



# Carbon–nitrogen association influences response of the microplankton food web to enrichment

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**ABSTRACT:** In aquatic ecosystems, there are 2 major forms of N available at the base of the planktonic food web: dissolved organic N (DON) and dissolved inorganic N (DIN). In DON, N is associated with organic C, which may promote both heterotrophs and autotrophs. In environments where DIN nitrate is the prevailing N form and dissociated dissolved organic C (DOC) is available, heterotrophs may also be promoted, but they may compete with the autotrophs for DIN. The influence of associated or dissociated CN nutrient sources on the interaction between organisms and the food web function is poorly known and has not been studied before. To approach this question, we performed a microcosm experiment with a coastal microbial food web, where N and C nutrient sources were provided either associated in 1 molecular compound (DON), or dissociated in 2 separate molecular compounds (DIN and DOC). The results showed that association or dissociation of C and N input had marked effects on all trophic levels, most probably through its effect on bacteria–phytoplankton interaction, which switched between increased coupling and increased competition. The biomass of all components of the food web benefitted from the association of C and N in a single DON molecule. Our study indicated that the degree of association between C and N is an important factor affecting the productivity and efficiency of the microbial food web. Therefore, the C and N association should be considered when studying aquatic systems.

**KEY WORDS:** Organic nitrogen · Inorganic nitrogen · Organic carbon · Microbial food web · Ecological stoichiometry · Bacteria–phytoplankton interaction

## 1. INTRODUCTION

Aquatic systems are facing multiple nutrient supplies simultaneously, which influences living organisms in the food web in a complex way. Part of the complexity comes from the nutrients themselves. For example, N occurs in 2 main forms: dissolved organic N (DON) (e.g. amino acids) and dissolved inorganic N (DIN) (e.g. ammonium and nitrate). As organisms have preferences for different forms of N, the N form could influence food web dynamics by promoting

different types of organisms at the base of the food web. In general, phytoplankton contribute to most of the uptake of DIN, while bacteria can utilize both DIN and DON simultaneously. Noticeably, when bacteria utilize DON with a low C:N ratio, they regenerate DIN (mostly in the form of ammonium), which can then benefit phytoplankton growth (Goldman et al. 1987). However, when DON is depleted in the ecosystem or when its C:N ratio is high, bacteria strongly compete for DIN with phytoplankton (Joint et al. 2002).

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In natural aquatic environments, dissolved N is either dissociated or associated with dissolved organic C (DOC). While DIN supplies solely N to osmotrophic organisms, DON allows for simultaneous assimilation of both C and N. Hence, when bacteria take up DIN, they need to acquire C from other sources, e.g. from DOC. In contrast, when bacteria consume DON, they simultaneously assimilate both C and N. These 2 types of association between N and organic C (DON or DIN + DOC) can strongly influence the interaction between bacteria and phytoplankton. Since bacteria have a higher affinity for DON than do phytoplankton and can mineralize the assimilated organic N as ammonium, utilization of DON can generate commensalism, a positive effect of bacteria on phytoplankton (Bratbak & Thingstad 1985, Bronk et al. 2007), which may promote a mutualistic interaction, leading to a relatively high autotroph to bacteria (AU:BA) biomass ratio (Danger et al. 2007). In contrast, if the bulk of DOC does not contain N, bacteria turn to DIN uptake, and both bacteria and phytoplankton compete for DIN (Daufresne & Loreau 2001). The interaction between bacteria and phytoplankton then turns into competition for N nutrition, in which both bacterial and phytoplankton growth are constrained.

Bacterial utilization of DON does not always result in commensalism, because the ammonium regeneration only occurs when the taken-up N exceeds the bacterial N demand. It is only when the C:N ratio of DON is lower than a specific threshold that the excess N is excreted as ammonium. Previous experimental and theoretical studies show that the threshold C:N ratio is around 10, which is very similar to the C:N ratio of aquatic bacteria (5–7) corrected with the respiration coefficient (around 0.6) (Parnas 1975, Goldman et al. 1987, Goldman & Dennett 1991, Cherif & Loreau 2007). When the DON has a ratio larger than the bacterial C:N ratio (>10), net N uptake and C excretion by bacteria may occur; thus, the interaction between bacteria and phytoplankton may turn into competition. However, in some cases, the bacterial C:N ratio may also align with the C:N ratio of their resource, either by adapting their cellular physiology (e.g. Egli 1991) or through a change in the species composition of the whole bacterial community (Cotner et al. 2010, Lee et al. 2017). If such a stoichiometric alignment between bacteria and the C:N ratio of DON happens, then net N uptake by bacteria does not occur, and competition with phytoplankton cannot be promoted by DON with large C:N ratios. Hence, the interaction of bacteria and phytoplankton is greatly dependent on whether bacteria contribute to the pool of ammonium or not.

As fundamental basal producers, autotrophs and bacteria are both important food sources for higher trophic levels, and the complexity of bacteria–phytoplankton interactions results in changes in basal production and further creates alternative pathways of energy flow to higher trophic levels (Legendre & Rassoulzadegan 1995, Fonte et al. 2013). Under a commensalistic interaction, the consumers would gain energy channeled via both the bacterial and the phytoplankton pathways, while under a competitive interaction, the consumers would mostly gain energy from either the phytoplankton or the bacterial pathway. Given that both pathways often show different trophic transfer efficiency (Degerman et al. 2018), we may expect different food web energy transfer when the basal interaction is switched between commensalism and competition.

Studies within the field of ecological stoichiometry have focused on how the ratio of limiting chemical elements (e.g. P, N and C) affect bacteria–phytoplankton interactions and associated processes (Mindl et al. 2005, Cherif & Loreau 2007, Danger et al. 2007). However, these earlier studies considered the relative amounts of the various chemical elements but have not yet addressed the question of how association or dissociation of N and C affects microbial interactions and how these effects transfer to higher trophic levels, e.g. heterotrophic grazers. To increase the understanding of how dissociated or associated C and N affect the interaction between bacteria and phytoplankton and the microbial food web transfer efficiency, we performed a microcosm experiment. We isolated a natural microplanktonic food web (<50  $\mu\text{m}$ ) from a coastal site in the northern Baltic Sea and enriched the food web in C and N with varying degrees of association. In the DON-addition treatment, C and N were associated in 1 organic molecule (glutamic acid). In contrast, C and N were fully dissociated in the DIN/DOC-addition treatment, consisting of nitrate and  $\alpha$ -ketoglutaric acid. In Baltic Sea coastal waters, the microbial food web shows many features that complicate the prediction of responses to different N enrichments, as it is rich in mixotrophs and small heterotrophs (Andersson et al. 1996) and receives high supplies of terrestrial matter that contains DON and DIN in various proportions according to the season (Savchuk 2018). Thus, the balance of bacterial to phytoplankton biomass is likely to be key to its functioning. The main objectives of this study were not to look at the nutrient enrichment effects but to elucidate if the addition of dissociated and associated C and N results in different types of bacteria–phytoplankton interactions

and if the food web structure is affected. Further, we addressed the impacts of DIN/DOC and DON uptake on the C:N:P stoichiometric response of different size fractions of the food web.

## 2. MATERIALS AND METHODS

### 2.1. Microcosm setup

Seawater for the experiment was collected from the northern Baltic Sea (63° 34' N, 19° 54' E, Bothnian Sea, Sweden) at a depth of 5 m on 12 October 2016. Before incubation, the water was pre-filtered through 50 µm mesh to exclude large plankton.

To compare the impacts of dissociated CN and associated CN addition on the microplankton food web, 12 microcosms were set up for 2 treatments (6 replicates in each treatment): DIN/DOC and DON (Table 1, Fig. 1). The DON treatment consisted of the addition of glutamic acid ( $C_5H_9NO_4$ ), in which the C and N are fully associated in 1 organic molecular compound, and the C:N ratio of DON was smaller than the threshold ratio of 10 to ensure that ammonium regeneration occurred in this treatment (Goldman et al. 1987). In the DIN/DOC treatment, in which the C and N are fully dissociated in 2 different molecular compounds, besides nitrate ( $NO_3^-$ ), the DOC  $\alpha$ -ketoglutaric acid ( $C_5H_6O_5$ ) was added at the same C concentration as in the DON treatment (Table 1, Fig. 1). Glutamic acid and  $\alpha$ -ketoglutaric acid were selected because these molecules commonly exist in nature and are highly bioavailable for bacteria and phytoplankton. Moreover, the molecular structures of these 2 organic compounds are similar to minimize potential utilization preference. Nitrate was selected as a source of DIN, because significant nitrification–deni-

trification was unlikely to occur under experimental conditions. Hence, all increases in ammonium after the addition of nitrate were interpreted as a result of mineralization by organisms present in the microcosms. The purpose of the experiment was also to get the most contrasted situations, with one favoring mostly bacteria and one mostly phytoplankton, as is often observed in natural ecosystems, where the phytoplankton dominance and bacterial dominance alternate across seasons. The addition of nitrate in the DIN/DOC treatment would favor phytoplankton over bacteria since bacteria have a low affinity for nitrate (Kirchman 1994), while the addition of DON would favor bacteria over phytoplankton (Bronk et al. 2007).

The original dissolved C, N and P concentrations in the collected water amounted to  $3800 \mu\text{g C l}^{-1}$  (DOC),  $215 \mu\text{g N l}^{-1}$  (total dissolved N [TDN]) and  $10 \mu\text{g P l}^{-1}$  (total dissolved P [TDP]). All the nutrients were added every second day (Days 0, 2, 4, 6, 8). Thus, the total addition reached  $100 \mu\text{M}$  of N,  $20 \mu\text{M}$  of P and  $500 \mu\text{M}$  of C at the end of the experiment. The C:N:P molar ratio of added nutrients was 25:5:1 for all

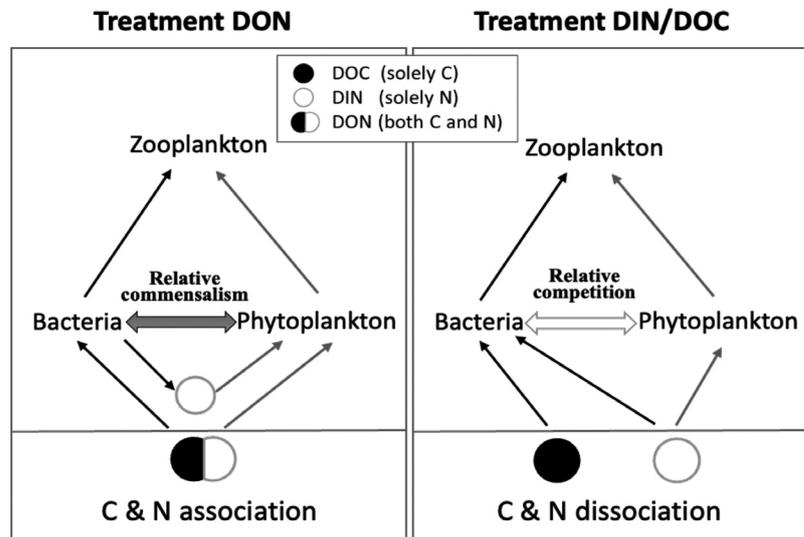


Fig. 1. Conceptual CN uptake models of the food web in the experiment. DON: dissolved organic N; DIN: dissolved inorganic N; DOC: dissolved organic C

Table 1. Nutrient additions to different treatments. DIN: dissolved inorganic N; DOC: dissolved organic C; DON: dissolved organic N; DIP: dissolved inorganic P; +: nutrient addition

Treatment	DIN ( $NaNO_3$ )	DOC ( $C_5H_6O_5$ )	DON ( $C_5H_9NO_4$ )	DIP ( $NaH_2PO_4$ )	Total C addition ( $\mu\text{M}$ )	Total N addition ( $\mu\text{M}$ )	Total P addition ( $\mu\text{M}$ )
DIN/DOC	+	+		+	500	100	20
DON			+	+	500	100	20

microcosms. To make sure that the microcosms were not limited by P, all microcosms were supplemented with the same amount of dissolved inorganic P (Table 1). We chose to add the nutrients in a protracted press perturbation fashion (Glasby & Underwood 1996) as a compromise. We wanted to ensure that the various organisms involved would still be growing at the end of the experiment, and not senescing, while at the same time avoiding the technical complexities of large-scale chemostat experiments. The effects of total N and C additions on planktonic food webs in mesocosms are well studied, notably in the Baltic Sea (Sörensson et al. 1989, Kivi et al. 1993, Balode et al. 1998, Traving et al. 2017). Hence, we did not focus our experiment on the effects of N and C additions per se but used a comparative approach between the 2 treatments that differ in their degree of association of C and N. We decided as well to set only the 2 most contrasted possible treatments (100% associated C and N vs. 100% dissociated C and N) to increase the possibility to capture any potential effect of this largely unexplored factor so far.

For each microcosm, 10 l of experimental water were incubated in high-density polyethylene cubitainers (total storage capacity 15 l). All the microcosms were placed in a temperature-controlled room (15°C) under 100  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  light intensity and 12:12 h light:dark cycle to simulate natural conditions. Air (0.2  $\mu\text{m}$  filtered) was gently bubbled to each microcosm to ensure mixing. The experiment lasted 10 d.

During the 10 d experiment, samples were taken for analyses of water chemistry, phytoplankton/bacterial abundance, and phytoplankton/zooplankton taxonomy and seston size fraction stoichiometry. Water chemistry and abundance samples were taken every second day (Days 0, 1, 3, 5, 7, 9). Taxonomy samples were taken at the beginning, middle and end of the experiment (Days 0, 5, 9). Plankton stoichiometry samples were filtered only from the original water and at the end of the experiment.

## 2.2. Water chemistry

Concentrations of phosphate, N and C were analyzed following standard analytical methods described in Grasshoff et al. (1999). Briefly, concentrations of phosphate and DIN (nitrate + nitrite + ammonium) were analyzed using a Seal QuAAtro39 autoanalyzer. Samples for TDN and TDP were analyzed in the same way after filtration through 0.2  $\mu\text{m}$

Supor membrane syringe filters (non-pyrogenic, Acrodisc<sup>®</sup>, Pall) and an oxidation step using peroxydisulfate. DON concentration was determined by the difference between TDN and DIN. DOC was analyzed on 0.22  $\mu\text{m}$  filtered (Supor membrane syringe filter, non-pyrogenic, Acrodisc<sup>®</sup>) and acidified (18 mM HCl, final concentration) samples using a high-temperature combustion Shimadzu TOC-5000 analyzer.

## 2.3. Heterotrophic bacteria and picophytoplankton

Heterotrophic bacteria and picophytoplankton abundances were estimated by flow cytometry. The samples were preserved with 0.1% glutaraldehyde (final concentration) and immediately stored at  $-80^{\circ}\text{C}$ . All frozen samples were quickly thawed in a  $30^{\circ}\text{C}$  water bath before analysis with a BD FACVerse<sup>™</sup> flow cytometer (BD Biosciences). Heterotrophic bacteria samples were stained with SYBR Green I (Invitrogen) to a final concentration of 1:10 000 (Marie et al. 2005). The samples were run at a flow rate of 40  $\mu\text{l min}^{-1}$  for 1 min. When necessary, samples were diluted with filtered seawater to avoid coincidence. Microspheres of 1  $\mu\text{m}$  (Fluoresbrite Plain YG, Polysciences) were added to the samples as the internal standard. Picophytoplankton samples were analyzed at a flow rate of 100  $\mu\text{l min}^{-1}$  for 3 min with 3  $\mu\text{m}$  microspheres (Fluoresbrite Plain YG, Polysciences) as the internal standard. Picoeukaryotes and picocyanobacteria could be discriminated based on their red and orange fluorescence.

Samples for phytoplankton ( $>3 \mu\text{m}$ ) and microzooplankton identification and counting were preserved with 2% acidic Lugol's solution. Samples were settled for 24 h and counted using the inverted microscope method (Utermöhl 1958). Functional groups (autotrophs [AU], heterotrophs [HT] and mixotrophs [MX]) were assigned according to Olenina et al. (2006). The ciliate *Mesodinium rubrum* was included in the autotrophs. The plankton cells were divided into different size classes ( $<3 \mu\text{m}$  [picophytoplankton],  $<10 \mu\text{m}$  [3–10  $\mu\text{m}$ ] and  $>10 \mu\text{m}$ ) based on measurements of the longest cell axis. Phytoplankton and ciliate biomasses were calculated from the geometric shape of cells following Olenina et al. (2006), and cell C content was calculated according to Menden-Deuer & Lessard (2000). The biomass of rotifers was calculated according to Pace & Orcutt (1981) and Andersen & Hessen (1991).

#### 2.4. CNP stoichiometry of different seston size fractions

Due to the technical restrictions that prevent the measurement of stoichiometry separately for each taxonomic group in natural communities, we used size fractionation to quantify the stoichiometry of different food web components. Water samples of seston were filtered into 3 different size fractions: (1) 0.3–0.7  $\mu\text{m}$ , with mostly bacterioplankton; (2) 0.7–10  $\mu\text{m}$ , with pico-/nanophytoplankton and heterotrophic nanoflagellates; and (3) 10–50  $\mu\text{m}$ , with larger nano-/microphytoplankton, ciliates and rotifers. The description of phytoplankton and zooplankton composition in the 0.7–10 and 10–50  $\mu\text{m}$  size fractions was based on microscopic examination of samples from this study and Baltic Sea phytoplankton size classification (Olenina et al. 2006) and zooplankton taxonomy (Telesh et al. 2009). We collected the 0.7–10 and 10–50  $\mu\text{m}$  size fractions by using GF/F glass fiber filters (25 mm, 0.7  $\mu\text{m}$  pore size). The 0.3–0.7  $\mu\text{m}$  size fraction was filtered on GF-75 glass fiber filters (Advantec, 25 mm, 0.3  $\mu\text{m}$  pore size). Both GF/F and GF-75 filters were pre-combusted at 450°C in a muffle furnace (Nabertherm LT 5/11/P33) for 5 h before filtration. The volumes filtered depended on the particle concentration of the water and were between 500 and 1000 ml. Two replicate filters were prepared for CNP analyses. Filters for particulate organic C and particulate organic N were analyzed using a CHN elementary analyzer (Leco, TruSpec Micro CHN/CHNS Determinator), and particulate organic P was quantified using the ash-hydrolysis method (Solórzano & Sharp 1980).

#### 2.5. Bacteria–phytoplankton interactions and transfer efficiency to higher trophic level

Measuring the interactions between bacteria and phytoplankton is not straightforward in planktonic food webs. In this study, we used the biomass ratio of autotrophs to heterotrophic bacteria, AU:BA, as an index to estimate the relative competition and coupling between treatments. For example, if the AU:BA ratio is higher in one treatment compared to the other, the dominance of autotrophs over bacteria could be due to ammonium regeneration by bacteria in this treatment. Besides the ratio of the overall autotroph biomass to bacteria, we also calculated the biomass ratio of different autotroph size fractions (e.g. autotrophs <3  $\mu\text{m}$  [Pico<3], autotrophs between 3 and 10  $\mu\text{m}$  [AU<10] and autotrophs between 10 and 50  $\mu\text{m}$  [AU>10]) to bacteria.

To assess food web transfer efficiency, we first calculated the biomass of the basal food web resource (Basal) by summing up the biomass of bacteria and picophytoplankton. We assumed that mixotrophs and heterotrophs were mainly grazing on small autotrophs (picophytoplankton) and bacteria. Then, we used the biomass ratios of mixotrophs and heterotrophs to the basal food web resource as the index of food web transfer efficiency. The heterotrophs were also classified into 2 size fractions, heterotrophs <10  $\mu\text{m}$  (HT<10) and heterotrophs >10  $\mu\text{m}$  (HT>10). For instance, if the resource ratio of heterotrophs >10  $\mu\text{m}$  to the basal food web (HT>10:Basal) is higher in one treatment compared to the other, this means that heterotrophic consumers gain relatively higher biomass per unit of available resource, indicating a higher food web transfer efficiency in this treatment.

#### 2.6. Statistical analyses

One-way ANOVAs were used to compare differences in the C:N, C:P and N:P ratios of each seston size fraction (0.3–0.7, 0.7–10 and 10–50  $\mu\text{m}$ ). We used a 3-way repeated-measure ANOVA to analyze how the AU:BA biomass ratios were affected by time (Days 5 and 9), treatment (DIN/DOC and DON addition) and size class of phytoplankton (Pico, AU<10 or AU>10). To test how the basal food resource could influence the biomass of their grazers, we performed a 3-way repeated-measure ANOVA to analyze how the ratio of the biomass of grazers to the biomass of their assumed resource (picophytoplankton + bacteria) was affected by time (Days 5 and 9), treatment (DIN/DOC and DON addition) and size class of grazers (MX, HT<10 or HT>10). The samples were taken from each microcosm over time repeatedly; therefore, it was necessary to apply ANOVAs for repeated measures. The main models used for 3-way repeated-measure ANOVAs consist of 1 between-group factor (Treatment) and 2 within-group factors (Day and Size class) (of phytoplankton or grazers).

All statistical analyses above were conducted using R (version 3.5.2) (R Core Team 2019). All data for ANOVAs were ln transformed *a priori*, according to the recommended practice for ratio data (Keene 1995, Isles 2020). The assumption for normality and homogeneity of variance was assessed on the residuals from the ANOVAs, through visual inspection of residual vs. fitted, square root residual vs. fitted, normal quantile–quantile and residual vs. leverage plots. The significance level was set at  $\alpha = 0.05$ .

### 3. RESULTS

#### 3.1. C, N and P dynamics in the water

Dissolved C, N and P concentrations in the water, measured just after collection, amounted to  $3800 \mu\text{g C l}^{-1}$  (DOC),  $215 \mu\text{g N l}^{-1}$  (TDN) and  $10 \mu\text{g P l}^{-1}$  (TDP) (Fig. 2). However, after the first nutrient addition on Day 0, the CNP concentrations in the water increased to approximately  $5000 \mu\text{g C l}^{-1}$ ,  $500 \mu\text{g N l}^{-1}$  and  $140 \mu\text{g P l}^{-1}$  in both types of CN additions. Even though there was no DIN addition to the DON-supplemented microcosms, DIN soon accumulated due to rapid ammonium regeneration (Fig. 2A,B). Hence, DIN was available during the whole experiment in both the CN-associated and CN-dissociated incubations. Nitrate was predominant where C and N were supplied separately (Fig. 2C), while ammonium was the prevailing inorganic N form where DON was added (Fig. 2B). The DON concentrations showed a strong decrease from Day 1 to Day 3 in the DON-supplemented microcosms (Fig. 2D), indicating significant DON consumption. In contrast, DON accumulated from the start to Day 7 in the DIN/DOC-associated microcosms (Fig. 2D), showing that DON was internally generated in this system.

The 2 types of CN additions induced large differences in the timing of C uptake. The major DOC reduction occurred already at Day 1 in the DON-supplemented microcosms, while the uptake mainly occurred after Day 3 in the DIN/DOC-associated microcosms. However, after Day 5, the DOC concentrations were relatively similar in both types of additions (Fig. 2F).

#### 3.2. Seston stoichiometry response

At the end of the experiment, the C:N ratio in the size fraction  $0.3\text{--}0.7 \mu\text{m}$  was slightly higher in the DIN/DOC treatment than in the DON microcosms ( $p = 0.00997$ , Table S1 in the Supplement at [www.int-res.com/articles/suppl/a088p187\\_supp.pdf](http://www.int-res.com/articles/suppl/a088p187_supp.pdf), Fig. 3A). No other stoichiometric CNP differences in seston were found between treatments (Table S1, Fig. 3A,B,C).

#### 3.3. Trophic dynamics within the food web

##### 3.3.1. Response of basal trophic levels (bacteria and phytoplankton) to DON and DIN/DOC additions

Heterotrophic bacteria and picophytoplankton constituted the lowest trophic levels in the system. The 2 groups showed significant temporal trends. Initially, the heterotrophic bacteria did not respond to any of the CN additions, but after Day 1, their abundance increased. The bacterial abundance increased rapidly until Day 5 and dropped dramatically to its original level after Day 7. An increase occurred on Day 9, with higher values in the DON treatment (Fig. 4A). Both picoeukaryotes and picocyanobacteria showed larger increases in the DON addition than in the DIN/DOC-associated microcosms during the first 5 d, followed by a marked decrease where the abundance of picoeukaryotes and picocyanobacteria dropped to 10 times their initial abundance (Fig. 4B,C).

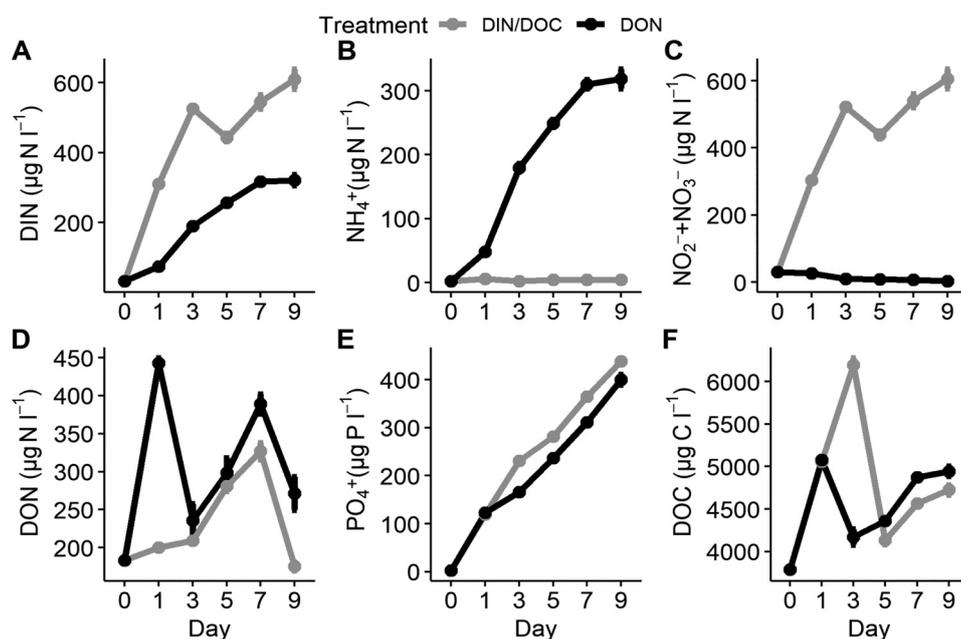


Fig. 2. Nutrient concentration (mean  $\pm$  SE) of (A) dissolved inorganic N (DIN), (B) ammonium ( $\text{NH}_4^+$ ), (C) nitrate + nitrite ( $\text{NO}_2^- + \text{NO}_3^-$ ), (D) dissolved organic N (DON), (E) phosphate ( $\text{PO}_4^{3-}$ ) and (F) dissolved organic C (DOC)

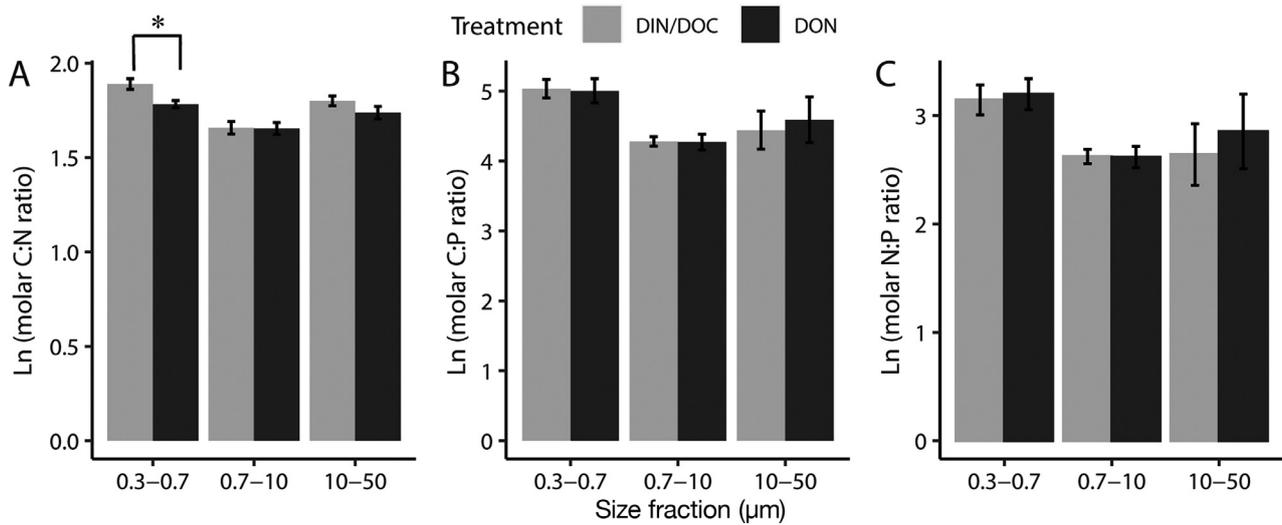


Fig. 3. Molar C:N:P ratios of different size fractions (mean  $\pm$  SE) at the end of the experiment: (A) C:N ratio, (B) C:P ratio and (C) N:P ratio. All ratios were Ln transformed. \*Significant at 5% level. Abbreviations as in Fig. 1

The AU:BA biomass ratios of different size classes showed variations in relation to CN addition and time of the experiment (Table S2). The overall AU:BA ratios were consistently higher in the DON addition than in the DIN/DOC-associated microcosms on both Days 5 and 9. However, the largest and significant difference on Day 5 was observed for the picophytoplankton fraction, while on Day 9, the difference appeared to be largest for the AU<10:BA ratio (Fig. 5A).

### 3.3.2. Response of higher trophic levels (mixotrophs and heterotrophs) to DON and DIN/DOC additions

Since the basal trophic levels responded differently to DON and DIN/DOC additions, we expected concomitant alterations of the grazers, e.g. mixotrophic and heterotrophic grazers. The ratio of grazer biomass to basal food resource showed similar responses for all higher trophic levels (MX, HT<10, HT>10); however, the ratios were higher in the DON addition than in the DIN/DOC-associated micro-

cosms (Table S3, Fig. 5B). The largest difference between treatments was observed for the MX:Basal and HT<10:Basal ratios on Day 5 and the HT>10:Basal ratio on Day 9 (Fig. 5B).

### 3.3.3. Whole food web response to DON and DIN/DOC additions

To synthesize the effect of the 2 different types of CN additions, we summarized the biomass structure of the whole food web (Fig. 6). In the original water, picophytoplankton and heterotrophic bacteria equally dominated the food web, while the biomass of larger phytoplankton and smaller heterotrophic grazers was low. In the middle of the experiment (Day 5), bacteria had doubled their C biomass in both the DON addition and DIN/DOC-associated microcosms. However, in the system receiving DON, there was significantly higher biomass of picophytoplankton, mixotrophs <10  $\mu$ m and larger phytoplankton (AU>10), while grazers such as rotifers (zooplankton >10  $\mu$ m, ZP>10) and ciliates (HT>10) started to

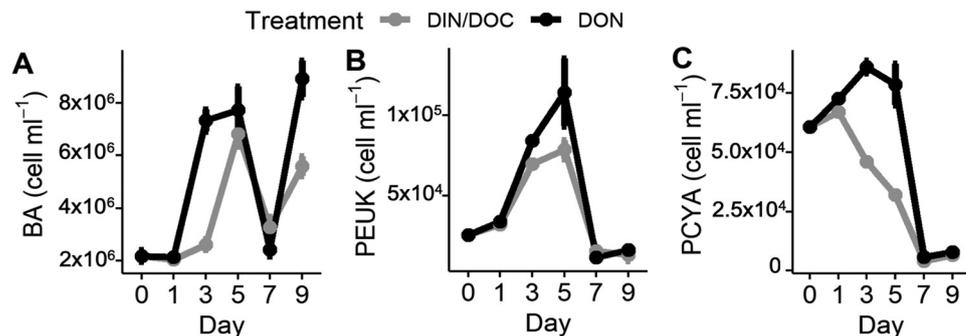


Fig. 4. Abundance (mean  $\pm$  SE) of (A) bacteria (BA), (B) picoeukaryotes (PEUK) and (C) picocyanobacteria (PCYA). Abbreviations as in Fig. 1

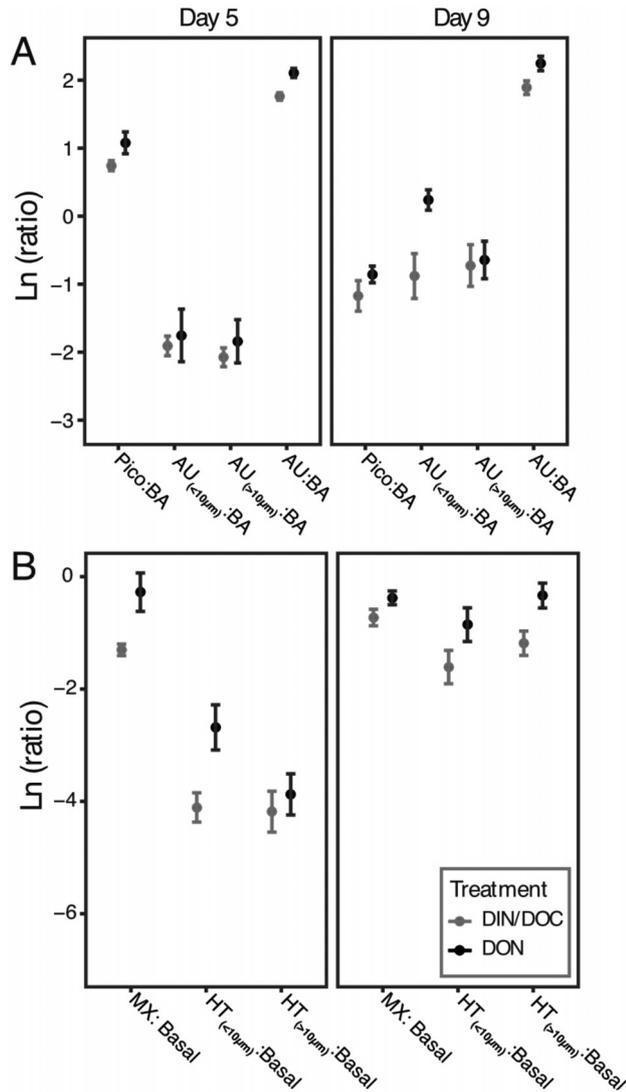


Fig. 5. (A) Ratio of autotroph to bacterial biomass (mean  $\pm$  SE) in different size fractions at Days 5 and 9. Pico:BA = picophytoplankton to bacteria biomass ratio;  $AU_{(<10 \mu m)}$ :BA = autotrophs  $<10 \mu m$  to bacteria biomass ratio;  $AU_{(>10 \mu m)}$ :BA = autotrophs  $>10 \mu m$  to bacteria biomass ratio; AU:BA = ratio of the biomass of all autotrophs to the biomass of bacteria. Cases where the ratio is higher in one of the treatments than in another indicate a greater coupling. (B) Food web transfer efficiency from basal food web resources to higher trophic levels: mixotroph and heterotroph biomass to basal food resource biomass (sum of picophytoplankton and bacterial biomass) ratios (mean  $\pm$  SE) in different size fractions at Days 5 and 9. MX:Basal = mixotroph to basal food resource biomass ratio;  $HT_{(<10 \mu m)}$ :Basal = heterotrophs  $<10 \mu m$  to basal food resource biomass ratio;  $HT_{(>10 \mu m)}$ :Basal = heterotrophs  $>10 \mu m$  to basal food resource biomass ratio. Cases where the ratio is larger in one of the treatments than in another indicate a higher food web transfer efficiency. All ratios were Ln transformed prior to plotting. Abbreviations as in Fig. 1

produce more biomass in the middle of the experiment (Day 5). At the end of the experiment (Day 9), the food web structure diverged even more between

the different types of additions. In the  $<10 \mu m$  fraction, heterotrophic bacteria gained more biomass in the DON than in the DIN/DOC addition. The same observation applies to the other components within this size fraction. To sum up, DON addition yielded higher C biomass in the whole food web compared to DIN/DOC addition (Fig. 6).

#### 4. DISCUSSION

To the best of our knowledge, this study is the first to try to go beyond nutrient enrichment to consider the food web effect of organic C associated with different forms of N. By using the 2 most contrasted possible treatments (100% associated C and N vs. 100% dissociated C and N), we increase the possibility to capture any potential effect of this elemental association. Of course, the study has a few limitations. For instance, (1) it was a short-term experiment, so the results will possibly represent temporary effects; (2) the continuous addition of DON or DIN/DOC probably prevents competition to strongly affect the basis of the food web; (3) C and N were added in similar amounts in all treatments, thus preventing us by design to test for an interactive effect between the amounts of nutrient addition and C and N association; and (4) the remineralization of the DON addition led to different DIN species in both treatments. In natural systems, nitrate is often the predominant form of N available in the water (Korth et al. 2014). Further, phytoplankton have a higher affinity for nitrate than do bacteria; therefore, using nitrate in the DIN addition allows testing the most contrasting sides of the bacteria–phytoplankton interaction.

Despite such limitations, the results of the study showed that changes in the food web between the treatments were rapid and significant. The repeated addition of C and N had marked positive effects on the growth of a nutrient-limited microplanktonic food web (Gruner et al. 2008) associated with delayed top-down responses that led to a subsequent biomass decrease in lower trophic levels. In addition to these effects of nutrient addition, the degree of association between C and N added further impacts on the productivity and efficiency of the microbial food web. The DON addition promoted the entire microplankton food web, and at the end of the experiment, the biomass of the whole food web was much higher than that in the DIN/DOC treatment. All trophic levels had been influenced by the addition of the associated or dissociated C and N, which could possibly, through altering the bacteria–phytoplankton inter-

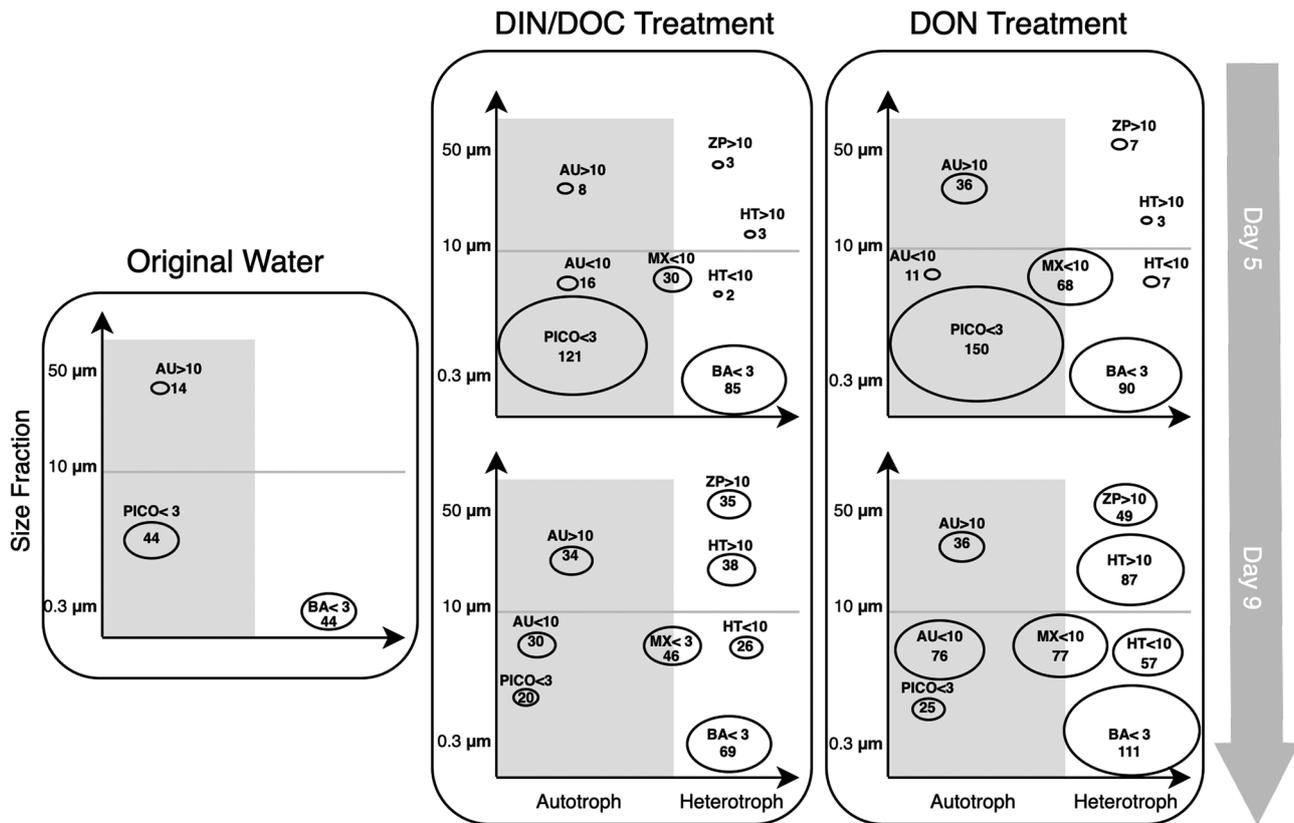


Fig. 6. Food web structure in the original water, and dissolved inorganic N/dissolved organic C (DIN/DOC) and dissolved organic N (DON) treatments at Days 5 and 9. The average C biomass of different functional groups is presented within ellipses ( $\text{mg C m}^{-3}$ ), and their size is proportional to the biomass. The grey area represents autotrophs, and the white area represents heterotrophs. BA: bacteria; Pico: picophytoplankton; AU: autotrophs; MX: mixotrophs; HT: heterotrophs; ZP: microzooplankton

action, switch between increased coupling or increased competition. More importantly, our study suggests that trophic interactions within microplankton food webs vary depending on whether the C and N elements are supplied associated in 1 molecule or dissociated in 2 different molecules, highlighting the importance of the chemical form of N inputs to the short-term dynamics of microplankton food webs. Although provided with the same total amount of C and N, almost all food web components, including bacteria, autotrophs, mixotrophs and heterotrophs, showed a marked increase in biomass when N and organic C were supplied associated in a single molecule (DON addition) compared to the addition where N and organic C were supplied in dissociated molecules (DIN/DOC addition). There are several lines of arguments converging to suggest that this positive effect results from a relative shift of the bacteria–phytoplankton interaction from competition to increased coupling with C and N in association: (1) ammonium concentrations were substantially higher when supplying DON, showing that large amounts of ammonium were internally regenerated from DON

in the microcosms, to support further phytoplankton growth, while in the DIN/DOC addition, phytoplankton and bacteria mainly competed for nitrate; (2) in the DON addition, bacteria had a C:N ratio that was slightly lower than that in the DIN/DOC addition, suggesting that bacteria were relatively C limited in this treatment; (3) bacterial and autotrophic biomasses were higher when adding DON, and the AU:BA biomass ratio also was higher. All these results suggest that bacteria synergistically benefitted the phytoplankton by utilizing DON and recycling N as ammonium. The effect of DON became, to some extent, the effect of ammonium. This important result may lead one to hypothesize that when N is added as ammonium instead of nitrate in the DIN/DOC treatment, the differences between the 2 treatments might have been much less. Our choice of nitrate in the DIN/DOC treatment is based on the importance of this nutrient as a source of N in coastal environments worldwide and singularly in the Baltic Sea (Korth et al. 2014). By using nitrate as the source of inorganic N, our experimental design helped uncover the recycling of organic N in a highly bioavailable form as one of the

mechanisms by which different degrees of CN association may affect coastal food webs. To uncover other mechanisms, it will be important to test other experimental designs, where, for example, DIN would be supplied either as ammonium or nitrate or as combinations of both nutrients. The commensalism interaction between bacteria and phytoplankton promoted by the recycling of ammonium was then transferred to mixotrophic and heterotrophic grazers, since the higher overall biomass of mixotrophic and heterotrophic grazers and higher grazer to prey biomass ratios were found in the DON addition.

#### 4.1. Effect of different associations of C and N on stoichiometry

Although the stoichiometric composition was different between size fractions, the only difference due to C and N association was found for the size fraction 0.3–0.7  $\mu\text{m}$ , i.e. the bacterial fraction. A previous study showed that the bacterial C:N ratio can be more flexible at the community level even though bacteria at the strain level can maintain a certain degree of homeostasis (Makino et al. 2003). In particular, bacterial strains with lower C:N ratios are more competitive under C limitation (Godwin & Cotner 2014). In our study, the supplied DON had a lower C:N ratio (= 5) than the bacterial fraction (~9 at Day 0 and ~5.5 at Day 9), suggesting excessive N uptake by bacteria when utilizing DON. An alternative mechanism for the lower C:N ratio might be physiological. In effect, classical experiments on the growth of bacteria under dual C and N nutrient limitation (e.g. Egli 1991) found that bacteria had lower C:N ratios under an excess of available N (as was likely the case in our DON-addition treatment in which ammonium accumulated). Irrespective of the mechanism, the observed change in stoichiometry was modest and not reflected in the C:P ratio. Thus, the question of its biological relevance remains open.

At the end of the experiment, there was no difference in the stoichiometric composition of the 2 other fractions, 0.7–10 and 10–50  $\mu\text{m}$ , even though the taxonomic compositions of these 2 size fractions were largely different: the 0.7–10  $\mu\text{m}$  fraction contained small mixotrophs, pico- and nanophytoplankton and nanoflagellates, and the 10–50  $\mu\text{m}$  fraction contained mostly ciliates, rotifers and large phytoplankton (but to a lesser extent). Ciliates and rotifers are known to be stoichiometrically homeostatic (Golz et al. 2015) and could be responsible for the similar stoichiometry of the 10–50  $\mu\text{m}$  fraction between treatments.

Smaller unicellular plankton are known to be more stoichiometrically flexible (Golz et al. 2015), except mixotrophs, which can better balance their assimilation of elements because they combine phototrophy and phagotrophy at the same time (Mitra et al. 2014, Ward & Follows 2016). Mixotrophs have intermediate stoichiometric homeostasis, more variable than strict heterotrophs but less variable than flexible phytoplankton (Katechakis et al. 2005, Chrzanowski et al. 2010). Since mixotrophs dominated the 0.7–10  $\mu\text{m}$  fraction in biomass, they may offer an explanation as to the relative stoichiometric stability of this size fraction between treatments. However, given the high stoichiometric flexibility observed within and between species that make functional groups, depending on environmental conditions and relative growth rate (see, e.g., Finkel et al. 2010, Godwin & Cotner 2017), only a detailed stoichiometric analysis at the species level could explain with certainty the patterns in stoichiometry observed in our experiment.

#### 4.2. Effect of different associations of C and N on bacteria–phytoplankton interaction

The molecular association between C and N yielded higher biomass of bacteria. Besides a substantial increase in the overall biomass at the end of the experiment in the DON addition, the average biomass of the autotrophs was more than twice as large as that in the DIN/DOC addition. These outcomes may result from the addition of C and N in associated form, which triggered early mineralization of the supplied DON releasing high amounts of ammonium. Due to the additional energy cost needed to reduce nitrate for assimilation, phytoplankton prefer to use ammonium above nitrate (Glibert et al. 2016). Hence, the increased availability of a better-quality N resource in the DON treatment could promote higher trophic transfer efficiency through the food web and might explain why the total autotroph biomass was significantly higher in the DON addition. The remarkable increases in both bacterial and phytoplankton biomass confirmed that enriching DON for the microbial food web could promote commensalism between bacteria and phytoplankton.

Different size classes of phytoplankton reacted differently to C and N association and thus interacted with bacteria differently. The smaller phytoplankton responded rapidly to the addition of resources to the point where all microcosms tended towards picophytoplankton dominance in the middle of the experiment, probably due to strong bottom-up control and

the quasi-absence of consumers. However, the picophytoplankton response also showed an earlier differential response to the additions, with higher biomass in the DON addition from the very first days, while the other phytoplankton size classes showed differences between treatments only near the end of the experiment (Fig. S1). An earlier study has shown that picophytoplankton are major users of regenerated ammonium (Chang et al. 1995), supporting their early response to accumulated ammonium concentration in the DON addition. It is also possible that the picophytoplankton may have used some of the DON directly, as a source of N. Previous studies showed that various groups of phytoplankton can take up DON using pinocytosis or cell surface enzymes (Berman & Bronk 2003, Bronk et al. 2007). However, such mechanisms are not specific to picophytoplankton; for instance, pinocytosis is also common in dinoflagellates and especially for ingesting dissolved macromolecules and thus cannot fully explain their early response.

Overall, our results are indicative of a strong coupling between bacteria and picophytoplankton when supplying C and N associated as DON, through the regeneration of ammonium by bacteria and a shift in food web transfer pathways. However, the response of the phytoplankton diverged between different size fractions, likely depending on the initial abundance and growth characteristics but also on different degrees of top-down control from higher trophic levels, as all the trophic levels of the food web showed significant responses to the treatment.

#### 4.3. Effect of different associations of C and N on whole food web dynamics

Besides phytoplankton and bacteria, the heterotrophic components of the food web also benefitted from the association between C and N; mixotrophs, nanoflagellates (HT < 10  $\mu\text{m}$ ), ciliates (HT > 10  $\mu\text{m}$ ) and larger zooplankton (ZP > 10  $\mu\text{m}$ ) all reached higher biomass in the DON treatment both in absolute terms and relative to the biomass of their resources. These increases at the higher trophic levels indicate that the benefits at lower levels from the commensalistic interaction between phytoplankton and bacteria were transferred up the food web. Hence, the whole microplankton food web benefitted from the DON addition. The higher overall biomass of the food web in the DON addition may partly result from the higher C content of the different size fraction of the food web (Fig. S2), suggesting that a large percent-

age of C supplied as a dissolved resource is locked in biomass and thus available for consumption by higher trophic levels.

Higher trophic levels exerted feedbacks on the bacteria–phytoplankton dynamics through top-down control. At the end of the experiment, the increase in heterotrophic grazers co-occurred with a collapse in the picophytoplankton biomass. The picophytoplankton biomass was reduced to levels below those at the start of the experiment, and autotrophs were dominated by cells between 3 and 10  $\mu\text{m}$ . The decline of picophytoplankton occurred despite continuous enrichment of resources in the microcosms but followed an increase in the abundance of consumers, suggesting that the picophytoplankton were significantly grazed at the end of the experiment in both treatments, while the 3–10  $\mu\text{m}$  autotrophs were released from grazing pressure and became dominant. According to previous studies, in both freshwater and marine systems, picophytoplankton in the size range of 0.3 to 3  $\mu\text{m}$  are the most important food source for microzooplankton (Šimek et al. 1995, Zingel et al. 2007, Grinienė et al. 2016). The most abundant consumers in our experiment were ciliates and rotifers larger than 10  $\mu\text{m}$  (Fig. S1), which graze on both bacteria and picophytoplankton but with a strong preference for the latter. Our data on stoichiometry composition also show that the size fraction 0.7–10  $\mu\text{m}$  (picophytoplankton) had a lower C:N ratio than the size fractions 0.3–0.7  $\mu\text{m}$  (bacteria) and 10–50  $\mu\text{m}$  (microplankton) in both treatments. Hence, picophytoplankton might represent a higher-quality food for ciliates and rotifers than do bacteria. Thus, differential consumer top-down control on the various basal trophic levels based on stoichiometric preferences may be another important factor controlling food web structure in response to C and N association.

## 5. CONCLUSIONS

Although utilizing different forms of N by the aquatic food web has been extensively studied, the concept of linking N to C upon different degrees of association for bacteria–phytoplankton interaction is novel. Our results show that the degree of association between N and C is an important factor to consider in the study of microplankton food web dynamics, at least when subjected to short-term perturbation in the form of nutrient additions. The association or dissociation of C and N inputs has important effects on all trophic levels, most probably through its effect on bacteria–phytoplankton interactions, which switch

from commensalism to competition. Indeed, the entire microbial food web benefitted when C and N were associated in a single DON molecule. The important role of DON in promoting the productivity and efficiency of the microbial food web calls for the inclusion of this factor in the study of the effects of inputs of allochthonous dissolved organic matter in coastal food webs. In the case of the northern Baltic ecosystem, terrestrial runoff and freshwater inputs are predicted to increase in the future as a consequence of climate change (Andersson et al. 2015), increasing N loads. Since terrestrial runoffs in northerly regions often bring organic C and N compounds in an associated form, coastal zones may be more affected by DON than other ecosystems in the near future. We thus recommend to evaluate and expand the results of this pioneer experiment with further experimentation. A more comprehensive gradient of coupling between C and N could be explored. Association between C and other nutrients, such as P and S, could be addressed, depending on the ecological system investigated. The use of isotopes as a tracer of elemental circulation among the various trophic levels, and of relevant molecular approaches for the monitoring of potential changes in the taxonomic composition of the various trophic levels, would be valuable extensions to our experimental approach.

*Acknowledgements.* This project was supported by funds from Umeå Marine Sciences Center to J.G. and by the Swedish marine strategic research environment EcoChange (the Swedish Research Council Formas) to A.A. We thank Jonas Forsberg for phytoplankton analysis; Joanna Paczkowska for helping in the lab; and Ann-Kristin Bergström, Sebastian Diehl and Göran Englund for reviewing the manuscript. We are grateful to the staff at the Umeå Marine Sciences Center for their expert assistance, in particular Henrik Larsson for chemical analyses and experimental setup. M.C. acknowledges financial support from the Knut and Alice Wallenberg Foundation via the project 'Climate change induced regime shifts in northern lake ecosystems.'

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*Editorial responsibility: Ilana Berman-Frank, Haifa, Israel*

*Reviewed by: C. M. Godwin, A. Lewandowska and 1 anonymous referee*

*Submitted: January 19, 2022*

*Accepted: November 3, 2022*

*Proofs received from author(s): December 12, 2022*