The biogeochemical role of zooplankton for nitrogen and phosphorus recycling in the ocean

Rol biogeoquímico del zooplancton en el reciclamiento de nitrógeno y fósforo en el océano

Tesis para optar al grado de Doctor en Oceanografía

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The biogeochemical role of zooplankton for nitrogen and phosphorus recycling in the ocean

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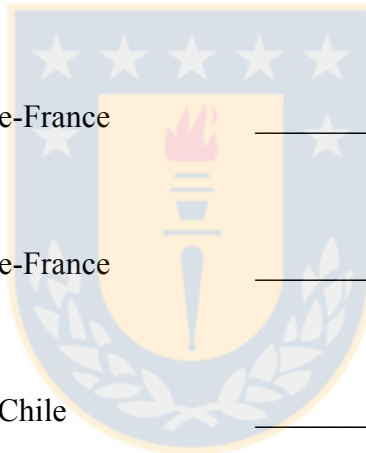Miembro Comité de Tesis
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PUBLICACIONES

- Escribano, R., Hidalgo, P., **Valdés, V** and Frederick, L. 2014. Temperature effects on development and reproduction of copepods in the Humboldt Current: the advantage of rapid growth. *Journal of Plankton Research*, 36(1): 104-116

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Abstract

"The biogeochemical role of zooplankton for nitrogen and phosphorus recycling in the ocean"

Valentina Paz Valdés Castro

PhD in Oceanography

Universidad de Concepción, 2017

The interaction between metazooplankton metabolism and the microbial loop is recognized as one of the principal mechanisms controlling the quantity, composition and cycling of DOM in the sea. Zooplankton and bacteria are usually considered as separate groups despite their temporal and spatial co-existence and close interaction. Zooplankton may release dissolved organic and inorganic matter through sloppy feeding, leaching from fecal pellets and excretion. However, there is a lack of knowledge on the impact of these dissolved compounds released by zooplankton on biogeochemical cycles and on the microbial community structure. An experimental approach with natural food sources and simulated *in situ* conditions was used to determine the role that trophic interactions involving primary and secondary producers in the recycling of nitrogen and phosphorus in the pelagic system of central-southern Chile. First, we quantified the nitrogen and phosphorus compounds excreted by copepods fed with natural sized-fractioned diets (20-150 µm and <20 µm). Then, we assessed the biogeochemical response of the microbial community to released nitrogen, including the nitrifying community, in terms of total and active cells, to the products excreted by copepods. Secondly, we explored the simultaneous interaction between copepod excretion and the biogeochemical response of active microbial community composition, over an oligotrophic gradient in the western tropical south Pacific (WTSP).

Our results show that DON was the main excretion product, with rates of up to 3.7 µmol $L^{-1} h^{-1}$ under autumn/winter conditions in central/southern Chile. Meanwhile, in spring/summer copepods actively excreted nitrogen and phosphorus compounds, mainly in the form of ammonium and dissolved organic phosphorus (DOP), reaching excretion rates of up to 2.6 µmol $L^{-1} h^{-1}$ and 0.05 µmol $L^{-1} h^{-1}$, respectively. In both cases the main products excreted were closely related to the main item food available for copepods (nanoplankton and microplankton,

respectively). In the autumn/winter experiments, ammonium was rapidly consumed by the microbial community. Ammonium consumption coincided with increased archaea (AOA) and bacteria ammonia-oxidizing (AOB) *amoA* transcript copies in copepods fed with the larger-sized fraction, whereas a different microbial community, probably heterotrophic, reacted to nitrogen input via excretion by copepods fed with the smaller-sized fraction in autumn. Moreover, during spring/summer a shift in the composition of active bacterial community was observed between periods and treatments, associated with the response of common-opportunistic seawater surface phyla Proteobacteria and Bacteroidetes.

In the WTSP, copepods contributed elevated levels of ammonium, DON and DOP highly labile. Copepod excretion can enhance the remineralization (ammonification-nitrification) process and reshape the diversity and composition of the active bacterial community. These changes were characterized by shifts in Alteromonadales and SAR11 in both study regions (Melanesian archipelago and subtropical gyre) and differed substantially from the *in-situ* community. Enhanced bacterial activity in presence of copepods would further increase the available substrates for microbial and phytoplankton growth in a highly stratified system and provide an alternative source of phosphorus for diazotrophs blooms in the WTSP.

We concluded that nitrogen and phosphorus excreted by copepods can be used directly by microbial communities, including nitrifying microbes, providing significant remineralized nitrogen for sustaining new and regenerated production in the upper ocean of different marine ecosystems.

Resumen

"Rol biogeoquímico del zooplancton en el reciclamiento de nitrógeno y fósforo en el océano"

Valentina Paz Valdés Castro

Doctorado en Oceanografía

Universidad de Concepción, 2017

La interacción entre el metabolismo del metazooplancton y el anillo microbiano es considerado uno de los principales mecanismos que controla la cantidad, composición y el reciclaje de la materia orgánica disuelta en el océano. Zooplancton y microorganismos suelen considerarse como grupos separados a pesar de su coexistencia temporal y espacial, y su estrecha interacción. El zooplancton libera materia orgánica e inorgánica disuelta a través del consumo incompleto de su presa, la disolución de sus pellets fecales y la excreción. Sin embargo, el impacto de estos compuestos liberados por el zooplancton en los ciclos biogeoquímicos y en la estructura de la comunidad microbiana ha sido pobremente estudiado. En este trabajo, a través de un enfoque experimental, con fuentes de alimentos naturales y condiciones *in situ* simuladas, se determinó el rol que juegan las interacciones tróficas involucrando productores primarios y secundarios en el reciclaje de nitrógeno y fósforo en el sistema pelágico en la zona de surgencia costera del centro-sur de Chile. En primer lugar, se cuantificaron los productos excretados por copépodos alimentados con dietas naturales de tamaño fraccionado. A continuación, se evaluó la respuesta biogeoquímica de la comunidad microbiana y su estructura, en términos de células totales y activas, incluyendo la comunidad nitrificante en respuesta a los productos excretados por copépodos. En segundo lugar, se exploró la interacción simultánea entre la excreción de copépodos y la comunidad microbiana, en términos de la respuesta biogeoquímica y la composición activa en un gradiente de oligotrofia en el Pacífico Sur Occidental Tropical (WTSP por sus siglas en inglés).

Nuestros resultados muestran que DON fue el principal producto de excreción, con tasas de hasta 3,7 μmol L^{-1} h^{-1} durante otoño-invierno en el centro-sur de Chile. Mientras que, en primavera/verano se excretó activamente compuestos de nitrógeno y fósforo, principalmente en forma de amonio y fósforo orgánico disuelto (DOP), alcanzando tasas de hasta 2,6 μmol L^{-1} h^{-1}

y 0,05 μmol L^{-1} h^{-1}, respectivamente. En ambos casos los principales productos excretados estuvieron estrechamente relacionados con el principal alimento disponible para copépodos (nanoplancton y microplancton, respectivamente). En los experimentos de otoño/invierno, el amonio fue rápidamente consumido por la comunidad microbiana. Este consumo coincidió con el aumento de los transcritos del gen *amoA* de arqueas (AOA) y bacterias amonio-oxidantes (AOB) en copépodos alimentados con la fracción de tamaño mayor, mientras que una comunidad microbiana diferente, probablemente heterotrófica, reaccionó a nitrógeno aportado vía excreción de copépodos, alimentados con la fracción de tamaño menor en otoño. Por otra parte, durante primavera/verano se observaron cambios en la composición de la comunidad bacteriana activa entre períodos y tratamientos, asociado a bacterias de aguas superficiales con características oportunistas como Proteobacteria y Bacteroidetes.

En el WTSP, los copépodos aportaron niveles elevados de amonio, DON y DOP altamente lábiles. La excreción de copépodos estimula el proceso de remineralización (amonificación-nitrificación) y puede modificar la diversidad y la composición de la comunidad bacteriana activa, caracterizada por cambios principalmente en Alteromonadales y SAR11 en ambas regiones de estudio (archipiélago Melanesio y giro subtropical). La actividad bacteriana en presencia de copépodos se intensificó, incrementando los sustratos disponibles para el crecimiento de microbios y fitoplancton, lo cual podría proporcionar una fuente alternativa de fósforo para la floración de diazotrófos en WTSP.

Concluimos que el nitrógeno y el fósforo excretados por los copépodos son utilizados directamente por las comunidades microbianas, incluidas las nitrificantes, proporcionando nitrógeno remineralizado para producción nueva y regenerada en el océano superficial en diferentes ecosistemas marinos.

Résumé

"Rôle biogéochimique du zooplancton sur le recyclage d´azote et phosphore dans l´océan"

Valentina Paz Valdés Castro

Science d´l´Environnement (ED129)

Université Pierre et Marie Curie, 2017

L'interaction entre le métabolisme du méso-zooplancton et la boucle microbienne est reconnue comme l'un des principaux mécanismes de contrôle de la quantité, la composition et le recyclage de la matière organique dissoute (DOM) dans l`océan. Le zooplancton et les bactéries sont généralement considérés comme de groupes distincts malgré leur coexistence temporelle et spatiale et leur étroite interaction. Le zooplancton peut libérer de la matière organique et inorganique par une consommation incomplète des proies, lessivage des pelotes fécales et l'excrétion. Cependant, il existe un manque de connaissances sur l'impact de ces composés dissous libérés par le zooplancton sur les cycles biogéochimiques ainsi que sur la structure de la communauté microbienne. Dans ce travail, on a utilisé une approche expérimentale pour déterminer le rôle que les interactions trophiques, impliquant les producteurs primaires et secondaires peuvent agir dans le recyclage de l'azote et du phosphore dans le système pélagique. Pour ce faire, nous avons utilisé des sources alimentaires naturelles et *in situ* simulées se rapprochant des conditions environnementales. Premièrement, nous avons quantifié l'azote et les composés phosphorés excrétés par des copépodes alimentés de régimes naturels fractionnés en taille. Ensuite, nous avons évalué la réponse biogéochimique de la communauté microbienne et sa structure, en termes de biomasse cellulaire totale et active (y compris la communauté nitrifiante) aux produits excrétés par les copépodes dans la région d'upwelling du Chili central. Deuxièmement, nous avons exploré l'interaction simultanée entre l'excrétion de copépodes et la communauté microbienne, en termes de la réponse biogéochimique et les changements de la composition de la communauté microbienne active, le long d`un gradient oligotrophique dans le Pacifique Sud tropical occidental (WTSP).

Nos résultats montrent que l'azote organique dissous (NOD) est le principal produit d'excrétion, avec des taux pouvant atteindre 3.7 μmol L^{-1} h^{-1} dans des conditions automne/hiver

dans le centre-sud du Chili. Pendant le printemps et l'été, les populations de copépodes excrètent activement l'azote et les composés phosphorés, principalement sous forme d'ammonium et de phosphore organique dissous (DOP), atteignant des taux d'excrétion allant jusqu'à 2.6 µmol L^{-1} h^{-1} et 0.05 µmol L^{-1} h^{-1}, respectivement. Dans les deux cas, les produits principaux excrétés étaient étroitement liés à la qualité de l'aliment principal disponible pour les copépodes (nanoplancton et microplancton, respectivement). Pendant les expériences d'automne/hiver, l'ammonium a été rapidement consommé par la communauté microbienne. La consommation d'ammonium excrété par des copépodes alimentés avec la fraction de plus grande taille a coïncide avec une augmentation des copies de transcription d'archaeal (AOA) et bactéries (AOB) ammonium oxydatrices alors qu'une communauté microbienne différente, probablement hétérotrophique, a réagi à l'entrée d'azote par excrétion par des copépodes nourris avec une fraction de taille plus basse. Au cours du printemps/été, un changement dans la composition de la communauté bactérienne active a été observé entre les périodes et les traitements, associé à la réponse du phylum bactérien opportuniste Proteobacteria et Bacteroidetes.

Dans le WTSP, les copépodes ont contribué avec des niveaux élevés d'ammonium, DON et DOP à forte labilité. L'excrétion des copépodes a favorisé le processus de réminéralisation (nitrification) et modifié la diversité et la composition de la communauté bactérienne active caractérisée principalement par les Alteromonadales et SAR11 dans les deux régions étudiées (archipel Mélanésien et gyre subtropical), différant ainsi considérablement de la communauté in situ. Une activité bactérienne améliorée en présence de copépodes augmenterait encore les substrats disponibles pour la croissance microbienne et du phytoplancton dans un système hautement stratifié et fournirait une source alternative de phosphore pour les proliférations de diazotrophes dans le WTSP.

Nous concluons que l'azote et le phosphore excrétés par copépodes peuvent être utilisés directement par des communautés microbiennes, y compris des nitrifiants, fournissant de l'azote reminéralisé pour soutenir la production nouvelle et régénérée dans l'océan supérieur des différents écosystèmes marins.

1. INTRODUCTION

1.1 Dissolved organic matter in marine environments

Large areas in the ocean are deficient in one or more nutrients necessary for life, such N, P, Si and other trace metals, and the distribution of life in the ocean depends on these bio-essential nutrient elements. This is especially true in the open ocean, known as oligotrophic zones, which can account for 40% of the global primary production (Carlson and Hansell, 2015). Trophic interactions in the pelagic ecosystems depend on primary and secondary production requiring these bio-elements, either as dissolved or as particulate forms. For several decades, research has focused on quantifying the inorganic concentration and on determining the stoichiometry of these bio-elements, which together with the Redfield anomalies are useful to describe the functioning of food webs, and to examine the processes that regulate marine biogeochemical cycles. The organic matter in marine systems is operationally divided into particulate organic matter (>0.7 µm; POM) and dissolved organic matter (<0.7 µm; DOM).

DOM is an important reservoir of nutrients in the upper ocean and is composed by a complex and heterogeneous mixture of molecules. However, only a small fraction has been identified and little is known about their reactivity. Most DOM is immediately respired by marine micro-heterotrophs, oxidized by photochemical process, or permanently buried in sediments (Ridgwell and Arndt, 2015).The euphotic zone is the principal site of organic matter production in the ocean and the main process of DOM production are: extracellular release by phytoplankton, grazers-mediated release and excretion, cell lysis (both viral and bacterial), solubilization of detrital and sinking particles and release from prokaryotes (Carlson and Hansell, 2015). All production, removal and transformation processes leave an imprint on the composition of DOM and how it is cycled in the water column. Thus, all the processes that influence the reactivity of organic matter in the ocean, have gained attention in the last decades (Strom et al, 1997; Zubkov et al, 2008; Wawrik et al, 2009).

Nitrogen and phosphorus are fundamental components of DOM. The cycle of nitrogen includes a variety of forms and a series of complex transformations, involving electron shifts between the most oxidized form (nitrate; NO_3^-) and the most reduced one (ammonium; NH_4^+). Inorganic nitrogen species include nitrate, nitrite (NO_2^-) and ammonium, and organic forms are present as amino acids, nucleic acids, amino sugars and urea, as well as their polymers (DNA,

RNA, proteins). Primary production in marine ecosystems can be partitioned in according to the source of nitrogen substrate utilized by phytoplankton (Dugdale and Goering, 1967). "New" production refers to the part of primary production that is fueled by nutrients inputs from outside the euphotic zone (mainly NO_3^- and N_2), while "regenerated" production is the fraction of primary production sustained by reduced compounds of nitrogen (mainly NH_4^+ and dissolved organic nitrogen; DON), produced *in situ* through the food web (Dugdale and Goering, 1967). Another element which is part of DOM is phosphorous. This has been much less studied than nitrogen, although it is as essential as N for growth and it can exist as dissolved and particulate forms. Dissolved fractions include inorganic phosphorous, existing in the environment almost exclusively as orthophosphate (PO_4^{3-}) and organic phosphorus compounds like P-esters, P-diesters and phosphonates (Paytan and McLaughlin, 2007).

1.2 Release of dissolved organic matter by zooplankton in pelagic system

An important component of the pelagic food web is zooplankton. This group controls the secondary production and hence the transfer of nutrients through the pelagic food web. Zooplankton is mainly represented by crustacean and gelatinous organisms, among which the microcrustacean copepods appears as the most abundant group for all regions, accounting for up to 55 to 95% of total numerical abundance of zooplankton (Harris et al., 2000; Escribano et al., 2007).

The four main processes by which zooplankton can release DOM (Figure 1) are direct excretory release, egestion, breakage of preys during feeding (sloppy feeding) and leaching from fecal pellets. Further studies on the relative importance of these processes demonstrated that sloppy feeding and excretion are the dominant modes of DOM production, being a minor role the leaching from the fecal pellets (Vincent et al., 2007; Saba et al., 2011). Mesozooplankton can remove from 10% to 40% of daily primary production in the coastal and open ocean, respectively (Calbet 2001). Complementary to the control exerted on the structure of food webs through direct predator-prey interactions, exerting a control on primary production (PP), mesozooplankton also play an important role in the active transport of DOM, and are capable of fueling the microbial loop (Richardot et al., 2011; Steinberg et al., 2002).
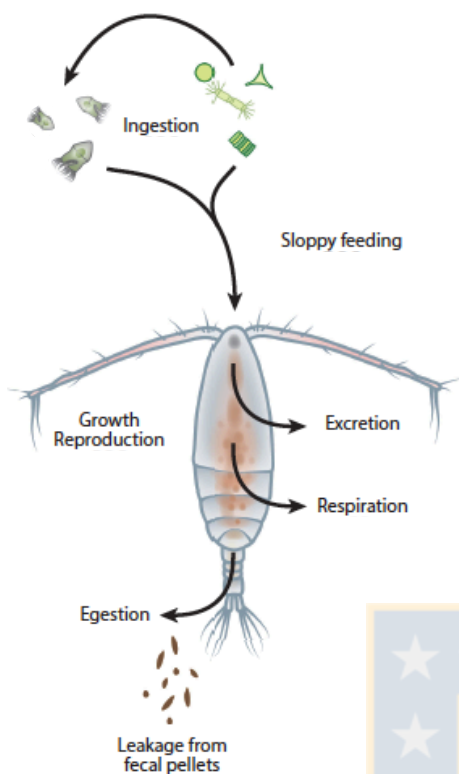
Figure 1: Particulate organic consumption and metabolism by zooplankton. Prey is consumed by zooplankton. Resulting of sloppy feeding some of dissolved organic compounds in the prey become lost to the surrounding water, while the rest of the prey is ingested. Some of the ingested prey is assimilated and used for growth and reproduction. The rest of the compounds that were not absorbed can be egested as fecal pellets, and released as waste from excretion. Extracted from Steinberg and Landry 2017.

In general, it has been reported that ammonium is the primary nitrogenous product excreted by marine zooplankton, comprising up to 75% of the total nitrogen excreted, followed by urea and amino acids which can account for 25% (Bidigare, 1983). Ammonium supplied by zooplankton in the pelagic ecosystem is believed to represent the largest input of this element in marine ecosystems. A global estimation of rates of ammonium by Hernández-León et al. (2008) showed that excretion rates were highest in equatorial waters and decreased rapidly poleward. Furthermore, 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). This finding has also been reported in oligotrophic zones with a 60% of total dissolved nitrogen excretion as DON (Le Borgne and Rodier, 1997). However, relatively few studies of DON release by excretion support those results (Conover and Gustavson, 1999; Miller and Glibert, 1998). Most of the research has been focused on nitrogen excretion and only a few studies are focused on phosphorus compounds, mainly on orthophosphate (Pomeroy et al., 1963; Ikeda et al., 2001;

Saba et al., 2009; Alcaraz et al., 2010). One of the first reported on phosphorus excretion demonstrated that one-third of soluble phosphorus released by zooplankton (amphipods) was in the organic form, also they found a tight coupling between dissolved organic phosphorus (DOP) production and uptake by bacteria (Johannes 1964). The same pattern was found by Satomi and Pomeroy (1965), where zooplankton excreted a high proportion (60%) of organic phosphorus.

1.3 Effects of feeding on excretion products

Because of the high variability in the composition of phytoplankton prey items, zooplankton can be affected in their growth and reproduction (Jónasdóttir et al., 2002; Aguilera and Escribano, 2012), but also the products excreted with consequences in the composition of bioavailable regenerated products. In this regard, it has been reported that composition and concentration of nitrogen excreted by zooplankton are affected by the quantity and quality of the food ingested (Saba et al., 2009). Miller and Roman (2008) reported that the N: C ratio of ingested food in copepods varied drastically in the various diets, ranging from 0.26 to 0.04 for ciliates and detritus respectively, affecting the nutritional status of organisms and with significant effects on nitrogen excretion. Thus, copepods fed food with N: C ratios resembling their own body composition, such as log-phase diatoms and ciliates, excrete more ammonium when fed in higher concentration. In the same context, other studies showed that ammonium was a high portion of total nitrogen release when copepods were feeding carnivorously (Corner et al., 1976), and Bidigare (1983) proposed that an increase in the release of urea by zooplankton may reflect the proportion of phytoplankton ingested in their diet, since phytoplankton contains a high content of arginine, a precursor of urea, although Miller and Roman (2008) suggested that urea excretion rates of *Acartia tonsa* were higher when fed with ciliates compared to a diatom diet.

On the other hand, Saba et al., (2009) showed a high dependency of the prevailing compound being excreted on the food provided to copepods, showing that DOP release was only detectable in diets containing micro-zooplankton. The available information however is contradictory and not always reflect the environmental conditions in terms of the food offered to zooplankton and only there are a few studies that reflect the omnivorous behavior of copepods in the natural environment.

1.4 The biogeochemical role of zooplankton in the marine system

Phytoplankton prefer ammonium over other sources of nitrogen, since it is in the reduced form, and therefore requires less energy to assimilate, but ammonium is not always the dominant substrate to be used by phytoplankton. It has long been recognized that urea uptake can support a significant amount of regenerated production in a wide variety of environments (Sipler and Bronk 2015). Furthermore, DON consumption can satisfy a large proportion of the requirements of nitrogen for the phytoplankton (Wawrik et al., 2009; Bronk et al., 2006), although little is known about its reactivity. The contribution of nitrogen to phytoplankton community has been more studied than the phosphorus. Pérez-Aragón et al. (2011) showed that copepods in a coastal upwelling zone of Chile can sustain up to 26.7% of the phytoplankton ammonium demand. In other upwelling zones, e.g. in northwest of Africa, it was found that zooplankton supplied an average of 44% of the ammonia demand (Smith and Whitledge, 1977). Moreover, in oligotrophic zones, such as the Mediterranean Sea, the nitrogen regenerated by mesozooplankton accounted for 43% of the nitrogen requirements by phytoplankton, and even in some coastal sites the ammonia excreted by zooplankton exceeded the phytoplankton nitrogen demand (Alcaraz et al., 1994). However, on a global basis, nutrient regeneration by mesozooplankton (between 200 to 2000 μm) was estimated to be in the range of 12-23% of the requirements for phytoplankton growth (Hernández-Leon et al., 2008). Alcaraz et al. (2010) studied the contribution to the regeneration of inorganic phosphorus via zooplankton excretion in the Artic zone and found that zooplankton contributed with 53% of the phosphorus requirement by phytoplankton.

On the other hand, the microbial community, as responsible for approximately half of the overall global primary production, plays an important role in nutrient cycling (Arrigo, 2005). Furthermore, it is the dominant biological component in the marine ecosystem exhibiting the most diverse metabolisms. The dominant nitrogen sources and their relative concentrations are important in determining the microbial community structure in aquatic environments. Bacteria and zooplankton are usually considered as separate group despite their temporal and spatial co-existence (Tang et al., 2010). However, recent research has established a direct relationship between the microbial and the zooplankton community with a consequent effect on the different biogeochemical pathways linked to nitrogen and phosphorus cycles (Calbet and Landry, 1999; Grossart et al., 2009; Titelman et al., 2008; Richardot et al., 2011; Tinta et al., 2012; Aristegui

et al., 2014). Vargas et al. (2007) showed that the copepod *Acartia tonsa* play an important role influencing bacterial production through the production of significant amounts of bacterial substrate by means of grazing activity. Furthermore, in addition to the classical contribution of zooplankton to food webs, their role as environmental reservoirs for a high diversity of microorganism, including pathogens, is increasingly recognized (Tang et al., 2010).

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 Farias 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 zone (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).

A recent study on the impact of krill excretion on microbial activity demonstrates that the addition of krill excretion products stimulates bacteria growth and production (Arístegui et al., 2014). Also, studies on jellyfish showed that the bloom of these organisms can provide significant amounts of dissolved organic matter (DOM) due to the jellyfish degradation and excretion, facilitating the mineralization of dissolved organic carbon and dissolved organic

nitrogen (DON), followed by a high accumulation of ammonium and orthophosphate available for microbial communities (West et al., 2009).

On the other hand, recent research has demonstrated that bacterial community composition can change in response to the addition of organic matter (Landa et al., 2013; Sarmento et al., 2013) DOM release during phytoplankton blooms has been associated with an important change in the microbial diversity and their metabolism (Mc Carren et al., 2010; Sarmento and Gasol, 2012). These results, was also found in studies on degradation of jellyfish, showed a rapid shift in community composition from unculturable Alphaproteobacteria to culturable species of Gammaproteobacteria and Flavobacteria (Tinta et al., 2012). However, the response of bacterial community structure to the compounds release by copepod excretion has not been studied yet.

1.5 The scientific problem

At present, the processes and mechanisms through which nitrogen and phosphorus compounds released by zooplankton can determine and influence the food web structure of the microbial community are unclear. At one end of the food web, zooplankton nutrition may depend on quality of their food resources, and the interaction between zooplankton organisms and their food may determine the quality of nitrogen and phosphorus organic compounds. Meanwhile, variable species of organic nitrogen and phosphorus can determine the structure of the microbial community. Although previous studies have shown responses of the bacterial community to input of DOM, the interaction among zooplankton feeding conditions, zooplankton excretion, and the associated microbial loop has not yet been examined in the pelagic system. All these processes however may vary depending on the environmental forcing, of which seasonal and regional effects can be highly significant. The complex relationships among biological components, nutrients and related cycling processes can be sketched for pelagic systems as shown in Figure 2.
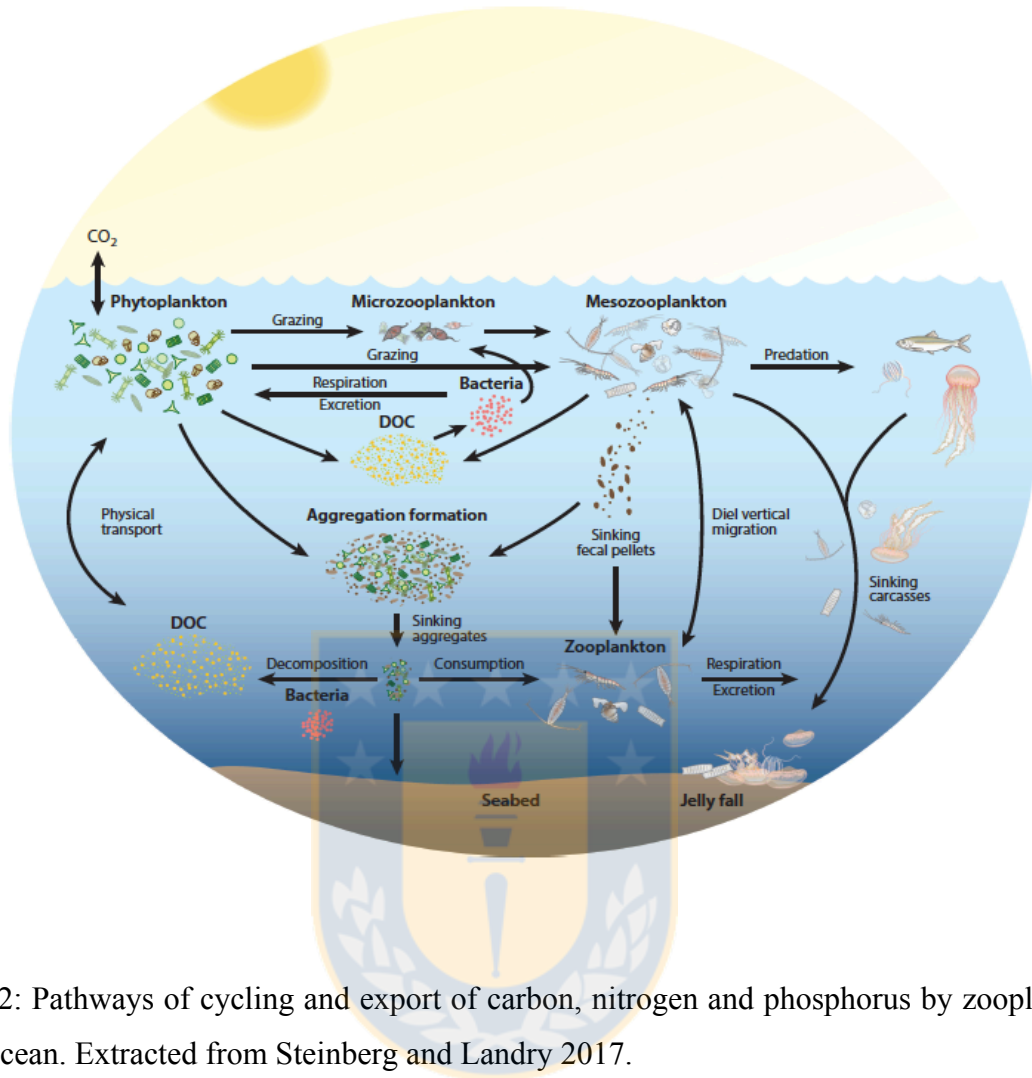
Figure 2: Pathways of cycling and export of carbon, nitrogen and phosphorus by zooplankton in the ocean. Extracted from Steinberg and Landry 2017.

1.6 Working Hypothesis

Hypothesis 1:

The type of food ingested (based on size fraction and composition) by zooplankton can determine the main nitrogen and phosphorus compounds to be excreted in a highly productive upwelling system off central-southern Chile.

Hypothesis 2:

Zooplankton excretion in different marine systems (coastal and open ocean), can significantly reshape the microbial community structure and activity.

1.7 General goal

This project aims at enhancing our comprehension on the role that trophic interactions involving microbial and metazoan food webs of the ocean can play in the recycling of nitrogen and phosphorus in the pelagic environment.

1.8 Specific goals

1) Identifying the nitrogen and phosphorus compounds excreted by zooplankton under different diets (prey composition), depending on seasonal variability in central-southern Chile and along an oligotrophic gradient in the western tropical south Pacific.

2) Quantifying the contribution of nitrogen and phosphorus excreted by zooplankton to the potential nitrogen and phosphorus demand of phytoplankton community and evaluate the impact of excreted products on local regenerated production.

3) Determining the role of nitrogen and phosphorus compounds excreted by zooplankton on nitrifying communities and in shaping the structure of the microbial community and its seasonal and regional variability in the central-southern Chile and western tropical south Pacific.

2. MATERIALS AND METHODS

The present study took place in the coastal area of Central Chile and in the oligotrophic region in the Western Tropical South Pacific (WTSP). The thesis work was done in order to evaluate two main issues: the excretion of copepods and the impact of the excreted compounds on microbial communities. The study was developed as based on experimental approaches with a total of seven experiments.

2.1 Study area

2.1.1 Central Southern Chile

Four oceanographic campaigns were conducted during 2012 and 2014. Two campaigns, named as CopeMOD (Copepods and Dissolved Organic Matter) during austral autumn/winter season (10th May 2012 and 9th September 2012, respectively), and two named as PROMO (Provenance and Reactivity of Dissolved Organic Matter), were conducted during the austral spring/summer season (23rd March 2014 and St 18 on 12th December 2014), onboard of the R/V Kay Kay II. The sampling sites were station 12 and station 18, located at ~22 km and ~33 km from shore, as indicated in Figure 3.

The productivity in the coastal zone off southern-central Chile is stimulated by upwelled water that fertilize the photic zone with inorganic nutrients during the spring-summer season. In contrast, the productivity is much lower in winter, and the system is mostly sustained by regenerated production (Fernandez and Farias 2012), and the input of new nutrients mainly comes from river runoff and mixing (Escribano and Schneider 2007; Sobarzo et al., 2007).
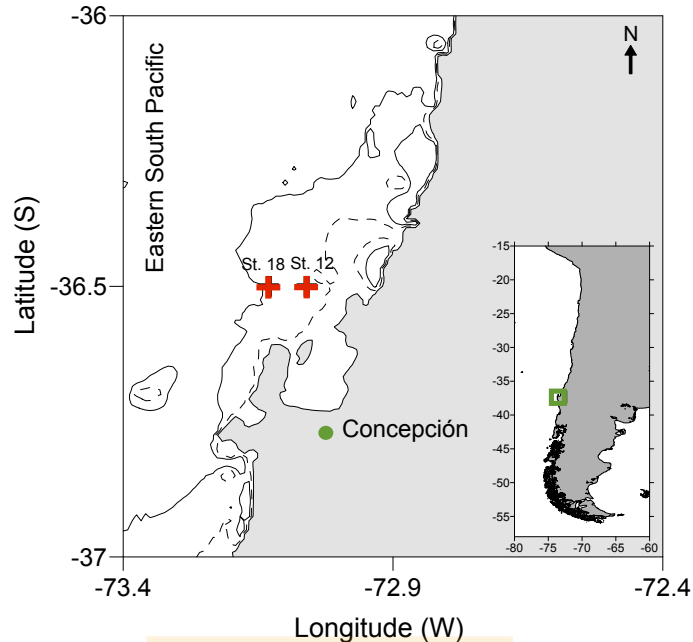
Figure 3: The eastern south Pacific and the coastal upwelling zone off Concepcion, illustrating the sampling stations. St 12 for CopeMOD and PROMO (late summer) experiments and St. 18 for PROMO experiment (spring). The solid contour line indicates 100 m depth and the long-dashed contour line indicates 50 m depth. Source: elaborated by the author.

2.1.2 Western Tropical South Pacific

Sampling was conducted during austral summer 2015 (18 February to 3 April) during the OUTPACE cruise (Oligotrophic to UlTra-oligotrophy PACific Experiments), onboard the R/V *L`Atalante*. The transect beginning in west off New Caledonia (18º S-159.9º E) and ending near Tahiti (17.56º S-149.05º W). Experiments were conducted in three long duration stations: LD A, B and C, which are located in two geographic regions: LD A and B in the Melanesian archipelago (MA) and LD C in the subtropical gyre (SG; Figure 4). These areas are characterized by low phytoplankton biomass with a deep chlorophyll-a maxima (DCM) and low nutrients surface concentrations. The WTSP harboring many islands with Fe rich sediments and is influenced by multiple ocean currents which promote shearing instabilities and strong eddies (Qiu et al., 2009) reducing their oligotrophic condition in the MA region. Furthermore, the WTSP has recently been considered a hotspot for N_2 fixation (Bonnet et al., 2017), which is considered to the largest external nitrogen source to the ocean (Sohm et al., 2011).
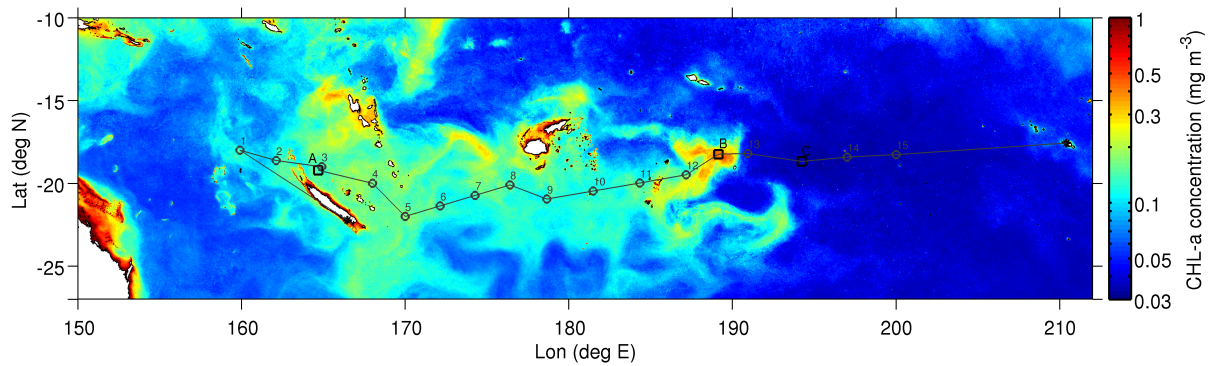
Figure 4: Transect during OUTPACE cruise with the sampling stations. The long duration stations are A, B and C. LD A and B are located in the Melanesian archipelago (MA) and LD C in the Subtropical Gyre (SG). Extracted from Moutin *et al*., 2018

2.2 Chapter I: Nitrogen excretion by copepods and its effect on ammonia-oxidizing communities from a coastal upwelling zone

During autumn and winter 2012, two experiments were performed in order to determine the main nitrogen compounds excreted by dominant species of copepods and its possible effect 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 DON and ammonium by copepod excretion. For this, at each sampling date we monitored the hydrological variables, such as temperature, salinity and dissolved oxygen, using a CTD-O profiler (SeaBird SBE 25 Plus). In order to examine the available food for copepods at this sampling site seawater samples were collected with 10-liter Niskin bottles for total and size fractioned chlorophyll-a (Chl-a), particulate organic carbon (POC), particulate organic nitrogen (PON), microplankton and nanoplankton. See methodological details in the results section in chapter I.

2.2.1 Experimental procedures

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 and 50 m depth under daylight conditions. Live samples were immediately transferred to coolers and diluted with surface seawater until arrival (~1 h after sampling) to the laboratory at the Dichato Marine

Station of the 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 (Postel et al. 2000; Turner 1979).

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 4 steps: acclimatizing copepods, feeding, excretion and microbial response (Figure 5), as follows: The acclimation phase (1) consisted of maintaining 4 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. 12º C). Acclimatization was followed by the copepod-feeding phase (2), to assess 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 (12º C). During this phase copepods were maintained in 200 μm mesh-tubes as the acclimation 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. 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 one-hour intervals (t0= 0h, t1 = 1h and t2 = 2h) to determine 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 (0.22 μm).

The 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 distributed into ten 1 L glass bottles under a peristaltic pump (1 L; Duran Schott). 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 two hours (t0 = 0h, t1 = 2h, t2 = 4h and t3 = 6h) 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. Quantitative measurements of AOA and AOB were determined by using complementary DNA (cDNA) templates and analyzed by quantitative polymerase chain reaction (qPCR).

Methodological details of nutrients analysis (NO_3^-, NO_2^-, NH_4^+), DON, bacterioplankton abundance and molecular analysis were provided in the results section in chapter I.

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 size-fractions)

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

3. EXCRETION
Time 2h (subsample 0, 1, 2 h)
Volume 6 L
Incubation in FSW 0.22 μm
Control without copepods

Treatment   Treatment   Control
20-150 μm   < 20 μm

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   Treatment   Control
20-150 μm   < 20 μm

Figure 5: 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. Source: elaborated by the author.

2.2.2 Data analysis

Average control concentrations (NH_4^+ and DON) during the excretion phase were subtracted from treatment values to obtain the corresponding concentration related to excretion during the incubation. Negative values indicated that control values were higher than those of the treatment, whereas positive values indicated that treatment values were higher than those of the control, indicating that copepods were indeed excreting. Copepod excretion rates for NH_4^+ and DON were determined using the endpoint approach for the time periods t0 to t1 and t1 to t2. For the entire incubation time (from t_0 to t_2) these rates were obtained from the slope of the linear regression of concentration as a function of incubation time. This because it has been reported that some N compounds excreted by zooplankton can be released in spurts of high concentration in short periods (~ 1 h; Gardner and Paffenhöfer 1982; Steinberg et al. 2002). Similarly 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 ANOVA and a 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.

2.3 Chapter II: Dissolved compounds excreted by copepods reshape the active marine bacterioplankton community composition

2.3.1 Field sampling

At each sampling station, an autonomous oceanographic profiler CTD-O SeaBird 19 was deployed to obtain temperature, salinity and dissolved oxygen measurements. Seawater samples were obtained at 10 m depth with a Niskin bottle (10L) for incubations and for chemical-biological measurements. Incubation seawater was maintained in carboys, in dark conditions, until arriving to the Dichato Marine Station of University of Concepcion (~1 h). Seawater samples for NH_4^+, NO_3^-, NO_2^-, PO_4^{3-} and bacterioplankton abundance were taken on board, DNA and RNA samples (the latter was taken only in spring) were immediately filtered upon arrival to the laboratory. Also, total and size fractioned chlorophyll-a (Chl-a), microplankton and nanoplankton abundance, were obtained at each site in order to determine the *in-situ* food available for copepods. See methodological details in the results section, chapter II.

2.3.2 Experimental procedures

Zooplankton were captured at each site by means of vertical hauls of a WP-2 net with 200 μm mesh-size. The net was equipped with a non-filtering cod end to capture undamaged individuals. Once onboard, the samples were poured into coolers and diluted with surface seawater (10 m depth) to maintain the organisms alive and in properly conditions until sorting at the laboratory (~1 h).

To explore the interaction between dissolved compounds excreted by copepods and bacteria, we designed an experiment consisting in four phases: (1) Copepods selection and acclimation, (2) Copepods feeding, (3) Copepod excretion and (4) Microbial response to dissolved compounds excreted in the previous phase (Figure 6). The experimental design of each phase is as follows:

Phase 1. Live zooplankton samples for each sampling event were sorted under a stereomicroscope at low light. The number of individuals used in both experiments was determined as based on the mean abundance observed in the St. 18 of the COPAS time series (Escribano et al., 2007; Hidalgo et al., 2010). In late summer, a mix of two abundant adult

copepods, *Acartia tonsa* (71%) and *Paracalanus* cf *indicus* (29%) were sorted, whereas, in spring, a mix of three adult copepods was sorted, *P.* cf *indicus* (37.5%), *Calanoides patagoniensis* (45%) and *Spinocalanus* sp. (17.5%). In both sampling dates, four equal groups of copepods, containing the same community, were isolated from surrounding water using 200 μm mesh-tubes and maintained in two incubators (previously washed with 10% HCl) filled with 10 L of 0.7 μm filtered seawater (GF/F; Whatman). The acclimation was carried out under dark conditions for 6 hours.

Phase 2. The 4-copepod groups selected were then transferred for feeding purposes into two 10 L incubators containing different size fractions of natural food, <20 μm and 20-150 μm (2 groups for each incubator), obtained earlier. This feeding phase was performed under dark conditions (12º C) and lasted 4 hours.

Phase 3. The copepod groups were transferred to a different set of incubators containing 0.2 μm filtered seawater (Durapore; Millipore). This was done to minimize the presence of microbial assemblages during the excretion phase. The incubator system consisted in six chambers (6 L each), two for the treatment fed with 20-150 μm size fraction, two for the treatment fed with <20 μm size fraction, and two incubators as controls (without copepods). Copepods were isolated in the chamber in tubes using 200 μm mesh-tubes, as feeding and acclimation phase. Each chamber was equipped with a sampling faucet to retrieve subsamples with minimal disturbance. At time intervals of 0 h (T0), 1 h (T1: T0+1h) and 2 h (T2: T0+2h) samples were collected in duplicate for NH_4^+, NO_3^-, NO_2^-, PO_4^{3-}, DON, dissolved organic phosphorus (DOP) and dissolved free amino acids (DFAA) from the sampling faucet with 0.5 L glass flask (acid-washed and autoclaved; Duran Schott). Bacterioplankton abundance was monitored during all incubation time to evaluate potential influence of small size microbial communities.

Phase 4. To evaluate the microbial response to dissolved compounds provided by copepod excretion, 10 glass bottles with 850 mL of seawater with natural microbial assemblages (10 m depth), were enriched with 150 mL aliquots of the excretion products derived from phase 3. Only one control chamber was used.

Samples were collected in duplicate at time intervals of 0 h (T0), 2 h (T1: T0+2h), 4 h (T2: T0+4h) and 6 h (T3: T0+6h) in duplicate for NH_4^+, NO_3^-, NO_2^-, PO_4^{3-}, DON, DOP, DFAA, RNA, bacterioplankton, cyanobacteria and picoeukaryotes abundance. DNA and RNA samples

were taken to assess the changes in the bacterial community structure, so that the assessment included the whole bacterial assemblages and the active one. The same protocol was applied for DNA samples (duplicated), but in 4 L polycarbonate bottles (Nalgene). In that case, 3.4 L of natural microbial assemblages was inoculated with 600 mL aliquots of dissolved compounds excreted by copepods from each chamber of the previous phase. DNA samples were taken at initial (T0) and final incubation time (T3: T0+6h) and RNA samples during all time intervals.
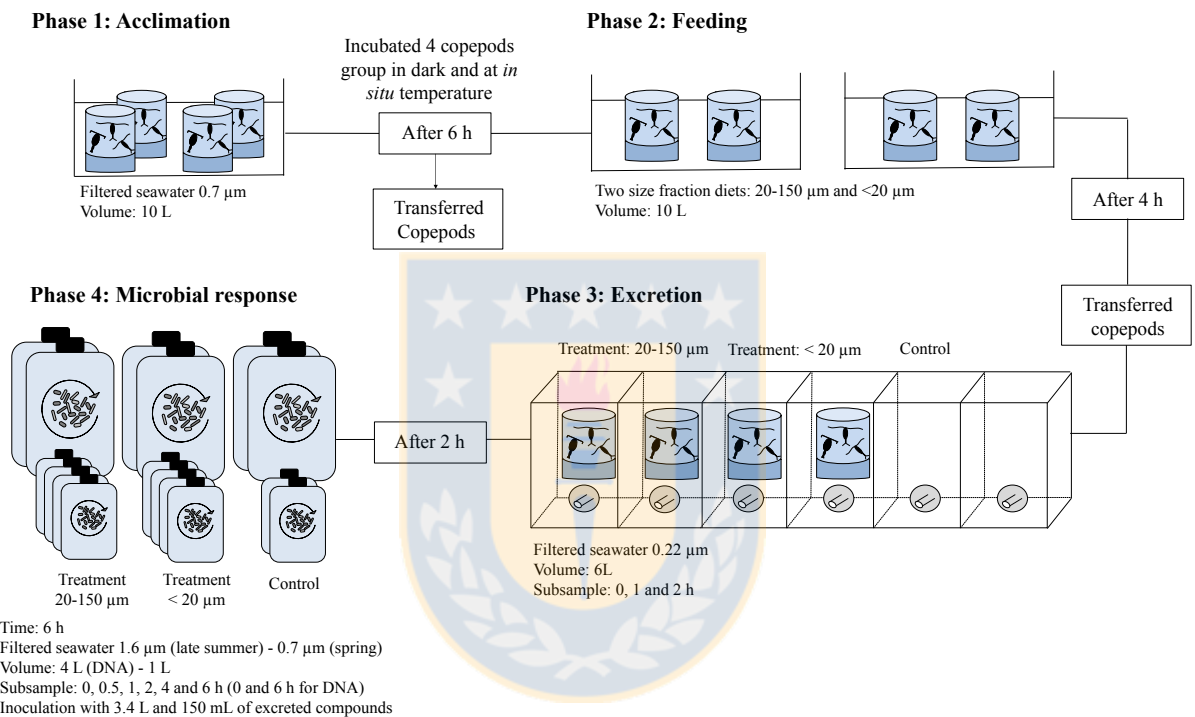
Figure 6: Experimental design used in PROMO experiments. The design consisted of four phases done sequentially. Each copepod group was acclimated for at least 6 h (phase 1) inside cylinders isolated from the surrounding water with a 200 µm mesh. Cylinders are then transferred to two glass containers for feeding (phase 2) under two natural size fractions of food: 20-150 µm and <20 µm during 4 h. Copepod excretion (phase 3) had replicated treatments and controls (without copepods), in 10 L chambers equipped with a faucet near the bottom for sampling aliquots. Finally, the microbial response (phase 4) was carried out in glass flasks and each treatment had four replicates. Source: elaborated by the author.

Mean control concentration of NH_4^+, PO_4^-, DON, DOP, DFAA was subtracted from the treatments, in order to obtain a concentration related to excretion during incubation periods. Then excretion rates of each nitrogen and phosphorus compounds (NH_4^+, PO_4^-, DON, DOP, DFAA) were estimated using the slope of the linear regression fitted to the concentration as a function of incubation time. The concentrations obtained in the controls during phase 4 were averaged and subtracted from the treatments at each time (T0, T1, T2, T3), as we did for the excretion phase. From these values, we estimated the turnover rate NH_4^+, NO_3^-, NO_2^- PO_4^-, DON, DOP and DFAA every two hours (T0-T1, T1-T2 and T2-T3). The rates were estimated using an end-point approach in according with the following equation:

Nutrient rate (μmol $L^{-1}h^{-1}$) = [Final nutrients]/Time(2h)

A positive value indicates a net accumulation, whereas a negative value indicates a net nutrient consumption.

Bacterial community structure was analyzed in terms of both total and active fraction by Illumina MiSeq sequencing method from 16S rRNA, using cDNA as template, and 16S rDNA (Campbell and Kirchman, 2013). See molecular methods, nutrient analysis, bacterioplankton abundance in the results section in chapter II.

2.3.3   Statistical analyses

Statistical analysis of treatments on chemical parameters associated with excretion products was performed using a paired t-test, and during the microbial response phase a two-way ANOVA was applied using treatments and time as effect levels, after testing for normality assumptions (Kolmogorov-Smirnov test) and homoscedasticity (Levene´s test). Pairwise multiple comparisons were performed using a Tukey test as a posteriori analysis.

Bacterial community structure was compared using ordination Bray-Curtis similarities, visualized in UPGMA dendrograms, whose nodes were further tested using a bootstrap analysis. Multivariate analysis was also applied to relate the variability in biological responses and environmental variables, using PRIMER v.6 and the add-on PERMANOVA+ software package. The permutational multivariate analysis of variance (PERMANOVA) with fixed factors was applied to investigate the differences of bacterial community composition for treatments and

control in both experiments. Principal co-ordinate analysis (PCO) was performed to visualize patterns of the bacterial community OTUs in response to different treatments.

2.4 Chapter III: Nitrogen and phosphorus recycling mediated by copepods in Western Tropical South Pacific

Live zooplankton samples were captured at night conditions at each LD station (day 2) with a WP2 zooplankton net with a 120 µm mesh size, equipped with a non-filtering cod-end to obtain undamaged individuals, between 0 to 100 m depth under night conditions in the three LD stations. Live samples were immediately transferred to coolers until sorting at the laboratory.

The experiment design involved three steps: (1) Copepod acclimation, (2) feeding and (3) copepod recycling (Figure 7). Seawater for incubations (30 L) was collected into clean (10% HCl rinsed) polycarbonate carboys from DCM using Niskin bottles (12 L) arranged on a CTD rosette. For acclimatizing (2) and copepod recycling (3) steps, seawater (22.5 L) was immediately filtered onto a 0.7 µm (GF/F; Whatman) using a peristaltic pump. The 7.5 L remaining was used in the feeding phase. Seawater for the different steps was maintained in a cold room (in situ temperature ~25º C) until the beginning of the experiment.

Undamaged individuals were sorted from the live samples and identified using a stereomicroscope at low light. Copepods samples in the three experiments consisted in a mix of the most representative copepods (adults) in the sample (Fig. S1). In acclimatization phase (1), 15 groups of 10 copepods were incubated in 500 mL Nalgene bottles, maintained for 4-6 h in filtered seawater previously filtered (GF/F Whatman) in darkness and at controlled temperature (in situ). In feeding step (2) copepods were removed from the bottles used in the previous phase, with a sieve (20 µm) and maintained in 500 mL polycarbonate bottles (Nalgene) with <150 µm filtered seawater (polycarbonate membrane) for feeding. This step lasted 4 hours and, as the acclimatization phase, was under controlled temperature. Thereafter, each copepod group (15 groups) was placed in 500 mL polycarbonate bottles (Nalgene) with the seawater previously filtered (0.2 µm; Millipore). Also, other 15 bottles without copepods were incubated as control. In total, 30 bottles were used in this step. The seawater used was filtered to avoid the presence of phytoplankton and small protist, that they can prey on bacteria, and ensure the presence of natural microbial assemblages. At the end of each step copepods were observed and checked by direct observation for ensuring swimming behavior in the bottles.

At time intervals of 0 h (T0), 0.5 h (T1: T0+0.5 h), 1 h (T2: T0+1 h), 2 h (T3: T0+2h) and 4 h (T4: T0+4 h) samples were collected for each bottle for NH_4^+, NO_3^-, NO_2^-, PO_4^{3-}, DON, DOP, bacterioplankton abundance and RNA. An additional time at 15 min (T0′: T0+0.25h) was added for NH_4^+ in LD B and C station. Six bottles were sacrificed at each time, three for treatment with copepods and three without copepods (control). The three bottles sacrificed per time were triplicates in the treatment with copepods and control. At the end of the experiment copepod samples were preserved immediately with formalin buffered by sodium borate (5% final concentration) for zooplankton composition analysis and biomass measurements.

Figure 7: Experimental design. The design consisted of three phases done sequentially. Each bottle with copepods was acclimated for at least 6 hours (phase 1), followed by feeding (phase 2) with the Deep Chlorophyll-a Maximum (DCM) seawater for 3 hours. Finally, copepod recycling (phase 3) has treatment and control (without copepods). Three bottles were sacrificed at each sampling time. Source: elaborated by the author.

Bacterial community structure was analyzed by Illumina MiSeq sequencing method from 16S rRNA (samples from incubation), using cDNA as template, and 16S rDNA (*in situ*;

Campbell and Kirchman, 2013). Methodological details for nutrients, bacterioplankton abundance and molecular methods were provided in the results section in chapter III.

2.4.1 Statistical analysis

Statistical analysis of treatments effects on chemical and biological parameters were performed using a two-way analysis of variance (ANOVA) after checking normality assumptions (Kolmogorov-Smirnov test) and homoscedasticity (Levene´s test). Pairwise multiple comparisons were performed using a Tukey test as a posteriori analysis. Statistical significance was set a p=0.05 and analysis was computed using R software.

The bacterial community structure was compared using the ordination Bray-Curtis similarities which were also used to build dendrograms by the unweighted pair group method with arithmetic averages (UPMG). For multivariate statistical analysis, the software package PRIMER v.6 and the add-on PERMANOVA+ was used. The permutational multivariate analysis of variance (PERMANOVA) with fixed factors was applied to investigate the differences of bacterial community composition for treatments and control in both experiments. Non-metric multidimensional scaling and principal co-ordinate analysis (PCO) was performed to visualize patterns of the bacterial community in response to different treatments.

3. RESULTS

3.1 Chapter I: Nitrogen excretion by copepods and its effect on ammonia-oxidizing communities from a coastal upwelling zone

Valentina Paz Valdés Castro

PhD in Oceanography

Universidad de Concepción

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 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.

Nitrogen excretion by copepods and its effect on ammonia-oxidizing communities from a coastal upwelling zone

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

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
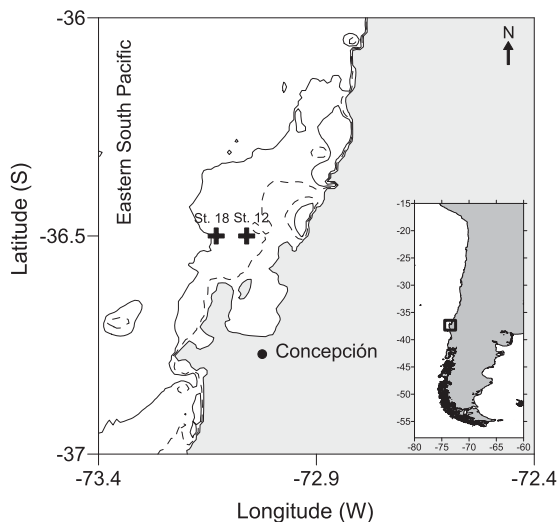
Fig. 1. The eastern south Pacific and the coastal upwelling zone off Concepcion, illustrating the sampling stations. Sta. 12 for CopeMOD experiments and Sta. 18 (COPAS Time Series) is included as a reference. The solid contour line indicates 100 m depth and the long dashed contour line indicates 50 m 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

3

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   Treatment 2   Control
20-150 μm   < 20 μm

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 ($\sim$ 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 ($\sim$ 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 $\sim$ 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
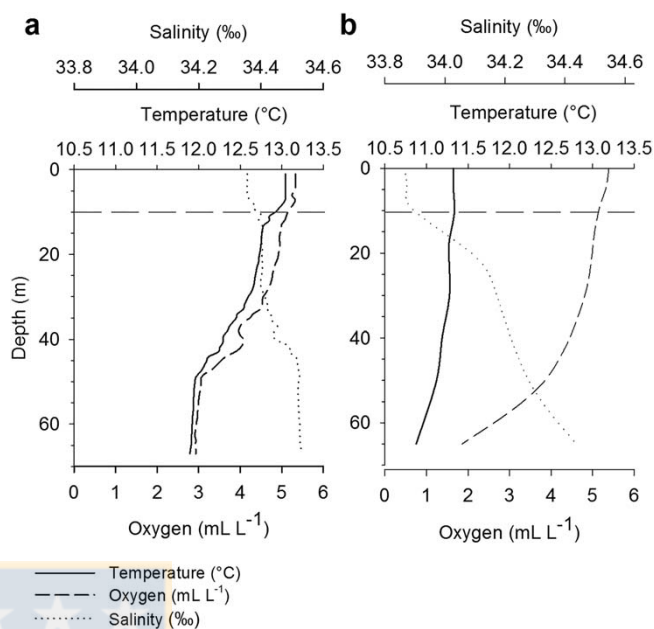
Fig. 3. Temperature (°C), salinity (‰), and dissolved oxygen (mL L^{-1}) in CopeMOD experiments: May 2012 (**a**) and September 2012 (**b**). Vertical profiles were obtained by continuous CTD-O measurement. The horizontal dashed lines indicate the collection depth of water samples (10 m depth).

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
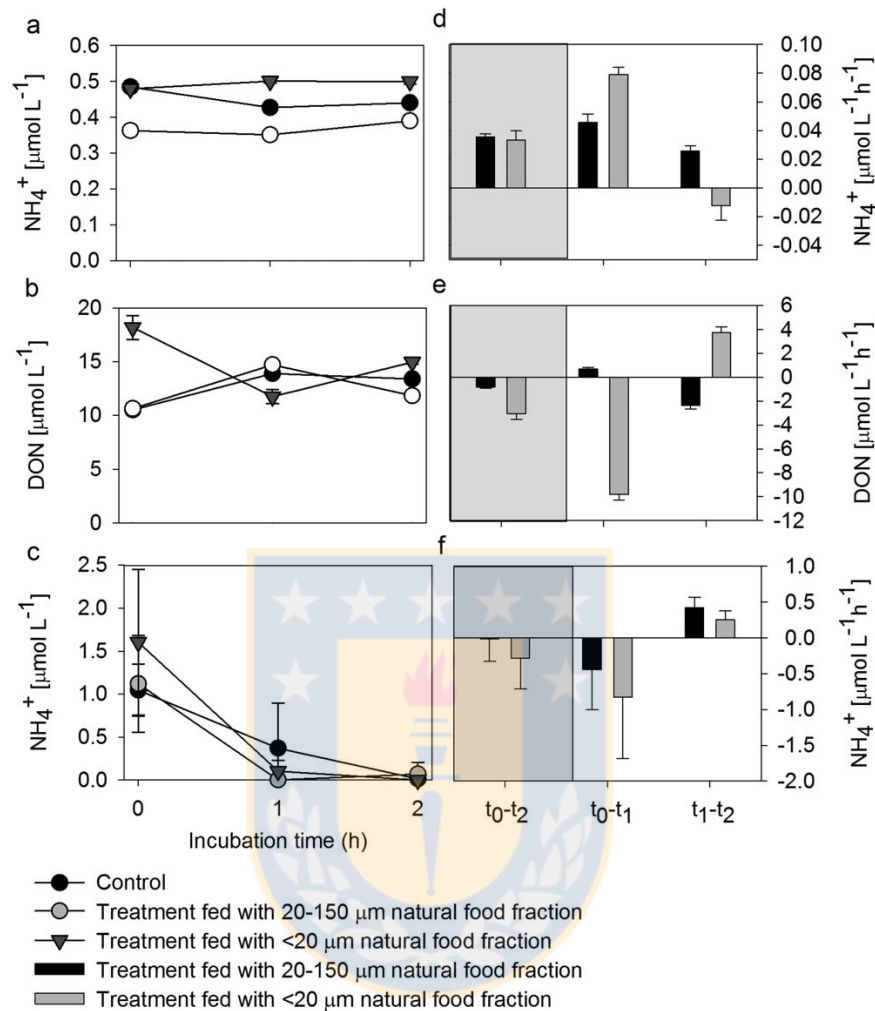
Fig. 4. Changes in nitrogen compounds over time during the excretion phase (left panel) and estimated excretion rates (right panel). (**a**) and (**d**) ammonium, (**b**) and (**e**) DON, in autumn. (**c**) and (**f**) ammonium in winter. Shaded panel indicates the excretion rates throughout the incubation (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).
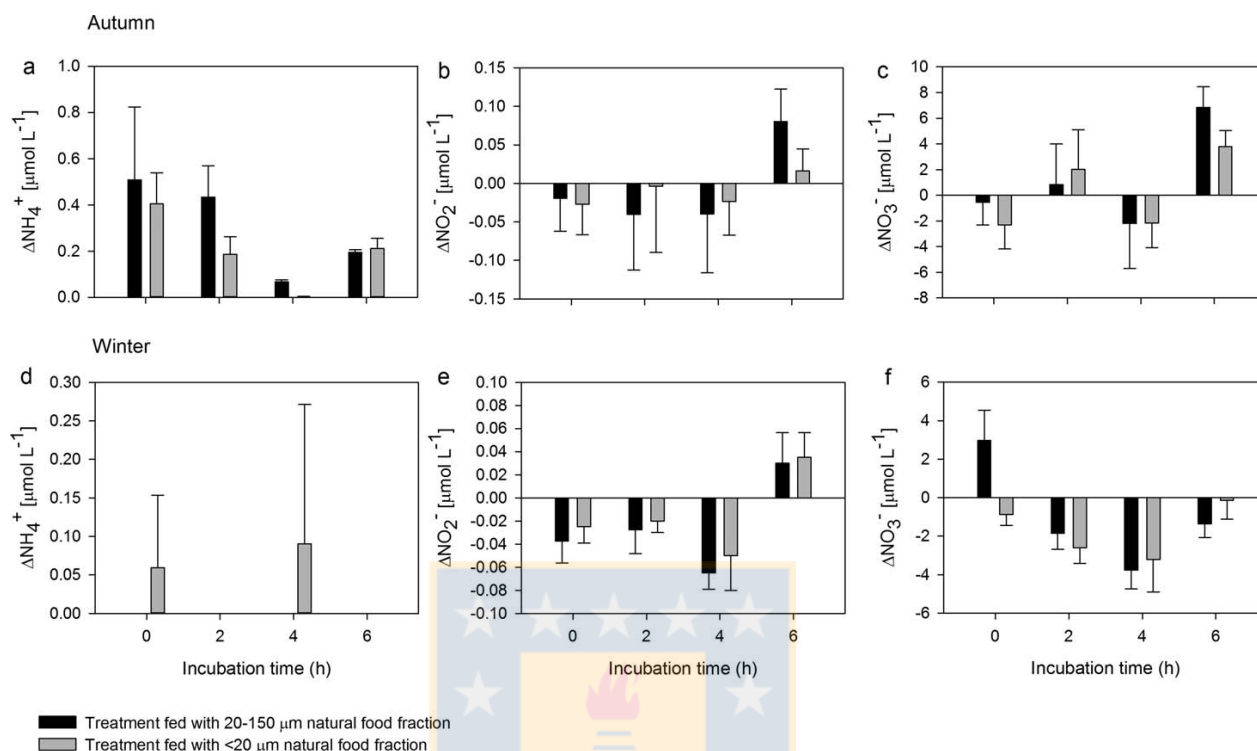
Fig. 5. Turnover of ammonium (**a**) and (**d**), nitrate (**b**) and (**e**), and nitrite (**c**) and (**f**) by microbial communities in CopeMOD experiments. The errors bars are standard deviations ($n = 4$).

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 ($\mu mol\ L^{-1}$)				Treatments ($\mu mol\ L^{-1}$)			
			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^+	20–150 μm	0.26±0.00	0.25±0.00	0.24±0.00	0.25±0.00	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^-	20–150 μm	16.8±4.61	15.4±4.10	17.4±3.47	10.5±4.23	−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^-	20–150 μm	0.47±0.01	0.44±0.08	0.47±0.06	0.36±0.03	−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^+	20–150 μm	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	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^-	20–150 μm	6.33±1.78	9.44±0.28	9.70±0.42	7.79±1.05	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^-	20–150 μm	0.36±0.04	0.38±0.01	0.41±0.02	0.35±0.01	−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 $< 20\ \mu$m 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 $< 20\ \mu$m 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 $< 20\ \mu$m 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 $< 20\ \mu$m) 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 $< 20\mu$m 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
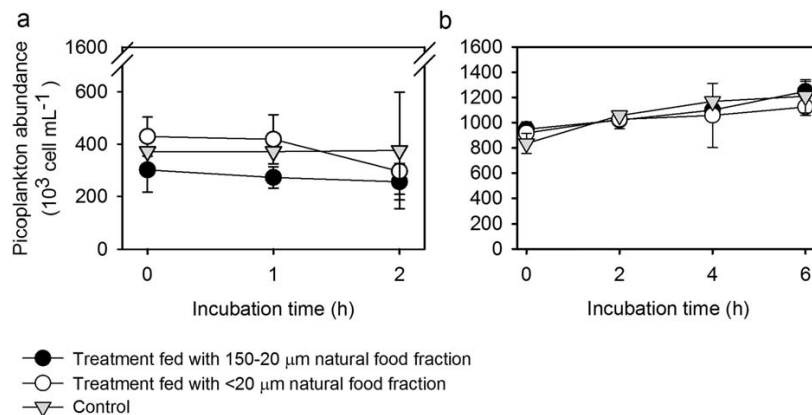
Fig. 6. Picoplankton abundance during the excretion (**a**) and the microbial response to the compound excreted by copepods phase (**b**) in autumn experiment. The errors bars are standard deviations ($n = 4$).
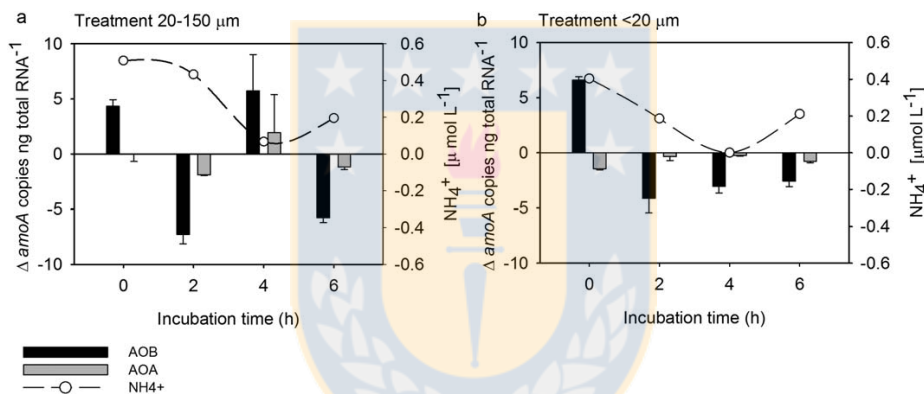
Fig. 7. Contribution of archaeal and bacterial *amoA* qPCR transcript counts and ammonium concentration over time in autumn experiment. (**a**) Treatment derived from copepods fed with 20–150 μm size fraction of natural food and (**b**) treatment derived from copepods fed with < 20 μm sized fraction 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.
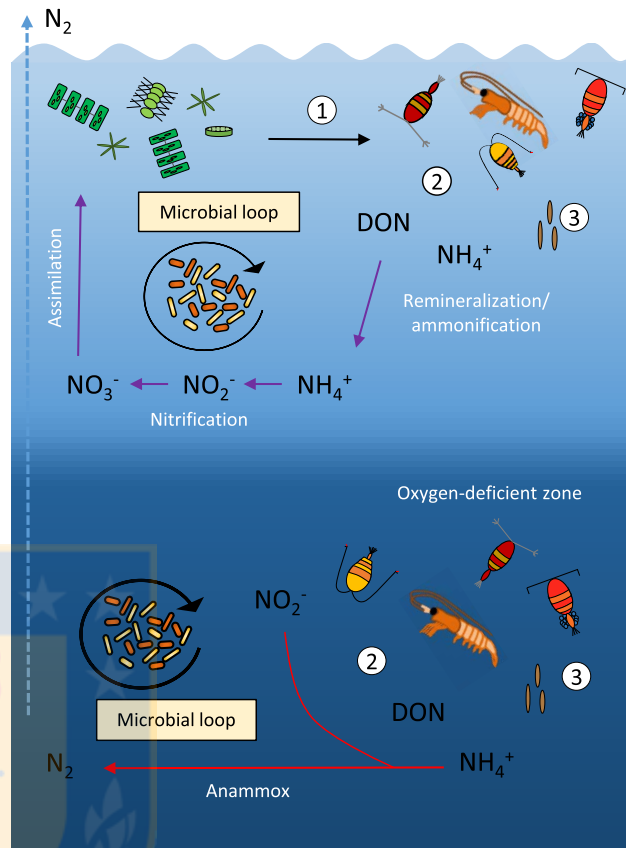
Fig. 8. Conceptual model representing the role of zooplankton for N cycling in the water column of an upwelling system, linked to the autotrophic community and the microbial loop. Three major processes are considered: (1) DON input by zooplankton through sloppy feeding, (2) DON and NH_4^+ input via zooplankton 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.

Supplemental Information

Nitrogen excretion by copepods and its effect on ammonia-oxidizing communities from a coastal upwelling zone

Valentina Valdés, Camila Fernandez, Veronica Molina and Ruben Escribano

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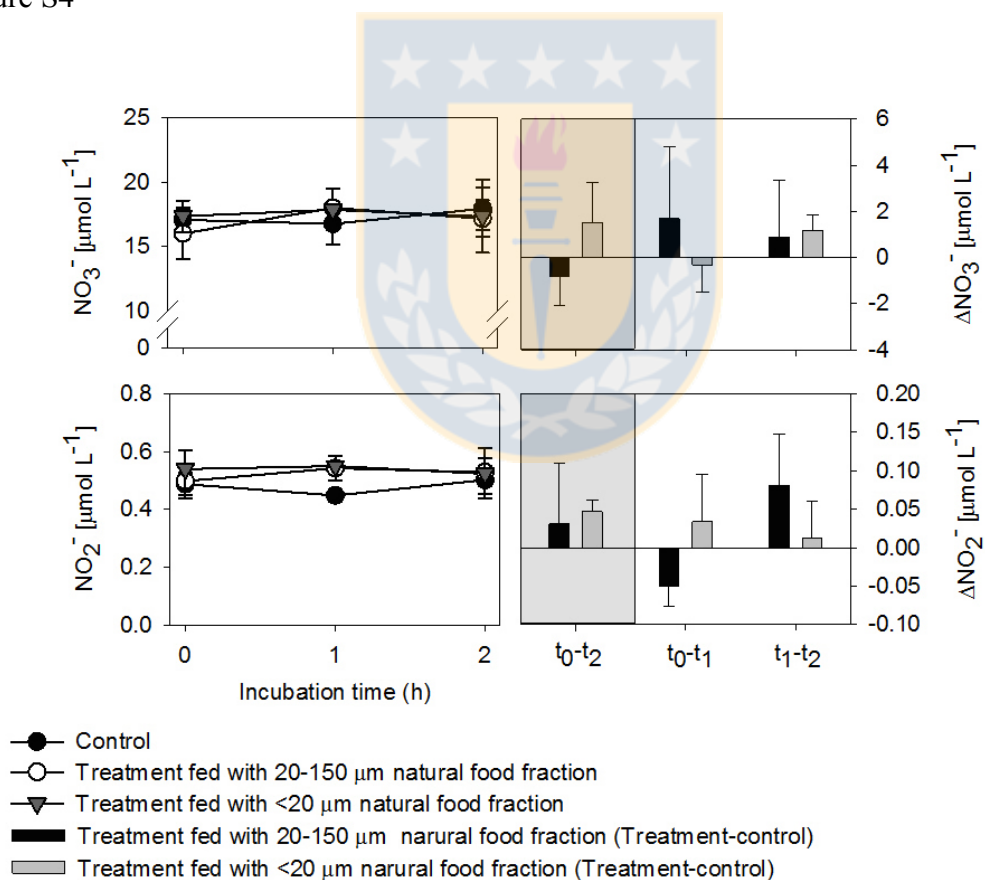
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Figure S1: Budget of nitrate and nitrite compounds over time in the excretion phase. The errors bars are standard deviations (n=4).

Artificial ammonium enrichment experiments

The predominant ammonia-oxidizers active in the study area were identified and transcriptional activity was assessed by ammonium enrichment experiments with in situ communities in the austral winter and summer (August 2008 and January 2009). To do so, seawater (20 L) was collected at different depths (surface and oxycline) at Station 18 (Fig. 1), considering that ammonia-oxidizing groups could be favored in the oxycline than in surface waters in the study area (Molina et al 2010). The oxycline depth was determined based on the oxygen profiler obtained from the COPAS time series at Station 18. Seawater was kept in carboys until reaching the laboratory. Seawater was then distributed into 1 L glass bottles (Duran Schott), with two replicates for controls and ammonium enrichment experiments for each sampling time, consisting of three hours in total t_0: 0h (carboy subsampling) and then t_1:1h, t_2:2h and t_3:3h. Thus, a total of 12 bottles were used per depth for the entire experiment. The number of bottles was selected to do the experiment in a timely manner considering the number of filtration units available to stop each experiment time. The ammonium enrichment treatment used a NH_4Cl solution designed to obtain a final concentration of 0.1 and 1 $\mu mol\ L^{-1}$ in winter and summer, respectively. These values were based on the expected ammonium concentration during the respective seasons considering the data available for the time series at Station 18. In situ ammonium concentrations obtain during our sampling varied between the surface and oxycline, 0.1-0.9 and 0.3-1.2 $\mu mol\ L^{-1}$ in the winter and summer experiments, respectively. The bottles were then incubated in darkness with a controlled temperature (12ºC). The incubation was completed by water filtration using a peristaltic pump. To avoid saturating the filters and to do the filtering in a timely manner, the water was pre-filtered through 1.6 μm GF/A (Whatman) and then filtered onto 0.22 μm Durapore filters (Millipore). The filters were soaked in 300 μL of RNAlater reagent (Ambion), frozen and kept at -80ºC until analysis (see the Molecular Methods section).

Identification and quantification of ammonia-oxidizing groups in artificial ammonium enrichment experiments. Specific RNA transcription assays were carried out using the reversed primers for archaeal *amoA*, Arch-amoAR (Francis et al. 2005), and bacterial *amoA*, amoA2R (Rotthauwe et al. 1997), with Sensiscript Reverse Transcriptase (Quiagen). All the reactions

included a control without the template. The archaeal and bacterial *amoA* were then amplified by PCR and qPCR using cDNA as template and the following primers Arch-amoAF and Arch-amoAR (Francis et al. 2005), amoA-1F and amoA-2R primers (Rotthauwe et al. 1997). A 25 µL PCR reaction was done using the following reagents: 1 µL template DNA (5-10 ng) and a final concentration of 1X PCR buffer minus Mg, 1.25 and 4 mmol L^{-1} of MgCl$_2$ (archaeal *amoA* and bacterial *amoA*, respectively), 200 µmol L^{-1} of deoxynucleotide triphosphate, 0.1 µmol L^{-1} of each primer (forward and reverse), and 1 U of Taq DNA polymerase (Promega). The thermocycler program consisted of the following steps and was used for archaeal *amoA* gene amplifications: 5 min at 95 °C, then 35 cycles of 1 min at 95 °C, 1 min at 53 °C or 56 °C (archaeal *amoA* and bacterial *amoA*, respectively), and 1.5 min at 72 °C, without a final extension. PCR products were visualized on agarose gels using standard electrophoresis procedures. The specificity of the PCR primer products was also evaluated by direct sequencing using the corresponding forward primers, only as a verification of the potential actively transcribed group and not as a community composition analyses since that had been reported previously in the study area (Molina et al. 2010). These sequences were aligned and phylogenetic relationship analyses were conducted using the Bosque program available from http://bosque.udec.cl (Ramírez-Flandez and Ulloa 2008). The phylogenetic analyses were based on maximum likelihood using the Fast Tree 2.0 method (Price et al. 2010) with bootstrap set of 1000 in Bosque. A consensus tree was generated using branches with bootstrap values higher than 70%. For AOA and AOB tree reconstruction *N. multiformis* and *N. maritimus* were used as outgroups, respectively. Interactive Tree of Life iTOL (http://itol.embl.de; Letunic and Bork 2016) was used to enhance tree visualization. All the sequences generated were deposited in the GeneBank public database under the accession numbers KU940408-KU949419 for AOA and KX449552 for AOB.

Reactions for archaeal *amoA* qPCR assays were carried out with the primers Arch-amoAF and Arch-amoAR. The primers amoA-1F and amoA-2R were used for experiments to quantify bacterial *amoA* genes. The qPCR reactions were done in a StepOnePlusTM Real-Time PCR System (Applied Biosystems), using a 20 µL reaction mixture with 5-10 ng of template DNA, (quantified in a NanoDrop ND-1000 Spectophotometer and the Quant-iTTM High-Sensitivity DNA assay with the Qubit Fluorometer (Invitrogen)), 0.4 M of primers and with the Power SYBR® Green PCR Master Mix (Applied Biosystems). All reactions were run in triplicate. The

qPCR protocol was similar for all the assays based on (Mosier and Francis 2008), which was as follows: 10 min at 95ºC, then 40 cycles for 30 s at 95ºC, 45s at 56ºC and 1 min at 72ºC with a detection step at the end of each cycle. A melting curve stage was run at the end of all the qPCR assays. The detection limit of the standards was always observed at a CT mean <30. The specificity of qPCR assays was verified by checking the dissociation curves and running the PCR products in agarose gels.

Plasmids containing cloned and sequenced *amoA* gene fragments were used as qPCR standards. The plasmids were extracted using Wizard Plus SV Minipreps (Promega) and quantified using the Quant-iTTM Broad Range DNA assay (Qubit Fluorometer, Invitrogen). The clones ST010550.E7 (FJ615376) and ST080550.F7 (GU066855) archaeal and bacterial *amoA*, respectively, were used as standards. The standard curves were prepared using 10-fold dilution series from $2 \times 10^7 - 2 \times 10^1$. The PCR efficiencies (E) and correlation coefficients (r_2) for the standard curves were as follows: archaeal *amoA* (E=91%, r2=0.989) and bacterial *amoA* (E=84%, r2= 0.994).
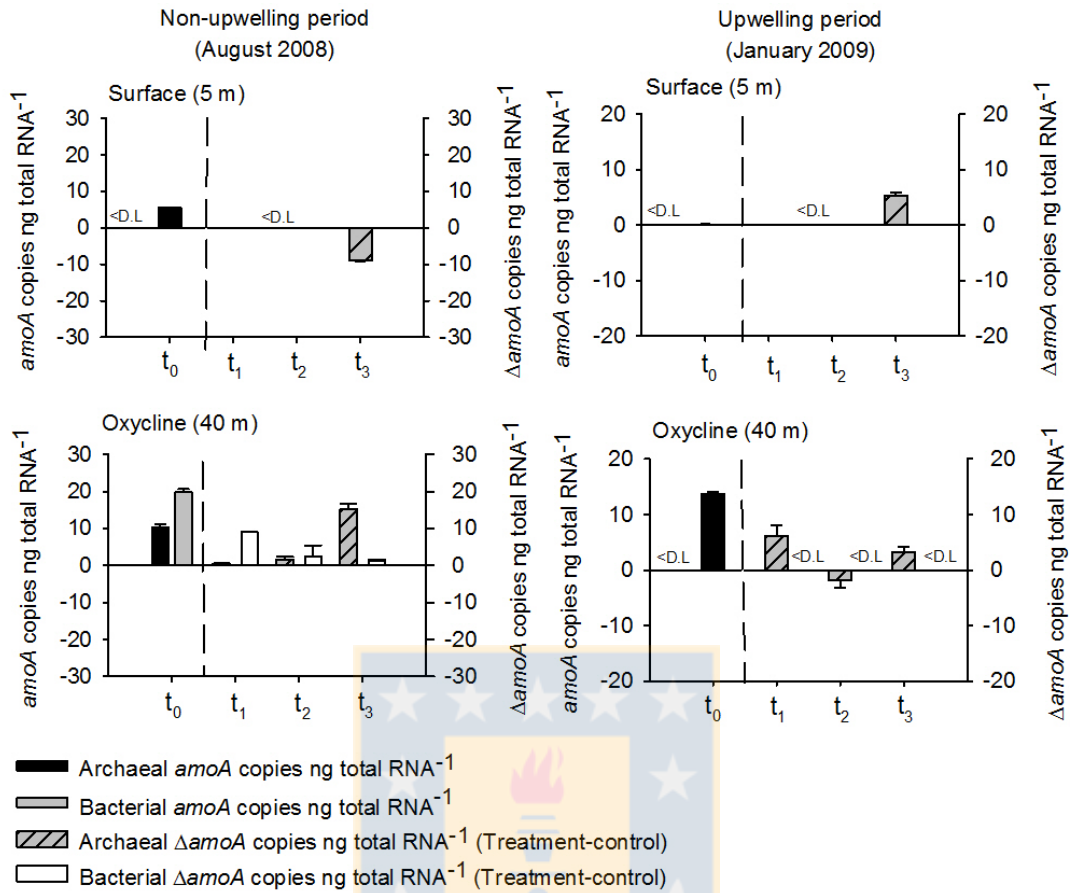
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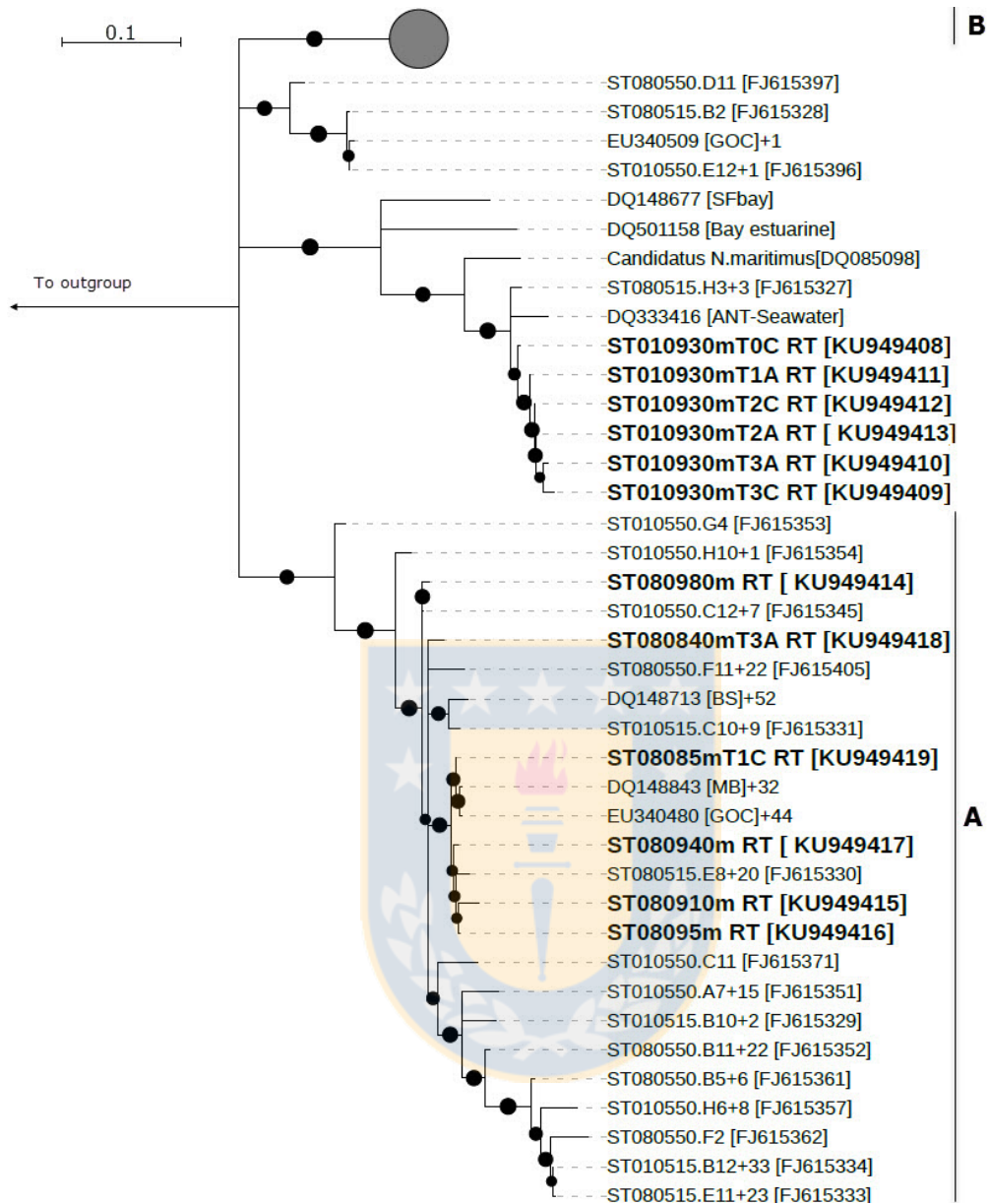
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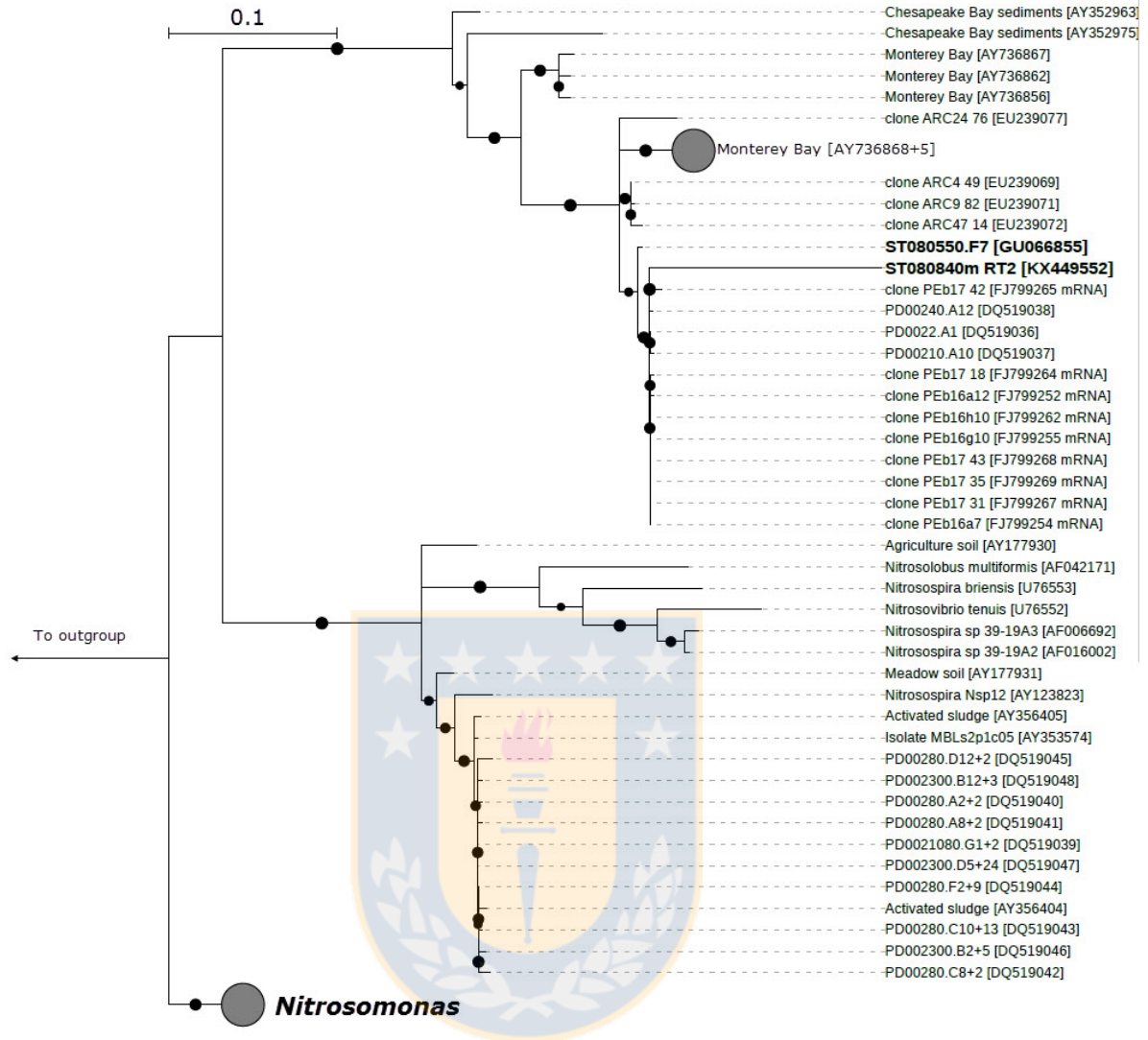
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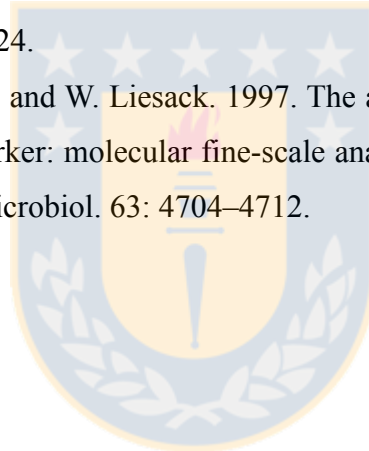
3.2 Chapter II: Dissolved compounds excreted by copepods reshape the active marine bacterioplankton community composition

Manuscript published in in Frontiers in Marine Science

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PhD in Oceanography

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Abstract

Copepods are important suppliers of bio-reactive compounds for marine bacteria through fecal pellet production, sloppy feeding and excretion of dissolved compounds. However, the interaction between copepods and bacteria is poorly understood in the marine environment. Herein, we investigated the nitrogen and phosphorus compounds excreted by copepods fed with two natural sized-fractioned diets (<20μm and 20-150μm) in the upwelling zone of central/southern Chile in late summer and spring. We then assessed the biogeochemical response of the bacterial community and its structure, in terms of total and active cells, to the enrichment with copepod excreted dissolved compounds. Results revealed that copepods actively excreted nitrogen and phosphorus compounds, mainly in the form of ammonium and dissolved organic phosphorus (DOP), reaching excretion rates up to 2.6 μmol L-1 h-1 and 0.05 μmol L-1 h-1, respectively. Maxima excretion rates were associated in both periods with the 20-150μm size fraction, but particularly during spring, where also a higher organic matter quality was observed in this excretion products compared to late summer. However, dissolved free amino acids (DFAA) excreted revealed higher excretion rates associated with copepods fed with <20μm size fraction, mainly by Histidine in late summer and Glutamic acid in spring. A shift in the composition of active bacterial community was observed between periods and treatments, associated with the response of common seawater surface phyla Proteobacteria and Bacteroidetes. Moreover, specific bacterial activity (16S rRNA: rDNA) suggested a differential response associated with the two size fraction diets; in late summer, Betaproteobacteria and Bacteroidetes were stimulated in the treatment enriched with excretion products derived from 150-20μm and <20μm size fraction, respectively. In spring, Alphaproteobacteria were active in

the treatment enriched with excretion products of copepods fed with <20µm size fraction while they were inhibited in the treatment enriched with excretion products of 20-150µm size fraction. Our findings indicate that different diets provided to copepods can have a significant impact on the quantity and quality of their excretion compounds, which can generate shifts in the active bacterial composition. The bacterial response is probably associated with common-opportunistic sea surface microbes adapted to rapidly react to environmental offers.

Dissolved Compounds Excreted by Copepods Reshape the Active Marine Bacterioplankton Community Composition

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Copepods are important suppliers of bioreactive compounds for marine bacteria through fecal pellet production, sloppy feeding, and the excretion of dissolved compounds. However, the interaction between copepods and bacteria in the marine environment is poorly understood. We determined the nitrogen and phosphorus compounds excreted by copepods fed with two natural size-fractionated diets (<20- and 20–150-μm) in the upwelling zone of central/southern Chile in late summer and spring. We then assessed the biogeochemical response of the bacterial community and its structure, in terms of total and active cells, to enrichment by copepod-excreted dissolved compounds. Results revealed that copepods actively excreted nitrogen and phosphorus compounds, mainly in the form of ammonium and dissolved organic phosphorus (DOP), reaching excretion rates of 2.6 and 0.05 μmol L^{-1}h^{-1}, respectively. In both periods, the maximum excretion rates were associated with the 20–150-μm size fraction, but particularly during spring, when a higher organic matter quality was observed in excretion products compared to late summer. There were higher excretion rates of dissolved free amino acids (DFAAs) from copepods fed with the <20-μm size fraction, mainly histidine (HIS) in late summer and glutamic acid (GLU) in spring. A shift in the composition of the active bacterial community was observed between periods and treatments, which was associated with the response of the common seawater surface phyla Proteobacteria and Bacteroidetes. The specific bacterial activity (16S rRNA:rDNA) suggested a different response to the two size-fractionated diets. In late summer, Betaproteobacteria and Bacteroidetes were stimulated by the treatment enriched with excretion products derived from the 20–150-μm and <20-μm size fractions, respectively. In spring, Alphaproteobacteria were active in the treatment enriched with the excretion products of copepods fed with the <20-μm size fraction, whereas they were inhibited in the treatment enriched with excretion products in the 20–150-μm size fraction. Our findings indicate that different

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copepod diets can have a significant impact on the quantity and quality of their excretion compounds, which can subsequently generate shifts in the active bacterial composition. The bacterial response is probably associated with common-opportunistic sea surface microbes that are adapted to rapidly reacting to environmental offers.

INTRODUCTION

Zooplankton are responsible for removing up to 40% of the primary production in marine environments (Calbet, 2001). A portion of the organic material assimilated by zooplankton is used for growth and reproduction. The portion that is not assimilated is released as dissolved compounds, via excretion (Møller, 2007; Saba et al., 2011). There is a lack of knowledge regarding the impact of dissolved compounds released by zooplankton in biogeochemical cycles and in the microbial community structure. Experiments designed to explore the use of these compounds by the microbial community would therefore provide valuable information regarding the role of zooplankton in nitrogen and phosphorus turnover. A recent study of the impact of krill excretion on microbial activity demonstrated that the addition of krill excretion products stimulates bacterial growth and production (Arístegui et al., 2014). Studies have shown that a jellyfish bloom can provide significant amounts of dissolved organic matter (DOM) due to jellyfish degradation and excretion, facilitating the mineralization of dissolved organic carbon (DOC) and dissolved organic nitrogen (DON), followed by a high accumulation of ammonium (NH_4^+) and phosphate (PO_4^{3-}) that becomes available for microbial communities (West et al., 2009; Blanchet et al., 2015).

Ammonium is recognized as the main excretion product of zooplankton (Bidigare, 1983). However, several studies have recognized DON as an important excreted material, accounting for between 7 and 90% of the total dissolved nitrogen (Miller and Glibert, 1998; Conover and Gustavson, 1999; Steinberg et al., 2000, 2002; Pérez-Aragón et al., 2011). Most previous studies have focused on nitrogen excretion, while only a few studies have focused on phosphorus compounds, and these have mainly considered PO_4^{3-} (Pomeroy et al., 1963; Ikeda et al., 2001; Saba et al., 2009; Alcaraz et al., 2010). The composition and quantity of the compounds excreted by zooplankton are highly dependent on the quantity and quality of the food that they consume. The effect of the food consumed on the content of zooplankton excreta has been reported by several authors (e.g., Miller and Roman, 2008; Alcaraz et al., 2010). However, some of the results are controversial and more studies are needed. Saba et al. (2009) observed that the release of inorganic nitrogen by copepods is variable and dependent on the type of diet, showing that the rate of NH_4^+ release was highest when feeding on a carnivorous diet, and lowest while feeding omnivorously. In contrast, organic nitrogen, such as urea, can account for a higher proportion of the total dissolved nitrogen when copepods feed on a mixed diet (omnivorously). Moreover, inorganic phosphorus excretion was higher when the copepod diet was herbivorous.

The importance of excretion products in biogeochemical cycles in the upper ocean has been identified in several studies. These products are recognized as a source of dissolved compounds for the microbial community and phytoplankton, and they also contribute to the vertical transport of bio-elements via diel vertical migration (Steinberg et al., 2002; Escribano et al., 2009; Pérez-Aragón et al., 2011). There have been many studies of the bioreactivity of DOM in recent decades, with DOM recognized as the largest reservoir of nutrients in marine environments. This reservoir can therefore be available for phytoplankton, with the microbial community being rapidly utilized over timescales of minutes to weeks for the most labile fraction (Bronk et al., 2007; Carlson and Hansell, 2015). Recent studies have shown that bacterial community composition can change in response to the addition of organic matter (Landa et al., 2013; Sarmento et al., 2013). DOM release during phytoplankton blooms is associated with important changes in microbial diversity and metabolism (McCarren et al., 2010; Sarmento and Gasol, 2012). Titelman et al. (2008) showed that copepod feeding can enhance organic phosphorus uptake and bacterial activity in a phosphorus-limited system. Therefore, the importance of these predator–prey interactions, as a resource for the microbial food web, depends on the type of DOM substrate and on the prevailing limitations.

Although previous studies have shown the responses of the bacterial community to the input of DOM, the interactions among zooplankton feeding conditions and excretion and the bacterial community have not been examined in the pelagic system. Here, we used an experimental approach, with natural food sources, and simulated in situ conditions to test the hypothesis that zooplankton excretion can significantly reshape the bacterial community structure, with such effects conditioned by the type of food available under a seasonal upwelling situation. For this, we offered sized-fractionated diets to the dominant zooplankton (copepods) and then assessed the effects of copepod excretory products on changes in organic and inorganic nutrients, and on the activity and composition of natural microbial assemblages.

MATERIALS AND METHODS

Study Area

The study area was located in the coastal upwelling system in central/southern Chile (Supplementary Figure 1A). Sampling was conducted on board the R/V Kay Kay II (University of Concepcion). The sampling stations were located ~22 km from the coast (Station 12; 36° 30′S, 73°04′W) and ~33 km from the coast (Station 18; 36° 30.8′S, 73°08′W). Station 12 was visited

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on March 23, 2014 and Station 18 on December 12, 2014, which corresponded to late summer and spring, respectively.

Sampling Strategy

At each sampling station, an autonomous oceanographic profiler (CTD-O SeaBird 19, Sea-Bird Scientific, Bellevue, WA, USA) was used to obtain temperature, salinity, and dissolved oxygen measurements. Seawater samples were obtained at 10-m depth, with a Niskin bottle (10 L) for incubations and chemical-biological measurements. Seawater used for incubation was stored in acid-cleaned carboys, in dark conditions, for transfer to the Dichato Marine Station at the University of Concepcion ($\sim$1 h). Seawater samples were analyzed on board for NH_4^+, nitrate (NO_3^-), nitrite (NO_2^-), PO_4^{3-}, and bacterioplankton abundance. Samples collected for DNA and RNA analysis (the latter was only analyzed in spring) were immediately filtered upon arrival at the laboratory ($\sim$1 h). Measurements of total and size-fractionated chlorophyll-a (Chl-a), and microplankton and nanoplankton abundance, were obtained at each site to determine the *in situ* food available for copepods (see Materials and Methods in the "Chemical and biological analysis" and "Molecular methods" sections).

Chemical and Biological Analysis

Samples for the analysis of Chl-a were taken in triplicate (300 mL) and filtered through GF/F filters (Whatman, Maidstone, UK). Samples used for the size-fractionated Chl-a analysis were pre-filtered onto a polycarbonate membrane (20-μm mesh). Both samples were measured by a fluorometric method (Parson et al., 1984). Nanoplankton abundance and composition were analyzed by epifluorescence microscopy. Samples were taken in duplicate in centrifuge tubes (50 mL) and immediately preserved with glutaraldehyde (2% final concentration). Microplankton samples were collected in duplicate (250 mL) and preserved with Lugol solution and analyzed by the Utermöhl method, based on inverted microscopy (Villafañe and Reid, 1995). Samples for the determination of bacterioplankton, cyanobacteria, and picoeukaryote abundance were taken in duplicate (1,350 μL in sterile cryovials), fixed with glutaraldehyde (150 μL of 1% glutaraldehyde) and stored at -80°C prior to laboratory analysis. Samples were analyzed by flow cytometry (FACScan, Becton Dickinson) according to Marie et al. (1997).

Duplicate samples for the analysis of nutrients (NO_3^-, NO_2^-, and PO_4^{3-}) were filtered through 0.7-μm filters (GF/F; Whatman), placed in 11-mL HDPE plastic tubes (acid-washed), and stored at -20°C prior to laboratory analysis. Concentrations of NO_3^-, NO_2^-, and PO_4^- were determined using standard colorimetric techniques (Grasshof, 1983). Duplicate samples for NH_4^+ determination (50 ml) were analyzed immediately according to Holmes et al. (1999) using a Turner Designs fluorometer. Samples for DON and dissolved organic phosphorus (DOP; 30 mL) were filtered through pre-combusted (450°C, 6 h) GF/F filters (Whatman), and analyzed by the wet oxidation procedure (Pujo-Pay and Raimbault, 1994). DON and DOP concentrations were corrected for NO_3^-, NO_2^-, and PO_4^3, respectively. Dissolved free amino acid (DFAA) samples (15 mL) were filtered through pre-combusted GF/F filters (Whatman),

dispensed in duplicate in acid-cleaned and pre-combusted (450°C, 6 h) glass flasks, and stored immediately at -20°C prior to analysis. DFAA quantification and identification were determined using high-performance liquid chromatography (HPLC; LC-10TA, Shimadzu, Kyoto, Japan) according to Pantoja and Lee (2003).

Zooplankton Collection

Zooplankton were captured at each site by means of vertical hauls of a WP-2 net of 200-μm mesh-size. The net was equipped with a non-filtering cod end to obtain undamaged individuals. Once onboard, the samples were poured into coolers and diluted with surface seawater (10-m depth) to keep the organisms alive and maintain them in a suitable condition prior to sorting at the laboratory ($\sim$1 h).

The Collection of Food and the Microbial Community for Use in Experiments

Seawater (20 L), collected at 10-m depth, was used as the medium to evaluate the impact of the type of food ingested by copepods on the composition of dissolved compounds being excreted, and the subsequent impact on bacterial community structure. Seawater was first filtered through a 150-μm sieve to remove large plankton particles and then through a 20-μm sieve. The fraction retained by the 20-μm sieve was re-suspended in filtered seawater (GF/F; Whatman) to produce the 20–150 μm food fraction, while the media passing through the 20-μm sieve was used as the <20-μm food fraction. Both size-fractions were maintained in the dark in a cold room (12°C) until the experiments were conducted ($\sim$7 h later).

Different microbial assemblages were targeted for the late-summer and spring experiments. In late summer, a larger-sized microbial community was used for incubations (1.6-μm-filtered seawater; APFA04700 pre-filters; Millipore). This medium enabled potential predation effects to occur during the experiment, which was not possible with the smaller microbial community used in the spring (0.7-μm-filtered seawater; GF/F; Whatman) that was assumed to be free of grazers. The bottles with natural microbial assemblages were prepared before the experiment ($\sim$4 h earlier) and kept under similar conditions as the food (12°C).

Experimental Procedures

To explore the interaction between the dissolved compounds excreted by copepods and bacteria, we designed an experiment consisting of four phases: (1) copepod selection and acclimation, (2) copepod feeding, (3) copepod excretion, and (4) the microbial response to dissolved compounds excreted in the previous phase (Supplementary Figure 2). The experimental design of each phase was as follows:

Phase 1. For each sampling event, live zooplankton samples were sorted using a stereomicroscope under low-light conditions. The number of individuals used in both experiments was determined based on the mean abundance observed at Station 18 in the Center for Oceanographic Research in the eastern South Pacific (COPAS) time series

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(Escribano et al., 2007; Hidalgo et al., 2010). In late summer, a mix of two abundant adult copepods, *Acartia tonsa* (71%) and *Paracalanus* cf *indicus* (29%) was sorted, whereas, in spring, a mix of three adult copepods was sorted, *P.* cf *indicus* (37.5%), *Calanoides patagoniensis* (45%), and *Spinocalanus* sp. (17.5%). On both sampling dates, four groups of copepods, containing the same community, were isolated from the surrounding water using cylinders with a 200-μm mesh inside to prevent the escape of copepods, and were maintained in two glass incubators (previously washed with 10% HCl) filled with 10 L of 0.7-μm-filtered seawater (GF/F; Whatman). The acclimation was conducted under dark conditions for 6 h.

Phase 2. The four copepod groups selected were then transferred for feeding purposes into two 10-L incubators containing different size fractions of natural food, <20-μm and 20–150-μm (two copepod groups for each incubator). This feeding phase was performed under dark conditions (12°C) and lasted 4 h.

Phase 3. The copepod groups were transferred to a different set of duplicate incubators containing 0.2-μm-filtered seawater (Durapore; Millipore). This was done to minimize the presence of microbial assemblages during the excretion phase. The incubator system consisted of six chambers (6 L each), two for the treatment fed with the 20–150-μm size-fraction diet, two for the treatment fed with the <20-μm size-fraction diet, and two incubators used as controls (without copepods). For the feeding and acclimation phase, copepods were isolated in the chamber using cylinders with a 200-μm mesh inside. Each chamber was equipped with a sampling faucet to retrieve subsamples with minimal disturbance. At time intervals of 0 h (T_0), 1 h (T_1: T_0+1h), and 2 h (T_2: T_0+2h) samples were collected in duplicate from the sampling faucet with a 0.5-L glass flask (acid-washed and autoclaved; Duran Schott) for the analysis of NH_4^+, NO_3^-, NO_2^-, PO_4^{3-}, DON, DOP, and DFAA. Bacterioplankton abundance was monitored during all incubation times to evaluate the potential influence of small size microbial communities.

Phase 4. To evaluate the microbial response to dissolved compounds provided by copepod excretion, 10 glass bottles containing 850 mL of seawater with natural microbial assemblages (10-m depth), were enriched with 150-mL aliquots of the excretion products derived from phase 3. The ten glass bottles consisted of four bottles for the treatments derived from copepods fed with the 20–150-μm size-fraction diet, four bottles for the treatments derived from copepods fed with the <20-μm size-fraction diet, and two control bottles that were derived from one of the chambers without copepods from the previous phase 3. We discarded one of the control chambers from the previous phase because of the limited time available to handle the samples.

Samples were collected in duplicate at time intervals of 0 h (T_0), 2 h (T_1: T_0 +2 h), 4 h (T_2: T_0 +4 h), and 6 h (T_3: T_0+6 h) for the analysis of NH_4^+, NO_3^-, NO_2^-, PO_4^{3-}, DON, DOP, DFAA, RNA,

bacterioplankton, cyanobacteria, and picoeukaryote abundance. DNA and RNA samples were taken to assess the changes in the bacterial community structure, so that the assessment included the whole bacterial assemblage and the active assemblage. The same protocol was applied for DNA samples, but in 4-L polycarbonate bottles (Nalgene). In this analysis, 3.4 L of the natural microbial assemblage was inoculated with 600-mL aliquots of dissolved compounds excreted by copepods from each chamber of the previous phase. In total, six bottles were inoculated (Supplementary Figure 2). DNA samples were taken at the initial (T_0) and final incubation time (T_3: T_0+6h), whereas RNA samples were taken at all-time intervals.

The mean concentration of NH_4^+, PO_4^-, DON, DOP, and DFAAs in the control was subtracted from the results for the treatments so as to obtain the concentration related to excretion during the incubation periods. The excretion rates of each of these nitrogen and phosphorus compounds were estimated using the slope of the linear regression fitted to the concentration as a function of incubation time. The concentrations obtained in the controls during microbial response phase 4 were averaged and subtracted from the treatments at each time (T_0, T_1, T_2, and T_3), as was done for excretion phase 3. From these values, we estimated the turnover rate of NH_4^+, NO_3^-, NO_2^- PO_4^-, DON, DOP, and DFAAs every 2 h (T_0-T_1, T_1-T_2, and T_2-T_3). The rates were estimated using an end-point approach as follows:

$$\text{Nutrient rate } (\mu\text{mol L}^{-1}\,\text{h}^{-1}) = [\text{Final nutrients}]\,/\text{Time}\,(2\text{h}) \tag{1}$$

A positive value indicated a net accumulation, whereas a negative value indicated a net nutrient consumption.

Molecular Methods

DNA samples for the initial characterization of bacterial communities were collected at 10-m depth at each sampling site and were maintained in acid-cleaned carboys prior to arrival at the laboratory. Samples (2 L) were filtered through cellulose ester filters (0.22 μm; Millipore) using a peristaltic pump and stored with RNAlater solution (Ambion, Waltham, MA, USA) at −20°C until extraction procedures. DNA samples (2 L) obtained during phase 4 (microbial response) were filtered (0.22 μm; Millipore) directly from 4-L carboys (see "Experimental procedures" section). Samples were taken in duplicate and stored as indicated above. However, only one of the duplicate samples was extracted for each incubation time. DNA extraction was conducted as described in (Levipan et al., 2014), using the PowerSoil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA) in accordance with the manufacturer's specifications. For the late-summer experiment, samples were analyzed by spectrophotometry (NanoDrop ND-1000 Spectrophotometer, Thermo Fisher Scientific, Waltham, MA, USA), whereas for the spring experiment the sample concentrations were obtained from Qubit assays (Invitrogen, Carlsbad, CA, USA). Details are given in Supplementary Table 1.

For the initial characterization of the active bacterial community at 10-m depth, RNA samples were collected only in the experiment performed in the spring, whereas for the microbial response phase, RNA samples were collected in

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both experiments (late summer and spring). Using a sterilized syringe and a 25-mm Swinnex filter, seawater (100 mL) was filtered through 0.22-μm hydrophilic PVDF filters (Millipore). The filters were preserved with RNAlater solution (Ambion) and stored at −80°C prior to RNA extraction. RNA samples were extracted using a Mirvana kit (AM1560; Ambion) in accordance with the manufacturer's instructions, including a mechanical disruption step and homogenization using 200-μm diameter zirconium beads (low-binding zirconium beads, OPS Diagnostics, Lebanon, NJ, USA) and homogenized twice at ~3,000 rpm for 30 s using a Mini-Beadbeater-8™ (Biospec Products, Bartlesville, OK, USA). To remove traces of DNA, RNA was treated with a TURBO DNA-free kit™ (Ambion). Finally, for the samples from the late-summer experiment, the concentration and quality (A_{260}/A_{280} ratio) of RNA and DNA extracts were determined by spectrophotometry (NanoDrop ND-1000 Spectrophotometer). For the spring experiment, the sample concentrations were obtained using a Qubit fluorometer (Invitrogen), following the manufacturer's instructions. The details are given in Supplementary Table 1.

Bacterial 16S rRNA Gene Analysis Using the Illumina MiSeq Platform

Bacterial community structure was analyzed in terms of both total and active fractions by the Illumina high-throughput sequencing method, using cDNA as the template and 16S rDNA (Campbell and Kirchman, 2013). The cDNA was generated using random primers provided by the ImProm-II™ Reverse Transcription System (Promega, Madison, WI, USA). Bacterial 16S rRNA genes from the V1–V3 region (27F-519R) were generated at the Research and Testing Laboratory (RTL, Lubbock, TX, USA).

The 16S rRNA and rDNA gene sequences were processed using Mothur software v1.35.1 (Schloss et al., 2009). Sequencing data sets were curated by quality filtration, to minimize the effects of random sequencing errors, by eliminating sequence reads <200 bp and trimming sequences that contained more than one undetermined nucleotide and sequences with a maximum homopolymer length of eight nucleotides. Thereafter, chimeric sequences were identified using the chimera UCHIME algorithm (Edgar et al., 2011) and removed to so as to retain high-quality reads.

The 16S rRNA and rDNA gene sequences were taxonomically classified using the automatic software pipeline SILVAngs available from https://www.arb-silva.de/ (Quast et al., 2013). The libraries were deposited in the European Nucleotide Archive (ENA) under study accession PRJE21106, with the following run access numbers: ERS1770858 and ERS1775609-ERS1775646.

The composition was analyzed at the phylum, class, and order taxonomic levels and categorized as abundant (>0.5%), semi-rare (0.1–0.5%), and rare (<0.1%) in the total sequences retrieved from each library (Pedrós-Alió, 2012). The Chao, Shannon, and Evenness indices were calculated using Past3 software. Previously, because the number of sequences per sample was variable, the diversity indices were normalized to the same number of sequences per sample (3,320 in late summer and 757 in spring) using Mothur software, version 1.35.1 (Schloss et al., 2009).

Statistical Analyses

For results obtained during the excretion phase, the statistical analysis of the effect of different treatments on the chemical parameters associated with excretion products was performed using a paired t-test. During microbial response phase, a two-way analysis of variance (ANOVA) was applied using treatments and time as levels, after checking for normality assumptions (Kolmogorov-Smirnov test) and homoscedasticity (Levene's test). A pairwise multiple comparison was performed using a Tukey test as an a posteriori analysis.

Bacterial community structure was compared using ordination Bray–Curtis similarities, visualized in Unweighted Pair Group Method with Arithmetic mean (UPGMA) dendrograms, whose nodes were further tested using a bootstrap analysis. A multivariate analysis was also used to analyze the variability of biological and environmental variables, using PRIMER v.6 and the add-on PERMANOVA+ software package. The permutational multivariate analysis of variance (PERMANOVA) with a fixed factor was used to investigate the differences in bacterial community composition for treatments and as a control in both experiments. Principal co-ordinate analysis (PCO) was performed to visualize patterns of the operational taxonomic units (OTUs) of the bacterial community in response to different treatments.

RESULTS

Hydrographic, Biogeochemical, and Biological Background

The hydrographic conditions during sampling events are shown in Supplementary Figure 1. In late summer 2014 (March; Supplementary Figure 1B), the temperature varied between 12.5°C in surface waters and 10.6°C near the bottom layers. Salinity varied between 34.6 and 34.9, with a surface minimum value at 10-m depth. The oxycline was very sharp, and was characterized by a strong reduction in oxygen at 20-m depth, reaching a concentration of <0.4 mL L^{-1} from 20-m depth to the bottom layers. In spring 2014 (December; Supplementary Figure 1C), the thermocline and oxycline were shallower than in the previous period, and were characterized by a similar temperature range (13°C in surface waters to 10.6°C in the bottom layers), but with a higher oxygen concentration in the surface and bottom layers. Salinity was less variable (34.6 and 34.7) than in the previous period, and the salinity minimum observed in the spring was absent.

Nutrient analyses at 10-m depth are shown in **Table 1** for both periods. The NH_4^+ concentration was similar between both periods, while NO_3^-, NO_2^-, and PO_4^{3-} concentrations were higher under late-summer conditions compared to spring. During spring, the DFAA concentration reached 1.7 μmol L^{-1} and was dominated by isoleucine (ILE), followed by histidine (HIS) (see details in Supplementary Table 2).

During late summer, a greater Chl-a concentration was associated with the 20–150-μm size fraction, and the <20-μm size fraction and phaeopigments accounted for 10.4 and 29% of the total pigment stock, respectively. Unfortunately, there was no data available for comparison during spring

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TABLE 1 | Initial nutrient concentration, dissolved free amino acid (DFAA) concentration, photosynthetic pigments, and the abundances of nanoplankton, microphytoplankton, bacterioplankton, cyanobacteria, and picoeukaryotes s in late summer and spring (mean ± standard deviation).

	Late summer (March)	Spring (December)
NH_4^+ (μmol L^{-1})	1.33 ± 0.07	1.40 ± 0.51
NO_3^- (μmol L^{-1})	8.61 ± 4.69	5.49 ± 0.02
NO_2^- (μmol L^{-1})	0.60 ± 0.21	0.27 ± 0.04
PO_4^- (μmol L^{-1})	1.74 ± 0.52	1.01 ± 0.02
DFAA (μmol L^{-1})	n. d	1.68 ± 0.13
Chl-a (mg m^{-3})	7.76 ± 1.01	n. d
Chl-a <20μm (mg m^{-3})	0.81 ± 0.03	n. d
Phaeopigments (mg m^{-3})	6.55 ± 1.44	n. d
Phaeopigments <20μm (mg m^{-3})	1.90 ± 0.13	n. d
Microplankton (cell mL^{-1})	15,302 ± 4,569	5,924 ± 1214
Autotrophic nanoplankton (cell mL^{-1})	149.25 ± 1.61	114.84 ± 7.73
Heterotrophic nanoplankton (cell mL^{-1})	840.82 ± 164.35	481 ± 139.21
Bacterioplankton abundance (10^3 cell mL^{-1})	533.04 ± 16.61	1,532 ± 47.20
Synechococcus abundance (10^3 cell mL^{-1})	4.87 ± 0.24	n. d
Picoeukaryotes abundance (10^3 cell mL^{-1})	0.79 ± 0.12	n. d

n.d. not determined.

(**Table 1**). Microphytoplankton abundances were higher during late summer compared to spring, and there was a low abundance of heterotrophic and autotrophic nanoplankton (0.9 and 5.2%, respectively). The microphytoplankton composition was dominated by *Thalassiosira* sp. and *Chaetoceros* sp. in both sampling seasons, and the bacterioplankton abundance was approximately three times higher in spring than in late summer (**Table 1**).

Excretion Phase: Evolution of Nitrogen and Phosphorus Compounds

During late summer (March 2014), the initial incubation time (T_0) was characterized by a high concentration of NH_4^+, NO_3^-, NO_2^-, and PO_4^{3-} compared to the *in situ* concentrations (**Figure 1** and **Table 1**). The NH_4^+ concentrations in the control were higher compared to the treatments and this difference became smaller at the end of incubation (**Figure 1A**). The difference between the control and treatments was only significant in the treatment fed with the 20–150-μm size fraction (*t*-test, $P = 0.03$). There was a significantly higher concentration of DON (**Figure 1C**) in the control than in the treatment fed with the 20–150-μm size-fraction diet (*t*-test, $P = 0.04$), and this difference increased at 2 h of incubation. The PO_4^{3-} concentrations (**Figure 1E**) did not change significantly between the treatments (20–150μm and <20μm) and control (*t*-test, $P = 0.13$ and $P = 0.17$, respectively), but there were significant differences between the two treatment diets (*t*-test, $P = 0.04$).

The initial concentrations of DOP (**Figure 1G**), were higher in the treatment fed with the 20–150-μm size-fraction diet (1.18 ± 0.6 μmol L^{-1}) compared to the control and the second treatment (<20 μm). However, this difference was not significant between treatments or between the control and treatments (20–150 μm and <20 μm; *t*-test, $P = 0.32$, $P = 0.21$, $P = 0.14$, respectively). A significantly higher concentration of DFAAs (**Figure 1I**) was found in control compared to the treatments (20–150 and <20 μm; *t*-test, $P < 0.001$ and $P < 0.001$, respectively), but there were no significant differences between treatments (*t*-test, $P = 0.06$).

In spring 2014, NH_4^+ concentrations (**Figure 1B**) were significantly higher in the treatment in which copepods were fed with the 20–150-μm size-fraction diet than in the treatment fed with the <20-μm size-fraction diet, or the control (*t*-test, $P < 0.001$ and $P < 0.001$, respectively); and NH_4^+ concentrations for the treatment fed with the <20-μm size fraction were also significantly higher than the control (*t*-test, $P = 0.02$). It is noteworthy that the initial concentration in the treatment fed with the 20–150-μm size fraction was 18 times higher in spring (27 μmol L^{-1}) compared with the late-summer experiments (1.5 μmol L^{-1}). The DON concentrations (**Figure 1D**) did not change significantly between the treatments and control (*t*-test, 20-150 μm $P = 0.22$, <20 μm $P = 0.18$), but DON in the treatment with the 20–150-μm size-fraction diet was significantly higher than in the treatment with the <20-μm size-fraction diet (*t*-test, $P = 0.04$). The PO_4^{3-} concentrations (**Figure 1F**) were significantly higher in the treatment with the 20–150-μm size-fraction diet compared to the treatment with the <20-μm size-fraction diet (*t*-test, $P < 0.001$), or the control, $P < 0.001$). The DOP concentrations were significantly higher in both treatments (20–150 and <20 μm) than in the control (*t*-test, $P = 0.01$ and $P = 0.02$, respectively), but there were no significant changes between treatment diets (*t*-test, $P = 0.78$). Finally, there were significantly higher DFAA concentrations (**Figure 1J**) in the treatment with the 20–150-μm size-fraction diet than in the treatment with the <20-μm size-fraction diet (*t*-test, $P = 0.03$), but this difference became smaller by the end of incubation. At this stage, significant differences were only observed for the treatment with the <20-μm size-fraction diet compared to the control (*t*-test, $P < 0.001$).

In both experiments, NH_4^+ was the main product excreted by copepods and was associated with the treatment fed with 20–150-μm size-fraction (**Table 2**). When standardized to the number of copepods in the incubations, the excretion rates per individual were 0.0028 and 0.0648 μmol L^{-1} h^{-1} in late summer and spring, respectively. DFAAs were excreted in late summer and spring by the copepods fed with the <20-μm size-fraction diet, with an excretion rate of 0.0003 μmol L^{-1} h^{-1}. Copepods fed with the 20–150-μm size-fraction diet only excreted DFAA during late summer (0.001 μmol L^{-1} h^{-1}). In both treatments, the organic form of phosphorus, DOP, was excreted only during late summer, with excretion rates of 0.0013 μmol L^{-1} h^{-1} for copepods fed with the 20–150-μm size-fraction diet and 0.001 μmol L^{-1} h^{-1} for copepods fed with the <20-μm size-fraction diet. The inorganic form of phosphorus, PO_4^{3-}, was excreted to a different extent in both experiments. Under late-summer conditions, it

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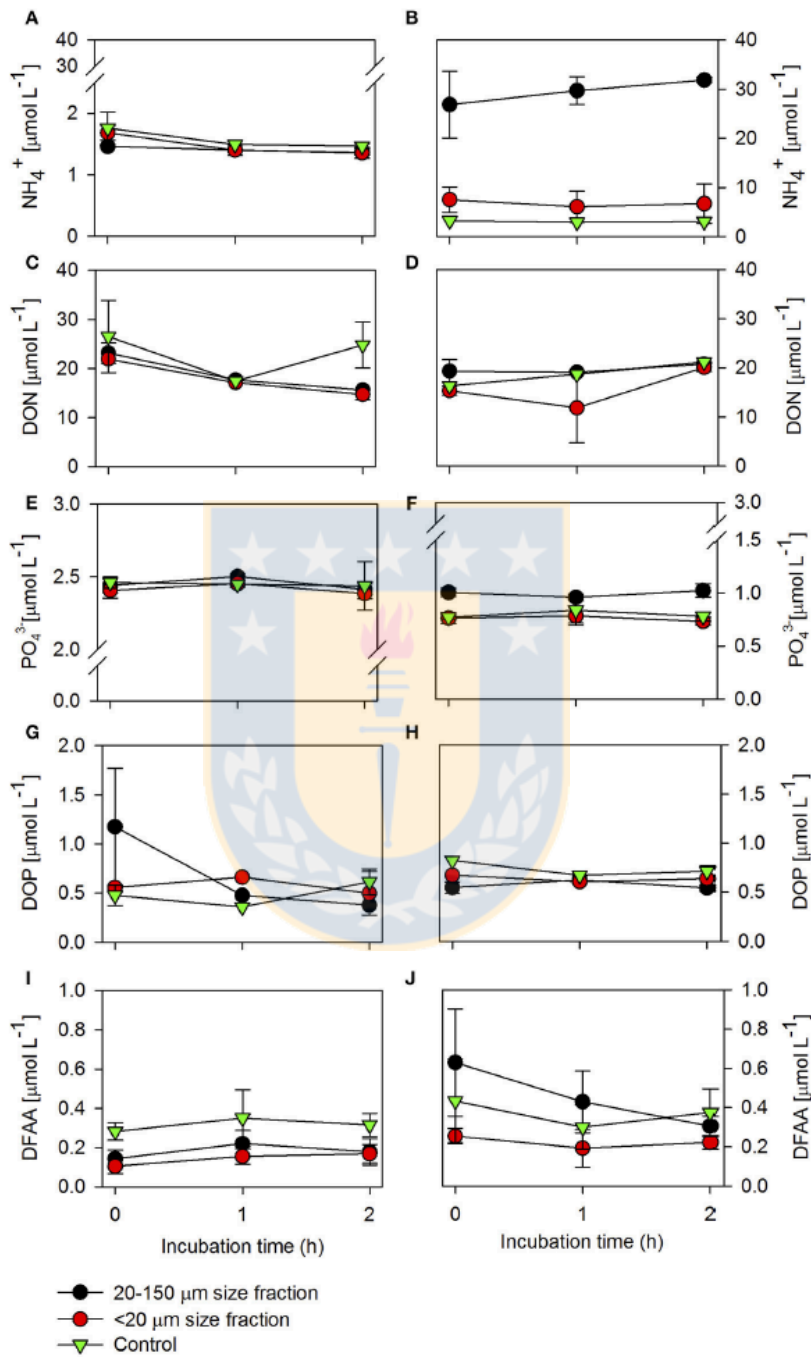
FIGURE 1 | Ammonium (NH_4^+) **(A,B)**, dissolved organic nitrogen (DON) **(C,D)**, phosphate (PO_4^{3-}) **(E,F)** dissolved organic phosphorus (DOP) **(G,H)**, and dissolved free amino acid (DFAA) **(I,J)** concentrations throughout incubation in the excretion phase for both treatments and controls in late summer and spring. Errors bars show the standard deviation ($n = 4$).

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TABLE 2 | Total excretion rates for ammonium (NH_4^+), dissolved organic nitrogen (DON), dissolved free amino acid (DFAA), phosphate (PO_4^{3-}), and dissolved organic phosphorus (DOP), in late summer and spring.

Treatments		Total excretion rate (μmol L^{-1} h^{-1})	
		Late summer (March)	Spring (December)
NH_4^+	20–150 μm	0.096	2.592
	<20 μm	−0.016	−0.295
DON	20–150 μm	−2.89	−1.726
	<20 μm	−2.74	−0.044
DFAA	20–150 μm	0.001	−0.132
	<20 μm	0.012	0.013
PO_4^{3-}	20–150 μm	−0.002	0.004
	<20 μm	0.001	−0.029
DOP	20–150 μm	−0.469	0.054
	<20 μm	−0.098	0.038

was excreted only by copepods fed with the <20-μm size-fraction diet, while under spring conditions it was excreted by copepods fed with the 20–150-μm size-fraction diet, which corresponded to 3.57×10^{-5} and 1×10^{-4} μmol L^{-1} h^{-1} per copepod, respectively.

The composition of the DFAAs excreted during the different periods and under the different treatments was variable (**Figure 2**). During the late-summer experiment, there was a larger accumulation of HIS in the DFAAs excreted by copepods fed with the <20-μm size-fraction diet, compared to the DFAAs excreted by copepods fed with the 20–150-μm size-fraction diet, in which serine (SER) was accumulated (**Figure 2A**). During the spring experiment, the higher excretion rate was found to be related to glutamic acid (GLU) production in both treatments, but this was three times higher when copepods were fed with the <20-μm size-fraction diet (**Figure 2B**).

Despite our efforts to avoid losses of excreted nitrogen and phosphorus due to microbial metabolism during the incubation, negative excretion rates were observed in the experiments (e.g., DON; **Table 2**). Flow cytometry data indicated that bacterioplankton abundances were lower and constant in both the treatments and the control during late-summer incubation ($27 \pm 2.5 \times 10^3$ cell mL^{-1}; Supplementary Figure 3). In spring, both the control and the treatment in which copepods were fed with the 20–150-μm size-fraction diet had lower and constant bacterioplankton abundances during all incubations (control: $299 \pm 64 \times 10^3$ cell mL^{-1}; 20–250-μm size-fraction diet: $537 \pm 14 \times 10^3$ cell mL^{-1}), but the treatment with copepods fed with the <20-μm size-fraction diet had higher abundances, exceeding the *in situ* conditions ($2,637 \pm 67 \times 10^3$ cell mL^{-1}).

Microbial Response Phase: Turnover Rate of Nitrogen and Phosphorus Compounds

There were variable patterns of consumption and accumulation in the microbial response to enrichments with copepod excretion products from the previous phase, which were dependent on the incubation time and origin of the excretion products (**Figures 3, 4**, Supplementary Figures 4). In late summer

(**Figure 3**), the microbial response was characterized by a significant accumulation of NH_4^+ and DON at first hour of incubation compared with the other incubation periods (ANOVA and Tukey's test; $P < 0.05$; Supplementary Table 3), while DOP and PO_4^{3-} were consumed during the first hour of incubation in the treatment amended with excretion products from the <20-μm size-fraction diet. During the second period of incubation (T_1-T_2), a high consumption of nitrogen and large amounts of phosphorous production occurred. During the T_2-T_3 incubation period, differences between the enrichment treatments were observed for almost all the nitrogen and phosphorous compounds analyzed, although these differences were not significant (ANOVA; $P > 0.05$). Both NO_3^- and NO_2^- were rapidly recycled, shifting from accumulation to consumption every 2 h, but only NO_3^- showed a significant consumption in T_1-T_2 compared to the initial and final periods (ANOVA and Tukey's test, $P < 0.05$; Supplementary Table 3).

During spring (**Figure 4**), the only clear difference between the enrichment treatments was observed for DON, with an accumulation of DON during the first incubation time (T_0-T_1) in the treatment enriched with excretion products from the <20-μm size-fraction diet. However, the differences in DON between enrichment treatments were not significant (ANOVA; $P > 0.05$). The NO_2^- and DFAAs were the only compounds for which there were significant differences during the incubation in both enrichment treatments; the behavior was characterized by an accumulation during T_0-T_1 (**Figure 4**), when there were higher production rates in the treatment with the 20–150-μm size-fraction diet, and a marked consumption in the final incubation period, in both enrichment treatments (ANOVA and Tukey's test, $P < 0.05$; Supplementary Table 3).

A non-metric multidimensional scaling analysis (MDS) of amino acid composition from the treatments in both experiments is shown in **Figure 5**. In late summer, a large dissimilarity between the DFAA composition was observed, which was associated with the incubation time. The contribution of specific amino acids was analyzed based on a superimposed correlation, which revealed a strong correlation between methionine (MET) and the first period of incubation in both treatments during the late-summer experiments. During this period, in both treatments, MET was accumulated, while during the other periods it was consumed (Supplementary Figure 5). Additionally, threonine (THR) was correlated with the third time-period (T_2-T_3) in the treatment with the <20-μm size-fraction diet. In the spring experiment, as in late summer, the DFAA composition differed according to incubation-time treatment, but only in the first period (T_0-T_1, **Figure 4B**). The correlation analysis indicated that glycine (GLY) was associated with the treatment enriched with compounds excreted by copepods fed with the 20–150-μm size-fraction diet, and GLU was associated with the treatment derived from copepods fed with the <20-μm size-fraction diet (**Figure 5** and Supplementary Figure 5).

The bacterioplankton abundance during the microbial response phase is presented in Supplementary Figure 6. During both experiments, bacterioplankton abundance increased throughout the incubation time, both in treatments and controls (Supplementary Figures 6A,D). In late summer, differences

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FIGURE 2 | Excretion rates obtained for aspartic acid (ASP), glutamic acid (GLU), serine (SER), histidine (HIS), glycine (GLY), threonine (THR), arginine (ARG), alanine (ALA), tyrosine (TYR), valine (VAL), phenylalanine (PHE), isoleucine (ILE), leucine (LEU), lysine (LYS), and methionine (MET) in **(A)** late summer and **(B)** spring for both treatments. Errors bars show the standard deviation ($n = 4$).
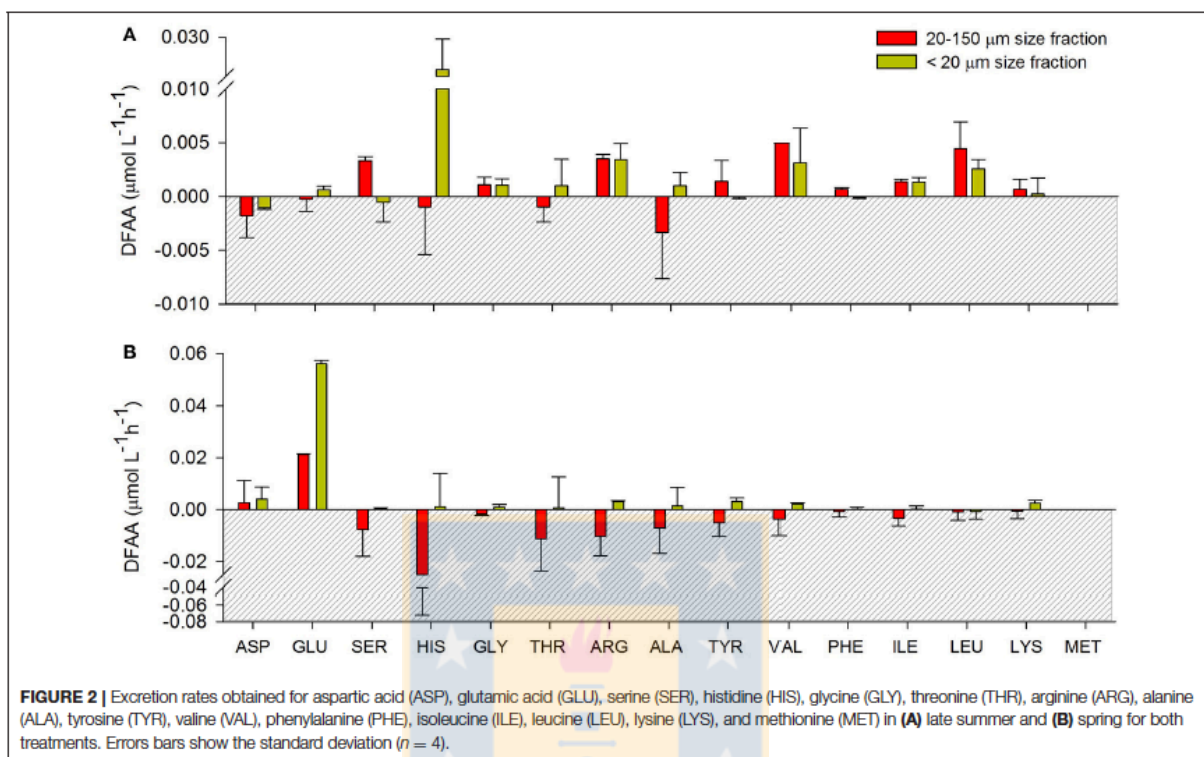
between the enrichment treatments and control were only observable for the treatment inoculated with compounds excreted from the 20–150-μm size-fraction diet, with lower bacterioplankton abundances being found compared to the control and to the treatment derived from copepods fed with the <20-μm size-fraction diet (t-test, $P < 0.05$). Cyanobacteria and picoeukaryotes were found throughout the incubation, with a high degree of variability in late summer (Supplementary Figures 6B,C) and overall higher abundances in late summer compared to spring (Supplementary Figure 6D).

Microbial Response Phase: Changes in Total and Active Bacterial Community Structure

During late summer, a total of 166,480 sequences were analyzed, ranging between 3,225 and 29,856 sequences for each library (Supplementary Figure 7A). The high number of sequences retrieved corresponded to DNA samples at the initial time (T_0) in the treatment inoculated with compounds excreted by copepods fed with the <20-μm size-fraction diet. For the second experiment, in spring, a total of 113,979 sequences were analyzed, with the higher number of sequences corresponding to the sample cDNA 20–150 μm at T_1 (Supplementary Figure 7B). Almost none of the rarefaction curves reached a plateau. Hence, our sequencing efforts did not completely cover the bacterial diversity. Thus, our results underestimated the alpha diversity

expected in the water column; however, because the sequence data were normalized to compare libraries of different size, we were able to compare the potential response of the active community using this index throughout the experiment. The alpha diversity derived from normalized 16S rDNA libraries is shown in **Figures 6C–F** (late summer) and **Figures 7C–F** (spring) for comparison, with relevant taxa and clustering analyses. Compared with the *in situ* bacterial community, the taxonomic richness that was determined (i.e., OTUs) and expected based on the Chao1 index was lower in the treatments in the late-summer and spring experiments. In accordance, the Shannon index diversity showed that the bacterial community obtained *in situ* and at the initial time had higher values than those observed throughout the incubation in both experiments (**Figures 6C,F**).

The bacterial community composition was analyzed by comparing the relative abundances of the major phylogenetic groups (**Figures 6B,E**). The *in situ* community present at Station 18 in late summer (**Figure 6B**) was dominated by Bacteroidetes (51%), followed by Gammaproteobacteria (26%), Alphaproteobacteria (17%), Cyanobacteria (3%), and Verrucomicrobia (1%). A similar composition was observed at Station 12 during spring, although there was a higher contribution of Cyanobacteria (12 times higher than that during the late-summer sampling) and a lower contribution of Gammaproteobacteria (10%) and Alphaproteobacteria (4%). In both cases, these communities changed substantially

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FIGURE 3 | Turnover rates of ammonium (NH_4^+), nitrate (NO_3^-), nitrite (NO_2^-), phosphate (PO_4^{3-}), dissolved organic nitrogen (DON), dissolved organic phosphorus (DOP), and dissolved free amino acids (DFAAs) estimated for every 2 h of incubation, during the microbial response phase in late summer. Each point represents four replicates (mean ± standard deviation).
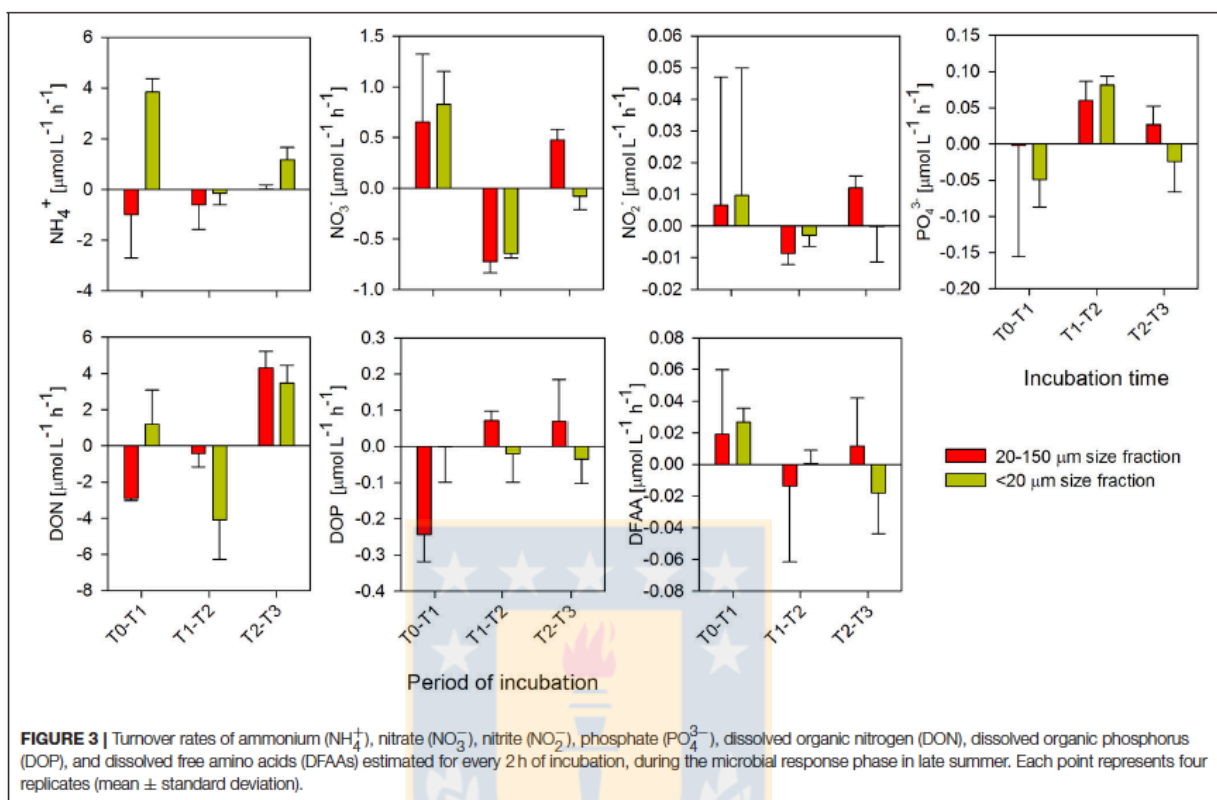
during the incubation, and were characterized initially by an increase in the relative contribution of Alphaproteobacteria and Gammaproteobacteria, and a decrease in the contribution of Bacteroidetes and the disappearance of Cyanobacteria and Verrucomicrobia.

The cluster analysis associated with the contribution of the orders in the different libraries indicated high levels of dissimilarity between the *in situ* bacteria and the community associated with the different treatments in both experiments (**Figures 6A,D**). Additionally, the total bacterial community from the experiments was mainly grouped according to the incubation time, T_0 vs. T_3 in both experiments.

The alpha diversity derived from the normalized 16S rRNA libraries (similar sequence numbers) for comparison is shown in **Figures 7A–C** (late summer) and **Figures 7D–F** (spring), together with the relevant taxa and results of the clustering analyses. The active OTUs were slightly different between treatments and controls, with no visible trend in the diversity index (**Figure 7C**) during the late-summer experiments. In spring, the number of OTUs and Chao1 index values were lower under *in situ* conditions than in the incubations (**Figure 7F**). The diversity, based on the Shannon index, was lower under *in situ* conditions than during incubation in the spring experiment. Both the control and the treatment inoculated with dissolved compounds excreted by copepods fed with the 20–150-μm size-fraction were characterized by a decreasing trend in diversity

over time. In contrast, in the treatment inoculated with dissolved compounds excreted by copepods fed with the <20-μm size-fraction diet, there was an increase in the Shannon index followed by a decrease every 2 h (**Figure 7F**).

The active *in situ* bacterial community composition was only available during the spring (**Figure 7E**) and was dominated mainly by Cyanobacteria (79%), followed by Bacteroidetes (11%), and Alphaproteobacteria (9%). There were substantial changes in composition throughout the incubation, which presented significant changes during the incubation. These were characterized by an increase in Bacteroidetes and Alphaproteobacteria, and a substantial decrease in Cyanobacteria (>75%). In late summer, the bacterial community was mainly represented by Alphaproteobacteria, Gammaproteobacteria, and Bacteroidetes (**Figure 7B**).

Cluster analysis results for the late summer (**Figure 7A**) indicated that the higher dissimilarities were observed at the initial time for the treatment fed with the 20–150-μm size-fraction diet and no significant differences were found between times and treatments. In contrast, in spring, more dissimilarities were observed for the *in situ* bacteria compared with the community associated with the different enrichment treatments (**Figure 7D**).

The 16S rRNA:rDNA ratio (**Figure 8**) showed a different response between treatments and periods. Compared to spring, in late summer (**Figure 8A**) a larger number of bacteria taxa

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FIGURE 4 | Turnover rates of ammonium (NH_4^+), nitrate (NO_3^-), nitrite (NO_2^-), phosphate (PO_4^{3-}), dissolved organic nitrogen (DON), dissolved organic phosphorus (DOP), and dissolved free amino acids (DFAAs) estimated for every 2 h of incubation, during the microbial response phase in spring. Each point represents four replicates (mean ± standard deviation).
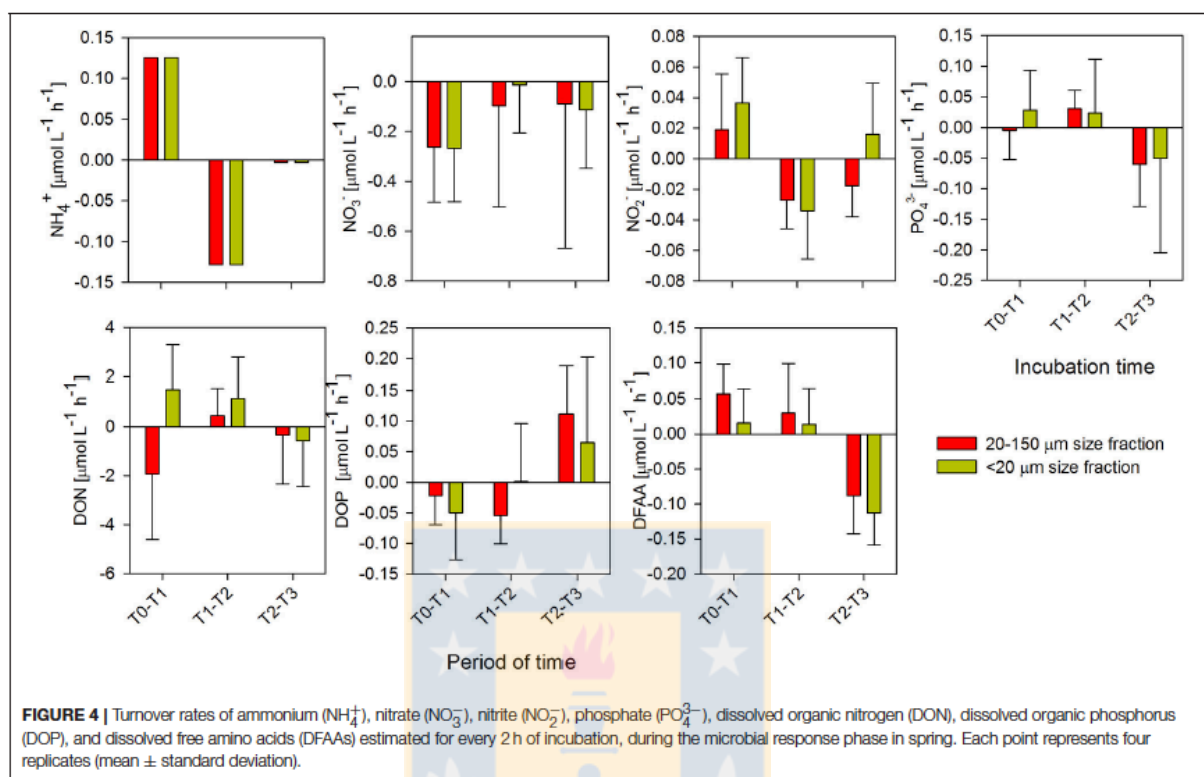
increased their activity after the enrichments, particularly in relation to the excretion products from copepods fed with the 20–150-μm size-fraction diet. The activity of Betaproteobacteria and Deltaproteobacteria increased at the end of the experiment (T_3), while the activity of Verrucomicrobia and Bacteroidetes decreased. In the enrichment derived from copepods fed with the <20-μm size-fraction diet, the activity of Bacteroidetes increased. Conversely, during spring (**Figure 8B**), a slightly higher activity of Alphaproteobacteria was observed at the end of the experiment in the treatment inoculated with compounds excreted by copepods fed with the <20-μm size-fraction diet at the end of the experiment, whereas this Proteobacteria class had a low activity in the treatment derived from copepods fed with the 20–150-μm size-fraction diet at the end of the incubation.

The responses of the initial (*in situ* abundant >0.5%, semi-rare 0.5–0.1% and rare <0.1%) bacterial class to the enriched treatment compared to the controls are shown in **Figures 9, 10,** for late summer and spring, respectively. Considering the initial composition of the bacterial community, it was mainly the abundant and semi-rare bacteria that responded to the treatment (generally positive values after subtraction of the control), whereas rare groups were not favored by copepod enrichment products (generally negative values after subtraction of the control). The initially abundant bacteria (**Figure 9**) were characterized by an increase in the contribution of Alphaproteobacteria in both treatments. On the other hand, the active class had a high relative abundance of

Gammaproteobacteria at T_0, which decreased throughout the incubation. A large increase in Flavobacteria (Bacteroidetes) was observed in the treatment derived from copepods fed with the 20–150-μm size-fraction diet, which was also observed between T1 and T2 in the treatments derived from copepods fed with the <20-μm size-fraction diet. The semi-rare class was characterized by a large increase in the contribution of ARKICE-90 (Proteobacteria) in the treatment derived from copepods fed with the 20–150-μm size-fraction diet compared to the control, while in the treatment derived from copepods fed with the <20-μm size-fraction diet, the contribution of Betaproteobacteria showed a slight increase compared to the control. Furthermore, the active semi-rare classes in both treatments were characterized by a rapid response (2 h) of ARKICE-90 (Proteobacteria) and an increase in the relative contribution of Sphingobacteria compared to the controls. The response of the rare bacteria (*in situ*) to the enrichment was characterized by a higher contribution in the controls compared to the treatments (**Figure 9**). In addition, many rare classes were not detected in the libraries when compared to the *in situ* conditions, including Bacteroidetes BD2-2, Melainabacteria, derived from Gracilibacteria, Elev-16S-509 (Proteobacteria), TA18 (Proteobacteria), and the OPB35 soil group (Verrucomicrobia). The specific contribution of the orders is shown in Supplementary Figure 8.

During spring, only abundant and semi-rare bacteria responded to the enrichment associated with copepod excretion

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FIGURE 5 | Non-metric multidimensional scaling analysis (MDS) of the amino acid profile based on Euclidean distance in late summer **(A)** and spring **(B)**. In **(A)** the treatment was enriched with dissolved compounds excreted by copepods fed with a 20–150-μm size-fraction diet. In **(B)** the treatment was enriched with dissolved compounds excreted by copepods fed with a <20-μm size fraction diet.

products (**Figure 10**). This was characterized by an increase in the contribution of Flavobacteria and Alphaproteobacteria in the treatment inoculated with compounds excreted by copepods fed with the 20–150-μm size-fraction diet, while in the active semi-rare class there was a large increase in the contribution of Betaproteobacteria (at 2 h) and ARKICE-90 (Proteobacteria) (at 4 h). In the second treatment (<20 μm), there was a large increase in the contribution of Betaproteobacteria after 6 h of incubation. The specific contribution of each is shown in Supplementary Figure 9.

A similarity percentage analysis (SIMPER) indicated that different bacteria contributed most to the dissimilarities following the addition of excreted compounds by copepods fed with the <20 and 20–150-μm diets (Supplementary Table 3). In late summer, 20 OTUs explained 70% of the dissimilarity between the total (DNA) and active (cDNA) orders in treatments (<20 and 20–150-μm). The contributions of Alteromonadales (Gammaproteobacteria) and Flavobacteriales (Bacteroidetes) could explain many of the dissimilarities in both the DNA and cDNA pools, whereas the contribution of Rhodobacterales (Alphaproteobacteria) explained many of the dissimilarities in cDNA. During spring, the same bacteria, as well as Oceanospirillales, were identified as potential contributors, which could explain the differences between treatments (Supplementary Table 4).

In a principal coordinate analysis (PCA) plot, the two first axes explained 61.9% of the variance in the late summer experiment

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FIGURE 6 | (A–D) Unweighted Pair Group Method with Arithmetic mean (UPGMA) clustering of the bacteria community structure in late summer and spring. Bootstrap values obtained from 1,000 interactions are depicted as circles on nodes (black: >80% and white: <70%), **(B–E)** taxonomic composition of abundant (>0.5%) phyla and Proteobacteria classes, comparing treatments and controls in late summer and spring, respectively and **(C–F)** the richness, coverage of specific libraries, Shannon (H') index, and evenness in the late summer and spring experiments.

and 56.6% in the spring. During late summer and spring (**Figures 11A,B**), the active bacterial community structure within the experiment was not associated with the different treatments or times. Analysis by PERMANOVA did not identify significant differences among all samples regarding the different treatments (20–150 and <20 μm; PERMANOVA, $P = 0.2$ and $P = 0.1$) and times (PERMANOVA, $P = 0.6$ and $P = 0.5$). However, the 16S rRNA/rDNA ratio clearly differed between times and treatments (**Figure 8**) and the overlaid correlation analyses associated with organic and inorganic compounds, which changed throughout the experiments, indicated that the active bacterial community structure from the late incubation period was associated with the DFAA and PO_4^{3-} concentration, though mainly during spring.

DISCUSSION

During our study, the hydrological characteristics were typical of late-summer and spring conditions (March and December, respectively), and were associated with an active upwelling period. In the study area, the upwelled water is nutrient-rich and has an oxygen deficiency, generating high photoautotrophic activity and consequently reaching high levels of primary production. However, this pattern varies throughout the upwelling period due to alongshore southerly winds during September–October, which vary over different time scales (Daneri et al., 2000; Montero et al., 2007; Sobarzo et al., 2007). In accordance with the seasonal pattern in this region (Sobarzo et al., 2007), both experiments were conducted under upwelling conditions. One was conducted during the upwelling season (December), which was close to the high solar radiation maximum (January), and the other was conducted at the end of the upwelling period (March). In fact, during late summer, NO_3^-, NO_2^-, and PO_4^{3-} were found at higher concentrations compared to spring, possibly due to the higher remineralization at the end of the upwelling season. These nutrient concentrations were within the range of values previously reported by the time-series program at Station 18 (Supplementary Figure 1; Cornejo et al., 2007; Montero et al., 2007; Morales and Anabalón, 2012).

FIGURE 7 | (A–D) Unweighted Pair Group Method with Arithmetic mean (UPGMA) clustering of active bacteria community structure in late summer and spring. Bootstrap values obtained from 1,000 interactions are depicted as circles on nodes (black: >80% and white: <70%), **(B–E)** taxonomic composition of abundant (>0.5%) phyla and Proteobacteria classes, comparing treatments and controls in late summer and spring, respectively and **(C–F)** the richness, coverage of specific libraries, Shannon (H') index, and evenness in the late summer and spring experiments.

Zooplankton are mostly represented by copepods in this zone (70% of the total zooplankton abundance; Escribano et al., 2007). *Acartia tonsa*, *P*. cf *indicus*, and *C. patagoniensis* are representative species of this area, with higher abundances during spring/summer, accounting for more than 30% of the relative copepod abundance (Escribano et al., 2007; Hidalgo et al., 2010). In contrast, *Spinocalanus* sp. is not a species commonly found at this site, and has been reported as inhabiting deeper waters (>1,000-m depth) in the western North Pacific (Ikeda et al.,

2006). However, in our study this species was present with a high abundance in shallow waters, and was a representative species for this particular sampling event.

Excretion of Nitrogen and Phosphorus Compounds

During our study, the copepod excretion products were associated with the different size fractions of food provided,
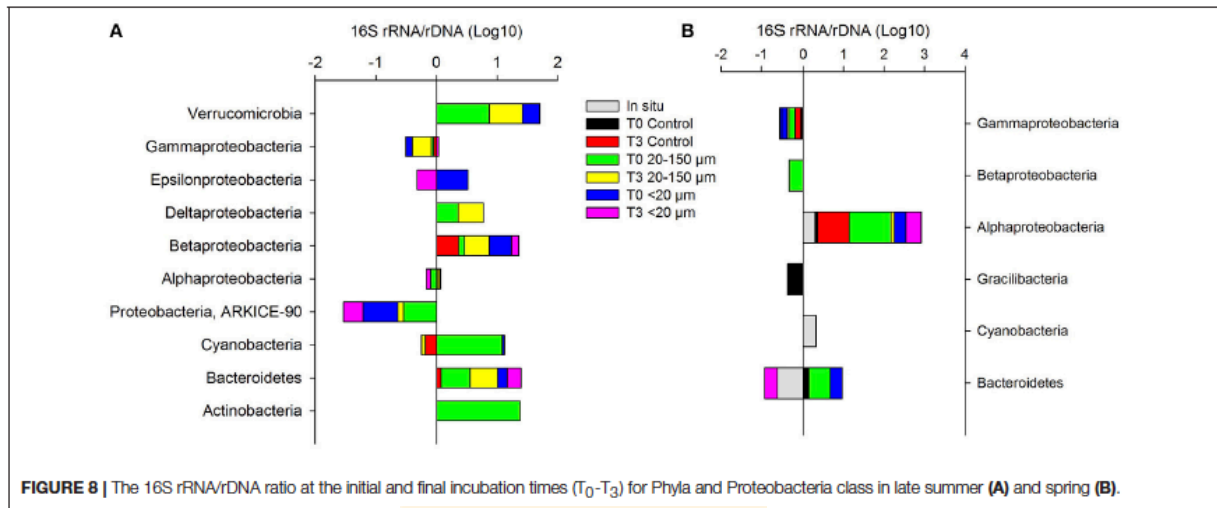
FIGURE 8 | The 16S rRNA/rDNA ratio at the initial and final incubation times (T_0-T_3) for Phyla and Proteobacteria class in late summer **(A)** and spring **(B)**.

influencing the type and magnitude of inorganic and organic compound excreted, including the specific composition of DFAAs. When the copepods were fed with the 20–150-µm size-fraction diet a higher accumulation of nitrogen and phosphorus compounds was observed at higher excretion rates, mainly in the form of NH_4^+ and DOP, compared with copepods fed with the <20-µm size-fraction diet. Studies of nitrogen excretion in this study area have shown that the main product excreted by copepods in spring and winter is DON (Pérez-Aragón et al., 2011), although DON excretion was six times higher in winter than spring. In the same study, NH_4^+ production was not detected in spring, in contrast with our study, in which NH_4^+ was the main compound excreted by copepods. Additionally, the NH_4^+ excretion rates observed in the current study were higher than those reported in this area and were also in the range of those reported in other upwelling zones (Fernández-Urruzola et al., 2016; Kiko et al., 2016).

The type and magnitude of the excretion products observed in our experiment compared with previous investigations in the study area and other upwelling zones could be related to the type of diet the copepods have. In the study area, copepods are capable of shifting their diet throughout the year, feeding on heterotrophic flagellates in winter and on chain-forming diatoms in spring (Vargas et al., 2006, 2010). During our study, which was conducted in spring and summer, the prevailing food available for copepods was microplankton, mainly diatoms, followed by heterotrophic nanoplankton (**Table 1**). The effect of diet on the main excreted compounds has been studied by several authors (Miller and Glibert, 1998; Miller and Roman, 2008; Saba et al., 2009), and it has been reported that copepods excrete more NH_4^+ when fed on a diet based on diatoms (Miller and Roman, 2008). Regarding the potential effects of food quantity on the excretion products, it may be possible that size-fractioned food can result in a variable biomass content of phytoplankton. However, studies in this upwelling zone have shown that the biomass in terms

of the carbon content is similar for the <20 and 20–150-µm size-fractions (Vargas et al., 2006, 2010).

Organic and inorganic phosphorus excretion has been less extensively studied than that of nitrogen compounds. Our results indicate that DOP was the main product excreted, accounting for 93% of the total dissolved phosphorus under late summer conditions in copepods fed with the 20–150-µm size-fraction. These results were in agreement with Johannes (1964), who reported that one-third of soluble phosphorus released by zooplankton (amphipods) was in the organic form. The same pattern was found by Satomi and Pomeroy (1965), who found that a large proportion (60%) of the phosphorus excreted by zooplankton was in the organic form. It has recently been shown that the prevailing compound being excreted by copepods is strongly dependent on the food consumed, with DOP release only being detectable in diets containing microzooplankton (Saba et al., 2009). However, these results include the nutrients released by sloppy feeding and leaching of fecal pellets, which are two other ways in which zooplankton can release dissolved compounds (Saba et al., 2011). Due to the large variability of these findings and the scarce information available, more studies of phosphorus release by zooplankton are necessary.

During our study, the specific composition of DON associated with DFAAs was determined, indicating shifts in the profile and magnitude between the treatment fed with the <20- and 20–150-µm size-fraction diets, mainly during spring. The information available about the composition of DFAAs is scarce and they are considered secondary excretory products. They account for between 5 and 48% of the total dissolved nitrogen excreted (Steinberg and Saba, 2008). The composition of the DFAA in the copepod excretion products was also variable during our experiments, with higher excretion rates in the treatment with copepods fed with the <20-µm size-fraction diet. In the late-summer experiment, the predominant amino acid excreted was HIS, while in spring it was GLU. It has been reported that GLY and alanine (ALA) are the

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FIGURE 9 | Abundant (>0.5%), semi-rare (0.1–0.5%), and rare (<0.1%) classes, classified according to their initial *in situ* contribution throughout the whole incubation period during late summer (March). The relative abundances of each class in the control were subtracted from the treatments. Positive values indicate a higher presence in treatments than in the control, and vice versa for negative values. **(A)** Treatment inoculated with dissolved compounds excreted by copepods fed with the 20–150-μm size-fraction diet, and **(B)** treatment inoculated with dissolved compounds excreted by copepods fed with the <20-μm size-fraction diet. The dashed lines indicate the composition of the total bacterial community (DNA-based).

predominant amino acids excreted, while cysteine (CYS) was absent in almost all estimations (Webb and Johannes, 1967), in contrast with our results in which ALA was excreted, but at low rates, and MET was absent in both experiments

(**Figure 2**). As with phosphorus, there are few studies available regarding DFAA excretion. Thus, further studies are required to improve our understanding of surface nitrogen and phosphorus turnover.

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FIGURE 10 | Abundant (>0.5%), semi-rare (0.1–0.5%), and rare (<0.1%) classes, classified according to their initial *in situ* contribution throughout the whole incubation period during spring (December). The relative abundances of each class in the control were subtracted from the treatments. Positive values indicate a higher presence in treatments than in the control and vice versa for negative values. **(A)** Treatment inoculated with dissolved compounds excreted by copepods fed with 20–150-μm size-fraction diet, and **(B)** treatment inoculated with dissolved compounds excreted by copepods fed with the <20-μm size-fraction diet. The dashed lines indicate the composition of the total bacterial community (DNA-based).

Negative excretion rates were found during the excretion phase, mostly for organic compounds (DON, DOP, and DFAAs). These negative rates indicate that the consumption was higher than the input provided to the copepods. The presence of these negative rates for DFAAs suggested a significant uptake of certain DFAAs. For example, in the late-summer experiment, a higher consumption of ALA and aspartic acid (ASP) was observed when copepods were fed with the 20–150-μm and <20-μm size-fraction diets, respectively. In contrast, in the spring experiment, a higher consumption of almost all DFAAs was observed in copepods fed with the 20–150-μm size-fraction diet, with the highest consumption rate for HIS (0.05 μmol L^{-1} h^{-1}). This could reflect the high lability of these compounds, which should be easily assimilated by microbial communities.

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FIGURE 11 | Principal coordinate analysis (PCA) based on Bray–Curtis similarity for the active bacterial community structure, at the order level, for different treatments and the control. **(A)** Late summer and **(B)** spring. Vectors indicate the best environmental variables (normalized transformed) correlated with ordination coordinates and vector lengths correspond to the correlation values.

Despite the attempts to minimize bacterioplankton abundance in the excretion phase, i.e., filtering the seawater to $<0.2\,\mu m$, bacterioplankton were present (Supplementary Figure 3), and could rapidly use these compounds.

Potential Fate of Dissolved Organic and Inorganic Compounds Derived from Copepod Excretion

The excretion products of copepods generated changes in the composition of the active bacterial community, and the extent of organic and inorganic nitrogen and phosphorus recycling. Our results suggested a rapid recycling of all nitrogen and phosphorus compounds, shifting from accumulation to consumption (or vice versa) after every 2 h of incubation. In the late-summer experiment, NH_4^+ was consumed throughout the incubation in the treatment with copepods fed with the $20–150$-μm size-fraction diet. DON, NO_2^-, and NO_3^- were accumulated toward the final period of the experiment. These results suggest a coupled uptake–remineralization process of NH_4^+ and DON, which is probably associated with heterotrophic microbial activities. The NO_3^- and NO_2^- accumulation in the treatment with copepods fed with the $20–150$-μm size-fraction diet suggested that nitrification processes occurred during the incubations. This was expected considering the previous findings of a tight coupling between excretion and ammonia oxidation (Molina et al., 2005, 2012) and the functional microbial groups involved (Valdés et al., 2017). During the late summer experiment, the treatment with copepods fed with the <20-μm size-fraction diet was

characterized by a high consumption of nitrogen compounds between the initial period and 4 h of incubation, with an NH_4^+ and DON accumulation in the final period. These results suggest a high bacterioplankton demand for DON remineralization/ammonification. This resulted in the accumulation of NH_4^+ at the end of the experiment, which was not present in the treatment with copepods fed with the $20–150$-μm size-fraction diet.

In addition, the DOM quality, evaluated by changes in the DON:DOP ratio (Supplementary Figure 10), could influence the microbial response to copepod excretion product enrichments. During spring, copepod excretion products contained a higher quality of DOM compared with late summer, generating a high turnover and consumption of DFAAs during the microbial response phase. This result supports the relevance of DFAAs as a relatively small but highly labile pool of DOM, which can support a large fraction of bacterial production in the ocean (Nagata, 2000). Moreover, the specific composition of DFAAs also indicated differences in the microbial response phase associated with the enrichment treatments. MET was the only amino acid accumulated during the first hour of incubation in both treatments, whereas THR was accumulated in the treatment enriched with compounds derived from copepods fed with the <20-μm size-fraction diet. DFAA concentrations are low in the marine system and their turnover can be quite fast, e.g., <30 min (Fuhrman, 1987). Our results showed that GLU and HIS were actively used by microbial communities at a low rate in late summer compared to spring, when the concentration throughout the incubation was higher and the uptake of DFAAs was mainly THR and HIS.

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In relation to phosphorus excretion and use, our results showed that DOP in the treatment inoculated with compounds excreted by copepods fed with the 20–150-μm size-fraction diet in the late-summer experiment had high consumption rates during the first 2 h, while for the inorganic form there was a higher accumulation between T_1-T_2. However, the estimated rate of DOP consumption was four times higher than that of PO_4^{3-} production. The same pattern was found in the treatment derived from copepods fed with the <20-μm size-fraction diet, but the difference between the DOP consumption and PO_4^{3-} accumulation rates was close to zero, with negative DOP consumption rates during all incubations. In addition, a lower DOP concentration and rate of change over time were observed in late summer than in spring. This could be in response to the quality of the food provided to copepods. A coupling between PO_4^{3-} uptake and DOP production was also observed at the end of the incubation for both treatments. There was an accumulation of PO_4^{3-} in the first 4 h of incubation, while at the same time DOP was consumed. However, in the final 2 h (T_2-T_3) PO_4^{3-} was consumed at a similar rate at which DOP was produced (**Figure 3**). In accordance with our results, the rapid utilization of phosphorus compounds (less than a few hours), and the coupled DOP production–utilization had been reported by other studies using labeled phosphorus in experiments in marine environments (Dolan et al., 1995; Lovdal et al., 2007).

Bacterial Response to the Copepod Excretion Products

During our experiment, we determined the response of the microbial community in terms of any changes in abundance and specific bacterial composition. Increases in the abundance of bacterioplankton communities were predominantly associated with the <20-μm enrichment, and this was particularly true for the spring experiment, in which there was an increase of ~400 × 10^3 cell mL^{-1} between T_0 and T_3. These results are in agreement with a recent study of the addition of compounds excreted by krill to bacterial assemblages, which showed that they stimulated bacterial growth and production in terms of biomass production, oxygen consumption, and growth (Arístegui et al., 2014).

In this study area, Bacteroidetes, Proteobacteria, Cyanobacteria, and Verrucomicrobia were the most abundant total (DNA) and active (RNA) phyla detected *in situ* during both sampling periods, and in late summer they were mostly represented by Flavobacteria, Gammaproteobacteria, Alphaproteobacteria, Sphingobacteria, Chloroplast, Cytophagia, and Verrucomicrobiae. In spring, all these classes were detected, with a greater contribution by Cyanobacteria and a smaller contribution by Gammaproteobacteria and Alphaproteobacteria, compared to late summer. Our results were in agreement with surface water diversity surveys in this study area based on metagenomics and an analysis of Station 18 during upwelling conditions (Murillo et al., 2014), and also the results of another study based on active bacteria 16S rRNA (Levipan et al., 2016)

The PERMANOVA results did not reveal significant differences between the treatments derived from copepods fed with the different size-fractionated diets. However, there were differences in the response of the composition of active bacteria to the compounds excreted by copepods based on the changes in their relative contribution, 16S rRNA:rDNA ratio, and the SIMPER analysis results. This suggests that the bacterial degradation of dissolved compounds is conducted by phylogenetically diverse communities, some generalist and others more specialized, that are associated with the upwelling periods. In the late-summer experiment, the treatment inoculated with dissolved compounds excreted by copepods fed with the 20–150-μm size-fraction diet had a higher contribution from Alphaproteobacteria in the first hour of incubation. This was mainly Rhodobacterales, which together with Flavobacteriales from the Bacteroidetes phyla, are very active bacterial communities. In the enrichment derived from copepods fed with the <20-μm size-fraction diet, Alphaproteobacteria were responsible for the differences found among treatment enrichments and controls during late summer. In contrast, in the spring experiment, Alphaproteobacteria were active in the enrichment derived from copepods fed with the <20-μm size-fraction diet, but were inhibited in the enrichment derived from copepods fed with the 20–150-μm size-fraction diet. A high 16S rRNA:rDNA ratio was also observed in spring. Alphaproteobacteria are aerobic heterotrophs that preferentially use monomers, such as amino acids and N-acetylglucosamine, and are therefore important competitors for amino acids in the ocean (Cottrell and Kirchman, 2000). In addition, copepods fed with the <20-μm size-fraction diet had higher DFAA excretion rates compared to the copepods fed with the 20–150-μm size-fraction diet, and during the microbial response phase these treatments were associated with THR in late summer, and with GLU and arginine (ARG) in spring. These results suggest that the response of microbial communities was more strongly associated with the quality of the food provided than with the amount of the compounds excreted, e.g., DON.

The uptake of specific DOM compounds by marine microorganisms has been studied by microautoradiography combined with *in situ* hybridization (MAR-FISH), and several studies have reported different uptake patterns of simple, low-molecular-weight compounds (Ouverney and Fuhrman, 1999; Cottrell and Kirchman, 2000; Vila et al., 2004). These results have demonstrated that Alphaproteobacteria are responsible in large part for the uptake of low-molecular-weight DOM, while Bacteroidetes are more specialized in the uptake of high-molecular-weight DOM. Alonso-Saez and Gasol (2007) demonstrated that DFAAs were readily taken up by Alphaproteobacteria and Gammaproteobacteria, but not by Bacteroidetes. Furthermore, the same authors showed that ATP was consumed in high proportions by Alphaproteobacteria, Gammaproteobacteria, and Bacteroidetes. Our experiment was conducted in late summer, and was characterized by the presence of complex structural biopolymers (cell wall components) associated with the large size (1.6 μm) of the organisms in the microbial community. The decay of this community by predation or autolysis could provide complex substrates in our incubation, enhancing the activity of Bacteroidetes and other groups, including semi-rare classes (e.g., Betaproteobacteria). There were differences in the 16S rRNA:rDNA ratio between treatments.

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Betaproteobacteria (semi-rare) were significantly active at the end of the experiment with copepods fed with the 20–150-μm size-fraction diet in late summer. Betaproteobacteria (e.g., Bourkholderiales, Hydrogenophilales, and Methylophilaceae) are commonly found in productive coastal waters (Rappé et al., 2000), including the area studied here (Levipan et al., 2016), and some are methylotrophs that can use C1 compounds, such as methanol, as a source of carbon and energy. Moreover, in the treatments derived from copepods fed with the <20-μm size-fraction diet, Bacteroidetes were active at the end of the incubation, and were related to the Flavobacteria. This group is one the most abundant phyla in coastal areas, where they represent between 10 and 30% of the total relative abundance of the bacterial community (Alonso-Saez and Gasol, 2007). They are able to degrade complex organic matter and are specialized in the degradation of biopolymers, such as proteins (Pinhassi et al., 1999; Teeling et al., 2012; Fernández-Gómez et al., 2013).

However, many groups were shifted during the incubation, including Gammaproteobacteria, which are mainly associated with the Alteromonadales, Cellvibrionales, and Oceanospirillales orders, particularly during spring. These groups are considered to be opportunistic bacteria in surface waters, and they responded rapidly to the enrichment derived from copepod excretion. Several studies have suggested that enrichment experiments tend to enhance the abundance of microorganisms rarely found in nature, but that have opportunistic and copiotrophic qualities that allow them to rapidly adapt to changes in environmental conditions, outcompeting abundant groups in the field (Nelson and Wear, 2014; Pedler et al., 2014; Logue et al., 2016). Lauro et al. (2009) argued that copiotroph bacteria have a higher genetic potential to sense and rapidly respond to a sudden nutrient influx, compared to the more widely distributed oligotrophic bacteria. Stocker et al. (2008) demonstrated that bacteria can gain growth advantages if they can exploit ephemeral nutrient patches, for example, those originating from phytoplankton photosynthetic products, or cell lysiate and organic matter leaking from particles and zooplankton excretion.

CONCLUSION

We conclude that NH_4^+ and DOP are the main compounds excreted by the copepod community in the coastal upwelling zone of central Chile under active upwelling conditions. Our data also suggests a direct relationship between NH_4^+ and DOP excretion and the dominant food available to copepods during this season (i.e., microplankton in the 20–150-μm size-fraction), but this is not true for DFAAs.

Our results suggest a tightly coupled remineralization and uptake, potentially linked to the assimilation process over short response times (<2 h), and particularly associated with the <20-μm size fraction. The results suggest that the response of microbial communities is associated with the quality of the compounds provided by copepod excretion. The response was characterized by a high turnover of DFAAs and the contribution of active typical surface water fast-responsive bacteria to the new source of organic matter available during late summer and spring, i.e., Alphaproteobacteria and Bacteroidetes, respectively.

Further studies of organic excretion (e.g., DOP and DFAA) and the design of experiments to explore the use of specific compounds released by zooplankton by the bacterial communities will provide valuable insights into the role of zooplankton in fueling the microbial loop.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2017.00343/full#supplementary-material

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Dissolved Compounds Excreted by Copepods Reshape the Active Marine Bacterioplankton Community

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1. Supplementary Figures and Tables

1.1 Supplementary Figures
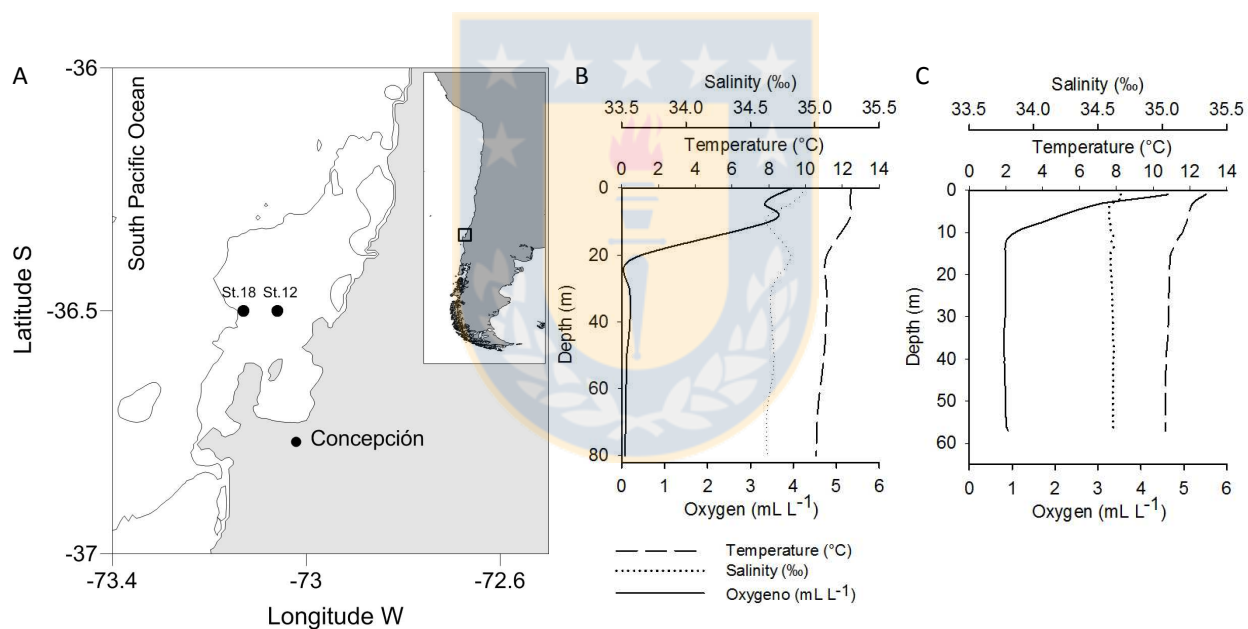
Supplementary Figure 1. (A) Study area and hydrographic conditions of salinity (‰), temperature (°C) and oxygen (mL L^{-1}) during both sampling events. Profiles were carried out in (B) late summer (March 2014) and (C) spring (December 2014). The contour line indicated 100 m depth.

Phase 1: Acclimation

Incubated 4 copepods group in dark and at *in situ* temperature

After 6 h

Transferred Copepods

Filtered seawater 0.7 µm
Volume: 10 L

Phase 2: Feeding

Two size fraction diets: 20-150 µm and <20 µm
Volume: 10 L

After 4 h

Transferred copepods

Phase 4: Microbial response

Treatment 20-150 µm   Treatment < 20 µm   Control

After 2 h

Time: 6 h
Filtered seawater 1.6 µm (late summer) - 0.7 µm (spring)
Volume: 4 L (DNA) - 1 L
Subsample: 0, 0.5, 1, 2, 4 and 6 h (0 and 6 h for DNA)
Inoculation with 3.4 L and 150 mL of excreted compounds

Phase 3: Excretion

Treatment: 20-150 µm   Treatment: < 20 µm   Control

Filtered seawater 0.22 µm
Volume: 6L
Subsample: 0, 1 and 2 h

Supplementary Figure 2. 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 fractions 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.
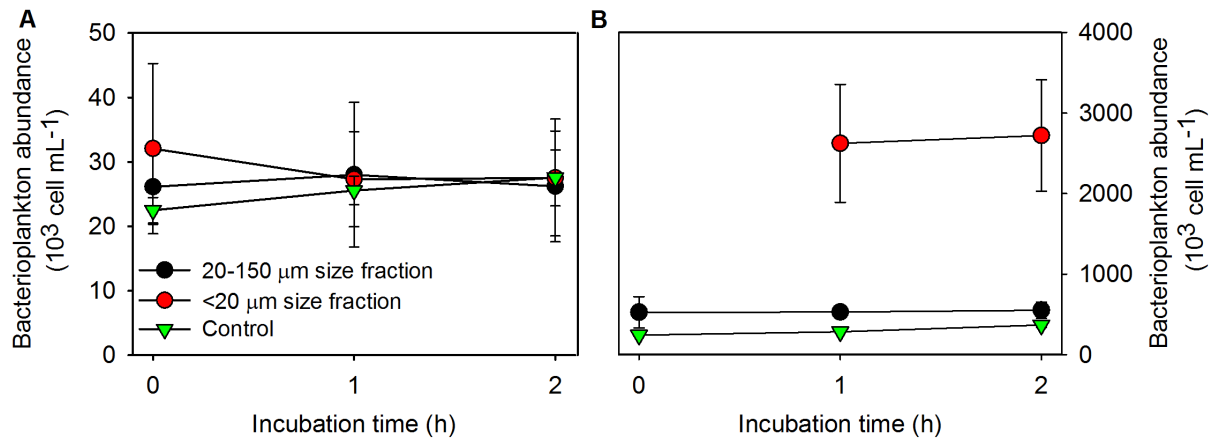
Supplementary Figure 3. Bacterioplankton abundance through excretion phase in both experiments, (A) late summer and (B) spring.
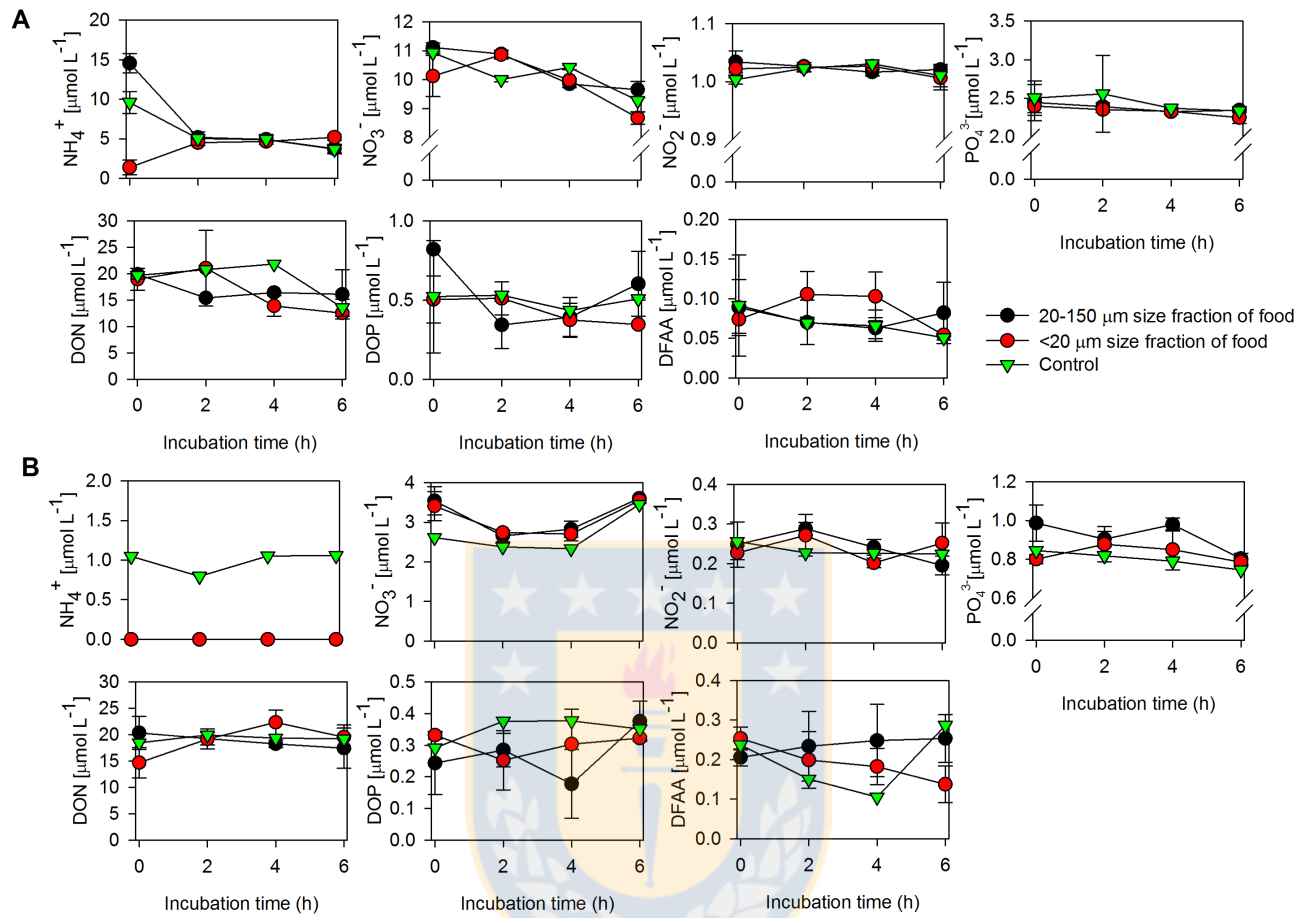
Supplementary Figure 4. Evolution of nitrogen and phosphorus compounds during microbial response phase in both experiments, (A) late summer and (B) spring.
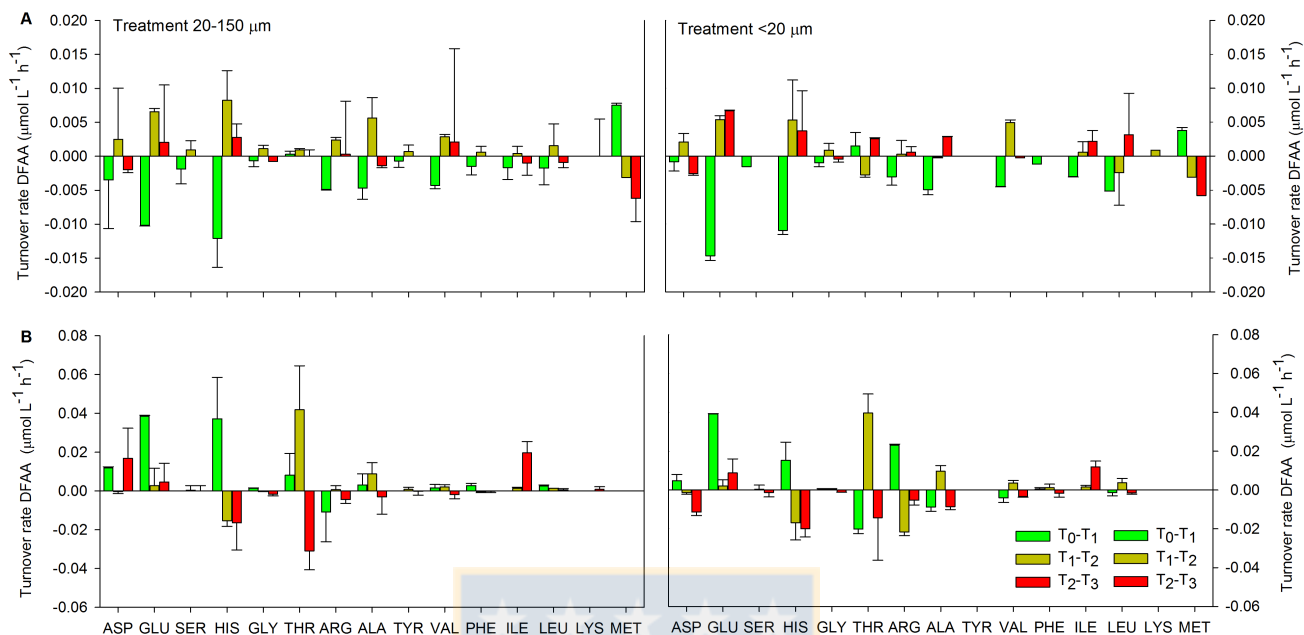
Supplementary Figure 5. Turnover rates of aspartic acid (ASP), glutamic acid (GLU), serine (SER), histidine (HIS), glycine (GLY), Threonine (THR), arginine (ARG), alanine (ALA), threonine (TYR), valine (VAL), phenylalanine (PHE), isoleucine (ILE), leucine (LEU), lysine (LYS), methionine (MET) estimated every two hours of incubation, for both sampling event and treatments. (A) Late summer and (B) spring experiment.
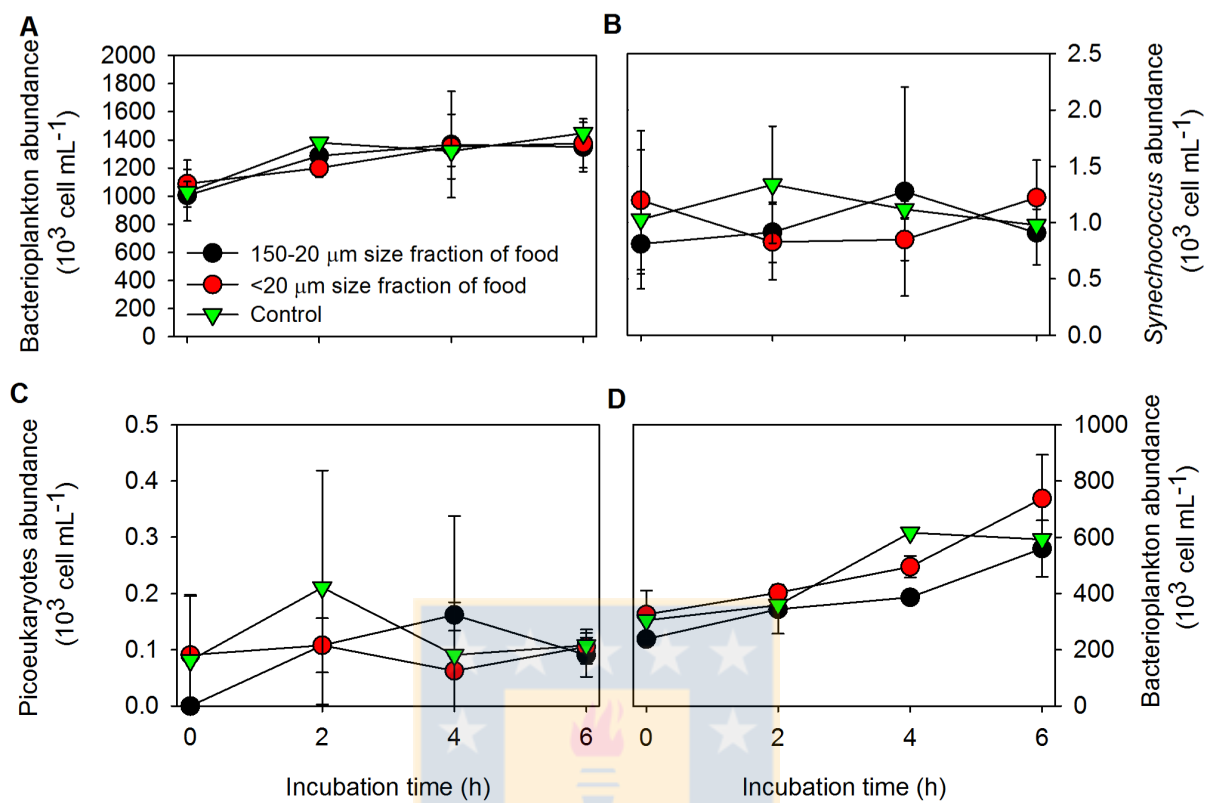
Supplementary Figure 6. (A) Bacterioplankton, (B) Cyanobacteria and (C) picoeukaryotes abundance during the microbial response phase in late summer and (D) bacterioplankton abundance during the microbial response phase in spring.
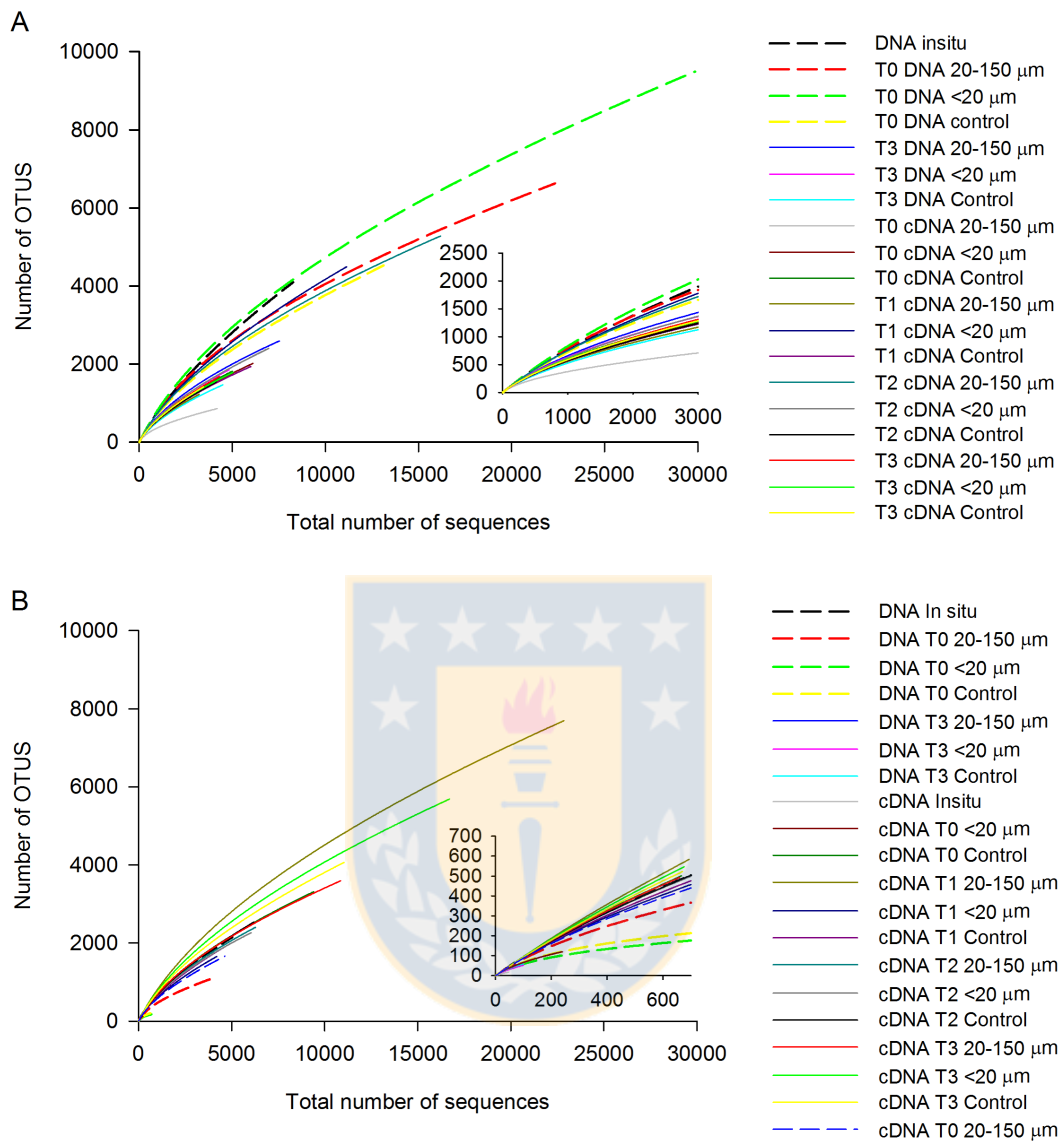
Supplementary Figure 7. Rarefactions curves for both experiments. (A) Late summer and (B) spring.
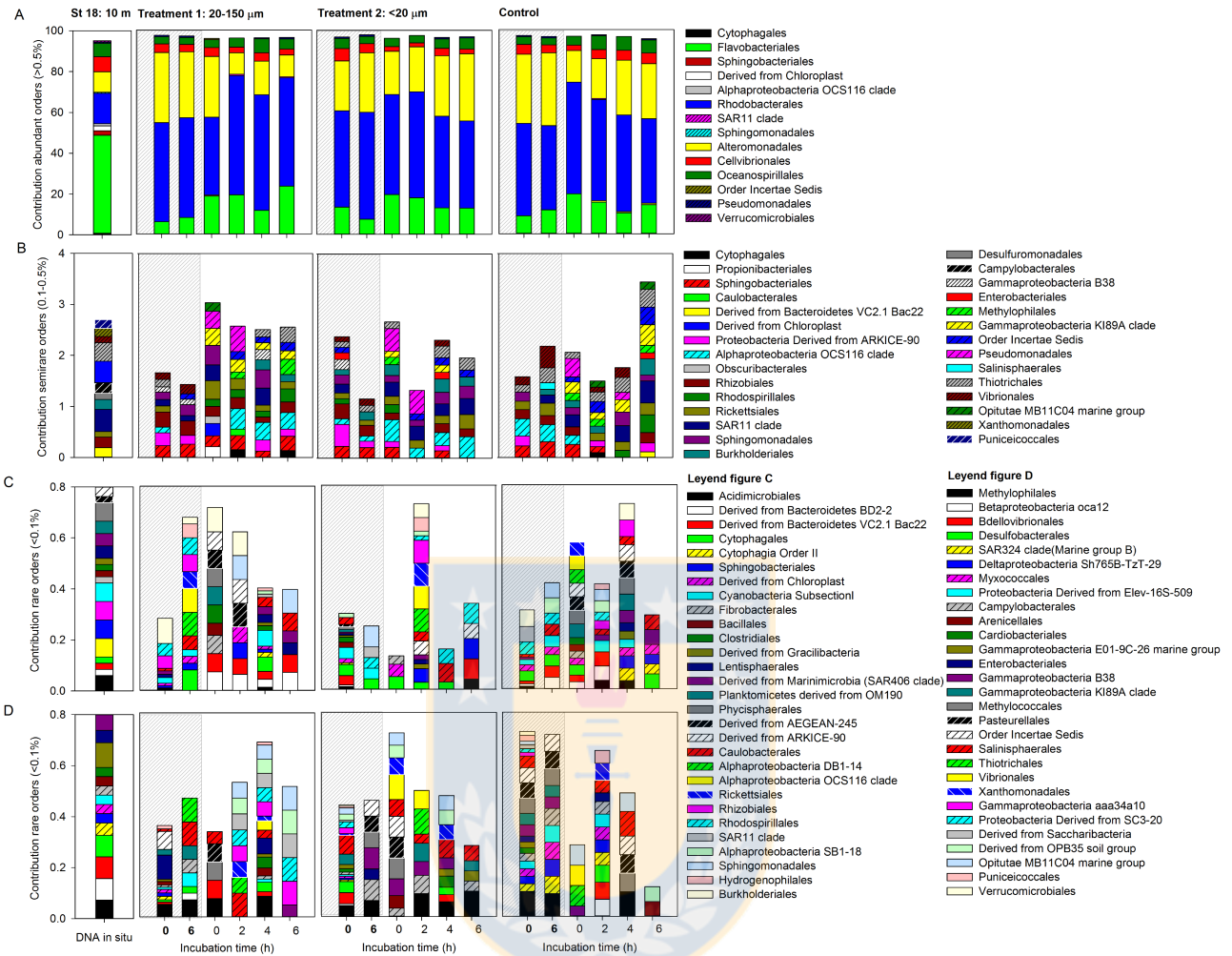
Supplementary Figure 8. (A) Abundant (<0.5%), (B) semirare (0.5-0.1%) and (C) rare (<0.1%) Orders in late summer (March) for *in situ* conditions and throughout the experiments.
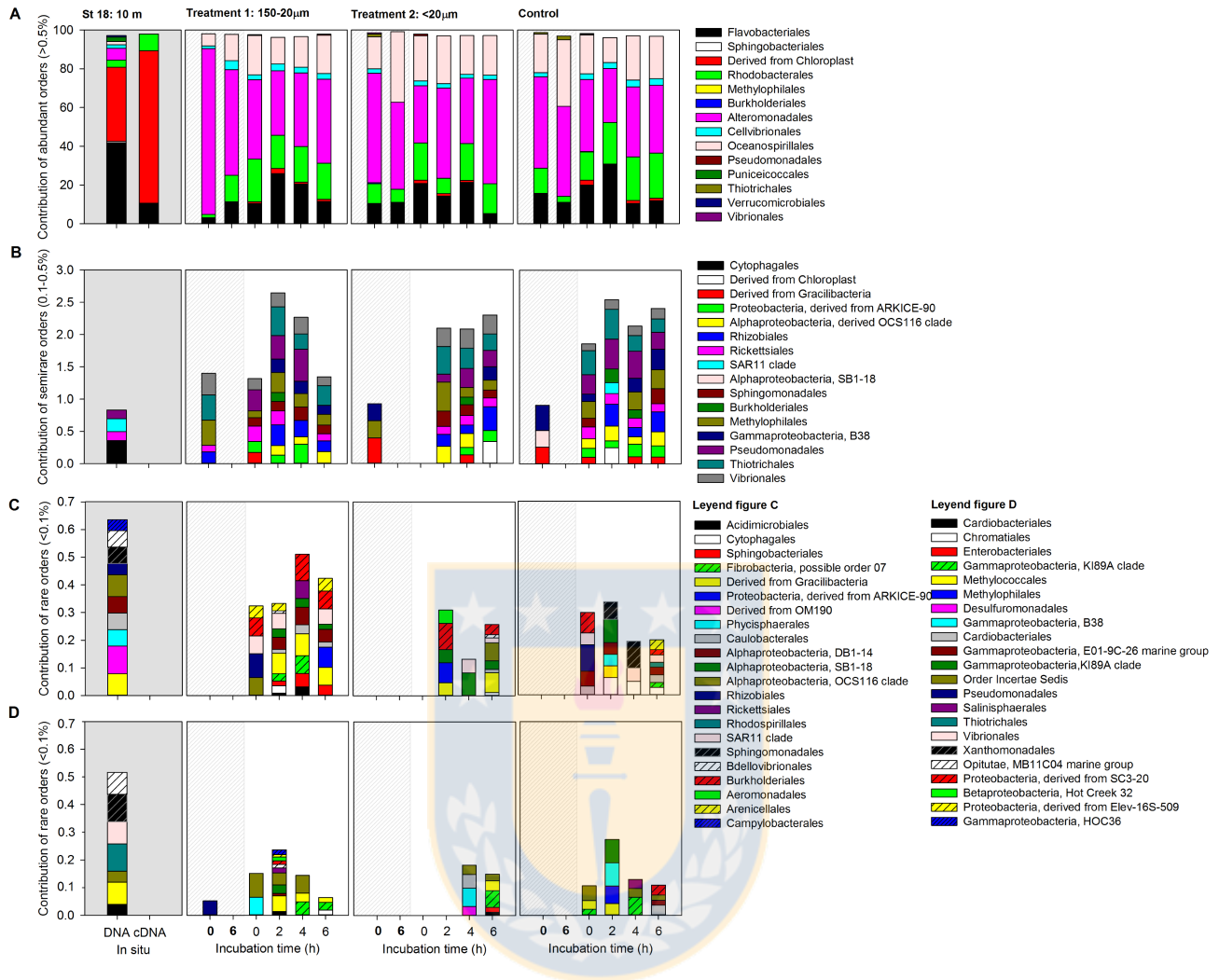
Supplementary Figure 9. (A) Abundant (<0.5%), (B) semirare (0.5-0.1%) and (C) rare (<0.1%) Order in spring (December) for *in situ* conditions and throughout the experiments.
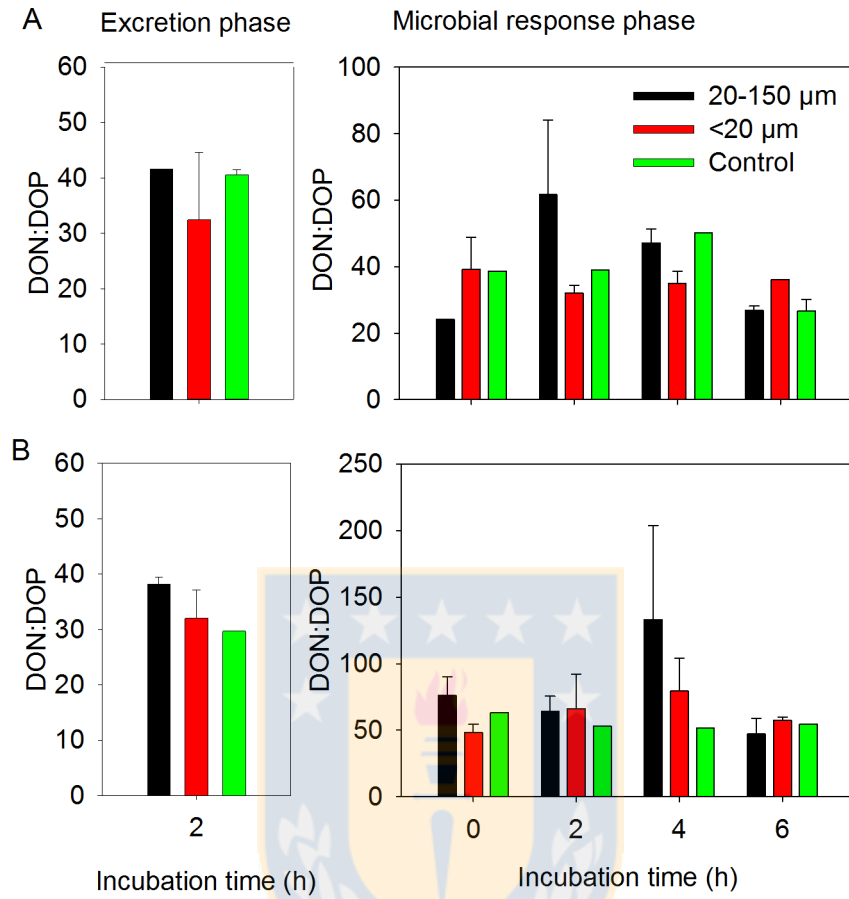
Supplementary Figure 10. DON: DOP ratio during the last hour of incubation in copepod excretion phase and though microbial response phase. (A) late summer and (B) spring.

1.2 Supplementary Tables

Supplementary Table 1. DNA and cDNA concentration and quality (260:280 ratio) for all samples obtained from the experiments.

Experiment	DNA/cDNA	Samples	Concentration (ng μL^{-1})	260:280 ratio
Late summer	DNA	T0_20-150 μm	20.80 ± 1.42	1.70 ± 0.17
		T0_<20 μm	29.58 ± 0.69	1.80 ± 0.07
		T0_Control	27.95 ± 1.92	1.59 ± 0.20
		T3_20-150 μm	29.35 ± 0.60	1.93 ± 0.04
		T3_<20 μm	24.52 ± 0.35	1.81 ± 0.04
		T3_Control	21.95 ± 0.65	1.69 ± 0.10
		In situ	7.19 ± 0.01	2.13 ± 0.14
	cDNA	T0_20-150 μm	10.27 ± 0.19	2.33 ± 0.16
		T0_<20 μm	5.27 ± 0.56	1.51 ± 0.16
		T0_Control	9.16 ± 0.09	1.71 ± 0.14
		T1_20-150 μm	8.39 ± 0.23	1.71 ± 0.12
		T1_<20 μm	6.94 ± 0.54	1.65 ± 0.08
		T1_Control	4.24 ± 0.14	1.18 ± 0.05
		T2_20-150 μm	7.99 ± 0.82	1.58 ± 0.10
		T2_<20 μm	6.72 ± 0.35	1.61 ± 0.06
		T2_Control	9.28 ± 1.69	1.99 ± 0.48
		T3_20-150 μm	8.07 ± 0.83	4.24 ± 2.87
		T3_<20 μm	9.51 ± 0.56	2.23 ± 0.09
		T3_Control	6.53 ± 0.25	2.21 ± 0.43
Spring	DNA	T0_20-150 μm	10.4	-
		T0_<20 μm	4.79	-
		T0_Control	5.56	-
		T3_20-150 μm	6.4	-
		T3_<20 μm	4.14	-
		T3_Control	15.2	-
		In situ	17.7	-
	cDNA	T0_20-150 μm	6.36	-
		T0_<20 μm	7.8	-
		T0_Control	5.84	-
		T1_20-150 μm	6.96	-
		T1_<20 μm	3.22	-
		T1_Control	7.8	-
		T2_20-150 μm	5.32	-
		T2_<20 μm	7.56	-
		T2_Control	11.5	-
		T3_20-150 μm	6.44	-
		T3_<20 μm	5.44	-
		T3_Control	12.3	-
		In situ	25.2	-

Supplementary Table 2. DFAA composition in station 12 at 10 m depth during spring (December). Each values represent two replicates (mean ± standard deviation).

DFAA composition	Concentration ($\mu mol\ L^{-1}$)
GLU	0.20
SER	0.05 ± 0.06
HIS	0.60 ± 0.12
GLY	0.08
THR	0.24 ± 0.07
ARG	0.02 ± 0.02
ALA	0.25 ± 0.01
VAL	0.09 ± 0.02
ILE	173

Supplementary Table 3. ANOVA followed by the Tukey´s Honest Significant Difference (HSD), statistical comparison was performed on nutrients turnover rates in treatments and between times throughout the experiments. The mean of HSD is recorded at 95% confidence level with its associated P value for this comparison. Significant P values are bolded.

	Late summer (March)		Spring (December)	
	20-150 μm	<20 μm	20-150 μm	<20 μm
NH_4^+				
T_0-T_1 – T_2-T_3	HSD=-1.02 P=0.67	**HSD=6.73** **P=0.00**	**HSD=0.13** **P=0.00**	**HSD=0.13** **P=0.00**
T_1-T_2 – T_2-T_3	HSD=-0.63 P=0.93	HSD=-1.33 P=0.40	**HSD=0.12** **P=0.00**	**HSD=0.12** **P=0.00**
T_1-T_2 – T_0-T_1	HSD=0.39 P=0.99	**HSD=-8.06** **P=0.00**	**HSD=0.25** **P=0.00**	**HSD=0.25** **P=0.00**
NO_3^-				
T_0-T_1 – T_2-T_3	HSD=0.17 P=0.06	**HSD=0.90** **P=0.01**	NA	NA
T_1-T_2 – T_2-T_3	**HSD=-1.2** **P=0.00**	HSD=-0.56 P=0.16	NA	NA
T_1-T_2 – T_0-T_1	**HSD=-1.37** **P=0.00**	**HSD=-1.47** **P=0.00**	NA	NA
NO_2^-				
T_0-T_1 – T_2-T_3	NA	NA	HSD=0.04 P=0.50	HSD=0.02 P=0.91
T_1-T_2 – T_2-T_3	NA	NA	HSD=-0.01 P=0.99	HSD=-0.05 P=0.19
T_1-T_2 – T_0-T_1	NA	NA	HSD=-0.05 P=0.26	**HSD=-0.07** **P=0.03**
DON				
T_0-T_1 – T_2-T_3	**HSD=-7.20** **P=0.00**	HSD=-2.26 P=0.30	NA	NA
T_1-T_2 – T_2-T_3	**HSD=-4.72**	**HSD=-7.56**	NA	NA

	P=0.00	P=0.00		
T_1-T_2 – T_0-T_1	HSD=2.48 P=0.28	**HSD=-5.30 P=0.00**	NA	NA
DFAA				
T_0-T_1 – T_2-T_3	NA	NA	HSD=0.12 P=0.06	**HSD=0.14 P=0.02**
T_1-T_2 – T_2-T_3	NA	NA	HSD=0.09 P=0.22	**HSD=0.13 P=0.03**
T_1-T_2 – T_0-T_1	NA	NA	HSD=-0.03 P=0.97	HSD=-0.001 P=1
PO_4^{3-}				
T_0-T_1 – T_2-T_3	NA	NA	NA	NA
T_1-T_2 – T_2-T_3	NA	NA	NA	NA
T_1-T_2 – T_0-T_1	NA	NA	NA	NA
DOP				
T_0-T_1 – T_2-T_3	**HSD=-0.32 P=0.00**	HSD=0.03 P=0.99	NA	NA
T_1-T_2 – T_2-T_3	HSD=2.7E-17 P=1	HSD=0.01 P=0.99	NA	NA
T_1-T_2 – T_0-T_1	**HSD=0.32 P=0.00**	HSD=0.02 P=0.99	NA	NA

Supplementary Table 4. Similarity percentage analysis (SIMPER) showing the contribution and taxonomic affiliation of OTUs explaining 70% of the dissimilarity between treatments through time for present and active bacterial community in both sampling date.

Dissimilarity: Present bacterial community, late summer experiment.

Factors	Av. Diss	Phyla/Class	Diss/SD	Contribution (%)
20-150 µm &<20 µm	9.50	Alteromonadales	2.33	11.58
		Flavobacteriales	1.26	11.09
		Cellvibrionales	1.51	5.81
		Oceanospirillales	1.00	3.86
		Rhodobacterales	1.81	3.57
		Enterobacteriales	1.39	3.44
		Salinisphaerales	1.27	2.96
		Derived from ARKICE-90	2.23	2.88
		Cytophagales	1.88	2.71
		Burkholderiales	1.29	2.49
		KI89A clade	1.20	2.27
		Caulobacterales	1.41	2.25
		Hydrogenophilales	0.86	2.20
		SAR11 clade	2.28	2.08
		Desulfobacterales	2.04	2.02
		Campylobacterales	1.15	1.87
		Derived from Bacteroidetes VC2.1 Bac22	0.87	1.76
		Sphingomonadales	1.33	1.70
		Derived from Chloroplast	1.28	1.60

		oca12	1.18	1.56

Dissimilarity: Active bacterial community, late summer experiment.				
20-150 µm &<20 µm	14.36	Alteromonadales	1.70	16.51
		Rhodobacterales	2.12	7.93
		Flavobacteriales	1.37	7.09
		Cellvibrionales	1.69	4.25
		Oceanospirillales	1.23	3.07
		Vibrionales	1.58	2.55
		Derived from Chloroplast	1.16	2.44
		Thiotrichales	1.34	2.42
		Derived from Bacteroidetes VC2.1 Bac22	1.97	2.35
		MB11C04 marine group	2.31	2.31
		Cytophagales	1.62	2.27
		B38	2.12	2.12
		KI89A clade	1.55	2.08
		Caulobacterales	1.24	1.93
		Rhodospirillales	1.47	1.91
		Sphingomonadales	1.91	1.87
		Campylobacterales	1.55	1.86
		Sphingobacteriales	1.50	1.85
		SAR11 clade	1.31	1.81
		Order *Incertae Sedis*	0.85	1.63

Dissimilarity: Present bacterial community, spring experiment.				
20-150 µm &<20 µm	24.75	Oceanospirillales	1.56	18.97
		Alteromonadales	1.37	17.86
		Rhodobacterales	1.54	13.90
		Flavobacteriales	1.17	11.78
		Cellvibrionales	1.21	8.82

Dissimilarity: Active bacterial community, spring experiment.				
20-150 µm &<20 µm	12.46	Flavobacteriales	1.45	15.87
		Rhodobacterales	1.09	10.64
		Alteromonadales	1.73	10.34
		Oceanospirillales	1.22	7.17
		Derived from Chloroplast	1.20	4.95
		Cellvibrionales	2.30	3.63
		B38	1.21	2.81
		Pseudomonadales	1.63	2.66
		DB1-14	1.63	2.43
		Arenicellales	1.59	2.13
		Sphingobacteriales	1.52	1.95
		Order *Incertae Sedis*	1.45	1.94
		Methylophilales	1.24	1.91
		KI89A clade	1.45	1.85

3.3 Chapter III: Nitrogen and phosphorus recycling mediated by copepods in the Western Tropical South Pacific

Manuscript in preparation (Biogeoscience)

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PhD in Oceanography

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Abstract

Zooplankton plays a key role in the regeneration of nitrogen and phosphorus in the ocean through grazing and metabolism. In this study, we explore the role of zooplankton release of organic and inorganic nitrogen and phosphorus compounds on the microbial community, over an oligotrophic gradient in the Western Tropical South Pacific. Three microcosm experiments were performed in the long duration stations (LD) in the Malanesian archipelago region (LD A and B) and in the subtropical gyre (LD C) during OUTPACE cruise. For this, using a mix of epipelagic copepods were feed and then incubated with marine microbial assemblages. Results revealed that in the treatment with copepods a significant increase in ammonium, DON and DOP was observed, stimulated remineralization (ammonification and nitrification) in the treatment with copepods than control. A shift in the composition of active bacterial community between the treatment incubated with copepod and control was characterized by an increase in Alteromonadales and SAR11 in LD A and B, respectively, associated with the nutrients concentration at each experiment, higher in LD A and lower in LD B. In the most oligotrophic station, both groups were increased but at different period of incubation. Bacterial remineralization of the dissolved organic matter derived from copepods metabolism could be highly efficient mechanism to maintain the nutrients in the upper layer support the phytoplankton and microbial growth.

Nitrogen and phosphorus recycling mediated by copepods in the Western Tropical South Pacific

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Abstract

Zooplankton plays a key role in the regeneration of nitrogen and phosphorus in the ocean through grazing and metabolism. In this study, we explore the role of zooplankton release of organic and inorganic nitrogen and phosphorus compounds on the microbial community, over an oligotrophic gradient in the Western Tropical South Pacific. Three microcosm experiments were performed in the long duration stations (LD) from the Melanesian Archipelago region (LD

A and B), which correspond to the oligotrophic region, to the western boundary of the South Pacific Gyre (LD C), which correspond to the ultra-oligotrophic region, during OUTPACE cruise. Using a mix of epipelagic copepods that were feed and then incubated with marine microbial assemblages. We revealed that in the treatment with copepods a significant increase in ammonium, dissolved organic nitrogen (DON) and dissolved organic phosphorus (DOP) was observed and remineralization (ammonification and nitrification) was enhanced in the treatment with copepods compared to controls. A shift in the composition of active bacterial community between the treatment incubated with copepod and control was characterized by an increase in Alteromonadales and SAR11 in LD A and B, respectively, associated with the nutrients concentration at each experiment, higher in LD A and lower in LD B. In the most oligotrophic station, both groups were increased but at different period of incubation. Bacterial remineralization of the dissolved organic matter derived from copepods metabolism could be highly efficient mechanism to maintain the nutrients in the upper layer support the phytoplankton and microbial growth.

1. **Introduction**

The Tropical South Pacific is considered one of the most oligotrophic regions in the world ocean. Its biogeochemical characteristics include nitrogen and phosphate limitation which modulate the low biological productivity(Moutin et al., 2008). In addition, the strong thermal stratification, enhanced by current global warming, decreases nutrient supply to the euphotic zone. The Western Tropical South Pacific (WTSP) was recently recognized as a hot spot of N_2 fixation (Bonnet et al., 2017) and the input of new nitrogen to the surface ocean through this process sustain over 50 % of primary productivity (Carpenter et al., 2004; Karl et al., 1997). However, it has been reported that predator-prey interactions can provide substantial amounts of nitrogen and phosphorus, and supply an alternative substrate for phytoplankton and microbial loop growth in a variety of areas, contributed to the regenerated production (Richardot et al., 2001; Vargas et al., 2007; Arístegui et al., 2014; Valdés et al., 2017).

Metazooplankton metabolism is recognized as one of the principal mechanisms controlling abundance, composition and cycling of DOM in the sea (Sipler and Bronk 2014). Zooplankton may release dissolved organic and inorganic matter through sloppy feeding (Møller, 2004, 2007), leaching of fecal pellet (Hasegawa et al., 2000; Steinberg et al., 2002) and excretion

(Saba et al., 2011). Along with bacterial remineralization, it determines the amount of regenerated nitrogen and phosphorus available for phytoplankton production (Steinberg and Landry, 2017).Copepods excrete up to 53% of its body nitrogen per day, mainly in the form of ammonium, thereby recycling much of the nitrogen in the water column (Steinberg and Saba 2008). Studies are reported that zooplankton could sustain between 40-50% of the nitrogen requirements of phytoplanktonic oceanic areas as North Pacific central gyre (Eppley et al., 1973), and in the oligotrophic Sargasso Sea, diel vertical migrating zooplankton can sustain between 2 to 19% of the nitrogen requirements by phytoplankton through ammonium excretion (Steinberg et al., 2002). Furthermore, zooplankton can also excrete substantial amounts of dissolved organic nitrogen (DON) amid total excreted dissolved nitrogen (Steinberg et al., 2002). Even less studied than nitrogen excretion is the phosphorus excretion by zooplankton. It has been reported, that one-third of the phosphors released by zooplankton (amphipods) was in the organic form (Johannes, 1964; Satomi and Pomeroy, 1965). Furthermore, Johannes, (1964) reported a tight coupling between DOP production and uptake by bacteria. Isla et al (2004) estimated that mesozooplankton phosphate excretion can sustain 21.7 % of phytoplankton phosphate requirements in oceanic water of the NW Iberian.

The quantity and quality of the dissolved compounds excreted are highly dependent of the type of food ingested (Miller and Roman, 2008; Saba et al., 2009). It has been documented a direct grazing on *Trichodesmium* spp for the harpacticoid copepods *Macrosetella gracilis*, *Miracia efferata* and *Oculosetella gracilis* in oligotrophic areas (O`Neil and Roman, 1994; O'Neil et al., 1996). Also, Hunt et al (2016) recently demonstrated during[15]N$_2$ labelled grazing experiment in New Caledonia lagoon, that copepods can graze on symbiotic and unicellular cyanobacteria (UCYN-C) and on cyanobacteria associated with diatoms based on. O'Neil et al (1996) estimated that harpacticoid copepods could consume 45% of total colony nitrogen, or 100% of the new nitrogen fixed each day.

The importance of quality and quantity of dissolved organic matter in structuring bacterioplankton community has been increasingly reported (Alonso-Saez and Gasol, 2007). However, the processes and mechanisms through which N and P compounds released by zooplankton can determine and influence the food web and the structure of the microbial community are unclear. Recent research has demonstrated that bacterial community

composition can change in response to the addition of DOM (Landa et al., 2013; Sarmento et al., 2013). Studies on zooplankton-produced DOM by bacterial communities are scarce. Some recent studies are demonstrated that the addition of excretory products released by zooplankton (krill) stimulates bacterial growth and production in the Southern Ocean (Arístegui et al., 2014). Valdés et al., (2017) documented that the nitrogen excreted by copepods can be used directly by the nitrifying community in southern/central Chile. However, studies of the impact nitrogen and phosphorus released derived from copepods metabolism on microbial community can provide valuable information about of the recycling of this nutrients in the ocean.

We studied the role of organic and inorganic dissolved compounds release by copepods along a gradient of oligotrophy in the WTSP, we also explore the role of recycling and the role of zooplankton-derived DOM in structuring the bacterioplankton community.

2. Materials and methods

2.1. Study area and sampling strategy

Sampling was conducted in Western Tropical South Pacific during austral summer 2015 (18 February -3 April), on board the R/V *L`Atalante*. The transect beginning in west of New Caledonia (18º S-159.9º E) and ending near Tahiti (17.56º S-149.05º W). Experiments was conducted in three long duration stations: LD A, B and C, which are located in two geographic regions: LD A and B in the Malanesian archipelago (MA) and LD C in the subtropical gyre (SG). These stations chosen based on the sea surface chlorophyll-a concentration, which was maxima in LD A and LD B, and minimum in LD C station. At each sampling station seawater samples at the Deep Chlorophyll Maximum (DCM), for ammonium, nitrate, nitrite, phosphate, DNA and RNA were immediately obtained for chemical and biological initial characterization. The DCM depths correspond to 80 m, 34 m and 140 m in LD A, B and C, respectively, and seawater samples were obtained using Niskin bottles (12 L) arranged on a CTD rosette.

2.2. Mesozooplankton sampling

Live zooplankton samples were captured at night conditions at each LD station (day 2) with a WP2 zooplankton net with a 120 μm mesh size, equipped with a non-filtering cod-end to obtain undamaged individuals, between 0 to 100 m depth under night conditions in the three LD stations. Live samples were immediately transferred to coolers until sorting at the laboratory.

2.3. Preparation of the microcosm and experimental setup

The experiment design consists in three steps: (1) Copepod acclimatizing, (2) feeding and (3) copepod recycling (Fig. 1). Seawater for incubations (30 L) was collected into clean (10% HCl rinsed) polycarbonate carboys from DCM using Niskin bottles (12 L) arranged on a CTD rosette. For acclimatizing (2) and copepod recycling (3) steps, seawater (22.5 L) was immediately filtered onto a 0.7 μm (GF/F; Whatman) using a peristaltic pump. The 7.5 L remaining was used in the feeding phase. Seawater for the different steps was maintained in a cold room (*in situ* temperature ~25º C) until the beginning of the experiment.
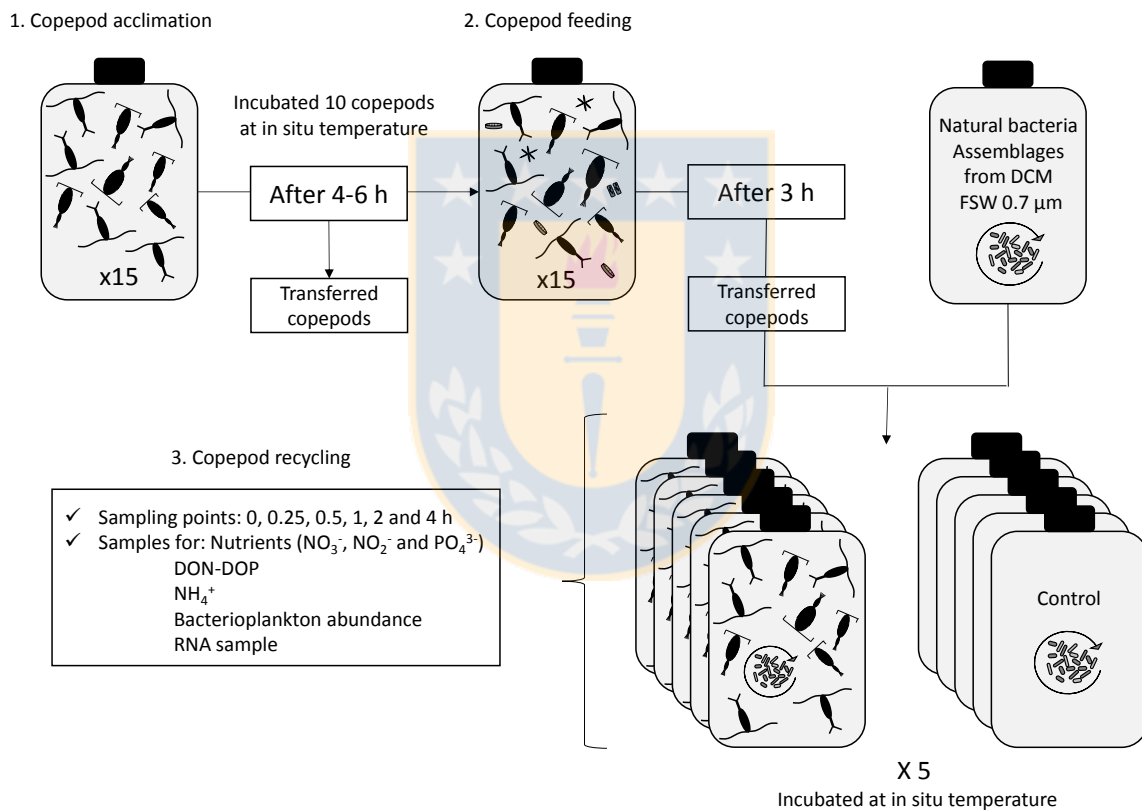
Figure 1: Experimental design. The design consisted of three phases done sequentially. Each bottle with copepods was acclimated for at least 6 hours (phase 1), followed by feeding (phase 2) with the Deep Chlorophyll-a Maximum (DCM) seawater for 3 hours. Finally, copepod recycling (phase 3) has treatment and control (without copepods). Three bottles were sacrificed at each sampling time

Undamaged individuals were sorted from the live samples and identified using a stereomicroscope at low light. Copepods samples in the three experiments consisted in a mix of the most representative copepods (adults) in the sample (Fig. S1). In acclimatization phase (1), 15 groups of 10 copepods were incubated in 500 mL Nalgene bottles, maintained for 4-6 h in filtered seawater previously filtered (GF/F Whatman) in darkness and at controlled temperature (*in situ*). In feeding step (2) copepods were removed from the bottles used in the previous phase, with a sieve (20 μm) and maintained in 500 mL polycarbonate bottles (Nalgene) with <150 μm filtered seawater (polycarbonate membrane) for feeding. This step lasted 4 hours and, as the acclimatization phase, was under controlled temperature. Thereafter, each copepod group (15 groups) was placed in 500 mL polycarbonate bottles (Nalgene) with the seawater previously filtered (0.2 μm; Millipore). Also, other 15 bottles without copepods were incubated as control. In total, 30 bottles were used in this step. The seawater used was filtered to avoid the presence of phytoplankton and small protist, that they can prey on bacteria, and ensure the presence of natural microbial assemblages. At the end of each step copepods were observed and checked by direct observation of ensuring swimming behavior in the bottles.

At time intervals of 0 h (T_0), 0.5 h (T_1: T_0+0.5 h), 1 h (T_2: T_0+1 h), 2 h (T_3: T_0+2h) and 4 h (T_4: T_0+4 h) samples were collected for each bottle for NH_4^+, NO_3^-, NO_2^-, PO_4^{3-}, DON, DOP, bacterioplankton abundance and RNA. An additional time at 15 min (T_0': T_0+0.25h) was added for NH_4^+ in LD B and C station. Six bottles were sacrificed at each time, three for treatment with copepods and three without copepods (control). The three bottles sacrificed per time were triplicates in the treatment with copepods and control. At the end of the experiment copepod samples were preserved immediately with formalin buffered by sodium borate (5% final concentration) for zooplankton composition analysis and biomass measurements.

2.4. Dissolved inorganic and organic nutrients measurements

Nutrient samples (NO_3^-, NO_2^- and PO_4^{3-}) were taken in duplicate in 20 mL HDPE bottles, filtered through 0.7 μm filters (GF/F; Whatman) and poisoned with $HgCl_2$ to a final concentration of 20 μg L^{-1}. Samples were stored at -20 °C and analyzed on board determined by standard colorimetric techniques (Aminot and Kérouel 2007), using a SEAL Analytical AA3 HR system (SEAL Analytica, Serblabo Technologies, Entraugues Sur La Sorgue, France). Samples for NH_4^+ determination (40 ml) were taken in duplicate and analyzed on board

according to Holmes et al., (1999) using a Turner Designs fluorometer. Samples for DON and DOP (30 mL) were filtered through pre-combusted (450º C, 6h) GF/F filters (Whatman). Samples were collected in Teflon bottles and analyzed immediately on board by the wet oxidation method(Pujo-Pay and Raimbault, 1994). DON and DOP concentrations were determined by sample oxidation (30 min, 120º C) and corrected for NO_3^-, NO_2^- and PO_4^3 concentrations, respectively.

2.5. Bacterioplankton abundance, DNA and RNA extraction.

Bacterioplankton abundance was determined by flow cytometry. From each bottle 1350 µL samples were fixed in sterile cryovials with glutaraldehyde (at 0.1% final concentration). The samples were stored at -80º C until laboratory analysis. Samples were analyzed by flow cytometry (FACScan, Becton Dickinson). The abundance of non-fluorescent picoplankton was estimated from samples previously stained with SYBR green I (Molecular probes) according Marie et al., (1997). DNA samples for the initial characterization of bacterial communities were collected at DCM depth of each sampling site. Samples (9-10 L) were filtered through cellulose ester filters (0.22 µm; Millipore) using a peristaltic pump and stored with RNAlater reagent (Ambion) at -20º C until extraction procedures. DNA extraction was carried out as described in Levipan et al., (2014), quantified by spectrophotometry (NanoDrop ND-1000 Spectrophotometer) and isolated using the PowerSoil DNA Isolation Kit (MoBio Laboratories) in accordance with the manufacturer´s specifications. RNA samples were collected for initial characterization of active bacterial community at DCM at each LD station. Seawater (100 mL) was filtered using a sterilized syringe and 25 mm swinnex through 0.22 µm hydrophilic PVDF filters (Millipore) and the filters were preserved with RNAlater solution (Ambion) and stored at -80º C until RNA extraction procedures. RNA samples were extracted using Mirvana kit (AM1560; Ambion) in accordance with manufacturer´s instructions including a mechanical disruption step and homogenization using 200 µm diameter zirconium beads (Low Binding Zirconium Beads, OPS Diagnostic) and homogenized twice at ~3,000 rpm for 30 s by using Mini-Beadbeater-8™ (Biospec Products). In order to remove DNA traces, RNA was treated with TURBO DNA-free kit (Ambion). Finally, concentration and quality (A_{260}/A_{280} ratio) of RNA extracts as well with DNA extracts were determined by spectrophotometry (NanoDrop ND-1000 Spectrophotometer)

2.6. Bacterial community structure analysis

Bacterial community structure was analyzed by Illumina MySeq sequencing method from 16S rRNA (samples from incubation), using cDNA as template, and 16S rDNA (*in situ*; Campbell and Kirchman, 2013). cDNA was generated using random primers provided by the ImProm-II[TM] Reverse Transcription System (Promega). Bacterial 16S rRNA gene libraries from V1-V3 region (27F-519R) were generated at the Molecular Research (www.mrdnalab.com, Shallowater, TX, USA).

The 16S rRNA and rDNA gene sequences were processed using Mothur software v1.35.1 (Schloss et al., 2009). Sequencing data sets were curated by quality filtration to minimize the effects of random sequencing errors, by eliminating sequence reads <200 bp and trimming of sequences that contained more than one undetermined nucleotide (N) and sequences with a maximum homopolymer length of 8 nucleotides. After chimeric sequences were identified using the Chimera UCHIME algorithm (Edgar et al., 2011) and removed to retain high quality reads.

The 16S rRNA and rDNA gene sequences retrieved were taxonomically classified using the automatic software pipeline SILVAngs available from https://www.arb-silva.de/ (Quast et al. 2013), and the libraries were deposited in the European Nucleotide Archive (ENA) under study accession PRJEB21648 with the following run access numbers: ERS1810581-ERS1810616.

The composition was analyzed at Phyla, Class and Order taxonomic level as abundant (>0.5%), semirare (0.1-0.5%) and rare (<0.1%) in total sequences retrieved from each library (Pedrós-Alió, 2012). The Chao, Shannon and Evenness indices were calculated using Past3 software (Hammer et al., 2001). Previously, since the number of sequences per sample was variable, we normalized the different libraries sizes by subsampling routine in Mothur software version 1.36 (Schloss et al., 2009), to reflect the lowest number of sequences encountered (LD A:10,831; LD B: 15,260 and LD C: 13,699).

2.7. Statistical analysis

Statistical analysis of treatments effects on chemical and biological parameters were performed using a two-way analysis of variance (ANOVA) after checking normality

assumptions (Kolmogorov-Smirnov test) and homoscedasticity (Levene´s test). Pairwise multiple comparisons were performed using a Tukey test as a posteriori analysis. Statistical significance was set a p=0.05 and analysis was computed using R software.

Bacterial community structure were compared using ordination Bray-Curtis similarities and used to build dendrograms by the unweighted pair group method with arithmetic averages (UPMG). For multivariate statistical analysis, the software package PRIMER v.6 and the add-on PERMANOVA+ was used. The permutational multivariate analysis of variance (PERMANOVA) with fixed factor was applied to investigate the differences of bacterial community composition for treatments and control in both experiments. Non-metric multidimensional scaling and principal co-ordinate analysis (PCO) was performed to visualize patterns of the bacterial community in response to different treatments.

3. Results

3.1. Biogeochemical *in situ* conditions

Inorganic nutrients concentrations, obtained at the DCM depth, varied substantially between the sampling sites (Table 1). Lower concentrations of NH_4^+, NO_3^-, NO_2^- and PO_4^{3-} were observed in LD B (0.01, 0.05, 0.02 and 0.03 μmol L^{-1}, respectively), compared to LD A and C. The highest concentrations were observed in LD A, with particularly high NO_3^- (1.53 μmol L^{-1}). The resulting N: P ratio showed greater values in LD A (9.97 ± 0.70) than LD B and C, and the lowest N: P ratio (1.21 ± 0.11) was found in LD C.

Table 1: Ammonium, nitrate, nitrite, phosphate *in situ* concentration and N: P ratio at each sampling site.

	LD A	LD B	LD C
NH_4^+ [μmol L^{-1}]	0.025 ± 0.001	0.011 ± 0.00	0.017 ± 0.000
NO_2^- [μmol L^{-1}]	0.064 ± 0.002	0.023 ± 0.02	0.048 ± 0.004
NO_3^- [μmol L^{-1}]	1.53 ± 0.008	0.051 ± 0.021	0.247 ± 0.012
PO_4^{3-} [μmol L^{-1}]	0.16 ± 0.012	0.03 ± 0.025	0.243 ± 0.008
N: P	9.97 ± 0.70	4.52 ± 4.07	1.21 ± 0.11

3.2. Changes in inorganic nutrients, DON and DOP during the incubations

Inorganic nitrogen and phosphorus concentrations changed substantially over the course of the experiment at the three LD stations (Fig. 2 and 3). In particular, ammonium concentrations changed extensively, which increasing significantly (four- and seven-fold) in the presence of copepods in all experiments (LD A: $F_{1,20}$: 93.2, p<0.001; LD B: $F_{1,18}$: 61.7, p<0.001; LD C: $F_{1,20}$: 108.9, p<0.001). The largest increase in ammonium concentration with respect to the controls was associated with T0 in LD A, T4 in LD B and T4 in LD C. These increases were significantly higher than controls (ANOVA and Tukey`s test p<0.001; Suppl. Table 1). Nitrite did not vary significantly through time in the experiments compared to controls (LD St. A: $F_{1,16}$: 0.50, p=0.49; LD St. B: $F_{1,18}$: 0.13, p=0.7; LD St. C: $F_{1,20}$: 0.44, p=0.51). In contrast, nitrate showed significant differences between the treatment with copepods and control in LD B and C. The highest differences were at T2 in LD B (ANOVA and Tukey`s test p=0.02; Suppl. Table 1), and at T4 in LD C experiments (ANOVA and Tukey`s test p=0.009; Suppl. Table 1), observed a significant increment in the treatment with copepods than controls (Fig. 2b and 2c) in nitrate concentrations. DON (Fig. 2) showed significant differences between the treatment and control in LD A and C (LD A: $F_{1,20}$: 8.99, p=0.007; LD C: $F_{1,20}$: 29.0, p<0.0001), and this difference was associated with the increment at T1 in LD A and T0 in LD C in the treatment with copepods. At LD B we did not find significant differences between treatments and controls trough the time (ANOVA and Tukey`s test p<0.01; Suppl. Table 1).

Phosphate in LD A showed a higher concentration during the first hour of incubation, followed by a sharp decrease (from 0.17 to 0.05, approximately; Fig. 3) in the treatment with copepods as well as control ($F_{1,20}$: 17.7, p<0.0001). However, the difference between treatment and control was not significant ($F_{1,20}$: 4.5, p=0.06). The same result was found in LD B and C (LD B: $F_{1,18}$: 0.13, p=0.7; LD C: $F_{1,20}$: 0.44, p=0.51). DOP concentrations (Fig. 3) did not vary significantly trough time in the three experiments (LD A: $F_{1,20}$: 0.3, p=0.87; LD B: $F_{1,20}$: 0.5, p=0.43; LD C: $F_{1,20}$: 0.70, p=0.60). Moreover, in the treatment with copepods DOP showed significant differences between the treatment with copepods and control in LD B and C (LD A: $F_{1,20}$: 0.43, p=0.52; LD St. B: $F_{1,20}$: 8.92, p=0.008; LD St. C: $F_{1,20}$: 7.94, p=0.01).
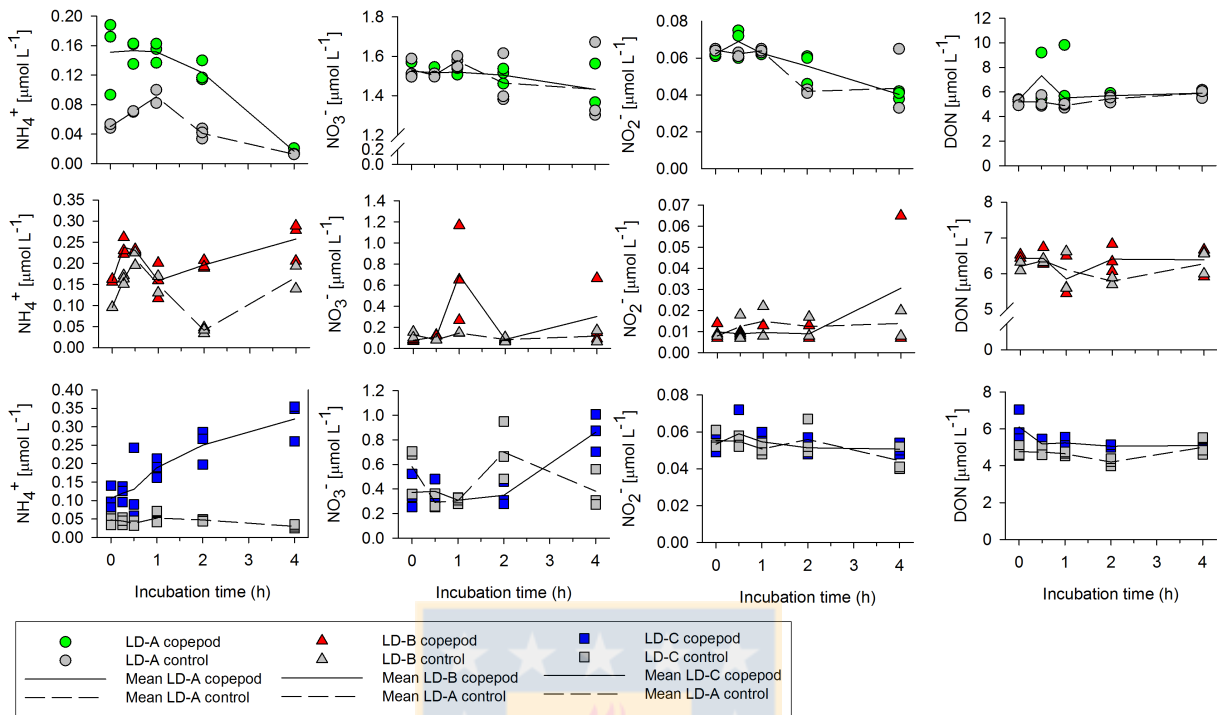
Figure 2: Ammonium, nitrate, nitrite and dissolved organic nitrogen variability through the incubation for treatments with copepods and control in the three LD station experiments.
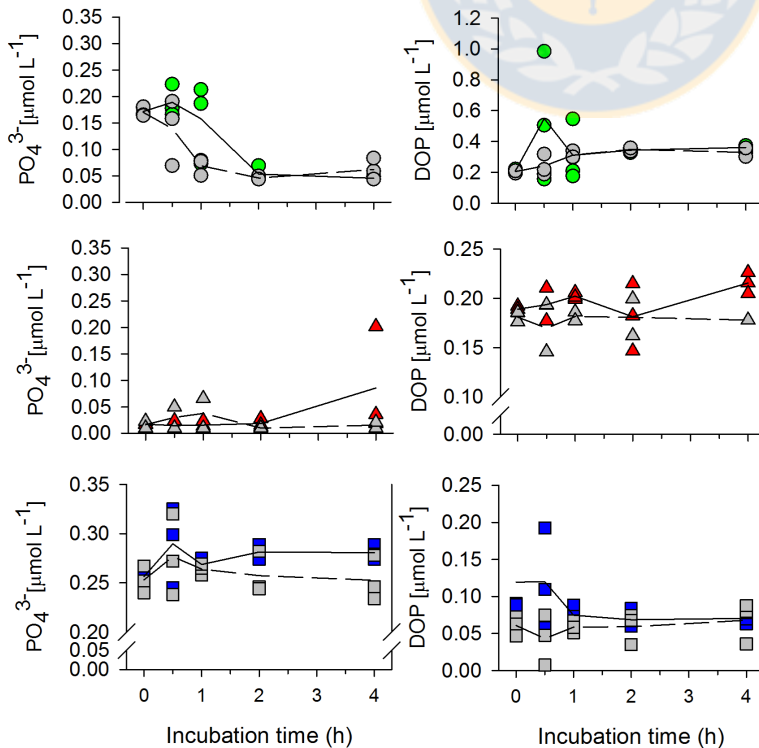
Figure 3: Phosphate and dissolved organic phosphorus variability through the incubation for treatments with copepods and control in the three LD experiments. See legends in Figure 2.

During the experiments, the resulting N: P ratios (NO: PO_4^{3-}) of the dissolved inorganic nutrients generally increased through the incubation associated with a decrease in dissolved organic ratios (DON: DOP) and vice versa (Fig. 4). In LD A experiment the inorganic N: P ratios (Fig. 4A and 4C) were lower than Redfield (N: P= 16) during the first hour of incubation followed by an increased from 12.5 to 32.4 at the end of incubation in the treatment with copepods. Meanwhile, in the control we observed the same pattern but at the end of incubation a decrease in N: P ratios was observed. At the same time DON: DOP ratio (Fig. 4B and 4D) decreased from 25 to 16.4. In LD B experiment, lower N: P than Redfield ratio was found through the experiment, suggesting a N deficiency relative to P, except at 1 h of incubation when a higher N: P ratio was observed (2.6 times higher than Redfield) in treatment with copepods. The DON: DOP ratios was close to the Redfield ratio in both treatment and control in both cases and then to decreased from 1 h of incubation to the end. Meanwhile, in LD C the organic dissolved nutrients ratio showed a substantial accumulation of N with respect to P from the first hour in incubation at the end of incubation in the treatment with copepods, showed a substantial deviation from the Redfield ration (> 16:1).
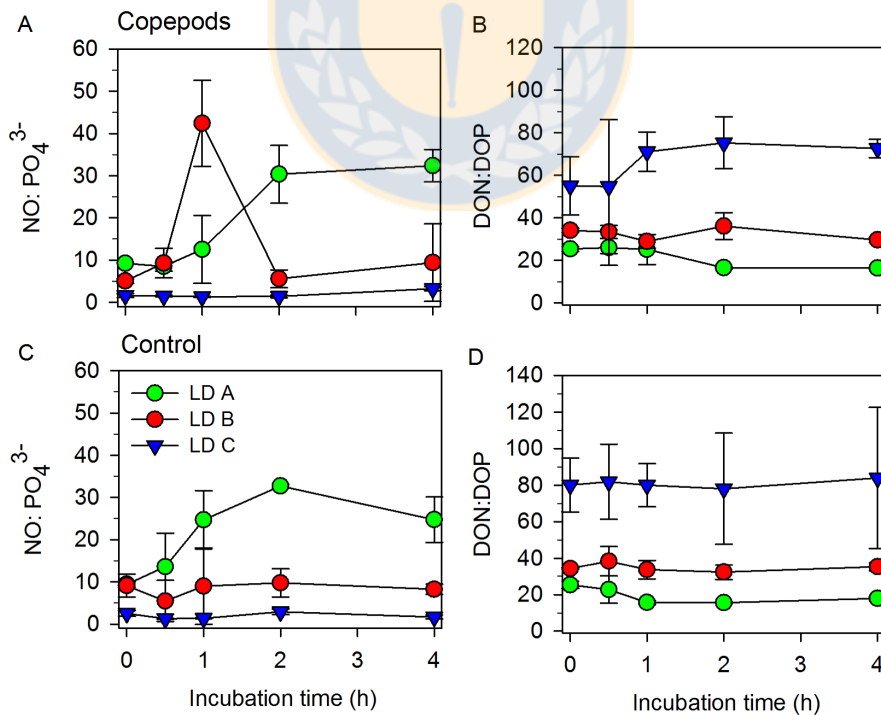
Figure 4: N: P ratios of the organic and inorganic nutrients through the incubation for the three experiments.

3.3. Changes in bacterioplankton abundance during the experiment evolution

Changes in bacterioplankton abundance are showed in the Fig. 5. Smallest abundance was found in LD A compared to the B and C experiments. In LD A (Fig. 5A) the abundance showed statistical differences between the time ($F_{1,20}$: 13.5, p<0.001), which increase through time after 1 hour of incubation, from $100x10^3$ to $200x10^3$ cell mL^{-1}. However, did not observed significant differences between the treatment with copepods and control ($F_{1,20}$: 0.004, p=0.95). In LD B (Fig. 5B), significant differences were found between treatments with copepods and control ($F_{1,16}$: 7.69, p=0.014) and between time ($F_{1,16}$: 33.8, p<0.000). A decrease in bacterioplankton abundance were observed in the second period of incubation (0.5 to 1 h) in the treatment with copepods as well as control but between 1 h and 2 h of incubation. In LD C (Fig. 5C) a strong difference was observed between times ($F_{1,20}$: 9.93, p<0.000), and between treatments and control ($F_{1,20}$: 6.22, p=0.02). A higher increase around $100x10^3$ cell mL^{-1} between 1 and 4 hours of incubation was observed in both treatment with copepods and control.
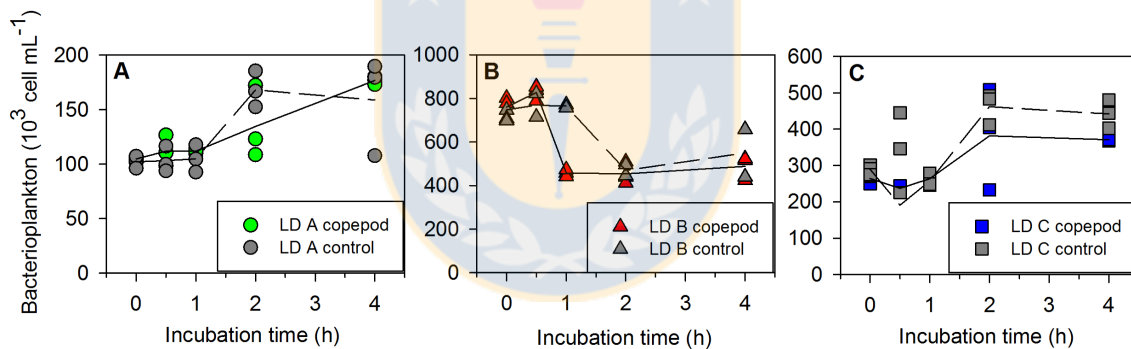
Figure 5: Bacterioplankton abundance through the incubation for the three LD station experiments: (A) LD A, (B) LD B and (C) LD C.

3.4. Active bacterial community composition and their response during the incubation

In LD A station, a total of 178,097 sequences were analyzed, ranging between 10,831 and 19,703 sequences for each library (Suppl. Fig. 1). In LD B station a total of 219,019 sequences were analyzed from 15,260 to 20,966, and in LD C a total of 230,805 sequences were analyzed, ranging between 13,699 and 22,104 sequences for each library. In the three LD station the higher number of sequences correspond to DNA *in situ*.

The alpha diversity derived from normalized 16S rDNA and rRNA libraries is shown in the Fig. 6. The taxonomic richness determined (OTU) and expected on the Chao1 index was higher in DNA *in situ* compared to the cDNA *in situ* and the samples related to the experiment. In accordance, Shannon index diversity showed that the bacterial community obtained *in situ* from 16S rDNA presented higher values than the observed through the incubation in the three LD station. Through the incubation the alpha diversity showed slight differences between treatments with copepods in the three experiments. However, the Shannon index showed higher values at the beginning the incubation, followed by a decrease between T2-T3, while at T4 an increase was observed in the treatment with copepods (Fig. 6).
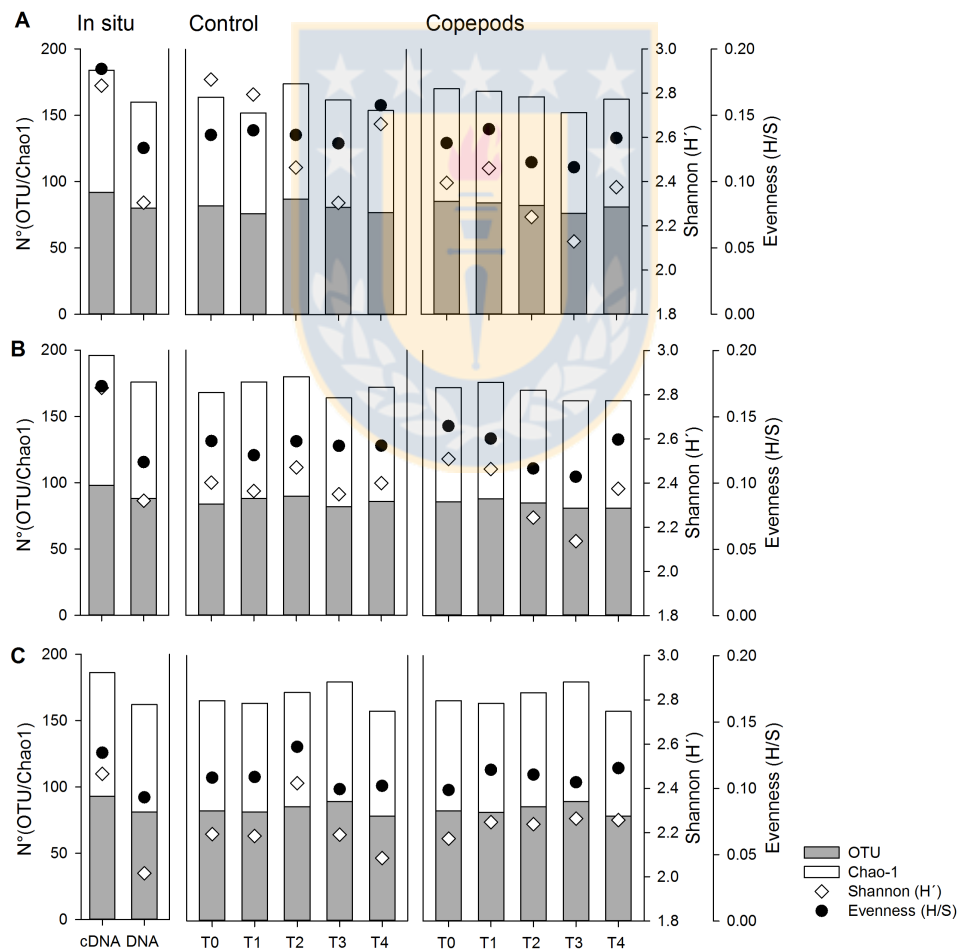
Figure 6: Richness, Chao1, Diversity (Shannon H´) and Evenness from the total and active *in situ* bacterial community, and through the incubation for the active bacterial community.

The total *in situ* bacterial community composition (Fig. 7) at each LD station was represented by a higher contribution of Alphaproteobacteria, mainly by SAR 11, dominating in LD C (49.8%) than in LD A (31.1%) and B (41.1%; Figure 7B). Cyanobacteria were also present in our libraries with a higher contribution in LD C (13.5%) than in A and B. In addition, a high number of abundant phyla were observed in LD A compared to the other stations. On the other hand, the active *in situ* bacterial composition (Fig. 7C) was characterized by a higher contribution of Alphaproteobacteria in the three LD stations (Fig. 7B and 7C), as total bacterial composition this class were mainly represented by SAR 11, with higher contribution in LD C (35.5%) than LD A (21.7%) and B (29.1%).
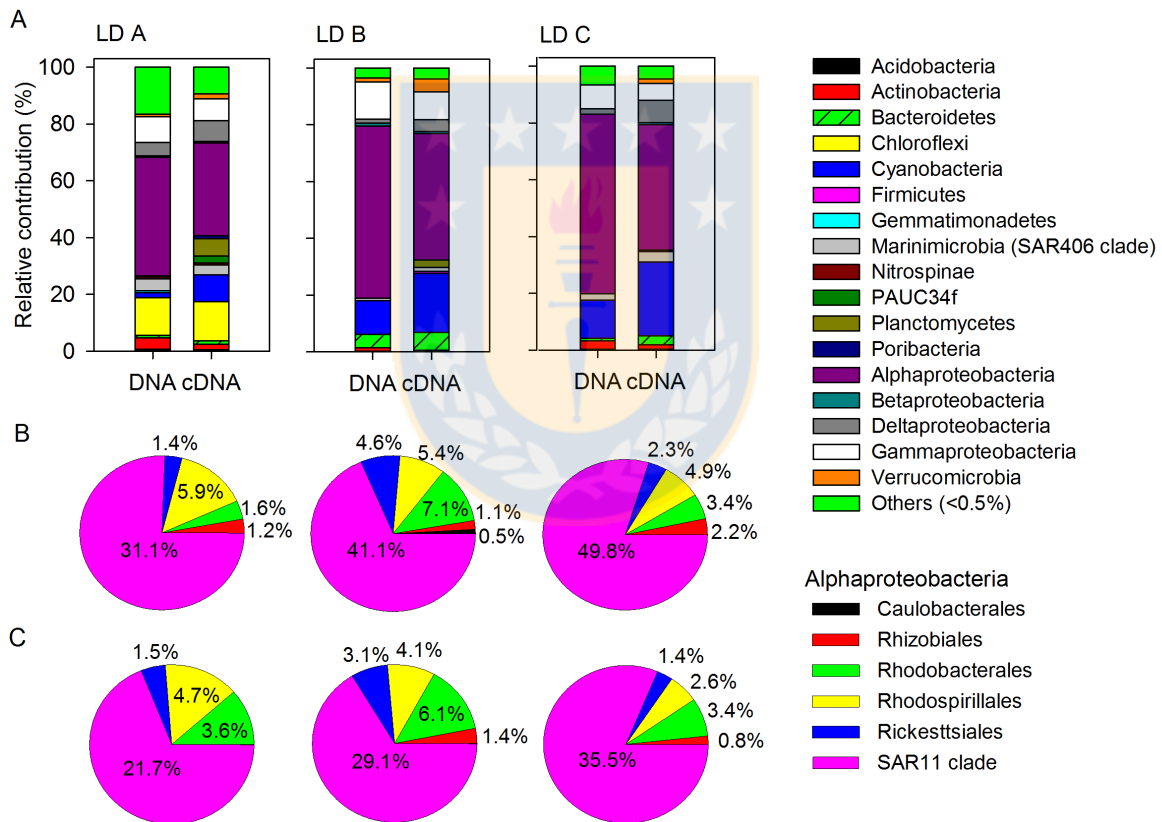
Figure 7: Relative abundance of major bacteria phyla and Proteobacteria class with a zoom in Alphaproteobacteria order at initial *in situ* conditions for the three-sampling site.

In general, the active bacterial community composition varied significantly during the incubation compared to the *in-situ* community in the three experiments carried out as indicated by the cluster (Fig. 8). During LD A (Fig. 8A) incubation, the bacteria community composition were characterized by an increase of Gammaproteobacteria between 39% to 68% of contribution between T0 and T4 in the treatments with copepods. Also, a decrease in Cyanobacteria, Chloroflexi and other taxa (semirare and rare) were found. In LD B (Fig. 8B) smaller differences in taxa contribution through the incubation was observed. Gammaproteobacteria, increased their contribution through the incubation from 28% at T0 to 32% at T4. Unlike the LD A and B, LD C experiment (Fig. 8C) showed a decrease in Gammaproteobacteria contribution and an increase in Alphaproteobacteria. The cluster analysis (Fig. 8) associated with the Orders contribution in the different libraries indicates that the bacterial community from the experiments was mainly associated according to the incubation time, T0, T1 and T2 versus T3 and T4, in LD A and LD B experiments. However, in LD C experiment was mainly grouped by treatments (with and without copepods; Fig. 8C).
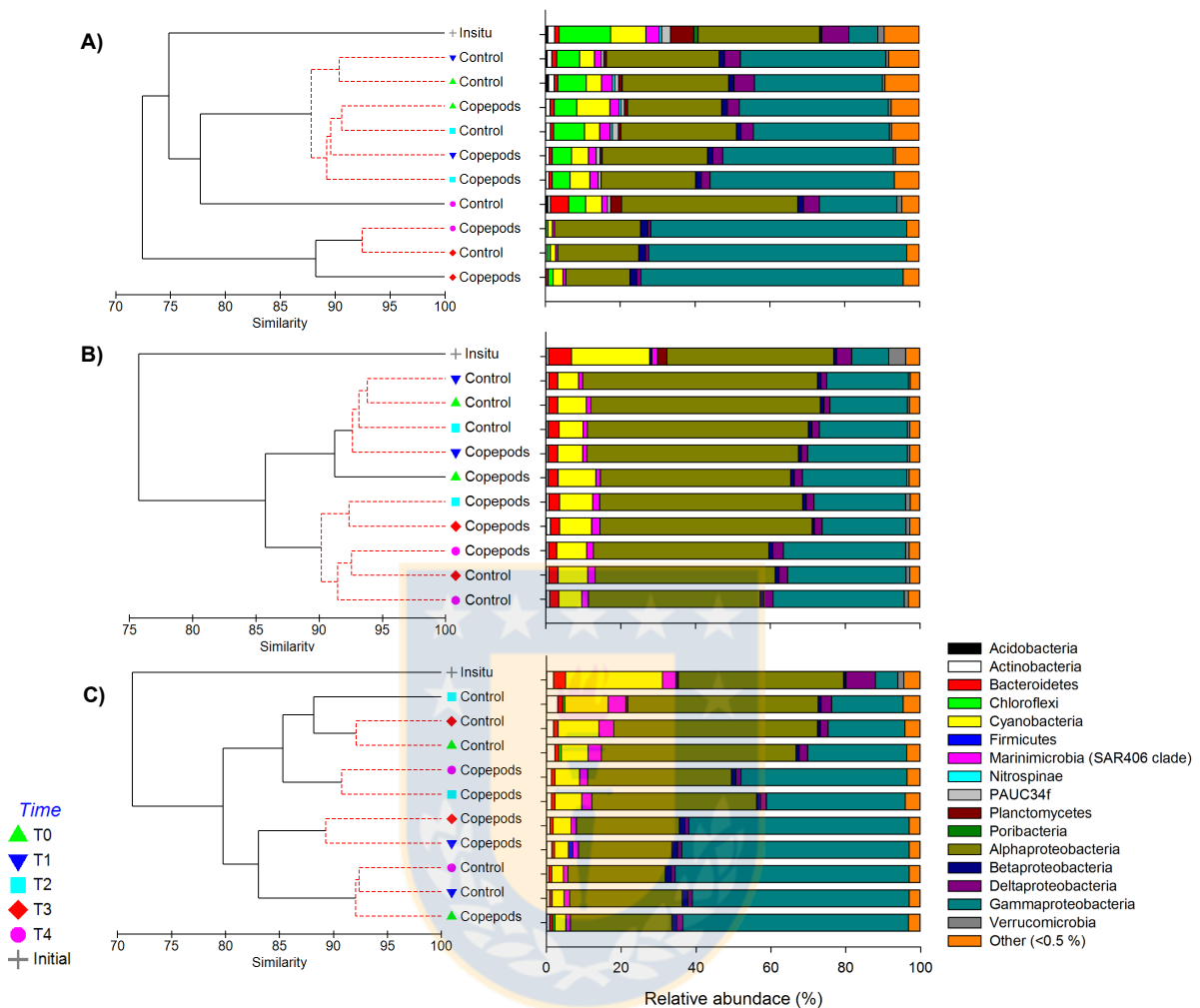
Figure 8: Similarity dendrograms based on the 16S rRNA and relative contribution of the abundant bacteria phyla and Proteobacteria subclasses for the initial *in situ* community, treatments with copepods and controls (left panel) through the incubation for the three experiments. Clustering in one the basis of a distance matrix computed using the Bray-Curtis index of similarity. The dendrogram was inferred with the unweighted pair-group average algorithm (UPGMA). Bacterial communities in the samples connected with red branch lines are not significantly different (SIMPROF test, p<0.05).

In addition, the greatest dissimilarities, based on SIMPER analysis (Fig. 9; Suppl. Table 2), between copepod treatments and control in LD A (Fig. 9A), was mainly due to an increase in Alteromonadales and Oceanospirillales in the treatment with copepods, whereas SAR11 and

Rhodobacterales increase their relative abundance in controls. At LD B experiment (Fig. 9B) smaller dissimilarities between treatment and control (9.5%) were observed and mainly due to a decrease of Alteromonadales, while the contribution of SAR11 increased in the treatment with copepods. In the LD C station (Fig. 9C), the dissimilarities were mainly due to a higher increase in Alteromonadales, however at the end of incubation decrease their contribution in the treatment with copepods and increase in the control, meanwhile the contribution of SAR11 increased in the treatment with copepods at the end of incubation. The specific order contribution for the three experiments are shown in Figures S2, S3 and S4.
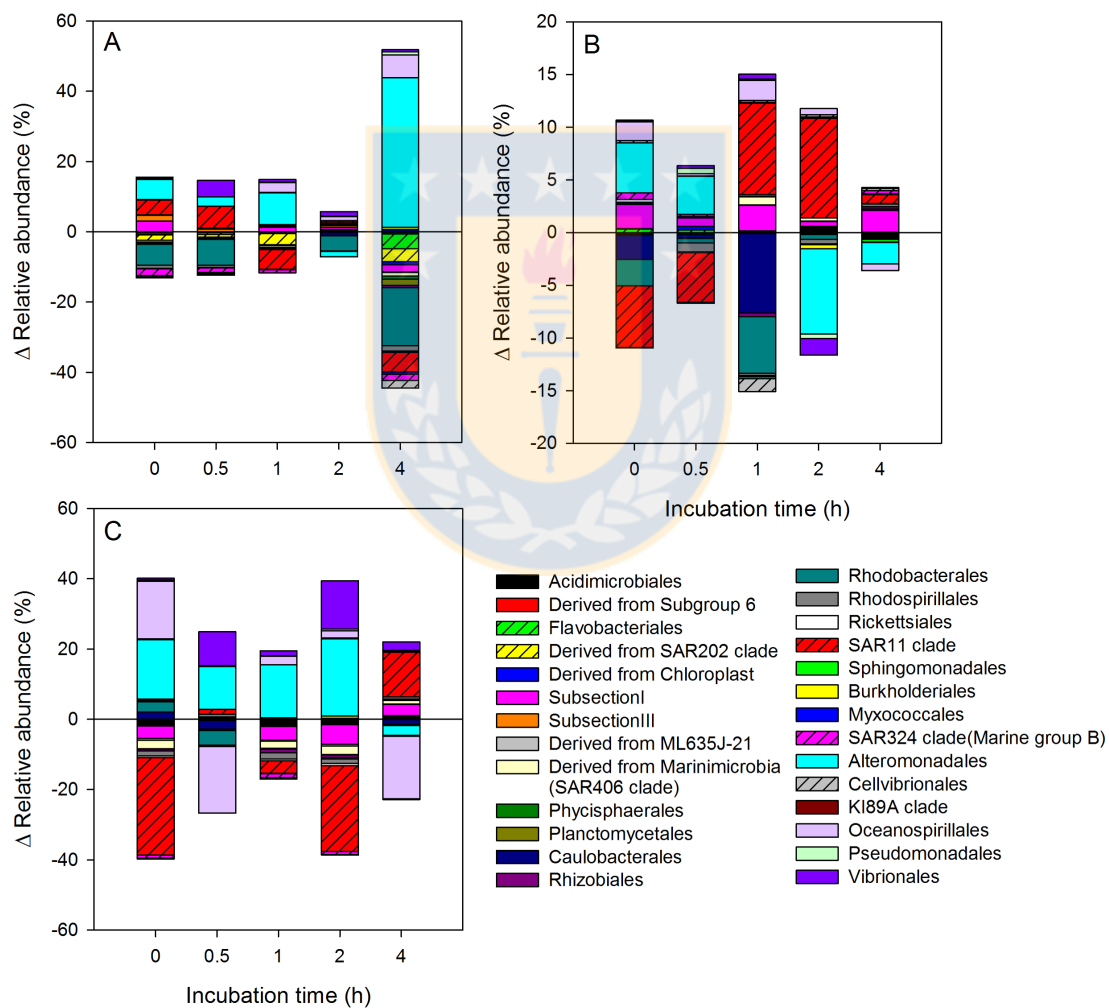
Figure 9: Contribution of active Orders through the incubation based on the SIMPER results. List of the OTUs explaining 50% of the dissimilarity observed through the experiment between treatment with copepods and control for each LD experiment.

PCO analysis with Orders contribution and environmental parameters indicates that in LD A experiment that the initial sampling points (T0, T1 and T2) were associated with inorganic compounds (NH_4^+, NO_3^-, NO_2^- and PO_4^{3-}; Fig. 10A) for both treatment with copepods and control. Meanwhile, nitrite concentrations were associated with the latter time-points in LD B (Fig. 10B). In LD C experiment (Fig. 11), no visual association was observed between environmental parameters and Order contribution. Non-metric multidimensional scaling analysis of 16S rRNA gene sequences (Fig. 12) from the experiments and *in situ* revealed consistency between experiments and *in situ* condition at each sampling site and between times in LD A and LD B experiments. In addition, the PERMANOVA main test revealed significant differences among all samples regarding the different experiments (pseudo-F=12.7, p=0.001). However, differences between treatment with copepods and control, and between sampling points did not showed significant differences (PERMANOVA, pseudo-F=2.1-1.3, p=0.12-0.25).
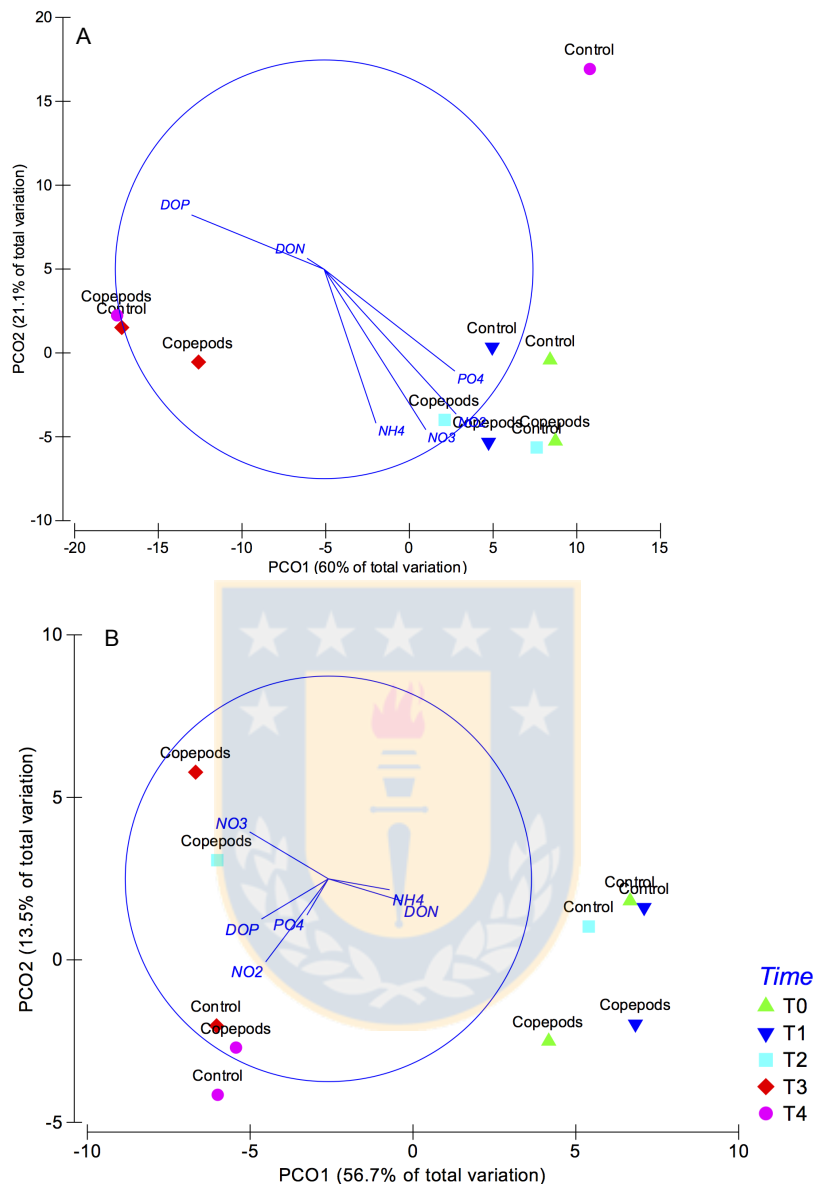
Figure 10: Principal component ordination (PCO) based on Bray-Curtis similarity at order taxonomic level, of treatment with copepods and control (A) LD A and (B) LD B experiments. Vectors indicate the best environmental variables (normalized transformed) correlated with ordinations and vector lengths correspond with the correlation values.
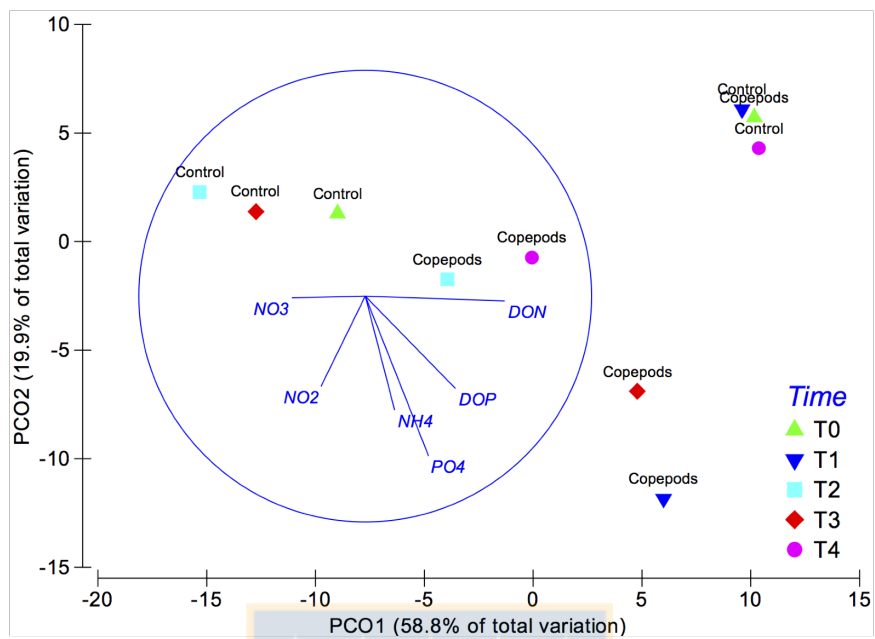
Figure 11: Principal component ordination (PCO) based on Bray-Curtis similarity at order taxonomic level, of treatment with copepods and control in LD C experiment. Vectors indicate the best environmental variables (normalized transformed) correlated with ordinations and vector lengths correspond with the correlation values.
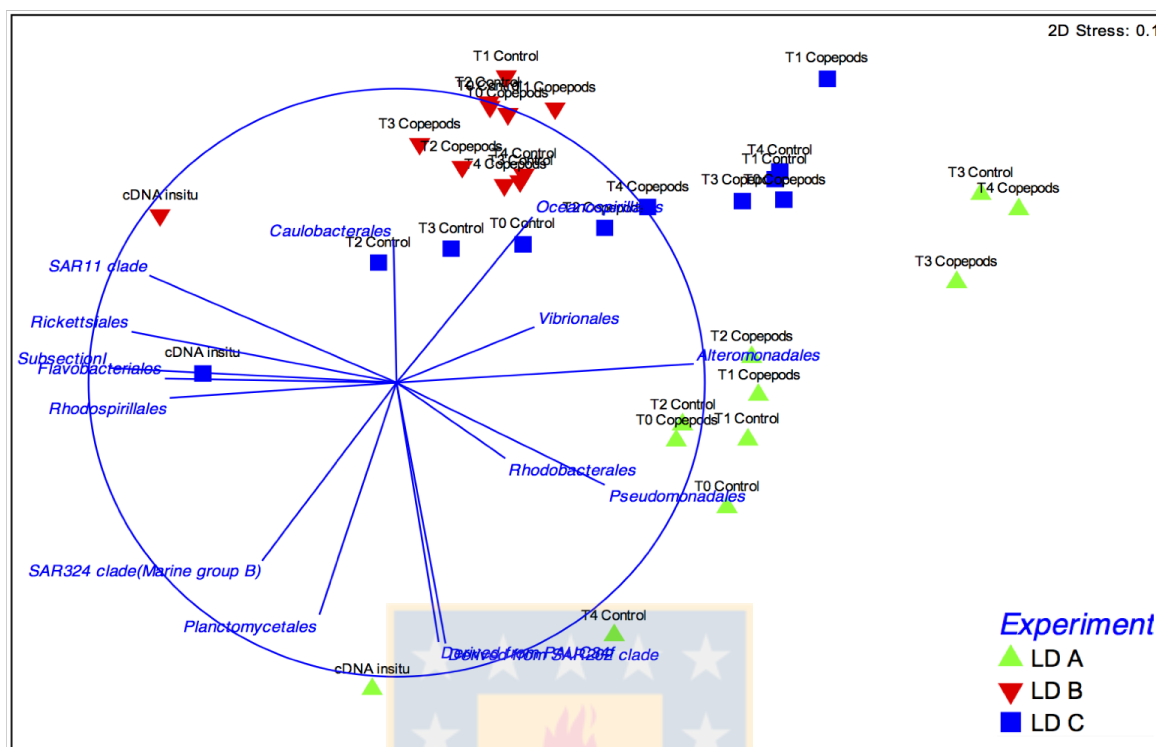
Figure 12: Non-metric multidimensional scaling analysis of 16S rRNA libraries based on Bray Curtis similarity for the three experiments carried out in the long duration station (LD).

4. Discussion

4.1. Methodological considerations

In this study, we did not monitor possible leaching from fecal pellets defecated during the experiment. The leaching is also one of the possible pathways that zooplankton can release dissolved compounds, along with the sloppy feeding and excretion. However, it has been demonstrated that the concentration provided by leaching is insignificant compared to excretion, less than 6% in the case of the release of DON, and for ammonium was not detected (Saba et al., 2011; Steinberg et al., 2000). Other studies suggest that the fecal pellet must be broken up mechanically to release substantial amount of DOM (Lampitt et al., 1990; Strom et al., 1997). Thus, we believe that our results can be mostly attributed to the excretion process than the leaching from the fecal pellets.

Zooplankton abundance was lower at LD C station compared to LD A and B, which was ~1,400 ind. m^{-3}, ~1,600ind. m^{-3} and ~ 500 ind. m^{-3}, for LD A, B and C, respectively. However, during the three experiments we used the same number of copepods for each incubation, since the lower ammonium concentration found at *in situ* conditions (Table 1). Our results, in special for the experiments carried out in LD C station, can be overestimated because it did not reflect the real abundance found in the environment. Copepods in the three LD station were mainly represented by *Clausocalanus sp*, *Oncaea sp* and *Oithona sp*. In addition, the higher abundance of *Microsetella sp* was associated to the MA region, possibly associated whit the *Trichodesmium* bloom (Carlotti et al., this issue). Copepod incubated in the three experiments consist in a mix of epipelagic copepods (Suppl. Table 5), in which *Clausocalanus* sp was the genera most represented in our incubation, in contrast to *Oncaeasp* and *Oithona sp* which was not used.

4.2. Biogeochemical changes in response to the dissolved compounds release by copepods

During our study, the biogeochemical changes observed at each experiment to the addition of compounds release by copepods showed significantly higher ammonium concentration in the treatment incubated with copepods compared to the controls (without copepods). However, these concentrations not always were associated with DON concentration as we expected. In the experiment carried out in the LD A station, higher DON concentrations were observed at initial times (between 0 and 1 h) in the treatment with copepods, and was actively consumed between 1 and 2 hours of incubation. This higher DON consumption (3.62 μmol L^{-1} h^{-1}; Supplementary Table 3) was not reflected in ammonium concentrations. In addition, at the same time the bacterioplankton abundance increase in 22,8x10^{3} cell mL^{1}, these results suggest that a higher demand of DON, and could be attributed to heterotrophic bacteria. The second experiment, was carried out in the LD B station, in the same region than LD A station (MA). At initial time points a decrease in ammonium concentration through the second period of incubation (0.5 and 1 h) could be related to the increase in nitrate concentration (highly variable between the triplicates) in the treatment with copepods, but this was not observed in the control, suggesting that in the treatment with copepods nitrifying communities could be actively used the ammonium. Besides, the increase in ammonium concentration related to the end points of incubations is followed by a decrease in DON concentration in the last period of incubation (1 and 2 h) at a rate of 0.12 μmol L^{-1} h^{-1} (Supplementary Table 3), as LD A this demand could be probably associated to

heterotrophic bacterioplankton, however, the demand in considerably lower in this experiment than LD A.

The highest differences in ammonium concentrations between copepods treatments and control was observed at the LD C experiment, up to 0.3 µmol L^{-1}, several times higher than *in situ* concentration. This increase through the incubation could be associated the consumption of DON observed through the first 2 h of incubation in the treatment with copepods, suggest a higher remineralization/ammonification of DON. In addition, at the end of incubation nitrate concentration increase in the treatment with copepods to the control. Our results, suggest that the release of DON from copepods can stimulate ammonium regeneration by bacteria and also the increment in nitrate concentration suggest strong nitrification occurring, providing an additional substrate for microbial and phytoplankton growth in LD C station, which correspond to the ultra-oligotrophic station in the SG.

The highest differences in DON concentration between the treatment with copepods and the control were found at initial time points, whit a higher DON concentration in the treatment with copepods. Zooplankton can excreted DON in highly concentrated spurts lasting up to an hour (Gardner and Paffenhöfer, 1982; Steinberg et al., 2002).

On the other hand, in the treatment with copepods DOP showed significantly higher concentrations respect to the control in LD B and C experiment at the end and at the initial point of incubation, respectively. In addition, in LD A experiment phosphate was found in higher concentration compared to the control among 0 and 1 h of incubation. Our results suggest that copepods could potentially contribute with substantial amount of DOP and provide an alternative source of phosphorus by phytoplankton and microbial loop in this study area. During LD B station, a bloom of *Trichodesmium* was observed, and the satellite data suggest the bloom was already two months old by the time of sampling at station LD B (de Verneil et al., this issue), which are in according with our lower nutrients concentration obtained *in situ* and trough our incubation. DOP concentration was significantly higher in the treatment with copepods than the control in this station. The contribution of DOP by copepods could contribute to maintain the duration of the bloom in this region (MA), provided an additional source of phosphorus to growth. It has been reported that *Trichodesmium* is capable to hydrolyzing DOP compounds (Mulholland et al., 2002), so zooplankton metabolism could potentially supply a significant

portion of the cellular phosphorus necessary for growth. Furthermore, during outpace transect we fed copepods with water from the DCM and the depth was different in the three stations, the deepest was LD C at 140 m, followed by LD A (80 m) and LD C (34 m). In the station in MA region, *Trichodesmium* spp and UCYN-B dominated in the upper photic zone (0-50; Stenegren et al., this issue) and it has been reported that copepods as *Macrosetella* sp can feeding on both diazotrophs (O'Neil et al., 1996; Hunt et al., 2016).

The food source can affect the relative organic and inorganic nutrient release and the stoichiometry of the resulting DOM. Stoichiometry theory indicated that the grazer and prey elemental composition are crucial parameters influencing ratios of nutrient release (Elser and Urabe, 1999). In the three LD station the inorganic N: P ratios *in situ* revealed a substantial decrease from Redfield ratio, suggesting a P-limitation relative to N under *in situ* conditions. This ratio was very lower in LD C station which coincide with the most oligotrophic station. In LD A station, an accumulation of inorganic nitrogen relative to phosphorus was observed. In contrast, with LD B and C the inorganic form showed a strong limitation of P relative to N through the incubation, except in the organic form suggest a higher accumulation of N relative to P, this is especially high in the experiment carried out in most oligotrophic station (LD C).

4.3. Changes in bacterial community composition induced by dissolved compounds release by copepods

In general, direct dissolved compounds release by copepods produce a moderate increase in bacterial abundance, which was significantly different from control in the LD B and C. However, the addition of compounds derived from copepod metabolism can greatly stimulated some bacterioplankton taxa. Through the incubations, the bacterioplankton community were characterized by changes mainly in Gammaproteobacteria and Alphaproteobacteria in all LD stations. A dominance of Gammaproteobacteria is rapidly observed in LD A experiment, mainly by Alteromonadales and Oceanospirillales, increased their contribution in the treatment with copepods compared to the control in 42 % and 7%, respectively (Fig. 9). The faster response in Alteromonadales (Gammaproteobacteria) has been documented and are frequently observed in microcosm studies, due to their high growth rate and their ability to exploit DOM rapidly when is available (Alonso-Saez and Gasol, 2007; Fuchs et al., 2000; Landa et al., 2013). The high

demand for DON found in this incubation (LD A) could be related to the increase in Alteromonadales. It has been reported that zooplankton metabolism can release highly labile compound and can be rapidly assimilated by microbial communities (Arístegui et al., 2014; Vargas et al., 2007).

In contrast, in the experiment carried out in LD B station, a substantial decrease in Alteromonadales and Oceanospirillales was observed in the treatment with copepods compared to the control. The higher contribution through the time was due Alphaproteobacteria, in which SAR11 increase their relative abundance (~8%) at 1 hour of incubation and Caulobacterales (<0.1%) at the end of incubation. SAR 11 was one of the major constituent of rRNA (*in situ* and microcosm incubation) and rDNA *in situ.* During our incubations SAR11 increased their contribution only in LD B experiment which coincided with the station with lowest nutrients concentrations. Also in this experiment, the bacterioplankton abundance showed a strong decrease in both treatment and control at 1 hour of incubation. SAR11 is the most abundant plankton in the ocean, is well adapted to lower concentration of nutrients, and are specialized to oxidize many labile and low-molecular-weight compounds produced by other plankton (Giovannoni, 2017).

By other hand, in the LD C experiment, an increment in Alteromonadales and Vibrionales were observed. Alteromonadales decrease their contribution in the treatment with copepods at the end of incubation, meanwhile increase their contribution in control. The reverse pattern was observed for SAR11 which increase their contribution at the end of incubation. In base with our observations copepods excreted mainly between 0 to 2 h of incubation, time in which Alteromonadales increase rapidly their contribution and when the nutrients were lowest, SAR 11 seem take advantage increase their contribution in the treatment with copepods at the end of incubation. Peduzzi and Herndl (1992) observed high monomeric carbohydrate concentration and bacterial activity in experiments were copepods were included. Furthermore, these authors observed that bacterial communities living in oligotrophic areas can be efficient to utilize the newly available substrate source, in according with our results.

Our results suggest that copepods can provide substantial amounts of nitrogen and phosphorus (as NH_4^+, DON, DOP) which microbial communities can directly use in a short period of time enhanced the bacterioplankton remineralization in the WTSP.

Bacterial remineralization of the dissolved organic matter derived from copepods metabolism could be highly efficient mechanism to maintain the nutrients in the upper layer support the phytoplankton and microbial growth. In this sense, the response associated to the different regions in study suggest that copepods in MA region could provide substantial amounts of DOP as an alternative substrate for growth or extend the life time of the diazotrophs bloom. The enhanced remineralization by copepods could be more important in the SG region, which is the most oligotrophic area.

5. Acknowledgement

6. References

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7. Supplemental information

Figure S1: Rarefaction curves derived from classified total and active bacteria OTUs detected from the 16S rDNA and rRNA libraries generated from samples collected *in situ* and the following experimental incubation treatment and control.

Figure S2: Contribution of total and active Classes classified according to their initial *in situ* contribution as abundant (A) and semi-rare (B) and rare (C-D) in percentages (>0.5%, 0.1–0.5% and <0.1% of libraries, respectively), and their changes through the time of the experiment at the treatment with copepods and control in LD A station experiment.

Figure S3: Contribution of total and active Classes classified according to their initial *in situ* contribution as abundant (A) and semi-rare (B) and rare (C-D) in percentages (>0.5%, 0.1–0.5% and <0.1% of libraries, respectively), and their changes through the time of the experiment at the treatment with copepods and control in LD B station experiment.

Figure S4: Contribution of total and active Classes classified according to their initial *in situ* contribution as abundant (A) and semi-rare (B) and rare (C-D) in percentages (>0.5%, 0.1–0.5% and <0.1% of libraries, respectively), and their changes through the time of the experiment at the treatment with copepods and control in LD C station experiment.

Table S1: ANOVA followed by the Tukey´s Honest Significant Difference (HSD), statistical comparison was performed on nutrients concentration in treatment with copepods and control.

The mean of HSD is recorded at 95% confidence level with its associated P value for this comparison. Significant P values are bolded.

LD A	T0	T1	T2	T3	T4
NH_4^+					
Copepod-Control	**HSD=0.1 p<0.0001**	**HSD=0.08 p=0.001**	**HSD=0.06 p=0.02**	**HSD=0.08 p<0.0001**	HSD=0.003 p=0.9
NO_2^-					
Copepod-Control	HSD=-0.01 p=0.99	HSD=0.006 p=0.98	HSD=-0.001 p=1	HSD=0.013 p=0.4	HSD=-0.008 p=0.93
NO_3^-					
Copepod-Control	NA	NA	NA	NA	NA
PO_4^{3-}					
Copepod-Control	HSD=0.003 p=1	HSD=0.05 p=0.72	HSD=0.09 p=0.1	HSD=0.006 p=0.99	HSD=-0.01 p=0.99
DON					
Copepod-Control	HSD=0.03 p=0.99	**HSD=0.28 p=0.01**	HSD=0.1 p=0.87	HSD=0.04 p=0.99	HSD=0.001 p=1
DOP					
Copepod-Control	NA	NA	NA	NA	NA
Bacterioplankton					
Copepod-Control	HSD=2.64 p=1	HSD=8.63 p=0.99	HSD=6.92 p=0.99	HSD=-33.6 p=0.55	HSD=17.82 p=0.97
LD B					
NH_4^+					
Copepod-Control	HSD=0.07 p=0.1	HSD=0.02 p=0.99	HSD=0.01 p=0.99	**HSD=0.16 P<0.001**	**HSD=0.09 p=0.006**
NO_2^-					
Copepod-Control	NA	NA	NA	NA	NA
NO_3^-					
Copepod-Control	HSD=-0.05 p=0.99	HSD=0.003 p=0.99	**HSD=0.37 p=0.02**	HSD=-0.002 p=1	HSD=0.13 p=0.89
PO_4^{3-}					
Copepod-Control	NA	NA	NA	NA	NA
DON					
Copepod-Control	NA	NA	NA	NA	NA
DOP					
Copepod-Control	HSD=0.008 p=0.99	HSD=0.02 p=0.74	HSD=0.02 p=0.85	HSD=0.008 p=1	HSD=0.04 p=0.35
Bacterioplankton					
Copepod-Control	HSD=42.7 p=0.99	HSD=57.9 p=0.97	**HSD=-307 p=0.001**	HSD=-16.8 p=0.99	HSD=-61.4 p=0.96
LD C					
NH_4^+					
Copepod-Control	HSD=0.06 p=0.76	HSD=0.09 p=0.22	**HSD=0.14 p=0.02**	**HSD=0.20 p<0.0001**	**HSD=0.29 p<0.0001**
NO_2^-					
Copepod-Control	NA	NA	NA	NA	NA
NO_3^-					
Copepod-Control	HSD=0.21 p=0.66	HSD=0.09 p=0.99	HSD=0.004 p=1	HSD=0.35 p=0.10	**HSD=0.48 p=0.009**
PO_4^{3-}					

Copepod-Control	NA	NA	NA	NA	NA
DON					
Copepod-Control	**HSD=0.21 p=0.006**	HSD=0.07 p=0.82	HSD=0.10 p=0.50	HSD=0.15 p=0.07	HSD=0.02 p=0.99
DOP					
Copepod-Control	HSD=0.05 p=0.46	HSD=0.07 p=0.15	HSD=0.01 p=0.99	HSD=0.01 p=0.99	HSD=0.002 p=1
Bacterioplankton					
Copepod-Control	HSD=-25.2 p=0.99	HSD=-101 p=0.56	HSD=5.96 p=1	HSD=-80.1 p=0.81	HSD=-71.4 p=0.89

Table S2: Similarity percentage analysis (SIMPER) showing the contribution and taxonomic affiliation of OTUs explaining 60% of the dissimilarity between treatments through time for present and active bacterial community at each LD station experiments.

LD A				
Factors	Av. Diss	Phyla/Class	Diss/SD	Contribution (%)
Copepods & Control	15.84	Alteromonadales	0.69	7.15
		Rhodobacterales	1.49	5.62
		SAR11 clade	1.59	4.15
		Vibrionales	1.27	3.65
		Derived from SAR202 clade	1.13	3.23
		SubsectionIII	1.32	3.01
		SAR324 clade (Marine group B)	1.54	2.84
		Flavobacteriales	0.61	2.63
		SubsectionI	1.31	2.61
		Oceanospirillales	0.80	2.28
		Planctomycetales	0.62	1.95
		Derived from PAUC34f	1.18	1.57
		Rhodospirillales	1.33	1.56
		Cellvibrionales	0.75	1.49
		Derived from Subgroup 6	1.23	1.43
		Caulobacterales	1.30	1.32
		Derived from Marinimicrobia (SAR406 clade)	0.14	1.26
		Phycisphaerales	0.72	1.22
		Derived from Arctic97B-4 marine	1.15	1.14
		Rhizobiales	1.39	1.09
		Derived from Poribacteria	1.16	1.09
		Derived from Chloroplast	0.81	0.98
		Pseudomonadales	0.84	0.97
		Sphingobacteriales	1.47	0.94
		Myxococcales	0.87	0.89
		E01-9C-26 marine group	1.35	0.86
		Puniceicoccales	1.35	0.86
		Nitrospinales	0.95	0.82
		Nitrospirales	1.62	0.81

		Cytophagales	0.92	0.78
LD B				
Copepods & Control	9.50	Alteromonadales	1.16	6.53
		Caulobacterales	0.58	5.47
		SAR11 clade	1.77	5.18
		Rhodobacterales	0.78	3.69
		SubsectionI	1.88	3.32
		Vibrionales	0.91	2.98
		Pseudomonadales	1.05	1.83
		Oceanospirillales	1.28	1.60
		Cellvibrionales	0.78	1.45
		Derived from Poribacteria	4.13	1.44
		Derived from ARKICE-90	1.62	1.36
		SAR324 clade (Marine group B)	1.26	1.34
		Sphingomonadales	0.25	1.30
		Desulfobacterales	1.74	1.19
		Hydrogenophilales	9.87	1.15
		Acidimicrobiales	1.74	1.14
		Chlorobiales	1.37	1.14
		Campylobacterales	1.44	1.13
		Unknown Order	1.19	1.13
		Acanthopleuribacterales	1.67	1.05
		Chromatiales	1.38	1.03
		MB11C04 marine group	3.00	1.02
		Derived from Marinimicrobia (SAR406 clade)	0.82	1.01
		Rhodospirillales	0.96	0.94
		Phycisphaerales	1.41	0.93
		Kordiimonadales	0.94	0.91
		NB1-j	4.36	0.90
		Bacillales	1.35	0.89
		A714019	1.84	0.88
		Derived from Chloroplast	1.09	0.88
		Derived from OPB35 soil group	1.21	0.86
		Rhizobiales	1.86	0.85
		Chlamydiales	1.40	0.85
		Derived from TK10	1.02	0.83
		Puniceicoccales	1.22	0.83
		Bdellovibrionales	1.56	0.79
		Aeromonadales	1.09	0.79
LD C				
Copepods & Control	18.37	Alteromonadales	1.75	8.94
		Vibrionales	1.24	8.66
		Oceanospirillales	1.46	7.92
		SAR11 clade	1.20	7.61
		SubsectionI	1.77	4.12

Derived from Marinimicrobia (SAR406 clade)	1.59	2.89
Caulobacterales	1.11	2.87
Acidimicrobiales	2.16	2.37
Rhodospirillales	3.65	2.17
Rhodobacterales	0.82	2.16
SAR324 clade (Marine group B)	1.54	1.77
Rhizobiales	2.05	1.70
Sphingomonadales	1.97	1.16
Derived from ML635J-21	1.45	1.13
Rickettsiales	1.21	1.10
Pseudomonadales	1.68	1.04
Rhizobiales	1.50	1.03
Burkholderiales	0.95	0.99
Micrococcales	0.57	0.97

Table S3: Consumption and production rates obtained in the treatment incubated with copepods. The turnover rates were obtained for each compound studied between incubation times and were estimated using an end-point approach. A positive value indicates a net accumulation and negative values indicates a net nutrient consumption.

Period of incubation		NH_4^+ ($\mu mol\ L^{-1}\ h^{-1}$)	NO_3^- ($\mu mol\ L^{-1}\ h^{-1}$)	NO_2 ($\mu mol\ L^{-1}\ h^{-1}$)	PO_4^3 ($\mu mol\ L^{-1}\ h^{-1}$)	DON ($\mu mol\ L^{-1}\ h^{-1}$)	DOP ($\mu mol\ L^{-1}\ h^{-1}$)
LD A	T_0-T_1	0.004 ± 0.117	0.016 ± 0.031	0.013 ± 0.015	0.035 ± 0.049	4.03 ± 5.02	0.68 ± 0.84
	T_1-T_2	-0.003 ± 0.045	0.005 ± 0.077	-0.013 ± 0.014	-0.063 ± 0.129	-3.62 ± 4.82	-0.28 ± 0.54
	T_2-T_3	0.028 ± 0.027	0.015 ± 0.027	-0.007 ± 0.008	-0.142 ± 0.035	0.20 ± 0.04	0.03 ± 0.20
	T_3-T_4	-0.053 ± 0.008	-0.036 ± 0.045	-0.008 ± 0.005	0.001 ± 0.001	0.11 ± 0.12	0.01 ± 0.01
LDB	T_0-$T_{0.25}$	0.331 ± 0.132	0.067 ± 0.050	-0.002± 0.006	-0.005 ± 0.017	-0.005 ± 0.37	0.01 ± 0.04
	$T_{0.25}$-$T_{0.5}$	-0.015 ± 0.045	-	-	-	-	-
	$T_{0.5}$-T_1	-0.095 ± 0.055	1.171 ± 0.910	0.001 ± 0.006	0.002± 0.029	-1.157 ± 1.429	0.02 ± 0.03
	T_1-T_2	0.027 ± 0.027	-0.612 ± 0.460	-0.001 ± 0.006	0.003 ± 0.014	0.557 ± 0.206	-0.02 ± 0.04
	T_3-T_4	0.031 ± 0.021	0.110 ± 0.162	-0.011 ± 0.016	0.034 ± 0.054	-0.010 ± 0.117	0.02 ± 0.02
LD C	T_0-$T_{0.25}$	0.056 ± 0.202	0.019 ± 0.318	0.011 ± 0.026	0.067 ± 0.067	-1.85 ± 1.18	0.001 ± 0.18
	$T_{0.25}$-$T_{0.5}$	0.043 ± 0.398	-	-	-	-	-
	$T_{0.5}$-T_1	0.117 ± 0.193	-0.143 ± 0.210	-0.009 ± 0.025	-0.042 ± 0.075	0.13 ± 0.84	-0.09 ± 0.16
	T_1-T_2	0.061 ± 0.050	0.040 ± 0.079	-0.003 ± 0.004	0.013 ± 0.012	-0.19 ± 0.29	-0.01 ± 0.02
	T_3-T_4	0.036 ± 0.041	0.256 ± 0.081	-0.001 ± 0.003	0.000 ± 0.005	0.02 ± 0.13	-0.001 ± 0.004

Table S4: Consumption and production rates obtained in the control (without copepods). The turnover rates were obtained for each compound studied between incubation times and were estimated using an end-point approach. A positive value indicates a net accumulation and negative values indicates a net consumptionof nutrient.

Period of incubation		NH_4^+ (μmol L^{-1} h^{-1})	NO_3^- (μmol L^{-1} h^{-1})	NO_2 (μmol L^{-1} h^{-1})	PO_4^3 (μmol L^{-1} h^{-1})	DON (μmol L^{-1} h^{-1})	DOP (μmol L^{-1} h^{-1})
LD A	T_0-T_1	0.040 ± 0.004	-0.054 ± 0.114	-0.004 ± 0.002	-0.061 ± 0.114	-0.01 ± 0.88	0.073 ± 0.123
	T_1-T_2	0.041 ± 0.027	0.143 ± 0.036	0.005 ± 0.001	-0.140 ± 0.095	-0.60 ± 1.26	0.142 ± 0.089
	T_2-T_3	-0.046 ± 0.009	-0.111 ± 0.111	-0.022 ± 0.001	-0.023 ± 0.019	0.55 ± 0.39	0.036 ± 0.034
	T_3-T_4	-0.014 ± 0.003	-0.016 ± 0.038	0.001 ± 0.009	0.008 ± 0.008	0.32 ± 0.04	-0.009 ± 0.009
LDB	T_0-$T_{0.5}$	0.143 ± 0.011	-0.094 ± 0.076	0.009± 0.016	0.027 ± 0.038	0.323 ± 0.207	-0.02 ± 0.08
	$T_{0.5}$-T_1	0.086 ± 0.032	-	-	-	-	-
	T_1-T_2	-0.121 ± 0.098	0.129 ± 0.004	0.005 ± 0.035	0.016 ± 0.136	-0.519 ± 1.322	0.02 ± 0.06
	T_3-T_4	-0.109 ± 0.018	-0.062 ± 0.025	-0.003 ± 0.004	-0.028 ± 0.040	-0.316 ± 0.593	-0.001 ± 0.09
	T_4-T_5	0.103 ± 0.081	0.017 ± 0.024	0.001 ± 0.001	0.003 ± 0.004	0.244 ± 0.267	0.04 ± 0.01
LD C	T_0-$T_{0.5}$	-0.012 ± 0.057	-0.581 ± 0.344	-0.001 ± 0.005	0.047 ± 0.098	-0.03 ± 1.01	-0.04 ± 0.08
	$T_{0.5}$-T_1	-0.025 ± 0.061	-	-	-	-	-
	T_1-T_2	0.029 ± 0.021	0.027 ± 0.086	-0.009 ± 0.005	-0.025± 0.072	-0.18 ± 0.72	0.03 ± 0.06
	T_3-T_4	-0.005 ± 0.016	0.394 ± 0.261	0.005 ± 0.012	-0.007 ± 0.027	-0.46 ± 0.09	0.0003 ± 0.03
	T_4-T_5	-0.009 ± 0.004	-0.159 ± 0.048	-0.006 ± 0.002	-0.002 ± 0.003	0.40 ± 0.15	0.004 ± 0.01

Table S5: Epipelagic copepod composition used at each experiment.

Specie	LD A	LD B	LD C
Haloptilus longicornis	X		
Pleuromamma sp	X	X	X
Acartia sp	X		
Saphirina sp	X		
Temora sp		X	
Miracia efferata		X	
Microsetella sp	X	X	
Copilia sp		X	X
Clausocalanus sp	X	X	X
Lucicutia sp			X

Figure S1
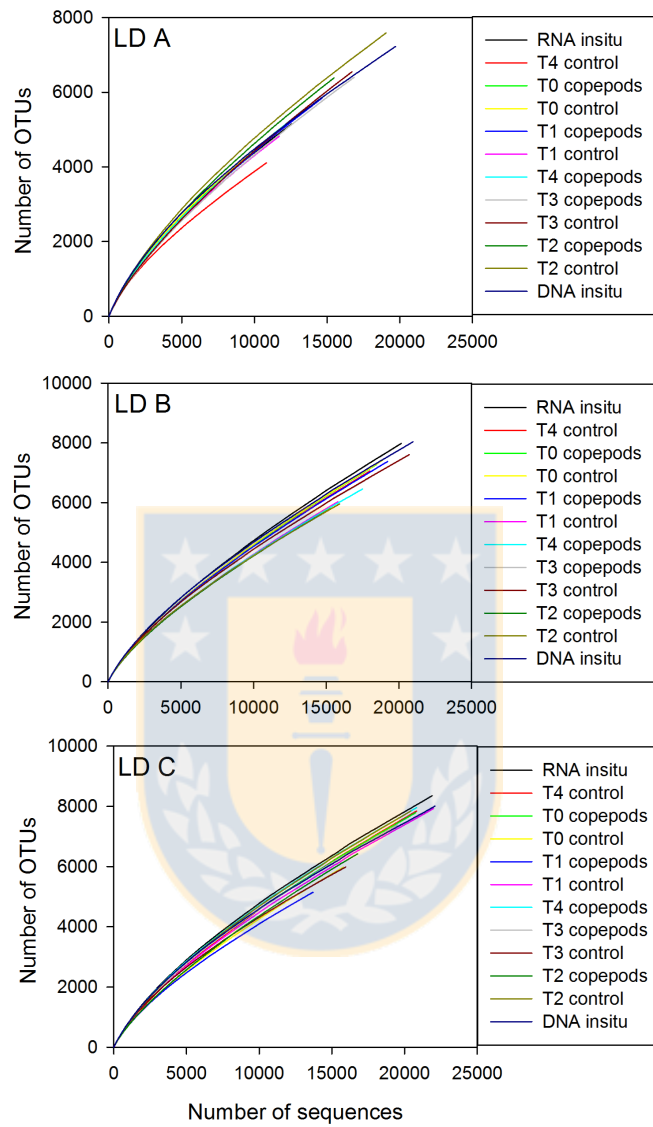
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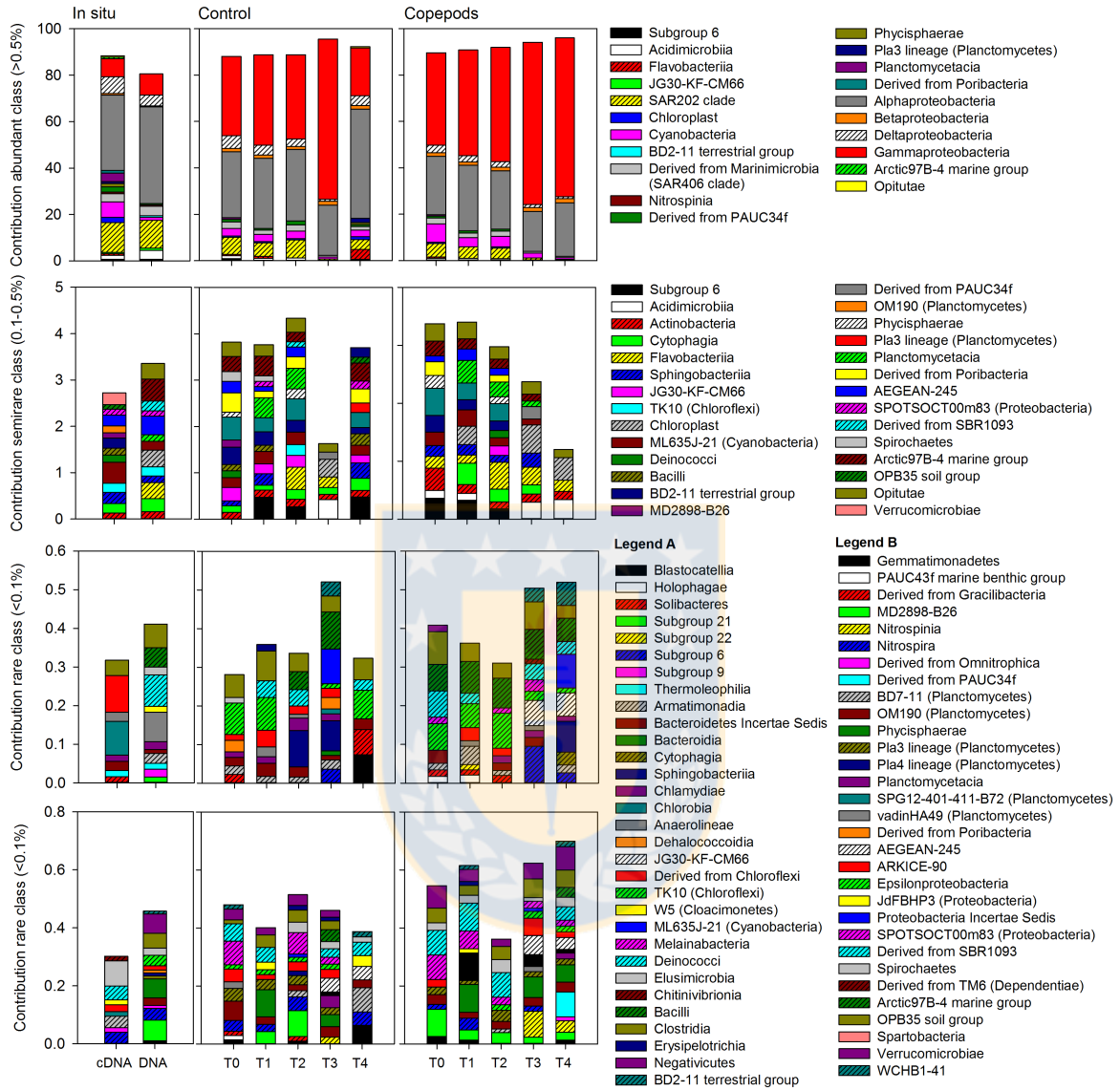
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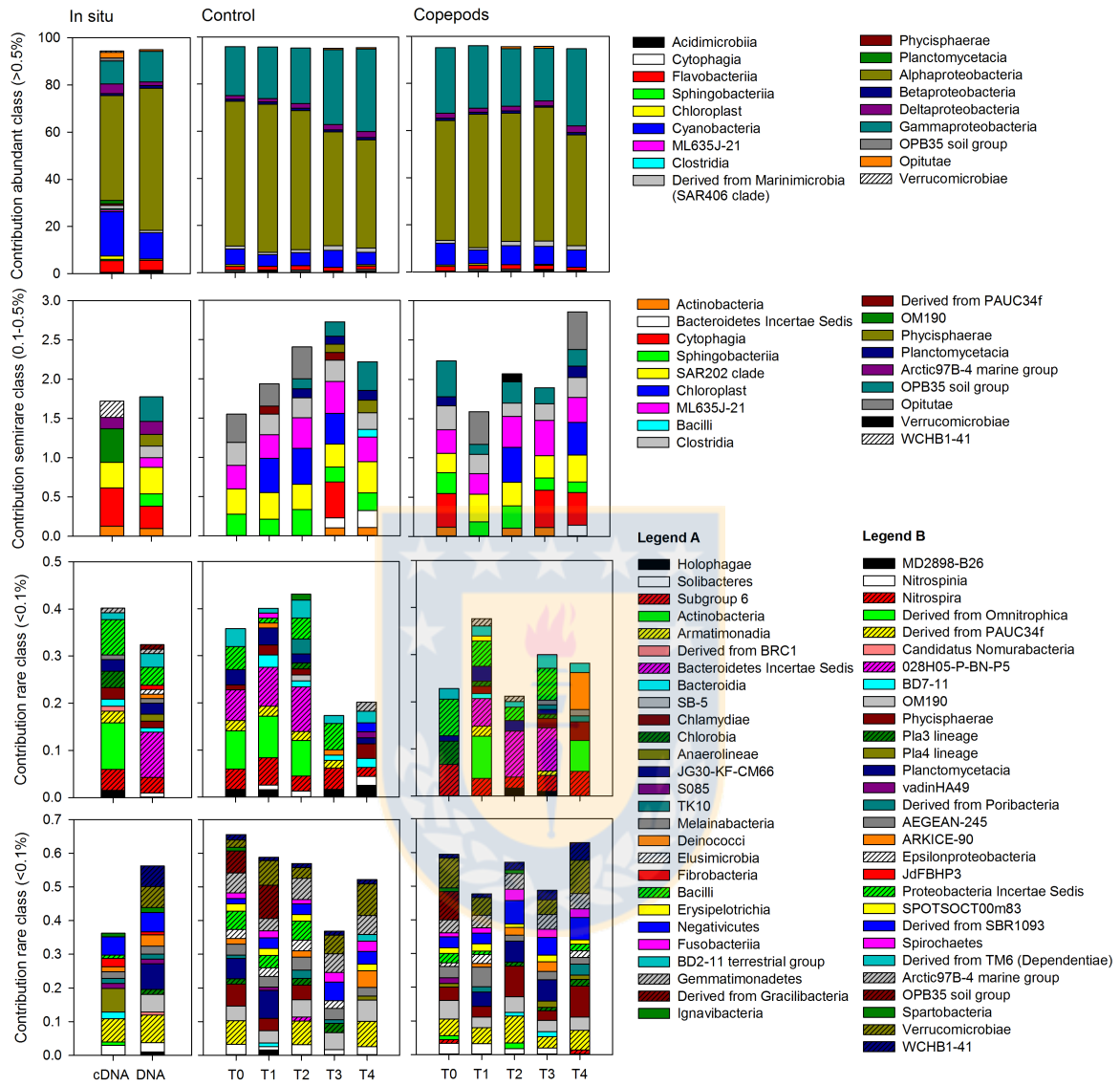
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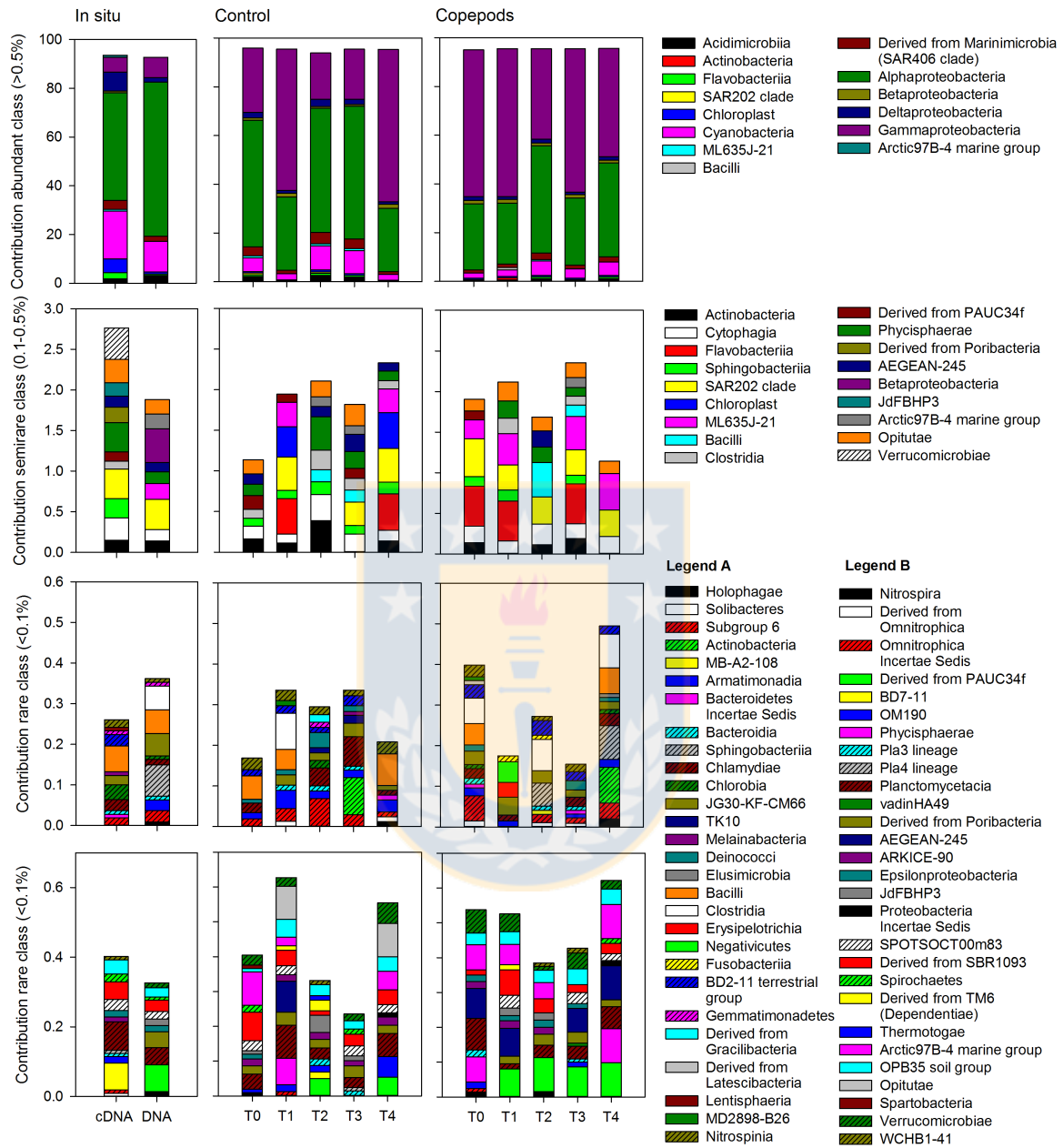
4. DISCUSION

Zooplankton excretion processes are extensively studied in the literature. Several works have been focused on ammonium excretion. However, the organic compounds (DON and amino acids) excreted by zooplankton are less studied and even less attention has been placed about the organic and inorganic phosphorus compounds. This issue is tackled in the Chapter II. Zooplankton excretion is considered a major recycling pathway for nitrogen, phosphorus and trace elements, and along bacterial remineralization determine the amount of regenerated nitrogen and phosphorus available for phytoplankton production (Steinberg and Saba, 2008). Our experimental approaches were designed in order to evaluate the compounds excreted by zooplankton and the impact on microbial loop in each experiment performed (7 in total). First, we assessed the dissolved compounds excreted by copepods quantified and identified and second we evaluated the subsequent utilization of these compounds by the microbial community. Also, we evaluated how such effect is conditioned by the type of food available by zooplankton. The impact on microbial community was studied as based on changes in nutrients concentration and shifts in the bacterial community composition throughout the incubation. In Chapter I we evaluated the response of the nitrifying community and in Chapters II and III we evaluated changes in the composition and the activity of the natural bacterioplankton community.

4.1 Nitrogen and phosphorus excretion by copepods

Zooplankton excretion has been studied for decades for different taxa and environments. Ammonium has been recognized as the primary nitrogenous product excreted, comprising up to 75% of the total dissolved nitrogen (TDN) excreted, followed by the organic forms (urea and amino acids; Bidigare, 1983). However, there is increasing evidence that DON excretion is an important excretory product. The few measurements of DON excretion showed a high variability, from 7% to 80% of TDN, even exceeding inorganic nitrogen excretion (Conover and Gustavson, 1999; Miller and Glibert, 1998; Steinberg et al., 2000, 2002). At central/southern Chile our results showed a high excretion of DON during the austral autumn with rates of up to 3.7 μmol L^{-1} h^{-1} (Chapter I). However, during the spring higher ammonium excretion rates were observed, nearly 2.6 μmol L^{-1} h^{-1} (Chapter II). The excretion of DON in winter is within the

range reported in this area (Pérez-Aragón et al., 2011). However, our ammonium excretion rates were higher than those reported in this area in austral spring and for other upwelling zones (Kiko et al., 2015; Pérez-Aragón et al., 2011). The effect of the diet of zooplankton on the main product excreted has been studied by several authors (Miller and Glibert, 1998; Miller and Roman, 2008; Saba et al., 2009). Changes on diet may be related to seasonality of the food sources (autotrophic and heterotrophic components). In this regard, Vargas et al (2006) reported that in the upwelling area of central-southern Chile copepods are capable of shifting their diet over the year cycle, feeding on heterotrophic flagellates in winter and on a diet based in diatoms in spring. Furthermore, Miller and Roman (2008) demonstrated that copepods excreted more ammonium when they had fed on a diet based on diatoms, which is in accordance with our results. This impact of the diet provided to copepods in the excreted compounds was also observed in the amino acids composition (Chapter II). During late summer experiment the predominant amino acid excreted was Histidine, while in spring was Glutamic acid. However, the composition of amino acids excreted by zooplankton are poorly studied for comparative analysis. However, further studies should confirm these results.

Phosphorus excretion has been much less studied that nitrogen excretion. Our results indicate that between inorganic and organic phosphorus excretion, DOP was the main product excreted, reaching the 93% of the total release of phosphorus under late summer conditions (Chapter II).

In spite of the fact that during OUTPACE experiments (Chapter III) we could not obtain an estimate of the excretion rate, a higher accumulation of organic compounds than inorganic compounds was observed in the three experiments over different incubation periods (Chapter III, supplementary information). We estimated that copepods could release substantial amounts of DON, which was the main compound released by copepods in the three experiments, with rates of 4.0 µmol L^{-1} h^{-1}, 0.9 µmol L^{-1} h^{-1} and 0.7 µmol L^{-1} h^{-1} for LD A, B and C, respectively.

4.2 Effects of DOM derived from copepods excretion on the microbial community: implication for coastal and open ocean regions

The release of dissolved compounds excreted by copepods is considered one of the principal mechanism controlling the quantity, composition and cycling of DOM in the sea

(Carlson and Hansel 2014). The impact of copepod excretion on the microbial community is highly dependent on the bacterial community structure and on the biogeochemical characteristic prevailing at each sampling site. Firstly, central-southern Chile is a highly heterogeneous system, characterized by seasonal wind-driven upwelling of subsurface nutrient-rich and poor-oxygen waters, which influence the chemical and biological components of the upper layer. By contrast, the WTSP is largely recognized as the most oligotrophic region of the world ocean, characterized by a limitation of nitrogen and phosphorus, which determine a low biological productivity.

4.2.1 Central-Southern Chile

Our results demonstrated that copepods excretion can provide a highly labile substrate for bacterioplankton growth, as evidenced by the rapid utilization (<2 h) through the incubation in the 4 experiments. The results showed coupled uptake-remineralization of ammonium and DON. In chapter I, the treatment derived from copepods fed with the smaller sized-fraction resulted in changes in DON accumulation-consumption that were observed over time, and this resulted in the accumulation of ammonium at the end of the incubation (Chapter I, Figure 5). These results were also found during the austral spring/summer (Chapter II). In fact, in the late summer experiment, ammonium was consumed throughout the incubation (treatment derived from copepods fed 20-150 μm), meanwhile DON was accumulated at the end, along with nitrate and nitrite. Other studies in the same area have revealed a tight coupling of ammonium production and consumption in winter, including ammonia oxidation and potential uptake by eukaryotic organisms (Molina et al., 2005, 2012), which is in agreement with our results. These results have been also demonstrated for phosphorus compounds. Johannes (1964), reported a coupled DOP production and uptake by bacteria, being capable to utilize the 80% of the DOP released by amphipods, based on radionuclide P^{32}. During the incubations, bacterioplankton rapidly uses the excreted phosphorus which was rapidly recycled (Chapter II; Figure 3). The coupled DOP production-utilization has been also reported in other studies with algae and bacteria using labeled phosphorus experiments in marine environments (Dolan et al., 1995; Lovdal et al., 2007).

During the incubations, nitrifying communities were transcriptionally active and could respond to the addition of ammonium derived from copepod excretion (Chapter I), both AOA and AOB were active in our incubations. However, in the inoculum derived from copepods fed with the smaller sized-fraction of food, presented lower *amoA* gene counts when ammonium was consumed (Chapter I; Fig. 7). In addition, lower accumulation of nitrite and no visible accumulation of nitrate (Chapter I; Fig. 5), suggesting that a distinct microbial community contributed to ammonium consumption, probably heterotrophic. Our study demonstrated and provided additional evidence that copepods make nitrogen compounds available to nitrifying communities in the surface layer (Yool et al., 2007; Zehr and Ward, 2002) in the central-southern Chile in autumn/winter, which in turn contribute with oxidized inorganic nitrogen to primary productivity in the system (Chapter I). One implication of this is overestimating regenerated production and underestimating new production in this system.

To determine the potential contribution of ammonium excretion by copepods to primary production we compared our results with the ammonium demand by phytoplankton at St. 18 in the euphotic layer (Fernandez and Farías, 2012). Excreted ammonium could potentially sustain 10% and 30% of phytoplankton ammonium demand in autumn/winter and spring/summer, respectively. Notably, it could also sustain up to 216% and 129% of surface (20–30 m depth) nitrification at St. 18 during autumn/winter and spring/summer, respectively (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.

Recent research has demonstrated that bacterial community composition can change in response to the addition of different sources of DOM (Landa et al., 2013; Sarmento et al., 2013). This has been studied mainly experimentally with amendments of DOM from phytoplankton bloom (McCarren et al., 2010; Sarmento and Gasol, 2012) and a few studies are focused on the impact of DOM derived from the jellyfish degradation on bacterial composition (Blanchet et al., 2015; Tinta et al., 2012, 2016). These studies have demonstrated that the DOM derived from jellyfish can promote changes in microbial diversity and their metabolism. During summer/spring, a shift in the composition of active bacterial community was observed between

periods and treatments, associated with the response of common seawater surface phyla Proteobacteria and Bacteroidetes (Chapter II). Moreover, specific bacterial activity (16S rRNA: rDNA) suggested a differential response associated with the two size fraction diets provided to copepods. Betaproteobacteria and Bacteroidetes were stimulated in the treatment enriched with excretion products derived from larger and smaller sized-fraction diets, respectively, in the late summer experiment. In spring, Alphaproteobacteria were active in the treatment enriched with excretion products of copepods fed with <20 μm sized-fraction, while they were inhibited in the treatment enriched with excretion products of 20-150 μm sized-fraction. The bacterial response is probably associated with common-opportunistic sea surface microbes adapted to rapidly react to the environmental offer. In this sense, Alphaproteobacteria use preferentially monomers, such as amino acids, and so being important competitors in the ocean and largely responsible for the uptake of low molecular weight DOM (Cottrell and Kirchman, 2000). Besides, Bacteroidetes seem to be specialized in uptake of high molecular weight DOM. In our experiment carried out in late summer, we expected the presence of complex structural biopolymers (cell wall components) associated with the larger size of the microbial community used (1.6 μm). The decay of this community by either predation or autolysis could provide complex substrates in our incubation, enhancing Bacteroidetes activity, in contrast with the spring experiment, in which Alphaproteobacteria were active.

The biogeochemical impacts of copepod excretion are not limited to the upper layers, as zooplankton can move through the water column by diel vertical migration. Le Borgne and Rodier, (1997) estimated that the 60% of the total nitrogen excreted by vertical migrators is in the form of DON. In this context, zooplankton can be an important source of DON for microbial communities at greater depths stimulating ammonium regeneration by bacteria (Miller et al., 1997; Steinberg et al., 2002). Furthermore, in central-southern Chile some copepods species are able to cross and inhabit the oxygen deficient zone (Escribano et al., 2009). 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 oxygen minimum zone of the eastern south Pacific, daily ammonium excretion by copepods, on occasions, could sustain up to 86% of the ammonium demand by the anammox process, reaching values close to 500 nmol L^{-1} d^{-1} in spring near St. 18 (Galán et al., 2012).

4.2.2   Western Tropical South Pacific

The high demand for DON found in the central-southern Chile was also observed in a contrasting area, as the oligotrophic zone in the WSTP (Chapter III). Our results showed a strong recycling of nutrients, in special in the experiment carried out in the LD A station where DON was accumulated at higher rates in the first period of incubation ($\sim$4 µmol L^{-1} h^{-1}). However, this was not reflected in the ammonium concentration, suggesting a high heterotrophic bacterioplankton demand for DON remineralization/ammonification. The bacterioplankton community was characterized by a change mainly in Gammaproteobacteria and Alphaproteobacteria in all LD stations. A dominance of Gammaproteobacteria was rapidly observed in LD A experiment, mainly accounted by Alteromonadales and Oceanospirillales. In contrast, in the experiment carried out in LD B station, a higher contribution through the time was due Alphaproteobacteria, in which SAR11 increased their relative abundance ($\sim$8%) at 1 hour of incubation and Caulobacterales (<0.1%) at the end of incubation. In the third experiment (LD C station), which corresponded to the most oligotrophic station, a higher increase in Alteromonadales and Vibrionales was observed. Bacterial degradation of dissolved compounds enrichments is carried out by phylogenetically diverse communities, some generalist and other more specialized. The faster response in Gammaproteobacteria (Alteromonadales; Chapter III) has been documented and are frequently observed in high abundance in incubation experiment, due to their high growth rate and their ability to exploit DOM rapidly when is available (Alonso-Saéz and Gasol, 2007; Fuchs et al., 2000; Landa et al., 2013). The high demand of DON in the experiment carried out in the LD A station could be associated to the high increase in the relative abundance of Alteromonadales through the incubation.

On the other hand, SAR11 is the most abundant plankton in the ocean, is well adapted to lower concentration of nutrients, and are specialized to oxidize many labile and low-molecular-weight compounds produced by other plankton, being an excellent competitor at ultralow nutrient concentration (Giovannoni, 2017). SAR 11 was one of the major constituent of rRNA (*in situ* and microcosm incubation) and rDNA *in situ* in the WTSP and during our incubations, SAR11 increased their contribution only in LD B station which coincided with the lowest concentration of inorganic nitrogen and phosphorus (Table 1; Chapter III). This station

coincides with a two-month-old *Trichodesmium* bloom which explain the lower inorganic *in situ* nutrients concentration observed. Which provide to SAR11 a competitive advantage over other microbes.

Our results suggest that copepods can greatly stimulate remineralization process, providing substantial amounts of DON and DOP. In addition, during all incubations ammonium was several times higher in the treatment with copepods than the concentrations found *in situ* and in controls. Steinberg et al. (2002) demonstrated that migrating zooplankton can contribute substantial amounts of DON through active transport to deeper layers and make it available for use by the microbial community. This could be especially important in stratified systems, such as the SG in the WTSP, in which the ongoing global warming has enhanced the stratification and decreased the nutrient availability. Furthermore, bacterial remineralization of the dissolved compounds derived from copepods metabolism could be a highly efficient mechanism to maintain the nutrients in the upper layer supporting the phytoplankton and microbial growth. In this sense, the response associated with the different regions under study in the WTSP suggests that copepods in the MA region could provide substantial amounts of DOP as an alternative substrate for growth or extending the life time of the diazotrophs bloom.

4.3 General considerations on the experimental approaches

Experiments were performed under controlled temperature conditions, corresponding to the *in situ* temperatures at each sampling site and in darkness to avoid potential interferences by light. The experiments performed in central-southern Chile were designed to evaluate separately the excretion and the subsequent utilization by the microbial community. During the excretion phase, copepods were incubated with filtered seawater (0.22 μm) to avoid the presence of the large-sized microbial community. During this phase, we followed bacterioplankton abundance and the results showed a low and constant number of bacterioplankton (Chapter I and II). However, during the experiments negative excretion rates were found. These negative values are the result from a dynamic process by which compounds excreted by copepods can be rapidly utilized at the same time, since these compounds excreted are highly labile. In other words, a negative value reflects that in our incubations there was a higher consumption of certain

compounds compared to the input provided by copepods. This could also be explained by presence of heterotrophic, chemoautotrophic, and also by small photoautotrophic communities. In the sample it could be as a reflect of the carryover of the microbiome associated with the copepod exoskeletons and intestines (Gerdts et al., 2013; Tang et al., 2010).

During our incubations, we evaluated the excretion of copepods, however we did not monitor possible leaching of dissolved compounds from fecal pellet produced during the experiments. In this regard, Steinberg et al., (2000) found that the leaching of dissolved organic carbon (DOC) from fecal pellets was insignificant compared to changes in DOC observed in excretion experiments. The same result was observed by Saba et al., (2011) which evaluated the relative importance of sloppy feeding, excretion and leaching from fecal pellets in the release of dissolved carbon and nitrogen in *Acartia tonsa,* and thus demonstrated that the leaching of DON was less than 6% while ammonium was not detected. Thus, we assumed that the leaching from fecal pellets is insignificant compared to the compounds released by excretion for all experiments performed.

The number of copepods incubated was determined as based on the average abundances observed in the coastal area in central-southern Chile in previous studies (Hidalgo et al., 2010). However, during the OUTPACE cruise the number of copepods was determined in order to obtain a readable signal in ammonium concentrations, since the general concentrations found during the cruise were at nanomolar levels. Zooplankton abundance was lower at LD C station compared to LD A and B, which was ~1,400 ind. m^{-3}, ~1,600 ind. m^{-3} and ~ 500 ind. m^{-3}, for LD A, B and C, respectively (Carlotti et al., unpublished). However, during the three experiments we used the same number of copepods for all incubations. Therefore, our results, specially for the experiment carried out in LD C station, can be overestimated because it may not reflect the real abundance likely to be found in the field.

4.4 Perspectives for future research

There has been a considerable progress in the last decade on our understanding of the role of marine zooplankton in nitrogen cycling. However, gaps still remain about the organic nitrogen compounds excreted by zooplankton and even more on phosphorus excretion. Further studies are needed to understand the use of specific compounds which are part of DON and DOP

release. Studies on both composition and lability of this material are needed to understand the different pathways by which DOM becomes utilized. The preferential uptake of some compounds by microbial community, in special for amino acids, indicates that they are selectively used. For this, a microcosm experimental approach designed to explore this issue and how it can affect the nutrient recycling in the pelagic system should be necessary.

Phosphorus cycle investigation, conducted in the coastal areas, are rare, especially when comparing with the relatively high level of knowledge on nitrogen cycling. Studies on all the processes that impact the phosphorus cycling in coastal areas are necessary too.

5. CONCLUSION

This thesis work concludes that the role that the pelagic trophic interactions, involving the microbial and classical food webs of the ocean, can play in the recycling of nitrogen and phosphorus in the central-southern Chile and the western tropical south Pacific can be stated as follows:

1. Planktonic copepods can provide substantial amount of DON in winter with copepods fed with the smaller sized-fraction diet and during spring/summer copepods actively excreted ammonium and DOP in copepods fed with higher sized-fraction. The prevailing excreted compounds by copepods is highly dependent to the type of food available for copepods, suggesting a seasonal influence on the excretion by copepods in central-southern Chile.

2. The excreted nitrogen compounds can be directly used by microbial communities in a short response time and revealing a tight coupling of ammonium production and consumption associated with a high demand for DON by heterotrophic bacteria in central-southern Chile and in western tropical south Pacific.

3. Archaea and bacteria ammonia-oxidizing (AOA-AOB) can respond to the ammonium excreted by the dominant copepods in central-southern Chile, in which AOA may compete more successfully under limited ammonium concentration than AOB.

4. The addition of copepod excreted products can stimulate bacterioplankton community, resulting in an increase in their abundance, and can reshape the diversity and the bacterial community composition. Proteobacteria and Bacteroidetes were the main phyla that respond to the release of compounds by copepods, and they are associated with common-opportunistic sea surface microbes in central-southern Chile and in the western tropical south Pacific.

5. The impact of copepod excretion on biogeochemical cycles, mediated by microbial community, is highly dependent on the environmental characteristic at each site. In central-southern Chile copepods can be an important source of nitrogen and phosphorus for regenerated production, complementing the *in-situ* nitrate uptake during spring/summer season, which is mostly upwelled, and such impact could be more important during non-active upwelling. Bacterial remineralization of DOM derived from copepods metabolism could be a highly efficient mechanism to maintain the nutrients in the upper layer in a highly stratified system, as in the subtropical Gyre region in the western tropical south Pacific, contributing to phytoplankton and microbial growth.

In summary, it is concluded that the results obtained from the study provide sufficient support for the proposed hypotheses, such that they cannot be rejected.

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