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Abstract

The role of zooplankton in microbial nitrogen turnover in marine environments is poorly understood. Here, we present results from two experiments designed to determine the excretion rate of ammonium and dissolved organic nitrogen (DON) by dominant copepods, *Acartia tonsa* and *Paracalanus* cf *indicus*, fed with two natural sized-fractioned diets (20–150 μm and < 20 μm), and its possible effects on the transcriptional activity of ammonia monooxygenase subunit A (*amoA*), a functional marker for ammonia-oxidizing archaea (AOA) and bacteria (AOB), as a response to the input of ammonium and DON by copepod excretion, during autumn and winter in central/southern Chile. Our results reveal that DON was the main excretion product, with rates up to 3.7 μmol L^{-1} h^{-1}. DON production increased in copepods fed with the small-sized food. Ammonium was also excreted, with rates up to 0.08 μmol L^{-1} h^{-1} in autumn and 0.4 μmol L^{-1} h^{-1} in winter, and rapidly consumed by the microbial community, decreasing down to 0.07 μmol L^{-1} between the initial time to 4 h. Ammonium consumption coincided with increased AOB and AOA *amoA* transcript copies in copepods fed with the larger-sized food, while a different microbial community, probably heterotrophic, reacted to nitrogen input via excretion by copepods fed with the smaller-sized food in autumn. AOA-A was transcriptionally active in winter with nearly zero ammonium concentration, suggesting that AOA outcompete AOB when ammonium becomes limited. We conclude that nitrogen excreted by copepods can be used directly by microbial communities, including nitrifying ones. Zooplankton excretion may thus provide significant remineralized nitrogen for new and regenerated production in the upper ocean.

The contribution of zooplankton to the biogeochemical cycles has traditionally been viewed as the transfer of nitrogen and carbon from photoautotrophic to larger heterotrophic organism, and its role for the sink of carbon and nitrogen to deep water. However, studies on the process producing dissolved compounds in the euphotic zone in marine environments have demonstrated that zooplankton also play an important role in the active transport of dissolved organic matter (DOM), and are capable of fueling the microbial loop (Richardot et al. 2001; Steinberg et al. 2002). In this sense, zooplankton can substantially contribute to environmental DOM through excretion, sloppy feeding (Møller 2004, 2007), and leakage from fecal pellets (Hasegawa et al. 2000; Steinberg et al. 2002). The importance of zooplankton excretion in the euphotic zone has been reported by several authors (Alcaraz et al. 1994; Le Borgne and Rodier 1997; Ikeda et al. 2006; Pérez-Aragón et al. 2011), but there is scarce research on the effect of excreted DOM by zooplankton, including its role as nitrogen supplier for microbial communities (Richardot et al.

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2001; Titelman et al. 2008). The importance of predator-prey interaction as a contribution for microbial food webs depends on the type of DOM substrate produced and prevailing limitations. For example, copepod feeding has been shown to enhance organic phosphorus uptake by bacterial activity in a phosphorus-limited system (Titelman et al. 2008).

It has generally been found that ammonium is the primary nitrogenous product excreted by marine zooplankton, comprising up to 75% of the total nitrogen, followed by urea and amino acids, which can account for 25% (Bidigare 1983). However, other studies on zooplankton excretion have shown that dissolved organic nitrogen (DON) in the form of urea and amino acids is a significant excretion product and can range from 7% to 80% of total dissolved nitrogen release, exceeding even inorganic nitrogen excretion (Steinberg et al. 2000, 2002; Steinberg and Saba 2008). Zooplankton diet can play an important role in determining the type of compounds released via excretion. For instance, studies have shown that organic nitrogen excretion increases with respect to inorganic excretion when zooplankton is starved (Miller and Glibert 1998; Miller and Roman 2008). Also, Saba et al. (2009) observed higher ammonium and dissolved organic carbon excretion when copepods were fed with a carnivorous rather than an omnivorous diet, while in the latter case more urea was released. Furthermore, the quality of food ingested, in terms of the N : C ratio, can affect the nitrogen compounds excreted. For example, Miller and Roman (2008) reported that ammonium excretion was 30% lower with copepods fed senescent diatoms and detritus (with lower N : C ratios than in copepods themselves) compared to copepods fed with diatoms and ciliates, which have similar N : C ratios to those of copepods. In nature, zooplankton may indeed experience these variable chemical conditions of their food resources. The coastal upwelling zone in southern central Chile represents a good example for such variation. It is a highly heterogeneous system, characterized by seasonal wind-driven upwelling of subsurface nutrient-rich and oxygen-poor waters, which influences the chemical and biological components of surface waters, i.e., in austral summer there is an abundance of chain forming diatoms. In contrast, small nanoflagellates are numerically more important in winter (Anabalón et al. 2007). Therefore, oceanographic variability affecting the availability of food for zooplankton can determine their excreted compounds in the euphotic zone.

Ammonium is the most commonly regenerated product released during bacterial DOM remineralization (Bronk et al. 2007), zooplankton excretion (Alcaraz et al. 1994), and photochemical breakdown of DOM (Rain-Franco et al. 2014). It promotes regenerated production in marine ecosystems (Dugdale and Goering 1967) and it is rapidly consumed in surface waters through assimilation, by phototrophic and heterotrophic plankton, including microbial communities with different life styles, and also by aerobic ammonia-oxidizing bacteria (AOB) and archaea (AOA) (Fernandez and Farías 2012; Molina

et al. 2012), and possibly by anaerobic oxidizers by anammox, eventually occurring in surface waters (Galán et al. 2012). Nitrifying microorganisms can couple aerobic chemoautotrophic metabolism with ammonia oxidation via nitrite to nitrate conversion in two steps: ammonia-oxidation and nitrite-oxidation, carried out by two functional groups of microorganisms, i.e., ammonia and nitrite oxidizers. Although a recent study showed that a single nitrite oxidizing bacteria belonging to the genus *Nitrospira* can do both steps and its relevance in marine pelagic environments is still unknown (Daims et al. 2015). This conversion plays an important biogeochemical role in the pelagic system by remineralizing organic matter and then supplying oxidized forms of nitrogen to photosynthesis, affecting our estimation of new vs. regenerated primary production (Yool et al. 2007). In fact, it has been reported that nitrification is active in marine euphotic zones (Wankel et al. 2007; Santoro et al. 2010; Shiozaki et al. 2016) and a recent study found that ammonium excretion and microbial nitrification affect the capacity of epipelagic ecosystems to retain their nutrients (nutrient retention efficiency [NRE]), and thus to maintain high levels of regenerated production (Fernández-Urruzola et al. 2016).

The first and usually limiting step in nitrification is carried out by archaea and mainly Betaproteobacterial ammonia-oxidizers (AOA and AOB, respectively) in the coastal zone of southern central Chile. This has been suggested based on their abundance and potential activity (Molina et al. 2010, 2012). The distribution of AOA and AOB in various environments suggests that these communities respond differently to environmental factors such as pH, sulfide, phosphate and ammonium levels (Erguder et al. 2009), light, and micronutrients availability (probably cooper) (Shiozaki et al. 2016). Physiological studies of *Nitrosopumilus maritimus* SCM1 have shown a greater affinity for ammonium (lower K_m) than does AOB, thus this AOA is well adapted to grow at low ammonium levels, which provides it with a competitive advantage (Martens-Habbena et al. 2009). Despite these important findings, the impact of different predator-prey processes on the composition of organic matter and nutrient release and the subsequent impact on nitrifying communities are largely unknown.

In this study, we determined the main nitrogen compounds excreted (DON and ammonium) by dominant species of copepods and its possible effects on the transcriptional activity of ammonia monooxygenase subunit A (*amoA*), a functional marker for AOA and AOB, as a response to the input of ammonium and DON by copepod excretion, during the non-upwelling season in southern central Chile.

Methods

Study area

The study was conducted in the coastal area of southern central Chile in 2012 (Fig. 1). Two experiments, named as
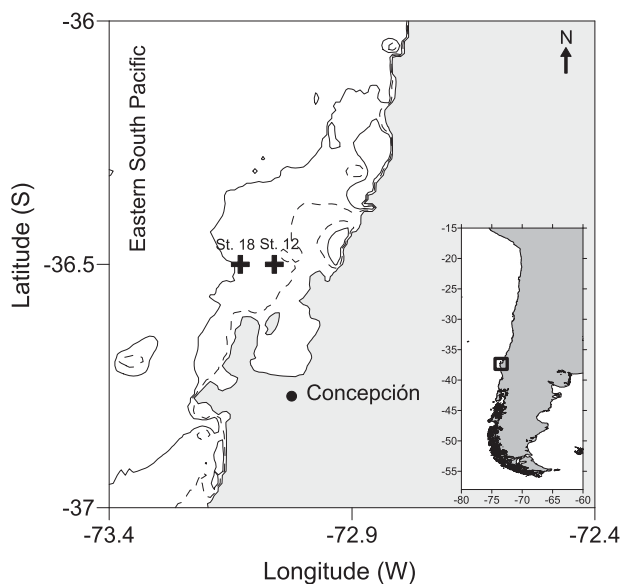
Fig. 1. The eastern south Pacific and the coastal upwelling zone off Concepcion, illustrating the sampling stations. Sta. 12 for CopeMOD experiments and Sta. 18 (COPAS Time Series) is included as a reference. The solid contour line indicates 100 m depth and the long dashed contour line indicates 50 m depth.

CopeMOD (copepods and dissolved organic matter), were conducted to assess the nitrogen compounds excreted by copepods and their subsequent utilization by microbial communities, including AOA and AOB, under non-upwelling conditions. The first associated with autumn and the second with winter conditions (10th May 2012 and 9th September 2012, respectively). The sampling site for the CopeMOD experiments was Sta. 12, located at 12 miles from shore, as is indicated in Fig. 1.

Physical, chemical, and biological in situ measurements during CopeMOD experiments

During the sampling, we first assessed physical and chemical parameters, temperature (°C), salinity (‰), and oxygen (mL O_2 L^{-1}) with a CTD-O profiler (SeaBird SBE 25 Plus). Water samples were taken at 10 m depth at Sta. 12, in order to examine the available food for copepods at this sampling site. At this depth, a chlorophyll a maximum is commonly found in this area (Escribano and Schneider 2007). Seawater samples were collected in 10-liter Niskin bottles for total and fractioned Chl a, particulate organic carbon (POC), particulate organic nitrogen (PON), microplankton and nanoplankton abundance as follows:

Samples for total Chl a were taken in duplicate (500 mL) and filtered through GF/F filters (0.7 μm; Whatman). Samples for size fractioned Chl a were pre-filtered by a 20 μm sieve and filtered through GF/F filters (0.7 μm; Whatman; 500 mL in triplicate). Both samples were measured by the fluorometric method of Parsons et al. (1984). Total and fractioned POC and PON samples (500 mL) were taken in duplicate using precombusted (450°C, 6 h) GF/F filters (Whatman) and analyzed

following Bodungen et al. (1991), using mass spectrometry elemental analysis (Finnigan IRMS Delta Plus at Universidad of Concepción). Seawater samples for phytoplankton composition were collected and analyzed in two size fractions, nanoplankton ($< 20\,\mu$m) and microplankton (20–150 μm), according to Anabalón et al. (2007). Samples for nanoplankton were taken in centrifuge tubes (50 mL in duplicate) and immediately preserved with glutaraldehyde (2% final concentration; Merck). Samples were analyzed by epifluorescence microscopy (Porter and Feig 1980). Microplankton samples were collected in duplicate, in 250 mL plastic tubes and preserved with Lugol's solution (Merck) and analyzed using the Utermöhl inverted microscopy method (Villafañe and Reid 1995).

CopeMOD experiments

Live copepod samples for experiments were collected with a WP2 zooplankton net with a 200 μm mesh size, equipped with a 5 L non-filtering cod-end, between 0 m and 50 m depth under daylight conditions. Live samples were immediately transferred to coolers and diluted with surface seawater until arrival ($\sim$ 1 h after sampling) to the laboratory at the Dichato Marine Station of University of Concepcion. One sample was also taken with a filtering cod-end and preserved immediately with formalin buffered by sodium borate (5% final concentration) for zooplankton composition analysis (Turner 1976; Postel et al. 2000).

Nitrogen excretion by copepods

In the laboratory, undamaged zooplankton individuals were sorted from the live samples and identified using a stereomicroscope at low light. The number of copepods used in the two experiments was determined based on the average abundances observed in the coastal area in previous studies (Escribano and Schneider 2007; Hidalgo et al. 2010). Copepod samples in the two experiments consisted of *Paracalanus* cf. *indicus* (80%) and *Acartia tonsa* (20%).

The experimental design consisted of four steps: acclimatizing copepods, feeding, excretion, and microbial response (Fig. 2), as follows: The acclimation phase (1) consisted of maintaining four copepod groups (20 individuals per group), for 6 h in filtered seawater (0.7 μm; GF/F Whatman), which had been obtained previously from 10 m depth at the sampling site and filtered with a peristaltic pump. Copepods were isolated from surrounding water using 200 μm mesh-tubes and were acclimatized in darkness at controlled temperatures simulating in situ conditions (ca. 12°C). Acclimatization was followed by the copepod-feeding phase (2), to assesses the impact of the type of food ingested by copepods, two size fractions of food were considered: microplankton and nanoplankton which were obtained by filtering natural food through sieves (20–150 μm and < 20 μm, respectively). The feeding phase lasted 4 h under controlled temperature (12°C). During this phase, copepods were maintained in 200 μm mesh-tubes as acclimation
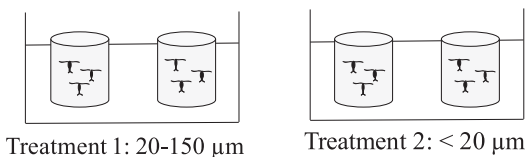
1. ACCLIMATION
Time 6h
Volume 10L
Incubation in FSW 0.7 μm
Container tube with mesh (200μm)
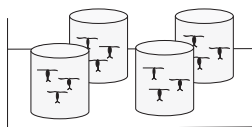
2. FEEDING
Time 4h
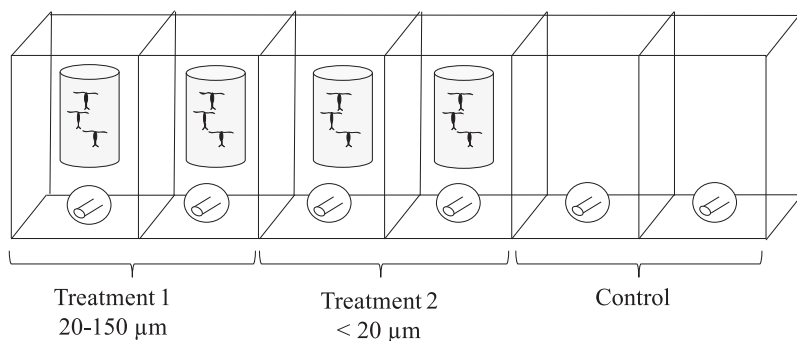Volume 10L
Natural food (2 fractions)

Treatment 1: 20-150 μm   Treatment 2: < 20 μm

3. EXCRETION
Time 2h (subsample 0, 1, 2 h)
Volume 6L
Incubation in FSW 0.2 μm
Control without copepods

Treatment 1
20-150 μm

Treatment 2
< 20 μm

Control

4. MICROBIAL RESPONSE
Time 6 hours (subsample 0, 2, 4, 6 h)
Natural bacterial assemblages 850 mL FSW 0.7 μm
Inoculation with 150 mL of excretion water
Total volume 1 L

Treatment 1   Treatment 2   Control
20-150 μm   < 20 μm
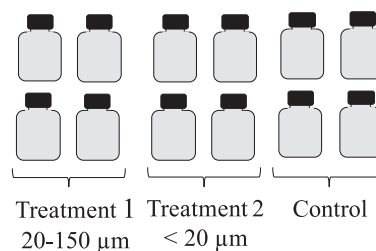
Fig. 2. Experimental design used in CopeMOD experiments. The design consisted of four phases done sequentially. Each copepod group was acclimated for at least 6 h (phase 1), followed by feeding (phase 2) under two natural size fraction of food: 20–150 μm and < 20 μm during 4 h. Copepod excretion (phase 3) had treatment and control (without copepods) in two replicated chambers. Finally, the microbial response (phase 4) was carried out in glass flasks and each treatment had four replicates. FSW, filtered seawater.

phase. Food quality for these size fractions was determined by analyzing species composition and abundance for nanoplankton and microplankton (*see* "Physical, chemical, and biological in situ measurements during CopeMOD experiments" section).

Thereafter, each copepod group was placed in an incubator for the excretion phase (3). The incubation system consisted of six chambers filled with 6 L of seawater previously filtered (0.2 μm; Millipore) through a peristaltic pump, to avoid the presence of large microbial communities. Four of the chambers contained groups of copepods each, and two chambers were without copepods and used as controls (Fig. 2). The incubation system was previously washed with 10% HCl to remove any residual organic matter. Each chamber was equipped with a sampling faucet to retrieve subsamples for chemical and biological analysis during the incubation period. Water samples were collected in duplicate from each chamber at 1-h intervals ($t_0 = 0$ h, $t_1 = 1$ h, and $t_2 = 2$ h) to determine ammonium (NH_4^+), nitrate (NO_3^-), nitrite (NO_2^-), and DON. Additionally, samples for picoplankton abundance were taken to evaluate the potential occurrence of small-sized

bacteria, although efforts were made reduce this influence by using filtered seawater (0.2 μm).

NH_4^+ samples were taken in duplicate using 50 mL glass bottles (Duran Schott) and analyzed by the fluorometric method described by Holmes et al. (1999), using a Turner Designs fluorometer. The standard error of this technique is less than 5%. Samples for NO_3^- and NO_2^- were stored in high-density polythylene (HDPE) plastic bottles (11 mL in duplicate) at -20°C until analyses by standard colorimetric techniques (Grasshof 1983). DON samples were filtered through pre-combusted (450°C, 6 h) GF/F filters (0.7 μm; Whatman), taken in duplicate in Teflon flasks (30 mL) and samples were determined by wet oxidation procedures following Pujo-Pay and Raimbault (1994). DON ($\pm$ 0.1 μmol L^{-1}) concentrations were determined by sample oxidation (30 min, 120°C) and corrected for NH_4^+, NO_3^-, and NO_2^-. Picoplankton abundance was analyzed by flow cytometry. To do this, samples of seawater (1.35 mL) were fixed with glutaraldehyde (0.1% final concentration; Merck) and analyzed at PROFC laboratory at Universidad de Concepcion, Chile, with a FACSCalibur flow cytometer (Becton Dickinson).

Abundance of nonfluorescent picoplankton was estimated from samples previously stained with SYBR green I (Molecular probes) (Marie et al. 1997).

Microbial response to copepods excretion (4)

To assess the response of ammonia-oxidizing communities to the DON and ammonium excreted by copepods, 8.5 L of seawater were filtered (0.7 μm; GF/F Whatman) evenly into ten 1-liter glass bottles under a peristaltic pump (1 L; Duran Schott) (Fig. 1). The seawater was filtered to avoid the presence of phytoplankton and small protist that can prey on bacteria, and ensure the presence of natural microbial assemblages. Subsamples (150 mL) were taken from each incubation chamber (excretion phase) to inoculate microbial assemblages in the 1 L glass bottles. In total, 10 bottles were inoculated and incubated at 12°C for 6 h. Every 2 h ($t_0 = 0$ h, $t_1 = 2$ h, $t_2 = 4$ h, and $t_3 = 6$ h), subsamples were collected for NH_4^+, NO_3^-, NO_2^-, picoplankton abundance, and RNA. Additionally, a DNA sample was collected in situ during winter experiment.

Molecular methods
DNA extraction

DNA samples (3 L) were pre-filtered serially through a 20 μm mesh and 3 μm filter, and then filtered onto a 47 mm diameter 0.22 μm membrane Nucleopore filter under a peristaltic vacuum. After filtering, each filter was immersed in 500 μL of RNAlater reagent (Ambion), frozen and kept at -20°C until analysis. DNA extraction was carried out as described in Levipan et al. (2014). The DNA was quantified by spectrophotometry (NanoDrop ND-1000 Spectrophotometer) and isolated using the PowerSoil DNA Isolation Kit (Mo Bio Laboratories) in accordance with the manufacturer's specifications.

RNA extraction

RNA samples (50 mL) were filtered using a sterilized syringe (60 mL) and 25 mm swinnex through 0.22 μm Durapore filters (Millipore) and were immersed into 300 μL of RNAlater reagent (Ambion), frozen and kept at -20°C until analysis. RNA samples were thawed on ice and RNAlater was carefully removed and discarded after initiating the RNA extraction using the mirVana isolation kit (Ambion). The RNA extraction protocol provided with the kit was followed with the minor modification consisting of mechanical disruption using (~ 100 μL) low binding zirconium beads (200 μm diameter OPS Diagnostic) and beadbeater (BioSpec), with two 30 s agitations (30 Hz) with a 30 s pause in the cell lysis step. RNA was treated in order to remove DNA traces using the Turbo DNA-free kit (Ambion).

Quantification of ammonia-oxidizing groups in CopeMOD experiments

Previous to CopeMOD experiments, the predominant active ammonia-oxidizers in the study area were identified as a response to artificial ammonium enrichment. Methods and figures are described in the Supporting Information.

In CopeMOD experiments, quantitative measurements of AOA and AOB were determined by using complementary DNA (cDNA) templates. The cDNA was synthesized with Improm-II Reverse Transcription System using 20 ng of total RNA and the following reverse primers. In the autumn experiment, ammonia-oxidizers were studied through the specific amplification of AOA and *Betaproteobacteria* AOB *amoA* gene marker using the primers Arch-amoAF, Arch-amoAR (Francis et al. 2005) and amoA1F-amoA 2R (Rotthauwe et al. 1997), respectively. In winter, AOA of different surface ecotypes were studied, *N. maritimus* was specifically targeted using the 423F forward primer (Elizondo-Patrone et al. 2015), and the AOA-A surface ecotype was amplified using Arch-amoAFA (Beman et al. 2010), both in combination with the Arch-amoA R reversed primer. The quantitative polymerase chain reaction (qPCR) reactions were done in a Strategene Mx3000P Real-Time PCR System (Thermo Fisher Scientific) and the data were analyzed using the MxPro-Mx3000P v4.10 Build 389 Schema 85 Stratagene software package, using a 20 μL reaction mixture with 5–10 ng of template DNA, (quantified in a NanoDrop ND-1000 Spectrophotometer and using the Quant-iT High-Sensitivity DNA assay with the Qubit Fluorometer [Invitrogen]). Quantitative PCR reactions were carried out on a volume of 20 μL, containing 1 μL of cDNA, Power SYBR Green Master Mix (Applied Biosystems) and the corresponding forward and reverse primers (0.4 μL final concentration). All reactions were run in triplicate. The qPCR protocol was determined according to Levipan et al. (2016) and the PCR efficiencies (E) and correlation coefficients (r^2) for the standard curves were as follows: general archaeal *amoA* ($E = 96.5\%$; $r^2 = 0.986$), general bacterial *amoA* ($E = 76\%$; $r^2 = 0.998$), AOA-A ($E = 68\%$; $r^2 = 0.97$), *N. maritimus* ($E = 65\%$; $r^2 = 0.99$), and AOB ($E = 63\%$; $r^2 = 0.99$). The detection limit of the standards was observed at a threshold cycle (CT) mean ≤ 31 in all cases.

Data analysis

Average control concentrations (NH_4^+ and DON) during the excretion phase were subtracted from treatment values to obtain the concentration related to excretion during the incubation period. Negative values indicate that control values were higher than those of the treatment, while positive values indicate that treatment values were higher than those of the control, indicating that copepods were excreting. Copepod excretion rates for NH_4^+ and DON were determined using the endpoint approach for the time periods t_0 to t_1 and t_1 to t_2. For the entire incubation time (from t_0 to t_2), these rates were obtained from the slope of the linear regression between concentration and time, since it has been reported that some N compounds excreted by zooplankton can be released in high concentration spurts in short periods (~ 1 h; Gardner and Paffenhofer 1982; Steinberg et al. 2002). As with the excretion phase, control

concentrations (NH_4^+, NO_3^-, and NO_2) in the microbial response phase were subtracted from treatments to obtain the production or consumption due to microbial activity at the respective sampling times. Finally, a two-way analysis of variance (ANOVA) and Tukey's post hoc test were applied to the copepod excretion and microbial response phases of the experiment.

The responses of AOA and AOB were evaluated as *amoA* gene copy number transcripts in the different treatments (20–150 μm and < 20 μm). This estimation is positive when the AOA and AOB responses increase and negative when they are the opposite compared to the control.

Results

### Hydrographic conditions during CopeMOD experiments

A well-mixed water column was observed in both CopeMOD experiments (May and September; Fig. 3a,b). Temperatures were higher in May than in September at 10 m depth, 12.9°C and 11.3°C, respectively. Salinity values were lower at the surface and increased with depth in both dates. Salinity in May ranged between ∼ 34.35 and 34.55, while in September it ranged from 33.85 to 34.55 between 10 m and 60 m, respectively. The water column was overall well oxygenated, with values around 5 mL L^{-1} at 10 m depth.

Copepod feeding conditions in CopeMOD experiments

Values of Chl *a* were below 1 mg m^{-3} for both periods (Table 1). However, Chl *a* concentrations were higher in autumn (May) than in winter (September). Fractioned Chl *a* (< 20 μm) represented a higher average concentration compared to the total size range. This indicates that in both experiments the upper layer was dominated by the smallest fraction of phytoplankton. Additionally, total POC in autumn had a concentration of 306 μg C L^{-1} while the fractioned (< 20 μm) POC had a concentration of 302 μg C L^{-1}. Total and fractioned (< 20 μm) PON concentrations were around 54 μg N L^{-1} and 47 μg N L^{-1}, respectively. Microphytoplankton presented low abundance in autumn (228 cell mL^{-1}), and *Cylindrotheca closterium* and *Skeletonema* sp. cells were predominant. Moreover, autotrophic nanoflagellates represented 96% of total nanoplankton abundance.

Changes in nitrogen compounds in the CopeMOD experiments: excretion phase

NH_4^+ concentrations in autumn were higher in the control than in the treatment fed with smaller-sized food, (Fig. 4a). This yields negative values throughout the incubation when the control is subtracted from the treatment. However, the difference between the treatment (20–150 μm) and the control decreased with time, resulting in a significantly higher excretion rate in the first period (t_0–t_1) of 0.05 μmol L^{-1} h^{-1} than in the second (t_1–t_2) 0.03 μmol L^{-1} h^{-1} (Tukey's test, $p = 0.003$; Fig. 4d). In contrast, copepods fed with the < 20 μm food fraction had values greater than those
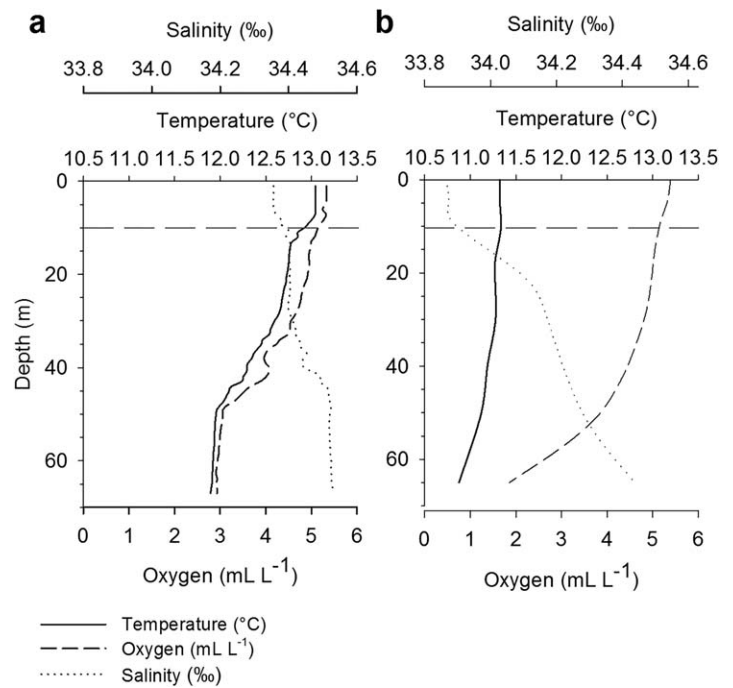
**Fig. 3.** Temperature (°C), salinity (‰), and dissolved oxygen (mL L^{-1}) in CopeMOD experiments: May 2012 (**a**) and September 2012 (**b**). Vertical profiles were obtained by continuous CTD-O measurement. The horizontal dashed lines indicate the collection depth of water samples (10 m depth).

Table 1. Initial contents of photosynthetic pigments, POC, PON concentration, and phytoplankton abundances in duplicate during autumn (May 2012) and winter (September 2012), respectively. n.d, not determined.

Parameters	May 2012	Sep 2012
Total Chl *a* (mg m^{-3})	0.616/0.670	0.092/0.081
Chl *a* < 20μm (mg m^{-3})	0.618/0.606	0.060/0.076
Total phaeopigments (mg m^{-3})	n.d	0.111/0.126
Phaeopigments < 20μm (mg m^{-3})	n.d	0.116/0.128
Total POC (μg C L^{-1})	348.48/263.86	n.d
POC < 20μm (μg C L^{-1})	329.57/274.48	n.d
Total PON (μg N L^{-1})	62.00/46.75	n.d
PON < 20μm (μg N L^{-1})	49.31/45.45	n.d
Total C:N	5.63	n.d
C:N < 20 μm	6.37	n.d
Total microphytoplankton (cell mL^{-1})	228.58	n.d
Autotrophic nanoplankton (cell mL^{-1})	197.44	n.d
Heterotrophic nanoplankton (cell mL^{-1})	6.58	n.d

of the controls, except at t_0 (Fig. 4a). As with the treatment fed with the larger food fraction, NH_4^+ accumulated significantly (Tukey's test, $p = 0.000$) during the first phase (between t_0 and t_1), with excretion calculated at 0.08 μmol L^{-1} h^{-1}, followed by NH_4^+ consumption in the last phase
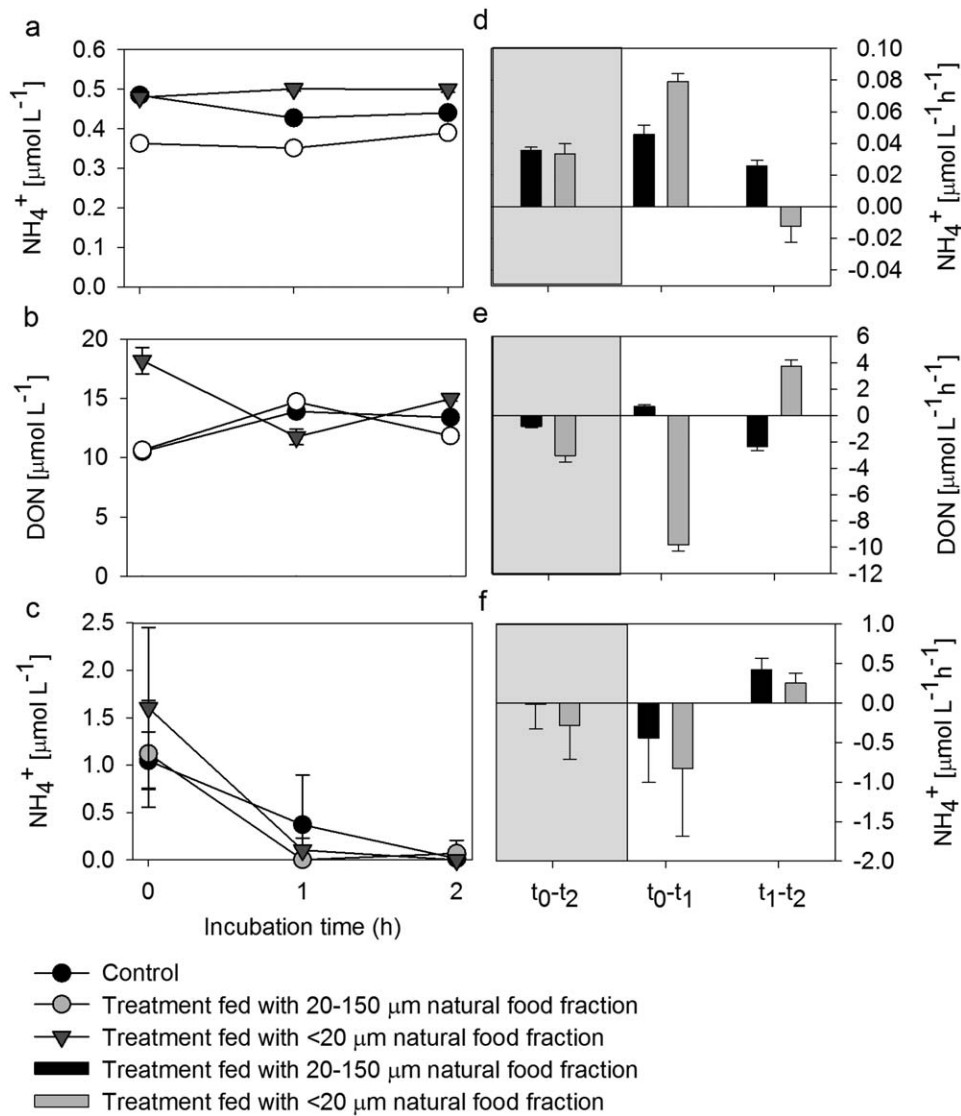
Fig. 4. Changes in nitrogen compounds over time during the excretion phase (left panel) and estimated excretion rates (right panel). (**a**) and (**d**) ammonium, (**b**) and (**e**) DON, in autumn. (**c**) and (**f**) ammonium in winter. Shaded panel indicates the excretion rates throughout the incubation (between t_0 and t_2) for ammonium and DON in the CopeMOD experiments. The errors bars are standard deviations ($n = 4$).

(between t_1 and t_2; Fig. 4d). We did not observe significant differences between treatments diets ($F_{2,18} = 1.08$, $p = 0.312$). DON concentrations were higher than NH_4^+ concentrations and changes over time showed that the treatments fed with the larger food fraction (20–150 μm) varied either positively or negatively with respect to the control (Fig. 4b), with a slight but nonsignificant accumulation in the first incubation period (Fig. 4e; $F_{1,8} = 0.759$, $p = 0.409$) and an excretion rate of 0.7 μmol L^{-1} h^{-1}. DON concentrations were initially higher in copepods fed with the < 20 μm fraction than in the control (Fig. 4b), but the concentration decreased over time. Consequently, the budget of excreted DON showed net consumption between t_0 and t_2, with a negative excretion rate (Fig. 4e). However, in the last period, DON excretion was observed,

with a rate of ~ 4 μmol L^{-1} h^{-1} for the treatment fed with the < 20 μm food fraction. However, there were no significant differences between treatment diets for DON excretion rates ($F_{1,8} = 0.813$, $p = 0.394$). Finally, NO_3^- and NO_2^- concentrations of the control were higher than those of the treatments (Supporting Information, Fig. 1) and the budget of NO_3^- and NO_2^- showed net accumulation throughout the incubation period, except for NO_3^- in the treatment fed with the < 20 μm food fraction (between t_0 and t_2; Supporting Information Fig. S1). However, concentrations varied substantially, and no significant differences were found in NO_3^- and NO_2^- concentrations between treatments ($F_{1,20} = 0.042$, $p = 0.840$ and $F_{1,20} = 0.114$, $p = 0.739$, respectively) or times ($F_{1,20} = 0.035$, $p = 0.853$ and $p = 0.3$, respectively).
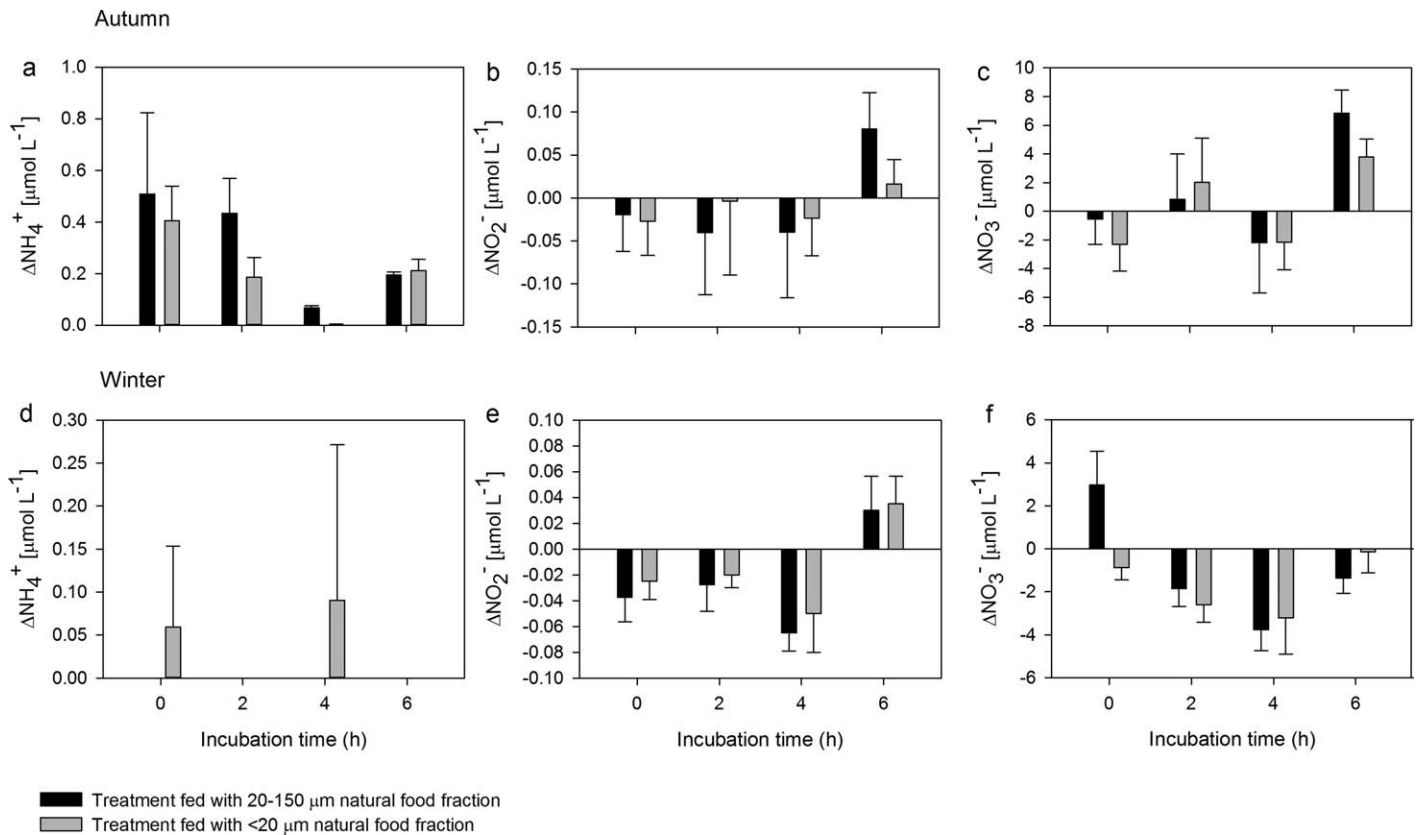
Fig. 5. Turnover of ammonium (**a**) and (**d**), nitrate (**b**) and (**e**), and nitrite (**c**) and (**f**) by microbial communities in CopeMOD experiments. The errors bars are standard deviations ($n = 4$).

Under winter conditions, NH_4^+ concentrations were higher in both treatments and control at the beginning of incubation, (t_0; Fig. 4c), although with a higher standard deviation. These high values were followed by a decrease in concentrations for treatments and control. Thus, the treatments yielded negative values when they were subtracted from the control (Fig. 4c). NH_4^+ in both treatments was mainly excreted in the second phase (between t_1 and t_2) of the experiment, 0.42 μmol L^{-1} h^{-1} and 0.25 μmol L^{-1} h^{-1} for the treatments fed with 20–150 μm and < 20 μm, respectively (Fig. 4c), but the differences between treatments ($F_{1,20} = 1.313$, $p = 0.265$) and time ($F_{1,20} = 2.708$, $p = 0.115$) were not significant.

Finally, we used the rate estimated throughout incubation (between t_0 and t_2) to standardize NH_4^+ and DON excretion. In autumn, NH_4^+ excretion rates were similar between treatments 0.036 μmol L^{-1} h^{-1} and 0.033 μmol L^{-1} h^{-1} for copepods fed with 20–150 μm and < 20 μm fraction, respectively (Fig. 4d). These rates were 0.0018 μmol L^{-1} h^{-1} ind.$^{-1}$ and 0.0017 μmol L^{-1} h^{-1} ind.$^{-1}$ after being standardized by the number of copepods in the sample. DON excretion rates were negative, −0.830 μmol L^{-1} h^{-1} and −3.049 μmol L^{-1} h^{-1} for the treatments fed with 20–150 μm and < 20 μm fraction, respectively (Fig. 4e). These rates were −0.042 μmol

L^{-1} h^{-1} ind.$^{-1}$ and −0.152 μmol L^{-1} h^{-1} ind.$^{-1}$ when standardized to the number of individuals in the sample. In winter, NH_4^+ excretion rates were negative, −0.010 μmol L^{-1} h^{-1} and −0.286 μmol L^{-1} h^{-1} for the treatments fed with the 20–150 μm and < 20 μm fractions, respectively (Fig. 4f). These rates were −0.0005 μmol L^{-1} h^{-1} ind.$^{-1}$ and −0.0143 μmol L^{-1} h^{-1} ind.$^{-1}$ after being standardized to the number of copepods in the sample. An ANOVA analysis was applied to NH_4^+ excretion rates (between t_0 and t_2) for autumn and winter, and found no significant differences between experiments and treatments ($F_{1,12} = 1.924$, $p = 0.191$ and $F_{1,12} = 1.097$, $p = 0.316$, respectively). Unfortunately, DON information for the winter is not available for comparisons.

Changes in nitrogen compounds during the CopeMOD experiments: microbial response phase

In autumn, NH_4^+ concentrations were higher in the treatments than in the controls, with an accumulation of over 0.5 μmol L^{-1} at the beginning of the experiments in both treatments (t_0; Fig. 5a). This contribution decreased significantly over the course of the incubation (within the first 4 h of incubation; Tukey's test, $p = 0.015$ and $p = 0.000$) and NH_4^+ was almost completely consumed at 4 h of incubation, reaching concentrations of 0.07 μmol L^{-1} and 0.0003 μmol

Table 2. Changes in concentration of nitrogen compounds, for controls and treatments, during microbial response phase in CopeMOD experiments. Average control values were subtracted from the treatments, and values were presented with the standard deviation ($n = 4$).

| Experiment	Parameters	Control (μmol L^{-1})					Treatments (μmol L^{-1})			
		t_0 (0 h)	t_1 (2 h)	t_2 (4 h)	t_3 (6 h)		t_0 (0 h)	t_1 (2 h)	t_2 (4 h)	t_3 (6 h)
May (autumn)	NH$_4^+$	0.26 ± 0.00	0.25 ± 0.00	0.24 ± 0.00	0.25 ± 0.00	20–150 μm	0.51 ± 0.31	0.43 ± 0.13	0.07 ± 0.01	0.20 ± 0.01
						<20 μm	0.40 ± 0.13	0.19 ± 0.08	0.0003 ± 0.00	0.21 ± 0.03
	NO$_3^-$	16.8 ± 4.61	15.4 ± 4.10	17.4 ± 3.47	10.5 ± 4.23	20–150 μm	−0.55 ± 1.7	0.84 ± 3.16	−2.20 ± 3.50	6.85 ± 1.60
						<20 μm	−2.31 ± 1.87	2.02 ± 3.08	−2.16 ± 1.92	3.79 ± 1.24
	NO$_2^-$	0.47 ± 0.01	0.44 ± 0.08	0.47 ± 0.06	0.36 ± 0.03	20–150 μm	−0.02 ± 0.04	−0.04 ± 0.07	−0.04 ± 0.08	0.08 ± 0.04
						<20 μm	−0.03 ± 0.04	−0.0004 ± 0.09	−0.02 ± 0.04	0.02 ± 0.03
Sep (winter)	NH$_4^+$	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	20–150 μm	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
						<20 μm	0.06 ± 0.09	0.00 ± 0.00	0.09 ± 0.18	0.00 ± 0.00
	NO$_3^-$	6.33 ± 1.78	9.44 ± 0.28	9.70 ± 0.42	7.79 ± 1.05	20–150 μm	2.97 ± 1.58	−1.84 ± 0.84	−3.60 ± 0.98	−1.35 ± 0.72
						<20 μm	−0.88 ± 0.56	−2.60 ± 0.82	−3.22 ± 1.68	−0.14 ± 0.98
	NO$_2^-$	0.36 ± 0.04	0.38 ± 0.01	0.41 ± 0.02	0.35 ± 0.01	20–150 μm	−0.04 ± 0.02	−0.03 ± 0.02	−0.07 ± 0.01	0.03 ± 0.03
						<20 μm	−0.03 ± 0.01	−0.02 ± 0.01	−0.05 ± 0.03	0.04 ± 0.02

L^{-1} for the treatment fed with the 20–150 μm and <20 μm sized food fractions, respectively (t_2; Table 2). In the last 2 h of incubation (t_3), a significant accumulation of NH$_4^+$ was observed for the aliquot derived from copepods fed with <20 μm size fractions (Tukey's test, $p = 0.02$; Fig. 5a). In addition, slight but significant differences were found between treatments in NH$_4^+$ concentrations ($F_{3,24} = 4.543$; $p = 0.044$). NO$_2^-$ (Fig. 5b) was mainly consumed during the first 4 h of incubation (between t_0 and t_2) and a significant difference was observed between the first 4 h and the end of the experiment for the aliquots derived from copepods fed with the 20–150 μm and <20 μm fractions (Fig. 5b, Tukey's test, $p = 0.04$). However, this difference was not significant between treatments ($F_{1,24} = 0.056$, $p = 0.8$). Nitrate (Fig. 5b) shifted significantly from consumption to accumulation every 2 h of incubation ($F_{3,24} = 16.064$, $p = 0.000$) and there was a significant increase of 40% in both treatments compared to the initial concentration in the experiment (Table 2; Tukey's test, $p = 0.008$). Nitrate and nitrite concentrations were not significantly different between treatments ($F_{1,24} = 1.129$, $p = 0.29$ and $F_{1,24} = 0.056$, $p = 0.81$, respectively). Picoplankton abundance increased consistently throughout the incubation in treatments and controls to nearly 300×10^3 cells mL^{-1}. However, we did not observe significant differences between treatments (20–150 μm and <20 μm) and controls (Tukey's test, $p = 0.29$ and $p = 0.80$, respectively), but a significant increase was observed in the treatment derived from copepods fed with the larger size fraction compared to the treatment fed with the smaller size fraction (Tukey's test, $p = 0.03$; Fig. 6b).

The response of AOB and AOA were determined based on qPCR *amoA* transcript counts during the experiments in response to the input of NH$_4^+$ and DON excreted by copepods. The qPCR *amoA* transcript counts over time for AOA and AOB were generally similar in the treatment derived from copepods fed with 20–150 μm size fraction, with significant shifts every 2 h in both treatments ($F_{3,11} = 4.522$, $p = 0.026$ and $F_{3,15} = 26.01$, $p = 0.000$, respectively), highlighting a notable increase in AOB activity at 4 h of incubation (Fig. 7a). At the same time, NH$_4^+$ concentrations decreased. In contrast, in the treatment spiked with excretion products by copepods fed with the <20μm fraction (Fig. 7b), only AOB qPCR *amoA* transcript counts increased significantly (Tukey's test, $p = 0.000$) at the initial time (t_0), and both AOB and AOA contributions decreased during the rest of the incubation period, with no visible increase in activity after 4 h (when NH$_4^+$ decreased). No significant differences were found for AOA and AOB qPCR *amoA* transcript counts between treatments ($F_{3,11} = 0.674$, $p = 0.429$ and $F_{3,15} = 0.802$, $p = 0.384$, respectively).

In winter, the microbial response to the NH$_4^+$ excreted by copepods showed minor differences from that of the control, with an accumulation at the beginning of the experiment, and then at hour 4 (t_2) of incubation. However, these accumulation levels were not significantly different from zero
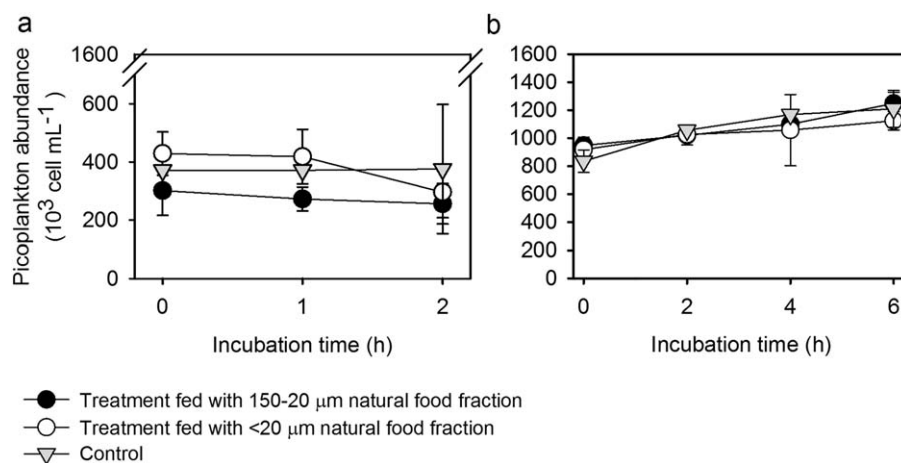
Fig. 6. Picoplankton abundance during the excretion (**a**) and the microbial response to the compound excreted by copepods phase (**b**) in autumn experiment. The errors bars are standard deviations ($n = 4$).
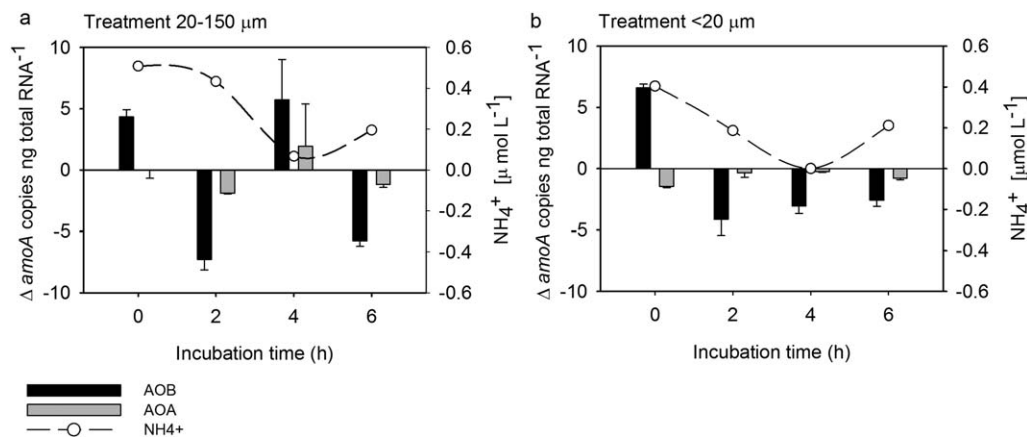
Fig. 7. Contribution of archaeal and bacterial *amoA* qPCR transcript counts and ammonium concentration over time in autumn experiment. (**a**) Treatment derived from copepods fed with 20–150 μm size fraction of natural food and (**b**) treatment derived from copepods fed with < 20 μm sized fraction of natural food. Control values were subtracted from the treatments. The errors bars are standard deviations ($n = 3$).

(control value) and between treatments ($F_{1,24} = 0.779$, $p = 0.517$ and $F_{3,24} = 2.150$, $p = 0.156$; Fig. 5d). Control nitrite values were higher than those of the treatments, with net consumption during the first 4 h (t_2) of incubation (Fig. 5e; Table 2). Accumulation was significant at the final time (t_3) in both treatments compared to the initial concentrations, reaching over 0.03 μmol L^{-1} in both (Tukey's test, $p = 0.000$). However, there were no significant differences in concentration between treatments ($F_{1,24} = 0.056$, $p = 0.81$). Nitrate was mainly consumed throughout the incubation period with significant decreases in concentrations between initial and final incubation times in the treatment fed with 20–150 μm (Tukey's test, $p = 0.000$), However, there were no significant differences in concentrations between treatments ($F_{1,24} = 1.129$, $p = 0.299$; Fig. 5f).

During this period, AOA-A, *N. maritimus*, and AOB qPCR *amoA* genes were detected in situ in DNA, but only AOA *amoA*

transcripts from AOA-A and *N. maritimus* were detected in the last hours of incubation (t_2 and t_3). The only transcripts that increased were those of AOA-A *amoA* (Table 3).

Discussion

Nitrogen excretion by copepods

In the coastal zone of southern central Chile, copepods contributed up to 70% relative abundance of the entire zooplankton community and were mostly represented by *Paracalanus* cf. *indicus*, *A. tonsa*, *Calanus chilensis*, and *Oithona* spp. (Escribano et al. 2007). The first two species were used in the CopeMOD experiments and contribute to 40% of the relative abundance of copepods in this zone (Escribano et al. 2007; Hidalgo et al. 2010). In autumn, the excretion of copepods was dominated by DON. However, a high degree of variability in DON concentrations was observed during the

Table 3. Bacterial and archaeal *amoA* gene counts (ecotype AOA-A and *N. maritimus*) in winter experiment. Average values were presented with the standard deviation ($n = 3$). <D.L, low detection limit.

Time	Treatment	DNA/ cDNA	AOB	AOA-A	*N. maritimus*
			(gene copies ng total DNA or RNA^{-1})		
Initial	In situ	DNA	72.2 ± 9.40	89.5 ± 0.99	<D.L
T_3	150–20 μm	DNA	<D.L	63.1 ± 6.21	344 ± 62.8
T_3	<20 μm	DNA	<D.L	76.3 ± 4.48	<D.L
T_3	Control	DNA	<D.L	89.5 ± 0.99	<D.L
T_0	150–20 μm	cDNA	<D.L	<D.L	<D.L
T_0	<20 μm	cDNA	<D.L	<D.L	<D.L
T_0	Control	cDNA	<D.L	<D.L	<D.L
T_1	150–20 μm	cDNA	<D.L	<D.L	<D.L
T_1	<20 μm	cDNA	<D.L	<D.L	42.1 ± 5.40
T_1	Control	cDNA	<D.L	<D.L	<D.L
T_2	150–20 μm	cDNA	<D.L	<D.L	<D.L
T_2	<20 μm	cDNA	<D.L	62.9 ± 1.22	<D.L
T_2	Control	cDNA	<D.L	35.4 ± 1.72	<D.L
T_3	150–20 μm	cDNA	<D.L	60.7 ± 5.49	<D.L
T_3	<20 μm	cDNA	<D.L	<D.L	<D.L
T_3	Control	cDNA	<D.L	52.0 ± 7.80	98.1 ± 6.9

incubation, resulting in a negative budget of DON. In contrast to ammonia, which is released continuously by crustaceans (Regnault 1987), DON and other compounds excreted by zooplankton (e.g., amino acids) can be released in highly concentrated spurts lasting up to an hour (Gardner and Paffenhofer 1982; Steinberg et al. 2002), such that its net production is masked by the ups and downs of concentrations between observation intervals (*see* Fig. 4). Indeed, our results showed DON accumulation in both treatments, although at different times during the incubation, 0.7 μmol L^{-1} h^{-1} in the first period (between t_0 and t_1) for the copepods fed with 20–150 μm fraction, and 3.7 μmol L^{-1} h^{-1} in the second period (between t_1 and t_2) for copepods fed with <20 μm fraction. Although no information was available for DON excretion in winter for comparison, previous experiments in this area showed that the excretion by copepods was mainly in the form of DON (mostly non-ureic) in winter, with more accumulation in the first hour of incubation with an excretion rate of 3.22 μmol L^{-1} h^{-1}, which is close to our estimate for the second period of the treatment fed with smaller-sized fraction (Pérez-Aragón et al. 2011).

It is known that the food ingested by copepods can affect the dominant excretion composition (Elser and Urabe 1999; Miller and Roman 2008; Saba et al. 2009). However, most studies have used monocultures of microalgae or a mix of them, and so they may not reflect real field conditions. The CopeMOD experiments considered two natural sized food fractions that represent two types of diets available to copepods in southern central Chile. In this context, Vargas et al. (2006) suggested that copepods can shift their diet over the course of the year. In summer, they feed almost exclusively on chain-forming diatoms, and then change their diets in autumn to ciliates and dinoflagellates when diatoms are in low abundance. Escribano and Pérez (2010) reported that nanoplankton, which are represented by our <20 μm food fraction, are a major part of the copepod diet in winter in southern central Chile, coinciding with the main food available for copepods in our study, predominantly small-sized organisms such as nanoflagellates, and to a lesser extent microplankton (Table 1). Our results show that copepods feeding on the smaller food fraction (<20 μm) yield a greater accumulation of DON and ammonium compared to those feeding on the larger fraction of food (20–150 μm). However, our treatment with the larger size fraction did not necessarily reflect the abundance of microphytoplankton expected for a spring-summer situation, which is usually much greater than what we used in our experiments (Anabalón et al. 2007). Nevertheless, DON has previously been identified as the main compound excreted by copepods in winter and spring in the study area, although the rate estimated in spring was 83% less than that of winter (Pérez-Aragón et al. 2011).

Control concentrations of NH$_4^+$ were higher than those in the treatments fed with the 20–150 μm size fraction. However, the difference between control and treatment decreased with incubation, revealing that copepods were excreting this compound. Our estimated rates are close to those reported in this zone in winter (Pérez-Aragón et al. 2011), but lower than those reported for other upwelling zones (Isla et al. 2004; Fernández-Urruzola et al. 2016; Kiko et al. 2015). In the second experiment, NH$_4^+$ decreased with incubation for both treatments and controls, which could be due to the high degree of lability of NH$_4^+$ in natural environments since it is actively used by phytoplankton and microbial communities.

DON and ammonium during the incubation showed some negative excretion rates (or higher consumption compared to NH$_4^+$ input), this could be explained by the presence of heterotrophic, chemoautotrophic, and also by small photoautotrophic communities. Despite our attempts to exclude these communities in the experiments, by using filtered seawater (0.22 μm), a low and constant number of picoplankton was found (Fig. 6a). Besides, this could be because of the carryover of the microbiome associated with copepod exoskeletons and intestines (Sochard et al. 1979). In this sense, in addition to the classical contribution of zooplankton to food webs, their role as environmental reservoirs for a high diversity of microbes, including pathogens, is increasingly recognized (Tang et al. 2010; Martinelli Filho et al. 2011; Gerdts et al. 2013). It has also been reported that temporal changes in environmental conditions, including

food type, is a key factor controlling the composition of bacterial communities associated with copepods (Moisander et al. 2015) and the copepod microbiome cannot be easily separated or distinguished from the bacterial community of the surrounding environment, and there is an active exchange of bacteria between water and copepods (De Corte et al. 2014). Therefore, we suggest that NH_4^+ was actively used by microbial communities in winter, which explains the abrupt decrease in NH_4^+ at 1 h of incubation (t_1), since the input from copepod excretion was not sufficient to match NH_4^+ demand. In addition, the presence of active chemoautotrophic communities could explain the accumulation of NO_2^- and NO_3^- during the last incubation period, detected in autumn experiment (Supporting Information Fig. S1).

In addition to the potential effect of microbiomes associated with copepods in our excretion rates estimations, other factors could also affect excretion rates, such as the light-dark cycle. Our study was conducted under simulated night conditions, but higher NH_4^+ excretion rates by *A. tonsa* have been observed during the day than at night (Miller and Glibert 1998). Thus, our NH_4^+ excretion rates in darkness could be an underestimation. There are other uncertainties in the experiments, one of which is the estimates of DON that depend on the analytical method used (Letscher et al. 2013). Comparison of procedures indicates variability that can give rise to inaccuracy. However, no clear conclusion has been drawn as to whether different methods yield significantly different results. Meanwhile, several authors continue to use wet oxidation of total dissolved nitrogen (Sharp et al. 2002).

Biogeochemical implications of copepods excretion for bacterial communities

The transformation of the PON consumed by copepods to DON and ammonium derived from excretion can provide nitrogen to fuel the microbial loop in surface and subsurface waters. There are several possible pathways for the utilization of these compounds in our experiments such as: bacterial uptake, dark phytoplankton uptake, ammonification, and the focus of this study, the chemoautotrophic ammonium oxidation, as part of the nitrification process.

In autumn, the excreted products resulted in different responses by microorganisms depending on the food provided to the copepods. When copepods were fed with 20–150 μm plankton fraction, stimulated the microorganism to consume NH_4^+ in the first 4 h (Fig. 5a). Then, NO_3^- and NO_2^- accumulated as a result of microbial nitrification and concurrently the *amo*A gene transcripts of ammonia-oxidizers increased (Fig. 5b,c). The response was higher in the AOB than in the AOA communities (Fig. 7a). In contrast, the inoculum derived from copepods fed with < 20 μm size fraction, presented lower accumulation of NO_2^- and there was not visible accumulation for NO_3^- (Fig. 5e,f). Additionally, lower *amoA* gene counts was observed (Fig. 7b), suggesting that a distinct microbial community contributed to NH_4^+

consumption, probably heterotrophic. There is still little information regarding the impact on the microbial community of compounds excreted by zooplankton. Nevertheless, other studies in the same area and further north have revealed tight coupling of NH_4^+ production and consumption in winter, including ammonia oxidation and potential uptake by eukaryotic organisms (Molina et al. 2005, 2012). In addition, the treatment with the < 20 μm fraction resulted in changes in DON accumulation-consumption that were observed over time in association with copepod excretion. This finding suggests high heterotrophic picoplanktonic demand for DON remineralization/ammonification, which resulted in the accumulation of NH_4^+ at the end of the experiment. On the other hand, the microbial response in winter resulted in very lower ammonium concentration throughout the incubation, with no visible difference between the controls and treatments. In this experiment, AOA and AOB were detected by finding qPCR in the *amoA* gene from in situ DNA. This finding corroborated the presence of these ammonia-oxidizing communities. However, only AOA were transcriptionally active, mainly toward the end of the experiment. These results support the idea that AOA compete more successfully under limited NH_4^+ concentrations than AOB and heterotrophic bacteria.

Productivity in the coastal zone off southern central Chile is stimulated by upwelled water that fertilizes the photic zone with inorganic nutrients during the spring-summer season. In contrast, (non-upwelling period) the productivity of the system is lower in winter, the system is sustained by regenerated production (Fernandez and Farías 2012) and the input of new nutrients was mainly for river runoff and mixing (Escribano and Schneider 2007; Sobarzo et al. 2007). The nitrogen compounds (ammonium and DON) excreted by zooplankton could be an important source of regenerated nitrogen, becoming more relevant in situations of low biological production (non-upwelling season). Several authors have reported that zooplankton provide over 20% of the requirements for primary production (Hernandez-Leon et al. 2008; Pérez-Aragón et al. 2011). In addition, inputs of regenerated products provide substrate for microbial community growth. Packard and Gómez (2013) defined NRE as the capability of the plankton community to retain nutrients in the upper layer of the water column. They showed that it could be calculated from the ratio of zooplankton respiration to carbon flux in a water-column. Subsequently, Fernández-Urruzola et al. (2016) showed that the vertical nitrogen flux can be calculated from ammonium excretion rates and that the ratio of the ammonium excretion to the vertical nitrogen flux is another measured of the NRE that complements NRE calculations from respiration and carbon flux (Osma et al. 2014). Here, we are showing the mechanism by which this NRE is accomplished.

This study found that ammonia-oxidizing communities and probably heterotrophic picoplankton actively use DON

and NH_4^+ excreted by copepods on short time scales. However, we cannot rule out that small phytoplanktonic cells can use these compounds, even under dark conditions (Alaoui et al. 2001). While it is known that AOA have a high affinity for ammonia, recent studies have found that AOA and AOB can use urea to fuel nitrification (Alonso-Saéz et al. 2012). Additionally, AOA can use methylphosphonate as a source of P (Metcalf et al. 2012) and use other compounds, like cyanate, as the sole source of energy and nitrogen (Palatinszky et al. 2015).

Moreover, our findings provide additional evidence that copepods make nitrogen compounds available to nitrifying communities in the surface layer (Zehr and Ward 2002; Yool et al. 2007), which in turn contribute with oxidized inorganic nitrogen to primary productivity in the system. This will result in overestimating regenerated production and underestimating new production. To determine the potential contribution to primary production of ammonium excretion by copepods, we compared our results with the ammonium demand by phytoplankton at Sta. 18 in the euphotic layer (Fernandez and Farías 2012). Excreted ammonium could potentially sustain 10% of phytoplankton ammonium demand. Notably, it could also sustain up to 216% of surface (20–30 m depth) nitrification at Sta. 18 (Fernandez and Farías 2012; Molina et al. 2012). Copepod excretion could also sustain heterotrophic prokaryote ammonium demand. However, there is scarce information (Allen et al. 2002; Bradley et al. 2010) for the study area. This prevents us from estimating the possible percentage sustained by copepod excretion.

The biogeochemical impacts of copepod excretion are not limited to the upper layers as zooplankton move through the water column by vertical diel migration and some copepods species are able to cross and inhabit the oxygen deficient zone (Escribano et al. 2009). In this context, zooplankton can be an important source of DON for microbial communities at greater depths and stimulate NH_4^+ regeneration by bacteria (Miller et al. 1997; Steinberg et al. 2002). Some authors have suggested that zooplankton provide a missing source of ammonium to anoxic waters, fueling anaerobic ammonia-oxidation and decoupling it from denitrification (Bianchi et al. 2014). In the eastern South Pacific oxygen minimum zone, daily ammonium excretion by copepods on occasions could sustain up to 86% of the ammonium demand by the anammox process, which can reach values close to 500 nmol L^{-1} d^{-1} in spring near Sta. 18 (Galán et al. 2012), and 2.11 nmol L^{-1} d^{-1} in winter, as estimated in northern Chile (Galán et al. 2009). The contribution to anammox by excretion could be lower because the metabolism of organisms migrating to the oxygen minimum zone may decrease in response to low oxygen and temperature (Seibel et al. 2016). However, estimates of nitrogen excretion should be substantially higher when considering the entire zooplankton community.
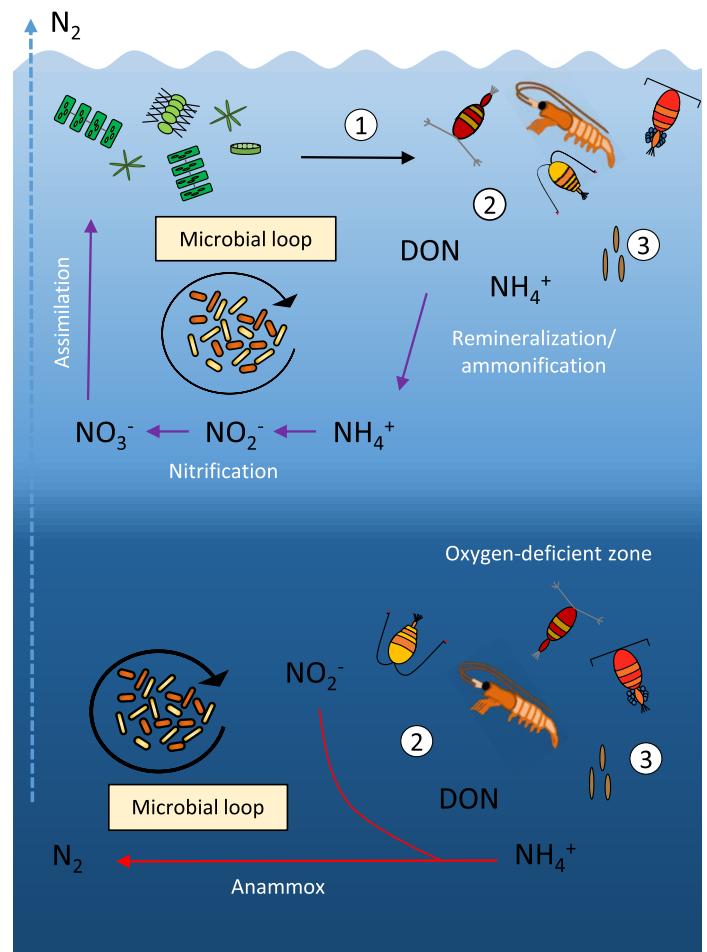
Fig. 8. Conceptual model representing the role of zooplankton for N cycling in the water column of an upwelling system, linked to the autotrophic community and the microbial loop. Three major processes are considered: (1) DON input by zooplankton through sloppy feeding, (2) DON and NH_4^+ input via zooplankton excretion, and (3) DON input by leaching of zooplankton fecal pellets. These compounds are then used by heterotrophic and chemoautotrophic bacteria and archaea to transform organic nitrogen into inorganic forms (microbial loop), making them available for new uptake by phytoplankton. It should be noted that nitrification can indeed occur in the upper illuminated layer. In subsurface water, an oxygen deficient zone is present mostly during the spring and summer. Some zooplankton able to migrate daily to this zone also contribute to anaerobic ammonium oxidation through processes (2) and (3).

Given the importance of excretion by copepods and other zooplankton in the euphotic layer, more studies are necessary to measure organic nitrogen release by excretion, sloppy feeding, and leakage of fecal pellets, with special attention to other less studied zooplankton groups. Also, more studies on lability and fate of these compounds will provide useful information on the role of zooplankton in fueling specific groups in the microbial loop. We summarize in Fig. 8, the key processes by which zooplankton can contribute to nitrogen cycling in the upwelling zone in a conceptual model.

Although most zooplankton in the upwelling zone inhabit the upper illuminated layer (< 50 m), apparently constrained by the presence of a shallow oxygen deficient zone (Escribano et al. 2009), some organisms are capable of crossing and inhabiting the low-oxygen layer by vertical diel migration (Escribano et al. 2009; Donoso and Escribano 2014), thus contributing NH_4^+ to anaerobic ammonium oxidation (Bianchi et al. 2014).

In conclusion, this study shows that DON was the main product excreted by the dominant copepods in southern central Chile in the non-upwelling season. We argue that the release of DON and ammonium is higher when the copepod food supply is comprised by a small sized (< 20 μm) fraction (e.g., nanoflagellates). These nitrogen compounds excreted by copepods can be directly used by microbial communities, including nitrifying communities, in which AOA compete more successfully under limited ammonium concentration than AOB, and other microbial communities having different metabolism such as, chemoautotrophic and heterotrophic (Fig. 8). Nitrifying and heterotrophic communities can rapidly respond to DON and NH_4^+ pulses excreted by copepods, hence sustaining the growth of microbial communities, with direct consequences for regeneration and new production in the photic zone and potentially contributing to nitrogen recycling in the subsurface oxygen minimum zone.

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Conflict of Interest

None declared.