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Appendix C: from S. R. Hall et al., "Inedible Producers in Food Webs: Controls on Stoichiometric Food Quality and Composition of Grazers" (Am. Nat., vol. 167, no. 5, p. 000)

Mesocosm Experiment: Details of Empirical and Statistical Methods

In this appendix, we more thoroughly describe the empirical and statistical methods used in the pond mesocosm experiment and provide associated references cited only here.

Experimental Methods

We used a fully factorial experimental design to examine the simultaneous effects of light availability, absolute nutrient (nitrogen and phosphorus) availability, relative N : P availability, and trophic structure on zooplankton community composition and the abundance and stoichiometry of algal producers. During May–June 2000, we created gradients of light and nutrient supply and predation risk in 300-L cattle tanks. To each tank, we added silica sand substrate, well water, and inorganic nitrogen (N) and phosphorus (P; $NaNO_3$ and NaH_2PO_4, respectively) to raise nutrient concentrations to target N : P ratios (nutrient ratio treatments: 5 : 1, 14 : 1, and 50 : 1) and supply (nutrient supply level treatments: high was 10 times the low value). Also, we reduced light availability in half of the tanks (light treatment) by 90% using neutral shade cloth. In other studies, we report results from other treatments of this experiment (one with predators and one without grazers; Hall et al. 2004, 2005), but these are not relevant here. We inoculated each mesocosm with diverse assemblages of algae and zooplankton at fortnightly intervals. These inoculae were collected along wide natural light–nutrient gradients from local ponds (proximate to Kellogg Biological Station and within Barry and Middleville State Game Areas, Kalamazoo and Barry Counties, MI, respectively). Each cattle tank also received 30 *Physa* spp. snails and 30 *Rana catesbeina* tadpoles to graze and remineralize nutrients bound in benthic algae. Finally, we added nutrients weekly to approximately maintain target levels (TN and TP levels of 700 and 14, 370.4 and 26.5, and 221.4 and 44.3 μg nutrient L^{-1} for 50 : 1, 14 : 1, and 5 : 1 nutrient ratio treatments, respectively; high-nutrient-supply-level treatments received 10 times these levels) by assuming a 5% day^{-1} loss rate from the water column (M. A. Leibold and V. H. Smith, unpublished data). These target levels of N and P correlated perfectly with each other on log scales ($R = -1$) and with N : P ratio ($R = -1$ for P, $R = 1$ for N). The factorial experimental design was

$$\text{replicates}_3(\text{nutrient ratio}_3 \times \text{nutrient supply level}_2 \times \text{light}_2).$$

We sampled this experiment at the end of summer to characterize final composition of grazer assemblages and algal stoichiometry. We collected 8.5-L samples using tube samplers and a fixed sampling regime during three sampling periods: September 15–17, September 29–October 1, and October 19–21, but data from these samples were averaged before analyses. During each period, zooplankton samples were sieved through 88-μm Nitex mesh and preserved in acid Lugol's solution. Zooplankton was identified and counted (Pennak 1978) to species for *Daphnia* and *Simocephalus* but to genus for all other taxa, and up to 25 individuals $species^{-1}$ $sample^{-1}$ were measured using a $\times 40$ dissecting microscope and converted into dry mass using published length-weight regressions (McCauley 1984). In addition, during the first sampling period only, we sieved water through 35-μm Nitex mesh (Wildlife Supply, Saginaw, MI) for subsequent C : P analysis of the edible algal fraction (Cottingham 1999). We filtered edible seston (which is mostly algae but also contains any suspended detritus and bacteria) on precombusted, acid-rinsed GF/F filters (Whatman, Florham Park, NJ). We then dried (60°C) one sample per mesocosm to measure C : N content (using a CHN autoanalyzer; Carlo-Ebra Instruments, Milan, Italy), and froze (−80°C) a second sample for particulate phosphorus measurements (APHA 1980; Prepas and Rigler 1982).

Finally, during each sampling period, we also measured biomass of "edible" (<35 μm) algae and "inedible" (>35 μm) algae using chlorophyll *a* as a proxy (by extracting samples on GF/F filters in chilled ethanol [Webb et al. 1992] and by using narrowband fluorometry [Welschmeyer 1994]).

Statistical Methods

We analyzed data from this experiment using three reasonably standard techniques, but each was sufficiently nuanced to deserve some attention here. First, we analyzed the response of inedible producers using univariate ANOVA where each factor was fixed. Here we determined significance of the *F* ratios after 9,999 randomizations (following [Anderson 2001*a*, 2001*b*] and using Matlab code we wrote [MathWorks 1999]). Second, we used a robust iteratively reweighted least squares (IRLS) procedure to estimate the strength of the partial linear regression (Neter et al. 1996, pp. 418–420; Hilborn and Mangel 1997) relating proportional abundance of *Daphnia* (arcsine–square root transformed) with the natural log of nutrient quota while controlling for log (N : P). This IRLS procedure dampens the influence of outlying observations by downweighting them. The magnitude of this downweighting depends on a user-defined weighting function (here the Huber function; Neter et al. 1996, pp. 419–420). Then, following Hilborn and Mangel (1997, pp. 161–162), we weighed each point in a modified simple sum of squares of errors (SSE), where smaller weighted SSE indicated better fit. This weighted SSE of the entire model was minimized by searching through parameter space using the downhill simplex (Matlab 5.3; MathWorks 1999). Then the statistical significance of the partial regression model with best-fitting, maximum likelihood–estimated parameters was calculated by randomizing the dependent variable 999 times and using the weighted SSE calculated from the partial model as the pivotal statistic. Finally, we bootstrapped 95% confidence intervals around each regression parameter by randomly sampling cases (1,000 times with replacement) and using Neter et al.'s (1996, p. 430) "reflection method" (which chooses the 97.5 and 2.5 percentiles). We repeated this IRLS procedure to estimate coefficients of a weighted partial regression model relating log (biomass of inedible algae) to log (phosphorus quota of edible algae) while controlling for both N : P ratio and light (categorized as -1 for shaded treatments and $+1$ for unshaded). Finally, we analyzed L-shaped relationships between *Daphnia* abundance and biomass of inedible producers, using tree regression (De'ath and Fabricius 2000). A regression tree algorithm (SYSTAT 8.0; SPSS 1998) recursively split inedible biomass into increasingly homogenous groups of *Daphnia* abundance. The final split point of the explanatory variable minimized within-group sum of squares.

Table C1
Results of nonparametric, univariate factorial ANOVA of log-transformed biomass of inedible and edible algae from the 2000 mesocosm experiment

		Inedible biomass (μg chl *a*/L)		Edible biomass (μg chl *a*/L)	
Source	df	*F* ratio	*P* value	*F* ratio	*P* value
Nutrient supply (*S*)	1	.02	.90	3.34[a]	.079[a]
N : P ratio (*R*)	2	1.31	.29	2.17	.14
Light (*L*)	1	2.69	.11	3.27[a]	.083[a]
$S \times R$	2	1.07	.36	.15	.86
$S \times L$	1	5.44[b]	.028[b]	.005	.94
$L \times R$	2	2.43	.14	2.48	.11
$S \times R \times L$	2	1.94	.17	.28	.76
Error	24				

Note: *P* values were based on 9,999 randomizations, following Anderson (2001*a*, 2001*b*); chl *a* = chlorophyll *a*. Mean square error of models = 0.43 and 0.070, and R^2 of the models = 47.4% and 41.2% for inedible and edible biomass, respectively.

[a] Marginally insignificant at $\alpha = 0.10$.

[b] Statistical significance at $\alpha = 0.05$.

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