Appendix D: Extended methods.

D1. Functional response experiments 1 and 2

Monoraphidium minutum was grown in batch cultures consisting of 2 L Erlenmeyer flasks with >80 µmol photons PAR s^{-1} m^{-2} (at a 16L:8D photoperiod) in WC medium (Stemberger 1981) at 20°C until a stationary phase was reached at approximately 50 mg algal C/L. All experiments were performed in Erlenmeyer flasks filled with 200 mL of algal food suspension. Algae were diluted to the desired food concentrations and transferred to experimental flasks immediately prior to the feeding trials. This resulted in 24 different algal concentrations ranging from 0.004 to 1.965 mg C per L in experiment 1, and 12 concentrations ranging from 0.023 to 1.847mg C per L in experiment 2. Each food concentration had three replicates in both experiments.

We cultured *Daphnia hyalina* in 30 L buckets with ADaM medium (Klüttgen et al. 1994) in a climate controlled chamber at 20°C and fed them daily with *M. minutum* at a concentration >1 mg C per L. For experiment 2 we acclimated *Daphnia* gradually (1.5°C per day) to the experimental temperature of 14°C and subsequently kept experimental animals at 14°C for at least another 3 days prior to the trials. In order to gradually habituate *Daphnia* to low food densities (which are most crucial to the accurate distinction between type II and III functional responses) we did not renew the food supply in the culture buckets on the day prior to a functional response experiment, which means that *Daphnia* were last fed 40 h before a trial. Animals were then filtered through a 250 µm mesh net, flushed with ADaM medium, transferred to experimental vessels (20 animals to each vessel), and were allowed to graze for 3 h in darkness to prevent algal photosynthesis. After

the experiments, *Daphnia* were removed from flasks with a net, and the remaining algal suspension was conserved with 0.5% Lugol solution. Initial (before grazing) and final (after grazing) algal densities were estimated from inverted microscope (Leica DMIL) counts in settling chambers (Utermöhl 1958). To convert algal counts to carbon concentrations we assessed the particulate organic carbon content of algal suspensions that had been filtered on pre-combusted and acid-washed GF/F glass fiber filters (Whatman) with a carbon analyzer (C-mat 500, Ströhlein). Moreover, 20 randomly selected *Daphnia* individuals from the pre-experimental culture were photographed under a microscope (Leica M32) and we measured their length from the upper eye border to the base of the tail spine using the software ImageJ. We then calculated the mass of a single *Daphnia hyalina* as

$$M = 5.59 \times L^{2.2}$$

where M is dry body mass of *Daphnia* (µg) and L is body length (mm) (Stibor & Lüning 1994). To calculate carbon mass of *Daphnia*, we assumed a fixed carbon to dry mass ratio of 45% (Hessen 1990).

The derived clearance rate was not corrected for algal growth since we lacked control flasks without *Daphnia* in these two experiments. However, the functional response experiments took place in the dark and in N- and P-free ADaM medium. Algae were also likely depleted of stored nutrients since they came from steady-state batch cultures. Finally, estimated growth increments of the same strain of *M. minutum* in experiment 4 (where algae came from steady-state continuous cultures and, thus, should have had higher growth rates than in the batch cultures used in experiments 1 and 2) never exceeded 1.2% per hour. Therefore, potential algal growth (cell division in the dark based on stored nutrients and photosynthate) can be expected to have been negligible.

D2. Functional response experiment 3

Cyclotella meneghiniana was grown in 2 L flow-through chambers (chemostats; dilution rate 0.15 per day) at 16°C in 3×L16 medium (Lindström 1991) at >80 µmol photons PAR s^{-1} m^{-2} (at a 16L:8D photoperiod), and mixed by continuous air bubbling and by a magnetic stirrer (15 min per h). Experimental dilutions of algae (9 different concentrations ranging from 0.006 to 0.885 mg C per L) were prepared from algae growing in stable-state chemostats (with approximate abundance of 26 mg algal C/L) and transferred to experimental vials (60 mL tissue flasks, Sarstedt). Each food concentration was replicated five times.

Daphnia hyalina was cultured in 1 L beakers at 16°C with 3×L16 medium and fed daily with *C. meneghiniana* at concentrations >1 mg C per L. For each trial 6 *Daphnia* individuals (kept in food-free medium 48 h prior to the experiment) were picked up manually using a glass pipette, flushed with N- and P-free 3×L16 medium, transferred to an experimental vial, and allowed to graze for 5 h in darkness. Additionally, each treatment had its *Daphnia*-free control to correct feeding rate calculations for algal growth. Flasks were mounted into a slowly rotating metal frame to keep algal cells in suspension. After the experiment, *Daphnia* were removed from flasks with a 250 µm mesh net and the remaining algal suspension was conserved with 0.5% Lugol solution. We estimated initial (before grazing) and final (after grazing) algal densities from inverted microscope (Nikon Eclipse TE 2000-U) counts in settling chambers (Utermöhl 1958). We assessed the carbon content of algal suspensions that had been filtered on pre-combusted and acid-washed GF/F glass fiber filters (Whatman) with a carbon analyzer (IL 500 TOC, Hach). We transferred

experimental animals from each replicate separately into a pre-weighted tin capsule, dried them for 12 h in 60°C, and weighed them.

The derived clearance rate was corrected for algal growth in the control flasks taken as an average over each treatment (global average 3.6% per h).

D3. Functional response experiment 4

This experiment was performed with *Daphnia magna* and *Monoraphidium minutum*. Culture conditions of *Daphnia* and algae were identical to experiment 3. Experimental dilutions of algae (9 concentrations from 0.001 to 2.898 mg C per L) were prepared from algae growing in stable-state chemostats (with approximate abundance of 30 mg algal C/L) and transferred to experimental vials (250 mL tissue flasks, Sarstedt). Each food concentration was replicated five times including one *Daphnia*-free control to correct feeding rate calculations for algal growth.

Prior to each trial, *Daphnia* were kept for 48 h at the same food concentrations as in the subsequent experiments with a single medium exchange after 24 h. At algal concentrations ≤ 0.1 mg C per L, for each trial 20 individuals of *D. magna* were picked up manually using a glass pipette, flushed with N- and P-free 3×L16 medium, transferred to an experimental vial, and allowed to graze in darkness for 1 h. The procedure was the same at algal concentrations > 0.1 mg C per L, except that we used 40 individuals and let them graze for 2 h. We gently inverted experimental flasks every 15 minutes to keep algal cells in suspension. Initial (before grazing) and final (after grazing) algal densities were estimated from particle counts in a flow cytometer (BD FACS Verse) using an excitation wavelength of 488 nm. Algal densities were converted to carbon biomass using an average

cell carbon content determined from stable-state chemostats as described for *C. meneghiniana* in experiment 3. We transferred experimental animals from each replicate separately into a pre-weighted tin capsule, dried them for 12 h in 60°C, and weighed them.

The derived clearance rate was corrected for algal growth in the control flasks taken as an average over all treatments.

D4. Pre-conditioning of *Daphnia*

There is a general concern in the literature about the impact of animal pre-conditioning, and in particular starvation, on the results of zooplankton grazing experiments (Chow-Fraser and Sprules 1992, Sarnelle and Wilson 2003). It has been suggested that long acclimation times to very low or zero algal concentrations before an experiment can result in decreased clearance rate due to exhaustion, which can bias the results towards a type III functional response (Muck and Lampert 1980). In contrast, other finding suggests that zooplankton filter feeders can increase their filtering speed almost instantly when exposed to higher food levels, even after a long starvation period (24-72h; Plath 1998). Moreover, compared to non-starved animals, starved *Daphnia* were shown to increase their ingestion rate when exposed to high food levels (i.e. levels not crucial to the accurate distinction between type II and type III responses), but this effect lasted only for several minutes (Lampert et al. 1988).

The above findings give little support for the notion that the exact pre-conditioning method, and in particular starvation, would strongly and consistently bias the results of functional response experiments. The three different methods that we used in our experiments reflect the variety of methods commonly reported in the literature. We believe

that the consistent observation of a type III response (regardless of the pre-conditioning method) supports the robustness of this finding.

D5. Estimation of algal phosphorus to carbon ratios

To measure the P:C ratios (= parameter Q_R in Table 1) of *Monoraphidium minutum* and *Cyclotella meghiniana* cells, we grew both species in 250 mL Erlenmeyer flasks in 3×L16 medium (Lindström 1991) with a phosphorus concentration of 0.93 mg/L, which gives a molar N:P ratio of 15. Light and temperature conditions were the same as in experiments 3 and 4 (>80 µmol photons PAR s^{-1} m^{-2}, 16L:8D photoperiod, 16°C). Flasks were mixed manually several times a day to prevent algal sedimentation. One-third of the total flask volume was exchanged daily. Algal density was monitored daily by measuring chlorophyll-a extinction at 680 nm in a V-560 spectrophotometer (Jasco). When cultures reached a steady state, which typically occurred at a biomass density of 15 mg C/L, flasks were sampled for cell counts. We conserved the samples in 0.5% Lugol solution and counted them under an inverted microscope (Nikon Eclipse TE 2000-U) in settling chambers (Utermöhl 1958). Another part of the flask volume was filtered on pre-combusted and acid-washed GF/F glass fiber filters (Whatman) and dried for 12 h in 60°C. From one set of filters we measured POC with a carbon analyzer (IL 500 TOC (Hach). From another set of filters we estimated the particulate phosphorus content using the molybdate-blue method after hot acid digestion with potassium persulphate (Menzel & Corwin 1965). From these data we calculated the C and P contents per cell and the P:C ratios of algal biomass.

D6. Estimation of algal growth parameters

We estimated algal growth parameters in additional experiments where *Monoraphidium minutum* and *Cyclotella meneghiniana* were grown separately in chemostats in a modified 3×L16 medium (Lindström 1991) with a phosphorus concentration reduced to 0.14 mg P/L, which gives molar N:P ratio of 100. Thus, all nutrients were provided in excess relative to phosphorus to ensure that no other element could become limiting.

The experimental setup was the same as for the algal cultures used in experiments 3 and 4. Each species was grown in three chemostats (volume 2 L, dilution rate of 0.15 per day) at 16°C and >80 µmol photons PAR s^{-1} m^{-2}) at a 16L:8D photoperiod and mixed by continuous air bubbling and by a magnetic stirrer (for 15 min per h). We monitored algal density every two days by harvesting 100 mL of the chemostat cultures, conserving them in 0.5% Lugol's solution, and counting them under an inverted microscope (Nikon Eclipse TE 2000-U) in settling chambers (Utermöhl 1958). Algal cultures were grown to steady state. Equilibrium algal carbon biomass R^* was then calculated as the product of algal cell density and average cell carbon content (determined as described for experiment 4), averaged over the last few sampling dates. Additionally, samples for dissolved phosphorus were taken every two days by filtering 10 mL of the chemostat cultures through a 0.2 µm syringe filter (Sarstedt). Thus acquired samples were measured for dissolved phosphorus using the molybdate-blue method (Menzel & Corwin 1965). Nutrient concentrations on the last few sampling dates were averaged to calculate equilibrium nutrient concentration N^*.

We calculated intrinsic algal growth rate (r) as

$$r = \frac{\ln R_{t2} - \ln R_{t1}}{t_2 - t_1} + D$$

where R_{t1} and R_{t2} are algal densities (cells/mL) at times t_1 and t_2, respectively, for the time interval $t_2 - t_1$ yielding the highest estimate of r (usually the first time interval except if algal growth showed an initial lag phase), and D is the dilution rate (1/d). To match functional response experiments that were performed at 14 and 20°C, we calculated algal growth rates for these temperatures based on the experimental estimates obtained at 16°C assuming a Q_{10} of 1.88 (Eppley 1972).

The half-saturation constant (N_H) for phosphorus limited algal growth was estimated by solving the equation describing nutrient dynamics at equilibrium

$$\frac{dN}{dt} = 0 = DN_{in} - DN - Q_R r \frac{NR}{N + N_H}$$

This yields

$$N_H = N^* \left(\frac{Q_A r R^*}{D(N_{in} - N^*)} - 1 \right)$$

where R^* and N^* are algal carbon biomass and nutrient concentration, respectively, at equilibrium, and other parameters are the same as in Table 1.

LITERATURE CITED

Chow-Fraser, P, and W. G. Sprules. 1992. Type-3 functional response in limnetic suspension-feeders, as demonstrated by *in situ* grazing rates. Hydrobiologia 232:175–191.

Eppley, R. W. 1972. Temperature and phytoplankton growth in the sea. Fishery Bulletin 70:1063–1085.

Hessen, D. O. 1990. Carbon, nitrogen and phosphorus status in *Daphnia* at varying food conditions. Journal of Plankton Research 12:1239–1249.

Klüttgen, B., U. Dülmer, M. Engels, and H. T. Ratte. 1994. ADaM, an artificial freshwater for the culture of zooplankton. Water Research 28:743–746.

Lampert, W., Schmitt, R.-D., and P. Muck. 1988. Vertical migration of freshwater zooplankton: test of some hypotheses predicting a metabolic advantage. Bulletin of Marine Science 43:620–640.

Lindström, K. 1991. Nutrient requirements of the dinoflagellate *Peridinium gatunense*. Journal of Phycology 27:207–219.

Menzel, D. W., and N. Corwin. 1965. The measurement of total phosphorus in seawater based on the liberation of organically bound fractions by persulfate oxidation. Limnology and Oceanography 10:280–282.

Muck, P., and W. Lampert. 1980. Feeding of freshwater filter-feeders at very low food concentrations: Poor evidence for "threshold feeding" and "optimal foraging" in *Daphnia longispina* and *Eudiaptomus gracilis*. Journal of Plankton Research 2:367–379.

Plath, K. 1998. Adaptive feeding behavior of *Daphnia magna* in response to short-term starvation. Limnology and Oceanography 43:593–599.

Sarnelle, O., and A. E. Wilson. 2008. Type III functional response in *Daphnia*. Ecology 89:1723–1732.

Stemberger, R. S. 1981. A general approach to the culture of planktonic rotifers. Canadian Journal of Fisheries and Aquatic Sciences 38:721–724.

Stibor, H., and J. Lüning. 1994. Predator-induced phenotypic variation in the pattern of growth and reproduction in *Daphnia hyalina* (Crustacea: Cladocera). Functional Ecology 8:97–101.

Utermöhl, H. 1958. Zur Vervollkommnung der quantitativen Phytoplankton – Methodik. Mitteilungen Internationale Vereinigung für theoretische und angewandte Limnologie 9:1–38.