Sustained stoichiometric imbalance and its ecological consequences in a large oligotrophic lake

James J. Elser[a,1] 🆔, Shawn P. Devlin[a], Jinlei Yu[b], Adam Baumann[a], Matthew J. Church[a] 🆔, John E. Dore[c] 🆔, Robert O. Hall Jr.[a] 🆔, Melody Hollar[d], Tyler Johnson[e], Trista Vick-Majors[a,f], and Cassidy White[g]

Considerable attention is given to absolute nutrient levels in lakes, rivers, and oceans, but less is paid to their relative concentrations, their nitrogen:phosphorus (N:P) stoichiometry, and the consequences of imbalanced stoichiometry. Here, we report 38 y of nutrient dynamics in Flathead Lake, a large oligotrophic lake in Montana, and its inflows. While nutrient levels were low, the lake had sustained high total N: total P ratios (TN:TP: 60 to 90:1 molar) throughout the observation period. N and P loading to the lake as well as loading N:P ratios varied considerably among years but showed no systematic long-term trend. Surprisingly, TN:TP ratios in river inflows were consistently lower than in the lake, suggesting that forms of P in riverine loading are removed preferentially to N. In-lake processes, such as differential sedimentation of P relative to N or accumulation of fixed N in excess of denitrification, likely also operate to maintain the lake's high TN:TP ratios. Regardless of causes, the lake's stoichiometric imbalance is manifested in P limitation of phytoplankton growth during early and midsummer, resulting in high C:P and N:P ratios in suspended particulate matter that propagate P limitation to zooplankton. Finally, the lake's imbalanced N:P stoichiometry appears to raise the potential for aerobic methane production via metabolism of phosphonate compounds by P-limited microbes. These data highlight the importance of not only absolute N and P levels in aquatic ecosystems, but also their stoichiometric balance, and they call attention to potential management implications of high N:P ratios.

phosphorus | nitrogen | stoichiometry | limnology | ecosystem

The emergence of the Anthropocene era has been marked by major changes in all of Earth's major biogeochemical cycles (1). For example, fluxes of carbon (C) (as CO_2) to the atmosphere have increased by ~14% during the last 120 y largely due to fossil fuel combustion. Fluxes of nitrogen (N) into the biosphere have increased by at least 100% due to application of the Haber-Bosch reaction for fertilizer production, land use change favoring N-fixing legumes, and conversion of atmospheric N_2 to available forms (NO_x) by high temperature combustion of petroleum and fossil gas (2). Finally, large-scale mining of phosphorus (P)-rich geological deposits for production of fertilizers has amplified rates of P cycling in the biosphere by ~400% (1). Each of these perturbations has biophysical and ecological impacts at differing time and space scales. For C, its accumulation in the atmosphere has altered Earth's radiative balance, warming the planet and perturbing precipitation patterns globally. Amplified inputs of reactive N to the Earth system enter the hydrosphere and, thus, potentially lead to overenrichment of lakes, rivers, and coastal oceans across broad regions. Amplifications of P inputs often impair water quality at watershed and local scales (3), stimulating phytoplankton production and contributing, along with N, to harmful algal blooms, fish kills, and "dead zones" (4, 5). These differential amplifications and their contrasting spatial scales indicate that ecosystems are experiencing not only absolute changes in biogeochemical cycling, but also perturbations in the relative inputs and outputs of biologically important elements (6). Studies of elemental coupling and uncoupling in ecosystems are not yet widespread, but emerging work has shown how C, N, and P are differentially processed as they pass through watersheds (7).

The potential for differential alteration in supplies of N and P to aquatic ecosystems suggests that understanding the nutrient status of a water body requires knowledge of not only absolute supplies of limiting nutrients, but also their relative proportions (i.e., their N:P stoichiometry). This work has been facilitated in recent years by the emergence of the theory of ecological stoichiometry (8). For example, seminal work by Redfield (9) found that N:P ratios in marine organic matter were tightly constrained around 16:1 (molar, here and throughout), a value that may represent the central tendency for the N:P ratio of phytoplankton undergoing balanced growth in which major

Significance

Both nitrogen (N) and phosphorus (P) drive water quality and are heavily altered by human activities that amplify their supplies to lakes, rivers, and oceans. Considerable attention is given to management of absolute nutrient levels but less to their relative abundances, their N:P stoichiometry. This study documents high N:P ratios in low-nutrient Flathead Lake that persist despite considerably lower N:P ratios in river nutrient inputs. The lake's elevated N:P ratios are associated with phytoplankton P limitation, impaired food quality for zooplankton, and potential production of the greenhouse gas methane by P-limited microbes. These findings highlight the need to consider not only absolute levels of N and P in aquatic ecosystems, but also their stoichiometric balance.

[1]To whom correspondence may be addressed. Email: jim.elser@umontana.edu.

pools of N (protein) and P (RNA) are produced at the same rate (10). In lakes, N:P ratios show much wider variation—around a value of ~30—perhaps reflecting the biogeochemical connections of lakes to terrestrial systems where N:P ratios have a similar value and range of variation (11). Nevertheless, this classic "Redfield ratio" of 16:1 can be thought of as representing a balanced nutrient supply for primary producers in pelagic ecosystems. When the ratios of N and P supplied deviate from this balanced ratio, primary limitation of growth by N (when N:P is low) or by P (when N:P is high) can occur. For example, phytoplankton growth in lakes with imbalanced total N: total P (TN:TP) ratios that exceed 30:1 is generally P limited (12). Disproportionate inputs of N relative to P from atmospheric deposition can increase lake TN:TP ratios and shift lake phytoplankton from N to P limitation (13), inducing P limitation in zooplankton (14). Imbalanced N:P ratios in nutrient supplies can also shift the competitive advantage among phytoplankton and enhance production of potentially toxic compounds during harmful algal blooms. For example, skewed supplies of N relative to P can increase production of N-rich secondary compounds by phytoplankton, while disproportionate inputs of P relative to N can induce production of C-based toxins (15). High N:P ratios can also enhance proliferation of fungal parasites of phytoplankton (16).

Imbalanced N:P ratios can impact aquatic ecosystems in other ways. For example, they can alter the functioning of food webs. In particular, shifts in nutrient supply regimes that enhance P limitation can impede energy flow in trophic interactions because biomass of P-limited primary producers is of low quality for animals due to its low P content (8). Ecosystem shifts to high N:P ratios and more prevalent P limitation can also impact the cycling of the greenhouse-active gas methane (CH_4) because phosphate limitation can result in production of methane under aerobic conditions in both marine and freshwater phytoplankton and bacteria (17, 18). Both chemoheterotrophic and photoautotrophic bacteria (e.g., *Pseudomonas*, SAR11, *Trichodesmium*, *Synechococcus*) can metabolize organic P compounds, called phosphonates, to acquire P. Microbial cleavage of one type of phosphonate, methylphosphonic acid (MPn), to acquire P results in formation of methane (17). While it is likely that anaerobic methane production due to oxygen depletion in response to P-driven eutrophication is the dominant process connecting P to methane dynamics, the significance of aerobic phosphonate metabolism to global methane cycles remains to be assessed. However, contributions are potentially large, given the prevalence of P limitation in both freshwater and marine ecosystems. In light of emerging trends that suggest overall increases in ecosystem N:P ratios due to human impacts (6), these trophic and biogeochemical impacts of stoichiometric imbalance show that it is critical to consider not only absolute levels of nutrients, but also their stoichiometry. In particular, high N:P ratios can accentuate P limitation, causing a suite of ecological impacts that, currently, are poorly described.

In this paper, we illustrate the utility of stoichiometric approaches by combining analyses of long-term records of nutrient supply and dynamics, together with contemporary experiments, to examine how imbalances in N:P stoichiometry (e.g., strong divergence from classic Redfield proportions) influence plankton ecology and biogeochemistry across multiple scales in Flathead Lake, a large lake in western Montana. The lake is itself relatively unperturbed by human impacts and, thus, maintains low overall nutrient levels. However, the strong stoichiometric imbalance that we describe makes Flathead Lake appropriate for assessing ecosystem consequences of what

appear to be general trends of increasing N:P ratios in global ecosystems (6). Numerous limnological properties of the lake and its inflow rivers have been monitored continuously for several decades, including concentrations of various forms of N and P. Thus, these time-series data allow us not only to assess long-term variability or stability in the stoichiometry of N and P in the lake and its river inflows over decadal time scales, but also to connect its stoichiometry with potential consequences for nutrient limitation, food web dynamics, and biogeochemical cycling under low-nutrient conditions.

Results and Discussion

Lake-Scale Stoichiometry. To assess the long-term status of N and P in Flathead Lake, we summarized data for average concentrations of TN and TP and their stoichiometric ratio in the upper 30 m of Flathead Lake since 1983 (Fig. 1). While exhibiting considerable seasonal variability, TN and TP concentrations, as well as the TN:TP ratios, showed no long-term trends over the 38 y of observation (Fig. 1). Overall, concentrations of TN and TP were low, falling in the lower 2.5% and 1%, respectively, of observations in the US Environmental Protection Agency's 2017 National Lakes Assessment (19). TN concentrations did decline
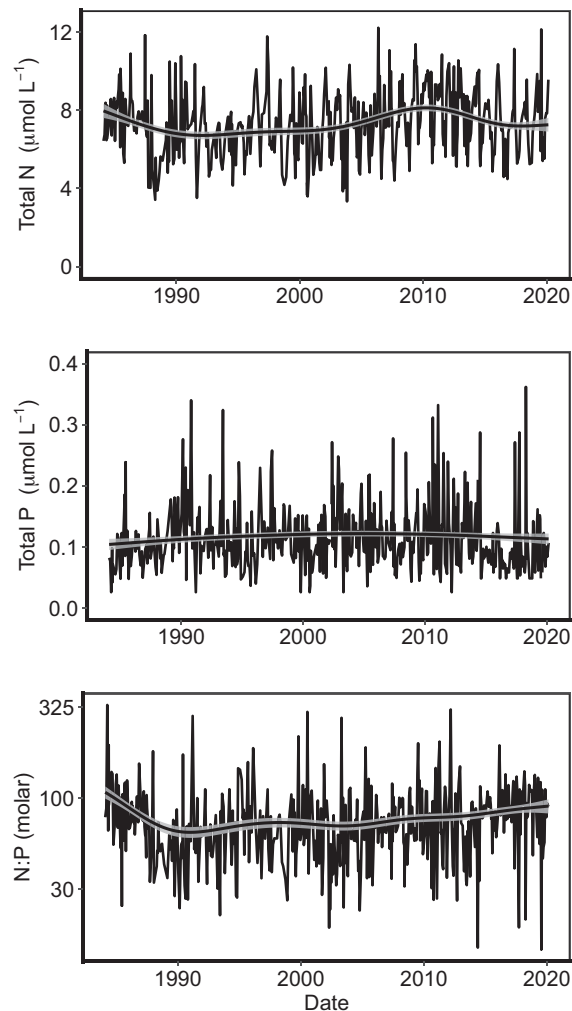
Fig. 1. (*A–C*) Biweekly or monthly concentrations of (*A*) TN, (*B*) TP, and (*C*) the TN:TP stoichiometric ratio (molar) in integrated samples of the upper 30 m of Flathead Lake from 1983 to 2019 (note natural log scale). GAMs were used to assess trends in the time series data, with solid black lines indicating the fitted model and gray shading indicating model error.

somewhat during a 5- to 6-y period during the late 1980s and had a temporary increase around 2010 (Fig. 1A), while TP concentrations were constant throughout (Fig. 1B). Following a modest decline at the start of the record (Fig. 1C), TN:TP ratios, while quite variable at short time scales, have held steady at high values (80 to 90:1) for the 30 y since 1990. Current TN:TP ratios of ~90:1 place Flathead Lake in the upper 87th percentile for TN:TP ratio in lakes of the United States (19). Thus, Flathead Lake has sustained a strong and steady stoichiometric imbalance for decades. This constancy of nutrient levels and ratios is notable, given that during the period of our observations, human population in the watershed—while still at relatively low density—doubled, and wildfires have burned sizable portions of the lake's watershed.

To connect the stoichiometric status of the lake to external inputs of N and P, we analyzed long-term data on river nutrient concentrations and discharge to estimate rates of nutrient loading and the N:P stoichiometry of that loading for the past 38 y. While highly variable, annual TN loading (Fig. 2A) has declined slightly during the study period. Annual loading of TP also displayed considerable year-to-year variability, declining modestly (~10%) over the study period (Fig. 2B). Reflecting this long-term stability in TN and TP loading, the TN:TP ratio of annual nutrient loading, while variable (range: 5 to 60 around an overall geometric mean of 21.5), showed no long-term trend (Fig. 2C). We note that the average loading N:P ratio from Flathead Lake's rivers is somewhat lower than national values of river and stream TN:TP ratios (median value

of 31:1; ref. 20). This relatively low N:P of loading likely reflects the status of the lake's watershed. Manning et al. (20) report that rivers and streams in forested watersheds generally have lower TN:TP ratios than those draining developed or agricultural watersheds. This is consistent with the relatively low TN:TP of loading to Flathead Lake, as the watershed is heavily forested with limited developed or agricultural land areas. Furthermore, much of this landscape lies within federally protected areas, including Glacier National Park, and federally designated wilderness areas. While there was no long-term trend in loading N:P, interannual variability in loading N:P ratios (Fig. 2C) was notable and appeared to be driven by changes in P loading (Fig. 2B). We hypothesized that this variation was driven by interannual changes in discharge. Indeed, loading N:P ratios (log-transformed) negatively correlated with annual discharge ($P < 0.001$, r = -0.65). That is, years when the loading N:P ratios were particularly low correspond to years with high absolute loading rates of N and, especially, P (see Fig. 2; 1990 to 1991, 1996 to 1997, 2002). This hydrologic variation itself may be due to decadal-scale climate variation (e.g., El Niño/La Niña cycles), as annual discharge into Flathead Lake is negatively correlated ($P = 0.03$, r = -0.34) with the Oceanic Niño Index. (Note that in western Montana, strong El Niño produces dry conditions, while La Niña is associated with wet conditions; ref. 21.) Following previous work (20, 22), we hypothesize that years of high discharge in the Flathead watershed include flood events in spring and early summer that mobilize and transport low N:P sediments from in-river or near-channel deposits in the floodplain.
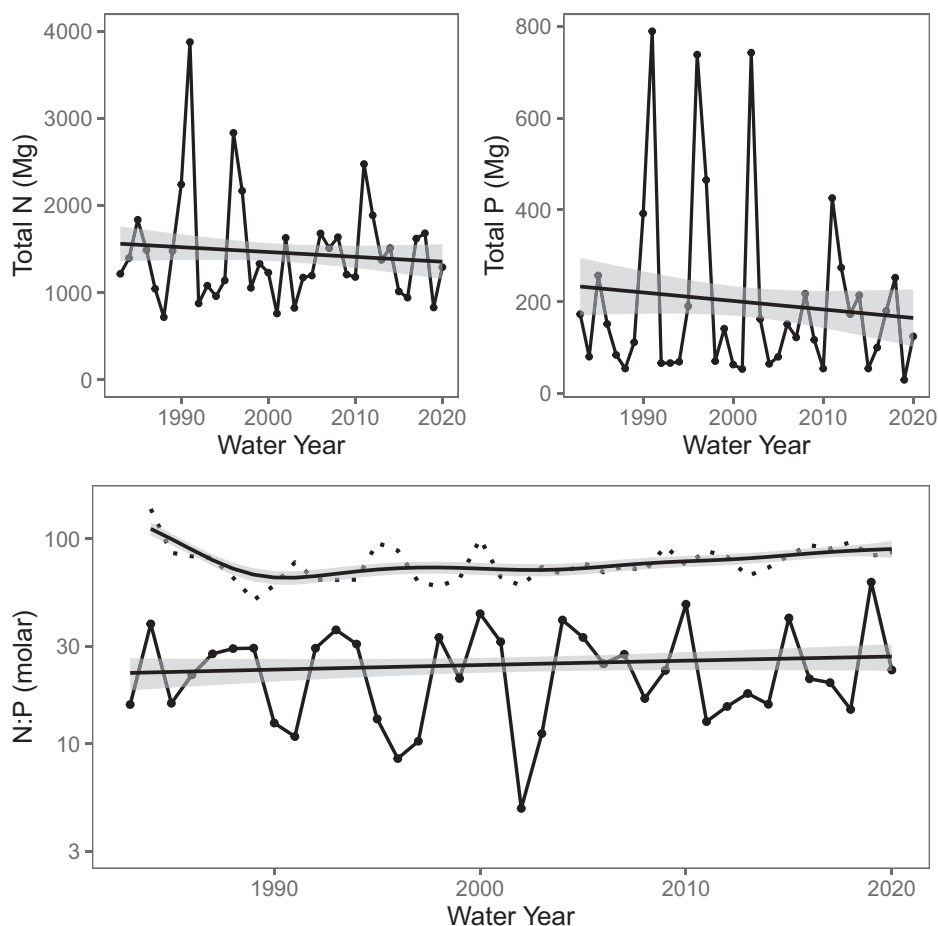

Fig. 2. (A–C) Annual riverine loading for (A) TN, (B) TP, and (C) TN:TP loading ratios (molar) since 1983. Inputs from the Flathead River dominate both the riverine hydrologic and nutrient loading to the lake. The dynamics of lake TN:TP ratio and associated GAM are shown in the upper dotted line to ease comparison.

Thus, a notable feature that emerges from this analysis of the long-term status of N:P stoichiometry in this coupled lake-watershed ecosystem is that the lake maintains unusually high N:P ratios (90:1) despite loading N:P ratios that are relatively low (~21:1). Various mechanisms might produce this discrepancy between N:P of the lake and its river inflows. One possible contributor is that river loading N:P ratios might not reflect what nutrients are available for biological use and subsequent processing in the lake water column. For example, based on measurements of nutrient pools in river inflows since 2016, at least ~50% of TP concentrations in river water are in particulate form versus only ~23% for N. This observation is consistent with earlier studies in the Flathead River showing that much P carried to Flathead Lake is in nonlabile forms associated with inorganic sediments (23). Thus, much inflowing P appears particle associated, entering Flathead Lake during periods of elevated spring and early summer runoff that is high in suspended particles. Much of this particulate load is likely lost to sedimentation soon after it enters the lake. Alternatively, low bioavailability of dissolved organic N (DON) may also increase TN:TP ratios. In a large study (348 watersheds) of organic N in runoff, 50% of exported N from forested watersheds was in the form of DON, and the bioavailability of this DON was only ~20% (24). Given that the Flathead Lake watershed is mostly forested, we expect DON to represent a large fraction of TN loading as well. While data needed to estimate DON contributions are lacking in the long-term record, assessments from spring 2021 indicate that DON contributed 49% to TN concentrations in river water, higher than nitrate (37%), a highly bioavailable form of N. Furthermore, dissolved organic P (DOP) carried by Flathead's inflows may be more bioavailable than its DON, as for rivers entering the Baltic Sea (75% of DOP bioavailable vs. 30% for DON) (25). Thus, riverine inputs of relatively refractory DON may allow N to accumulate in the lake. Unfortunately, long-term data on the relative bioavailability of DON and DOP in river loading to Flathead Lake are not available. High lake TN:TP ratios, despite comparatively low N:P ratios in river discharge, may also be explained if low river N:P ratios are offset by contributions from high N:P nutrient sources, such as atmospheric deposition to the lake surface. Atmospheric deposition in this region does have a high N:P ratio (~50:1) and has been an increasing contributor to the lake's nutrient budget (26). Furthermore, most of the deposited N is in the form of highly available NH_4^+, and direct aerosol deposition to lake surface waters may make it an important nutrient source of N during summer periods of low river discharge (26). Nevertheless, this direct deposition constitutes <10 and 4% to the lake's overall N and P inputs, respectively (26), and thus is unlikely to have a major influence on the overall nutrient regime. Thus, we hypothesize that in-lake processes are primarily responsible for the maintenance of Flathead Lake's imbalanced N:P stoichiometry.

These in-lake processes might include N_2 fixation. While taxa of cyanobacteria capable of N fixation are present in both the phytoplankton and periphyton of Flathead Lake (27), direct measurements of N fixation for the lake are lacking. Nevertheless, even relatively low rates of N fixation would accumulate in the lake's N inventory if rates of denitrification are also low, as would be expected, given that the entire water column and even upper sediment layers are well oxygenated year-round. Sedimentation processes might also contribute to high TN:TP in the water column of Flathead Lake, as particulate P contributes a greater fraction (~39 to 49%) of water column TP than particulate N contributes to TN (18%). Since particle-bound

nutrients sink but dissolved forms do not, sedimentation might differentially remove P relative to N from the water column, raising overall TN:TP ratios. Another internal process that might amplify or mitigate stoichiometric imbalance is eddy diffusion of nutrients from the hypolimnion into surface waters during stratified periods. To assess this, we examined N:P ratios in hypolimnetic (90 m) pools during summer for 2018 to 2020. These ratios were also high (TN:TP of 137:1 and dissolved inorganic nitrogen: soluble reactive phosphorus of at least 190:1), implying that internal resupply of nutrients to surface waters would further reinforce stoichiometric imbalance and potential P limitation in the lake. These hypothesized internal processes contributing to the high TN:TP ratios observed in Flathead Lake require investigation but are consistent with a recent global analysis of 573 lakes (28) indicating that deep lakes (in which mean depth exceeds summer mixing depth) such as Flathead Lake have high TN:TP ratios and, thus, more potential for P limitation of phytoplankton growth because of increased efficiency of P sedimentation and diminished influence of benthic denitrification.

Consequences at the Ecological Level: Phytoplankton Nutrient Limitation. Regardless of its underlying causes, what are the ecological consequences of Flathead Lake's sustained highly imbalanced TN:TP ratios? We do not have continuous long-term data on summer phytoplankton nutrient limitation or C:N:P stoichiometry of seston (suspended particulate matter) in Flathead Lake. However, high in-lake TN:TP ratios could cause phytoplankton P limitation (12), at least in summer when light intensities are high, potentially leading to high C:P and N:P ratios in lake seston. Consistent with this possibility, nutrient enrichment bioassays performed in early to midsummer 2018 and 2019 show evidence of phytoplankton growth response to addition of P alone, with larger responses to combined N and P enrichment but no significant response to the addition of N alone (Fig. 3). According to a taxonomy of N × P enrichment experiment outcomes (29), these results would be consistent with serial N&P limitation, with P as the primary limiting nutrient during these summer months. Unfortunately, experiments testing N versus P limitation of phytoplankton in Flathead Lake are quite limited for earlier periods relevant to our study. Nevertheless, a set of N × P enrichment experiments similar to ours were performed in 1987 and 1988 (30, 31). While these found no response of chlorophyll-*a* concentrations to enrichment with P alone (in contrast to our results for three of six experiments; Fig. 3*A*), they did demonstrate significant stimulation by combined N&P enrichment, consistent with the strong response to combined N&P enrichment that we observed in all six of our experiments. These midsummer bioassays support a primary P limitation of phytoplankton during this period; however, we note that the N:P ratio of dissolved organic nutrients in lake must be quite high since particulate N:P is generally lower than TN:TP, even after inorganic N (nitrate) is depleted as summer progresses (*SI Appendix*, Fig. *S1*). This observation suggests differential accumulation of organic N pools (relative to P) that are not bioavailable. Thus, N&P colimitation or even N limitation of phytoplankton growth seems possible once pools of available N (NO_3^-) are depleted by late summer (see Fig. S1 in *SI Appendix*). That N can colimit phytoplankton growth is supported by our experimental results that show a consistent secondary stimulation when N is added along with P (Fig. 3), suggesting that, concordant with our inferences about river-borne DON, much of the lake's DON is also not readily bioavailable. In fact, during
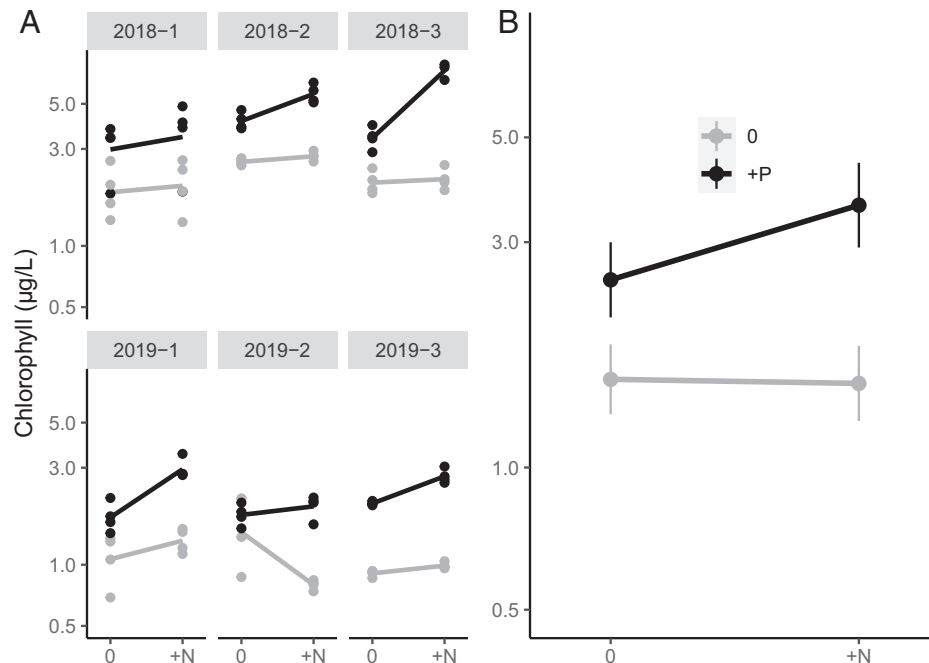
Fig. 3. P primarily limited Flathead Lake phytoplankton growth during summer, as estimated from factorial enrichment of N and P in six 5-d experiments testing response of chlorophyll-*a* concentration. (*A*) Raw data for three experiments in 2018 (Expt 1: 18 June; Expt 2: 2 July; Expt 3: 9 July) and 2019 (Expt 4: 24 June; Expt 5: 1 July; Expt 6: 8 July) (note log transformation of *y* axis). Lines connect means of the +P treatments. (*B*) Predicted values based on a mixed-effects linear model, with intercept as a random effect and N and P effects as fixed for all data combined. Model structure is $Y_{i,j} = \beta_{0,j} + \beta_1 N_{i,j} + \beta_2 P_{i,j} + \beta_3 N_{i,j}P_{i,j} + \varepsilon_{i,j}$ where $Y_{i,j}$ is ln (chlorophyll-*a* concentration) in replicate *i* in experiment *j*. $N_{i,j}$ and $P_{i,j}$ are dummy variables for N and P addition, and $\varepsilon_{i,j}$ is independent and identically distributed error. Parameter estimates (±SE) are P effect, $\beta_2 = 0.49 \pm 0.067$; N effect, $\beta_1 = 0.02 \pm 0.067$; and NP interaction, $\beta_3 = 0.39 \pm 0.096$. Overall, P addition increased chlorophyll-*a* when added alone or with N, but N addition only had an effect when added with P (*B*).

summer (June to September 2016 to 2021), DON contributed an average of 68% of TN in lake surface waters (DOP contributed 56% of TP). A contributing role of N in colimiting phytoplankton growth in oligotrophic Flathead Lake, despite its high TN:TP ratios, is consistent with earlier comparative analysis indicating that the N:P ratio at which phytoplankton growth shifts to primary P limitation is higher at low nutrient levels (32). Consistent with the lake's overall high TN:TP ratios and our bioassay data indicating that early to midsummer (June to July) phytoplankton growth is P limited, seston C:P and N:P ratios (Fig. 4) were also consistently high (>300:1 and >30:1, respectively) in the lake's surface layers during summer. Indeed, epilimnetic seston C:P ratios reached as high as 490, consistently surpassing the threshold elemental ratio for P limitation of the important herbivore *Daphnia* (~200 to 300; ref. 33).

Consequences for Trophic Interactions: Food Quality for Zooplankton. High C:P and N:P ratios such as those in Flathead Lake seston indicate potential P limitation of animal growth (34), but it remains unclear if such stoichiometric food quality limitations operate at low food concentrations (35) such as those in Flathead Lake (Fig. 4*A*). To test to what extent these high seston C:P ratios impose P limitation on *Daphnia* despite low overall food concentrations, we performed growth experiments involving manipulations of both lake seston concentration and C:P ratio (see *SI Appendix* for details) in summer 2019. In short, we incubated seston from the epilimnion of Flathead Lake (after screening out zooplankton as well as phytoplankton larger than 45 μm that are less edible for *Daphnia*) with or without PO_4^{3-} enrichment for several days, allowing P uptake to lower seston C:P ratios and to increase seston biomass concentrations above low ambient levels. On a daily basis, we then diluted (using filtered lake water) or

concentrated subsamples of that seston to achieve a wide gradient of food concentrations for both control (high C:P ratio) and +P treatments (low C:P ratio). Juvenile *Daphnia* were then grown in these various food concentration and P enrichment treatments using daily transfers for 5 d, after which body growth rate of each animal was recorded. P enrichment of lake seston lowered seston C:P ratios (from ~280 to 591 in control seston to ~83 to 104 in enriched seston) and improved growth of *Daphnia* across all food concentrations (Fig. 5). While vertical migration into deeper water to feed on seston with lower C:P ratios (Fig. 4*B*) might ameliorate this food quality effect, the current dominant daphnid in the lake, *Daphnia thorata*, prefers warmer waters and tends to remain in the upper 5 to 10 m (36). Together with phytoplankton bioassay results, these findings support the idea that imbalanced N and P ratios in Flathead Lake are associated with P limitation in the food web at both the first and second trophic levels, affecting filter-feeding *Daphnia* with potential impacts on its roles in modulating water quality (37) and transferring primary production to higher trophic levels. One implication of our data is that a more stoichiometrically balanced nutrient regime in the lake, achieved perhaps by adding N removal to wastewater treatment in the basin or by mitigating atmospheric N deposition, might alleviate P limitation; lower seston C:P ratios; and, thus, increase *Daphnia* abundances, imposing stronger water column filtering and further increasing the lake's already considerable water transparency.

Biogeochemical Consequences: Aerobic Methane Production. We also assessed whether the strong stoichiometric imbalance in Flathead Lake was associated with potential for aerobic production of methane via metabolic processing of methylphosphonates by P-limited lake microbes. Consistent with these mechanisms, experimental addition of MPn (together with nitrate and glucose)
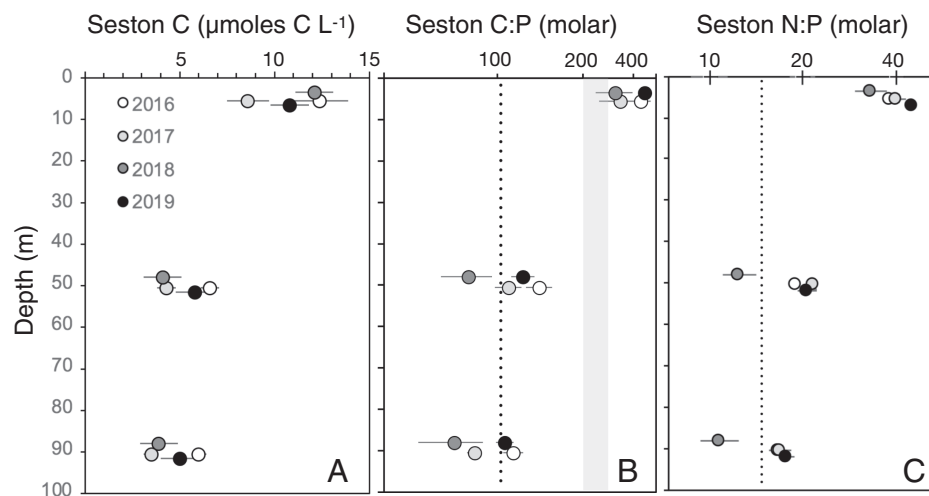
Fig. 4. (A–C) Average (A) seston C concentrations and (B) C:P and (C) N:P ratios (molar) at 5-, 50-, and 90-m depth during summer (June to mid-September) 2016 to 2019. Error bars indicate one SEM of five sampling dates within each year. Note that x axes in B and C are natural log-transformed. The gray bar in B indicates the range of values (200 to 300) for the threshold elemental ratio, above which *Daphnia* P limitation is predicted to occur. The dotted lines in B and C indicate the Redfield ratios for C:P (106) and N:P (16), respectively.

to epilimnetic water from Flathead Lake strongly stimulated methane production (Fig. 6), but this stimulation was suppressed when PO_4^{3-} was added to satisfy microbial P demand. These findings support the view that the high N:P stoichiometric ratios in Flathead Lake and associated P limitation could induce aerobic methane production, providing a feedback between lake nutrient stoichiometry and dynamics of this greenhouse gas. While P-sensitive aerobic methane production via phosphonate metabolism has been observed in oligotrophic Lake Saiko in Japan (18) and in mesotrophic Yellowstone Lake in the United States (38), data are needed from eutrophic lakes to establish the broader significance of our findings from oligotrophic Flathead Lake. However, it is likely that conventional anaerobic pathways of methane production dominate in eutrophic lakes due to oxygen depletion driven by high rates of organic matter production and burial.

Implications. Taken together, our observations indicate that strong imbalances in lake N:P stoichiometry can be sustained over long periods and that this imbalance can have a variety of ecological consequences. While high N:P ratios in Flathead Lake primarily reflect natural processes occurring in the lake

and in its relatively undisturbed watershed, our findings are timely given that various human activities can also accentuate or attenuate stoichiometric imbalances in aquatic ecosystems. For example, rapid population expansion, sewage inputs, and fertilizer use in China during recent decades lowered N:P ratios in its freshwater ecosystems (39). However, subsequent implementation of advanced wastewater treatment in China has counteracted those tendencies, leading to increasing TN:TP ratios (40). Increases in TN concurrent with decreases in TP have occurred in the Laurentian Great Lakes in recent decades and are attributed to implementation of P controls within the 1978 Great Lakes Water Quality Agreement (41). Similar processes also affect nutrient regimes in coastal oceans. Burson and colleagues (42) demonstrated strong declines in inorganic N:P ratios as well as seston N:P ratios moving offshore as phytoplankton nutrient limitation shifted from P limitation inshore to N limitation offshore in the North Sea. They attributed this pattern of inshore P limitation to high N:P ratios in river inflows due to anti-eutrophication efforts in northern Europe, which together have differentially reduced P relative to N supply to this ecosystem. In particular, PO_4^{3-} detergent bans and advanced wastewater treatment have effectively removed P, while N inputs from agricultural fertilizers have increased, and those from atmospheric deposition have declined only modestly. These shifts have likely affected phytoplankton community composition, toxin production during harmful algal blooms, and trophic interactions in coastal areas of the North Sea (42). Finlay et al. (43) have also linked P reduction to accumulation of N in lakes, resulting in stoichiometric imbalance in lake N:P ratios. While the mechanisms of this outcome are not completely established, Finlay et al. hypothesized that decreases in P availability (possibly due to mitigation of P pollution) weaken the ability of lakes to assimilate N and convey it to microhabitats (such as benthic sediments) for denitrification.

Our data on nutrient limitation of phytoplankton, seston C:N:P stoichiometry, and food quality for *Daphnia* provide direct evidence for some of the potential consequences of sustained decoupling of P and N proposed by Finlay et al. (43). While our food quality experiment in Flathead Lake (Fig. 5) highlights the role of stoichiometric constraints in a low-nutrient system, as seen in previous experiments (14, 34), food quality impacts of high seston C:P likely operate also in
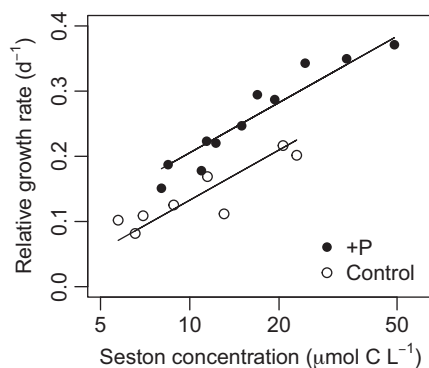

Fig. 5. *Daphnia* relative growth rate (g) response to seston concentration depended on both seston concentration and seston C:P ratio in Flathead Lake. Regression model was $g = -0.123 + 0.256\ln(C) + 0.073P$ ($R^2 = 0.92$), where C is the seston concentration, and P is a categorical value contrasting the control and P-enriched treatments. That is, this model showed that P enrichment of seston increased growth rate by 0.073 d^{-1} (CI 0.40 to 0.98 d^{-1}) compared to unenriched seston, independent of food quantity. Note log-scaled x axis.
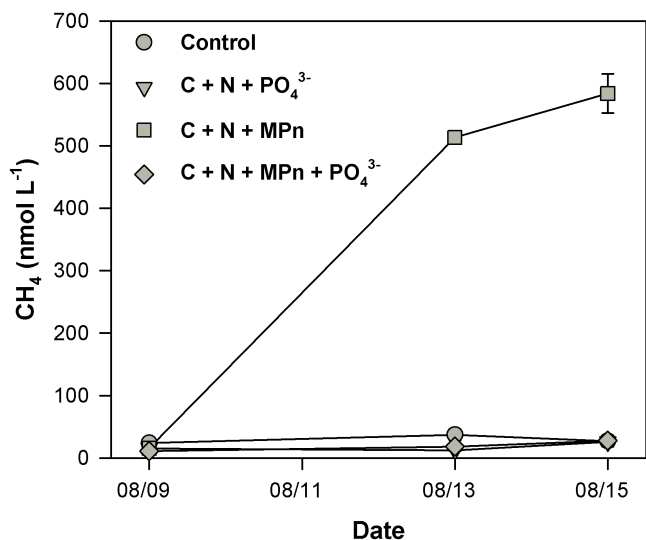
Fig. 6. Time-course experiment showing phosphate suppression of methane production by planktonic methylphosphonic acid use in Flathead Lake (August 2018). Experimental treatments were unamended (circles) or amended with 106 μmol C L^{-1} as glucose, 16 μmol L^{-1} nitrate (as a source of N), and either 1 μmol L^{-1} PO$_4^{3-}$ (triangles), 1 μmol L^{-1} MPn (squares), or 0.5 μmol L^{-1} PO$_4^{3-}$ + 0.5 μmol L^{-1} MPn (diamonds).

mesotrophic and eutrophic lakes, as shown in earlier work (44, 45). Furthermore, our finding that imbalanced N:P stoichiometry in Flathead Lake is accompanied by the potential for aerobic methane production is also important, given that inland waters contribute ~50% of global methane inputs to the atmosphere (46). While most aquatic methane flux derives from anaerobic processes that, themselves, are amplified by P-driven eutrophication (47), our data raise the possibility that aerobic methane production may be accentuated by establishment of high N:P ratios in lakes and other aquatic ecosystems. Overall, we propose that while much attention has been productively focused on processes that sustain or disrupt a lake's overall nutrient concentrations and thus its trophic status, aquatic scientists should also seek to better document and understand the stoichiometric status of aquatic ecosystems, given the various consequences that sustained imbalance in N:P stoichiometry can impose. Our data on impacts of high N:P ratios pertain to the oligotrophic conditions of Flathead Lake; the relevance of these impacts in more nutrient-rich lakes requires further assessment. Nevertheless, given that human interventions now dominate both the N and P cycles (1), we contend that management of nutrients informed by stoichiometric understanding will be of increasing importance during the Anthropocene (6, 43, 48, 49). Nutrient balance matters.

Materials and Methods

Study Site. Flathead Lake is located in northwestern Montana (47.902° N, −114.104° W). The lake is fed by the Flathead and Swan Rivers (along with other minor tributaries, <5% total load) and flows out to the Flathead River. The lake has a short residence time (~2.2 y). Lake water level is held at a stable elevation (881 m above sea level) throughout the summer months by operation of a dam at the outlet; however, lake elevation can vary by as much as 3 m on an annual cycle. At full pool, the mean depth is 39 m, and the maximum depth is 105 m. Much of Flathead Lake's watershed lies in Glacier National Park and adjacent wilderness areas (33%) and in managed national forest land in the United States and Canada (28%), while the remainder of the watershed (39%) lies in intermountain lowlands (prairie, pasture, farmlands, and expanding urban and suburban land use). The current year-round population in Flathead County (the largest in the watershed) is ~104,400. In addition, the area experiences considerable and expanding tourism visitation, especially in summer. Midsummer

water transparency (Secchi depth) ranges from 10 to 15 m, and epilimnetic chlorophyll-*a* concentrations are consistently less than 3 μg L^{-1} during the period of record (37). Nutrient concentrations are summarized in the main text. The lake's upper food web is dominated by introduced fish species, especially lake trout (*Salvelinus namaycush*), whose abundance has increased following invasion by *Mysis diluviana* (opossum shrimp) in the early 1980s (37).

Lake Monitoring and Nutrient Loading.
Lake monitoring. The Flathead Lake Monitoring program has regularly measured physical, chemical, and biological parameters at the deepest point of the lake (Midlake Deep) and in tributaries since 1977. Dissolved and total nutrient concentrations were measured ~15 times per year at Midlake Deep and each of the major lake tributaries. Nutrient concentrations were determined using standard colorimetric segmented flow methods with alkaline persulfate digestions to assess organic pools. Samples for dissolved nutrient analyses (total dissolved P, [NO$_2^-$ + NO$_3^-$]-N, NH$_4^+$-N) were filtered to 0.45 μm, while total nutrient concentrations were determined on unfiltered water samples. In addition, a limited set of samples were assessed for total dissolved N. Detailed methodology and metadata regarding the Flathead Lake Monitoring program's sampling and analysis protocols are available on the Flathead Lake Biological Station (FLBS) Public Data Portal (https://flbs.umt.edu/PublicData) and have been described previously in the literature (26, 37, 50). River discharge data were obtained from the United States Geological Survey's National Water Information System for both the Flathead River (USGS 12355500) and the Swan River (USGS 12370000) gauges.

Close inspection of the time series of nutrient chemistry in the lake called attention to a potentially anomalous period in the data for TP concentrations (beginning 2013); this period coincided with a change in the analytical methods used to measure the time series P pools. Beginning in July 2013, the colorimetric P determinations shifted to include a correction for potential silica interference (*SI Appendix*). The lack of silica correction in the pre-2013 analysis led to an overestimation of TP concentrations by 1.6 μg L^{-1} (51.6 nmol L^{-1}) in lake samples and an Si-proportional overestimate of P in river samples. Hence, for this study, we transformed data prior to 2013 accordingly (see *SI Appendix* for details). Because TP levels in river samples were relatively high, the Si correction did not have a large effect on estimates of riverine TP loading, but the 51.6 nmol L^{-1} correction of lake TP concentrations for the early periods represented a ~25 to 30% reduction in values reported for this observation period. Corrected data are displayed in Figs. 1 and 2. See *SI Appendix* for additional information.

Nutrient concentration time series. Generalized additive models (GAMs) and mixed-effect models were used to identify trends for unaggregated Midlake Deep nutrient concentrations through time. Additive models are used because the Flathead Lake Monitoring program dataset time series contains missing data, has intermittent changes in the frequency of and intervals between sampling events, and likely has autoregressive tendencies that preclude standard linear models (51). Models included a random effect parameter accounting for the month sampled, thus preserving inherent variation due to seasonality or variable sampling frequencies throughout the dataset. To examine trends in the long-term time series data, GAMs were constructed with the R package *mgcv* structured with concentration as a function of date with a splining smoother term. Model optimization (i.e., the number of nodes used in the smoothing function) was based on a restricted maximum likelihood estimate approach. Models were assessed (52) using the package *mgcv* in R (53).

Nutrient loading time series. Annual loads of TP and TN were estimated using the *rloadest* and *loadflex* packages (54) in R (55). *rloadest* is based on the USGS LOADEST program (56), and *loadflex* allows for composite models accounting for variation in concentration to hydrograph relationships through time. These packages were used to estimate loads based on time series data of hydrologic regimes and nutrient concentrations. Total mass daily loadings were estimated and summed to calculate the annual load for the entirety of the Flathead Lake Monitoring program dataset. Models were automatically selected based on maximum likelihood estimation or least absolute deviation when residuals were not normally distributed. The TN:TP ratio was calculated daily and averaged annually. GAMs were then used to assess patterns of change in nutrient concentrations, loading, and stoichiometric balance through time as indicated above.

Nutrient Enrichment Experiments. Six *in situ* incubation experiments were performed to test for nutrient (N, P) limitation of phytoplankton growth via factorial enrichment of N and P in June and July of 2018 and 2019. All water samples were collected from Yellow Bay, a deep embayment on the eastern side of the lake. Water was collected along a 5-m column using an integrated tube sampler (2018) or a Kemmerer sampler (2019) at four random sites within Yellow Bay. All samples were filtered through a 125-μm Nitex mesh to remove macrozooplankton prior to enrichment. Initial water samples were reserved to determine the initial chlorophyll-*a* concentrations.

For each factorial N × P experiment, 16 clear 500-mL bottles were rinsed and then filled with water from Yellow Bay, four each for a control (C), N, P, and a combined NP treatment. The N and P treatments were 8 μmol L^{-1} N (equimolar as NH$_4$Cl + NaNO$_3$) and 0.5 μmol L^{-1} P (as Na$_2$HPO$_4$), respectively, while the N&P treatment received both 8 μmol L^{-1} N and 0.5 μmol L^{-1} P enrichment (16:1 atomic ratio). In 2019, bottles used in experiments 2 and 3 all received the same level of potassium (K) as part of a separate study. All bottles were suspended at a depth of 1.5 m (equivalent to ~50% of incident light) in Yellow Bay. After 4 d, 250 mL from each bottle were filtered onto a glass fiber filter (Whatman GF/F) that was frozen until extraction. Chlorophyll-*a* was extracted by holding filters in the dark at 4 °C for 12 to 24 h in either methanol or 90% acetone. Extracts were analyzed following EPA method 445.0 (57) using a Turner Designs fluorometer and a hydrochloric acid correction for phaeophytin.

We estimated the effect of N, P, and N&P additions in multiple experiments by fitting a random intercept model predicting chlorophyll-*a* concentration for replicate *i* in experiment *j* ($chl_{i,j}$) with slopes for N, P, and N&P as fixed effects. Values for N and P were 0 if not added and 1 if added. This model was

$$log(chl_{i,j}) = \beta_{0,j} + \beta_1 N_{i,j} + \beta_2 P_{i,j} + \beta_3 N_{i,j}P_{i,j} + \varepsilon_{i,j}$$

where $\varepsilon_{i,j}$ is normally distributed error. We fit this model using *lmer*() in package *lme4* in R.

Daphnia Food Quality Experiments.

Seston manipulation. We used a 5-L Van Dorn horizontal water sampler to collect lake water from depths of 0.5, 3, 5, and 6 m in Yellow Bay. Water was combined into a single sample, passed through a 45-μm nylon net to remove zooplankton and phytoplankton inedible to *Daphnia*, and transferred to 20-L polycarbonate carboys. We conducted two experiments (21 August and 16 September 2019) to assess *Daphnia* growth at different food concentrations for ambient seston (high C:P ratio) and for P-enriched seston with low C:P ratio. In both experiments, food (seston) quantity and quality were manipulated by enriching N to 0.45 μmol L^{-1} (as NaNO$_3$) to control carboys and P to +0.16 μmol L^{-1} and +0.5 μmol L^{-1} P (as K$_2$HPO$_4$) in the first experiment and +0.48 μmol L^{-1} P in the second experiment to P-enriched carboys. Carboys were then placed in an outdoor incubator circulated with surface lake water to maintain temperature. The incubator reduced light intensity to 30% of ambient levels using blue Plexiglas shading. The carboys were held for 3 to 5 d to allow seston concentration to increase before the start of the *Daphnia* growth experiment, allowing us to examine animal growth with high and low seston C:P and covering a range of food concentrations after dilution (see below).

Seston C:P ratio and concentration. Seston C concentrations and C:P ratios in both the lake and the carboys were measured daily. Seston was filtered onto precombusted and acid-washed Whatman glass fiber filters (GF/F grade), dried for at least 24 h at 60 °C, and then digested using modified persulfate oxidation followed by infrared detection (for C measurement) and the ascorbic acid method (for P measurement) (58). In the first experiment, seston concentration for each treatment carboy was measured daily by filtering seston onto a GF/F filter and measuring C concentration prior to dilution with filtered lake water (see below). In the second experiment, seston concentration in each individual jar used for animal growth was estimated daily by measuring turbidity (using a Hach 2100N turbidimeter) and then converting the turbidity measurement to C concentration based on a previous calibration.

Food concentration gradient preparation. In the first experiment, gradients of three food concentrations were established for seston from the control, +0.16 μmol L^{-1} P, and +0.5 μmol L^{-1} P treatments. For all the treatments, food concentrations were set at 25, 50, and 100% of the actual *in situ* concentration and prepared by diluting ambient seston (100%) with Whatman GF/F-filtered (nominal pore size: 0.7 μm) lake water. In the second experiment, gradients of five food concentrations were established for both control and P-enriched

(+0.5 μmol L^{-1} P) seston. For the P-enriched treatments, food concentrations were set at 6.25, 12.5, 25, 50, and 100% of the initial concentration. For the control treatment, food concentrations were lower, and, thus, levels of seston were set at 12.5, 25, 50, 100, and 200% of the initial concentration in the carboy. To prepare these treatments, a 5-μm Nitex net was used to concentrate seston from the control carboy to 200% of the ambient level, and then the resulting concentrated seston preparation was diluted to different target concentrations with Whatman GF/F-filtered water. For the enriched treatment, food concentration gradients were prepared by diluting ambient seston (100%) with GF/F-filtered water.

Daphnia culture. A population of *Daphnia pulicaria* isolated from Flathead Lake was maintained in the laboratory in modified COMBO media (59) on a diet of nutrient-sufficient *Scenedesmus acutus*. Adult *Daphnia* were separated from the main culture to produce offspring used in the experiments. Neonates were collected between 24 and 48 h in age, imaged for initial body size using a digital camera on a dissecting microscope, and placed individually into 60-mL glass vials with 60 mL prepared seston (see above). Each food treatment had eight replicate animals for the first experiment and seven replicates for the second experiment. Vials were kept on a rotating plankton wheel to prevent food particles from setting. The wheel was kept inside a growth chamber at 19 to 20 °C with continuous illumination. Every day, animals were transferred individually into freshly prepared seston. The experiments ran for 5 d, after which animals were individually reimaged. Relative growth rate (*g*) of each *Daphnia* was calculated from the pre- and postexperimental body sizes as g (d^{-1}) = ln (S_2/S_1)/dt, where *S* is individual body area at beginning (S_1) and end (S_2) of the experiment, and dt is the duration of the experiment (5 d).

Data analysis. In the first experiment, daily measurements of seston concentration in each jar were averaged across all replicates for that food concentration treatment, and the resulting mean 5-d seston C concentration was used for analysis of *Daphnia* relative growth rate. In the second experiment, daily measurements of seston C concentrations of each treatment were averaged and used in analysis. Examination of data indicated that P enrichment resulted in similar C:P ratios in the two experiments and that growth rate data for the two experiments also collapsed onto a similar response function with seston C concentration. So, data for the two experiments were combined for comparison of growth rate of animals fed P-enriched seston with growth data for animals receiving unenriched seston, while controlling for seston concentration.

To do so, we fit the following regression model to the data:

$$g = \beta_0 + \beta_1 log(C) + \beta_2 P + \varepsilon \qquad [1]$$

where *g* is relative growth rate, *C* is seston concentration (log transformed to facilitate linearity), *P* is a dummy variable designating P-enriched or control treatments, and ε is normally distributed error. This model assumes a variable intercept and a common slope and is equivalent to an Analysis of Covariance without an interaction term. Models with an interaction term (i.e., $\beta_3 log(C) \times P$) had $P = 0.07$ for β_3 and Akaike information criterion values three units higher than obtained for Eq. **1**, indicating an overfitted model.

Methane Experiment. A time-course experiment to examine potential plankton production of CH$_4$ based on the use of MPn was conducted in August 2018. Near-surface Flathead Lake water was collected into four 20-L polycarbonate carboys and transported to the laboratory. In the laboratory, carboys were amended with treatment-specific nutrient substrates (glucose, N, P, and MPn). Experimental treatments were compared to unamended lake water controls. Nutrients (final concentrations) were added as C + N + PO$_4$$^{3-}$ = 106 μmol L^{-1} C (as glucose) + 16 μmol L^{-1} NO$_3$$^{-}$ (as NaNO$_3$) + 1 μmol L^{-1} PO$_4$$^{3-}$ (as Na$_2$PO$_4$); C + N + MPn = 106 μmol L^{-1} C (as glucose) + 16 μmol L^{-1} NO$_3$$^{-}$ + 1 μmol L^{-1} MPn; C + N + MPn + PO$_4$$^{3-}$ = 106 μmol L^{-1} C (as glucose) + 16 μmol L^{-1} NO$_3$$^{-}$ + 0.5 μmol L^{-1} MPn + 0.5 μmol L^{-1} PO$_4$$^{3-}$. Water was subsampled from each carboy into 160-mL glass serum bottles, and bottles were crimp-sealed with Teflon-lined silicone stoppers. Incubations were conducted at *in situ* temperatures in the dark. Triplicate serum bottles from each treatment (and control) were harvested at each sampling time point for quantification of CH$_4$ concentrations as described above. Samples were killed by injecting 200 μL of an 8 M NaOH solution and analyzed by gas chromatography via headspace (20 mL) gas introduction.

Author affiliations: [a]Flathead Lake Biological Station and Division of Biological Sciences, University of Montana, Polson, MT 59860; [b]Nanjing Institute of Geography and Limnology, Chinese Academy of Sciences, Nanjing 210008, China; [c]Department of Land Resources and Environmental Sciences, Montana State University, Bozeman, MT 59717; [d]Department of Ecosystem and Conservation Sciences, University of Montana, Missoula, MT 59812; [e]Department of Integrative Biology, Oklahoma State University, Stillwater, OK 74078; [f]Department of Biological Sciences, Great Lakes Research Center, Michigan Technological University, Houghton, MI 49931; and [g]Ecology, Evolution, and Organismal Biology program, University of Montana, Missoula, MT 59812

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