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Food nutrient availability affects epibiont prevalence and richness in natural *Daphnia* populations

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Abstract

The increased input of nutrients into biological systems has been shown to result in altered biotic interactions through changes in food availability. The aim of this study was to test for an association between phytoplankton nutrient content and epibiont variables in natural zooplankton populations. Via a field survey, we studied how a gradient in food quantity and quality impacted host population density and epibiont variables in *Daphnia pulex*. We found a significant decrease in epibiont prevalence and infracommunity richness, which could mainly be attributed to a changing phytoplankton N : P ratio (caused by P-limitation). We performed a lab experiment in which we exposed *Daphnia magna* to different algal nutrient ratios and the epibionts detected in the field study. P-limitation in the algae affected *D. magna* performance and resulted in similar trends of food quality effects in the epibiont variables. The experiment, however, also reflected subtle differences between different epibiont species.

Eutrophication is a persistent threat to biodiversity in natural ecosystems and especially in freshwater ecosystems (Jones and Brett 2014). Nitrogen (N)- and phosphorus (P)-rich pollutants are released in ponds and streams, strongly influencing ecosystem functioning (Gulati and van Donk 2002). Changes in these nutrients are particularly relevant when considering the effect of anthropogenic-induced nutrient inputs in natural ponds and biotic and abiotic interactions (Elser et al. 2010; Peñuelas et al. 2012; Frenken et al. 2017; Narr et al. 2019). Global N : P ratios in aquatic ecosystems have changed since the industrial revolution, mainly due to the disproportional increase in C- and N-concentration relative to P-concentration (Peñuelas et al. 2012). Changed nutrient supply rates to an aquatic ecosystem often result in important modifications of food quality for invertebrate consumers (Sterner and Elser 2002; van de Waal et al. 2010; Hessen et al. 2013). Zooplankton, for example, have been found to be negatively affected by both the relative limitation or excess of either N or P in their food resources (Boersma & Elser 2006, Zhou and Declerck 2019). For instance, a high phytoplankton N : P ratio can serve as low quality food for zooplankton due to relative excess of nitrogen (Reyserhove et al. 2017*a*,*b*), or limitation by phosphorus (Andersen and Hessen 1991; Sterner et al. 1997; Acharya et al. 2004; Zhou et al. 2018).

We here focus on the effect of food quality on one type of biotic interaction, in particular the zooplankton-epibiont interaction. Epibionts are organisms that live attached to its host body surface and are known to affect the zooplankton host. Positive effects (Barea-Arco et al. 2001; Fernandez-Leborans 2010) as well as negative effects of epibionts have been documented (Burris and Dam 2014; Conde-Porcuna et al. 2014; Pauwels et al. 2014). Epibionts mainly use the host as a substratum for attachment, but can mechanically interfere and compete for nutrient intake with the host (Pauwels et al. 2014). Studies have shown that epibiont burden is positively dependent on host body size (Threlkeld and Willey 1993; Krasnov et al. 2004), and transmission rate is expected to be positively dependent on host population density (Decaestecker et al. 2005; Ebert 2005). Knowledge on the

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Additional Supporting Information may be found in the online version of this article.

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effect of environmental changes in N : P ratios on epibiont variables and which mechanisms are involved is lacking. Increases in N- or P-loading may not always have a detectable effect on biotic interactions because of the complexity of the ecosystem and the multitude of possible drives (Aalto et al. 2015). Other biotic factors such as predation pressure (Guttierez et al. 2016), interspecific competition (Decaestecker et al. 2015), or abiotic factors such as temperature changes (Jeppesen et al. 2014; Brans et al. 2017; Hessen et al. 2017) may interact with studied variables in generating the detected associations (van de Waal et al. 2018).

We investigated the effect of a stoichiometric mismatch on the performance of *Daphnia* and demographic features of its epibionts. *Daphnia* is a widespread keystone genus in freshwater ecosystems as it provides a link between primary producers and the upper levels in the food chain (Ebert 2005; Lampert 2006). In particular, we studied how phytoplankton N : P ratio impacts *Daphnia* epibiont prevalence, association intensity, and species richness in natural *Daphnia* populations and in experimental *Daphnia* cohorts. We focus on this food quality variable as it is particularly relevant in the context of increasing N to P supply rates to aquatic ecosystems (Peñuelas et al. 2012, 2013) and as nutrient availability (both N and P) have been suggested to impact *Daphnia* – endoparasite interactions (Aalto et al. 2014; Reyserhove et al. 2017*a*,*b*; Narr et al. 2019). We expect that the effect of stoichiometric imbalance in the elemental supply of the environment will have an effect on the species composition and relative abundance of the epibionts. Whether the epibionts are autotrophic, saprophytic or suspension feeders feeding on algae being plastic with respect to elemental composition. Given that epibionts feed on seston, it can be expected that they will be affected under a stoichiometric mismatch. However, some epibionts do not directly feed on algae and may depend on *Daphnia* food sources (and performance) as well.

Materials and methods

We performed a field survey in combination with a laboratory experiment to investigate the effect of food quality on epibiont variables. For the field survey 12 ponds that contained *Daphnia pulex* populations (Table S1) were selected out of a large sampling campaign. Between May and June 2013, a total of 81 shallow ponds covering the central region of Flanders (Belgium; see Data SI1) were sampled as part of the Belspo Interuniversity Attraction Pole project SPEEDY (Spatial and Environmental determinants in Eco-Evolutionary Dynamics) investigating the effect of urbanization on organismal traits (Brans et al. 2017). The selected ponds were shallow, fishless and had a surface area smaller than 1 ha. As the ponds were sampled once, we cannot generalize our results for one pond over the field season. The different ponds were sampled over the field season ad random.

Characterization of abiotic and phytoplankton variables of SPEEDY ponds

For 57 of the 81 sampled ponds, phytoplankton total N-concentration (P_{TN}, % DW), phytoplankton total P-concentration ($P_{TP,}$ % DW) and phytoplankton C : N, C : P, and N : P (molar) ratios were determined as proxies for food quality (Data SI1 and Table S2). Chlorophyll a (Chl a) and phytoplankton total carbon concentration (P_{TC}, % per phytoplankton dry weight [DW]) were determined as proxies for food quantity. Pond physical variables (temperature, oxygen, pH, and conductivity) and chemical variables (total phosphorus, total nitrogen, dissolved organic carbon, and suspended matter) were also quantified (Data SI1).

Zooplankton and epibiont sampling and variables in selected SPEEDY ponds

D. pulex populations were sampled with a tube sampler at four different locations in the pond (in different quadrants from the limnetic part to the shore) in order to determine the population density and the mean body size. Forty liter were filtered over a 64 μm sieve and fixated with formalin to determine *D. pulex* population density. An additional sample was taken via a sweep net (64 μm mesh size) and stored on ice to randomly isolate 20–30 living female adults and check them for the presence of epibionts within 24 h of sampling. Epibionts were identified to taxa according to Decaestecker et al. (2005) and Ebert (2005) (see Table 1). Adult females were inspected using a light microscope with reflected (organisms were illuminated from the top against a dark background) and transparent light (from below through the transparent organisms). The prevalence of a particular epibiont species was defined as the number of infected *D. pulex* individuals with that epibiont species relative to the total number of screened *D. pulex* individuals for a given pond. Due to the high amount of zero prevalence samples for *Colacium* sp., and because *Brachiounus rubens*, *Vorticella* sp. and *Amoebidium parasiticum* prevalence roughly responded in a similar way to the changing food variables, the epibiont prevalence data was pooled into one single variable for all epibiont species. This "pooled epibiont prevalence" was determined as the number of infected *D. pulex* individuals with at least one epibiont species relative to the total number of screened individuals for a given pond. Epibiont infracommunity richness was defined as the number of epibiont species infecting one zooplankton individual. Association intensity of a particular epibiont was defined as the total number of epibiont individuals (number of propagules under the stereomicroscope) in or on a single infected *D. pulex* individual, excluding non-infected animals. We did not consider a pooled association intensity variable (i.e., a single value representing the association intensity of all epibiont individuals on one *D. pulex* individual) given that the variation in the number of spores or propagules among epibiont species was very large.

Table 1. Characteristics and prevalence of the detected epibionts. Prevalence is represented as mean value ± SE (*n* = number of *Daphnia* populations).

Epibiont species	Location of association	Type—taxon	Feeding mode	Prevalence (mean±SE) (*n*)
Vorticella sp.	Carapace/filter apparatus	Protist—Ciliata—Alveolata	Suspension feeder	27.34±7.27% (9)
Brachionus rubens	Carapace	Zooplankton—Rotiferia	Suspension feeder	9.29±4.61% (5)
Colacium sp.	Carapace/filter apparatus	Protist—Euglenozoa	Autotrophic	8.04±4.41% (5)
Amoebidium parasiticum	Carapace/filter apparatus/partly in gut	Protist—Mesomycetozoea	Parasitic—Saprohytic	22.29±9.39% (8)

Laboratory *Daphnia*–epibiont experiment

In order to confirm the effect of N : P ratio on *Daphnia* epibionts from the field survey, we performed an additional experiment in which the N : P ratio of *Chlamydomonas reinhardtii* was experimentally manipulated when fed to infected *Daphnia magna* (KNO 15.04 clone isolated from a small pond near Knokke, at the Belgian coast 51°20′05.62″N, 03°20′53.63″E) cohorts.

Using single stage chemostats, we established three *C. reinhardtii* cultures with different molar N : P ratios: a low N : P culture (mean: 16; SD: 3.0), a medium N : P culture (mean: 27; SD: 2.4), and a high N : P culture (mean: 74; SD: 4.44). Differences in N : P ratio were obtained by varying the concentrations of phosphorus in the culture medium. The algal food cultures were maintained in three continuous cultures using 2 liter-single stage chemostats that were continuously fed with modified WC

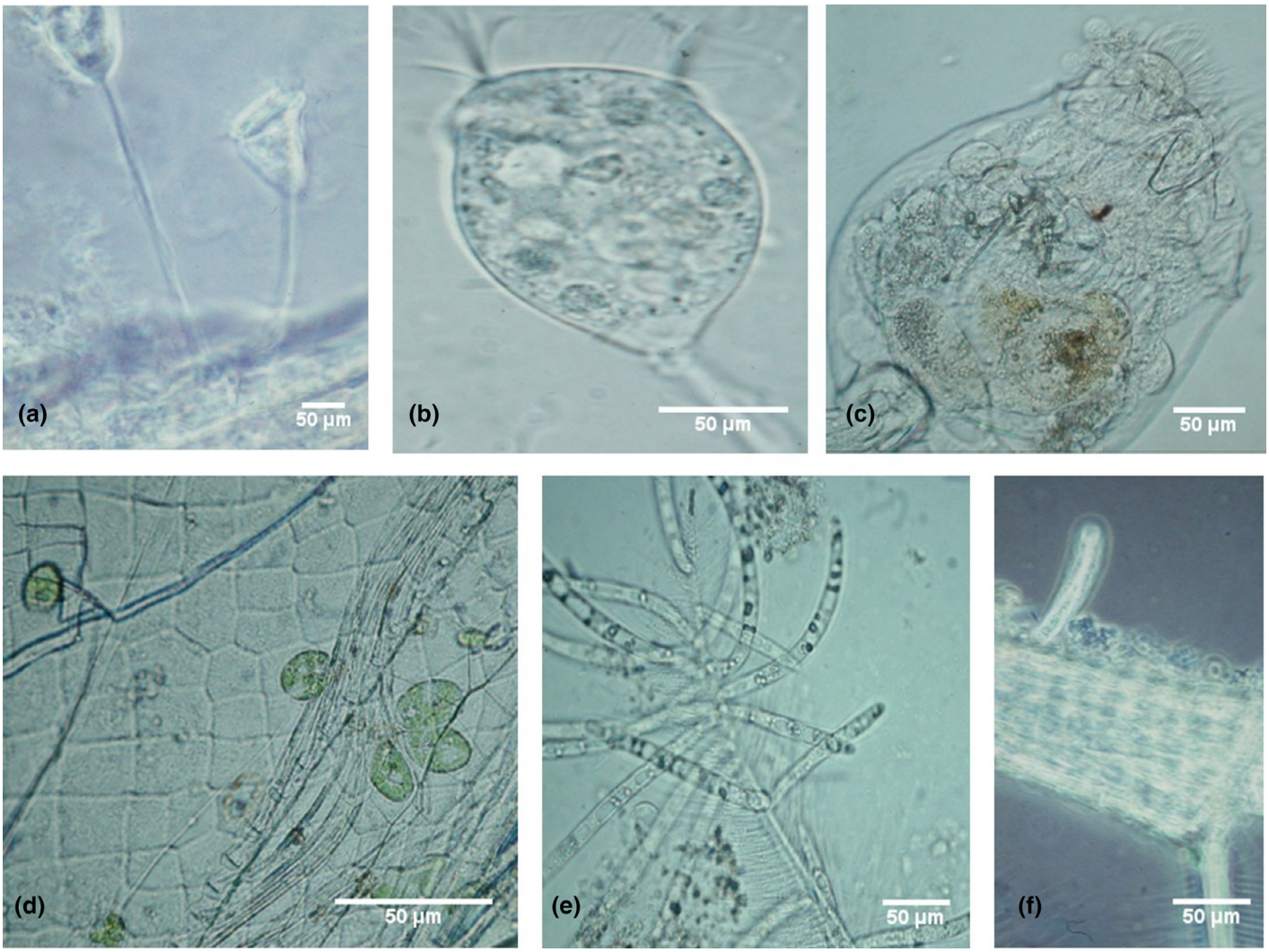

Fig. 1. Pictures of epibionts: (**a** and **b**) *Vorticella* sp., (**c**) *B. rubens*, (**d**) *Colacium* sp., (**e**–**f**) *Amoebidium parasiticum*. Pictures have been taken on 400X magnification.

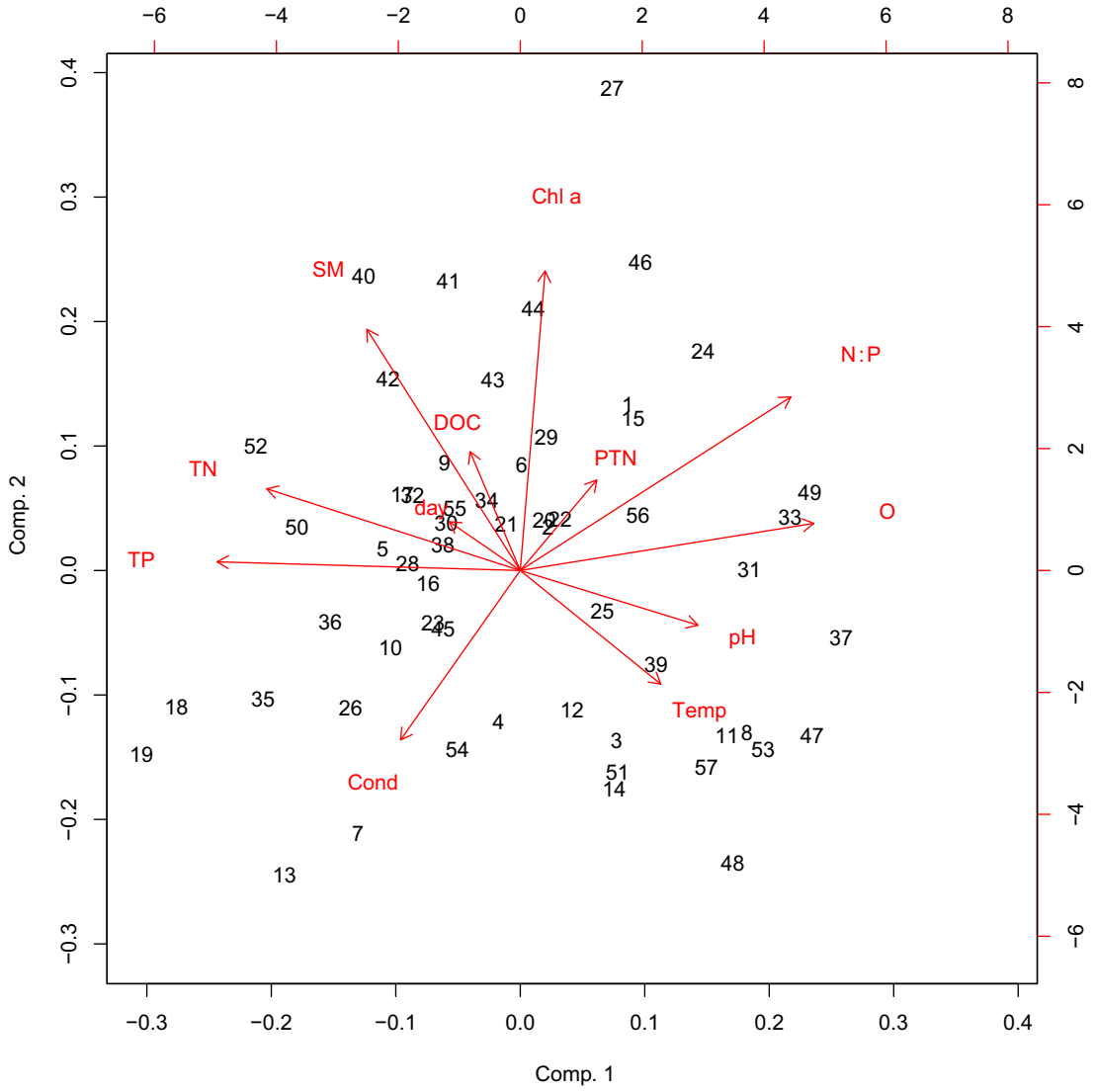

Fig. 2. PCA bi-plot on pond (**a**) biotic and phytoplankton variables. Temp, temperature; O, oxygen; cond, conductivity; TP, total phosphorus; TN, total nitrogen; SM, suspended matter; NP, phytoplankton N : P ratio; Chl a, Chlorophyll a; P_{TN}, phytoplankton total nitrogen.

Table 2. Result of the loading of the variables on the PCA-axes.

	Comp. 1	Comp. 2	Comp. 3	Comp. 4	Comp. 5	Comp. 6	Comp. 7	Comp. 8	Comp. 9	Comp. 10	Comp. 11	Comp. 12
N : P	0.418	0.343					0.291		0.613	0.2	0.35	0.276
Cond	−0.185	−0.335		0.251	0.501		0.637				−0.283	0.202
pH	0.275	−0.108	−0.56		0.195	−0.171		0.475	−0.147	0.336	0.221	−0.344
Temp	0.217	−0.224	−0.443	−0.279			0.171	−0.726				−0.223
O	0.454		−0.282			−0.279	−0.226		−0.185	−0.278	−0.362	0.573
Chl a		0.592		0.281	0.289	0.119	0.189		−0.249	−0.492	0.138	−0.319
SM	−0.237	0.476	−0.298	0.238	−0.217	0.146		−0.178		0.569	−0.37	
DOC		0.235		−0.653	−0.241	−0.212	0.512	0.179	−0.326			
TN	−0.392	0.161	−0.24	−0.153		−0.461	−0.114	0.102	0.576	−0.243	−0.258	−0.196
TP	−0.468		−0.228		0.203	−0.194	−0.154	−0.224	−0.151		0.606	0.428
dag	−0.11		−0.176	−0.445	0.343	0.689	−0.224	0.227	0.114			0.174
PTN	0.118	0.179	0.408	−0.268	0.591	−0.28	−0.197	−0.243	−0.133	0.365	−0.138	−0.143

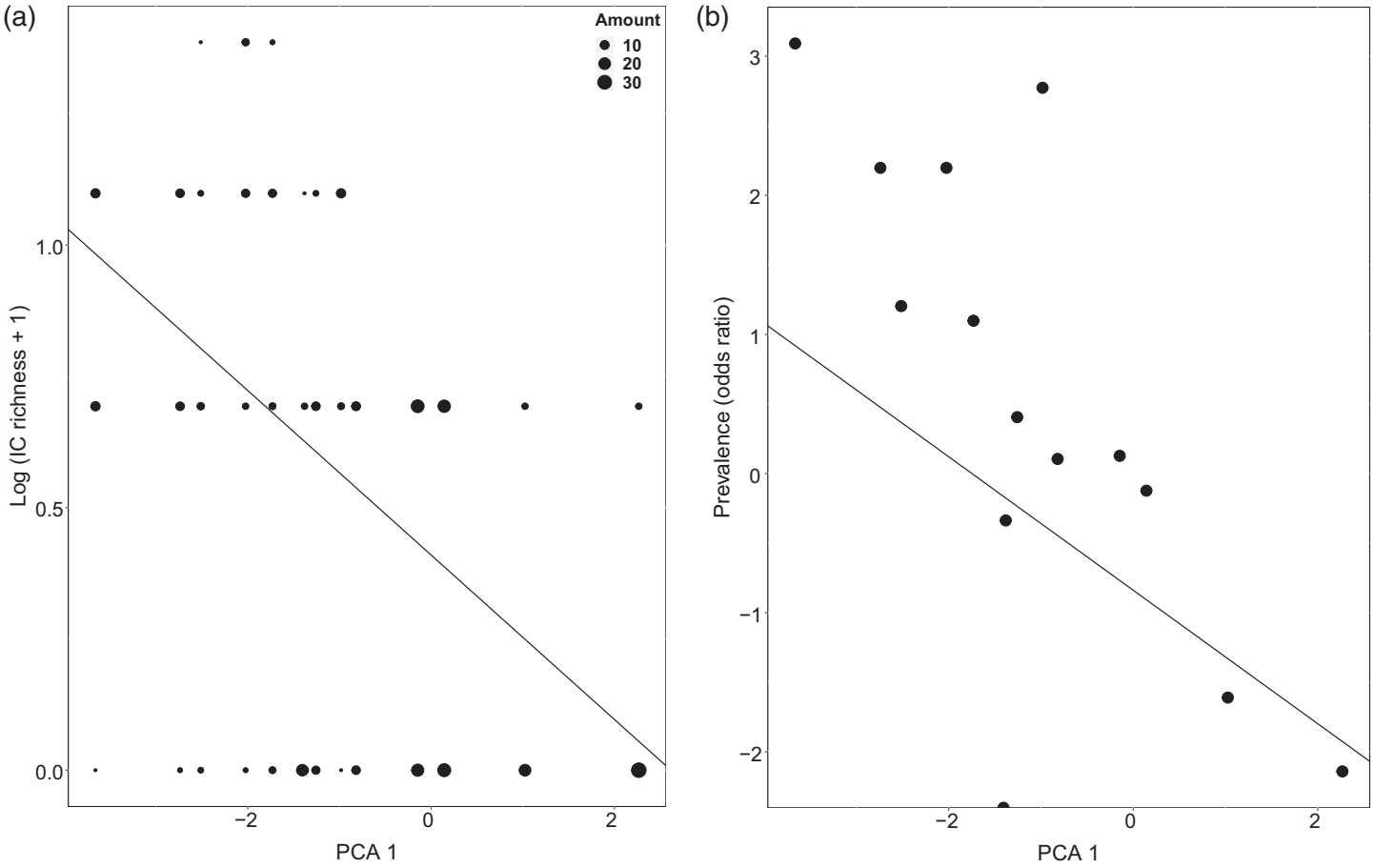

Fig. 3. Impact of PCA 1-axis on epibiont prevalence (**a**) and infracommunity (IF) richness (**b**). Epibiont prevalence is expressed as log(odds ratio). The size of the data points in plot a relates to the amount of host individuals recorded with a certain infracommunity richness. Data points plotted on the x-axis in plot b represent zero prevalence ponds, $n = 12$ (number of ponds).

medium at a temperature of $23 \pm 1°C$ and a dilution rate of $0.33\ d^{-1}$. We varied concentrations of dissolved inorganic nutrients (KH_2PO_4 and $NaNO_3$) between the chemostats to realize algae with different N : P ratios: medium of "High N : P" cultures contained $170\ mg\ L^{-1}$ $NaNO_3$ and $2.613\ mg\ L^{-1}$ KH_2PO_4; medium of "medium N : P" cultures contained $170\ mg\ L^{-1}$ $NaNO_3$ and $4.36\ mg\ L^{-1}$ KH_2PO_4; and medium of "low N : P" cultures contained $85\ mg\ L^{-1}$ $NaNO_3$ and $8.71\ mg\ L^{-1}$ KH_2PO_4.

We first created 18 cohorts of 30 *D. magna* juveniles of 3–4 d old. Each multifactorial combination of N : P food and epibiont treatments were replicated independently three times (cohorts of three independent maternal lines). Three maternal lines were set up. Five individuals per maternal line were transferred in 500 mL filtered tap water and fed every other day with a saturating amount of *Chlorella vulgaris*. Medium was refreshed every week. Juveniles from the first brood were discarded. Twenty juveniles from the second brood per maternal line were transferred in 2 liter-jars of filtered tap water. Parental *Daphnia* were discarded after releasing their second brood. This process was repeated for every new batch of 2nd brood juveniles up to three generations. From here on, we started to feed the *Daphnia* with an increasing ratio of *C. reinhardtii*/*C. vulgaris* (80/20–60/40–40/20) on every

Table 3. Statistical analysis on the impact of PCA1- and PCA2-axes on epibiont variables (prevalence, infracommunity—IF—richness and association intensity). *z*, *z* value; *p*, *p* value. Significant *p* values are indicated in italics.

		PCA 1			PCA 2				
	n	Slope	*z*	*p*	Slope	*z*	*p*	Marginal R^2	Conditional R^2
Prevalence	12	−0.830	−3.765	*<0.001*	−0.177	−0.767	0.443	0.285	0.462
IF richness	12	−0.153	−4.416	*<0.001*	−0.037	−0.970	0.332	0.105	0.105
Association intensity	12	−0.202	−0.718	0.4725	0.175	0.522	0.602		

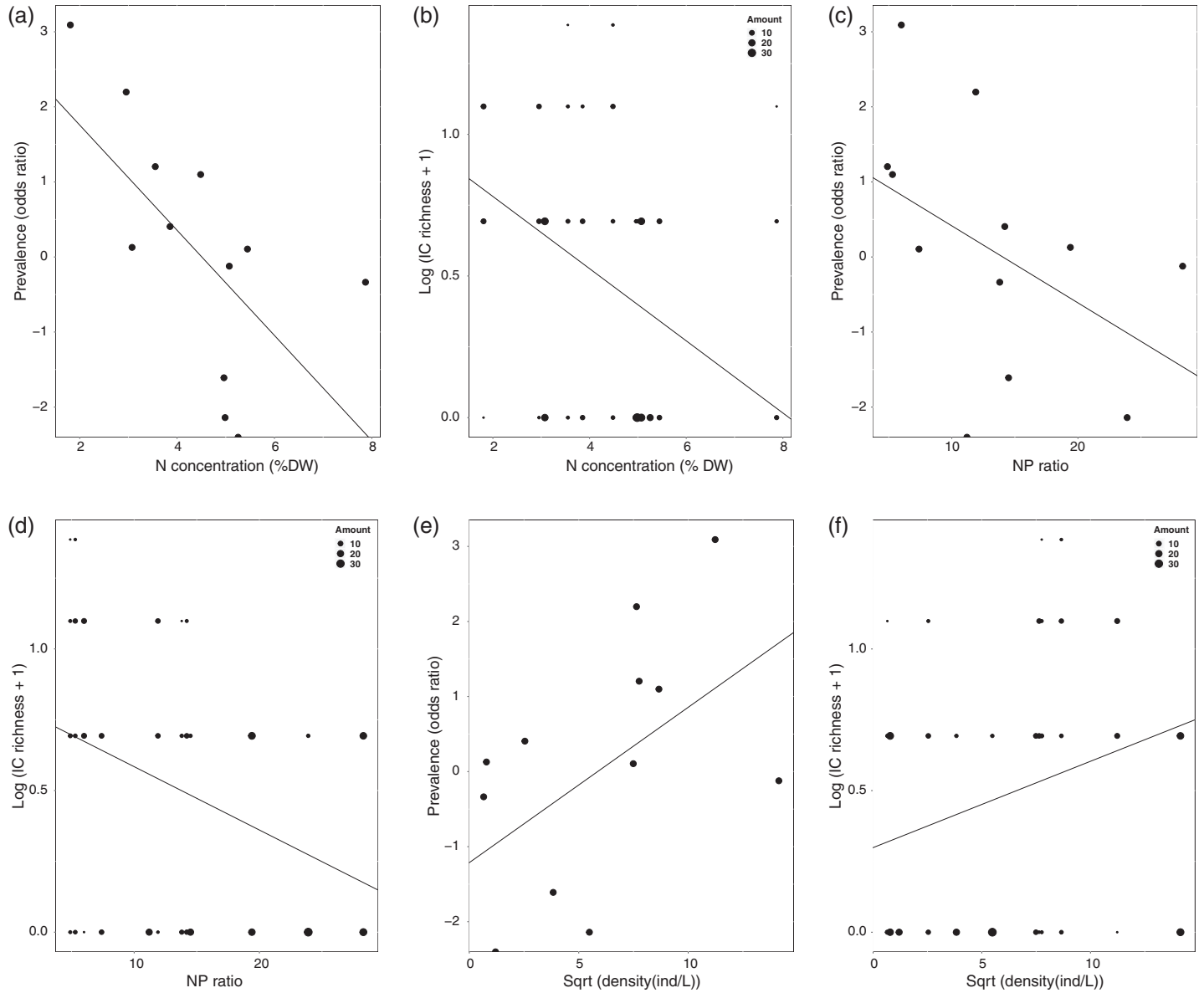

Fig. 4. Impact of phytoplankton N-concentration (**a**), N : P ratio (**c**) and *D. pulex* population density (**e**) on epibont prevalence, and impact of phytoplankton N-concentration (**b**), N : P ratio (**d**) and *D. pulex* population density (**f**) on infracommunity richness. Epibiont prevalence is expressed as log(odds ratio). Data points plotted on the *x*-axis (**a**, **c**, **e**) represent zero prevalence ponds. The size of the data points for plots (**b**), (**e**) and (**f**) relates to the amount of host individuals recorded with a certain infracommunity richness. $n = 12$ (total number of ponds sampled).

other day until the individuals were fed with pure *C. reinhardtii*. Hereafter the maternal lines were maintained by feeding every other day with a saturating amount of *C. reinhardtii*. For practical reasons related to monitoring, we divided all units from each maternal line per treatment combination in two technical replicates (with each a cohort of 15 juveniles) before exposure to the epibionts.

The experimental design consisted of three food N : P levels and two epibiont treatments (i.e., a control and a mixture of *A. parasiticum* and *Vorticella*). For the epibiont treatment, we exposed experimental individuals to a mix of *A. parasiticum* and *Vorticella* sp. For this, *Daphnia* individuals infected with epibionts were collected from a pond in Kortrijk, Belgium (50°48′05.7″N 3°16′32.9″E). These individuals were squashed and pooled per replicate prior to use in the experiment. *Daphnia* individuals of the experiment were each exposed to the equivalent of 1/3 infected individual. We thus created three squashed samples and applied part of each of these samples to two technical replicates of each of the factorial combinations. From 0–3/4 d old: the daphnids were fed every other day with a saturating amount of *C. reinhardtii*. From 4/5–6/7 d old, the daphnids were not fed (start-up of the experiment and stimulation uptake epibionts).

Table 4. Regression analysis on the impact of phytoplankton N : P ratio, total nitrogen concentration (P_{TN}) and *D. pulex* population density on epibiont variables (prevalence, infracommunity IF richness and association intensity). *z*, Wald's *z* value; *p*, *p* value. Significant *p* values are indicated in italics. IF richness: random effect = pond identity; variance = 0.0148; SD = 0.1217. Association intensity: random effect = pond identity; variance = 2.172; SD = 1.474. Epibiont species identity: chi-squared value: 91.26, $p < 0.001$.

	Epibionts		
	Slope	***z***	***p***
Prevalence			
N : P ratio	−0.10	2.05	*0.04*
P_{TN}	−0.70	2.22	*0.02*
D. pulex pop. density	0.20	2.04	*0.04*
IF richness			
N : P ratio	0.022	3.02	*0.002*
P_{TN}	0.127	2.43	*0.01*
D. pulex pop. density	0.030	2.43	*0.01*
Association intensity			
N : P ratio	0.041	0.32	0.75
P_{TN}	0.434	1.31	0.19
D. pulex pop. Density	0.032	0.12	0.90

From 7/8 d old until the end experiment: the daphnids were fed every day with 1 mg C L^{-1} of *C. reinhardtii*.

Daphnia life history traits and body size, epibiont association intensity and prevalence were examined on one of the two technical replicates on four time points: on day 13, day 15, day 20, and day 22. Epibiont prevalence and association intensity was determined by screening a random subsample of five individuals per cohort. Individuals were individually screened using a light microscope with reflected and transparent light. The prevalence of each epibiont species was defined as the number of infected *D. magna* individuals relative to the five inspected individuals per cohort. The association intensity of each epibiont species was defined as the number of individuals of the respective epibiont species present on the carapace of the host individual. Body size was determined by taking a picture of each screened individual and analyzing body size using the ImageJ software. Body size was defined as the distance between the head and the base of the tail. Survival was measured on six time points: on day 5, day 8, day 13, day 15, day 20, and day 22. Survival was defined as the percentage of surviving individuals per cohort relative to the number of individuals on day 1.

Statistical analyses epibiont field data

To determine the relevance of the phytoplankton food quality and food quantity variables in the larger set of biotic and abiotic pond variables (total nitrogen concentration, total phosphorus concentration, dissolved organic carbon concentration, suspended matter, pH, conductivity, temperature and oxygen level), and to reduce the amount of independent variables in the statistical analyses, a principal component analysis (PCA) was performed on the dataset of SPEEDY ponds (n = 57, originally there were 81 ponds in the SPEEDY data set, but only 57 contained all variable measurements needed for the PCA). For this analysis, all variables were standardized (scaled), and temperature, TP, TN, SM, DOC, Chl a, N : P ratio, and P_{TN} were log transformed to fulfill the assumptions of normality. We included the larger pond data set (57 ponds rather than the 12 selected *D. pulex* ponds) as this allowed us to carry out a more powerful analysis. This pond data set was inclusive the 12 ponds for which we could analyze epibiont prevalence and intensity on *D. pulex* (Table S1).

Epibiont prevalence, association intensity and infracommunity richness (log + 1 transformed) were analyzed by generalized linear mixed models (GLMMs) with a binomial (prevalence) and Poisson (intensity and richness) distribution respectively. We accounted for overdispersion by including the observation-level effect as a random effect (as in Harrison 2014). In the association intensity GLMM, values for each epibiont species were used and we accounted for epibiont identity (by including "epibiont species identity" as an extra explanatory variable). Multiple counts for association intensity per zooplankton individual per pond were available, as multiple epibionts can infect one host individual. Non-infected individuals were excluded from these association intensity analyses given the redundancy with prevalence. Host individual was nested in pond and pond identity was considered as a random factor. To account for interdependence, host individual was nested in pond and identity was included as a random effect. All statistical tests were performed in R 3.6.1 (R Core Team).

Statistical analyses *Daphnia*–epibiont experiment

Body size and survival was analyzed to confirm the effect of the N : P treatments on *Daphnia* performance. Body size was analyzed using a GLM assuming a Gaussian distribution of the data. Survival was analyzed for the last time point with a Log-rank (Mantel-Cox) test. All analyses on association intensity and prevalence were performed on each of the four time points independently, and for *A. parasiticum* separately, *Vorticella* sp. separately and both epibionts pooled. Association intensity was analyzed using a GLM assuming a Poisson distribution of the data and accounted for overdispersion when the observed residual deviance was higher than the degrees of freedom. Prevalence was analyzed using a GLM assuming a Binomial distribution of the data and accounted for overdispersion when the observed residual deviance was higher than the degrees of freedom. For the association intensity, non-associated individuals were left out of the analysis given the redundancy with prevalence. Analyses on association intensity for *Vorticella* sp. were not possible due to too few data points. In all these analyses, we evaluated the effect of the factor 'maternal line' by comparing AIC values of models with and without inclusion of this factor. Maternal line was included if the AIC value of the corresponding model was more than two units smaller than

the model without this factor. All statistical tests were performed in R 3.6.1 (R Core Team).

Results

Epibiont sampling in the SPEEDY field design

Four epibionts (*Vorticella* sp., *B. rubens*, *A. parasiticum*, and *Colacium* sp.) were detected in the *D. pulex* populations (Table 1 and Fig. 1). Epibionts were present in 11 of the 12 sampled ponds.

In the PCA-analysis on the studied ponds, the percentage of explained variation of PCA1 and PCA2 was 25.73% and 15.75%. Oxygen, total phosphorus and total nitrogen were the most pronounced variables according to the PCA1-axis, while Chl a correlated most with the PCA2-axis (Fig. 2; Table 2). PCA1-axis revealed a significant negative effect on epibiont prevalence and infracommunity richness, PCA2-axis (mainly food quantity) did not show any effect (Fig. 3; Table 3).

Regression analysis on the studied ponds (correlation on log-transformed data between N and N : P: $r = 0.055$, df = 21, and $p = 0.8$ and between P and N : P: $r = -0.62$, df = 21, and $p = 0.002$, $n = 12$) showed that variation in the seston N : P ratio was more strongly driven by P_{TP} than by P_{TN} indicating that seston N : P ratio mainly reflects P-limitation of the food resource rather than excess N.

A high phytoplankton N-concentration and N : P ratio (P-limitation) was significantly associated with a low epibiont prevalence (Fig. 4a,c; Table 4). High values of the seston N-concentration and N : P ratio (P-limitation) were associated with a low epibiont infracommunity richness (Fig. 4b,d; Table 4). A high *D. pulex* population density was significantly associated with a high epibiont prevalence (Fig. 4e; Table 4) and more epibiont species per host individual (Fig. 4f; Table 4). No significant effects were found on epibiont association intensity (Table 4). Food N : P ratio and *D. pulex* population density were uncorrelated.

Laboratory Daphnia*–epibiont experiment*

A high food N : P ratio (P-limitation) was associated with a smaller *Daphnia* body size (Fig. 5a, effect food N : P ratio on *Daphnia* body size: df = 13, $p < 0.001$). Throughout the experiment, food N : P ratio had a significant effect on the association intensities of *A. parasiticum* as well as of all epibionts pooled (Table 5). In both cases, association intensities decreased with increasing food N : P ratios, especially at the highest N : P level (Fig. 5c,d). Furthermore, food N : P ratio affected the prevalences of *A. parasiticum* and *Vorticella* sp., but only on day 20 (Table 5). On that day, prevalence of *A. parasiticum* was highest at intermediate food N : P ratio, whereas for *Vorticella* highest prevalence was found at the highest level of food N : P ratio.

Discussion

We investigated whether nutrient availability correlated with epibiont association intensity, prevalence, and richness in a field survey of natural *D. pulex* populations. We used phytoplankton N : P ratio (P-limitation) and N-concentration as proxies for food quality and found that food quality correlated with epibiont prevalence and infracommunity richness. An increasing phytoplankton N : P ratio (P-limitation) and N-concentration significantly reduced epibiont prevalence and infracommunity richness, i.e., the number of epibiont species infecting one zooplankton individual. The results of our field survey showed that averaged over all studied species, epibionts perform best in terms of prevalence and richness when P-availability in the environment is high and N-availability is low. The strong relationship with both nutrient availabilities shows that both an excess of N- and P-limitation contribute independently to the epibiont variables. Food quantity did not have a strong effect on the epibiont variables.

We next performed an additional laboratory experiment in which the obtained field results on epibiont variables were largely confirmed. Under P-limitation, food quality for the epibionts was less good, the *Daphnia* performed less well and epibiont association intensities were lower. For epibiont prevalence, the laboratory experiment yielded less clear evidence: significance in the results was limited to specific dates, and there were epibiont species specific effects. Prevalence of *Vorticella* increased under P-limitation. For *A. parasiticum*, the pattern was different with the highest prevalence being at intermediate N : P levels.

It is difficult to disentangle whether the detected nutrient change effect on epibiont variables is a direct effect of the algae on the epibionts, or an indirect effect of the algal nutrient changes on the epibiont community mediated by the *Daphnia* host. Interactions with the host may have played a role through different mechanisms. Host individual performance (Narr and Krist 2014; Reyserhove et al. 2017*a*), population density (Decaestecker et al. 2015) and community composition changes (Johnson et al. 2007; Aalto et al. 2015) can all impact host–epibiont interactions. First, changes in *Daphnia* performance mediated by stoichiometric constraints (Wagner et al. 2017; Reyserhove et al. 2017*a*,*b*) may have played a role in addition to direct effects of stoichiometry on the epibionts. This is very likely, given that some of the epibionts (e.g., *A. parasiticum* and *Colacium* sp.) do not directly feed on the algae. In addition, competitive interactions for the algae between *Daphnia* and epibionts such as *Vorticella* and *Brachionus* (e.g., less P-availability upon increased N) may have influenced the pattern (Aalto et al. 2014; Pauwels et al. 2014). Third, host population density and associated host-epibiont contact rates may have been altered, changing association dynamics (Aalto et al. 2015). Increasing host population density positively impacted epibiont prevalence and infracommunity richness in the field study. It can be expected that, when host population density increases, epibiont prevalence, transmission, and infracommunity richness increases as well (Kiffner et al. 2014; Sponchiado et al. 2016). Thus, the *D. pulex* dynamics may have driven the epibiont dynamics in

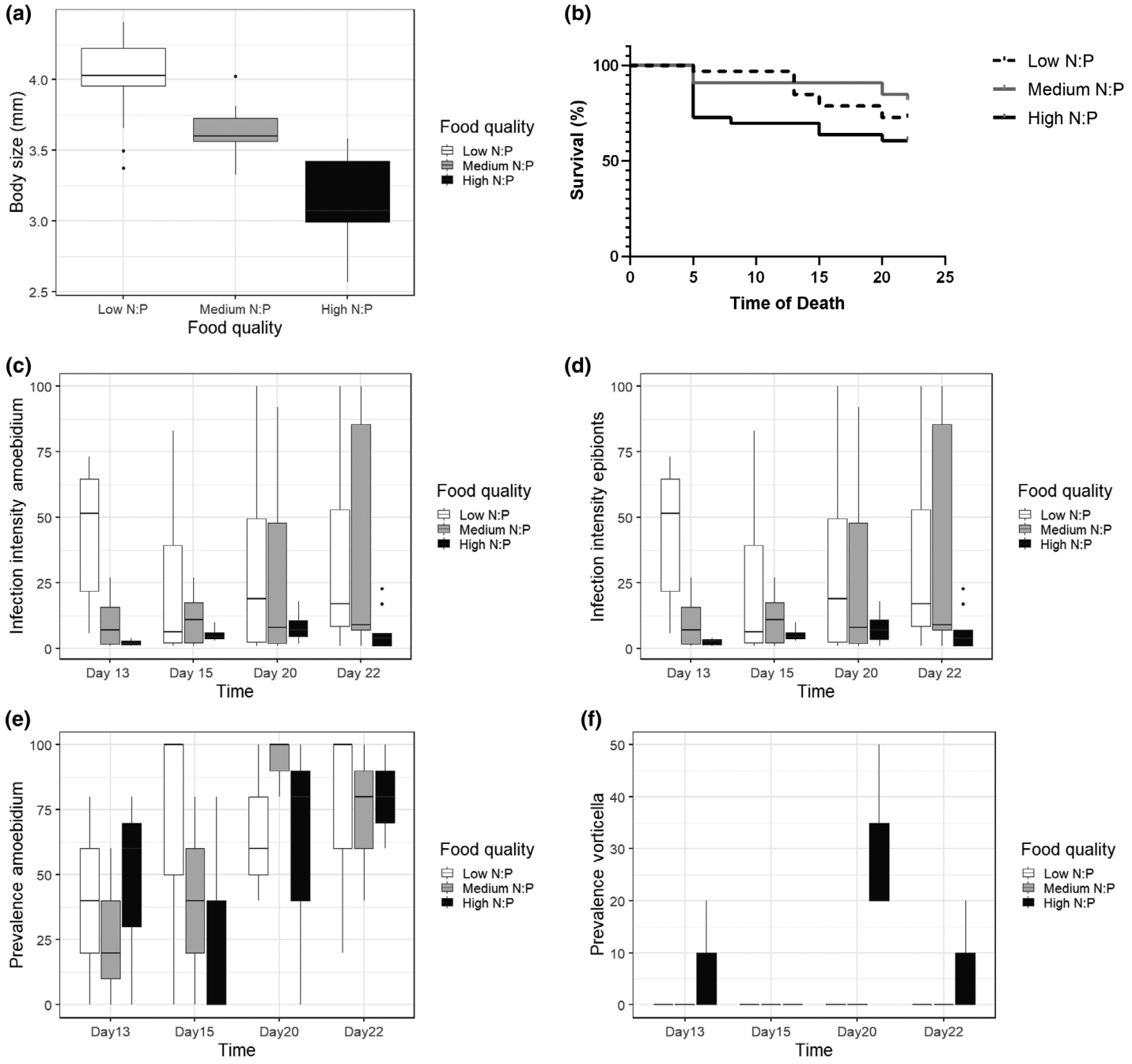

Fig. 5. Results of the laboratory *Daphnia* – epibiont experiment on the effect of algal N : P ratio on (**a**) average *Daphnia* body size, (**b**) *Daphnia* survival through time, (**c**) average association intensity of *A. parasiticum* through time, (**d**) average association intensity through time of all epibionts pooled, (**e**) prevalence of *A. parasiticum* through time, and (**f**) prevalence of *Vorticella* sp. through time. White bars, low food N : P ration; gray bars, medium N : P; black bars, high food N : P ratio.

the field. However, no significant correlation between algal nutrient content or N : P ratio and *D. pulex* population density could be detected in our data. Also, microbial symbionts have been described to play an important role in food acquisition (Sison-Mangus et al. 2015; Callens et al. 2016) and toxin degradation of cyanobacterial algae (Macke et al. 2017) in *Daphnia*. Especially with respect to nutrient acquisition in zooplankton, microbial symbionts and epibiotic microflora may play a structuring role as catalysts for biogeochemical reactions involved in nutrient recycling and eutrophication (Eckert and Pernthaler 2014; De Corte et al. 2018; van de Waal et al. 2018).

In conclusion, we here found an effect of a food quality gradient on epibiont prevalence and association intensity in a field survey, which was partly confirmed in a laboratory

Table 5. Statistical analysis on the impact of food N : P ratio on epibiont variables (prevalence and average association intensity of *A. parasiticum*, *Vorticella* sp., and both epibionts combined) in the *Daphnia* cohort experiment. Significant data ($p < 0.05$) is indicated in bold. Highly significant data ($p < 0.001$) is italics.

	Day 13		Day 15		Day 20		Day 22	
	df	*p*	df	*p*	df	*p*	df	*p*
Prevalence								
Amoebidium parasiticum	4	0.524369	4	0.1681	4	**0.0276565**	4	0.9938234
Vorticella sp.	—	—	3	1	3	**0.01726**	—	—
Epibionts	4	0.524369	4	0.1681	4	0.062058	4	0.9938234
Association intensity								
Amoebidium parasiticum	4	*<0.001*	4	*<0.001*	4	*<0.001*	4	*<0.001*
Vorticella sp.	—	—	—	—	—	—	—	—
Epibionts	4	**0.001683**	4	*<0.001*	4	*<0.001*	4	*<0.001*

experiment in which the nutrient ration in the algae was manipulated. Overall, a low phytoplankton N-concentration and N : P ratio/high P-availability was associated with higher epibiont prevalence, co-associations and association intensity. The discrepancy between laboratory results and results of the field survey for prevalence might indicate that the pattern for prevalence in the field is in part dictated by differences in *Daphnia* population density.

References

Aalto, S. L., T. Ketola, and K. Pulkkinen. 2014. No uniform associations between parasite prevalence and environmental nutrients. Ecology **95**: 2558–2568. https://doi.org/10.1890/13-2007.1

Aalto, S. L., E. Decaestecker, and K. Pulkkinen. 2015. A three-way perspective of stoichiometric changes on host-parasite interactions. Trends Parasitol. **31**: 333–340. https://doi.org/10.1016/j.pt.2015.04.005

Acharya, K., M. Kyle, and J. J. Elser. 2004. Biological stoichiometry of *Daphnia* growth: An ecophysiological test of the growth rate hypothesis. Limnol Oceanogr **49**: 656–665.

Andersen, T., and D. O. Hessen. 1991. Carbon, nitrogen, and phosphorus content of freshwater zooplankton. Limnol. Oceanogr. **36**: 807–814. https://doi.org/10.4319/lo.2004.49.3.0656

Barea-Arco, J., C. Pérez-Martinez, and R. Morales-Baquro. 2001. Evidence of a mutualistic relationship between an algal epibiont and its host, *Daphnia pulicaria*. Limnol. Oceanogr. **46**: 871–881. https://doi.org/10.4319/lo.2001.46.4.0871

Boersma, M., J. J. Elser. 2006. Too much of a good thing: on stoichiometrically balanced diets and maximal growth. Ecology **87**: 1325–1330.

Brans, K., L. Govaert, J. Engelen, A. Gianuca, C. Souffreau, and L. De Meester. 2017. Eco-evolutionary dynamics in urbanised landscapes: Evolution, species sorting and the change in zooplankton body size along urbanisation gradients. Philos. Trans. R. Soc. **B 372**: 20160030. https://doi.org/10.1098/rstb.2016.0030

Burris, Z. P., and H. G. Dam. 2014. Deleterious effects of the ciliate epibiont *Zoothamnium* sp. on fitness of the copepod *Acartia tonsa*. J. Plan. Res. **36**: 788–799. https://doi.org/10.1093/plankt/fbt137

Callens, M., E. Macke, K. Muylaert, P. Bossier, B. Lievens, M. Waud, and E. Decaestecker. 2016. Food availability affects the strength of mutualistic host-microbiota interactions in *Daphnia magna*. ISME J. **10**: 911–920. https://doi.org/10.1186/s40168-018-0444-1

Conde-Porcuna, J. M., E. Ramos-Rodriguez, and C. Pérez-Martinez. 2014. *In situ* production of empty ephippia and resting eggs by an obligate parthenogenetic *Daphnia* population. J. Plan. Res. **36**: 157–169. https://doi.org/10.1093/plankt/fbt072

De Corte, D., A. Srivastava, M. Koski, J. A. L. Garcia, Y. Takaki, T. Yokokawa, T. Nunoura, N. H. Elisabeth, E. Sintes, and G. J. Herndl. 2018. Metagenomic insights into zooplankton-associated bacterial communities. Environ. Microbiol. **20**: 492–505. https://dx.doi.org/10.1111/1462-2920.13944

Decaestecker, E., S. Declerck, L. De Meester, and D. Ebert. 2005. Ecological implications of parasites in natural *Daphnia* populations. Oecologia **144**: 382–390. https://doi.org/10.1007/s00442-005-0083-7

Decaestecker, E., D. Verreydt, L. De Meester, and S. A. J. Declerck. 2015. Parasite and nutrient enrichment effects on *Daphnia* interspecific competition. Ecology **96**: 1421–1430. https://doi.org/10.1890/14-1167.1

Ebert, D. 2005. *In* D. Ebert [ed.], Ecology, epidemiology and evolution of parasitism in *Daphnia*. Bethesda, MD: National Center for Biotechnology Information, US, Available from: http://www.ncbi.nlm.nih.gov/books/NBK2042

Eckert, E. M., and J. Pernthaler. 2014. Bacterial epibionts of *Daphnia*: A potential route for the transfer of dissolved

organic carbon in freshwater food webs. ISME J. **8**: 1019–1808. https://doi.org/10.1038/ismej.2014.39

Elser, J. J., A. L. Peace, M. Kyle, M. W. Wojewodzic, M. L. McCrackin, T. Andersen, and D. O. Hessen. 2010. Atmospheric nitrogen deposition is associated with elevated phosphorus limitation of lake zooplankton. Ecol. Lett. **13**: 1256–1261. https://doi.org/10.1111/j.1461-0248.2010.01519.x

Fernandez-Leborans, G. 2010. Epibiosis in Crustacea: An overview. Crustaceana **83**: 549–640. https://doi.org/10.1163/001121610X491059

Frenken, T., J. Wierenga, A. S. Gsell, E. van Donk, T. Rohrlack, and D. B. van de Waal. 2017. Changes in N:P supply ratios affect the ecological stoichiometry of a toxic cyanobacterium and its fungal parasite. Front. Microbiol. **8**: 1015. https://doi.org/10.3389/fmicb.2017.01015

Gulati, D. R., and E. Van Donk. 2002. Lakes in The Netherlands, their origin, eutrophication and restoration: State of the art review. Hydrobiologia **478**: 73–106. http://citeseerx.ist.psu.edu/viewdoc/download?doi=10.1.1.565.3867&rep=rep1&type=pdf

Guttierez, M. F., M. Devercelli, S. Brucet, T. L. Lauridsen, M. Sondergaard, and E. Jeppesen. 2016. Is recovery of large-bodied zooplankton after nutrient loading reduction hampered by climate warming? A long-term study of shallow hypertrophic lake Sobygaard, Denmark. Water **8**: 341. https://doi.org/10.3390/w8080341

Harrison, X. A. 2014. Using observation-level random effects to model overdispersion in count data in ecology and evolution. Peer J. **2**: e616. https://doi.org/10.7717/peerj.616

Hessen, D. O., J. J. Elser, R. W. and J. Urabe. 2013. Ecological stoichiometry: an elementary approach using basic principles. Limnol. Oceanogr. **58**: 2219–2236.

Hessen, D. O., O. T. Hafslund, T. Andersen, C. Broch, N. K. Shala, and M. W. Wojewodzic. 2017. Changes in stoichiometry, cellular RNA, and alkaline phosphatase activity of *Chlamydomonas* in response to temperature and nutrients. Front. Microbiol. **8**: 18. https://doi.org/10.3389/fmicb.2017.00018

Jeppesen, E., M. Meerhoff, T. A. Davidson, D. Trolle, M. Sondergaard, T. L. Lauridsen, M. Beklioglu, S. Brucet Balmaña, and others. 2014. A climate change impacts on lakes: An integrated ecological perspective based on a multi-faceted approach, with special focus on shallow lakes. J. Limnol. **73**: 84–107. https://doi.org/10.4081/jlimnol.2014.844

Johnson, P. T. J., J. M. Chase, K. L. Dosch, R. B. Hartson, J. A. Gross, D. J. Larson, D. R. Sutherland, and S. R. Carpenter. 2007. Aquatic eutrophication promotes pathogenic infection in amphibians. Proc. Natl. Acad. Sci. USA **104**: 15781–15786. https://doi.org/10.1073/pnas.0707763104

Jones, J., and M. T. Brett. 2014. Lake nutrients, eutrophication and climate change, p. 273–279. *In* P. Brimblecombe, R. Lal, R. Stanley, and J. T. Trevors [eds.], Handbook of global environmental pollution (vol. 1). Springer.

Kiffner, C., M. Stanko, S. Morand, I. S. Khokhlova, G. I. Shenbrot, A. Laudisoit, H. Leirs, H. Hawlena, and others. 2014. Variable effects of host characteristics on species richness of flea infra-communities in rodents from three continents. Parasitol. Res. **113**: 2777–2788. https://doi.org/10.1007/s00436-014-3937-2

Krasnov, B. R., G. I. Shenbrot, I. S. Khokhlova, and A. A. Degen. 2004. Flea species richness and parameters of host body, host geography and host 'milieu'. J. Anim. Ecol. **73**: 1121–1128.

Lampert, W. 2006. *Daphnia*: Model herbivore, predator and prey. Pol. J. Ecol. **54**: 607–620. https://doi.org/10.1111/j.0021-8790.2004.00883.x

Macke, E., C. Callens, L. De Meester, and E. Decaestecker. 2017. Host-genotype dependent gut microbiota drives zooplankton tolerance to toxic cyanobacteria. Nat. Commun. **8**: 1608. https://doi.org/10.1038/s41467-017-01714-x

Narr, C. F., and A. D. Krist. 2014. Host diet alters trematode replication and elemental composition. Freshw. Sci. **34**: 81–91. https://doi.org/10.1086/679411

Narr, C. F., D. Ebert, G. Bastille-Rousseau, and P. Frost. 2019. Nutrient availability affects the prevalence of a microsporidian parasite. J. Anim. Ecol. **88**: 579–590. https://doi.org/10.1111/1365-2656.12945

Pauwels, K., L. De Meester, H. Michels, E. Jeppesen, and E. Deceastecker. 2014. An evolutionary perspective on the resistance of *Daphnia* to the epizoic rotifer *Brachionus rubens*. Freshw. Biol. **59**: 1247–1256. https://doi.org/10.1111/fwb.12344

Peñuelas, J., J. Sardans, A. Rivas-Ubach, and I. A. Janssens. 2012. The human-induced imbalance between C, N and P in Earth's life system. Glob. Chang. Biol. **18**: 3–6.

Reyserhove, L., K. Muylaert, I. Vanoverberghe, and E. Decaestecker. 2017*a*. Synergistic effects of dual parasitism in *Daphnia magna* under nutrient limitation. Belg. J. Zool. **147**: 61–76. https://doi.org/10.1111/j.1365-2486.2011.02568.x

Reyserhove, L., G. Samaey, K. Muylaert, V. Coppé, W. Van Colen, and E. Decaestecker. 2017*b*. A historical perspective of nutrient change impact on an infectious disease in *Daphnia*. Ecology **98**: 2784–2798. https://doi.org/10.1002/ecy.1994

Sison-Mangus, M. P., A. A. Mushegian, and D. Ebert. 2015. Water fleas require microbiota for survival, growth and reproduction. ISME J. **9**: 59–67. https://doi.org/10.1038/ismej.2014.116

Sponchiado, J., G. L. Melo, T. F. Martins, F. S. Krawczak, F. C. Jacinavicius, M. B. Labruna, D. M. Barros-Battesti, and N. C. Caceres. 2016. Ectoparasites of small-mammals: Determinants of community structure in south American savannah. Parasitology **144**: 475–483. https://doi.org/10.1017/S0031182016001906

Sterner, R. W., J. J. Elser, E. J. Fee, S. J. Guildford, and T. H. Chrzanowski. 1997. The light: Nutrient ratio in lakes: The balance of energy and materials affects ecosystem structure

and process. Am. Nat. **150**: 663–684. https://doi.org/10.1086/286088

Sterner, R. W., and J. J. Elser. 2002. How to build an animal: The stoichiometry of metazoans, p. 135–179. *In* R. W. Sterner and J. J. Elser [eds.], Ecological stoichiometry: The biology of elements from molecules to the biosphere. Princeton, NJ: Princeton University Press.

Threlkeld, S., and R. Willey. 1993. Colonization, interaction, and organization of cladoceran epibiont communities. Limnol. Oceanogr. **38**: 584–591. https://doi.org/10.4319/lo.1993.38.3.0584

Van de Waal, D. B., A. M. Verschoor, J. M. Verspagen, E. vain Donk, and J. Huisman. 2010. Climate-driven changes in the ecological stoichiometry of aquatic ecosystems. Front. Ecol. Environ. **8**: 145–152. https://doi.org/10.1890/080178

Van de Waal, D. B., J. J. Elser, A. C. Martiny, R. W. Sterner, and J. B. Cotner. 2018. Editorial: Progress in ecological stoichiometry. Front. Microbiol. **9**: 1957. https://doi.org/10.3389/fmicb.2018.01957

Wagner, N. D., C. Prater, and P. Frost. 2017. Dynamic responses of phosphorus metabolism to acute and chronic dietary phosphorus-limitation in *Daphnia*. Front. Environ. Sci. **5**: 36. https://doi.org/10.3389/fenvs.2017.00036

Zhou, L., K. D. Lemmen, W. Zhang, and S. A. J. Declerck. 2018. Direct and indirect effects of resource P-limitation differentially impact population growth, life history and body elemental composition of a zooplankton consumer. Front. Microbiol. **9**: 172. https://doi.org/10.3389/fmicb.2018.00172

Zhou, L., and S. A. J. Declerck. 2019. Herbivore consumers face different challenges along opposite sides of the stoichiometric knife-edge. Ecol. Lett. **22**: 2018–2027. https://doi.org/10.1111/ele.13386

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Conflict of Interest

None declared.

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