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# Gut passage of phosphorus-limited algae through *Daphnia*: do they take up nutrients in the process?

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With 4 figures

**Abstract:** Nutrient-limited algae are known to be a food source of inferior quality for zooplankters. Three factors are thought to determine this poor quality: direct elemental limitations of the algae, biochemical limitations and an increased resistance to digestion because of an increase in cell wall thickness. Thus far, most studies have concentrated on the effect of the algae on the daphniids. It has recently been hypothesized, however, that while going through the digestive tract of herbivorous zooplankters the digestion resistant nutrient-limited algae might actually take-up nutrients, in a similar way as it has been described for gelatinous alga such as *Sphaerocystis*.

In this study, we present results of different experiments investigating whether nutrient-limited algae are indeed more resistant to digestion, and whether nutrient-limited algae take-up the limiting nutrient in the guts of their predators. We observed that digestion resistance is not very important, and that it can only be observed at high food levels. As a result, we could not find any evidence for nutrient uptake of these algae when they pass through the daphniids. We did find that animals adapted to low-P environments have a higher incorporation efficiency for P, and conclude that digestion resistance in nutrient stressed algae is of very limited ecological relevance.

**Key words:** Excretion, nutrient limitation, stoichiometry, homeostasis, *Sphaerocystis.*

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## Introduction

In recent years, the interest in the importance of food quality as a factor influencing growth and reproduction of freshwater zooplankton species has been large (Gulati & DeMott 1997, Sterner & Schulz 1998). Although it has been well established that algae, cultured under nutrient deficient conditions show decreased quality as food for zooplankters, the exact mechanisms of this phenomenon are still unclear. Consumers might be affected through direct elemental limitations (Sterner 1993), morphological changes reducing digestibility (van Donk et al. 1997) or changes in the content of essential components such as highly unsaturated fatty acids (Müller-Navarra 1995). Recent evidence suggests that, when comparing algae cultured under different conditions, mineral limitation plays the primary role in the determination of food quality (Urabe et al. 1997, DeMott et al. 1998, Boersma 2000, Elser et al. 2001, Becker & Boersma 2003, 2005), but that species specific differences in the content of essential fatty acids might be of higher importance when comparing a variety of different food species (Brett et al. 2000, Park et al. 2002). Indeed, many experiments with algae of different nutrient status as food for zooplankters have shown that when fed nutrient limited algae, zooplankters show reduced growth (Sterner et al. 1993, Sundbom & Vrede 1997, Boersma & Kreutzer 2002). An additional mechanism that may contribute to the reduced success of zooplankton feeding on nutrient-limited algae was proposed by van Donk & Hessen (1993). Following the observation that both *Daphnia pulex* and *D. magna* exerted lower grazing pressure on P-starved green algae relative to nutrient-saturated algae, these authors observed that some P-starved cells passed intact through the daphniid gut. Typically, P-starved algae increased their cell size, probably owing to arrested cell division and accumulation of intracellular glycogen compounds, and they increased the thickness of their cell wall (Tillberg & Rowley 1989, van Donk et al. 1997).

Planktonic algae are able to withstand grazing pressure from zooplankton in various ways. The most obvious way is through morphological features such as gelatinous sheaths, which may allow viable gut passage (Porter 1975), size (Bern 1994) or shape (Lampert 1977), which interfere with ingestion. Most likely, there is a trade-off between the metabolic costs associated with morphological changes produced for grazing protection and growth rate. With nutrient supply in excess, fast growth rates may to some extent compensate for grazing losses. In nutrient-deficient systems, however, growth rates are depressed and some morphological means of grazer protection would be more beneficial, even at the expense of growth rate (Cronin 2001). In the case of unicellular algae it is, however, unclear whether the observed increase in cell wall thickness under nutrient limitation is a way to store excess carbon, or

a life-history strategy to avoid predation when times are bad. VAN DONK et al. (1997) followed up the studies of VAN DONK & HESSEN cited above, studying the morphology of different strains of *Chlamydomonas reinhardti* and *Selenastrum capricornutum* under different nutrient conditions. They also carried out experiments on the digestibility of the different algae, and the viability of the algae that pass the gut of *Daphnia*, finding that nutrient-limited algal cells pass the gut of *Daphnia* intact and alive to a greater extent than algae that are not nutrient limited. At the end of their paper they suggest that viable gut passage may even be beneficial for the nutrient-limited algae, allowing them to take up nutrients from the *Daphnia* gut when pools of dissolved nutrients are depleted. VAN DONK et al. (1997) base this hypothesis on the work of PORTER (1973, 1975, 1976), who showed that as gelatinous phytoplankton pass the gut of *Daphnia* intact, in the process nutrients are taken up from non-gelatinous phytoplankters which are digested in the gut, or even from the animals. In fact, this phenomenon was recently also observed by LEWIN et al. (2003) for roach feeding on the cyanobacterium *Microcystis.* Many of the colonies passed the gut intact, and took up phosphorus in the process of gut passage.

In this study we aim to elaborate on the findings of VAN DONK et al. and PORTER, and to study whether nutrient limited algae do indeed take up nutrients while passing through the gut of daphniids, thus potentially benefiting from being taken up by their predator. The study of VAN DONK et al. was carried out with high amounts of food, and animals that were not nutrient limited themselves. These would have been the 'optimal' conditions to find algae surviving the guts intact, as there was no real need for the animals to digest all of the algae in the gut, and most likely as a result of the high food levels the gut passage time was short, and hence the assimilation rates lower. In this study, we expand on this by using two different food levels, and animals that have grown under nutrient limiting conditions. Stoichiometry theory (STERNER & HESSEN 1994, ELSER et al. 1996) predicts that zooplankton growth and nutrient recycling should be tightly coupled with the resource nutrient ratios, and that consumers should release much of the nutrients present in excess, while retaining the limiting nutrient (OLSEN et al. 1986, GULATI et al. 1995). This implies that one would predict that nutrient limited daphniids should not let anything pass through their guts intact (but see DEMOTT et al. 1998), and generally release as little of the limiting nutrient as physiologically possible.

## Material and methods

### Animals and algae

The daphniids (*Daphnia magna*) originated from long established stock cultures at the Max-Planck-Institute in Plön, Germany. During all phases of the experiments and the

pre-cultivation they were kept in an artificial, phosphorus-free medium (ADaM) (KLÜTTGEN et al. 1994). Prior to each experiment, several females were raised separately in 200 ml jars from neonates to adults and fed P-sufficient green algae, *Scenedesmus obliquus,* at concentrations above 1 mg C $l^{-1}$. The daphniids were transferred to clean jars at regular intervals and third brood neonates released within 24 h were used for the experiments. Neonates from several mothers were pooled and then distributed randomly to the experimental vessels.

During the experiments, the animals were fed *S. obliquus* grown either on low, (83.7 µg P $l^{-1}$) or high (1.395 mg P $l^{-1}$) phosphorus Z/4 medium (ZEHNDER & GORHAM 1960) in semi-continuous cultures, with a dilution rate of around 0.1 $d^{-1}$, yielding C : P ratios (molar) of around 200 for P-sufficient algae and > 1000 for P-limited algae. For the experiments with the radio-labelling, an aliquot of 100 ml was taken from the algal cultures, 900 ml of fresh medium (Z/4) and (Z/4 P-limited), and 7.4 MBq $^{33}PO_4$ added, and incubated for three days. Several experiments were carried out with the radio-labelled algae; first of all we established whether the assimilation and incorporation efficiencies of unlabelled *Daphnia* feeding on labelled P-sufficient and P-limited algae were different. Second, the labelled algae were fed to daphniids for four days, and then these animals were used with unlabelled algae to estimate the loss of P from P-limited and P-sufficient daphniids.

## Digestion resistance and assimilation efficiencies

Digestion resistance of P-sufficient and P-limited algae was first checked using a mixture of algae and fluorescent beads. Five adult *Daphnia magna* were fed a mixture of beads and algae in a ratio of five algal cells to one bead at two food concentrations (0.1 and 1.0 mg C $l^{-1}$). After four hours they were taken out of the feeding suspension, rinsed with ADaM medium, and transferred to 50 ml vessels without algae to defaecate for an hour, a method very similar to the one described by VAN DONK et al. (1997). Lugol's solution was added to this medium, and the ratio between undigested algae and beads counted, as digested and undigested algae can easily be distinguished, and *Daphnia* is known to ingest algae and beads nonselectively.

If digestion resistance plays an important role for nutrient limited algae, one would expect the incorporation efficiency for phosphorus to be lower in animals fed on phosphorus limited algae than in those fed on phosphorus sufficient ones. Furthermore, one would expect that animals adapted to P-limited conditions have higher incorporation efficiencies than those kept under P-sufficient conditions. We tested this using animals adapted to both conditions at a food concentration of 1.0 mg C $l^{-1}$, and labelled P-sufficient and P-limited algae. Neonate *Daphnia* were grown in batch cultures for six days in P-limited and P-sufficient conditions and fed 1 mg C $l^{-1}$. Ingestion was established by incubating 1–3 individuals for 10 minutes in radio-labelled P-limited and P-sufficient algae. After this, they were rinsed, dried and dissolved in 0.5 ml of tissue solubilizer (Soluene 350, Packard) and $^{33}P$ content counted with 10 ml of toluene scintillator (Permablend III, Packard), using a Packard Tri-Car-b 2300 TR scintillation counter. The incorporation of phosphorus was measured after three hours of incubation in the different media, and the incorporation efficiency computed as the amount of radioac-

tive label incorporated divided by the amount ingested. For every experimental treatment we had 10–18 replicates of 1–3 animals.

### Uptake of phosphorus by algae

Animals release phosphorus not only through defecation, but also through other metabolic processes, such as excretion. In order to be able to correct for these processes, we fed four-day labelled *Daphnia* either living algal cells or heat killed (15 min at 70 °C) *Scenedesmus*. The difference between radio-label outside the *Daphnia* after the incubation of animals fed living or dead algae would give a good indication of the active uptake of phosphorus by nutrient-stressed algae. Moreover, all of the radio-label outside the daphniids fed heat-killed algae should be dissolved. Hence, we carried out the incubation experiment with labelled P-sufficient *Daphnia* (fed $^{33}$P-*Scenedesmus* for four days), fed P-limited algae, which were either alive or heat killed. Eight replicates of five labelled *Daphnia* individuals were taken from the stocks, were fed unlabelled algae for two hours, and then starved for two hours to avoid the gut content of the labelled daphniids contaminating the incubations. Subsequently, they were incubated with unlabelled algae (1 mg C $l^{-1}$) in a total volume of 50 ml overnight. After the incubation the animals were taken out, washed in ADaM, and analysed for radioactivity using the methods described above. The algal solutions were filtered, and both the filter as well as the solution were analysed for radioactivity.

## Results

### Digestion resistance and assimilation efficiency

Two separate experiments were carried out with the mixture of algae and beads, one with high food (1.0 mg C $l^{-1}$), one with low food (0.1 mg C $l^{-1}$). As these experiments were not carried out together we could not carry out an ANOVA with food level as a factor, as food effects would also include the effects of the different experiments. Hence, it is possible only to test differences in cells per bead at the two different concentrations separately. It is clear that at both food levels the number of cells per bead decreased substantially, from five to less than two (Fig. 1). This indicates that both algae were eaten and digested. Differences between P-sufficient and P-limited algae were not significant for the low food level ($t_8 = 0.88$), but at the higher food levels we observed significantly more P-deficient algae in the faeces of *Daphnia* ($t_8 = 3.12$; $P < 0.05$). This indicates that there might be a difference in digestion resistance between P-limited and P-sufficient algae only when food conditions are high.

In the incorporation efficiency experiment we observed that both the preconditioning of the animals as well as the condition of the algae fed had a significant effect on the incorporation efficiency for P of the daphniids (2-way ANOVA; $F_{1,54} = 6.0$; $p = 0.02$ for the animal condition; $F_{1,54} = 12.3$; $p < 0.001$

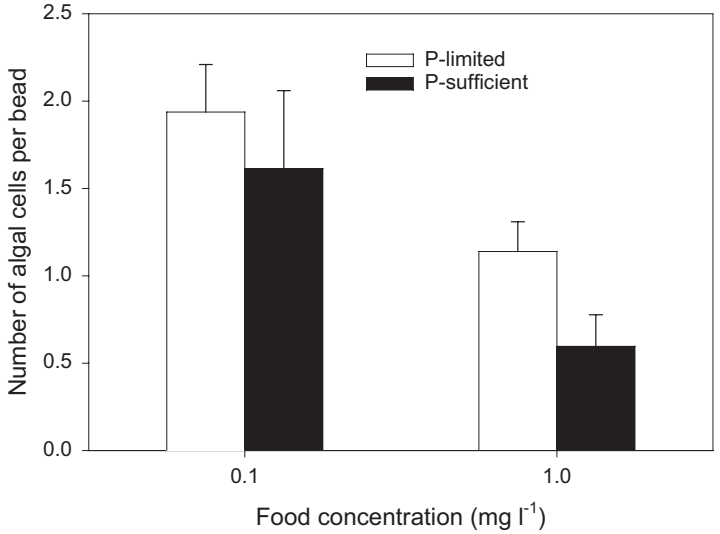


**Fig. 1.** Algal cells per bead after defaecation of *Daphnia*. Initial ratio of cells per bead was five. Error bars indicate standard errors of the mean, n = 5 for all treatments.

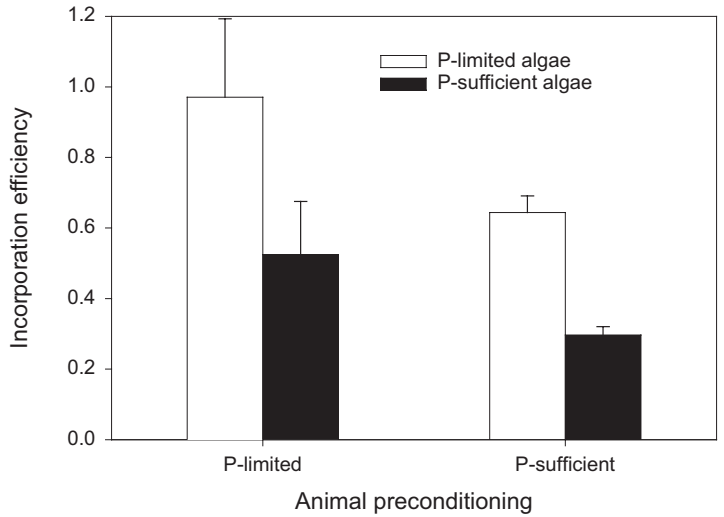


**Fig. 2.** Effect of animal preconditioning (P-limited, P-sufficient) and algal conditions on the incorporation efficiency for phosphorus. Error bars indicate standard errors of the mean, n = 10–18.

for algal background). The interaction between animal condition and algal condition was not significant ($F_{1,54} = 0.2$; $p = 0.67$). Animals grown under P-limited conditions showed consistently higher incorporation efficiencies for P than those grown under P-sufficient conditions. P from P-limited algae was

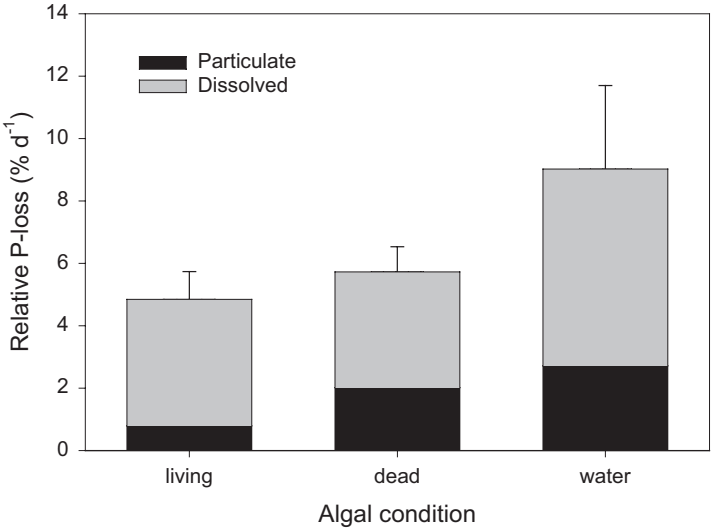


**Fig. 3.** Relative P-loss (% $d^{-1}$) of P-sufficient animals fed living and heat-killed algae, as well as non-fed animals. Labelled *Daphnia* were incubated overnight, and the $^{33}P$ in particulate (algae) and dissolved fractions was measured. Error bars represent standard errors of the total P-loss (n = 5–10).

incorporated with a higher efficiency by *Daphnia* adapted to both P-limited and P-sufficient conditions (Fig. 2).

### Phosphorus release from *Daphnia*

The difference in loss (percentage of the radiolabel present in the animals) in treatments with living P-limited algae, heat-killed algae and only ADaM was not significant ($F_{2,22} = 2.4$; $p = 0.11$; Fig. 3). In fact the losses were lowest for the living algae, and highest for the treatment with ADaM only. Contrary to our expectations, we did not observe more label in the particulate fraction in the living algae. Based on this result we combined all of our experimental datasets, including incubations with P-limited *Daphnia*, and investigated whether the relative P-loss (without differentiating between algal conditions, but omitting the ADaM treatment), was different for differently pre-conditioned animals. We observed that relative P-loss was not significantly different between P-sufficient and P-limited animals ($F_{1,98} = 0.02$; $p = 0.9$; Fig. 4), but that the difference was significant when considering only the dissolved ($F_{1,98} = 7.3$; $p = 0.008$), or the particulate fraction of the loss ($F_{1,98} = 20.5$; $p < 0.001$). P-sufficient animals lost more of their phosphorus in the dissolved form, whereas P-limited animals lost their P in the particulate form.

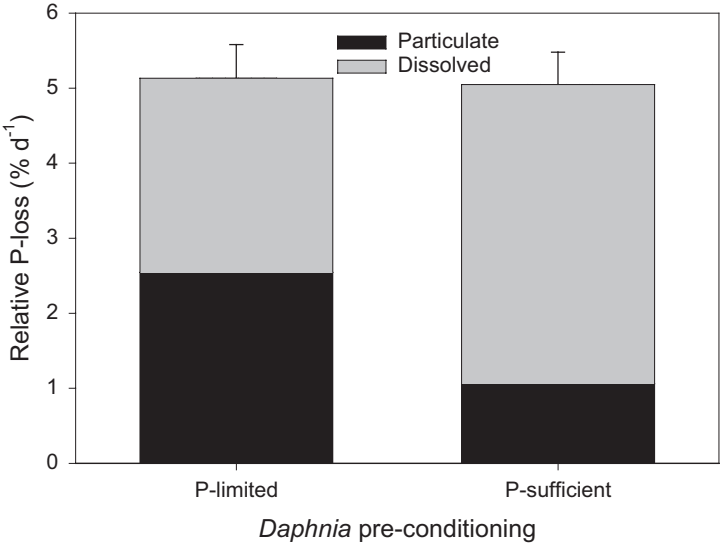


**Fig. 4.** Relative P-loss (% $d^{-1}$) of P-sufficient animals and P-limited *Daphnia* fed living and heat-killed algae. Labelled *Daphnia* were incubated overnight, and the $^{33}P$ in particulate (algae) and dissolved fractions was measured. Error bars represent standard errors of the total P-loss (n = 40–60).

## Discussion

Nutrient limited algae have been shown to have thicker cell-walls (VAN DONK et al. 1997), which prompted this study, investigating whether this would allow *Scenedesmus* to pass through the gut of *Daphnia* intact, and taking up nutrients in the process. Our results indicate that, even though theoretically this would be an excellent strategy for nutrient-stressed algae, the ecological relevance of this process, if at all present, is low. We only observed differences in digestion resistance between nutrient-stressed and nutrient-sufficient algae at higher food levels, although as a result of the fact that we had to do separate analyses for the two food levels this result needs to be interpreted with some care. The fact that in general the digestion resistance seemed to be lower at higher food levels is most likely caused by differences between experiments. In theory, higher digestion resistance at higher food levels is to be expected as gut passage time is dependent on the concentration of the food, with shorter gut-passage times when feeding on higher concentrations (PORTER et al. 1982). As a result of the shorter gut passage time the algae should be broken down with lower efficiency, and hence more algae going through the gut intact. The negligible effect of digestion resistance on low food concentrations is corroborated by the data of BOERSMA & KREUTZER (2002), who observed no difference in growth rates between juvenile daphniids fed on low concentra-

tions of P-limited algae and those fed on the same quantities of P-limited algae which had been spiked with P just before feeding. In fact, even at the very high food levels used by VAN DONK et al. (1997), one can estimate the percentage of algae that really survived gut-passage, and were still intact at the other end of the *Daphnia.* This was in the order of 2–4 %, based on what the animals were fed, and what was recovered. This is a very low percentage. Moreover, phosphorus from nutrient limited algae was incorporated with a significantly higher efficiency than from P-sufficient cells. This is in good agreement with the data presented by DEMOTT et al. (1998), who also observed very high assimilation efficiencies for P, and stated that their data also directly conflict with the notion that P-limited algae are digestion resistant. Furthermore, we observed that the animals adapted to low P conditions showed higher incorporation efficiencies for P than those preconditioned with P-sufficient food. Although this does intuitively make sense, as animals should develop mechanisms to retrieve as much P as possible from nutrient limited food these findings seem to contradict the findings of DEMOTT et al. (1998), who observed lower P assimilation efficiencies for animals grown under severely P-limited conditions. It is unclear what caused these differences, and more investigations are needed to clarify this discrepancy. So, in contrast to the gelatinous green algae *Sphaerocystis schroeteri* which can pass the gut of *Daphnia* with 90 % of the cells intact (PORTER 1975) it seems that viable gut passage of nutrient limited *Scenedesmus* is not an important ecological phenomenon.

This is also reflected in our findings with the living and heat-killed *Scenedesmus*. We took the combination that was most likely to yield a positive result (P-sufficient animals with P-limited algae), and did not observe significant differences in the P-release between the dead and the living cells. Moreover, the daphniids lost more of their P when they were not fed at all. Obviously, with the method we used we cannot distinguish between phosphorus taken up by the algae in the gut, and phosphorus that was excreted by *Daphnia* and subsequently taken up by the algae, except that in the second case we should not have found labelled particulate P in those treatments that were fed dead algae. However, if there had been active uptake from the daphniids, we should have seen differences in total proportions of P-released. Surprisingly, we did find that even in the treatments with dead algae some P was in the particulate form. Potentially, this still was egestion from the gut of the labelled *Daphnia*, although this is not very likely after two hours of feeding on non-labelled algae and two hours of starvation before the experiments were started. It is also possible that bacteria took up the P, but since the experiments were carried out in freshly prepared ADaM this is also not very likely. The most likely explanation is that the P excreted by the *Daphnia* as a result of normal metabolic processes, adsorbed to particulate matter. Since we still do not know how exactly excretion takes place in *Daphnia* (PETERS 1987), it could well be that some of

the excretion takes place in the gut. Interestingly, we did observe differences in the way P-limited and P-sufficient daphniids released P. They both released around 5 % of their total P-pool per day. Possibly, this is an overestimation, since we labelled the animals for four days only, and homogeneous labelling normally takes around a week (PORTER 1976). Nevertheless, the released fractions are in the same range as those published by DEMOTT et al. (1998), who measured P release rates of around 10 % per day even for severely P-limited animals, and indicates again that even those animals that are under severe P-stress do release this vital element in considerable amounts [compare OLSEN et al. (1986) and ANDERSON et al. (2005)]. In our study, we observed that P-sufficient animals released more of their P in dissolved form, which does indicate that they excrete more P, whereas the P-limited animals produce relatively more particulate P, which potentially indicates some egestion of labelled material still.

In conclusion, although it has been well established that more digestion resistant algae such as *Sphaerocystis* or *Oocystis* can indeed pass the gut of *Daphnia* in tact, and take up nutrients in the process, the ecological relevance of nutrient-stress induced digestion resistance in *Scenedesmus* is limited. Perhaps they pass the gut intact to some extent when food concentrations are very high, but this is not a situation that occurs regularly in nature. Moreover, we did not find any evidence that daphniids lose more P when fed living P-limited algae compared to heat-killed ones, even with P-sufficient daphniids, a situation that will also not occur in nature. Therefore, it is highly unlikely that, in normally edible algae such as *Scenedesmus,* uptake of nutrients in the gut of daphniids is an ecologically relevant process.

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