Rich in Phosphorus, Poor in Quality: Assessing

Daphnia Spp. Responses to a Multi-Species P-Enriched Diet

by

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A Thesis Presented in Partial Fulfillment
of the Requirements for the Degree
Master of Science

Approved July 2015 by the
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ARIZONA STATE UNIVERSITY

December 2015

ABSTRACT

Phosphorus (P), an essential nutrient for growth of all organisms, is often in limited biological supply for herbivore consumers compared to other elements, such as carbon (C). Ecological stoichiometry studies have assessed responses of filter-feeding zooplankton from the genus *Daphnia* to single and multi-species food resources that are P-limited, finding decreased growth as a result to changes in metabolic processes and feeding behavior. Conversely, recent laboratory studies have shown that P-rich algal food resources also result in decreased growth rates for *Daphnia*, though the possible mechanisms behind this maladaptive response is understudied. Moreover, no published study tests the existence of the "stoichiometric knife edge" hypothesis for low C:P under field conditions. To address this lack of information, I measured growth rate as well as respiration and ingestion rates for *D. magna*, *D. pulicaria*, and *D. pulex* that were fed natural lake seston experimentally enriched with different levels of PO_4^{3-}. I found heterogeneous effects of high dietary P across *Daphnia* species. Growth rate responses for *D. magna* were strong and indicated a negative effect of high-P, most likely as a result to decreased ingestion rates that were observed. The seston treatments did not elicit significant growth rate responses for *D. pulex* and *D. pulicaria*, but significant responses to respiration rates were observed for all species. Consumer body stoichiometry, differences in seston C:P for each experiment, or differential assimilation by producer types may be driving these results. My study suggests that the stoichiometric knife edge documented in laboratory studies under low C:P conditions may not operate to the same degree when natural seston is the food source; diet diversity may be driving complex nuances for consumer performance that were previously overlooked.

ACKNOWLEDGMENTS

First and foremost, I owe many thanks to my advisor, Jim Elser, for his invaluable mentorship and guidance throughout my research. I also thank my supervisory committee for their feedback, thought-provoking questions, insights and resource provision, and timeliness and availability to discuss my research: Jim Elser (Chair), Jon Harrison, and Susanne Neuer. In addition to the academic guidance I received from my committee, I am also thankful to Jennifer Learned, whose help, oversight, and laboratory assistance was critical for the success of my project. I am also grateful to Ze Ren, Joe Rittenhouse, Cecilia Laspoumaderes, and Anna Sell for assistance in the lab in maintaining *Daphnia* cultures and other contributions throughout my experiments. Additionally, input and advice on experimental design and trouble-shooting insights from other Elser Lab members was especially valuable throughout the beginning stages of my research. Numerous other people provided time, support, and feedback that greatly helped me with field and laboratory-related logistics (Hannah Heavenrich and Amalia Handler) and other logistics related to my time in the School of Life Sciences (Wendi Simonson and Yvonne Delgado). This work was supported by the National Science Foundation (NSF grant DMS-0920744) and Arizona State University Graduate and Professional Student Association (GPSA).

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INTRODUCTION

Elemental imbalance is defined in the stoichiometric framework as a measure of the dissimilarity in the elemental composition between an organism and its resources (Sterner and Elser 2002). All organisms have a specified range of nutritional requirements for optimal growth and metabolism (Sterner and Hessen 1994). In stoichiometric terms, a diet is considered balanced when an organism's food elemental composition is near the organism's body elemental composition (Sterner and Hessen 1994; Sterner and Elser 2002). Organisms may be considered rich in certain elements depending on body composition. Phosphorus (P), an essential nutrient for all life, may be higher in biomass for certain organisms compared to others depending on abundance of certain biomolecules, such as ribosomal RNA, that require higher allocation of P for rapid growth (Elser et al. 2003). In freshwater ecosystems, the effects of C:P imbalances between consumers and their food have been of particular interest given the observation that such systems are often P-limited (Hecky and Kilham 1988; Elser et al. 2007) and that P-limited algae are poor quality for consumers (DeMott et al. 1998; Elser et al. 2000; Sterner and Schulz 1998; Sterner 2008). While the effects of low-P diets on consumer physiology and performance are well studied, the effects of stoichiometric imbalance due to high-P food are relatively unknown.

Zooplankton from the genus *Daphnia* have proven to be excellent model organisms for stoichiometry studies and are important prey for higher-level consumers of aquatic food webs (Hessen et al. 2013). *Daphnia* are P-rich animals, with P content (calculated as total % dry mass of the organism) ranging from 1.0-1.6%, depending on the species (Andersen and Hessen 1991; Sterner and Elser 2002; Kyle et al. 2006). Thus, *Daphnia*

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generally require more P in their food compared to the requirements of other zooplankton for optimal growth. However, because P is often limiting to phytoplankton production in freshwater ecosystems, resulting in seston biomass with low P content (high C:P ratio; Sterner 2008), *Daphnia* growth can often be limited by P (Urabe et al. 1997). The effect of this stoichiometric constraint on *Daphnia* has been tested under both laboratory and field conditions (Hessen et al. 2013).

Both lab and field studies have shown that food low in P (high C:P) impairs *Daphnia* growth rates compared to moderate C:P food (Sterner 1993; Sterner and Robinson 1994; DeMott et al. 1998; Elser et al. 2001; Urabe et al. 2003). Laboratory studies have consistently showed reduced growth when various species of *Daphnia* are fed *Scenedesmus* (or other unialgal cultures) with high C:P ratio (> 300; Sterner and Robinson 1994; DeMott et al. 1998; Urabe et al. 2003; Hessen et al. 2013). In natural ecosystems, however, *Daphnia* are generally polyphagous, and thus food resources likely contain numerous other producer species (Urabe and Waki 2009). As a result, more realistic assessments of *Daphnia* performance under nutrient limitation of natural lake seston were warranted. Elser et al. (2001) showed that when *Daphnia* fed on seston collected from three lakes that was enriched with P, growth was significantly stimulated. Thus, P appears to limit *Daphnia* growth in both laboratory and field settings.

Potential mechanisms behind poor growth under low-P food conditions, such as changes in feeding behavior or metabolic processes, have been described in previous studies on *Daphnia* (Sterner and Robinson 1994; DeMott et al. 1998; He and Wang 2008; Bukovinnszky 2012). Indeed, to cope with low-nutrient food, consumers often increase ingestion rates to meet their nutrient demand (Bukovinnszky 2012), a mechanism coined

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"compensatory feeding" (Suzuki-Ohno et al. 2012). *Daphnia* are relatively non-discriminatory feeders; while selective particle ingestion can be achieved to some degree (DeMott 1986; Butler et al. 1989; Schatz and McCauley 2007), *Daphnia* are more likely to alter rates of ingestion under in response to food conditions (Sterner and Hessen 1994). Increases in feeding activity have been recorded for *Daphnia* consuming low-P, high C:P food in several studies (DeMott et al. 1998; Suzuki-Ohno et al. 2012). Early studies recognized that increased ingestion of P-deficient food resources would mean ingestion of more energy (C) than required for maintenance and growth of the consumer (Sterner and Hessen 1994; DeMott et al. 1998). Consistent with this, more recent work has found that *Daphnia* increase both respiration and excretion rates, but not carbon assimilation, when fed high C:P food with excess C (Darchambeau et al. 2003; Suzuki-Ohno et al. 2012). Changes to consumer physiology and behavior, therefore, can be driven by dietary stoichiometric imbalance in both laboratory and field studies of P-limited *Daphnia* (DeMott et al. 1998; He and Wang 2008; Hessen et al. 2013).

The previous examples all deal with stoichiometric imbalance in food C:P ratios that are higher than optimal for animal growth. How organisms respond to stoichiometric imbalance in the other direction, when food C:P ratio is considerably lower than needed by the consumer, is relatively understudied and little is known about behavioral and physiological responses to P-rich food. However, as human activity shifts aquatic systems from P-limited (high algae C:P) to P-rich (low algae C:P) states, the effects of high food P content on consumer production are increasingly relevant. Previously, it was thought that consumption of excess dietary P did not come with a physiological cost for consumers (Sterner and Schulz 1998; Sterner and Elser 2002; Boersma and Elser 2006).

Rather, increased availability of P in food resources was thought to increase excretion rates of the excess nutrient (Olsen et al. 1986; Elser and Urabe 1999) but without any penalty. Additionally, previous studies dismissed the possibility of lake seston reaching such low C:P values; thus, stoichiometry studies under excess-P conditions were not considered relevant (Boersma and Elser 2006). However, laboratory findings now challenge these assumptions, revealing surprising and seemingly maladaptive responses in *Daphnia* to high levels of dietary P and pointing to the existence of a "stoichiometric knife edge" in consumer response to food C:P ratio (Boersma and Elser 2006).

High dietary P has been found to decrease growth rates of *Daphnia magna* (Plath and Boersma 2001), predatory copepods (Laspoumaderes et al. 2015), zebra mussels (Morehouse et al. 2013), and several other invertebrates (Dy Peñaflorida 1999; Tan et al. 2001; Frost and Elser 2002; Elser et al. 2006). However, the underlying biological mechanisms for the negative effect of high-P food are still largely unknown. Plath and Boersma (2001) showed that *D. magna* greatly reduced its feeding rates in response to high dietary P, resulting in poor growth as energy demands could no longer be met. They also suggested that respiration rates could increase as a response to increased excretion or detoxification of excess dietary P, thus negatively affecting growth rates (Plath and Boersma 2001). Indeed, recent laboratory findings have found that increased respiration rates may drive reductions in growth under P-excess conditions (Elser et al., unpublished data), supporting the hypothesis that high levels of dietary P can alter consumer physiological performance. However, no published studies have manipulated C:P ratios of multi-species phytoplankton assemblages from nature to assess how P-rich food might affect *Daphnia* under more realistic conditions. Yet, most filter feeding zooplankton are

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polyphagous (Urabe and Waki 2009; Hood and Sterner 2010), and many freshwater ecosystems today face eutrophic conditions where low C:P ratios are likely (Boersma and Elser 2006; Sterner 2008). Thus, research is needed to understand the biological consequences of high dietary P on consumer performance under ecologically realistic conditions using field-collected plankton assemblages as food items.

Using three species of *Daphnia* (*D. magna*, *D. pulicaria*, and *D. pulex*) fed natural phytoplankton communities freshly collected from an urban lake, I carried out several experiments to study 1) how dietary P-enrichment above optimal levels affects *Daphnia* growth, and 2) which underlying processes (ingestion rates and respiration rates) might be governing changes in growth rates. Furthermore, based on the hypotheses that high dietary P content affects *Daphnia* growth and that shifts in feeding and respiration rates underpin such changes in animal growth rates, I predict that, as the P content of the phytoplankton is increased, 1) *Daphnia* growth rates will decrease, 2) ingestion rates will decrease, and 3) respiration rates will increase, reflecting results found in previous studies using unialgal cultures in the laboratory. This study moves beyond previous stoichiometric imbalance studies by considering the potential effects on *Daphnia* performance of high P content in naturally occurring, multi-species food resources. Thus, my results provide a more complete picture of the effects of stoichiometric imbalances in freshwater ecosystems.

METHODS

Daphnia spp. Cultures

Cultures of three species of *Daphnia* were cultured separately at Arizona State University: *D. magna*, *D. pulex*, and *D. pulicaria*. Clonal populations of each species were maintained in zooplankton artificial growth media (COMBO recipe after Kilham et al. 1998) that was replaced every two weeks and fed the green alga, *Scenedesmus acutus*, grown under phosphorus sufficiency (MON; Sterner 1993). Prior to experimentation, separate cultures of each species were established and raised on a diet of lake seston collected from the study reservoir (see below).

Study Site Selection and Chemistry

To study the effects of high dietary P in natural phytoplankton communities, the P content of seston present in water collected from a local desert reservoir, Tempe Town Lake (TTL) was experimentally manipulated. Tempe Town Lake is an urban lake maintained in the Salt River channel adjacent to downtown Tempe, Arizona (USA). It is approximately 3.2 km long with an average depth of 4.0 m and a maximum of 5.8 m (AGFD 2015). Nutrient concentrations and the molar C:P ratio of seston were assessed for TTL throughout the duration of the study period. In addition, phytoplankton samples were taken, preserved, and later analyzed to assess the community composition.

Nutrient (C and P) concentrations of surface water and seston collected from Tempe Town Lake (coordinates: 33°35'55.1" N, 111°56'04.3" W) were monitored from April 25 to June 24, 2015. Ambient seston C:P was measured throughout the duration of each experiment; seston was captured on a Whatman GF/F filter, dried for a minimum of 24 h at 60°C, and digested using a modified persulfate oxidation digestion (for C

measurement) and ascorbic acid determination method (for P measurement; APHA 2005). This method yielded a mean trueness value (as % of certified value) of 98.02% ± 4.62% for P concentrations (NIST Apple Leaf); % recovery of C was not measured, but it is assumed that 100% oxidation of organic C to CO_2 occurred. Seston C concentrations in TTL ranged from 101 to 282 μmoles C / L with a mean of 170 ± 6.0 μmoles C / L (mean ± SE). Ambient seston P concentrations in the surface water ranged from 0.50 to 4.1 μmoles C / L over this time, and the mean was 1.4 ± 0.14 μmoles C / L (mean ± SE). Molar C:P ratios in TTL ranged from 81 to 567 and had a mean of 210 ± 18 (mean ± SE).

Seston Treatment Preparation

Water collected from TTL was screened through a 35 μm mesh to remove inedible particles and other zooplankton. Based on the observed seston C concentration and C:P ratio, the screened lake water was then "spiked" with phosphorus by adding a volume of 500 μM KH_2PO_4 stock solution sufficient to reach the following target molar C:P ratios (assuming that all added PO_4^{3-} was assimilated): ambient (no added P), 100, 75, and 50. All treatments were gently bubbled with air for a minimum of 8 h in a dark incubator at 24°C to allow P assimilation. Seston C:P after enrichment was measured daily for each treatment throughout the duration of each experiment using the methods described above. The purpose was to ensure that food concentration (C, mg/L) provided to the *Daphnia* in the various P enrichment treatments remained constant throughout the duration of the experiment while target P content was reached.

Growth Rate

Broods were isolated from gravid females raised on lake seston from which clonally uniform neonates were obtained for use in each experiment. Neonates were collected

between 24 and 48 h in age, imaged for initial size measurements using ImagePro

software, and then placed individually into 100 mL polyethylene vials with

approximately 50 mL of P-spiked Tempe Town Lake seston treatment. Animals were

kept on in a rotating plankton wheel to prevent food particles from settling. Additionally,

the wheel was kept inside a growth chamber at 22°C with a dim light / dark regime of 12

/ 12 h. Treatments were refreshed daily: individual *Daphnia* were pipetted from their

vials, the TTL treatment suspension was replaced with freshly prepared lake seston of the

corresponding C:P ratio, and animals were placed back into the fresh treatment. The total

experimental period ran for five days, when animals were individually re-imaged.

Approximately 10-14 replicates (with 1 individual per replicate) per treatment were used

for measuring specific growth rates. Growth rate was obtained from the pre- and post-

experimental images. The specific growth rate for each animal was calculated as:

$$\mu = \frac{\ln\left(\frac{s_1}{s_2}\right)}{t_2 - t_1}$$

where μ = growth rate, s is individual surface area at beginning (s_1) and end (s_2) of the

treatment period, and t = time in days.

Respiration Rate

Following the growth cycle and post-imaging of *Daphnia* described above,

respiration rates were measured as oxygen consumption by individual animals using a

Unisense MicOx Microrespiration System and microelectrode. For each treatment, the

baseline seston respiration rate for each food C:P treatment was determined for a sample

containing mixed aliquots of food suspensions from each individual vial. Individual

Daphnia were pipetted into 800-µL vials with a miniature stir bar to continually mix the

suspension. Vials were stoppered, rendering them air-tight. All baseline seston and

individual *Daphnia* were allowed to equilibrate for 5 minutes before O_2 readings were

continuously recorded for 5 min. The entire procedure took place in the dark to eliminate

effects of photosynthetic activity. Respiration rates were calculated as the rate of change

in O_2 concentration, corrected by subtracting the baseline rate (determined in prey vials

only) from the rate observed for each *Daphnia* vial, and then normalized by mass of each

individual. Mass was calculated for each individual using species-specific surface area to

mass regressions measured previously in the Elser Lab (unpublished data).

Ingestion Rate

Animals were tested for feeding rate on day 5 of the treatment regime. ^{14}C labeling

of algal cells was used to measure ingestion rates (DeMott 1998; He and Wang 2006;

DeMott et al. 2010). The radioactive food source was prepared by first concentrating a

small volume of green algae, *Scenedesmus acutus*, by centrifuging and then re-

suspending the cells in media depleted of sodium bicarbonate ($NaHCO_3$) to enhance

uptake of ^{14}C by the algal cells. The solution was then spiked with 0.2 mCi of ^{14}C-

$NaHCO_3$ and allowed to incubate in the dark on a shaker table for a minimum of 8 h.

The radioactively-labeled *S. acutus* was then concentrated onto a filter, re-suspended in

COMBO growth media with $NaHCO_3$, and mixed with each TTL seston C:P treatment.

To estimate the amount of algal C contribution to the total radioactive mixture, this

process was repeated without the radioactive step; instead of mixing with each TTL

seston treatment, the algae was captured on Whatman GF/F filters and analyzed for C and

P using the methods described above. Based on this method, algal C contributed

approximately 20% of the total C concentration. This labeled food addition is relatively

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high for a tracer method; however, based on preliminary experiments, it was necessary to acquire a detectable radioactive signal in the animals. Ingestion rates obtained are likely meaningful because animal feeding behavior likely did not change in the short amount of time (6 minutes) given to feed on the labeled mixture. Furthermore, the C:P ratio of the mixture nearly matched that of their treatment seston food, and thus no major changes to the overall treatment C:P likely occurred.

Animals fed on the labeled mixture for 6 min. This feeding time ensured adequate ingestion of food before assimilation or egestion (DeMott et al. 1998; He and Wang 2006). Four individuals were used for each replicate to ensure a detectable radioactive signal, with 4 replicates per treatment. At the end of the feeding period, animals were rinsed with COMBO media, anesthetized with carbonated water, and immediately sorted into scintillation vials. Animals were dissolved in 0.5 mL of tissue solubilizer (Soluene 350) for 8 h at 60°C and counted with 5 mL of scintillation fluid (Solvable Optima Gold) using a Beckman LS 6500 liquid scintillation counter. For each trial, the radioactivity of 1 mL of the food suspension was also counted with 5 mL of scintillation fluid. Radioactivity measured in experimental animals was corrected by subtracting levels measured in non-treatment (control) *Daphnia* that did not ingest any radioactive material. Filtering rates were then determined based on the measured ^{14}C activity in the animals and in the food suspensions following Haney (1973). Finally, filtering rates were calculated as ingestion rates using the prey concentration in the treatments and normalized by the total mass of the animals in each vial (mg C mg dry weight^{-1} d^{-1}). Specific growth rates were also calculated for the *Daphnia* used for ingestion rate experiments by taking pre- and post-treatment photos of all individuals.

Statistical Analyses

One-way analysis of variance (ANOVA; Zar 2010) with post-hoc Tukey HSD analyses were used to test for effects of food P treatment (i.e. food C:P ratio) on the response variables a) growth rate, b) respiration rate, and c) ingestion rate, for each species of *Daphnia* used. All data were tested for normality and homogeneity of variance; all data met the assumptions of ANOVA. All statistics were performed in RStudio Version 0.98.1062.

RESULTS

Treatment Chemistry and Community Composition

I was successful in P enrichment of ambient TTL seston and obtained three distinct treatment C:P ratios for each experiment (Figure 1) while C concentrations (μmoles C / L) remained relatively constant (Figure 2). Treatments were categorized, in order of increasing P content (decreasing C:P ratio), as *a* (ambient), *b*, *c*, and *d*. For the *Daphnia magna* experiments, the treatments had mean (± SE) C:P values of *a)* 180 ± 12 (ambient), *b)* 110 ± 5.5 *c)* 71 ± 4.2, and *d)* 56 ± 1.3. Treatment mean (± SE) C:P during the *Daphnia pulex* experiments were: *a)* 190 ± 25 (ambient), *b)* 130 ± 11, *c)* 88 ± 5.1, and *d)* 67 ± 3.6. Lastly, treatment mean (± SE) C:P for the *Daphnia pulicaria* experiments were *a)* 300 ± 36 (ambient), *b)* 120 ± 5.7, *c)* 94 ± 5.9, and *d)* 71 ± 4.5. Differences in treatments were statistically significant (ANOVA, p < 0.05) for all experiments. The phytoplankton community composition of Tempe Town Lake was also assessed; lake phytoplankton used in the experiments was dominated by cyanobacteria and haptophytes, primarily those from the genus *Prymnesium*.

Growth Rates

All three species of *Daphnia* used for this study tended to have lower growth rates when the C:P of their seston diets was low (P-rich), but the difference in growth rates between treatments varied by species. All outcomes of statistical analyses are reported in Tables 1 (ANOVA results) and 2 (Tukey HSD results).

For *Daphnia magna*, specific growth rates declined as P increased in their food (Figure 3A, $p < 0.05$). *Post hoc* tests showed that *D. magna* that fed on ambient TTL seston (treatment *a*, C:P of ~185) and on seston with a mean C:P of ~108 (treatment *b*) had significantly higher growth rates (~0.36 and ~0.31 d^{-1}, respectively) than those that fed on the more P-rich treatments, *c* and *d* (~0.27 and ~0.25 d^{-1} respectively).

Specific growth rates for *Daphnia pulex* were somewhat lower than for the other two species and did not differ significantly among the P enrichment treatments (Figure 3B, $p = 0.48$), although rates tended to decline with higher dietary P, with the highest value (~0.20 d^{-1}) observed for *Daphnia* that fed on ambient seston and a decline to a value of ~0.15 d^{-1} for the highest-P treatment.

Growth rates in response to the TTL seston treatments for *Daphnia pulicaria* were qualitatively similar to those observed for the other species (Figure 3C). While specific growth rates for *D. pulicaria* that fed on the ambient seston (~0.35 d^{-1}) tended to be greater than rates for those that fed on the P-enriched seston (~0.30 d^{-1} for treatments *b*, *c*, and *d*), this pattern was not statistically significant ($p = 0.88$).

Respiration Rates

Responses in respiration rates were statistically significant for all species, but differed by treatment. Respiration rates ranged from ~170 to ~3.2 x 10^4 nmol O_2 mg^{-1} h^{-1}.

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D. pulicaria had the highest rates overall whereas *D. pulex* had the lowest, consistent

with its overall low growth rates. The statistical outcomes for respiration rates are

included in Tables 1 and 2.

   D. magna respiration exhibited a 'hump-shaped' pattern with highest respiration rates

occurring in the mid-P treatments (treatments *b* and *c*) and lowest respiration rates

observed for the ambient and highest-P treatment (Figure 4A; $p < 0.05$). *Post hoc* tests

showed that respiration rate in treatment *c* (C:P of ~ 71) was significantly higher than in

the highest-P treatment and in the ambient seston (C:P of ~56 and ~180, respectively).

   For *D. pulex*, respiration rates did not follow the same pattern observed for the other

two species, although results were statistically significant (Figure 4B; $p < 0.05$).

Individuals in the two higher C:P treatments (C:P of ~190 and ~130) had significantly

higher respiration rates compared to those in the two lower C:P treatments (~88 and ~67),

indicating that respiration rates decreased with increasing dietary P.

   Respiration rates for *D. pulicaria* also showed a significant response to treatment

(Figure 4C; $p < 0.05$). Like responses seen for *D. magna*, responses for *D. pulicaria*

exhibited an a strong hump-shaped pattern, with highest respiration rates occurring in the

mid-P treatments *b* and *c* (C:P of ~120 and 94, respectively). *Post hoc* tests showed that

significantly greater respiration rates for treatment *b* compared to the other treatments and

for treatment *c* compared to *a* were driving these differences.

Ingestion Rates

   The three species differed in their feeding responses to manipulation of seston C:P

ratio. Overall ingestion rates, across all species, ranged from ~0.14 to ~22 mg C mg^{-1} d^{-1}.

In general, all observed ingestion rates were higher than those recorded in laboratory

studies using unialgal diets of *Scenedesmus acutus* (Elser et al., unpublished data). Statistical results are reported in Tables 1 and 2.

Ingestion rates declined significantly for *D. magna* with increasing dietary P (Figure 5A; $p < 0.05$). *Post hoc* tests revealed that a difference between the ambient seston treatment and the other three P treatments drove this pattern. Individuals that fed on ambient TTL seston had a mean ingestion rate of ~22 mg C mg^{-1} d^{-1} but this declined strongly to ~8 and ~2 mg C mg^{-1} d^{-1} for treatment *b* and treatments *c* and *d*, respectively.

The effect of increased P in TTL seston on *Daphnia* feeding behavior was less apparent for the other two species. For *D. pulex*, a general U-shaped response of ingestion rates with seston C:P was observed (Figure 5B). Feeding rates for ambient seston were generally highest compared to the P-enriched treatments with a mean of ~3 mg C mg^{-1} d^{-1}; mid-P treatments (*b* and *c*) were generally lowest with a mean of ~2 mg C mg^{-1} d^{-1}. However, this difference was not statistically significant.

For *D. pulicaria*, ingestion rates also did not differ as a function of seston C:P ratio (Figure 5C). Contrary to observations for the other two species, feeding appeared to follow a hump-shaped pattern with lowest rates occurring for individuals that fed on ambient TTL seston and the highest-P treatment (~11 mg C mg^{-1} d^{-1} for both ambient and treatment *d*) and highest rates occurring for those that fed on the mid-P treatments (14 mg C mg^{-1} d^{-1} and 12 mg C mg^{-1} d^{-1}, for treatments *b* and *c* respectively), those these observations were not statistically significant.

DISCUSSION

My results support the existence of a "stoichiometric knife edge" for one species, *D. magna*, that showed decreased growth rate on a low C:P, multi-specific diet. For *D. magna*, these results show that even field-collected seston enriched with P can impose negative effects on consumer physiology and performance. For other species of *Daphnia*, however, these results show that effects of food P content in multi-species phytoplankton assemblages may not necessarily reflect observations found in mono-specific laboratory studies, thus highlighting the complex influence of diet composition on herbivores at low C:P ratios.

The impact of high C:P (P deficient) diets on herbivore performance is well known for both laboratory (Sterner and Robinson 1994; DeMott 2003; Hessen et al. 2013) and field (Elser et al. 2001) settings. However, only one published study has addressed low C:P (excess P) diets of *Daphnia* in the laboratory (Plath and Boersma 2001) and no published studies provide empirical results for *Daphnia* under field conditions. My results mirror the results for *D. magna* from the unialgal study conducted by Plath and Boersma (2001), as *D. magna* growth rate declined rates when given a multi-specific phytoplankton diet with low C:P ratios. The likely mechanism behind the significant decline in *D. magna* growth rates was a change in feeding behavior: *D. magna* lowered their ingestion rates with an increase in dietary P, demonstrating a reverse compensatory feeding behavior also reported by Plath and Boersma (2001). In this interpretation, as somatic P requirements for growth were met in the high-P treatments, individuals lowered their filtering rates and, therefore, their ingestion rates. The negative

consequence of this behavior is insufficient intake of C needed for metabolic processes that support growth.

Changes in respiration rates were observed for all species, but did not necessarily appear to be governing changes in growth rate. Since growth remained constant for *D. pulex* and *D. pulicaria*, changes in respiration rates may reflect metabolic consequences of eliminating excess P (c.f., Boersma and Elser 2006). Thus, for *D. magna* at least, this study further challenges the assumption that P-rich diets carry no physiological cost for zooplankton consumers by providing empirical support under more realistic dietary conditions with mixed phytoplankton communities. For *D. pulex* and *D. pulicaria*, however, the results are more nuanced and do not necessarily support the stoichiometric knife edge hypothesis under low C:P conditions, as decreasing C:P ratio brought about changes in respiration rates that were not clearly linked to growth rate. Therefore, mechanistic changes to *Daphnia* feeding behavior and physiology may depend on certain conditions, such as *Daphnia* species, the community composition of their diet, or the ambient seston C:P.

There are several possible explanations for the differences in responses observed between species. One explanation may relate to differences in *Daphnia* body C:P stoichiometry and thus each species' physiological requirements of energy (C) and P for optimal growth (Sterner and Elser 2002; Elser et al. 2003). It has previously been suggested that, all else being equal, higher-P animals may be more sensitive to changes in diet C:P when available P is low (Sterner and Hessen 1994; Sterner and Elser 2002). Conversely, when dietary C:P is reduced to very low levels, however, lower-P *Daphnia* species (*D. pulex* and *D. pulicaria*) might be expected to be less able to cope with excess

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P in their food (Elser et al. 2003; DeMott and Pape 2005). For example, they may have limited internal sinks (i.e., RNA) to accommodate extra P, and other physiological costs might be incurred as a result (Elser et al. 2003). This has been shown a limited number of studies that compare responses of low-P to high-P animals to elevated dietary P. For example, growth rates declined for *Bosmina* (a low-P animal) under high-P conditions whereas rates remained constant for high-P *Daphnia* (DeMott and Gulati 1999). Studies with low-P snails and high-P insects (Elser et al. 2000; Elser et al. 2005) also corroborate this hypothesis. In terms of % dry mass, *D. magna* are considered lower in P (~1.0%, Main et al. 1997, Kyle et al. 2006) compared to *D. pulex* (~1.4%, Andersen and Hessen 1991; ~1.3%, Kyle et al. 2006) and *D. pulicaria* (~1.5%, Elser et al. 2003, Kyle et al. 2006). Thus, this hypothesis may be supported by my data; *D. magna* showed significant responses to increased dietary P whereas *D. pulex* and *D. pulicaria* did not.

Another possible contributor to variation observed in species' responses could be differences in treatment C:P values for the various experiments. Ambient TTL seston C:P ranged from ~180 to ~300, depending on when Tempe Town Lake was sampled (notably, seston C concentrations remained relatively constant during this time whereas P concentrations varied). *D. pulicaria* fed on ambient TTL seston that was relatively low in P (C:P of ~300) while *D. pulex* and *D. magna* were given ambient TTL seston with a C:P of ~190 and ~180, respectively. Thus, treatments imposed on *D. magna* may have had the largest effect because the ambient TTL seston C:P for the *D. magna* experiment was the lowest for the three species. Furthermore, the highest-P treatments in the *D. magna* experiment achieved C:P ratios as low as ~56. However, for *D. pulex* and *D. pulicaria*, a lack of treatment effect may be due to the inability to reach such low C:P ratios as

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obtained for the *D. magna* experiment. Indeed, a C:P ratio of ~70 achieved in the *D. pulex* and *D. pulicaria* experiments may be considered "P-sufficient" (DeMott and Page 2005), and adverse effects of excess P on growth or other processes simply may not have been imposed. Ultimately, this identifies a limitation of the interspecific comparisons in this study in that I was unable to apply a uniform range of food C:P ratios for the three species. Nevertheless, the effects of low food C:P on *D. magna* growth and on respiration and feeding in other species suggests that further investigation is needed, both spatially and temporally, to better test the stoichiometric knife edge hypothesis for low C:P ratios in natural systems with multi-species food resources.

Existence of the stoichiometric knife edge at low C:P ratios is rapidly gaining support in laboratory experiments with simple food types for a variety of consumers (Plath and Boersma 2001; Frost and Elser 2002; Morehouse et al. 2013; Laspoumaderes et al. 2015). My data extend support for the stoichiometric knife edge in *Daphnia* by providing evidence for *D. magna* consuming diets of mixed phytoplankton. However, the relevance of these studies may be questioned, if the occurrence of such low food C:P ratios in lakes and reservoirs is rare. However, a global assessment of seston stoichiometry by Sterner et al. (2008) records that 47% and 52% of observations had C:P ratio values less than 180 in small and large lakes, respectively. Indeed, for Tempe Town Lake, ambient seston C:P ratios in the ~120-180 range were common and seston C:P declined ~300 to ~180 within one month during my observations. Moreover, this same study system underwent an extreme change in seston C:P from September to October 2014, following a large rain event (~10 cm of rain in 24 h; NOAA 2014). During the rain event, seston C:P ratios fell to ~51. Following the event, from late September through

October, C:P ratios remained constant with a mean of 75 ± 15 (Appendix A). Thus, these TTL data show that seston C:P ratios as low as ~75 can be observed under natural conditions, with potential effects on filter-feeding zooplankton.

Another important consideration for this type of study is the effect of food quality in mono-specific versus multi-specific resources on consumer physiology. Indeed, many studies in which herbivore consumers were fed mono-specific diets of poor quality demonstrate strong negative effects on growth and other physiological processes (Sterner and Hessen 1994; DeMott et a. 1998; Urabe et al. 2003). In nature, however, many herbivorous zooplanktons are polyphagous and, furthermore, the food items of different size fractions they eat may not be of uniform stoichiometric composition nor will all respond similarly to elevated nutrient concentrations in their environment (Urabe and Waki 2009). For example, faster growing organisms (i.e., bacteria) may assimilate elevated P more rapidly than other types of microplankton that make up the seston. Furthermore, differences in *Daphnia* species' filtering apparatuses may drive differential consumption of certain size fractions. For example, Geller and Müller (1981) measured the distances between the setulae of 11 Cladoceran species, finding distinct groups of species according to the 'mesh sizes' of their filtering anatomy. With respect to the three species I considered, *D. magna* are considered "fine mesh filter-feeders" whereas *D. pulex* and *D. pulicaria* are considered "medium mesh filter-feeders" (Geller and Müller 1981). The short distance between the setulae of *D. magna* allows for these individuals to feed efficiently on bacterial food, which may be richer in P.

In addition to potential differential consumption by different *Daphnia* species, differential digestion and assimilation may also occur to offset physiological trade-offs of

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consuming particular food items of poor quality (Hood and Sterner 2010). Studies on a variety of taxa have shown that herbivore regulatory behaviors for consumption of certain biomolecules, such as proteins and carbohydrates, correspond with individual performance (Logan et al. 2004; Mayntz et al. 2005; Raubenheimer and Simpson 2004). In stoichiometric terms, the same could be operating for filter-feeding zooplankton and individual elements in multi-species diets (DeMott 1998; Urabe and Waki 2009). This was true for one study in which *D. pulicaria* were given diets involving both unialgal and multialgal cultures raised at elevated CO_2 (Urabe and Waki 2009). When the animals were fed a single algal species, growth rates declined due to poor nutritional quality of the high CO_2-raised food. However, animals fed on the mixed culture food maintained high growth rates despite imbalanced P and N relative to C in the overall diet. Thus, Urabe and Waki (2009) concluded that higher diversity in herbivore diets can mitigate the effects of CO_2-induced stoichiometric imbalance on consumer performance. Similar mechanisms could also hold true for increased P in *Daphnia* diets. Adverse effects of low C:P ratios have been consistently observed for mono-specific culture studies but my study, the first of its kind to test effects of low C:P in multispecies assemblages, found weak effects on performance of two species of *Daphnia*. Thus, bulk measurements of seston C:P may not be informative for this type of study where differential allocation of nutrients can occur. The possible alleviatory effect of diet diversity needs investigation for the P-rich portion of the stoichiometric knife edge curve.

TABLES

Table 1. Statistical results for Analysis of Variance (ANOVA) for each response

variable and species. Results are given for responses of specific growth rate,

respiration rate, and ingestion rate to C:P ratio of TTL seston for *Daphnia magna*,

*Daphnia pulex*, *Daphnia pulicaria*. Significant values (p < 0.05) are bolded.

Species	Response Variable	F Test Statistic	DF	P-Value
D. magna	growth rate	9.788	3	**0.0002**
	respiration rate	3.758	3	**0.0173**
	ingestion rate	12.05	3	**0.0006**
D. pulex	growth rate	0.843	3	0.4790
	respiration rate	2.859	3	**0.0461**
	ingestion rate	679	3	0.5810
D. pulicaria	growth rate	1.47	3	0.2420
	respiration rate	30.54	3	**< 0.0001**
	ingestion rate	1.195	3	0.3530

Table 2. Statistical results for Tukey HSD Post Hoc tests. Results are given for difference between treatment groups for specific growth rate, respiration rate, and ingestion rate to C:P ratio of TTL seston for *Daphnia magna*, *Daphnia pulex*, *Daphnia pulicaria*. Significant values ($p < 0.05$) are bolded.

Repsonse Variable	Group Comparison	P- Value *D. magna*	*D. pulex*	*D. pulicaria*
growth rate	a - b	0.2099	0.7713	0.2986
	a - c	**0.0080**	0.9228	0.2618
	a - d	**0.0001**	0.4121	0.6246
	b - c	0.4741	0.9913	0.9919
	b - d	0.0845	0.9383	0.9491
	c - d	0.8490	0.8335	0.8752
respiration rate	a - b	0.3378	0.9614	**< 0.0001**
	a - c	**0.0229**	0.0656	**0.0213**
	a - d	0.9881	0.1717	0.1876
	b - c	0.5734	0.2626	**< 0.0001**
	b - d	0.5232	0.4928	**< 0.0001**
	c - d	**0.0508**	0.9627	0.7285
ingestion rate	a - b	**0.0153**	0.5912	0.3332
	a - c	**0.001**	0.7167	0.8357
	a - d	**0.0012**	0.9669	0.9880
	b - c	0.4157	0.9962	0.7887
	b - d	0.4583	0.8422	0.4956
	c - d	0.9998	0.9279	0.9536

FIGURES

Figure 1. Seston C:P ratios of Tempe Town Lake (TTL) P enrichment experiments for
each of the three *Daphnia* species in terms of (atomic). Means (± SE) are reported
for each treatment type, in order of increasing P: *a* (ambient), *b*, *c*, and *d*. Statistically
significant differences ($p < 0.05$) between treatment means for each experiment have
different letters above each bar.
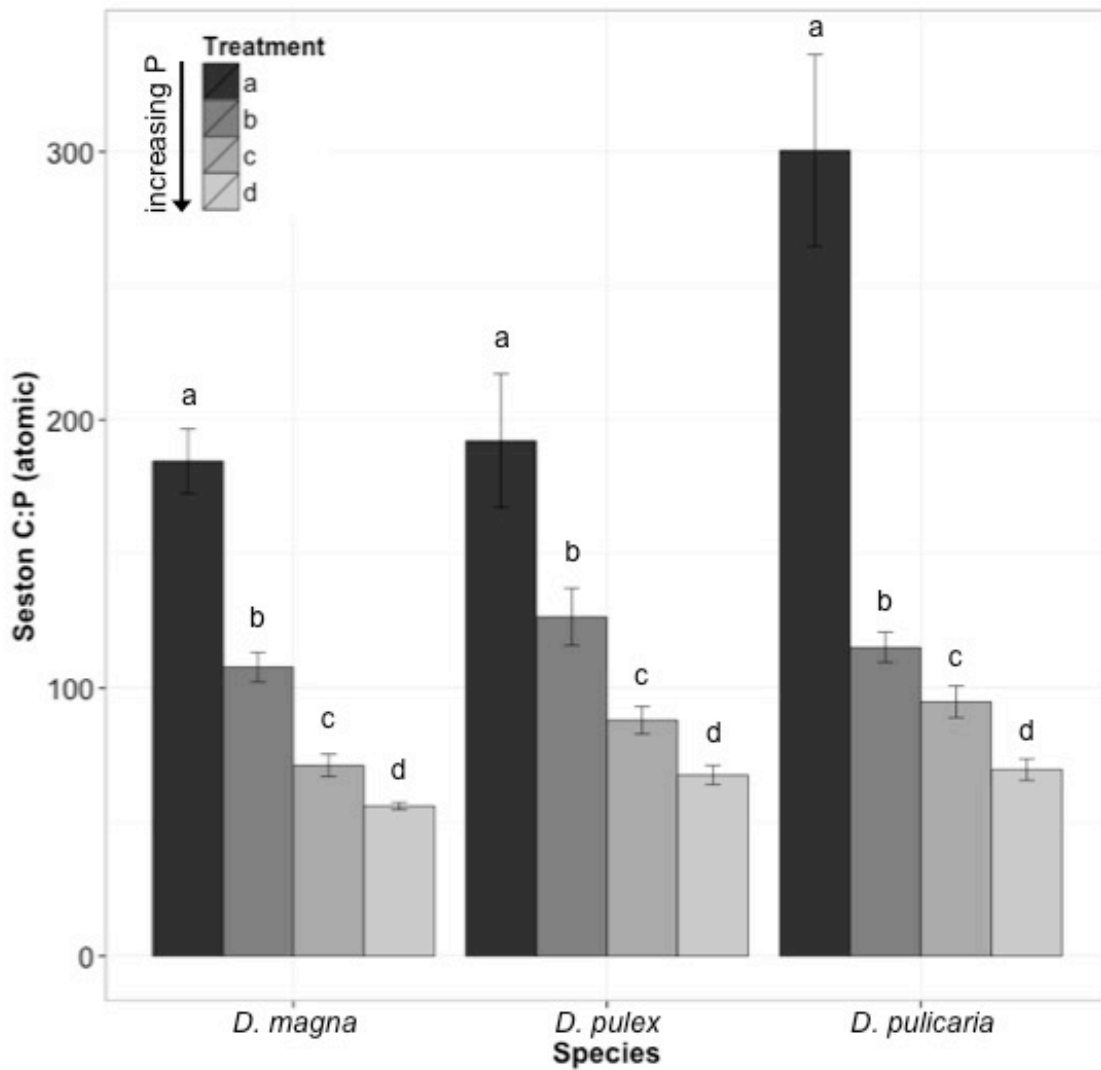
**Figure 2.** Mean C concentrations (μmoles / L) of Tempe Town Lake (TTL) enrichment experiments for each of the three *Daphnia* species. Means (± SE) are reported for each treatment type, in order of increasing P: *a* (ambient), *b*, *c*, and *d* as in Fig. 1. Statistically significant differences (p < 0.05) between treatment means for each experiment have different letters above each bar.
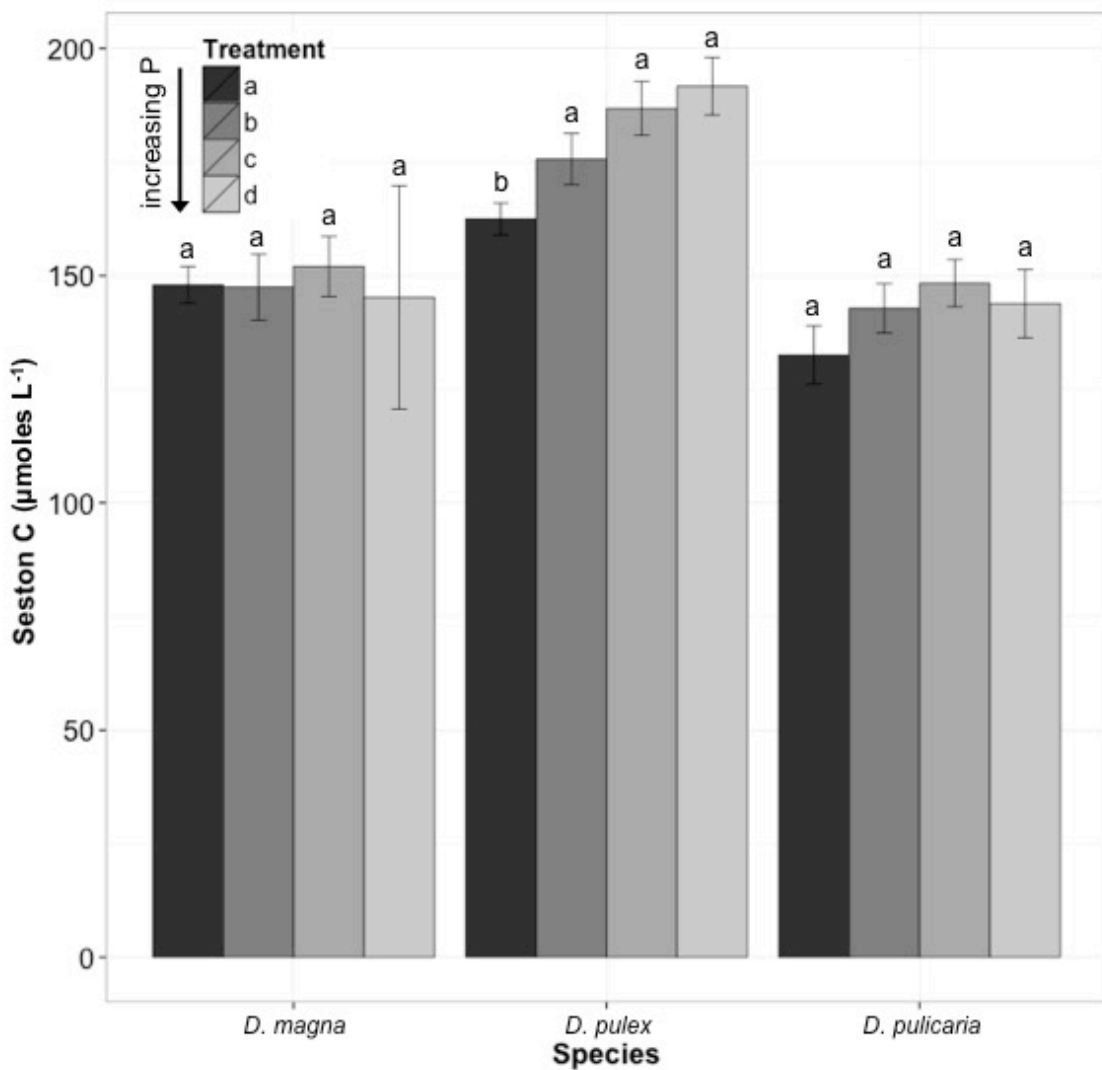
Figure 3. Mean (± SE) specific growth rates of *Daphnia magna* (A), *Daphnia pulex* (B),

Daphnia pulicaria (C) to variations in seston C:P ratios. Summaries of the statistical

analyses of these data are shown in Tables 1 and 2. Statistically significant

differences ($p < 0.05$) between treatment means for each experiment have different

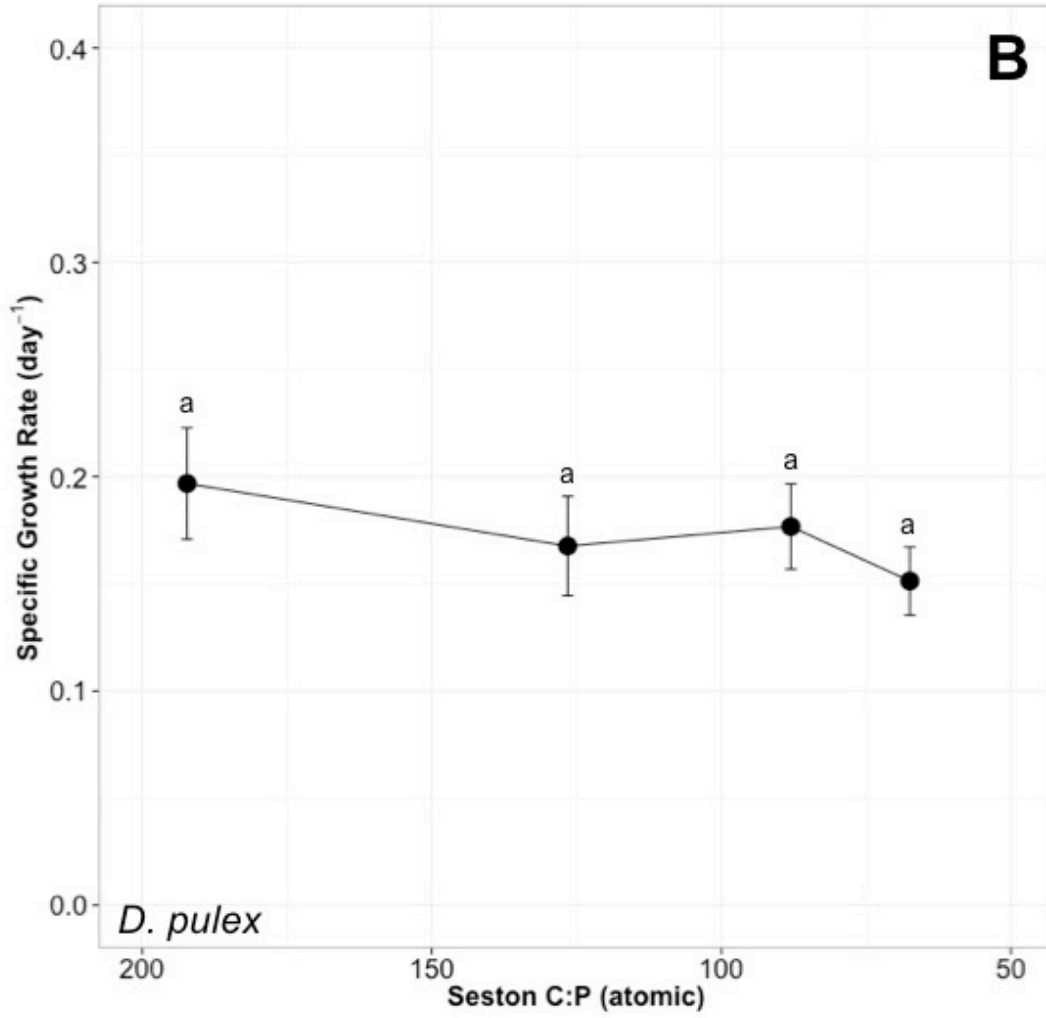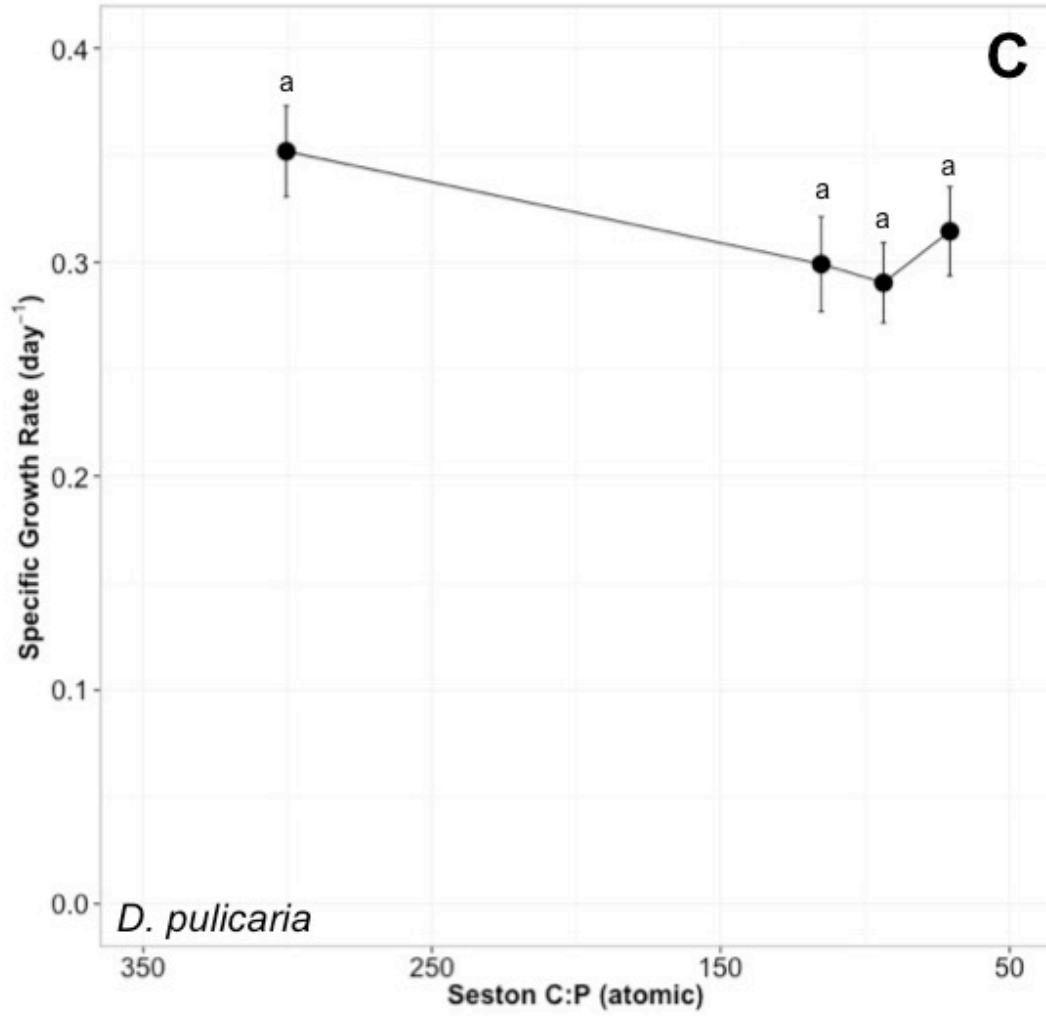letters above each bar.
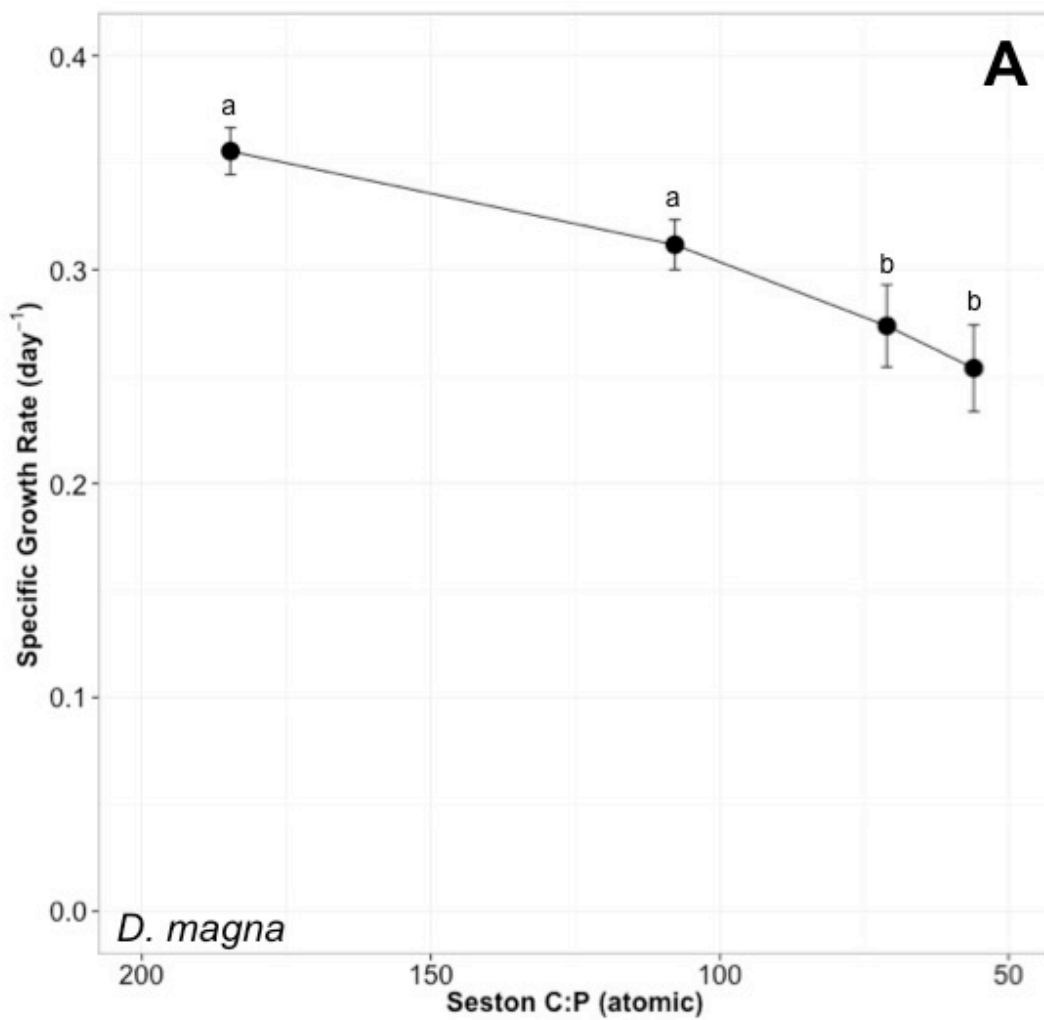
Figure 3 continued.

Figure 3 continued.

Figure 4. Responses of specific respiration rate to C:P ratio of TTL seston for *Daphnia magna* (A), *Daphnia pulex* (B), *Daphnia pulicaria* (C). Results reported as means (± SE). Summaries of the statistical analyses of these data are shown in Tables 1 and 2. Statistically significant differences (p < 0.05) between treatment means for each experiment have different letters above each bar.
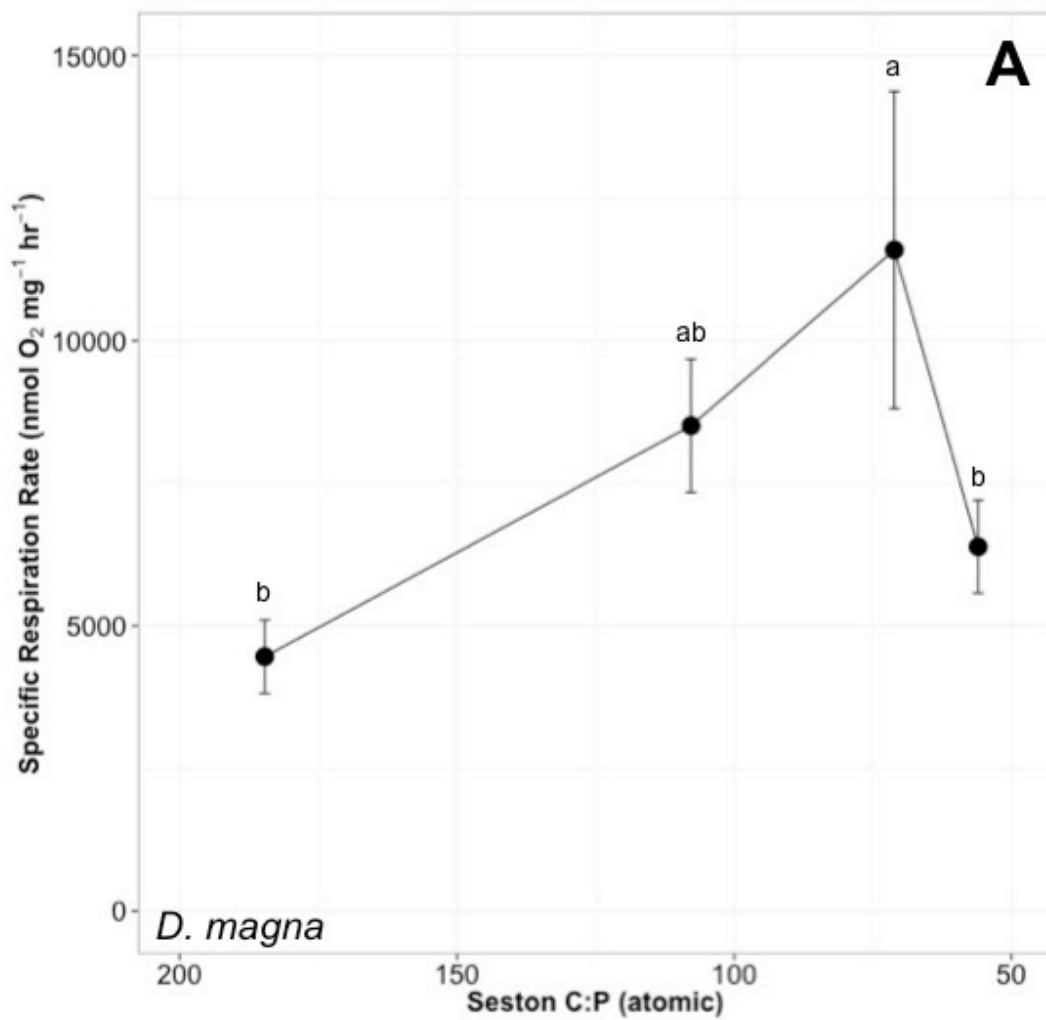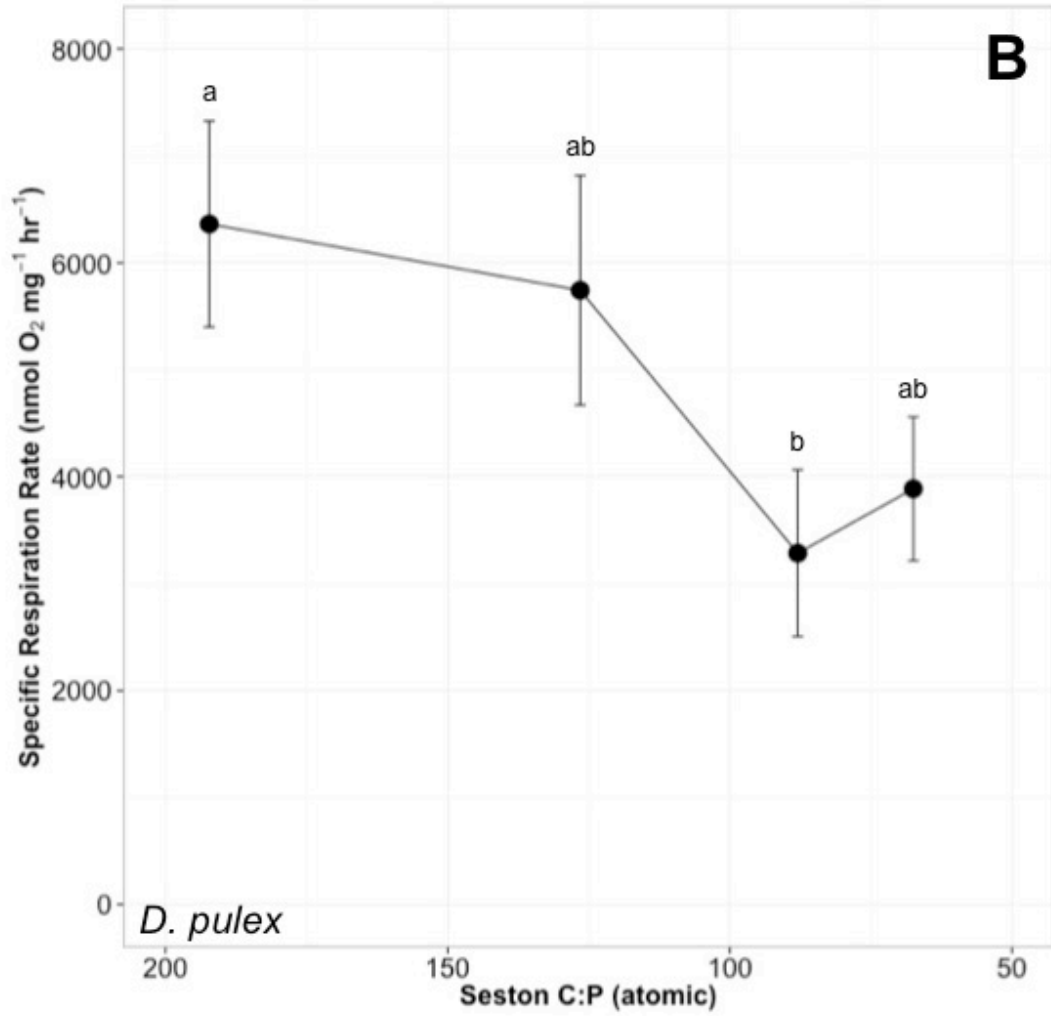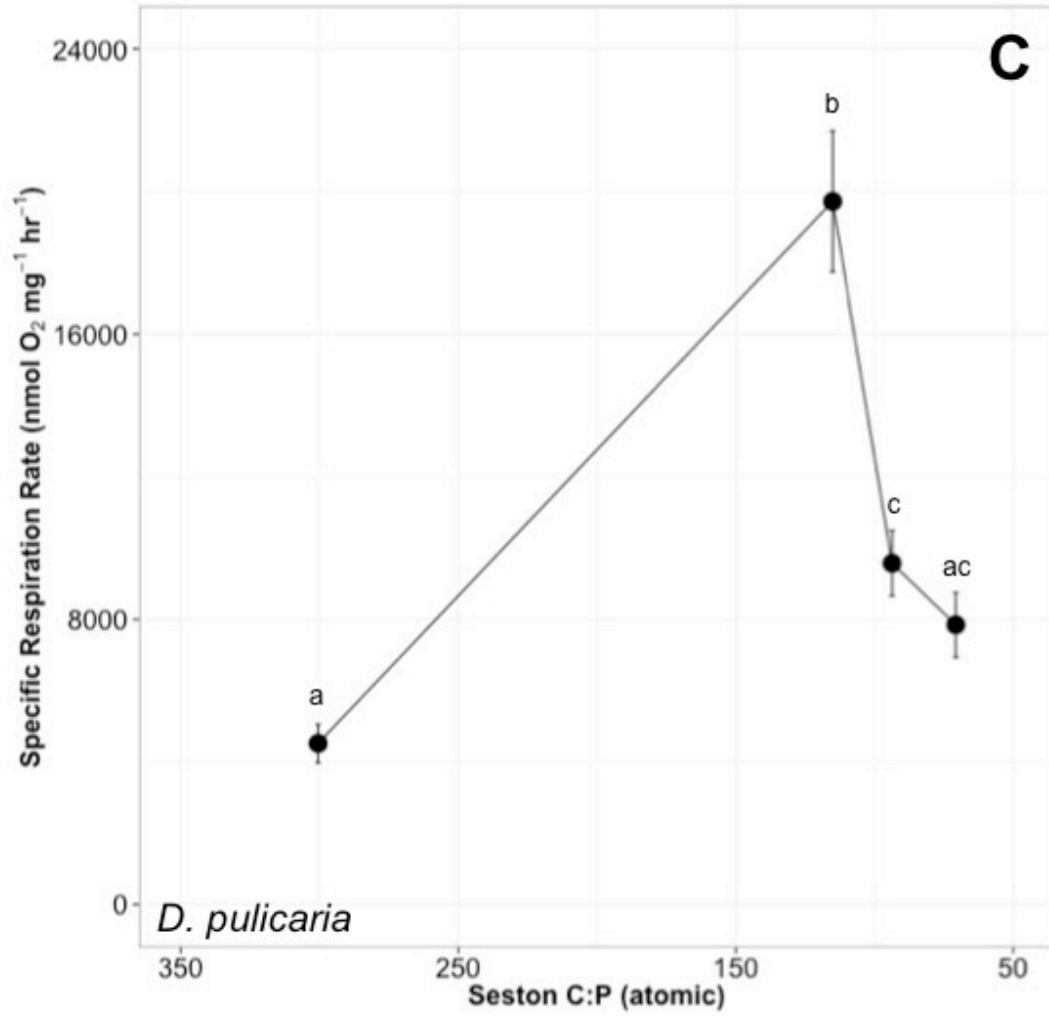
Figure 4 continued.

Figure 4 continued.

Figure 5. Responses of specific ingestion rate to C:P ratio of TTL seston for *Daphnia magna* (A), *Daphnia pulex* (B), *Daphnia pulicaria* (C). Results reported as means (± SE). Summaries of the statistical analyses of these data are given in Tables 1 and 2. Statistically significant differences ($p < 0.05$) between treatment means for each experiment have different letters above each bar.
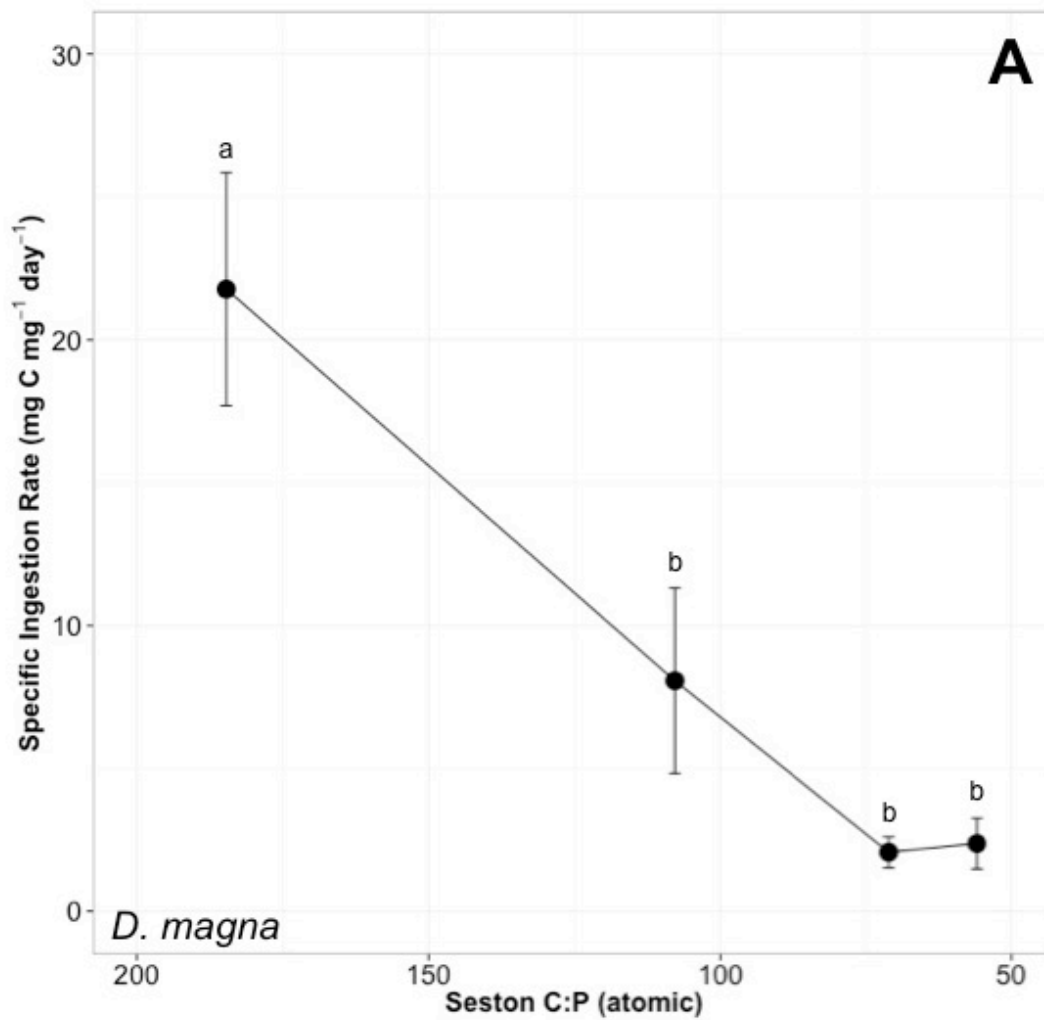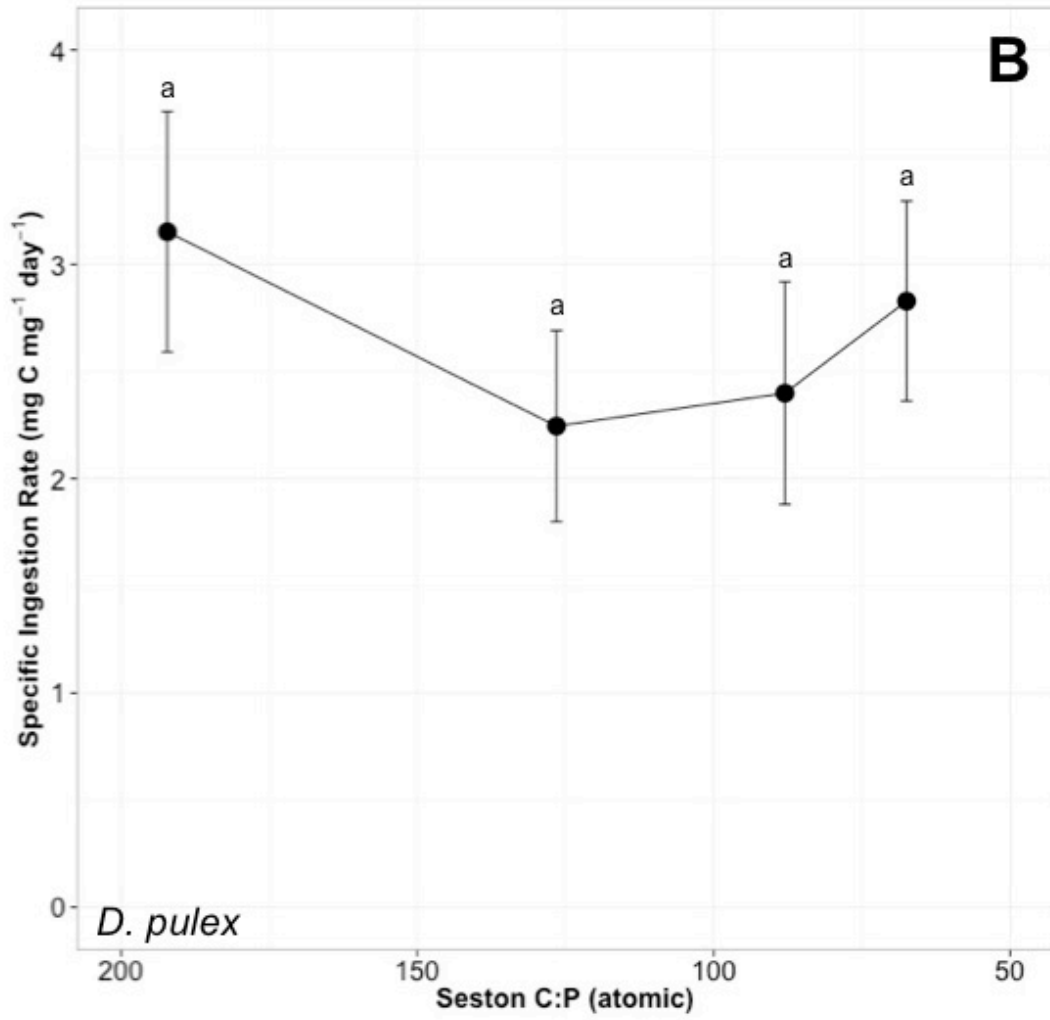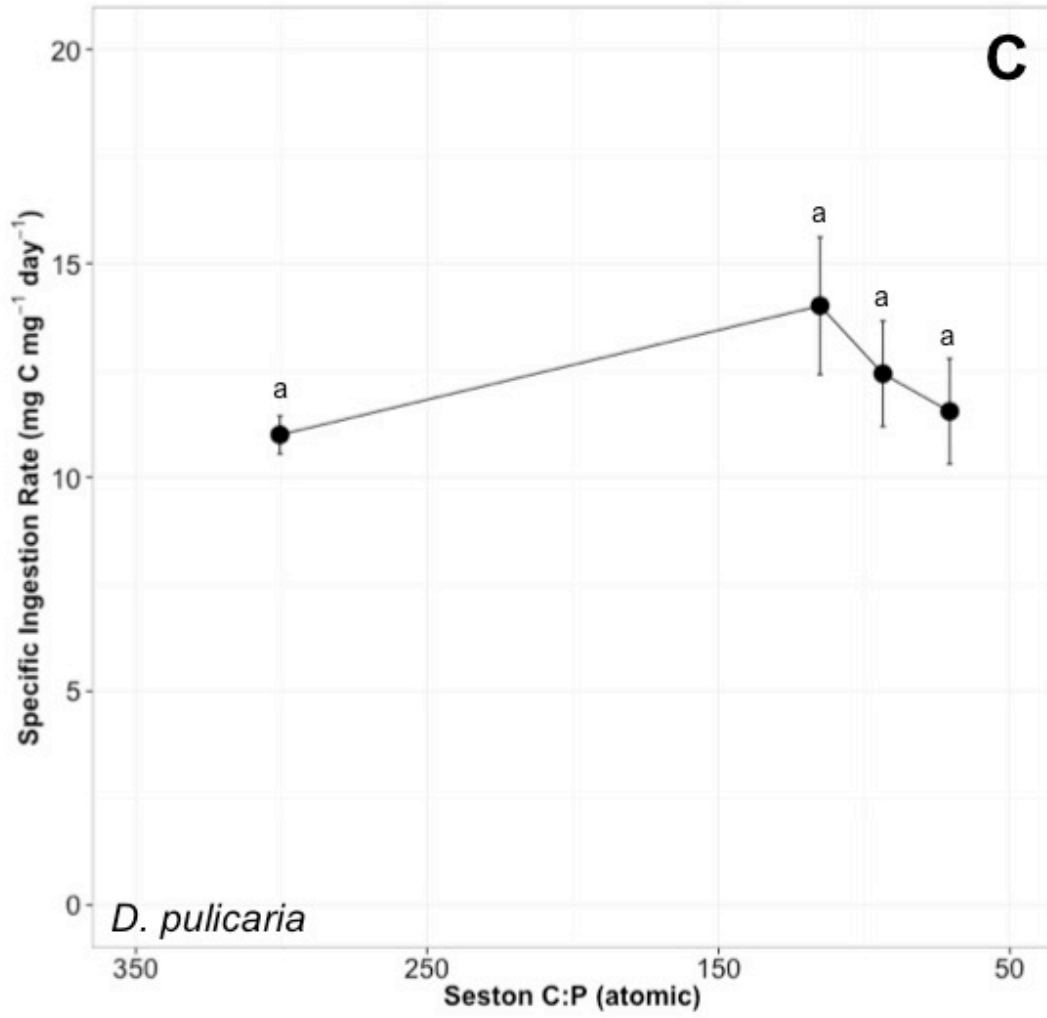
Figure 5 continued.

Figure 5 continued.

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APPENDIX A

DATA COLLECTED FEBRUARY – OCTOBER 2014

Figure A1. Mean (± SE) Tempe Town Lake (TTL) seston C:P (A), C concentration (mg C / L) (B), and P concentration (mg P / L) (C) measured from February 26, 2014 through October 16, 2015. The significant rain event that occurred on September 8, 2014, is indicated by the arrow.