

Species-specific stoichiometric effects of leaf litter on algal growth, production, and cell quotas

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Abstract

Dissolved organic matter and nutrients released from forest leaf litter are important cross-ecosystem resources that support freshwater ecosystems. Dissolved organic matter released from leaf litter is one of the allochthonous sources for heterotrophic organisms in freshwater communities. However, the role of macro- and micronutrients released from leaf litter in producing autochthonous organic matter in aquatic ecosystems is not necessarily well understood. In this study, therefore, we investigated how dissolved nutrients released from leaf litter affect the algal growth, biomass production, and cell elemental quotas. Specifically, we focused on the responses of the green algae to TDN, TDP, and micronutrients released from the leaf litter of 11 temperate tree species. We found that the algal growth rate increased with TDP when micronutrients were amended. In contrast, the algal biomass production increased with TDN, regardless of micronutrient amendment. Micronutrient limitation of algal growth rate was found in the leaf litter leachates from oak, Japanese elm, and Japanese hemlock. However, algal biomass production was limited by micronutrients only in the leaf litter leachate from Japanese hemlock. More importantly, leaf litter leachates from different tree species altered algal cell quotas and C:N:P ratios, which would affect secondary production. These results suggest that forest vegetation change or succession affect the quantity and quality of autochthonous organic matter and thus the mass transfer efficiency in the aquatic community by changing the stoichiometry of nutrient input from the leaf litter.

Introduction

Dissolved organic and inorganic matter released from forest leaf litter is an important cross-ecosystem resource that influences the communities in freshwater ecosystems. Numerous studies have shown that, in lakes and ponds, terrigenous dissolved organic carbon (DOC) can enhance heterotrophic microbial production and reduce underwater light intensity, thereby reducing the algal growth and primary production rates. However, because leaf litter contains various elements other than C, including nitrogen (N) and phosphorus (P), it is likely to promote autochthonous production through algal growth in aquatic ecosystems where leaf litter is introduced. Indeed, recent studies have shown that leaf litter stimulates algal growth and increases energy transfer from autochthonous organic matter to higher trophic levels through the grazing food chain in lakes.

However, the effects of leaf litter on aquatic organisms through the release of nutrient elements may vary among tree species because the elemental content of leaves is highly species-specific. In addition, the water-soluble fractions of nutrient elements in leaf litter also vary among tree species. In fact, our previous study showed that the concentrations of dissolved N and P in leaf litter leachates varied widely among 11 tree species because the release efficiencies of these nutrients differed among tree species (Ho et al. 2023). These results suggest that the production of autochthonous organic matter is affected by the change and succession of the surrounding terrestrial vegetation through the changes in the inputs of various dissolved nutrient elements from the leaf litter. To test this possibility, it is essential to investigate the response of algae to leaf litter leachates from different tree species.

In freshwater ecosystems, the algal production rate of is mainly limited by the supply rate of dissolved P, although at certain times it may be limited by N or co-limited by N and P . In addition, essential micronutrients, such as iron, are known to affect algal production and growth rates in aquatic ecosystems , because these trace metals are essential core elements of mechanoenzymes that play various functional roles in cell metabolism and catabolism . Therefore, if the relative and absolute levels of these micronutrients released from leaf litter differ among different tree species, algal production and growth rate may change depending on the dominant tree species growing around the freshwater ecosystems.

In autotrophs such as algae, the biomass production is related to its photosynthetic activities, cell division rates, i.e., growth rate, and cell quotas, i.e., cell-specific C, N, and P contents, are affected by various cell processes other than photosynthesis . Accordingly, the nutrient elements that affect algal production rates are not necessarily the same as those that affect growth rates . More importantly, the growth of herbivorous zooplankton is influenced by the cellular N and P contents relative to the C content in the algae . This fact suggests that not only the production rate of autochthonous organic matter but also its mass transfer to higher trophic levels may be affected by changes in the dominant tree species growing around the freshwater ecosystems via macro- and micronutrients supply from fallen and submerged leaf litter. However, although several studies have shown that leaf litter can stimulate algal growth and increase energy transfer to zooplankton , no study has yet examined whether such effects differ among leaf litter from different tree species.

In the present study, therefore, we investigated whether the algal response is different depending on the tree species providing the leaf litter to freshwater ecosystems. For this purpose, we prepared culture media using leaf litter leachates from 11 common tree species in the plain and mountainous regions of northeastern Japan. Then, we investigated the cell growth rate, carbon biomass production, and the C, N, and P cell quotas of a green alga *Scenedesmus obliquus* . We used *Scenedesmus* because this group of green algae is common freshwater phytoplankton and has been used in various experimental studies . Our specific objectives are to clarify (1) if N and P nutrient supply relative to algal requirements for growth and production differ among the leaf litter leachates of the 11 tree species, (2) if the leaf litter leachates provide sufficient micronutrients, and (3) if the cell C, N, and P quotas of the algal species vary depending on the type of leaf litter leachates. Based on these results, we discuss the possibility that the quantity and quality of autochthonous organic matter produced by algae and its mass transfer to higher trophic levels in aquatic ecosystems are influenced by the changes in tree species composition in the surrounding forests.

Materials and methods

Preparation of leaf litter leachates

We collected the leaf litter of 6 broadleaf and 5 coniferous species commonly found in the plains and mountainous of northeastern Japan. Although we did not check when they fell, we collected leaf litter on top of the leaf litter layer on the ground so that the litter did not have direct contact with the soil. These fallen leaves were collected at Zao (N38.122, E140.451) on October 3, 2020, at Kawatabi (N38.745, E140.757) on October 15, 2020, and at Aobayama (N38.259, E140.837) on November 19, 2020 in Miyagi Prefecture, Japan (Table 1).

Before use, leaf litter was dried at room temperature (20 °C) and was cut with scissors to mimic the early decomposition processes by detritivores. For each tree species, we weighed the cut leaf litter and placed it in two 1000 mL Nalgene bottles containing 800 ml of distilled water. The weight of leaf litter in each bottle was adjusted to control the final concentration of TDP to approximately 1-4 μM after 7 days of the leaching process based on the release efficiency (Table 1) estimated by Ho et al. (2023). The water in the two replicate bottles was aerated in the dark for 7 days at 20 °C and mixed before being used as leaf litter leachates.

We used *Scenedesmus obliquus* as the experimental algal species because it has been frequently used in various studies . Prior to the incubation of *Scenedesmus* , the leaf litter leachate was filtered through 40 μm mesh to remove litter particles. DOC and total dissolved N (TDN) concentrations in the leaf litter leachate were measured using a TOC/TN analyzer (multi N/C 3100, Analytik Jena GmbH, Jena, Germany). Total

dissolved P (TDP) concentrations were measured using the ascorbic acid-molybdenum blue method after persulfate oxidation .

Experiment

For leaf litter leachate from each tree species, we prepared “control” and “amended” treatments using 200 mL Nalgene incubation bottles. The control treatment consisted of 150 mL of filtered leaf litter leachate, and the amended treatment consisted of 147 mL of filtered leachate and 3 mL of basal COMBO medium, which contained macroelements (K, Ca, Mg, B), trace elements (Fe, Mn, Cu, Zn, Co, Mo, Se, and V), and vitamins (B12, biotin, and thiamin), but no inorganic N and P nutrients . We referred these chemicals as micronutrients. In each leaf litter leachate, we set up three replicates for each treatment.

We inoculated algal cells into each experimental bottle at an initial incubation cell density of 2.2×10^4 cells mL^{-1} . The algae were incubated for 7 days in the bottles, which were placed in an incubator at a temperature of 20 degC and light intensity of $100 \mu\text{E m}^{-2} \text{s}^{-1}$. The bottles were manually shaken every day to resuspend the cells. After 7 days, we preserved 20 mL of culture medium in Lugol’s solution for estimating the cell number by microscopic enumeration. We also filtered 5 mL of culture medium on two combusted GF/F filters (Whatman), which were dried for further analyses of the C, N, and P contents of *Scenedesmus obliquus* .

Measurements of algal growth rates and elemental contents

We enumerated the number of algal cells at the end of incubation using the Fuchs-Rosenthal counting chamber and an optical microscope (Olympus, Tokyo, Japan; eyepiece magnification 10x, objective magnification 10x) (cells mL^{-1}). Then, the algal growth rate was calculated from the difference between the cell number at the start (N_0) and the end of incubation (N_t) as follows,

$$\text{GR} = \frac{\ln(N_t) - \ln(N_0)}{t} \quad (1),$$

where $t = 7$ days.

The C and N contents of algal cells collected on GF/F filters ($\mu\text{g mL}^{-1}$) were measured using a CHN elemental analyzer (2400 Series II CHNS/O Elemental Analyzer, Perkin Elmer, Massachusetts, USA). The P content of algal cells was measured by the ascorbic acid-molybdenum blue method after potassium persulfate oxidation . The C, N, and P cell quotas of the algal cell were calculated as:

$$Q_E = \frac{\text{Scenedesmus C, N, or P biomass } (\mu\text{g } \mu\text{L}^{-1})}{\text{Scenedesmus density (cells } \text{mL}^{-1})} \quad (2).$$

Statistical analyses

We used ANOVA and Tukey’s multiple comparison test to examine if the algal growth rate, C biomass, and C, N, and P cell quotas were significantly different among the control treatments prepared from the leaf litter leachates of 11 tree species. We also tested for the algal response to micronutrient amendment in each leaf litter leachate using Student’s t -test. These analyses were performed using functions `aov` , `TukeyHSD` , and `t.test` in the R ver. 4.2.2 . We examined the effects of TDN and TDP on the algal growth rate and C biomass by the segmented linear regression model using functions `lm` and `segmented` in package `segmented` in the R ver. 4.2.2 .

Results

Growth rate and carbon biomass

The growth rate ($F = 59.65$, $p < 0.001$) and C biomass ($F = 286.9$, $p < 0.001$) of *Scenedesmus* were significantly different among the culture media prepared from 11 types of leaf litter leachates in the control treatment (Fig. 1a). The addition of micronutrients to the culture medium had a significant positive effect on the algal growth rate of in the leaf litter leachates of oak, Japanese elm, and Japanese hemlock. However, the micronutrient amendment had a negative effect on growth in the culture medium prepared from the

leaf litter leachate of hinoki cypress. For the algal C biomass, a significant positive effect of micronutrient amendment was detected only in the culture medium prepared from the leaf litter leachate of Japanese hemlock (Fig. 1b). However, algal C biomass was not affected by the micronutrient amendment in culture media prepared from other leaf litter leachates.

Because TDN and TDP concentrations varied among the different types of leaf litter leachates, we examined whether variations in the algal growth rate and C biomass of *Scenedesmus* were related to these nutrient concentrations in the culture media prepared from the leaf litter leachate. Due to the large variation of growth rate at TDN concentrations $< 5 \mu\text{M}$, the growth rate was not related to TDN concentration regardless of the micronutrient amendment (Fig. 2a and b). However, the algal growth rate tended to increase with TDP concentration in the amended treatment but not in the control, although the growth rate also varied widely and decreased when TDP concentration was $< 2 \mu\text{M}$ (Fig. 2c and d).

Unlike the growth rate, the algal C biomass increased significantly with TDN in the control treatment, although the increasing trend was attenuated when TDN exceeded $5 \mu\text{M}$, as shown by the segmented regressions with breakpoints at $5.31 \mu\text{M}$ (Fig. 2e and f). In the amended treatment, the relationship between the algal C biomass and TDN was not improved. It was almost the same as in the control treatment, as indicated by similar values in the segmented regressions (Fig. 2e and f). Similarly, algal C biomass tended to increase with TDP regardless of the micronutrient amendment, although the increasing trend was not significant due to low C biomass values relative to TDP in some culture media, such as those prepared from leaf litter leachates of Siebold's beech, Japanese elm and Japanese red pine (species ID 5, 6, 9 in Fig. 2g and h).

Cell quotas and stoichiometry

The C, N, and P cell quotas in *Scenedesmus* varied significantly among the culture media from different types of leaf litter leachates in the control treatment (Fig. 3; $F = 33.24$, $p < 0.001$ for C quota; $F = 40.27$, $p < 0.001$ for N quota; $F = 16.36$, $p < 0.001$ for P quota). The micronutrient amendment of the culture media had a significant negative effect on the C cell quota in the leaf litter leachates of oak and Japanese hemlock, and a significant positive effect was detected in the leaf litter leachate of hinoki cypress (Fig. 3a). The micronutrient amendment had a negative effect on the N cell quota only in the leaf litter leachate of Japanese hemlock (Fig. 3b). Similarly, the negative effect of the micronutrient amendment on the P cell quota was detected in the leaf litter leachate of Japanese hemlock, and a significant positive effect was found in the leaf litter leachates of Japanese red pine and hinoki cypress (species ID 9 and 11; Fig. 3c).

The molar C:N cell quota ratios of *Scenedesmus* varied from 6.6:1 to 21:1, and was less than 12:1 except for Japanese maple, oak, Japanese hemlock, Japanese red pine, and Japanese larch (species ID 1, 4, 7, 9, 10 in Fig. 4a). The molar C:P ratios of the algae varied from 106:1 to 300:1, except for cells in grown in the culture medium prepared from the leaf litter leachate of Japanese maple (species ID 1), which reached C:P = 600:1 (Fig. 4b). The molar N:P ratio of the algae was distributed between 10:1 and 30:1, and was lower than 16:1 in the leaf litter leachates of Siebold's beech, Japanese elm, Japanese red pine, and hinoki cypress (species ID 5, 6, 9, 11 in Fig. 4c).

Discussion

The effect of leaf litter leachates on the growth, production, and cell stoichiometry of the green alga *Scenedesmus* varied significantly among the leaf litter leachates of different tree species. As nutrient concentrations were not the same among the culture media, growth rates in different types of leachates could not be directly compared with each other. Therefore, we plotted the C biomass accumulated during the 7-day incubation period against TDN and TDP in the leachate. The result showed that the accumulated C biomass increased with increasing TDN in the leachate regardless of the micronutrient amendment, although the increasing trend was attenuated at higher TDN concentrations. In addition, C biomass was highly limited in the leachates of Siebold's beech, Japanese elm and Japanese hemlock regardless of high TDP concentrations (species ID 5, 6, 7 in Fig. 2g and h). These results suggest that the variation in C biomass among the

different culture media from leaf litter leachate was mainly caused by TDN concentration. In a previous study, Ho et al. (2023) suggested that nitrogen is the most deficient element in the leaf litter leachates relative to phytoplankton demand, because the percentage of release relative to leaf litter elemental content was lowest for TDN compared to DOC and TDP in all 11 tree species studied. The present study confirmed the validity of this suggestion for phytoplankton production.

However, the algal growth rate, estimated from temporal changes in cell abundance, differed from that of C biomass. In the present study, it ranged from 0.2 to 0.3 d⁻¹ in for most of the leaf litter leachates, except for Japanese elm and Japanese hemlock, where the growth rate was <0.2 d⁻¹. The result suggests that the nutrient conditions in the present study were moderate according to the growth rate of *Scenedesmus* under different culture conditions in previous studies. However, the growth rate was not closely related to either TDN or TDP in leaf litter leachate, especially when micronutrients were not amended. The result suggests that the factors regulating growth rate, i.e., cell division rate, and net C biomass accumulation rate, i.e., organic carbon production rate, are different in this algal species.

It should be noted that when micronutrients were added, the algal growth rate significantly increased in the leaf litter leachates of oak, Japanese elm and Japanese hemlock, although the opposite trend was found in the leachate of hinoki cypress. The results contrasted with the response of C biomass, which showed a significant difference between the culture medium with and without micronutrient amendment only in Japanese hemlock. These results suggest that the fitness of the present algal species in the terms of cell division rate is more sensitive to micronutrient supply than the net production rate. In fact, the algal growth rate was significantly correlated with TDP but not TDN when micronutrients were additionally supplied. This fact implies that the variation of algal growth rate among different types of leaf litter leachates was mainly regulated by P supply in concert with micronutrients, whereas that of net production rate was more regulated by N supply. The relative importance of P rather than N may be related to the fact that P is an essential element of DNA and RNA and can strongly limit cell division.

Micronutrients are known to control the enzyme activity that regulates the N and P use efficiency of phytoplankton. Indeed, in leaf litter leachates from Japanese red pine and hinoki cypress, a significant increase in N and P cell quotas was found when micronutrients were additionally supplied. In these types of leaf litter leachates, micronutrients may play a role in P and N use efficiency. In addition, micronutrients appear to play different roles in algal growth and C biomass accumulation. Several studies have shown that the factors affecting algal cell division rates are different from those affecting the photosynthetic production rates. For example, Li et al. showed that in the dinoflagellate *Amphidinium carterae*, the cell division cycle was halted but the photosynthesis continually accumulated C in cells under P deficiency. On the contrary, Negi et al. found that in the green alga *Chlorella sorokiniana*, N deficiency did not change the cell density, but decreased the photosynthetic rate.

As mentioned above, the addition of micronutrients tended to reduce the growth rate of *Scenedesmus* incubated in a culture medium from the leaf litter leachate of hinoki cypress. The result suggests that the leaf litter leachate of hinoki cypress may contain excessive amounts of some micronutrients that are harmful to *Scenedesmus*. Rueter et al. showed that Al and Cu tended to reduce the growth rate of *Scenedesmus* when supplied in excess. Because we added a mixture of micronutrients, we could not determine which micronutrients and trace elements were harmful when supplied in excess. However, the present study indicates that the water-soluble content of micronutrients and trace elements varies widely among the leaf litter of different tree species as suggested by Pourhassan et al.

Studies have shown that decomposition and nutrient release rates somewhat differ between coniferous and broadleaf tree species. However, we could not determine whether leaf litter leachates from broadleaf or coniferous trees had a better nutrient composition for the growth rate of *Scenedesmus* due to the large variation of growth rate within each of these two groups. In addition, we could not find significant difference in the C biomass production rate of this green alga between the culture media from broadleaf and coniferous leaf litter leachates due to the large variations in the rate within these tree species. These results suggest that the effect of leaf litter on aquatic organisms cannot be generalized across broad taxonomic tree groups.

Similar to the growth and C biomass production rates, leaf litter leachates from different tree species had different effects on the C:N:P ratio of the algae. It is well known that the elemental contents of algae are important determinants of the quality of food for herbivorous zooplankton. In this study, the molar C:N and C:P ratios of *Scenedesmus* varied from 6.6 to 21 and 106 to 600, respectively, depending on the leaf litter leachate. In general, algal cells with C:N and C:P ratios close to Redfield ratios are good quality food for zooplankton growth. In the leaf litter leachates of Siebold’s beech, Japanese elm, Japanese red pine and hinoki cypress, the molar C:N and C:P ratios ranged from 6.6 to 12 and 106 to 160, respectively, which are close to the Redfield ratios. Thus, algae grown in lentic waters receiving nutrients from leaf litter of these species would be suitable food for herbivorous zooplankton. However, in the leaf litter leachates from Japanese maple and Japanese cedar, the C:P ratio of *Scenedesmus* cells was greater than the threshold elemental ratio (>300) above which the growth rate of zooplankton such as *Daphnia* is limited by phosphorus. Urabe and Watanabe also suggest that algal cells with C:N ratios greater than 15 are suboptimal food for zooplankton. In this study, the C:N ratio of *Scenedesmus* cells in the leaf litter leachates of Japanese maple and Japanese hemlock was greater than this value. In addition, depending on the leaf litter leachate of different tree species, algae may alter the cellular content of other chemical substances, such as fatty acids and amino acids, that affect food quality for herbivores. Together with such a possibility, the present results suggest that the mass transfer from algae to herbivorous zooplankton in a lake may be influenced by the vegetation composition in the watershed via subsidies of nutrient inputs from leaf litter.

Conclusion

The present study showed that algal growth rate, biomass production, and elemental contents varied depending on the leaf litter leachates from different tree species, due not only to differences in N and P concentrations but also to those of micronutrients. These results suggest that the stoichiometry and composition of leaf litter leachate can cause substantial changes not only in algal fitness and primary production, but also in mass transfer from algae to herbivores. Although we conducted this study using only a single algal species, the responses to the changes in leaf litter leachate may differ between algal species and other heterotrophic organisms that utilize organic matter in leaf litter leachate. This possibility is worth investigating in future studies, as the effects of the change and succession in the surrounding vegetation may have extended to the composition of the entire community in the freshwater ecosystems. In this study, we investigated the short-term and direct effects of leaf litter using leaf litter leachate for 7 days of incubation. In nature, the effects of leaf litter on aquatic ecosystems may last longer through decomposition in soils and sediments. Therefore, to fully understand the effects of vegetation changes on the aquatic community, long-term monitoring studies are needed in aquatic ecosystems where the catchment vegetation has been changed.

References

Table 1. Stoichiometry of the leaf litter used in the present study. The standard deviation of the elemental contents from multiple measurements is given in parentheses. Release efficiencies of dissolved C, N, and P after 7 days of leaching are from Ho et al..

	Tree species	Tree species	Tree species	Tree species	Sampling site	Sampling s
	Common name	Species name	Tree type	Leaf type	Area	Latitude
1	Japanese maple	<i>Acer amoenum</i> var. amoenum	Broadleaf	Deciduous	Aobayama	N38.259
2	Zelkova	<i>Zelkova serrata</i>	Broadleaf	Deciduous	Aobayama	N38.259
3	Erman’s birch	<i>Betula ermanii</i>	Broadleaf	Deciduous	Zao	N38.129
4	Oak	<i>Quercus serrata</i>	Broadleaf	Deciduous	Zao	N38.122
5	Siebold’s beech	<i>Fagus crenata</i>	Broadleaf	Deciduous	Kawatabi	N38.745
6	Japanese elm	<i>Ulmus davidiana</i> var. japonica	Broadleaf	Deciduous	Kawatabi	N38.745
7	Japanese hemlock	<i>Tsuga diversifolia</i>	Coniferous	Evergreen	Zao	N38.122
8	Japanese cedar	<i>Cryptomeria japonica</i>	Coniferous	Evergreen	Kawatabi	N38.745
9	Japanese Red pine	<i>Pinus densiflora</i>	Coniferous	Evergreen	Kawatabi	N38.745
10	Larch	<i>Larix leptolepis</i>	Coniferous	Deciduous	Kawatabi	N38.745

	Tree species	Tree species	Tree species	Tree species	Sampling site	Sampling site
11	Hinoki cypress	<i>Chamaecyparis obtusa</i>	Coniferous	Evergreen	Kawatabi	N38.745

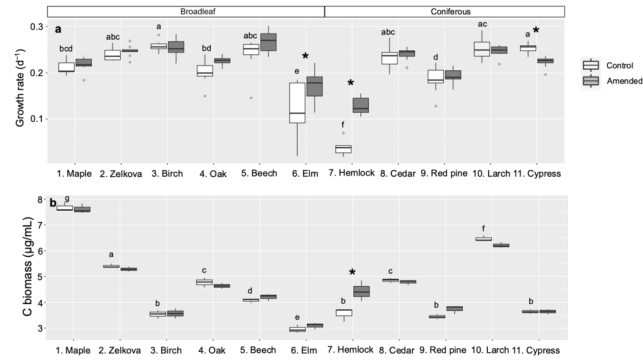


Figure 1. *G* growth rate (a), and *C* biomass (b) of algae incubated in the leaf litter leachates of 11 tree species. White boxes are incubations without micronutrient amendment (control treatment) and gray boxes are incubation with micronutrient amendment (amended treatment). Boxes are arranged according to the P content in leaf litter biomass from low to high within broadleaf and coniferous tree groups as in Table 1. Asterisks indicate that there is a significant difference between control and amended treatments ($p < 0.05$). Different letters indicate a significant difference among control treatments of different leaf litter leachates.

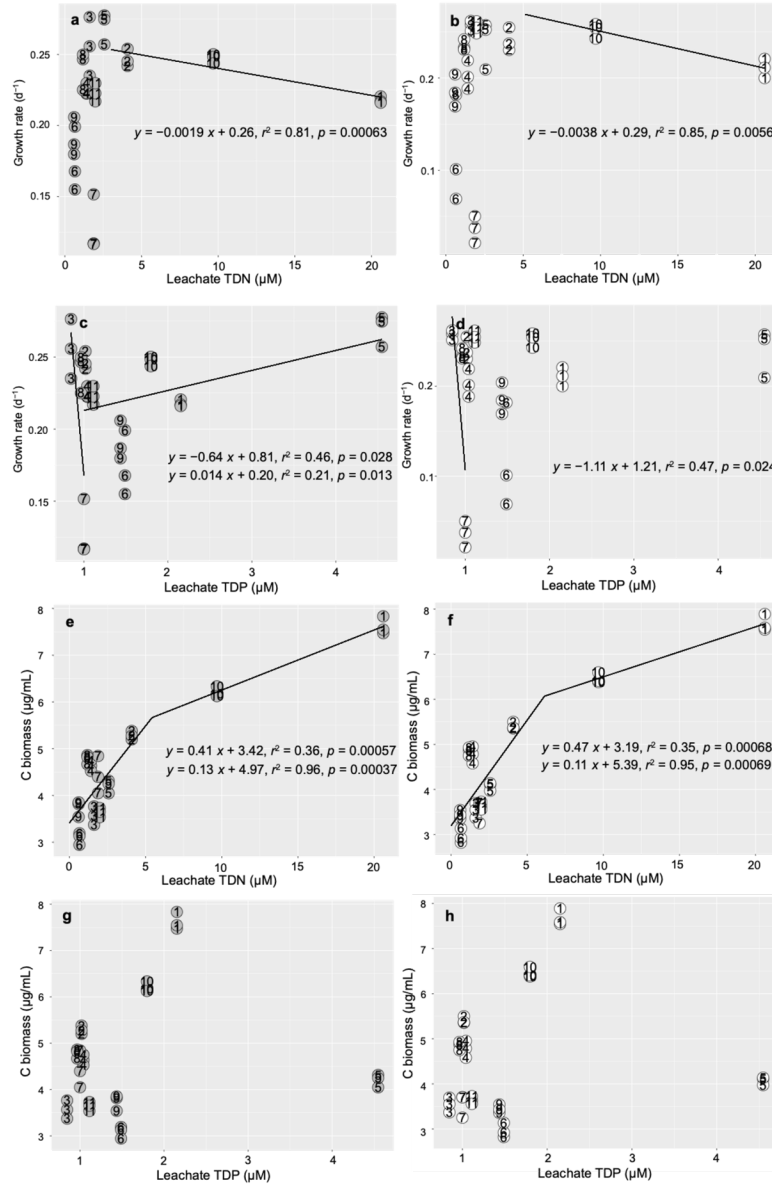


Figure 2. Algal growth rate (a-d) and accumulated C biomass (e-g) plotted against total dissolved N (TDN) and total dissolved P (TDP) concentrations in amended (a, c, e, and g) and control treatments (b, d, f, and h). The significant segmented linear regression is shown as solid lines. The numbers in the dots represent the tree species ID shown in Table 1.

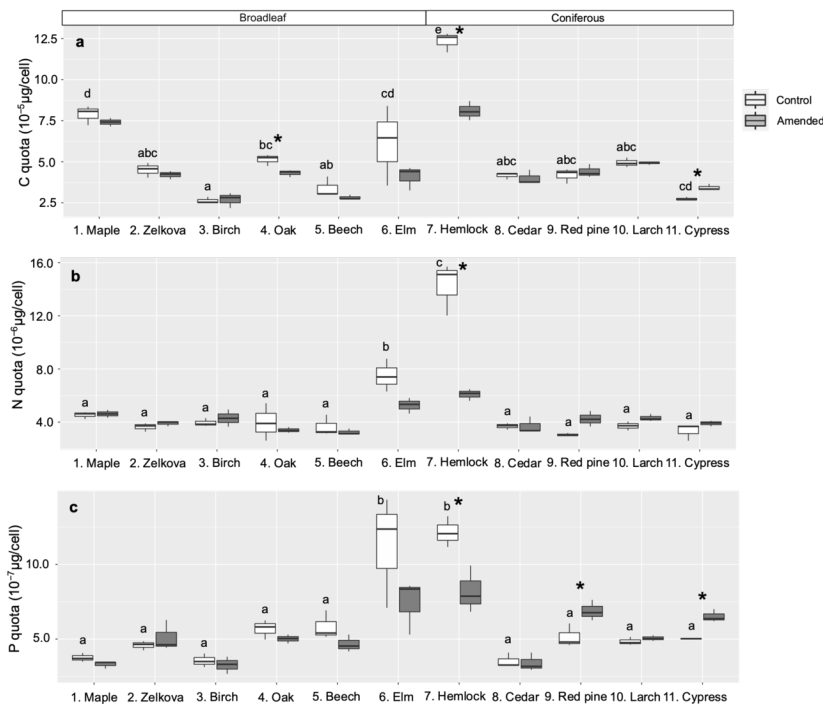


Figure 3. C (a), N (b), and P (c) cell quotas of algae incubated in culture media prepared from leaf litter leachates of 11 tree species. White boxes are control incubations and gray boxes are amended incubations. Boxes are arranged according to the P mass content in leaf litter from low to high within broadleaf and coniferous groups as in Table 1. Asterisks indicate that there is a significant difference between control and amended incubations ($p < 0.05$). Different letters indicate a significant difference between control treatments of different leaf litter leachates.

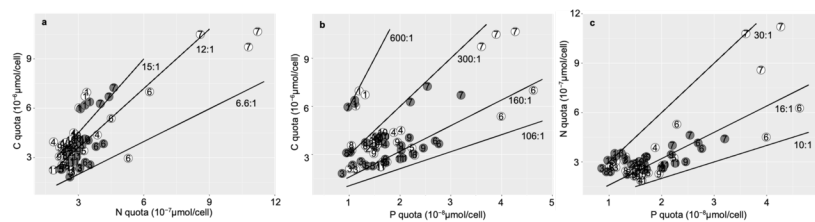


Figure 4. Scatter plots of C and N ratios (a), C and P ratios (b), and N and P ratios (c) of the algal cells. The molar C:N = 6.6:1, 12:1, 15:1 ratios (a), molar C:P = 106:1, 160:1, 300:1, and 600:1 ratios (b), molar N:P = 10:1, 16:1, and 30:1 ratios (c) are shown as solid lines. White dots represent control treatments and

gray dots represent amended treatments. The numbers in the dots represent the tree species ID shown in Table 1.