

## IDEA AND PERSPECTIVE

## Nutrient co-limitation of primary producer communities

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### Abstract

Synergistic interactions between multiple limiting resources are common, highlighting the importance of co-limitation as a constraint on primary production. Our concept of resource limitation has shifted over the past two decades from an earlier paradigm of single-resource limitation towards concepts of co-limitation by multiple resources, which are predicted by various theories. Herein, we summarise multiple-resource limitation responses in plant communities using a dataset of 641 studies that applied factorial addition of nitrogen (N) and phosphorus (P) in freshwater, marine and terrestrial systems. We found that more than half of the studies displayed some type of synergistic response to N and P addition. We found support for strict definitions of co-limitation in 28% of the studies: i.e. community biomass responded to only combined N and P addition, or to both N and P when added separately. Our results highlight the importance of interactions between N and P in regulating primary producer community biomass and point to the need for future studies that address the multiple mechanisms that could lead to different types of co-limitation.

### Keywords

Autotroph, co-limitation, meta-analysis, multiple nutrient limitation, nitrogen, phosphorus, plant biomass, plant community, stoichiometry, von Liebig.

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### INTRODUCTION

Synergistic interactions between limited supplies of nitrogen (N) and phosphorus (P) are widespread across aquatic and terrestrial systems (Elser *et al.* 2007). Indeed, various theories predict nutrient co-limitation and other kinds of interactions between limiting resources (Bloom *et al.* 1985; Chapin *et al.* 1987, 2002; Gleeson & Tilman 1992; Rastetter & Shaver 1992; Sinclair & Park 1993; Danger *et al.* 2008). However, our historical, conceptual understanding of nutrient limitation is essentially one of single-nutrient limitation that is derived from Liebig's Law of the Minimum (Liebig 1842; van der Ploeg *et al.* 1999; Craine 2009). The metaphor that is often evoked is that of a barrel with staves of unequal length; water in the barrel (i.e. plant yield) is limited by the shortest stave. Increasing the length of that shortest stave (i.e. adding the most limiting resource, and only that particular resource) will allow the barrel to hold more water (i.e. increase yield) until the next shortest stave (i.e. resource) becomes limiting. Addition of the second, new limiting resource will further increase plant yield, however, only a single resource is ever limiting at any given time. Liebig's Law of the Minimum was developed to describe the

constraints on the production of individual crop plants, but not the biologically diverse communities of plants and ecosystems to which Liebig's Law has been extended (Danger *et al.* 2008). The aim of this article is to synthesise and extend recently proposed definitions of nutrient co-limitation in marine systems (Arrigo 2005; Saito *et al.* 2008) and to summarise the interactive responses of primary producer communities to factorial N and P addition from 641 published studies in terrestrial, freshwater and marine systems.

Although multiple nutrient limitation of agronomic crops has been extensively studied (e.g. Fageria 2001), the term 'co-limitation' appeared relatively recently in the ecological literature (e.g. Price & Morel 1991). Due to the 'emerging paradigm' aspect of multiple limitation, (Kuhn 1962; Sinclair & Park 1993; Arrigo 2005; Sterner 2008) it is perhaps not surprising that the discipline lacks standard definitions for co-limitation and related resource interactions (Arrigo 2005; Sterner 2008). At the biochemical level, co-limitation necessarily requires a set of alternative definitions that take into consideration the unique chemical roles that different elements play at the molecular level (Saito *et al.* 2008). For example, elemental nutrients may be biochemically substitutable within the same enzyme or different enzymes may perform the same catalytic function, but use different

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elements, as has been shown for several metals (e.g. zinc and cobalt in some marine phytoplankton, Saito & Goepfert 2008). Organismal growth can be considered co-limited in the case of substitutable nutrients (Saito *et al.* 2008), because it can be characterised mathematically as a function of two or more nutrients (Pahlow & Oschlies 2009). Alternatively, biochemical function can depend on the simultaneous presence of two or more different elements (Saito *et al.* 2008; e.g. nickel and nitrogen, Price & Morel 1991). These biochemical definitions of co-limitation focus primarily on aspects of metallo-enzyme chemistry (Saito *et al.* 2008) but, because cellular growth can be limited by intrinsic enzymatic rates, biochemical definitions of co-limitation can be generally extended to higher cellular- or organismal-levels, recognising that multiple metabolic pathways might be limited by different elements and in different ways (e.g. simultaneously or substitutively). Thus, cellular and organismal growth can be constrained by multiple elemental nutrients that might play independent or interactive biochemical roles (Saito *et al.* 2008).

Definitions of co-limitation at the community-level partially mirror those at the biochemical level: multiple nutrients can be simultaneously or independently co-limiting (Arrigo 2005; Box 1). However, because communities are composed of multiple species with potentially shared or unique adaptations to limiting ecological factors, community-level co-limitation introduces a new layer complexity (North *et al.* 2007). At one extreme, a community might consist of functionally equivalent species (*sensu* Hubbell 2001) with all individuals identically co-limited by the same nutrients (i.e. complete niche overlap). As plant species share a limited number of potentially limiting nutrients as well as similar metabolic and physiological pathways (Hutchinson 1961), co-limitation at the community-level might thus simply reflect similar underlying biochemical co-limitation of all individuals in the community. At the other extreme, there might be species that are each limited by different nutrients (i.e. unique niches). However, stable co-existence of species competing for limiting resources does not require completely non-overlapping niches; just that species differ sufficiently in their resource requirements and impacts (Tilman 1982, 2004; Chesson 2000). In addition to species-specific resource limitation, plants can adjust their allocation towards acquiring different resource to better balance supply and demand or even to increase the availability of limiting nutrients (e.g. phosphatase production) (Chapin *et al.* 2002). The relative supply, and therefore limitation, of multiple resources can fluctuate over time (Chapin *et al.* 2002). Thus, co-limitation at the community-level is probably due to a combination of mechanisms, from those that cause species to be similarly limited by the same nutrients, and to niche differentiation mechanisms that cause species to be differently limited by different nutrients (Arrigo 2005).

Definitions for *simultaneous* and *independent co-limitation* allow clear, testable predictions of how plant communities might respond to nutrient additions and point to potential underlying biochemical, physiological and ecological mechanisms explaining patterns of nutrient limitation (Box 1a,b). However, these strict definitions of co-limitation definitions overlap other commonly used, but more general, definitions of *synergistic co-limitation* (i.e. a super-additive response to two or more added nutrients, e.g. Davidson & Howarth 2007; Sterner 2008). In addition, *serial limitation*, (*sensu* Craine 2009), whereby response to a second resource occurs only after prior addition of a 'primary' limiting resource, can result in a synergistic response (Box 1c,d). In this case, the nutrients are interactive and super-additive, but order-dependent. *Serial limitation* corresponds most

directly to the classical concept of 'Liebig limitation', which posits the presence of only a single limiting resource at a given time (Liebig 1842; van der Ploeg *et al.* 1999; Craine 2009). However, *simultaneous* co-limitation could also be considered a special case of Liebig limitation if two or more equally limiting resources behave as single collective resource. *Serial limitation* is not strict co-limitation (Box 1c,d), nor is super-additivity or synergy sufficient criteria to distinguish *serial limitation* from cases of 'true' co-limitation such as *simultaneous* co-limitation (Box 1a). In addition, *independent co-limitation* (Box 1b) need not be synergistic: independent responses to multiple nutrients might interact super-additively, additively or even sub-additively – independence here refers to each nutrient eliciting a biomass response rather than statistical independence (Box 1b). In short, multiple definitions are required, yet existing definitions overlap.

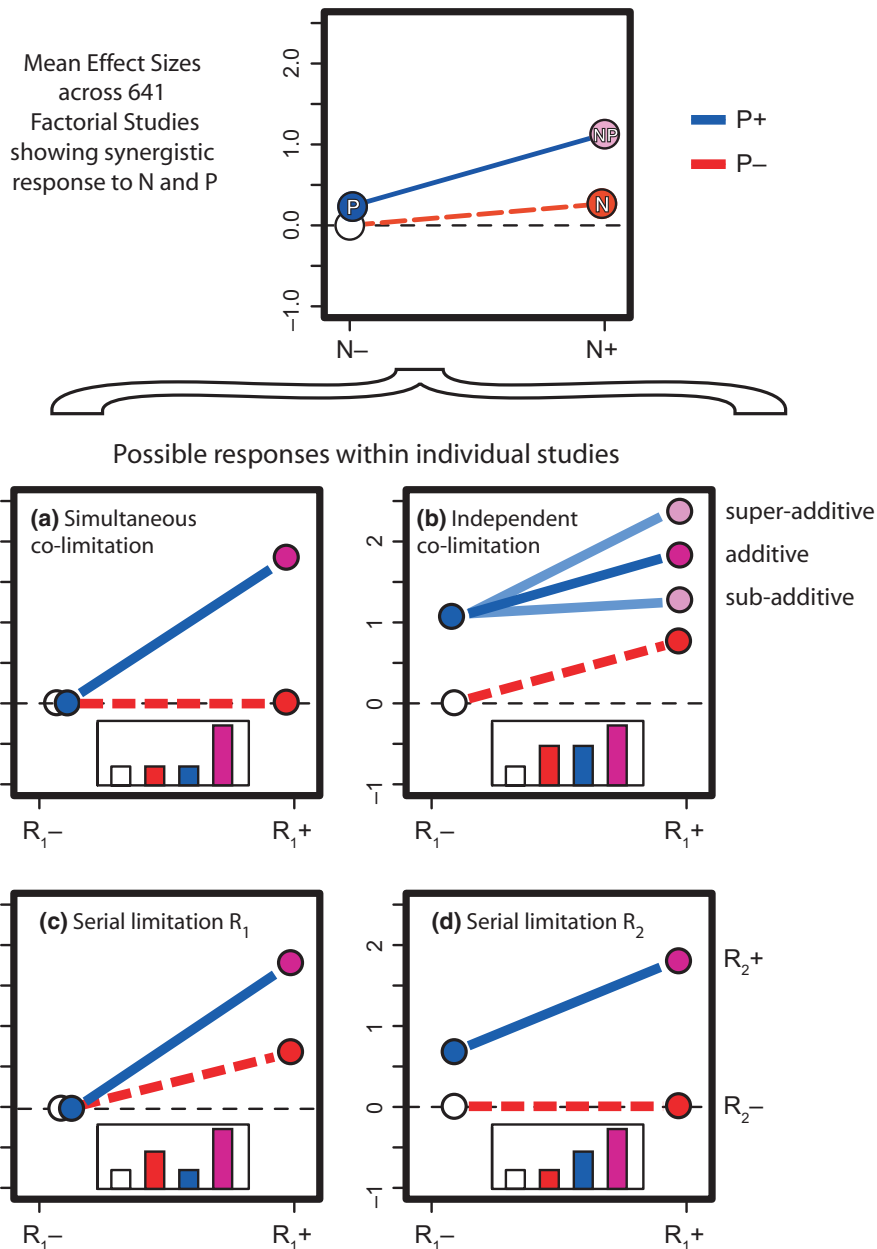
Herein, we define co-limitation as *simultaneous co-limitation* (Box 1a) or *independent co-limitation* (Box 1b), and distinguish these from *serial limitation* (Box 1c,d), but we also consider the established broader definition of *synergistic co-limitation* (Box 1a, b-super-additive, c and d), which emphasises the interactive potential of multiple limiting nutrients. We used a meta-analytic approach to test the prevalence of empirical evidence for alternative definitions of co-limitation in primary producer community biomass responses to factorial addition of N and P from 641 fertilisation studies in freshwater, marine and terrestrial systems. Using alternative response tests, we categorised individual factorial nutrient addition studies according to effect size and statistical criteria as indicative whether N and P were simultaneously *co-limiting* vs. independently *co-limiting* or suggested potential serial limitation. As the likelihood of a study being assigned to a particular response category depends on statistical power and effect size – greater statistical power is needed to detect smaller significant effects – we explored the sensitivity of our results to these issues. The relative response of N and P addition are predicted to depend on environmental factors such as ambient levels of N and P. For example, greater ambient total N and P should result in smaller N and P effects (Craine & Jackson 2009). Co-limitation should be more likely to be found, where ambient N : P is in closer balance with demand (Elser *et al.* 2007; Craine & Jackson 2009). As studies differ in experiment design and methodology, we also tested whether the type of nutrient limitation of individual studies was predicted by experiment-level covariates such as experiment duration or latitude.

## METHODS

Elser *et al.* (2007) reported significant interactions between N and P across freshwater, marine and terrestrial ecosystems in a meta-analysis of the effects of N and P addition on community-level primary production (i.e. communities of autotrophs including terrestrial plants and aquatic algae). However, this analysis included numerous non-factorial additions of N and P, where the nature of these interactions could not be explored at the study level. For criteria used in study selection and response units, see Methods and Appendices in Elser *et al.* (2007). These analyses focus on a specific subset of the 1069 experiments in the Elser *et al.* (2007) database: the 641 studies in which there was a factorial addition of N and P. The studies included in the current analysis are listed in the online Appendix, and are a subset of those available in the public data repository of the National Center for Ecological Analysis and Synthesis (<http://knb.ecoinformatics.org/knb/metacat/nceas.347/nceas>). Herein, we used two methods to categorise each study into one of eight response types

**Box 1 Alternative responses to multiple limiting resources**

Average response to factorial addition of nitrogen (N) and phosphorus (P) from 641 terrestrial and aquatic manipulations is shown in the top panel. The average responses, although significant, might either reflect a consistent response pattern shared by all studies or conversely might average over different study-level responses that themselves represent alternative forms of limitation, as in a–d below. Herein, we use interaction plots to illustrate possible responses to factorial addition of two resources,  $R_1$  and  $R_2$ , with  $R_1$  addition ( $R_1$  – control (white and dark blue points),  $R_1$  + added (red and purple points)) on the  $X$ -axis, and separate lines indicating  $R_2$  addition ( $R_2$  – control, dashed red line;  $R_2$  + added, dark blue line). The  $Y$ -axis represents log ratio effect size of the response of growth (i.e. biomass responses to nutrient addition relative to controls). Response category (a) represents simultaneous co-limitation in which biomass response only occurs if both resources are added simultaneously. Response category (b) represents independent responses to both resources when they added individually. Categories (c) and (d) represent serial type responses in which biomass responds only to a single resource when added individually, but synergistically to both resources when added together. Inset bar graphs illustrate an example of relative observed biomass values resulting from factorial addition of N and P (colours as defined above). Not shown are other alternative responses including single-resource response, negative responses or no response to nutrient addition.



(Figure S1). We evaluated these studies using log ratio effect-size criteria based on the mean treatment and control responses (control ( $N_0P_0$ ), N addition ( $N_1P_0$ ), P addition ( $N_0P_1$ ) and N + P addition ( $N_1P_1$ ) treatments).

A key goal of these analyses is to test for equivalence among treatments; however, the failure to reject a null hypothesis of equivalence arises both from statistical power and the differences among the means. Herein, we use an approach based on bioequivalence (*sensu* Dixon & Garrett 1994) as opposed to failing to reject the hypothesis of equivalence. To do this, we set a 'biological significance' or threshold of effect-size criterion, which is analogous to setting a probability threshold for accepting differences among means such as  $P < 0.05$  (Dixon & Garrett 1994; Dixon & Pechmann 2005). We use this effect-size criterion to test the logarithm of the ratio of treatment response relative to the control against a selected threshold level (1.385, see methods below) for determining whether responses to addition of N, P and N + P should be scored as significantly greater than the control value.

Use of effect-size criteria may also be preferable to the use of  $P$ -value significance criteria, because low replication or statistical power (i.e. Type II error) in experiments may obscure the ability to detect biologically meaningful responses (Johnson 1999). Log response ratios represent the proportional response to experimental treatment, but are unit-less, allowing response magnitudes from different studies measured in different units and magnitudes to be analysed on the same scale, and tend to be distributed normally (Hedges *et al.* 1999). The interpretation of log ratios is also intuitive: a log ratio of 0 represents a treatment response identical to the control value (i.e. no response); values greater than zero are positive treatment responses and values less than zero are negative responses; a value of 0.7 represents about a twofold or 100% increase in the treatment relative to the control. We calculated the following three response ratios:

$$\text{Nitrogen response} : \ln(N_1P_0/N_0P_0) \quad (1)$$

$$\text{Phosphorus response} : \ln(N_0P_1/N_0P_0) \quad (2)$$

$$\text{N + P response} : \ln(N_1P_1/N_0P_0) \quad (3)$$

Log response ratios greater than the chosen critical threshold effect size (or less than the negative critical value) were scored as significant; log response ratios less than the positive critical, but greater than the negative critical value were scored as non-significant.

In addition, we calculated an interaction ratio response index for each study that was also proportional to the control and centred on zero. Super-additivity is indicated by whether increased biomass (i.e. difference between treatment and control) from the addition of both N and P exceeds the summed biomass increase from single additions of N and P (note that this index uses untransformed data to avoid the effect of log-transformation making multiplicative relationships additive (Bland & Altman 1996):

$$((N_1P_1 - N_0P_0) - ((N_1P_0 - N_0P_0) + (N_0P_1 - N_0P_0)))/N_0P_0 \quad (4)$$

which simplifies, algebraically, to: Interaction:

$$((N_1P_1 + N_0P_0) - (N_1P_0 + N_0P_1))/N_0P_0 \quad (5)$$

Interaction ratio values greater than the threshold proportion (identical to the critical threshold effect size above, but not log-transformed because this index can include negative values)

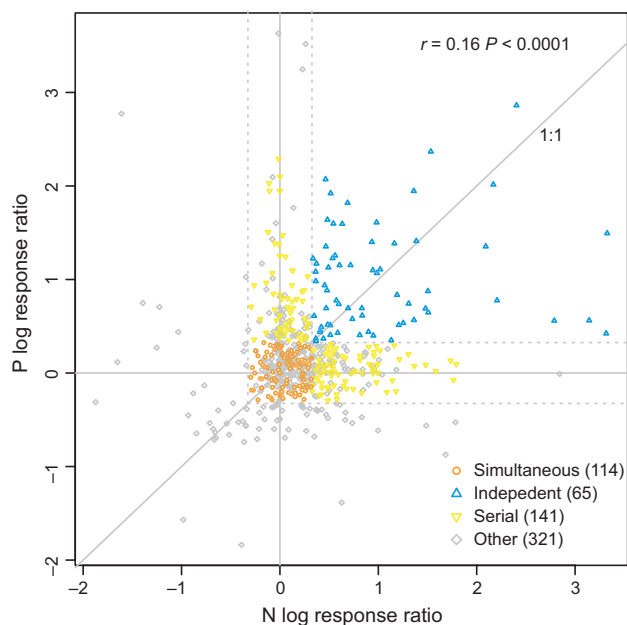
indicate a super-additive response, and values less than the negative of the threshold indicate sub-additivity. The combinations of significant positive, significant negative and non-significant scores – N, P, N + P and interaction effects – from equations 1, 2, 3 and 5 were used to distinguish the co-limitation category that each study was consistent with at that given critical threshold effect size (Box 1). We also categorised studies in terms of whether they showed no response, responded only to a single added nutrient or showed some type of negative response. A flow diagram of the logical tests used for our classification methodology is provided in Figure S1, and follows, sequentially, that of a typical statistical analysis of factorial data: first assessing potential interactions, followed by a priori contrasts.

We note here that the choice of the critical threshold level for such an effect-size criterion is arbitrary, as is the case of selecting critical thresholds for  $P$ -values (e.g.  $P = 0.05$ ). However, to choose an effect size that might be representative of more traditional statistical methods, we used a subset of the data consisting of 124 studies that reported estimates of both means and variance among replicates within a treatment using statistical  $z$ -score criteria at  $P = 0.05$  (see Supporting Information). We first categorised co-limitation responses according to the statistical  $z$ -score criteria, and then determined a corresponding effect-size threshold value by iteratively varying critical effect size values, testing the resulting categorisation against the  $z$ -score categorisation and selecting the effect-size value that minimised the deviance between the two categorisation methods. Categorisation using a critical effect size of 1.385 (38.5% greater than control values) best corresponded to, and was statistically indistinguishable from categorisations using  $z$ -scores at  $P = 0.05$ . In addition, we tested the sensitivity of our results to the selected value of the critical threshold by categorising all 641 factorial N and P addition studies across a range of critical effect sizes representing minimum significant treatment responses ranging from 1.01 to 32 times control values (minimum log response ratios ranging from 0.01 to 3.46).

We tested whether marine, freshwater and terrestrial systems differed in terms of co-limitation to N and P. In addition, we used generalised linear models with binomial error to test whether various experiment-level covariates affected a study's likelihood of being classified as alternative co-limitation categories. These covariates included latitude, total N and P and experiment duration.

## RESULTS

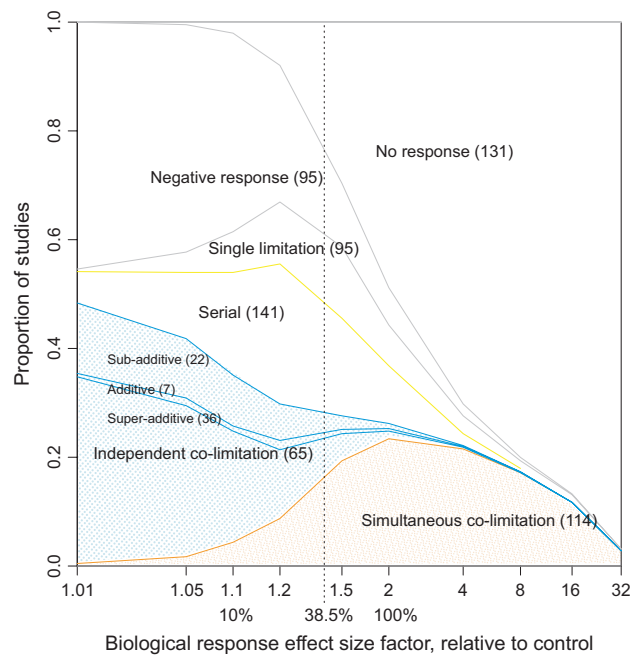
Terrestrial and aquatic systems showed synergistic effects of factorial N and P addition, on average, similar to the results of Elser *et al.* (2007), which included non-factorial studies. The mean responses to factorial N and P addition contain substantial study-level variation with respect to the relative effects of N and P (Fig. 1). Sixty five percent of the studies presented signs of nutrient limitation (either co-limitation, serial or single limitation). Overall, 28% of the studies appeared to be either *simultaneously* or *independently co-limited* by N and P (Fig. 1); thus, co-limitation was a more common form of nutrient limitation than serial limitation in this large-scale data set. Although our analysis revealed that studies varied considerably in terms of response categories, our categorisation was quite sensitive to the value we assigned as the critical effect size (Fig. 2). The strong sensitivity of these results to arbitrary critical effect sizes suggests that interpretation of results from individual studies might be highly sensitive to issues of



**Figure 1** Type of co-limitation response depends on the magnitude of N and P effect sizes (i.e. whether log response ratio exceed our critical effect size of 38.5% – corresponding to a critical z-score at  $P = 0.05$  – indicated by dashed grey lines), and whether the response to combined N and P is super-additive. *Simultaneous* co-limitation is indicated by orange symbols; *independent* co-limitation by blue symbols; *serial limitation* by yellow symbols; and other types of responses by grey symbols. Solid grey diagonal line is 1 : 1.

statistical power as well as various sources of error. Using smaller critical effect sizes, which treated smaller changes in biomass in response to a treatment as significant, strongly increased the proportion of independent co-limitation. Conversely, increasing the critical effect size, which requires a larger change in biomass for significance, increased the proportion of studies showing simultaneous co-limitation or no response. The use of greater critical effect sizes must eventually, and obviously, result in all studies showing no significant response (Fig. 2). This did not occur until a critical effect size was reached, where N + P additions had an effect over 32 times greater than controls, underscoring the strong synergistic – ‘hyper-additive’ – effects that combined N and P addition can have on biomass. We found synergistic responses to N and P addition in 54% of the 641 studies.

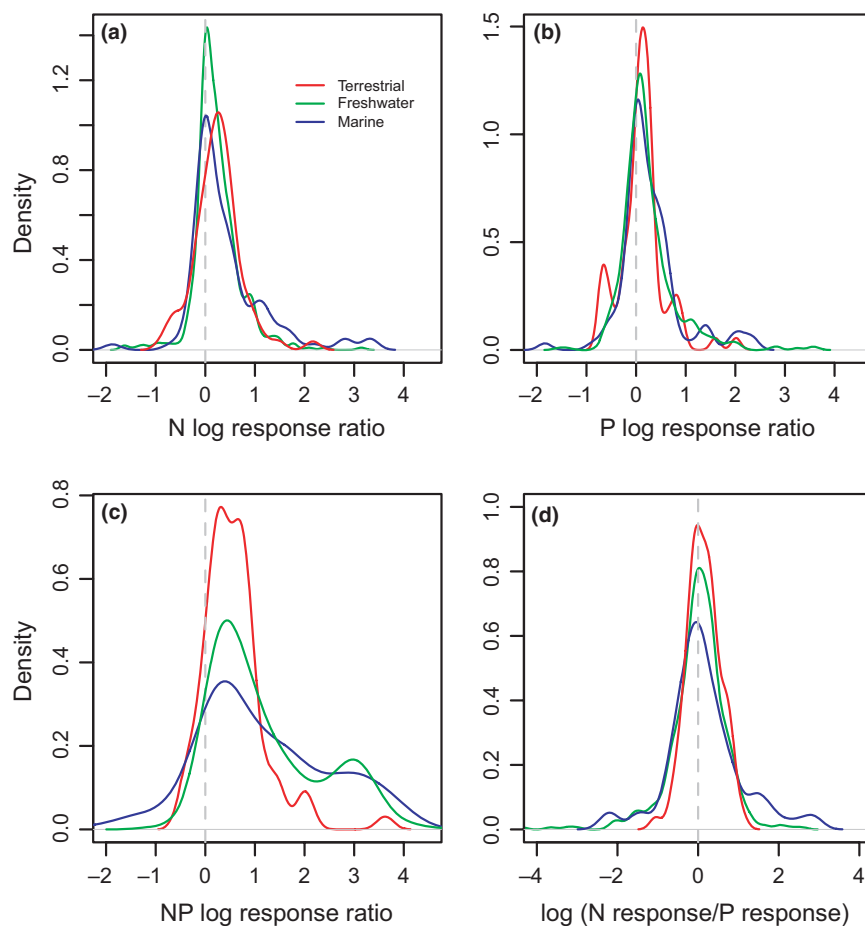
Freshwater, marine and terrestrial systems showed similar distributions of log-ratio responses to N, P and N and P addition (Fig. 3a-c). The log-response ratios from addition of N + P were right-skewed, especially in marine and freshwater systems, (Fig. 3c) consistent with ‘hyper-additivity’. Many studies showed disproportionately large effect sizes with combined N and P addition (note that log response ratios are shown on a multiplicative scale and therefore N and P response ratios cannot simply be added to indicate additivity). We found that freshwater, marine and terrestrial systems did not differ in terms of their relative responses to N vs. P; all systems showed fairly equal N and P effect sizes (Fig. 3d), which was mostly consistent with the larger data set analysed by Elser *et al.* (2007), but which found the mean response to N to be greater than the mean response to P in marine systems. Parallel to the earlier finding of mostly consistent N- and P-limitation patterns across systems (Elser *et al.* 2007), we found no significant system differences in the proportion of response



**Figure 2** Proportions of response categories as a function of critical effect-size criterion (the minimum observed percent biological response required for ‘significance’;  $n = 641$  studies total). Cumulative proportions of each category (corresponding to categories in Box 1 and including ‘other’ types of responses) that were found at varying levels of critical effect size, from bottom to top: *simultaneous* N and P co-limitation responses (orange region); *independent* effects of N and P (blue region); *serial limitation* responses to N and P (yellow region). Other responses (grey lines) include single response to only N or P, negative response or no significant response. Vertical dotted line shows 38.5% critical effect size, equivalent to a statistical z-score threshold at  $P = 0.05$ . Numbers in parentheses are the number of studies within each category at 38.5% critical effect size.

categories among freshwater, marine and terrestrial systems (Fig. 4,  $P = 0.22$ ). As a result of the lack of between-system differences, and to focus on the general topic of co-limitation, we present the remainder of our analyses pooled across systems.

We tested whether studies that differed in their response to N and P addition also differed with respect to various experiment-level covariates: experiment duration, latitude and total N and P. Studies showing independent co-limitation or negative responses tended to have been of longer duration (Fig. 5a). Experiments showing simultaneous co-limitation or no response tended to occur at higher latitudes (Fig. 5b). Co-limitation, whether simultaneous or independent, tended to be found in studies with lower environmental levels of total N and P (Fig. 5c,d) and co-limited studies occurred at lower levels of total N and P than did studies that appeared to be more strongly limited by a single nutrient (e.g. serial or single limitation categories; Fig. 5e,f). Total N and P were positively correlated with each other ( $r = 0.69$ ,  $P < 0.0001$ ). However, we found no difference between types of limitation and the log ratio of total N to total P (testing whether environmental N : P stoichiometry predicts the responses to N and P addition;  $P = 0.26$ ,  $n = 115$ ) or the absolute value of that ratio (testing whether co-limitation is less likely to be found if resources are generally imbalanced;  $P = 0.28$ ,  $n = 115$ ). Neither did the log ratio of total N to total P predict the likelihood of co-limitation vs. single limitation (simultaneous and independent vs. serial and single limitation categories;  $P = 0.88$ ,  $n = 75$ ).



**Figure 3** Distributions for (a) N, (b) P and (c) N + P log response ratios from factorial N × P experiments in terrestrial (red), marine (blue) and freshwater (green) systems. (d) Responses to N and P are similar in magnitude among systems (i.e. the average ratio of the response to N is equal to the response to P as indicated by the log of that ratio centred on 0).

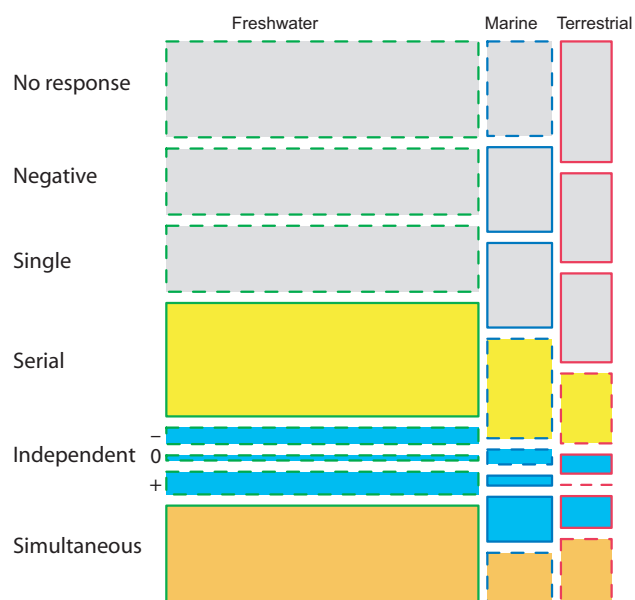
Negative responses to nutrient addition were surprisingly common, given that they are rarely discussed in the nutrient limitation literature. There were 95 (15%) studies that showed some type of negative response: 22 did not have treatment responses less than controls, but were simply sub-additive in response to N + P. The remaining 73 had at least one treatment response less than controls. Few of these negative responses were consistent with a potential toxicity effect; only nine studies showed decreased biomass with N + P addition and decreased biomass with either N or P addition, whereas only five studies showed decreased biomass with N + P, but no response to either N or P. In 59 of the negative-response studies, the reduction in biomass due to single addition of either N or P was reversed when N and P were added together.

## DISCUSSION

Our analysis of this more focused data set indicates that synergistic limitation of plant community biomass by N and P is common across aquatic and terrestrial systems, consistent with the results of Elser *et al.* (2007) that included non-factorial studies. Twenty eight percent of the 641 studies we examined displayed one of two specific types of co-limitation: *simultaneous* response to only N and P combined or *independent*, but super-additive response to both N and P separately (both types of strict co-limitation). Another 22% showed *serial limitation* (a synergistic response to a second nutrient only after addition of a 'primary' limiting nutrient). While fitting a general definition of *synergistic co-limitation*, *serial limitation* does not meet strict definitions of

co-limitation in which two or more resources are independently or simultaneously limiting. Nevertheless, most studies did not show strict co-limitation, although this may be partly due to issues of statistical power (see below). Our results are probably conservative in terms of finding evidence for co-limitation: many of the studies categorised as serial or single-resource limitation examples may have been limited by nutrients other than N and P or other factors that were not tested in most of the studies. Some individual studies identified co-limitation by other nutrients such as potassium in terrestrial systems (e.g. Appendix: Olde Venterink *et al.* 2001), and iron in aquatic systems (North *et al.* 2007). Nutrient limitation studies that manipulate greater numbers of added resources tend to find increasing effect sizes, which also suggest, conversely, that studies testing fewer potential limiting factors are more likely to find smaller or non-significant effects of multiple nutrient addition (Harpole *et al.* 2007a).

Our results add empirical weight to the argument of Danger *et al.* (2008) that questioned the applicability of Liebig's Law of the Minimum to plant communities and ecosystems (as opposed to individual plants, for which it was developed). Given that studies finding either serial or single limitation were more common than those showing strict co-limitation, Liebig's Law of the Minimum would seem to have some empirical utility (but see discussion of effect-size criteria below). Nevertheless, across a large range of critical effect sizes (5% to >200%), the percentage of strict co-limitation experiments (independent and simultaneous) ranged from about 40% to 25%, which we feel is sufficiently large to call into question the general adequacy of Liebig's Law of the Minimum for understanding



**Figure 4** Mosaic plot showing similar proportions of nutrient response categories among freshwater (green outlines), marine (blue outlines) and terrestrial (red outlines) systems. Size of rectangles is proportional to frequency of response category in the vertical direction and proportional to the frequency of studies among systems in the horizontal direction. *Simultaneous* co-limitation is represented by orange rectangles, *independent* co-limitation by blue (+, 0, - indicate super-additive, additive and sub-additive, respectively), *serial limitation* in yellow and other types of responses in grey. Solid outlines indicate positive standardised residuals, and dashed outlines indicate negative standardised residuals from contingency analysis, which found no support for system differences in the proportions of response categories ( $P = 0.22$ ).

multiple-resource limitation of plant communities (Craine 2009). Although *simultaneous* co-limitation by two equally limiting resources could be considered a special case of Liebig limitation, this does not fit the conventional definition of Liebig's Law of the Minimum. In particular, the Law of the Minimum cannot accommodate *independent* co-limitation (Box 1b). If Liebig's Law of the Minimum represents our most basic hypothesis for nutrient limitation, it can be viewed as a type of null hypothesis; our analysis would conservatively reject the hypothesis represented by the Law of the Minimum in at least one of four studies.

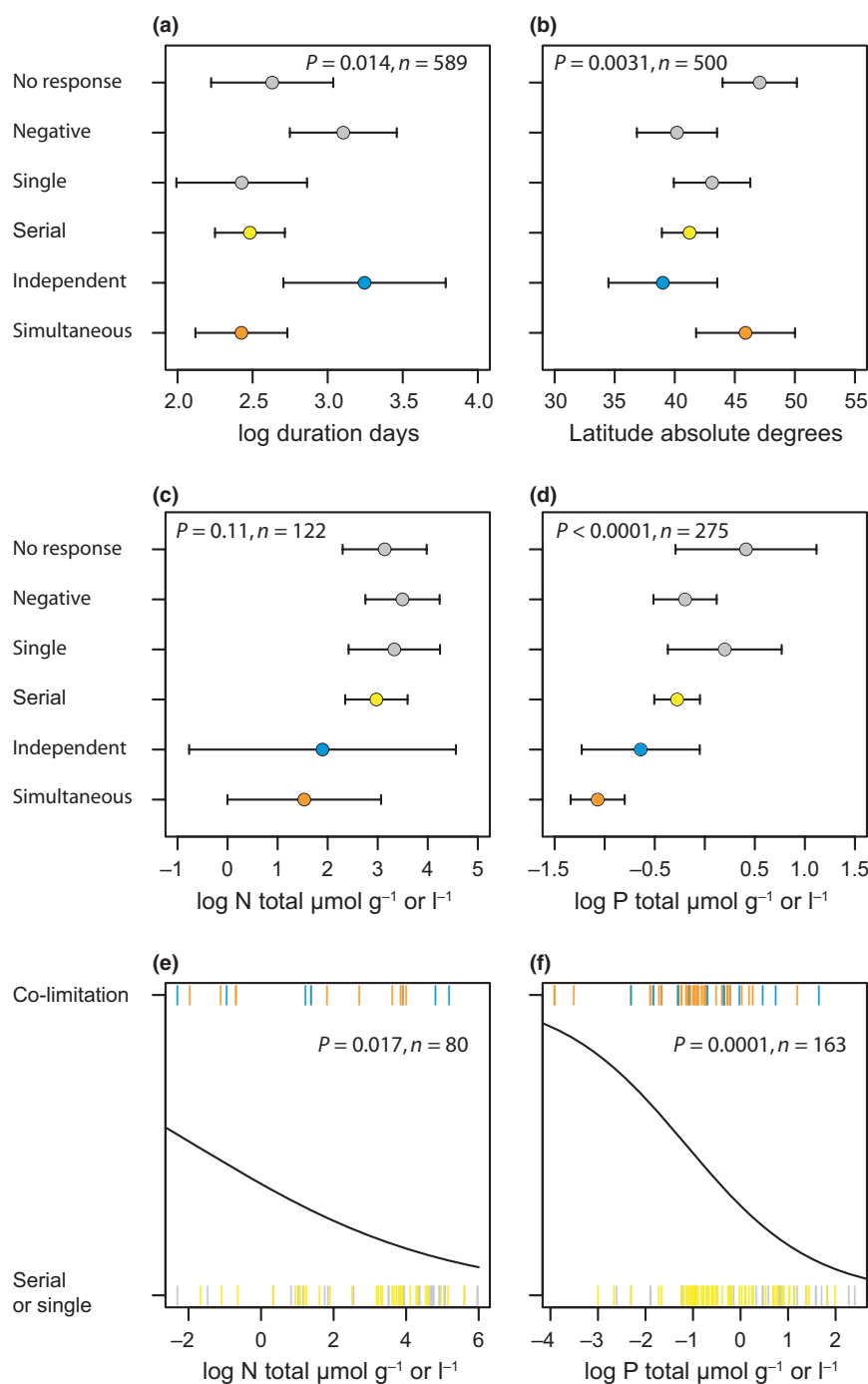
The other major finding of our study is that the type of study-level nutrient limitation response we found depended on experimental covariates, but not on system. Our finding of surprising similarity in N- and P-limitation of primary producers across systems, as with the results of Elser *et al.* (2007), is counter to previous suggestions of primary P-limitation in freshwater systems and N-limitation in marine and terrestrial systems. In addition, we found that the effect sizes of N and P within studies were on average equal to each other, and that their ratios were similar across systems. Elser *et al.* (2007) found equal effect sizes of N and P in freshwater and terrestrial systems, but greater N effect than P effect in marine systems. The fact that the proportion of co-limitation and other nutrient response categories did not differ between systems further supports the suggested importance of shared biochemical stoichiometry among all autotrophic organisms (Loladze & Elser 2011).

The type of co-limitation that might be observed appears to be sensitive to the length of the experiment: independent co-limitation

was associated with longer duration experiments, possibly reflecting changes in other limiting resources and changes in community composition over time. Changes in production can lag changes in limiting resources such as precipitation by several years (Lauenroth & Sala 1992). After an initial increase in plant biomass, the effect of N addition disappeared over the course of a 17-year experiment in hayed grasslands, possibly due to increasing limitation by potassium (van der Woude *et al.* 1994). Tundra plant community response to N and P addition increased over 15 years accompanied by strong shifts in species dominance (Shaver *et al.* 2001). The responsiveness of phytoplankton communities to nutrient addition can vary greatly over the course of a year, with peak production-related diatom blooms, Si concentration and grazing (Hecky & Kilham 1988). Negative effects also increased over time, suggesting that nutrient accumulation could lead to toxic effects, altered soil or water chemistry, changes in community composition and loss of diversity or increased herbivory in open systems. For example, The Park Grass Experiment in Rothamsted, UK, established by John Lawes in 1856, is the longest continually running ecological experiment. Application of combinations of N, P, K and micronutrients over the course of more than 150 years have led to dramatic loss of species diversity, nutrient-specific changes in plant functional and species composition, declines in soil pH, changes in trophic structure and evolutionary responses (Silvertown *et al.* 2006).

Simultaneous co-limitation or no limitation responses tended to occur more often at higher latitudes, which might suggest that those study sites were characterised by very low availability of both N and P or dominated by slow-growing species unable to respond strongly to nutrient enrichment (Chapin *et al.* 1986). A meta-analysis by Downing *et al.* (1999) found that phytoplankton growth rate response to N addition was positively correlated with latitude, but growth rate response to P addition was negatively correlated with latitude, consistent with the hypothesis that P-limitation should be greater than N-limitation at lower latitudes and vice versa. Our findings may differ because our analysis included both pelagic and benthic producers in marine systems. Soil age in terrestrial systems correlates with latitude, such that co-limitation by N and P would be expected on soils of intermediate age and latitude (Walker & Syers 1976; Vitousek & Farrington 1997), but without data on soil age from the terrestrial sites we were unable to test this hypothesis. Other factors could also constrain productivity responses to nutrient addition at high latitudes, including low temperatures, solar energy and moisture (Rosenzweig 1968).

Both simultaneous and independent co-limitation, suggesting more balanced resource limitation, tended to be found in studies with lower total N and P, whereas higher levels of total N and P were associated with studies characterised by more imbalanced limitation by a single nutrient (e.g. serial or single limitation categories). However, overall, total N and P were overall positively correlated with each other. Freshwater lakes have been found to switch from N-limitation to P-limitation with greater rates of N-deposition, and with balanced responses at intermediate ratios of N : P (Elser *et al.* 2009a), similar to studies finding co-limitation at intermediate N : P ratios (Vitousek & Farrington 1997; Olde Venterink & Gusewell 2010). However, in our study, co-limitation was not significantly more likely to occur at more balanced total N : P ratios, possibly because total N and P encompass both unavailable and available pools. Insight into the environmental factors promoting N and P community co-limitation will require better understanding of how these nutrients interact with each other



**Figure 5** Nutrient response categories differ in mean (a) experiment duration, (b) latitude of the study and (c, d) total N and P ( $P$ -values are for overall ANOVA,  $n$  indicates sample size; not all studies included all covariate data). (e, f) Probability of co-limitation response, either *simultaneous* or *independent*, decreases with greater total environmental N or total P, whereas probability of *serial* or single limitation increases (line shows fit of logistic regression).

to control community composition, and how they interact with other limiting factors including other nutrients, temperature, pH, water, light, microbial function and herbivory.

Multiple resource co-limitation (i.e. plant growth expressed as a function of two or more limiting resources) has been treated extensively in the theoretical literature (e.g. Droop 1973; Tilman 1982; Bloom *et al.* 1985; O'Neill *et al.* 1989; Sommer 1991; Gleeson & Tilman 1992; Rastetter & Shaver 1992; Huisman & Weissing 1999;

Klausmeier *et al.* 2004; Danger *et al.* 2008; and many others). Studies differ in how plant growth is characterised as a function of multiple nutrients, and some studies have contrasted alternative growth functions including traditional Liebig-minimum functions (e.g. O'Neill *et al.* 1989). However, Danger *et al.* (2008) showed that, because communities made up of species competing for resources change in composition and diversity with changes in resources, co-limitation necessarily emerges at the community level even if individual species'



growth is characterised by Liebig-minimum functions. Our results strongly indicate that models of nutrient-limited growth for plant communities, if they are to adequately represent the range of empirical results we demonstrated in our analysis, must be able to accommodate both simultaneous and independent responses to multiple nutrients, synergistic co-limitation and negative responses.

The distribution of response categories we found among 641 factorial N × P addition studies was quite sensitive to the effect-size criterion we used. Use of smaller critical effect sizes might suggest that increased experimental power would lead to greater detection of independent co-limitation (and negative responses), but also increasing probability of type I error. Simultaneous co-limitation occurred frequently even with very large critical effect sizes (>100%), emphasising that the synergistic response to N and P is ‘hyper-additive’ (i.e. multiplicative on a log scale; Fig. 4a). The number of co-limitation studies we found is probably a conservative estimate of the importance of co-limitation in primary producer communities for several reasons. The failure to find significant independent responses to either or both N and P might be due to type II statistical issues, methodology or to other nutrients or factors not manipulated in the study that were limiting. Furthermore, actual multiple independent limitation might appear to be single limitation even when species are in fact limited by multiple nutrients. This might occur when plant biomass response is not ‘equally limited’ by all nutrients (e.g. growth response to different nutrients depends on the relative costs, demands and acquisition associated with different limiting nutrients; Gleeson & Tilman 1992).

As nutrient addition is expected to increase limitation by other resources, the negative responses to nutrient additions we found might represent co-limited systems that are strongly stoichiometrically constrained; most of the negative response studies showed a positive response to the combined addition of multiple nutrients (N + P). The potential for nutrient addition to produce negative responses was acknowledged prior to von Liebig. In 1837, Carl Sprengel reasoned that identifying limiting factors required adding neither too little nor too much (Browne 1942). Liebig’s Law of the Minimum was modified by various researchers to account for possible negative or toxicity effects of nutrient addition on crop yields (Browne 1942). Rather than toxicity (in the sense of a poison) resulting from excess nutrient addition – especially in the case of metals – the studies we identified that showed negative responses to N or P were more consistent with Liebscher’s ‘law of the optimum’ (Browne 1942). In this case, unbalanced nutrient addition might lead to excess plant assimilation of the added nutrients and exacerbated internal stoichiometric imbalance of the non-added nutrients. Negative effects of unbalanced N : P supply ratios on plant growth have been shown (Güsewell 2005; Olde Venterink & Güsewell 2010). Restoration of balanced nutrient supply should result in enhanced growth, in contrast to toxicity effects, which should persist even in the presence of proportionately balanced nutrient supplies.

We found increasing frequencies of negative responses using smaller critical effect sizes, (especially below 38.5%), which suggests that the likelihood of a finding a negative response may be partly due to issues of sampling error. Besides toxicity and stoichiometric imbalance, other mechanisms might contribute to observing negative or no responses (or even single-nutrient responses) to fertilisation. Fertilisation might increase herbivory rates by changing the amount and nutritional quality of vegetation (see Gruner *et al.* 2008). Fertilisation has been shown to decrease soil water availability (Harpole *et al.* 2007b), which can limit net productivity. Changes in the ratios of available nutrients can drive changes in species composition

and the production of the ‘winning’ species under fertilisation, which may not exceed that of the original community. Fertilisation (e.g. with ammonium compounds) can lower soil pH, which can impact species composition and productivity. Nutrient addition may not significantly change the availability of nutrients (e.g. P) due to adsorption, and addition of one nutrient may interact to affect the availability or uptake of other nutrients (Havlin *et al.* 1999; Eviner *et al.* 2000). Although we focus here on limits to primary producer community biomass, production of biomass may be limited by different nutrients than are other processes of interest such as vital demographical rates or litter decomposition; thus, the lack of a biomass response to a nutrient may not reflect its potential limitation to other important biological functions.

Our objective with this analysis was to synthesise recent attempts to define co-limitation and to quantitatively review empirical evidence for alternative definitions of co-limitation. Various authors have provided multiple definitions or sets of definitions of multiple nutrient limitation, which correspond with our definitions (Box 1) of simultaneous co-limitation (Güsewell *et al.* 2003; Arrigo 2005; Craine 2009), independent co-limitation (Güsewell *et al.* 2003; Arrigo 2005; Niinemets & Kull 2005; Sterner 2008; Craine 2009), *synergistic co-limitation* (Davidson & Howarth 2007; Sterner 2008) and serial limitation, which does not meet strict definitions of co-limitation (Craine 2009). Recently, Allgeier *et al.* (2011) applied a metric intended to test for non-additive responses to N and P addition to a subset of the Elser *et al.* (2007) dataset. Their analysis confirmed the general results of Elser *et al.* (2007) that *synergistic* responses were frequent, but they also suggested that antagonistic responses were most common. However, general definitions of co-limitation based solely on non-additive responses (e.g. Allgeier *et al.* 2011) confound alternative definitions that have a strict biochemical interpretation (e.g. *simultaneous* vs. *serial*; Saito *et al.* 2008) or omit obvious types of co-limitation that are not necessarily super-additive (e.g. Box 1b *independent co-limitation*). In addition, testing for interactions or synergistic responses is highly sensitive to often unacknowledged issues of data transformation and the scale of measurement, especially a concern because log-transformations (e.g. Allgeier *et al.* 2011) can make multiplicative relationships (i.e. interactions) additive (Bland & Altman 1996). Our tests for simultaneous and independent co-limitation are less sensitive to such scale of measurement issues.

As the primary producer community biomass responses to factorial N and P addition that we analysed here potentially encompass a wide range of biochemical-, individual- and community-level mechanisms of co-limitation, the relative importance of particular underlying mechanisms cannot be easily inferred. Evidence of co-limitation or nutrient interactions does provide motivation for more detailed, stoichiometrically explicit experiments (e.g. Güsewell 2005; Olde Venterink & Güsewell 2010); response surface designs that manipulate the supply of multiple nutrients across combinations of nutrient ratios and total nutrient supply (e.g. Saito *et al.* 2008); species-specific growth response and physiological studies to identify resource-based traits and tradeoffs and their biochemical underpinnings (e.g. Litchman *et al.* 2006); and longer duration experiments to quantify the effects of nutrient addition on community composition, microbial nutrient cycling and ecosystem function (e.g. Clark & Tilman 2010).

Multiple nutrient co-limitation has important implications for understanding the impacts of increasing rates of nutrient pollution for all systems and for system-specific issues such as mitigating the effects of eutrophication on aquatic ecosystems. Conley *et al.* (2009) argued that wastewater treatment for abatement of both N and P is

necessary to prevent the adverse effects of excess production in freshwater and coastal ecosystems. Others have claimed that N-fixation by cyanobacteria prevents the occurrence of widespread N-limitation in phytoplankton, and therefore that P remains the main source of eutrophication (Schindler & Hecky 2009). Elser *et al.* (2009a,b) provide evidence for frequent N and P co-limitation of lake phytoplankton growth except under conditions of heavy atmospheric N-deposition. Our results support the paradigm that co-limitation occurs frequently, and nutrient interactions even more so, across a variety of aquatic and terrestrial ecosystems, and for reasons that have biological and theoretical basis.

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## AUTHORSHIP

JTN wrote the first draft of the manuscript; WSH wrote the subsequent and final drafts of the manuscript; all authors contributed substantially to revisions; JTN, EEC, EWS and WSH designed the study; WSH, EEC and EWS analysed the data; DSG and EWS assembled and managed the meta-database, with all authors contributing substantially to assembling the database.

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## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

**Figure S1** Flow chart of logical tests used to categorise each factorial N x P study. Simultaneous co-limitation in orange, independent co-limitation in blue and serial limitation in yellow. Y or N correspond to 'yes' or 'no' (logical true or false). Negative effects are whether N, P or N + P addition produces a significant reduction in biomass relative to the control.

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