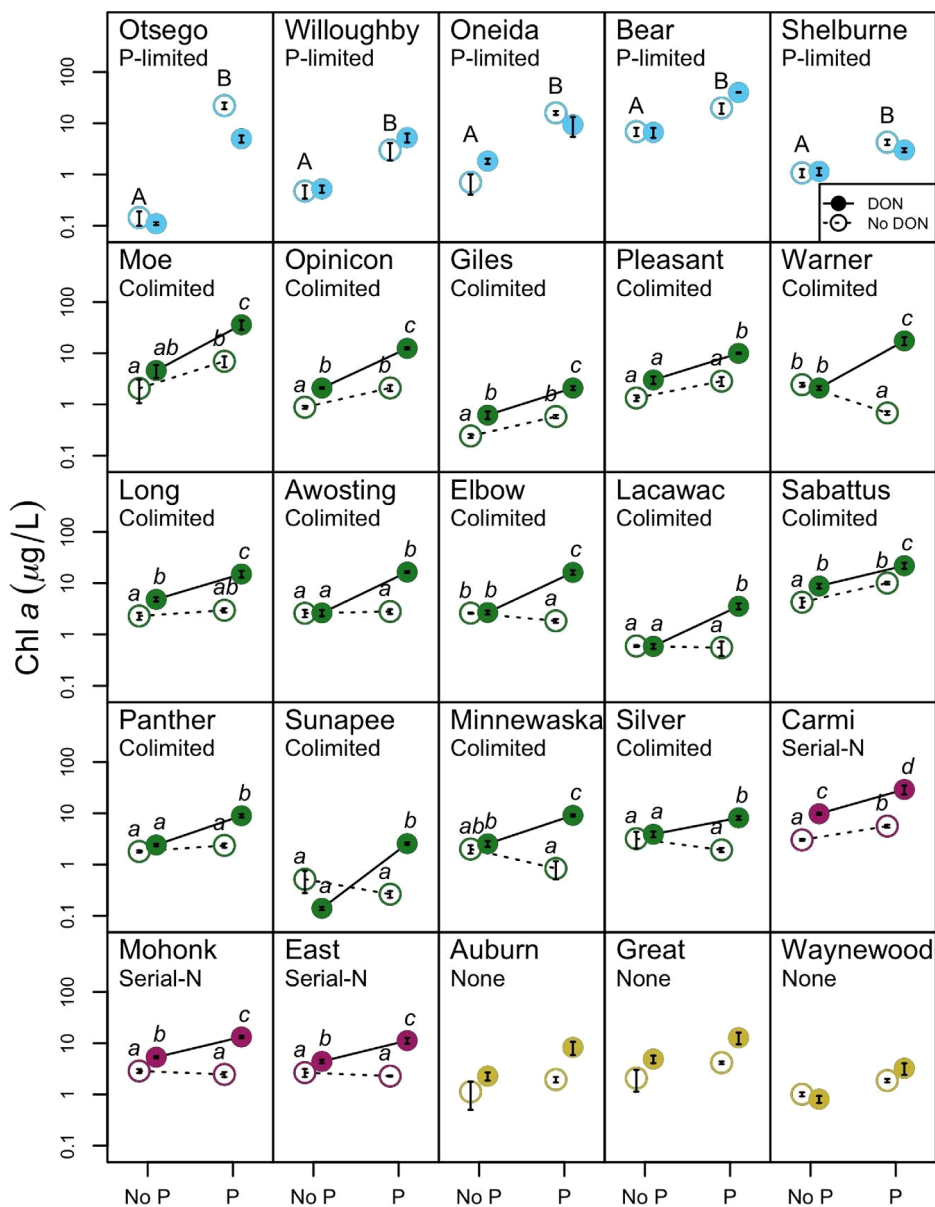
Fig. 3. Interaction plots for Chl *a* response to organic nitrogen (DON) and phosphorus (P) additions across 25 lakes. Similar plots for inorganic nitrogen and inorganic + organic nitrogen are in the Supporting Information. Filled circles are mean Chl *a* of ($\pm$SE) DON addition treatments, open circles are means of no DON addition treatments. Colors depict limitation type identified at top left of each panel. Colimitation refers to simultaneous colimitation, which was the only type of colimitation observed across our study lakes. Lowercase letters indicate significant interaction terms for colimited and serial limited phytoplankton communities. Uppercase letters indicate significant differences between the means of P vs. DON treatments for P-limited phytoplankton communities. Error bars represent standard error for each treatment. Note the *y*-axis log scale.

DIN, the addition of P stimulated a higher phytoplankton biomass response than without P addition (P main effect: $F_{1,96} = 23$, $p < 0.001$; Fig. 5). There was neither an interaction effect ($F_{1,96} = 0.55$, $p = 0.46$) nor a main effect of N form ($F_{1,96} = 0.01$, $p = 0.94$; Fig. 5).

Within each lake, phytoplankton communities had similar biomass responses, regardless of the N form provided. Without P additions, 88% of lake phytoplankton communities had the same effect size with DON and DIN; similarly, with P

additions, 72% of lakes had the same effect size regardless of whether DON or DIN was added (Fig. 6, open circles and triangles). Of the lakes with significantly different effect sizes, the majority experienced higher phytoplankton biomass with DON than DIN (above the gray line in Fig. 6). ES_{DIN} was significantly and positively related to ES_{DON} across all lakes ($ES_{DON} = 0.70 * ES_{DIN} + 0.10$, $F_{1,23} = 29$, $p < 0.001$, $R^2 = 0.54$; lower left solid line in Fig. 6). ES_{DIN+P} was significantly and positively related to ES_{DON+P} across all lakes
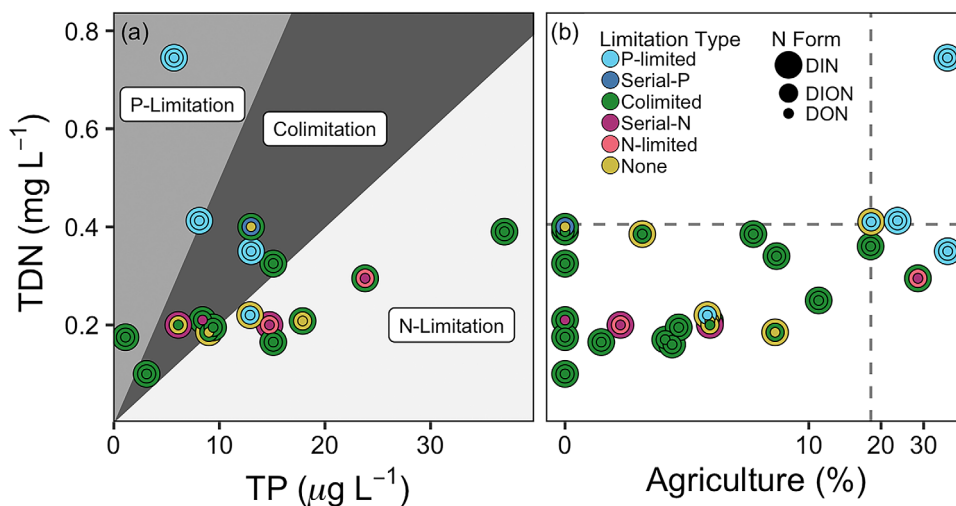
Fig. 4. (**a**) Comparison of ambient N : P ratios typical of N limitation (<44 by mole or <19.9 by mass; lightest gray section on bottom right), colimitation (darkest gray in middle section), and P limitation (>110 by mole or >49.7 by mass; medium gray section on top left) with limitation type as determined by in situ incubation. (**b**) Regression tree analysis for direct (*y*-axis) and indirect (*x*-axis) variables with only a single predictor of nutrient limitation type identified for each. TDN predicted a split in limitation types at 0.41 mg N L^{-1}. Percent agriculture predicted a split in limitation types at 18.2% agriculture of the surrounding watershed. Colors indicate limitation type for each lake; circle size depicts nitrogen additions (largest: DIN, intermediate: DION, smallest: DON).

($ES_{DON+P} = 0.49 * ES_{DIN+P} + 1.1$, $F_{1,23} = 26$, $p < 0.001$, $R^2 = 0.51$; upper right solid line in Fig. 6). However, the slopes of the relationships both with P and without P additions were statistically lower than a slope of 1, indicating that biomass growth using DON was higher than DIN at low effect sizes (without P: $t_{23} = 2.3$, $p = 0.033$, with P: $t_{23} = 5.3$, $p < 0.001$; Fig. 5).

Ambient TDN, TP, and TDN : TDP ratios were significant predictors of phytoplankton effect size when all nitrogen treatments with phosphorus were explored together (ES_{DIN+P}, ES_{DON+P}, ES_{DION+P}; Fig. 7), but did not relate to effect sizes in N-only treatments (ES_{DIN}, ES_{DON}, ES_{DION}; not shown). In the

first GAM, the main effects of ambient TP and TDN explained 77.2% of the deviance in $ES_{DIN+P,DON+P,DION+P}$ when N and P were experimentally added together (s[TP], wiggliness
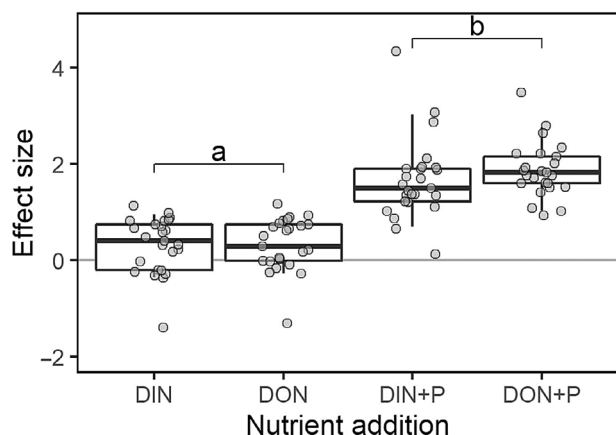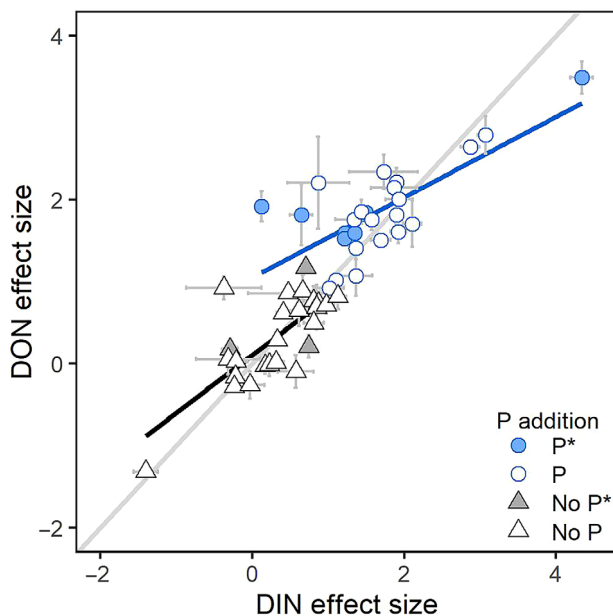

Fig. 5. Mean effect size for DIN, DON, DIN plus phosphorus (DIN + P), and DON plus phosphorus (DON + P) treatments across the 25 study lakes. The boxes indicate the median ± one quartile; the whiskers extend to the 5th and 95th percentile. Brackets and letters indicate significant main effect differences between treatments with and without P.

Fig. 6. Within-lake DIN effect size (ES_{DIN}) and DON effect size (ES_{DON}) in the absence of phosphorus (P) additions (triangles) and presence of P additions (circles). Each point represents an individual lake. Lakes that experienced significantly different DIN vs. DON effect sizes (*) are filled. Error bars represent standard error; some error bars are obscured by the point because they are small. The lines are linear regressions for the No P (black) and P (blue) treatments. The 1 : 1 line is drawn in a light gray diagonal line.
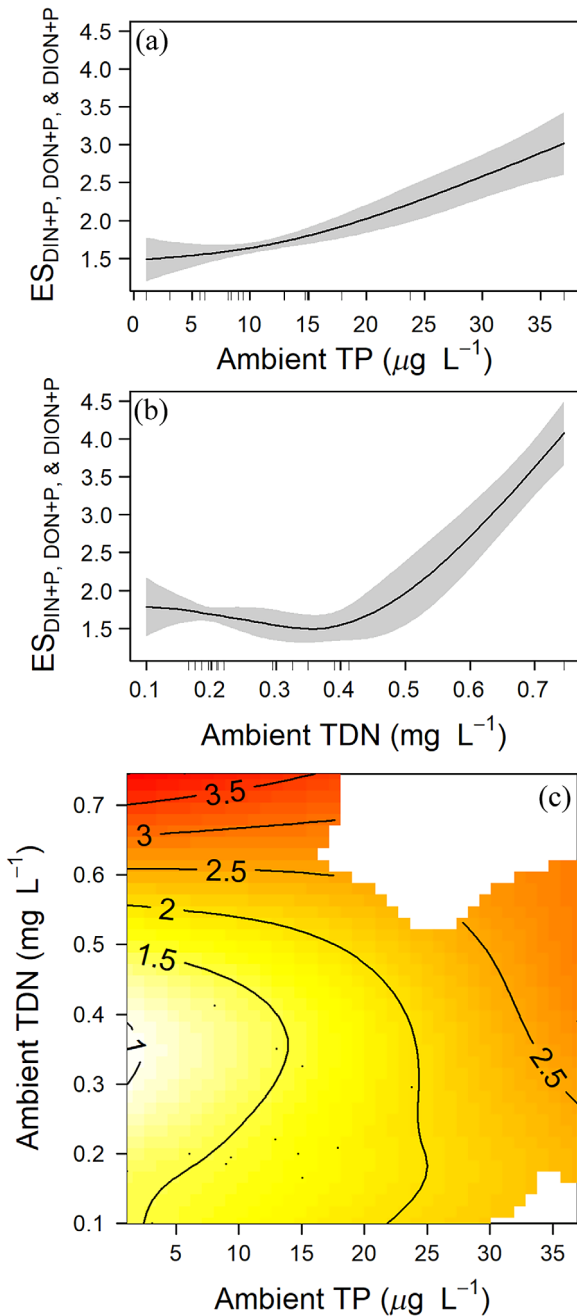
Fig. 7. General additive model analysis of ambient nutrient controls on phytoplankton effect size of conutrient addition (ES_{DIN+P}, ES_{DON+P}, ES_{DION+P}). *X*-axis inner tick marks indicate sample distribution. (**a**) Main effects of TP and (**b**) TDN. (**c**) Interaction effect of TP and TDN, contour lines represent phytoplankton effect size and heat map of interpolated effect size in the same units as contour lines.

[edf] = 2.1, F = 16.1, $p < 0.001$; s[TDN], edf = 3.2, F = 31.7, $p < 0.001$; Fig. 7a,b). In the second GAM, the interaction between TP and TDN explained 76.9% of the deviance in $ES_{DIN+P,DON+P,DION+P}$ when N and P were experimentally added together (edf = 5.6, F = 22.6, $p < 0.001$; Fig. 7c). In the third GAM, the main effect of TDN : TP ratio explained 70.6% of the deviance

in $ES_{DIN+P,DON+P,DION+P}$ when N and P were experimentally added together (edf = 4.5, F = 21.1, $p < 0.001$; not shown). Model fit and AIC determined that first (ES = s[TP] + s[TDN]) and second (ES = te[TP, TDN]) GAMs were the best models, both outperforming the third GAM (SE = s[TDN : TP]). GAM results indicated that phytoplankton effect size was lowest (<1.5) when TP was low (<10 $\mu g\,L^{-1}$) and TDN was between 0.3 and 0.5 mg L^{-1} (Fig. 7c). Effect size was moderate (1.5–2) when TP was 10–15 $\mu g\,L^{-1}$, TDN was <0.2 and when TDN was 0.5–0.6 mg L^{-1} (Fig. 7a,b). Effect sizes were the highest (>2) when TP was high (>20 $\mu g\,L^{-1}$) or when TDN was high (>0.6 mg L^{-1}; Fig. 7a,b).

Discussion

In this study, we found that phytoplankton communities experienced increased biomass in response to DON additions. Furthermore, the magnitude of phytoplankton effect size in response to DON inputs was similar to that of DIN, suggesting that the utility of DON as an N source matches DIN. The phytoplankton communities' ability to use DON caused some lakes to shift limitation type in response to relative availabilities of different N forms. The majority of phytoplankton communities were colimited, regardless of the N form used to assess limitation type, which is consistent with previous nutrient limitation studies (Harpole et al. 2011; Andersen et al. 2020; Cook et al. 2020; Lewis et al. 2020). Several lakes, however, had nutrient limitation types that did not match expectations based on ambient water column TDN : TP stoichiometric ratios (Fig. 4a). We also found that environmental variables could be used to understand differences in nutrient limitation patterns. High TDN concentrations and agricultural land use were associated with single nutrient P limitation and comparatively lower ambient TDN and TP concentrations were associated with dual nutrient limitation (colimitation, serial-N, or serial-P).

In aquatic ecosystems, the DIN pool (primarily nitrate and ammonia) has historically been thought to be preferred by autotrophs (Morris and Lewis 1988; Bronk et al. 2007; Bradley et al. 2010). However, DON pools in lakes are complex, comprising a variety of N forms with different reactivities, chemical complexities, and biological availabilities (Mackay et al. 2020; Wymore et al. 2021; Reinl et al. 2022). As a result, treating N pools as inorganic or total often oversimplifies the movement and transformation between various N pools and can mask the availability of DON as a primary N source for phytoplankton (Berman and Bronk 2003; Mackay et al. 2020; Wymore et al. 2021). The results of our study provide evidence that DON supported similar levels of phytoplankton community biomass as DIN in lakes (Fig. 5). At times, phytoplankton biomass was higher in DON treatments than it was with DIN for the same lake. These responses emerged when ambient TDN or TP concentrations were relatively low (Fig. 6). In oligotrophic and mesotrophic freshwater systems, those typically less anthropogenically impacted, DON often makes up the majority

of the TDN pool (Berman and Bronk 2003; Wymore et al. 2021). Therefore, DIN is an incomplete metric of N availability in lakes. Treating DIN as the preferred or only source of N for freshwater phytoplankton may result in overlooking a large component of what is actually accessible among all individual N pools.

Diversity among phytoplankton taxa, mutualism, and microbial nutrient transformations could explain why most lakes (60%) experienced identical nutrient limitation types across the different N forms (Fig. 2; Supporting Information Table S1). In these cases, both N forms were taken up equally well by phytoplankton in our microcosm incubations. Mutualism between heterotrophic bacteria and phytoplankton might help explain some of these similar responses. Organic nutrients can be mineralized by the phytoplankton's microbiome and rapidly assimilated by phytoplankton (Reinl et al. 2022). In addition, isolated or cultured phytoplankton taxa can have varying growth rates under different N forms with some taxa growing best in the presence of DON (Berg et al. 2003; Fiedler et al. 2015; Erratt et al. 2020). In diverse, natural phytoplankton communities, as in the experiment here, sufficient resource niches are likely available for different phytoplankton taxa to exploit multiple N forms. Concurrently, heterotrophic mineralization of N could be occurring in the bags during incubation, which can cause excess DON to be excreted as ammonium by heterotrophic or mixotrophic microorganisms (Callbeck et al. 2021). We added a small and labile organic molecule (glycine) here; however, other more complex and large organic molecules exist naturally in freshwaters (Berman and Bronk 2003; Bronk et al. 2007; Mackay et al. 2020). Extracellular enzymes produced by heterotrophic organisms could cleave DON from those larger organic molecules creating DIN and more labile DON molecules that could be directly assimilated by a diverse phytoplankton assemblage. Additional research is needed to focus on the nutrient transformational and uptake mechanisms that lead to similar phytoplankton growth on DON and DIN.

In the remaining 40% of lakes, N form did affect the limitation types observed (Fig. 3; Supporting Information Fig. S2; Table S1), suggesting that phytoplankton communities were better able to take advantage of some N forms over others, based on preferential uptake or differing microbial transformations (Donald et al. 2013; Cira et al. 2016; Mackay et al. 2020). Differential phytoplankton responses to DIN and DON could be facilitated by carbon limitation of heterotrophic bacteria. Bacteria in low DOC lakes would rapidly mineralize the added DON for carbon, leveling ammonium behind to stimulate phytoplankton growth. Under this hypothesis, low DOC lakes would see higher DON phytoplankton effect sizes than DIN effects size with the opposite being true of high DOC lakes. This hypothesis is plausible, but, in this study, there was no clear pattern between DOC concentration and the phytoplankton effect size of DON, DIN, and DION. With wide interspecific variation, different phytoplankton taxa such as dinoflagellates,

cyanobacteria, chrysophytes, and chlorophytes can survive and grow rapidly using DON (Berman and Bronk 2003). In fact, some taxa even exhibit preference for reduced forms of DON like urea and dissolved free amino acids over DIN (Antia et al. 1975, 1991; Erratt et al. 2020). Phytoplankton uptake and incorporation of DON depends on the form of N; phytoplankton growth is greatest when the N form is simple and reduced (Berman and Chava 1999; Berg et al. 2003; Bronk et al. 2007; Fiedler et al. 2015; Moschonas et al. 2017; Mackay et al. 2020). For example, glycine, the DON form in this study, is accessible to a variety of phytoplankton taxa based on its size and amine group (Antia et al. 1975; Wheeler et al. 1977; Mackay et al. 2020). Mixotrophic taxa can also assimilate DON via osmotrophy, generating phytoplankton biomass and complementing autotrophic production (Caron et al. 1990; Jansson et al. 1996; Granéli and Legrand 1999). Excess N could also be excreted in a usable form by autotrophic phytoplankton (Granéli and Legrand 1999). While we are limited in our knowledge of the phytoplankton diversity in our microcosms and lakes, mixotrophs are common across our study region and could have substantially contributed to the overall growth observed after DON additions (Princiotta and Sanders 2017).

Stoichiometric ratios were weaker predictors of nutrient limitation compared to ambient nutrient concentrations. This pattern may be because the relatively narrow range of TDN : TP (50 units) was not enough to provide a holistic understanding of the relationship between ratios and nutrient limitation type (Ptacnik et al. 2010; Moon et al. 2021). Moreover, many lakes, including those in our study, have N : P ratios of 10–200 and these stoichiometries have been linked to multiple phytoplankton community limitation types, depending on ambient conditions (Bergström 2010; Andersen et al. 2020; Lewis et al. 2020; Moon et al. 2021). More extreme values (e.g., TDN : TP < 10 or > 200) might be needed to predict phytoplankton growth reasons to nutrient additions from N : P ratios alone. Previously, in a subset of this study's lakes, when using DIN to predict limitation types, lakes that were N-limited generally fell within the expected stoichiometric range as predicted by ambient TDN : TP molar ratios, but colimitation and P-limitation were not as well constrained by stoichiometric ratios (Lewis et al. 2020). In this study, we found that lakes exhibited variability in limitation type in response to different N forms that were not easily explained by stoichiometry (Fig. 4a). These patterns could be due to the form of nutrients used to calculate stoichiometric ratios or limitations to the application of Redfield concepts under dual nutrient limitation (Ptacnik et al. 2010; Cook et al. 2020; Lewis et al. 2020). For example, using total N in calculating stoichiometric ratios may overestimate the availability of macronutrients for autotrophic uptake. Ultimately, ambient nutrient concentration was a better indicator of the phytoplankton community's nutrient requirements in these predominantly oligotrophic to mesotrophic study lakes than TDN : TP ratios (Fig. 7; Bergström 2010).

Colimitation was the dominant form of limitation type across our study lakes and with different N forms (Fig. 5). Colimitation is prevalent across a range of aquatic systems, including streams (Tank and Dodds 2003), lakes (Elser et al. 2009; Paerl et al. 2016; Cook et al. 2020; Lewis et al. 2020), and marine ecosystems (Elser et al. 2007). In addition, spatial patterns of nutrient limitation within a single aquatic ecosystem (Levine et al. 1997; Elser et al. 2007; Sterner 2008), as well as nutrient limitation studies across lakes with different land use patterns (Bratt et al. 2020) have further highlighted the importance of both N and P on phytoplankton biomass. The colimitation pattern could stem from both organismal and community processes. Additional nutrients of one type (e.g., N) could enable an organism to access the other nutrient (e.g., P), possibly through the production of particular enzymes that facilitate access to that nutrient (Bracken et al. 2015). Communities with a diversity of functional taxa, including obligate autotrophs, mixotrophs, and N-fixers could simultaneously use N or P depending on the stoichiometry needs and physiology of the taxa (Harpole et al. 2011; Andersen et al. 2020; Swarbrick et al. 2020).

While phytoplankton in most lakes were colimited under at least one N form, we did find three lakes that were exclusively P-limited regardless of N form (Fig. 2). Background TDN and land use were the best predictors of nutrient limitation type, where P-limited lakes occurred at higher TDN concentrations and agricultural land use (Fig. 4b). However, we had only a few single nutrient limited lakes with variable lake characteristics (Table 1); we would expect more single nutrient limited lakes in expansion to eutrophic and hypereutrophic lakes in more agriculturally dominated landscapes (Hayes et al. 2015). Agricultural landscapes can be the source of different nitrogen and phosphorus forms depending on the agriculture use (row crops vs. pasture), vegetation taxa, fertilizer application regime, and hydrologic flow-paths that generate high runoff and groundwater N concentrations (Bullerjahn et al. 2016; Andersen et al. 2020; Wymore et al. 2021). With low to moderate concentrations of P, phytoplankton can respond quickly to any additional P if P is rapidly cycled (Morris and Lewis 1988). If N concentrations were to decrease, we might expect seasonal shifts in these P-limited lakes to colimitation or even N limitation (Kolzau et al. 2014). Therefore, while P limitation is common among high TDN and agricultural land use, changing ambient N loads may result in shifts to colimitation.

The results of our experiment reveal that organic forms of N can support lake phytoplankton communities. DON additions caused a similar phytoplankton effect size as DIN, indicating that DON is a more important resource for phytoplankton growth than is typically believed (Bronk et al. 2007), especially in oligotrophic lakes where DIN can be limiting.

Our study lakes had mostly low to moderate ambient N and P concentrations. Phytoplankton communities responded to different N forms, especially in the presence of P additions, suggesting phytoplankton communities have flexibility in their nutrient demands and can capitalize on the DON pool across a range of nutrient conditions. In this region and others around the world, lakes are experiencing an increase in dissolved organic matter ("browning"; Williamson et al. 2015; Stetler et al. 2021) and increasing DON concentrations (Stetler et al. 2021). Expanding on these results to understand how various N forms, both labile and refractory, might affect phytoplankton might prove crucial for predicting algal blooms and nutrient limitation and setting load limits in freshwater ecosystems.

Data availability statement

Experimental data available by request through contacting one of the study's co-authors. Metadata for the study is available in Table 1.

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Conflict of Interest

None declared.

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