Trace metal–macronutrient colimitation of algal biofilms in streams with differing ambient inorganic nutrients

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Abstract: The supply of nutrients in streams is an important driver of biofilm production, ecosystem process rates, and basal resource availability. Current understanding of bottom-up drivers of microbial processes derives from studies of N and P, even though algal biofilms require a much larger set of elements to sustain growth. Studies in marine and lake ecosystems demonstrate that trace metals like Fe, Zn, Ni, and Mo can limit the growth of primary producers, but it is not known if these patterns hold in streams. We used trace metal nutrient diffusing substrata to experimentally enrich biofilms with N, P, Fe, Zn, Ni, and Mo alone and in specific combinations to test for macronutrient (i.e., N and P) limitation and trace metal–macronutrient colimitation. We completed enrichment experiments in 5 low-macronutrient streams in the Upper Peninsula of Michigan, USA, and 5 high-macronutrient streams in northeast Ohio, USA. As expected, biofilm chlorophyll *a* was most frequently colimited by N and P (40% of streams), with macronutrient limitation more common in the Upper Peninsula streams. At least 1 trace metal was limiting or colimiting with a macronutrient in 9/10 study streams, including streams that showed no evidence of N or P limitation. Trace metal colimitation with macronutrients was more frequent in streams with low inorganic N and P surface-water concentrations. In 4 streams, we observed algal biomass responses consistent with biochemically dependent colimitation, in which a trace metal alleviates N or P limitation by increasing access to an alternative source (e.g., organic P, N_2). In biochemically dependent, colimited biofilms, the growth enrichment was less for trace metals than the inorganic nutrient (<15%), which suggests a substantial energy trade-off when relying on alternate nutrient sources. Overall, we demonstrated that trace metals are critical nutrients for stream primary producers, and that trace metal limitation may be an overlooked bottom-up driver that can have unexplored consequences for the structure and function of streams.

Key words: nitrogen, phosphorus, metals, algae, periphyton, nutrient limitation, stoichiometry, nutrient diffusing substrates

Stream biofilms are at the interface between the water column and sediment, and these microbial communities play a critical role in the structure and function of stream ecosystems (Battin et al. 2016). If provided with adequate light, warm temperatures, and a balanced supply of nutrients, autotrophs dominate streambed biofilms and have primary control over nutrient cycles and food webs (Bernot et al. 2006, Larned 2010). However, when resources are insufficient in amount or unbalanced relative to biotic demand, biofilm growth rates slow, which reduces nutrient processing rates and limits basal resource provisioning. The growth of stream biofilms requires >20 elements in different amounts and ratios (Kaspari and Powers 2016), but most of what is understood about how nutrient supplies alter primary producers in freshwater streams focuses on 2 macronutrients, N and P (Francoeur 2001, Ardón et al. 2021). Although N and P are often limiting or colimiting in freshwater ecosystems (Elser et al. 2007), a meta-analysis of stream primary producers demonstrated that 43% of limitation experiments showed no growth response to N and P fertilization alone or in combination (Francoeur 2001). Light, streamflow, and grazing are frequently invoked as alternative environmental variables controlling biofilm growth (Francoeur 2001, Larned 2010), but we suggest that trace metals may also be an underappreciated driver of biofilm growth in streams.

Trace metals are used by microbes for a variety of metabolic functions via metalloproteins that function in nutrient acquisition enzymes, electron transfer reactions, and biomolecule hydrolysis and synthesis (Hecky and Kilham 1988, McKay et al. 2001, Twining and Baines 2012). Although these metals are needed in lower concentration than other elements, evidence from marine and lacustrine ecosystems demonstrates that growth of microbes can be stimulated by trace metals (North et al. 2007, Lewis and Wurtsbaugh 2008, Moore et al. 2013). Trace metals, like Fe, can be the primary limiting nutrient in NO_3^--rich areas of the ocean (Moore et al. 2013) and can generate a growth response in lakes that is of similar magnitude to P enrichment (Lewis and Wurtsbaugh 2008). Trace metal limitation has received considerably less attention in stream ecosystems (but see Kunza and Hall 2013, Larson et al. 2018), possibly because of a presumption that high particulate loads and contact with sediment would elevate streamwater metal concentrations above limitation thresholds. Although trace metal concentrations are higher on average in stream water than marine and lake water, concentrations are also highly variable and can be lower than potentially limiting concentrations (Gaillardet et al. 2003). Metal enrichment experiments in streams are rare, but biofilms in P-rich streams in

Costa Rica exhibited greater growth when supplied with a trace metal mixture (Pringle et al. 1986). A subsequent study of 8 Australian streams found no increased growth when enriched with a trace metal mixture (Chessman et al. 1992), but the mixture included Cu at doses that likely caused toxicity (Costello and Burton 2014). Overall, there is a need to determine whether microbial communities in streams can be limited by trace metals, as in other aquatic ecosystems. Furthermore, mechanistic understanding of trace metal limitation can be gained by moving away from a single trace metal mixture treatment to more targeted trace metal and nutrient combinations that target specific physiological pathways (e.g., Browning et al. 2017).

Trace metals (Fe, Mo, Ni, and Zn) can potentially be colimiting with macronutrients (i.e., N and P) through metalloenzymes used to acquire N and P and facilitate electron transfer in photosynthesis (Table 1). NH_4^+ and PO_4^{3-} are biochemically favored forms of N and P, and all other forms of N and P require chemical transformation before they can be incorporated into biomass. Although primary producers have the highest demand for N and P in well-lit streams, most of the N and P transformations are done by extracellular enzymes released by heterotrophic bacteria in exchange for C (Rier et al. 2007). Thus, nutrient limitation in biofilms is typically assessed at the community level, where both heterotrophic and autotrophic members of the biofilm can benefit from nutrient enrichment. Fe is used in many metalloproteins (Twining and Baines 2012), but the highest cellular demand is for proteins involved in electron transfer during photosynthesis and respiration (Sunda 1989, Raven et al. 1999). Mo is a cofactor for the enzymes NO_3^- reductase (Vega et al. 1971, Twining and Baines 2012) and, when paired with Fe, the N-fixing enzyme nitrogenase (Glass et al. 2012). Ni is a cofactor in urease, which converts urea to NH_3, and, as such, supports organic N assimilation (Rees and Bekheet 1982, Sunda 1989). Zn is also used in multiple metalloproteins (Twining and Baines 2012), and one of its most important roles is as a cofactor in alkaline phosphatase, which cleaves PO_4^{3-} groups from organic compounds (Morel and Price 2003). The physiological role of trace metals establishes the potential for biochemically dependent colimitation (sensu Saito et al. 2008), where the ability to acquire alternative forms of N and P depends on a sufficient supply of the trace metal.

Our study asked if trace metals could limit algal growth in streams and whether the type of limitation (e.g., biochemically dependent colimitation, simple limitation) depends on macronutrient concentrations and form. In oligotrophic streams with low inorganic nutrient concentrations, we expected to see biochemically dependent colimitation due to a reliance on alternative sources of N and P (i.e., not NH_4^+ or PO_4^{3-}). In streams with high ambient nutrient concentrations, we expected simple trace metal limitation or no nutrient limitation because macronutrient demands are met.

METHODS

To address our research questions about trace metal limitation and colimitation under different ambient conditions, we completed nutrient enrichment experiments in 2 regions that differed in anthropogenic activity and, thus, N and P concentrations in streams. We used the nutrient diffusing substrate (NDS) experimental approach to enrich biofilms with N, P, and select trace metals at the patch scale and measured biomass response. We measured chlorophyll *a* (Chl *a*) on the NDS as a measure of autotrophic biomass and used linear models to assess differences between biomass on single and multiple element enrichment.

Study sites

To understand conditions where trace metals alleviate nutrient limitation on biofilm growth, we investigated our research questions in distinct regions of the USA with variable ambient inorganic nutrients to encompass a variety of nutrient concentrations and ratios. We used 10 different streams (orders 2–4) to quantify biofilm nutrient and trace metal colimitation (Fig. 1). Five streams with low inorganic nutrient concentration (Elm Creek, Fisher Creek, Mountain Stream, Salmon Trout River, and Pine River) were located in Michigan's Upper Peninsula (UP) in the Dead-Kelsey watershed (hydrologic unit code 04020105), and 5 streams with high inorganic nutrient concentrations (Brandywine Creek, Breakneck Creek, Fish Creek, Mill Creek, and an unnamed tributary [hereafter Cicada Creek]) were in northeast Ohio (NEO) in the Cuyahoga River watershed (hydrologic unit code 04110002).

Trace metal NDS experimental design

To evaluate nutrient and trace metal colimitation, we elevated nutrients and trace metals either as single element additions (N, P, or Fe) or as multiple element additions for a total of 8 treatments/stream, including control (Table 1). We assumed that N and P colimitation would be the most common condition (Elser et al. 2007), so multi-element treatments targeted nutrient–trace metal combinations with either N or P and a trace metal that can be used to acquire the complementary nutrient (e.g., Ni, which may substitute for N via urease). Fe was also added to all multi-element treatments because of its broad role in various metabolic processes. Trace metal NDS (tNDS) were composed of agar amended with concentrated stock solutions of N as NH_4^+ (NH_4Cl), P as PO_4^{3-} (KH_2PO_4), or trace metals (Fe as $FeCl_3$, Zn as $ZnSO_4$, Mo as Na_2MoO_4, and Ni as $NiCl_2$) in a small polyethylene cup (30 mL, 2.2-cm diameter opening; Poly-Cons®, Cranford, New Jersey). We placed a glass fritted disk on the surface of the agar to provide an inorganic substrate for biofilm growth. Nutrient and trace metals diffused out of the cups and incorporated into biomass, thus alleviating nutrient or trace metal limitation by the microbial community that colonized the fritted disk (Costello et al. 2016, Tank et al. 2017). We based target concentrations for the tNDS (Table 1) on the general algal growth media COMBO (Kilham et al. 1998), which has the potential to stimulate growth without causing toxicity.

In each stream, we secured 40 to 60 tNDS cups to plastic L-bars on a small patch of the stream bed within canopy light gaps and incubated them for ~3 wk (NEO in August 2016, UP in July 2017). Water depth where L-bars were placed differed among streams (depth: 10–25 cm), but within a stream, L-bars were placed at a similar depth (±5 cm) to minimize spatial variation. Treatment combinations were replicated 5× except Ni (10×), Zn (10×), and control (15×) treatments in the UP, which had increased replication for ancillary measurements not reported here. Some tNDS cups were lost because of burial or dislodgement, but losses were minimal (0–4/stream in UP, 0–9/stream in NEO) and randomly distributed across treatments, apart from Cicada Creek where all N-alone tNDS were lost. All stream reaches selected for tNDS deployment had open canopies or large canopy gaps, which encouraged colonization by autotrophic biofilms.

Surface-water analysis

To establish nutrient and trace metal concentrations at the beginning and end of the experiment, we collected filtered (0.45-µm mesh, polyethersulfone) water samples from each stream (~100 mL) at deployment and retrieval of the tNDS cups. We digested water samples with alkaline persulfate (de Borba et al. 2014) to measure total dissolved N (detection limit [DL] = 150 µg/L) and total dissolved P (DL = 30 µg/L). We used ion chromatography (ICS-2100; Dionex™, Sunnyvale, California) to measure NO_3-N (DL = 10 µg/L) and PO_4-P (DL = 2 µg/L) from filtered and digested water samples, and we used the indophenol method to measure NH_4-N

(DL = 2 μg/L) colorimetrically with a flow injection analyzer (Quikchem 8000; Lachat Instruments, Milwaukee, Wisconsin). We measured concentrations of filtered Fe, Mo, Ni, and Zn (DL = 2, 10, 10, and 4 μg/L, respectively) in acidified samples (2% HNO_3) with inductively coupled plasma–optical emission spectrometry (Optima 8000; Perkin Elmer, Waltham, Massachusetts). We estimated dissolved organic N (DON) and dissolved organic P (DOP) as the difference between total dissolved N and dissolved inorganic N (DIN; combination of NO_3-N + NH_4-N) or total dissolved P and PO_4-P.

Algal biomass estimates

To characterize biofilm responses to nutrient and trace metal enrichment, we measured biomass growth as Chl *a*. We retrieved tNDS cups from the streams after 3 wk and removed the fritted disks from the agar. We placed all disks in black Whirl-Pak® (Atlanta, Georgia) bags to limit light degradation of photosynthetic pigments and froze them until analysis. We measured Chl *a* directly from the disk by using an overnight extraction in ethanol (4°C) and measuring the absorbance on a Genesys™ 10S spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts) and correcting for pheophytin via acidification with 2N hydrochloric acid) (Steinman et al. 2006).

Data analysis

We analyzed Chl *a* response to nutrient enrichment treatments for each stream individually and for all streams within a region. Nutrient enrichment was characterized as 1 of 3 predictor variables: N (factor levels: none or N added), P (factor levels: none or P added), and trace metals (factor levels: none, Fe added, Zn + Fe added, Mo + Fe added, Ni + Fe added). For data from each individual stream, we fit a 3-way analysis of variance model with N, P, and trace variables and the interaction between N and P. The experimental design was not fully crossed and, thus, not all treatment combinations could be analyzed, and typical post hoc tests comparing all groups were not appropriate. However, our experimental design allowed for planned comparisons (Ruxton and Beauchamp 2008, Davis 2010) of multi-element treatments to the most relevant single element enrichment by using simple linear contrasts (lm function in R; version 4.1.1; R Project for Statistical Computing, Vienna, Austria; Table 1). For the regional tests, we used the *lme4* package (version 1.1-31 Bates et al. 2015) in R to fit a linear mixed-effects model to all data from the UP or NEO with the above treatments as fixed variables and stream as a random variable. We log transformed all Chl *a* values to meet the assumption of homogenous variance among groups.

RESULTS

Water chemistry

In UP streams, filtered streamwater concentrations of Mo, Ni, and Zn were all below method detection limits (10, 10, and 4 µg/L, respectively), and concentrations of Fe were variable (Table 2). With the exception of Elm Creek, all Fe concentrations were low (≤20 percentile) compared with stream concentrations across the United States (NWQMC 2019) but exceeded concentrations that limit phytoplankton in the ocean. Mean DIN concentrations were <120 µg N/L and present as NO_3^- and NH_4^+ in relatively equal amounts (Table 2). DON concentrations were high relative to DIN (mean DON:DIN = 13; Table S1). Mean PO_4-P was below or near our DL of 2 µg/L. Mean DOP ranged from <30 to 60 µg/L, and mean DOP:PO_4-P = 18 (Table S1). DIN and PO_4-P were at concentrations known to limit biofilm growth, and the relative amount of N and P (molar DIN:PO_4-P = 80; Table 2) approached the N:P ratio where P limitation is more likely than N limitation (100:1; Keck and Lepori 2012).

In NEO streams, dissolved water-column concentrations of Mo, Ni, and Zn were also below the DL, with lower Fe than UP streams (Table 2). DIN concentrations (80–6200 µg/L) were high in most NEO streams, and NO_3^- concentrations (66–6100 µg NO_3-N/L) exceeded NH_4^+ (17–93 µg NH_4-N/L). DON concentrations (<150–2490 µg N/L) were comparable to UP streams except in Brandywine Creek, which was 3 to 5× greater than all other streams. Mean PO_4^{3-} ranged from 12 to 58 µg P/L, and mean DOP ranged from 110 to 210 µg P/L. DON concentrations were greater than DIN in Brandywine and Cicada creeks and was similar to the UP streams, but the remaining 3 NEO streams had inorganic N in excess of organic N. All NEO streams had organic P in excess of PO_4^{3-} (mean DOP:PO_4-P = 6; Table S1). The relative amount of inorganic N and P averaged across all NEO streams (DIN:PO_4-P = 103) was similar to the ratio observed in the UP streams, which also indicated potential P limitation (Keck and Lepori 2012). However, Cicada Creek and Mill Creek had much lower ratios (DIN:PO_4-P = 14 and 33, respectively), which suggests that either N or P could be limiting (Keck and Lepori 2012).

Regional tNDS response

Broadly, biofilms in oligotrophic UP streams responded to nutrient enrichment more frequently than biofilms in the NEO streams (Table 3). As expected, within both regions, the addition of N, P, and Fe together yielded the most growth of algal biomass (Figs 2F, 3F), but the response was greater in UP streams (+217% growth compared with single elements) than NEO streams (+130%). UP streams also responded to single nutrient enrichment by P and Fe but not N, which suggests that N is secondarily limiting. Addition of N + Zn + Fe caused greater growth of algal biofilms than N alone in both the UP and NEO, and the regional effect size was larger in NEO than the UP (79 and 58% greater growth than N alone, respectively). The response to nutrient treatments was more varied among individual streams in NEO than in the UP (random effect $R^2 = 0.54$ and 0.28, respectively; Table S2).

UP stream responses

Elm Creek PO_4^{3-} ($p = 0.003$) and Fe ($p = 0.01$) treatments increased algal biomass 105 and 62%, respectively, over the control conditions (Tables 3, S3, Fig. 2A). N addition and multiple element amendments did not increase Chl *a* above expected additive effects of P and Fe. Collectively, these results suggest that Elm Creek is independently colimited by P and Fe.

Fisher Creek Similar to Elm Creek, PO_4^{3-} ($p < 0.001$) and Fe ($p < 0.001$) treatments increased algal biomass, but the growth responses were much greater: 5.6 and 3.2× greater than controls for P and Fe, respectively (Tables 3, S3, Fig. 2B). N + Zn + Fe treatment increased algal biomass an additional 1.3× over the N treatment alone ($p = 0.03$), but our experimental design could not distinguish whether this was a result of Fe or Zn colimitation. N addition and trace metals associated with N uptake (Mo and Ni) did not increase Chl *a* beyond expected additive effects of P and Fe.

Mountain Stream N ($p = 0.003$) and P ($p < 0.001$) treatments increased algal biomass 57 and 83% over the controls, respectively, but Fe alone had a negligible effect on biomass (6%) (Fig. 2C). The combined N + P + Fe ($p < 0.001$) treatment increased algal biomass far in excess of additive effects (Tables 3, S3), which is indicative of N–P–Fe simultaneous colimitation. N + Zn + Fe treatment increased algal biomass an additional 53% over the N treatment alone ($p =$

0.008), and P + Ni + Fe treatment increased algal biomass an additional 74% over P treatment alone ($p < 0.001$).

Pine River Enrichment with N, P, or Fe alone yielded algal biomass that was no different from control conditions (Tables 3, S3), but the combined N + P + Fe treatment caused a large increase in algal biomass (Fig. 2D). This result indicates that Pine River biofilms were simultaneously N and P colimited. P + Ni + Fe increased algal biomass 43% over the P-alone treatment ($p = 0.03$), but no other trace metal treatments yielded a substantial increase in biomass growth (Table 3).

Salmon Trout River Similar to Pine River, single nutrient enrichments did not yield algal biomass greater than controls, but the N + P + Fe combination greatly increased biomass (Tables 3, S3, Fig. 2E), indicating simultaneous colimitation. P + Mo + Fe ($p = 0.03$), P + Ni + Fe ($p = 0.003$), and N + Zn + Fe ($p = 0.05$), treatments increased algal biomass 55, 68, and 50%, respectively, over the controls.

NEO stream responses

Brandywine Creek Fe enrichment increased algal biomass 56% over the control conditions ($p = 0.03$). No other single element or mixed element treatment stimulated growth of algal biomass (Tables 3, S3, Fig. 3A). Multi-element mixtures that included Fe had algal biomass similar to controls (Table 3), which contradicts the characterization of the biofilm as Fe limited.

Breakneck Creek Breakneck Creek did not have any single or multiple element treatments that yielded higher algal biomass than the control biofilms (Table S3, Fig. 3B). There was consistently lower algal biomass than controls on all tNDS supplying Fe, with the exception of the N + P + Fe treatment (Table 3).

Cicada Creek Single element enrichment with P and Fe yielded algal biomass no greater than controls, but biomass was much greater when exposed to the combination of N + P + Fe (Tables 3, S3, Fig. 3C). N-alone treatments were lost during high flow, but the N + Zn + Fe treatment did not substantially increase algal growth (Table 3), indicating that the biofilm was likely N–P colimited rather than N limited. The P + Mo + Fe treatment yielded algal biomass 2.4× greater than the P-alone treatment ($p = 0.02$).

Fish Creek Single element additions of N, P, and Fe and the combination of all 3 nutrients did not stimulate algal growth (Tables 3, S3, Fig. 3D), which indicates that the biofilms were not nutrient limited. However, the N + Zn + Fe combination increased algal biomass ~6× over the N treatment alone ($p < 0.001$).

Mill Creek Similar to Fish Creek, algal biofilms in Mill Creek did not grow to a greater biomass when exposed to N, P, Fe, or the combination of all 3 nutrients (Table S3, Fig. 3E). Biofilms enriched with the combination of N + Zn + Fe ($p = 0.01$) and Ni + P + Fe ($p < 0.001$) treatments increased algal biomass 2.1 and 3.9× over the N and P treatments, respectively.

DISCUSSION

The biofilm response to macronutrients was as expected, with evidence of N–P–Fe colimitation most frequently observed (4/10 streams), P limitation in 2 streams, and no evidence of N-alone limitation. The occurrence of macronutrient colimitation in the study streams was similar to what has been documented in meta-analyses of NDS experiments (Francoeur 2001, Ardón et al. 2021), which demonstrated that N–P colimitation was the most frequent state in rivers. Extreme surface-water DIN:PO_4-P (i.e., <1 or >100) have been shown to be predictive of N or P limitation (Keck and Lepori 2012), and the study streams mostly confirm those findings. No streams had surface-water DIN:PO_4-P < 1, and we also did not observe any strict N limitation. Two rivers (Elm Creek in UP and Breakneck Creek in NEO) had DIN:PO_4-P > 100, but only Elm Creek was limited by P alone, with Breakneck Creek showing no nutrient limitation. Fisher Creek (UP) had the next highest DIN:PO_4^{3-} (81) of all study streams and was the only other stream limited by P alone. The remainder of the study streams had DIN:PO_4-P between 14 and 80 and exhibited either N–P colimitation or no macronutrient limitation.

Independent limitation and colimitation

Fertilization with trace metals frequently caused greater algal growth; however, we could not determine if there was a link between low surface-water metal concentrations and the type of limitation. Evidence of limitation and colimitation by ≥1 trace metal was observed in 9/10 streams. The only exception was Breakneck Creek (NEO), which was not limited by any nutrient. Despite algal biofilms in Breakneck Creek not showing evidence of metal limitation, biofilms did respond to Fe enrichment with lower growth than controls. Although metal limitation or colimitation was the prevailing state, the exact metals limiting a stream differed among streams. Fertilization with Ni + Fe and Zn + Fe caused greater algal growth in 4/10 streams each, Fe alone stimulated growth in 3 streams, and Mo + Fe increased growth in 2 streams. For marine phytoplankton communities, metal concentrations in water can be predictive of metal limitation (Sunda 2012, Moore et al. 2013), but our results indicated no relationship between Fe concentration and limitation.

Fe concentrations were measurable in all streams but were not predictive of Fe limitation or colimitation with other trace metals. The highest Fe concentrations in the study regions were measured in Elm and Fisher creeks (UP) and Brandywine Creek (NEO), but these 3 streams all showed algal growth in response to Fe addition. However, Fe concentrations in these streams may not represent the amount available for microbial uptake because Fe in filtered water samples can be in forms that are not biologically available. Fe in surface water is often present as colloids or is otherwise strongly bound to organic matter that can pass through a filter but are not bioavailable to microbes (Gress et al. 2004, Imai et al. 2011). Fe colimitation with N and P was observed in streams with low surface-water Fe (i.e., Cicada Creek, Mountain Stream, and Pine River), but our experimental design did not allow us to isolate the effects of Fe in this multinutrient treatment (i.e., N–P colimitation potentially caused the response).

Biochemically dependent colimitation

Our experimental treatments were designed to identify biochemically dependent colimitation (sensu Saito et al. 2008) by comparing growth responses among multi-element treatments. For our experimental design, biochemically dependent colimitation would be manifest by maximum growth in a treatment supplying nutrients in their most bioavailable form and intermediate growth when either N or P is replaced with a trace metal. We found evidence for potential trace metal–macronutrient coupling in 4/10 streams, most commonly in the UP streams. The inorganic nutrient treatment (N + P + Fe) served as the theoretical maximum of Chl *a* concentration if biofilms were nutrient limited, and this treatment yielded the highest biomass in all 5 UP streams and Cicada Creek in NEO. Fe was added to all multi-element treatments; thus, Fe–N or Fe–P colimitation may be the cause of responses in the treatments with Mo, Ni, and Zn. However, if algal biofilms were using Fe to access N, we would see a response to both the P + Ni + Fe and P + Mo + Fe treatments. Indeed, we did observe such a response in the Salmon Trout River, where the increase in algal growth was similar between the P + Ni + Fe and P + Mo + Fe treatments (15 and 12%, respectively) and much less than the mixed treatment including NH_4^+ (463%). Thus, it is likely that Salmon Trout biofilms were N–Fe colimited because Fe can alleviate N limitation by increasing N fixation and N reduction (Twining and Baines 2012, Larson et al. 2018).

Several other streams exhibited results consistent with biochemically dependent trace metal–macronutrient colimitations. Mountain and Pine streams appeared to be N–Ni colimited. Substituting Ni for N in the mixed element treatment yielded a greater algal biomass than P alone but a lower biomass than the N + P + Fe mix. Ni can alleviate N limitation through greater urease activity (Rees and Bekheet 1982), but the amount of increased algal growth we observed was only 6 to 15% of the growth observed from NH_4^+ fertilization. Algal biofilms in Salmon Trout and Mountain streams showed growth responses consistent with biochemically dependent P–Zn colimitation, which we suggest may be caused by faster remineralization of organic P through increased phosphatase activity (Morel and Price 2003). Substituting Zn for P yielded greater algal biomass than N and Fe alone, but the increase in growth was only 8 and 11% (Salmon Trout and Mountain rivers, respectively) of the growth observed with PO_4^{3-} supplied. Finally, algal biofilms in Cicada Creek exhibited a growth response consistent with biochemically dependent N–Mo colimitation. Substituting Mo for N yielded greater algal biomass than P and Fe alone, but the increase in growth was just 7% of the growth enrichment observed for biofilms supplied with NH_4^+. The N–Mo colimitation we observed was potentially from an increase in N-fixation or NO_3^- reductase activity under N-limited conditions because both pathways have a high requirement for Mo and Fe (Glass et al. 2012).

Implications of trace metal–macronutrient colimitation

Although our study was limited in geographic scope, the data we generated suggest that trace metal limitation generally co-occurs with the more commonly studied N and P limitation. In total, we observed trace metal–macronutrient colimitation at 60% of the study streams, with biochemically dependent limitation in 4 streams and independent colimitation (Fe and P) in 2 streams. Importantly, each stream that was limited or colimited by macronutrients was also limited by ≥1, and frequently multiple, trace metals. The streams with the lowest ambient inorganic nutrient concentrations, all the UP streams and Cicada Creek in NEO, were most likely to be colimited by trace metals and nutrients. Given that trace metals function in enzymes related to nutrient acquisition (McKay et al. 2001), it is not surprising that trace metals and macronutrients are colimiting in streams with low ambient nutrient concentrations.

Our observation of biochemically dependent colimitation also suggests that trace metals can alleviate macronutrient limitation by increasing access to alternative nutrient sources. However, the growth enrichment from colimited trace metals was modest (<15% more algal biomass relative to the colimited macronutrient), which suggests that there is a steep energetic trade-off to relying on remineralized N and P or newly fixed N for growth. Energetic trade-offs are likely a consequence of primary producers relying on closely associated heterotrophic bacteria within the biofilm to remineralize N and P. For this study, we did not explore how heterotrophs responded to trace metals, and, thus, we cannot definitively test mechanisms related to microbial interactions. However, primary producers supply C to heterotrophic bacteria in exchange for extracellular enzymes that increase the availability of N and P (Rier et al. 2007), and this C subsidy reduces the amount of energy available for producing algal biomass. The connection between primary producers and heterotrophic bacteria is strong under nutrient limiting conditions but can be decoupled with increased inorganic nutrient supply (Scott et al. 2008). Our data suggest that the mechanisms leading to strong autotrophic–heterotrophic coupling in biofilms are related to trace metal supply in addition to macronutrients, and future work should more explicitly study these relationships under varying trace metal conditions.

Meta-analyses of nutrient limitation experiments in streams show that algal biofilms frequently exhibit no growth increase with N and P fertilization, particularly in streams with high nutrient concentrations (Beck et al. 2017, Ardón et al. 2021). Streams that do not respond to N or P are often classified as not nutrient limited (e.g., 43% of experiments in Francoeur 2001), and growth limits are often attributed to other environmental variables like streamflow, light, temperature, and grazing (Beck et al. 2017, Ardón et al. 2021). Likewise, most streams in NEO had high ambient N and P concentrations, and, unsurprisingly, biofilms did not respond to macronutrient additions. However, in all but Breakneck Creek, these high-nutrient streams had

≥1 trace metal combination that increased algal growth (e.g., Zn + Fe in Mill and Fish creeks). To our knowledge, micronutrients have scarcely been invoked as a potential alternate limiting factor for biofilms in streams that do not show a macronutrient response, yet our data suggest this may be common. Our study was not designed to identify potential mechanisms for increased algal biomass with trace metal combinations in streams that are not macronutrient limited, but trace metals are used in many enzymes for a range of metabolic functions (Twining and Baines 2012, Kaspari and Powers 2016). For example, Zn is used in enzymes for carbonic anhydrase, DNA and RNA synthesis, and protein hydrolysis (Twining and Baines 2012) in addition to its most frequently studied role in alkaline phosphatase. Thus, although trace metal limitation may occur in high-nutrient streams, further work should aim to understand the pathways by which trace metals may stimulate additional growth in these typically productive streams.

Overall, trace metal limitation may be an overlooked pathway in understanding bottom-up controls on algal production. In this study we observed independent limitation by trace metals and P, biochemically dependent trace metal–macronutrient colimitation, and high nutrient streams with diverse trace metal limitation. Although trace metal limitation has been previously observed in streams (Pringle et al. 1986) and other freshwater ecosystems (Lewis and Wurtsbaugh 2008), there is a dearth of literature on how these critical nutrients may be important drivers of biofilm biomass and function. Anthropogenic activities enrich streams with trace metals (Nriagu 1996, Pinter et al. 2022), and increasing urbanization and reliance on chemicals (Bernhardt et al. 2017) will likely elevate concentrations and expand the geographic scope of trace metal enrichment in surface waters. Algal biomass responses to trace metal fertilization were typically modest relative to growth response to macronutrients, and we saw no evidence in our study to indicate that increases in trace metal supply in streams are likely to cause eutrophication as does nutrient fertilization. However, the metabolic role of trace metals and their colimiting relationship with macronutrients means that functional rates of biofilms may be altered by trace metal availability. For example, the rate of internal N and P cycling in biofilms can influence retention and storage of macronutrients, and these processes may be influenced by trace metals. For a greater understanding of drivers of biofilm growth, there is a need to expand our concept of nutrient limitation beyond just N and P (Kaspari and Powers 2016) to more broadly focus on all of the elements required by stream biota.

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FIGURE CAPTIONS

Fig. 1. Stream sites where trace metal–macronutrient colimitation was investigated. All Upper Peninsula, Michigan, USA, streams were within subwatersheds that empty into Lake Superior, and northeast Ohio, USA, streams were within subwatersheds of the Cuyahoga River, Lake Erie.

Fig. 2. Biofilm algal biomass (µg chlorophyll *a* [Chl *a*]/cm^2) in response to macronutrient and trace metal enrichment via nutrient diffusing substrata in 5 Upper Peninsula (UP), Michigan, USA, streams (A–E). Individual nutrient diffusing substrata are shown as points, bar heights are means, and error bars are ±1 SE of the mean. Data from all streams are compiled to show the general pattern in the region (F). Note the log scale on the *y*-axis.

Fig. 3. Biofilm algal biomass (µg chlorophyll *a* [Chl *a*]/cm^2) in response to macronutrient and trace metal enrichment via nutrient diffusing substrata in 5 northeast Ohio (NEO), USA, streams (A–E). Individual nutrient diffusing substrata are shown as points, bar heights are means, and error bars are ±1 SE of the mean. Data from all streams are compiled to show the general pattern in the region (F). Note the log scale on the *y*-axis.

Table 1. Single and multiple element trace metal nutrient diffusing substrata treatment combinations used to assess nutrient and trace metal colimitation. For mixed-element treatments, the target concentration metal is bolded, and all other elements are at the same concentration as the single element treatments. APA = alkaline phosphatase.

Treatment	Target concentration (µM)	Planned contrast	Purpose of test
None	–	–	Control condition
N	50	Control	N limitation
P	50	Control	P limitation
Fe	3.7	Control	Fe limitation
N + P + Fe	–	N and P (additive)	N–P–Fe colimitation
N + **Zn** + Fe	0.08	N	Zn–P colimitation via organic P remineralization (APA)
P + **Mo** + Fe	0.09	P	Mo–N colimitation via N-fixation or NO_3^- reduction
P + **Ni** + Fe	0.16	P	Ni–N colimitation via organic N remineralization (urease)

Table 2. Mean surface-water nutrient and trace metal concentrations in streams measured at trace metal nutrient-diffusing substrata deployment and retrieval. Mo, Ni, and Zn were also quantified, but all were below method detection limits (10, 10, and 4 µg/L, respectively). Values reported as <*x* were below the method detection limit, where *x* = the detect limit for that specific analyte. Mean water chemistry for each region is also reported, with values of $^1/_2$ detection limit substituted for any solutes measured below detection. DIN = dissolved inorganic N, DON = dissolved organic N, DOP = dissolved organic P, NEO = northeast Ohio, UP = Upper Peninsula, Michigan.

Stream	NO_3-N (µg/L)	NH_4-N (µg/L)	DON (µg/L)	PO_4-P (µg/L)	DOP (µg/L)	DIN:PO_4-P (molar)	Fe (µg/L)
UP streams (mean)	34	28	580	2	31	80	50
Elm Creek	65	54	730	2	58	133	163
Fisher Creek	31	42	510	2	38	81	43
Mountain Stream	<10	17	580	<2	<30	49[a]	8
Pine River	18	12	560	<2	<30	66[a]	7
Salmon Trout River	49	15	540	2	28	71	31
NEO streams (mean)	1500	64	760	29	150	103	10
Brandywine Creek	330	80	2490	12	120	76	22
Breakneck Creek	6100	56	<150	40	210	343	6

Cicada Creek	66	17	440	13	110	14	2
Fish Creek	480	74	450	24	120	51	10
Mill Creek	760	93	350	58	190	32	12

606 [a] N:P ratio used ½ the detection limit in calculations.

Table 3. Biofilm growth effect sizes of single nutrient additions (alone, % > control) and multi-element enrichment (% > single element additions; Table 1). *P*-values are for specific a priori treatment contrasts. NEO = northeast Ohio, nd = no data, UP = Upper Peninsula, Michigan.

Stream	Fe alone		N alone		P alone		P + Mo + Fe		P + Ni + Fe		N + Zn + Fe		N + P + Fe (nonadditive)	
	%	*p*	%	*p*	%	*p*	%	*p*	%	*p*	%	*p*	%	*p*
UP streams[a]	31	0.03	14	0.32	64	<0.001	1	0.95	30	0.06	58	<0.001	217	<0.001
Elm Creek	62	0.01	5	0.79	105	0.003	22	0.45	–8	0.72	46	0.07	22	0.61
Fisher Creek	319	<0.001	17	0.67	557	<0.001	–33	0.38	–11	0.76	132	0.04	–30	0.64
Mountain Stream	6	0.71	57	0.003	83	<0.001	–30	0.05	74	<0.001	53	0.008	655	<0.001
Pine River	–23	0.08	4	0.79	–10	0.49	5	0.8	43	0.03	11	0.5	665	<0.001
Salmon Trout River	–27	0.06	15	0.48	–39	0.003	55	0.03	68	0.003	50	0.05	463	<0.001
NEO streams[a]	0	>0.99	–14	0.47	–11	0.56	13	0.57	28	0.23	79	0.009	130	0.04
Brandywine Creek	56	0.03	28	0.16	25	0.27	–12	0.60	11	0.65	–1	0.97	–48	0.14
Breakneck Creek	–24	0.32	4	0.88	10	0.73	–15	0.56	–45	0.02	–42	0.04	21	0.73

Cicada Creek	–46	0.22	nd[b]	nd[b]	–64	0.05	237	0.02	64	0.32	70	0.27	3552	<0.001
Fish Creek	12	0.73	–69	0.001	59	0.17	–12	0.69	–10	0.76	603	<0.001	55	0.50
Mill Creek	47	0.34	18	0.61	–54	0.03	15	0.69	394	<0.001	208	0.01	56	0.52

[a] Models for all streams included a random effect to account for different streams within the region.

[b] All N-alone trace metal nutrient-diffusing substrata were lost at Cicada Creek.

611 Figure 1

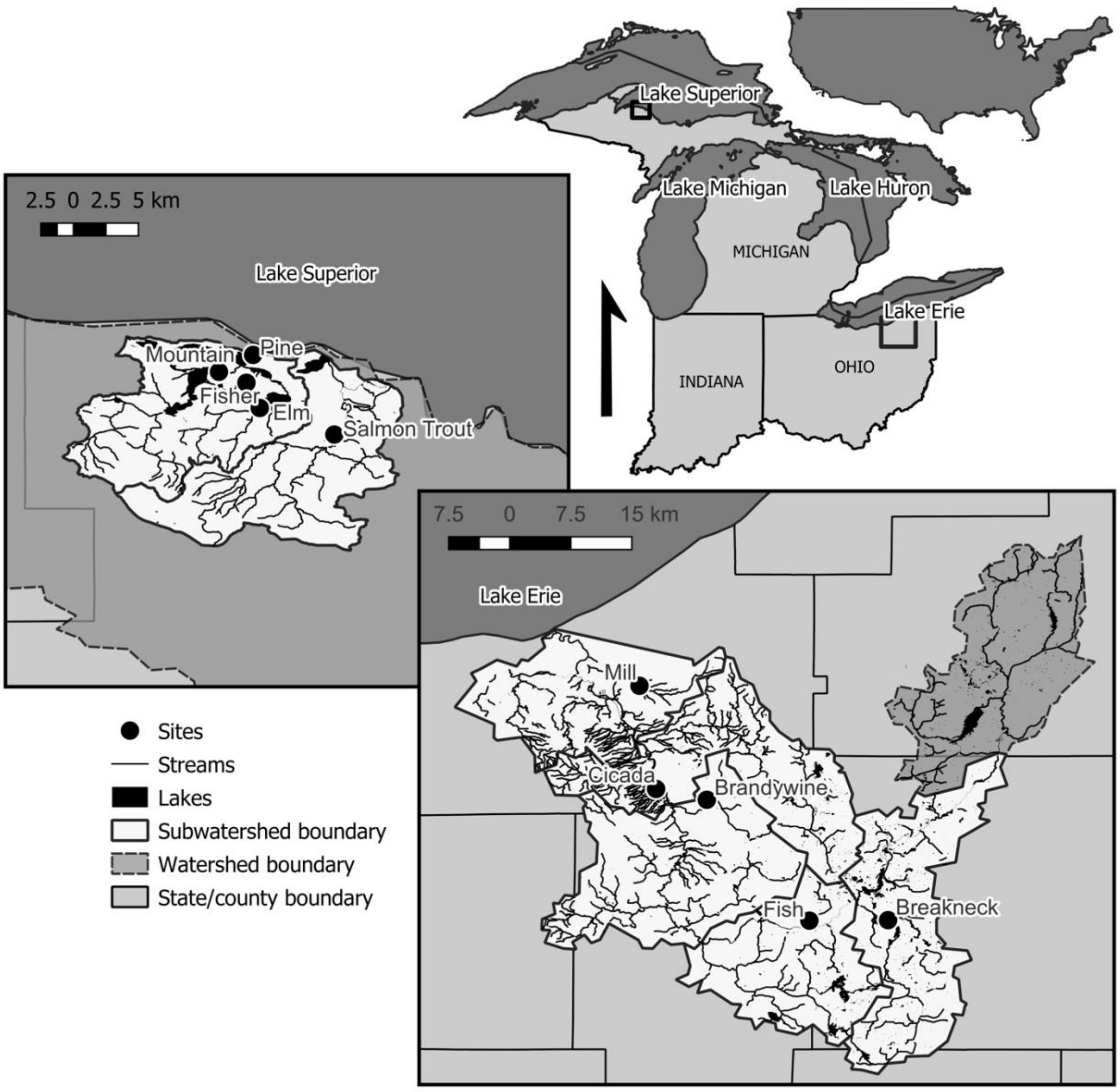

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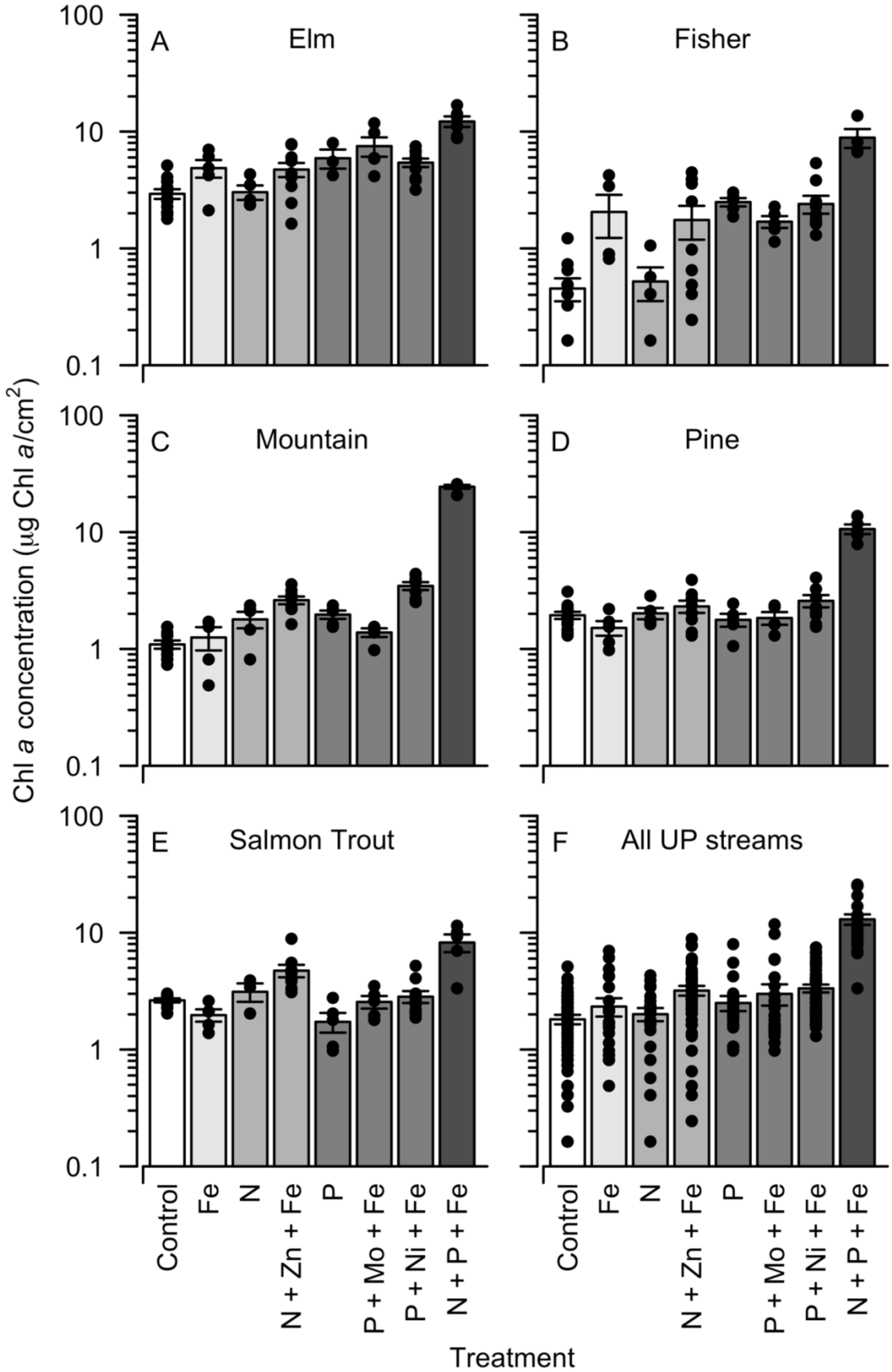
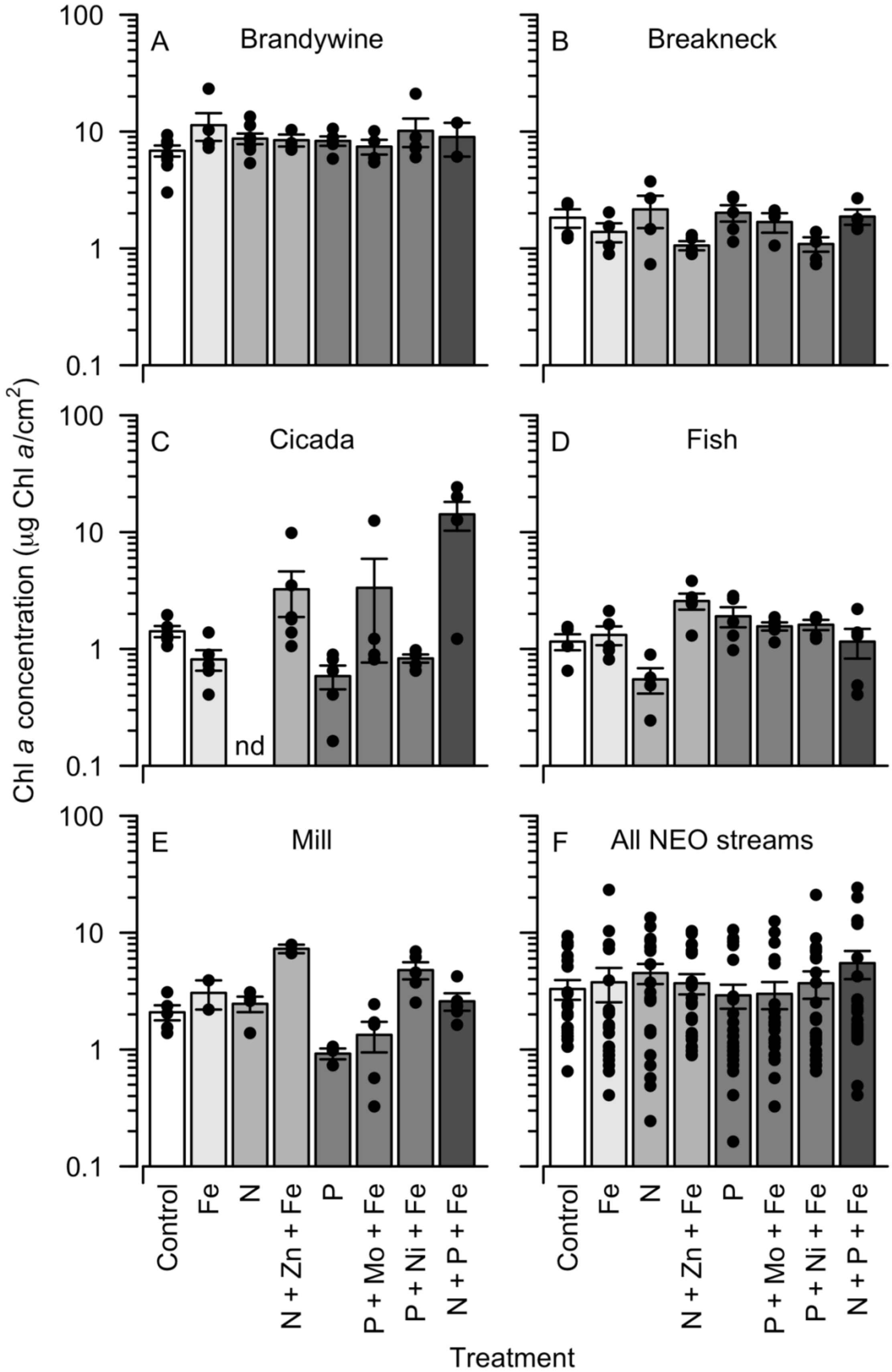
Figure 2


A
Elm
B
Fisher
C
Mountain
D
Pine
E
Salmon Trout
F
All UP streams
100
10
1
0.1
Chl a concentration (µg Chl a/cm^2)
Control
Fe
N
N + Zn + Fe
P
P + Mo + Fe
P + Ni + Fe
N + P + Fe
Treatment

617 Figure 3



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