Appendix B: Detailed methods for mesocosm preparation, animal collection, litter chemistry and biofilm measurements, detailed modeling methods, algal C:N sources, and statistical analysis.

Mesocosm preparation:

In the fall of 2011 freshly senesced green ash and red maple leaf litter was collected within sites at the Oakland University Biological Preserve, Bald Mountain State Recreation Area and the University of Michigan's Saginaw Forrest. Leaves from each species were homogenized after collection from these multiple sites. The litter was dried at 20ºC for 48 hours with circulating air. Percent moisture was determined by weighing ~5 g before and after being placed in a drying oven at 60ºC for 24 hours. Five 10-g subsamples of leaves from each tree species were then removed from the mixture and placed in a -20ºC freezer for later elemental analysis. We then weighed out 130 g (dry mass) to create the 5 litter mixtures. Leaf litter was added to the mesocosms in the second week of December 2011. Mesocosm froze within a week of litter addition and remained frozen until two weeks prior to tadpole addition.

Eighty plastic pools (110 L) were filled with 100 L tap water and were allowed to sit for one week (to off-gas chlorine), prior to the inoculation with pond water (1 L filtered through 0.5-mm Nitex, USA) obtained from a local wetland in the first week of December 2011. Four replicates of each litter treatment were then randomly assigned within each of four spatial blocks. To create variation in shade, lids consisting of two different shade cloth densities were constructed (polyethylene PAK knit shade cloth Hummert International, USA), producing high (77%) and low (27%) shade treatments (Williams et al. 2008). Ten pools of each shade treatment were then randomly assigned within each spatial block. Each mesocosm received one unglazed ceramic tile placed on the north-facing side of the mesocosm to assess biofilm growth. Additionally, one temperature loggers (I-button; Embedded Data Systems, KY, USA) was

assigned to two low light and two high light mesocosms within each of the spatial blocks in the Spring of 2012 as soon as the ice melted. I-buttons were set to record temperature every three hours and were removed on June 1. High light and low light mesocosms were on average 16.7 ± 0.8 ºC and 14.7 ± 0.7 ºC respectively through the sampling period.

Amphibian collection and measurements:

In the spring of 2012, 10 adult male and 10 female wood frogs (collected 2nd week of March) and American toads (collected 3rd week of March) were obtained during their breeding migrations. Wood frogs were collected along a drift fence surrounding a pond at the Saginaw forest (Ann Arbor, Michigan, USA), while American toads were collected from the Oakland University Biological Preserve (Rochester, Michigan, USA). Both amphibian collection sites contained red maple and green ash trees. The adults were allowed to amplex in lab and deposit eggs in 10 L containers of aged tap water. Eggs were allowed to hatch and the resulting tadpoles from the 10 clutches were pooled. Recently hatched wood frog tadpoles (30 per mesocosm) and American toad tadpoles (40 per mesocosm) were then each added to half of the pools (April 2 for wood frogs and April 9 for toads). The tadpole densities used in this experiment were well within the ranges observed for these two species in natural ponds (Berven, unpublished data; Morin, 1983), and were also densities that other researchers have used in mesocosm experiments (Maerz et al. 2010, Cohen et al. 2012, Stephens et al. 2013). Because of initial differences in mass between the two species, stocking densities were different to equalize initial amphibian biomass. The initial masses were estimated for each species by randomly selecting 10 tadpoles from the reservoir immediately prior to the initiation of the experiment and weighing them. Weighing was accomplished by gently blotting the animal with filter paper and carefully placing them on a scale. This procedure was followed for the intermediate (at day 47) and final mass. The tadpoles

were then allowed to develop until metamorphosis, and were removed from mesocosms on the emergence of their forelimbs (Gosner Stage 42). The metamorphs were kept in 1 cm of aged tap water in mesocosm-specific 14 L plastic containers until tails were resorbed (which took no more than two days), at which point wet mass was determined and time taken to complete metamorphosis was recorded. The metamorphs were killed in a lethal solution of MS-222 and were frozen. Amphibian survival was determined by dividing the number of juveniles to complete metamorphosis by the stocking density.

Litter chemistry measurements:

Litter chemistry was assessed based on samples taken prior to addition of litter to mesocosms. Litter was dried at 60 ºC for 24 hours, ground in a mill, and passed through a 0.5 mm mesh sieve. Percent N and C values were determined on the five ground subsamples of each litter species by combustion in a CHN analyzer (Leco, St. Joseph, MI, USA).

Litter C and N measurements were not obtained for later time points in the experiment. This posed a potential problem for predicting tadpole growth due to the potential for litter C and N to change following submergence in water, due to the leaching of soluble compounds and the colonization of microbes (Gulis and Suberkropp 2003, Gessner 1991). We therefore conducted a sensitivity analysis based on literature values for litter compositional changes in water, to determine what potential effects this might have on model predictions. Gulis and Suberkropp (2003) found little change in %N despite a 40% reduction in dry mass of maple leaf litter following 30 days of submergence in a non-nutrient-enriched stream. However, Gessner (1991) found a ~35% increase in N of alder leaf litter and Suberkropp et al. (1976) found a ~33% increase in N of hickory and a ~25% increase in N of oak litter following 4 weeks of inundation.

Because the mesocosms in our study froze soon after litter addition and thawed within a few weeks of amphibian addition, it is likely that the N concentration of the litter changed little prior to the beginning of the experiment. We therefore assumed for the sensitivity analysis that litter %N might have increased by as much as 20% prior to initiation of the experiment. According to the sensitivity analysis, such a change in litter %N would have increased our predicted tadpole growth rates by 23%, which would still have been well within the range of observed tadpole growth rates (Appendix C: Table C5).

Biofilm and chlorophyll a measurements:

Biofilm biomass and chlorophyll *a* (chl. *a*) content, conductivity, and dissolved polyphenolics were determined 30 days after addition of tadpoles. Conductivity [a surrogate measure for dissolved nutrients, (Iwai and Kagaya 2007)] was determined using a Hach probe (CDC40110; Hach Company, Loveland, CO, U.S.A.) Biofilm was assayed by removing a clay tile, brushing off all biofilm from the surface into an enamel pan, and concentrating the scraped material onto a pre-weighed 0.7 um G/F/F filter (Whatman Inc, Kent, U.K.). The filter was then dried at 80 ºC for 24 h, cooled in a desiccator, and weighed. Fluorometric analysis (Trilogy Model, Turner Instruments, Sunnyvale, CA, U.S.A.) was used to determine chl. *a* content of biofilm following a modified version of the EPA method 445.0 (Arar and Collins 1997). For dissolved polyphenolics, one 250 mL subsample sample was collected from four 0.5 L mesocosm water surface samples and was stored at -20 ºC until analysis using the amended colorometric Folin-Ciocalteu method as described by Stephens et al. (2013). We intended to assay periphyton quality directly for this experiment; unfortunately, the periphyton samples deteriorated before we were able to process them (due to equipment problems). To address this we conducted our meta-analysis of freshwater periphyton quality as described below.

Modeling methods

We constructed a model based on "the minimal model" created by Sterner and Elser (2002) to explore mass balance constraints of N on tadpole growth. The initial model was developed to explain growth of *Daphnia* feeding on algae of varying quality. We have kept the same form of the model, however some of the parameter units were slightly different to accommodate tadpoles feeding on litter/biofilm as opposed to filter feeding. We also followed Sterner and Elser (2002) by assuming that: 1) only one element (C or N) limits tadpole production at a time causing the loss of the limiting nutrient to be reduced to a finite minimum and 2) that the C:N of new production is homeostatic. Because the bulk of mass acquisition (aside from water) during growth is in the form of C we chose to model the accumulation of carbon as a result of increases in litter N. Following the second assumption, the ratio of the rate of C and N acquisition (growth rates) must be proportional to the initial stoichiometry of the organism:

$$\frac{g_C}{g_N} = \frac{Q_C}{Q_N} \qquad \text{(B.1)}$$

where g_C represents the growth based on C and Q_C represents the concentration of C bound in the tadpole dry mass. The same reasoning can be applied to N. All model variables and parameter units can be found in Appendix C, Table C2. Expanding upon this equation we can include the fact that growth is the difference between incorporation of new material and loss:

$$\frac{IA_C S_C - L_C Q_C}{IA_N S_N - L_{N(\min)} Q_N} = \frac{Q_C}{Q_N} \qquad \text{(B.2)}$$

In this equation I stands for the intake rate of total food, A_C and A_N represent the concentration of each respective element bound in the food (leaf litter for wood frogs and periphyton for toads), S_C and S_N are the assimilation efficiencies of each element, and the parameters marked

L_C and L_N are the specific loss rates of the respective elements. Rearranging Eq. B.1 we find that:

$$g_C = \frac{Q_C}{Q_N} \times g_N \qquad \text{(B.3)}$$

Substituting the full equation for g_N from eq. B.2 into eq. B.1 we find that:

$$g_C = \frac{Q_C}{Q_N} \times (IA_N S_N - L_{N(\min)} Q_N) \qquad \text{(B.4)}$$

which follows the second assumption that the rate of tadpole C accumulation must be proportional to the rate of N acquisition times the original stoichiometry.

For the model fitting we measured tadpole mass after a given amount of time and calculated growth rates of carbon as follows:

$$g_C = \frac{(final\ mass - innitial\ mass)}{innitial\ mass \times days} \times Q_C \qquad \text{(B.5)}$$

The initial masses were estimated for each species by randomly selecting 10 tadpoles from the reservoir immediately prior to the initiation of the experiment and weighing them. g_C was calculated for wood frogs and toads in this study, and for wood frogs exposed to 10 different species of leaf litter from Stephens et al. (2013).

Q_C and $Q_N$values for both species were originally reported as mg element · mg dry mass^{-1} (i.e., percentage element by dry mass). We were unfortunately unable to obtain C and N values from wood frogs and toads in this study due to the samples going bad through storage. Thus we derived parameter values from the literature. The mean C and N values for wood frogs were obtained from Stephens et al. (2013) which reported these values of metamorphic wood frogs.

The mean C and N values of four tropical stream dwelling *Bufonid* tadpole species reported by Vanni et al. (2002) were used for our American toad model. The C and N values were then converted to mmol element · mg dry mass^{-1}. Additionally, while it is known that some animals can change in stoichiometry through ontogeny [e.g. daphnia, fish; Pilati and Vanni (2007), Sterner and Elser (2002)], the degree to which tadpoles change stoichiometry through development is not known at this point. Thus, we held the assumption of the ES model that the consumer stoichiometry is homeostatic.

I for both species were taken from Richardson (2002). The values were originally reported in dry mass as mg food · mg tadpole^{-1} · hour^{-1} and converted to mg food · mg tadpole^{-1} · day^{-1} for use in our model. $L_{N(\min)}$ values for both species originated from Munro (1953). *Bufo Bufo* and *Rana temporaria* tadpoles (at ¾ hind leg development; used for American toads and wood frogs respectively) were fasted for 3 days and were excreted which should yield N excretion rates close to their absolute minimum. These tadpoles were the youngest developmentally used in Munro (1953) and, thus, were closest to the tadpole stages when we assayed growth in this study. The values used were originally reported as mg N · g tadpole (wet mass)$^{-1}$ · day^{-1}, which was then converted to mmol N · mg tadpole dry mass^{-1} · day^{-1}. A wet mass-to-dry mass conversion factor of 0.1 was used. While S_N can vary, we used the assumption by Sterner and Elser (2002) that S_N should be very close to 1 when N is limiting.

A_C and A_N of the leaf-litter mixture in this study and of the 10 different leaf litter species in Stephens et al. (2013) were directly measured. The values were initially calculated as mg element · mg dry mass^{-1} (i.e., percentage element by dry mass), and then converted to mmol element · mg dry mass^{-1}. Periphyton A_C and A_N for American toads were estimated using our meta-analysis as described below.

Periphyton C:N Meta-analysis

To gain an estimate of periphyton C and N values in the mesocosms, we conducted a meta-analysis of the literature reporting on periphyton C:N values in freshwater ecosystems. We used the Web of Science database and conducted a search as follows. All studies used had to report on both the concentration of C or N and C:N in the periphyton from freshwater sources. If a C:N value was given without an accompanying C or N value the study was discarded. In terms of search criteria selected, studies must have included "biofilm or periphyton or epilithon or aufwuchs and stoichiom* and nitrogen" as a topic. We then refined the number of studies by selecting only Marine and Freshwater Biology or Ecology or Limnology journals. This search yielded 74 studies that we then screened to isolate C and N values. Values were harvested from the sources using the program Data Thief (Tummers 2006). Of these we were able to use 7 studies producing a total of 68 periphyton C and N values (Appendix C: Table C6) with an average C of 22.0% (by dry mass) and an average N of 1.9% (by dry mass) yielding an average C:N of 13.9. These values were all converted into mmol element $\cdot$ mg^{-1} dry mass.

Statistical analysis

All statistical analysis was conducted in Program R (R Core Team 2012). We used the function "nls" contained within the base package in Program R to assess the fit of the wood frog model to the observed data. We then optimized the model allowing I and $L_{N(\min)}$ to vary and compared the AIC's of the original and optimized model, and conducted a sensitivity analysis to determine the degree to which the model changed when these values were optimized (Appendix C: Table C5). Our sensitivity analysis was conducted using an A_N and A_C of 1.26×10^{-3} and 3.86×10^{-2} respectively yielding a C:N of 30.6. We then independently increased or decreased I, $L_{N(\min)}$, or A_N by 20% and the resulting growth rate was then compared to the original (see

Appendix C, Tables C2–C3). We also included A_N in this analysis (holding all other parameters constant) because of the potential change in %N as a consequence of litter leaching and conditioning.

We used general linear models using the "lm" function in the base package of Program R to explain variation in growth, development and survival based on the main and interactive effects of %N, amphibian species, and light level. The effect of block was included in all analyses. Additionally, it appeared that litter quality was nonlinearly related to the three wood frog fitness traits so we also tested whether a polynomial term for litter quality significantly improved each model. Survival data was arcsine-square-root transformed prior to analysis.

We used path analysis [R package lavaan (Rosseel 2012)] to test which biotic and abiotic mechanisms most likely caused observed alterations in amphibian performance in response to litter %N. A separate path analysis (identical in structure) was conducted for each amphibian species at each of the two light levels. The potential paths included (1) the direct effect of leaf litter as a food source for tadpoles, and (2) the indirect effects of litter quality on food availability, in the form of bacteria and algae that might be influenced by dissolved nutrients and (potentially antimicrobial) polyphenolics leached from the leaf litter. It is possible that higher litter N might have directly caused an increase in the biomass of algae growing on litter. However, our periphyton measurements were based on algae growing on tiles propped against the side of the pool. Thus any litter nutrient effects on our periphyton and biofilm estimates should have been via soluble nutrients and carbon leaching out of leaves We also tested for direct effect of (possibly toxic) polyphenolics on tadpole growth. These mechanisms are all known to alter larval amphibian growth and development (Earl et al. 2012, Stephens et al. 2013, Stoler and Relyea 2013). We assessed relative differences in the standardized path coefficients as a

qualitative way to assess the strength of each relationship. We had fewer replicates for high light wood frogs ($N = 18$) and low light American toads ($N = 18$), because of four unexplainable die-offs in the mesocosms noticed early in the experiment.

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