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Zooplankton-derived dissolved organic matter composition and its bioavailability to natural prokaryotic communities

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Abstract

Zooplankton grazing onphytoplankton promotes the release of particulate and dissolved organic matter (DOM) into the water column and therefore plays a key role in organic matter cycling in aquatic systems. Prokaryotes are the main DOM consumers in the ocean by actively remineralizing and transforming it, contributing to its molecular diversification. To explore the molecular composition of zooplankton-derived DOM and its bioavailability to natural prokaryotic communities, the DOM generated by a mixed zooplankton community in the coastal Atlantic off Spain was used as substrate for a natural prokaryotic community and monitored over a ~ 5-d incubation experiment. The molecular composition of solid-phase extracted DOM was characterized via Fourier-transform ion cyclotron resonance mass spectrometry. After ~ 4 d in the zooplankton-derived DOM amended incubation, the prokaryotic community demonstrated a 17-fold exponential increase in cell number. The prokaryotic growth resulted in a reduction in bulk dissolved organic carbon concentration and the zooplankton-derived DOM was considerably transformed at molecular and bulk elemental levels over the incubation period. The C : N ratio (calculated from the obtained molecular formulae) increased while the functional diversity decreased over the incubation time. In addition, molecular indices pointed to a reduced bioavailability of DOM at the end of the experiment. These findings show that zooplankton excreta are a source of labile organic matter that is quickly metabolized by the prokaryotic community. Therefore, a fraction of carbon is shunted from transfer to secondary consumers similarly to the viral shunt, suggesting that the zooplankton–prokaryotic interactions play an important role in the ocean's carbon cycle.

Dissolved organic matter (DOM) is the largest reservoir of reduced organic carbon in the marine environment. Most DOM is autochthonously generated by primary producers in the ocean´s surface (Hansell 2013). The organic carbon bound by primary production can be released from the cell to the surrounding environment either directly by leakage or exudation (Thornton 2014) or by viral lysis (Wilhelm and Suttle 1999) or zooplankton grazing activities (Hygum et al. 1997; Thornton 2014; Steinberg and Landry 2017). The material released via zooplankton grazing is composed of DOM and particulate organic matter (POM) (Steinberg and Landry 2017). Crustacean zooplankton are the most abundant metazoan in the ocean and release a conspicuous amount of DOM via sloppy feeding, excretion, and leakage from fecal pellets, therefore contributing to the ocean's dissolved carbon and nitrogen

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

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pools (Møller et al. 2003; Steinberg et al. 2004; Møller 2007; Saba et al. 2011). The composition of the DOM released by zooplankton grazing activity is mostly dependent on the quality and quantity of the food consumed (Steinberg and Landry 2017). Marine DOM comprises a complex matrix of labile and recalcitrant molecular compounds and its reactivity influences the trophic interactions of the ecosystem (D'Andrilli et al. 2019). DOM bioavailability not only depends on its size-fractionated composition (Varela et al. 2020) but also on the environmental conditions (such as temperature and nutrient concentrations) and on the composition of the in situ prokaryotic consumer community (Thingstad et al. 1997; Dittmar 2015). A recent study showed that dissolved free amino acids, as well as taurine, and vitamins released by zooplankton can be actively metabolized by the prokaryotic community, comprising an important fraction of the labile DOM pool in marine ecosystems (Maas et al. 2020). Prokaryotes are the main DOM consumers in the ocean and affect the DOM molecular composition by transforming preferentially labile DOM (Ogawa et al. 2001; Osterholz et al. 2015), and therefore enhance the diversification of the organic matter pool of the ocean (Hach et al. 2020).

Zooplankton–prokaryotic interactions play a key role in the ocean carbon cycling (Tang et al. 2010). Therefore, characterizing the prokaryotic transformation of labile carbon released by zooplankton activity is key to understand the carbon fluxes in the ocean. This study investigates the temporal changes in the composition of zooplankton-derived DOM in relation to bulk prokaryotic community properties. We hypothesized that zooplankton activity results in the release of labile DOM that is quickly transformed by the prevailing prokaryotic community. To this end, we monitored changes in prokaryotic abundance, and the concentrations of dissolved organic carbon (DOC), dissolved organic nitrogen (DON), and inorganic nutrients resulting from the addition of zooplankton-derived DOM. The manipulation experiments were conducted with samples collected at a coastal upwelling site (RADIALES station) off A Coruña (North West [NW] Spain). Here, we took the analytical challenge to assess the molecular composition of DOM isolated by solid-phase extraction (SPE-DOM) during a ~ 5-d incubation experiment with and without zooplankton-derived DOM.

The SPE-DOM was analyzed by Fourier-transform ion cyclotron resonance mass spectrometry (FT-ICR-MS) to investigate the zooplankton-derived DOM molecular composition and in relation to its bioavailability for prokaryotic remineralization. Although FT-ICR-MS provides a highly detailed, untargeted representation of this SPE-DOM fraction, it should be noted that larger biopolymers such as carbohydrates and proteins, as well as ionic monomers such as individual amino acids, fall outside the analytical window of this approach. Therefore, in this experiment, we are focusing on the fraction of DOM that is captured by this routine analytical approach, representing > 60% of marine DOM (Dittmar et al. 2008).

Material and methods

Sampling and experimental setup

Zooplankton and water samples were collected at Station E4CO off the NW coast of Spain in September 2019. This station is routinely sampled in a long-term monitoring program of physical, chemical, and biological observations on the pelagic ecosystem (http://www.seriestemporales-ieo.net). Mesozooplankton (200–2000 μm) were captured by means of near-surface hauls (≈ 4 m maximum depth) using a bongo net of 50-cm diameter and a 200-μm mesh equipped with two non-filtering cod end collectors to obtain undamaged individuals. Once onboard, one of the collectors was immediately preserved with formaldehyde (10% final concentration) for subsequent taxonomic abundance information, while the content of the second collector was diluted with surface seawater (< 2 m depth) to keep the organisms alive and in a suitable condition during transport to the laboratory. The abundance and taxonomical composition of zooplankton was determined at the end of the zooplankton excretion phase (Supporting Information Fig. S1) by examination of aliquots using an Olympus stereomicroscope (Bode and Alvarez-Ossorio 2004). The sampled community was dominated by copepods (> 50% of total abundance), mainly *Acartia clausi* (adults and juveniles), and to a lesser extent *Paracalanus parvus* and *Temora longicornis* together with a mixture of several small copepods (Table 1). This mixed zooplankton community was split into two glass bottles (previously washed with 10% HCl) and kept in 0.2-μm-filtered seawater (the water was filtered with GTTP Whatman filters prerinsed in ultrapure water [MilliQ]). A CTD profile was made using a SBE-25 CTD, and seawater (≈ 80 liters) was collected at the surface in 20-liter polycarbonate carboys on board R/V Volandeira.

Briefly, the experimental setup to assess the interaction between the dissolved organic compounds excreted by zooplankton and prokaryotes comprised four phases: (1) zooplankton selection and acclimation, (2) zooplankton feeding, (3) zooplankton DOM release, and (4) the prokaryotic response to the dissolved compounds excreted in the previous phase (Supporting Information Fig. S1). The mixed zooplankton community (*see* above) amounted to a total biomass of 1591 mg (dry weight) and was initially acclimatized for 2 h in one 50-liter carboy containing 0.7-μm-filtered ambient seawater. Subsequently, the zooplankton was transferred into a 50-liter carboy containing 150-μm-filtered ambient seawater, with potential zooplankton food such as phytoplankton cells. This feeding phase was performed in the dark at in situ temperature for 4 h. Thereafter, the zooplankton was transferred to a carboy containing 10 liters of 0.2-μm-filtered seawater (Durapore; Millipore) to minimize the presence of prokaryotes during the excretion phase.

To evaluate the prokaryotic response to dissolved compounds derived from zooplankton excretion, 20-liter polycarbonate carboys containing 17 liters of 0.7-μm-filtered surface

Table 1. Taxonomic composition and abundance of the natural mesozooplankton community from the experiments used to generate the zooplankton-derived DOM.

Zooplankton taxa	Density (indiv. m^{-3})	Relative abundance (%)
Acartia clausi (V–VI)	1424.4	43.8
Acartia clausi (I–IV)	505.4	15.5
Paracalanus parvus	107.2	3.3
Copepods (I–IV)	436.5	13.1
Bryozoa (larvae)	153.2	4.7
Temora longicornis (I–IV)	91.9	2.8
Evadne nordmanni	91.9	2.8
Cnidaria	76.6	2.4
Cirripedia (larvae)	76.6	2.4
Gastropoda (larvae)	68.9	2.1
Podon intermedius	61.3	1.9
Appendicularia	45.9	1.4
Acartia discaudata	38.3	1.2
Pseudocalanus elongatus	23.0	0.7
Foraminifera	15.3	0.5
Bivalvia (larvae)	15.3	0.6
Temora longicornis (V–VI)	7.7	0.2
Siphonophora	7.7	0.2
Polychaeta (larvae)	7.7	0.2

seawater were collected, thus including only the natural free-living prokaryotic communities (the prefiltration step removed larger prokaryotes and other organisms that could have contributed to the DOM pool). These samples were subsequently amended with 3-liter aliquots of 0.2-μm-filtered water recovered at the end of the excretion phase of zooplankton. Control treatments consisted of 17 liters of 0.7-μm-filtered seawater and 3 liters of 0.2-μm-filtered seawater. Therefore, the treatments comprised both zooplankton excreta (collected during the egestion phase) and environmental DOM, while the controls consisted of environmental DOM.

Triplicates of DOM-amended and control treatments were monitored for 108 h in the dark at in situ temperature (~ 13°C). Subsamples for prokaryotic abundance, inorganic and organic nutrients, and DOM molecular composition were collected every 12/24 h (Supporting Information Fig. S1). A sample from ambient seawater (< 2 m depth) was collected as an environmental control.

Prokaryotic abundance

The abundance of prokaryotic cells during the incubation experiment was determined by flow cytometry as previously described (Gasol et al. 1999). Briefly, water samples (1.8 mL) were preserved with 1% paraformaldehyde plus 0.05% glutaraldehyde (final concentration), shock-frozen in liquid nitrogen for 5 min and stored at −80°C until further analysis. Samples were thawed and stained with Syto13 for 10 min in the dark. Fluorescent latex beads (approximately 1×10^5 mL^{-1}; Molecular Probes, Invitrogen) were added as internal standard. Prokaryotic cells were counted using a FACSCalibur flow cytometer (Becton Dickinson) according to their signature in right angle light scatter and green fluorescence.

Solid-phase extraction

SPE of DOM from the incubation experiment was conducted following the methodology of Dittmar et al. (2008). Briefly, 1 liter of sample was filtered through GTTP filters (Millipore, nominal pore size 0.2 μm, three times prerinsed in MilliQ water), acidified to pH 2 with HCl (analytical grade), and passed through a 1-g SPE cartridge (prerinsed twice with methanol, MilliQ and MilliQ at pH = 2) (PPL, Agilent). The cartridges were then desalted with 0.01 M HCl and dried with pure nitrogen gas. SPE-DOM samples were eluted from the cartridge using 6 mL methanol (99.9%, HPLC grade). Subsamples of 100 μL were dried for 24 h at 50°C and redissolved in 10 mL of ultrapure water to quantify the DOC concentration in the extract.

Nutrients, DOC, and DON

Samples for dissolved inorganic nutrients (nitrate, nitrite, orthophosphate, and silicate) were measured colorimetrically by using a QuAAtro (SEAL Analytical Inc.). The protocols from SEAL analytics Q-126-12 and Q-104-09 were used for nitrate and nitrite, and Q-125-12 for phosphate concentration analysis, respectively (Coverly et al. 2012).

DOC and total DON in the samples and in the SPE extracts were quantified on a Shimadzu TOC-V$_{\mathrm{CSH}}$ total organic carbon analyzer, equipped with an autosampler ASI-V, via high-temperature catalytic combustion as previously described by Catalá et al. (2018). DON values were obtained by subtraction of the dissolved inorganic nitrogen concentration from the total dissolved nitrogen concentration. The analyses were quality controlled using a DOC deep-sea reference material (Hansell Biogeochemistry Laboratory, University of Miami). The SPE extraction efficiency was determined by comparing total DOC before and after SPE extraction. The coefficient of variation for DOC measurements was ~ 5%. The extraction efficiency was 43% ± 23%.

DOM characterization by FT-ICR-MS

The SPE-DOM samples were analyzed by ultrahigh-resolution mass spectrometry on a Bruker Solarix 15 Tesla FT-IC-MS (Bruker Daltonik GmbH) with electrospray ionization in negative mode using an autosampler at a DOC concentration of 2.5 ppm in a 1 : 1 mixture of methanol and water. Mass spectra were accumulated with 200 scans. The scans were accumulated in a mass window ranging from 92 to 1000 Da. All spectra were calibrated internally using Bruker Daltonics Data Analysis software and further processed using ICBM-OCEAN, which applies a method detection limit to account for noise, excludes known contaminants and reduces systematic error to allow for precise formula attribution (Riedel and Dittmar 2014; Merder et al. 2020). The dataset we used here was termed "Likeliest match," which only considers the most likely molecular formulae based on the smallest distance from the reference mass, isotope ratio verifications, and a homologous series network (Merder et al. 2020). The selection criteria for the molecular formulae followed default settings in ICBM-OCEAN ($H \leq 4C$; $O < 1.2C$; $N \leq C + 1$; $S \leq C + 1$; $P \leq C + 1$; Merder et al. 2020).

All formulae in the instrumental blanks were considered contaminants and removed. All samples were run in triplicate apart from the treatment samples of time 1 and time 4, which were run in duplicate. In addition, all samples considered were run in technical duplicates. Formulae were assigned to chemical categories as implemented in ICBM-OCEAN (Merder et al. 2020). The bioproduction index (I_{BIO}) was previously determined by identifying five ubiquitous molecular formulae associated with fresh DOM release in a controlled marine mesocosm relative to peaks known to be recalcitrant and present in North Equatorial Pacific Intermediate Waters (Osterholz et al. 2015; Seibt 2017) and indicates the bioavailability of DOM. The molecular degradation index (I_{DEG}) consists of 10 ubiquitous molecular formulae in the marine environment that are correlated with $\Delta^{14}C$ content, and indicates the DOM degradation state (Flerus et al. 2012). Therefore, the higher the bioproduction index, the more bioavailable the DOM, while higher degradation index indicates more degraded DOM.

Total numbers of carbon, nitrogen, sulfur, and phosphorous atoms were derived for each sample by summing the number of each of these elements of all detected formula. A C : N ratio was then calculated for each sample by dividing the total number of C by the total number of N. Functional diversity of DOM was estimated following Mentges et al. (2017) based on H : C ratios of chemically distinct compounds and provides a quantitative measure for chemical diversity between samples.

Statistical analyses

All data processing and statistical analyses were conducted in R software v.3.4 Briefly, Student's *t*-tests and linear regression were performed in basic R, while principal component analysis (PCA) was computed with the "Vegan" package implemented in R (Oksanen J et al. 2018), the 95% confidence ellipse for both treatment and control are also shown. Statistical significance for all the analyses was set at *p*-value less than 0.05.

Results

Prokaryotic response to zooplankton-derived DOM

At the beginning of the incubation, in the zooplankton-derived DOM-amended treatment, concentrations of DOC and DON were ~ 19 and ~ 5 μM higher than in the control (Student's *t*-test, $p < 0.05$; Fig. 1). The DOC concentration decreased in the DOM-amended treatment from 90.3 ± 6.9 μM at the beginning of the experiment to 81.7 ± 1.6 μM at 60 h, while the control increased from 70.8 ± 7.9 μM at the beginning of the experiment to 91.7 ± 7.7 μM at 84 h, therefore showing an opposite trend. The highest DON concentration was found at 84 h in both DOM-amended treatment and control (Fig. 1).

At the beginning of the experiment, the concentrations of nitrate, nitrite, orthophosphate, and silicate were ~ 1.1, ~ 0.07, ~ 0.43, and ~ 2.9 μM higher, were higher in the DOM-amended treatment than in the control (Student's *t*-test, $p < 0.05$; Supporting Information Fig. S2), with no significant changes throughout the incubation in either the DOM-amended treatment or the control.

Prokaryotic abundance exponentially increased in the DOM-amended treatment from $1.6 \times 10^5 \pm 7.9 \times 10^3$ cells mL^{-1} at 12 h to $2.7 \times 10^6 \pm 5.1 \times 10^5$ mL^{-1} at 84 h, increasing 17-fold in 72 h (Fig. 1). In the control, the prokaryotic abundance also increased, but only sixfold, from $1.1 \times 10^5 \pm 1.3 \times 10^4$ mL^{-1} at 12 h to $6.2 \times 10^5 \pm 8.3 \times 10^4$ mL^{-1} at 108 h. The prokaryotic abundance in the DOM-amended treatment was fivefold higher than in the control at the end of the exponential phase.

Molecular composition of the zooplankton-derived DOM

The DOM-amended treatment exhibited a greater DOM molecular diversity (number of detected unique molecular formulae) than the control and the ambient seawater. Specifically, the DOM-amended treatment comprised 4163 ± 720 (average ± SD, calculated from triplicates) molecular formulae at the beginning of the incubation, while the control and the ambient seawater only 2968 ± 396 and 2655 molecular formulae, respectively (Table 2). The functional diversity showed a similar trend, with higher values in the DOM-amended treatment at the beginning of the incubation (0.105 ± 0.004) as compared to the control (0.095 ± 0.003) and the ambient sample (0.092). The C : N and C : P ratios calculated from the molecular formulae significantly differed at the beginning of the experiment between the DOM-amended treatment and the control (Student's *t*-test, $p < 0.05$), while C : S was not significantly different (Student's *t*-test, $p > 0.05$). At the beginning of the experiment, the C : N atomic ratio was lower in the DOM-amended treatment as compared to the control, while at the end of the incubation ratios were comparable to the ambient seawater (Table 2). The DOC : DON ratios were not significantly different at the beginning of the incubation (Student's *t*-test, $p > 0.05$) in the DOM-amended treatment as compared to the control (Table 2).

In the incubation experiment, the DOM mostly consisted of highly unsaturated and unsaturated molecular formulae. The DOM-amended samples at the beginning of the experiment had a lower proportion of highly unsaturated compounds and a higher proportion of unsaturated and aromatic compounds as compared to the control and the ambient seawater sample (Table 2). The average mass of the detected compounds did not change after the DOM addition. However, the DOM-amended samples had significantly different degradation and bioproduction indices at the beginning of the experiment than the control (Student's *t*-test, $p < 0.05$) (Table 2).

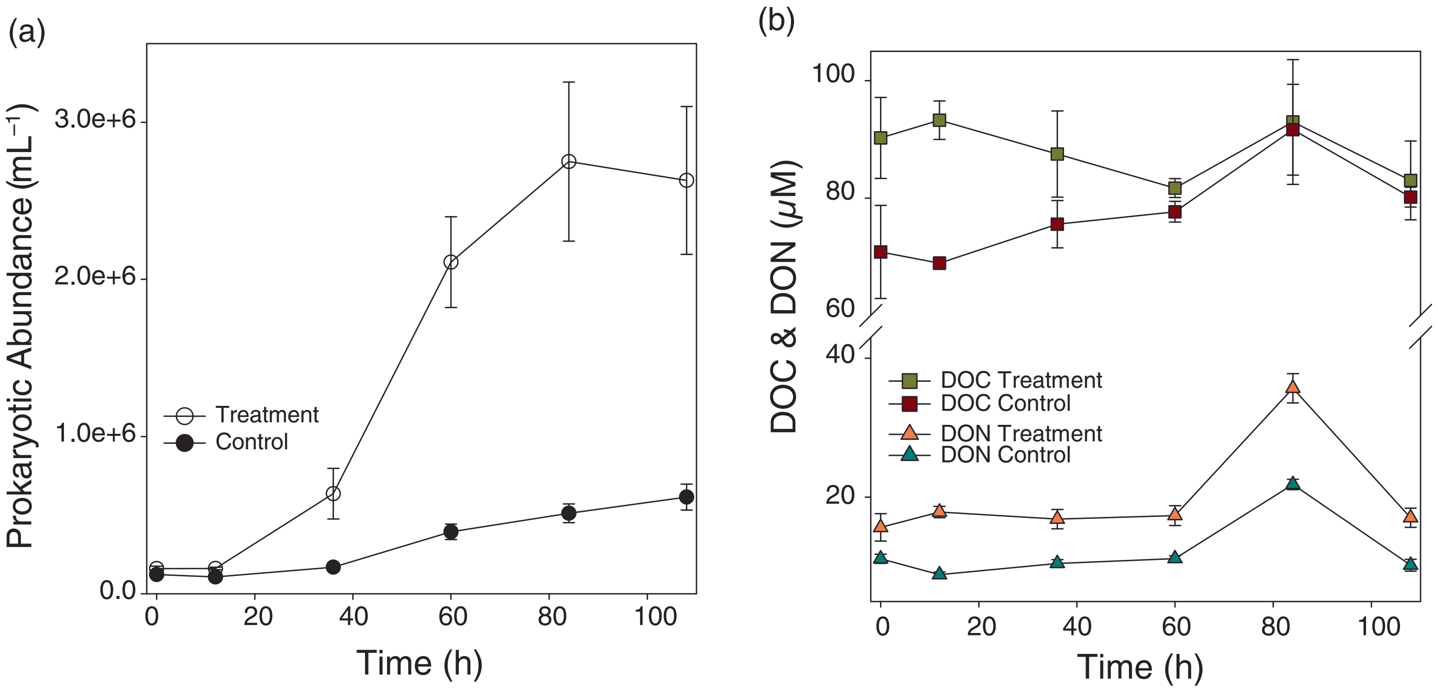

Fig. 1. Prokaryotic abundance (**a**), DOC and DON concentrations (**b**) over the incubation experiment.

Table 2. Average (± SD) molecular masses, number of molecular formulae, functional diversity (assessed from H : C ratios), atomic ratios, relative contribution of aromatic, highly unsaturated, saturated, unsaturated, unsaturated with nitrogen, degradation, and bioproduction indexes of the DOM assessed by FT-ICR MS and DOC : DON ratios assessed in the ambient seawater, control, and amended treatment at the beginning and end of the experiment. No replicates were analyzed from the ambient seawater.

		Control				Treatment			
		Time 0		Time final		Time 0		Time final	
	Environment	Average	SD	Average	SD	Average	SD	Average	SD
Molecular mass	414	410	116	418	122	414	123	413	115
Number of molecular formulae	2655	2968	396	3734	690	4163	720	2798	615
Functional diversity (H/C ratio)	0.092	0.095	0.003	0.092	0.002	0.105	0.004	0.090	0.001
C : N	31	31	1	29	1	27	1	31	1
C : P	576	500	83	407	60	381	47	508	144
C : S	194	184	16	168	17	159	17	194	13
% Aromatic	3.5	3.6	0.6	5.0	1.3	4.9	0.8	3.5	1
% Highly unsaturated	84	80	2.3	80	2.6	74	1.4	83	1.5
% Saturated	0.7	0.7	0.2	0.4	0.1	0.8	0.1	0.4	0.1
% Unsaturated	11	14	1.5	12	0.7	15	0.6	12	0.3
% Unsaturated with N	1.5	2.0	0.5	2.4	0.8	4.9	0.9	2.0	0.2
Degradation index (I_{DEG})	0.713	0.696	0.002	0.698	0.003	0.687	0.002	0.701	0.003
Bioproduction index (I_{BIO})	0.341	0.349	0.007	0.346	0.004	0.363	0.003	0.334	0.012
DOC : DON	7.6	6.4	1.0	7.9	0.7	5.8	0.3	4.9	0.8

Temporal changes in DOM molecular composition

PCA of the DOM molecular composition over the incubation time showed overlap of the samples of the DOM-amended treatment and those of the control (Fig. 2a). The first coordinate accounted for 28.5% and the second for 9.2% of the variation in DOM molecular composition. The values of principal component axis 1 (PC1) of DOM-amended samples significantly declined over the incubation time ($R^2 = 0.92$,

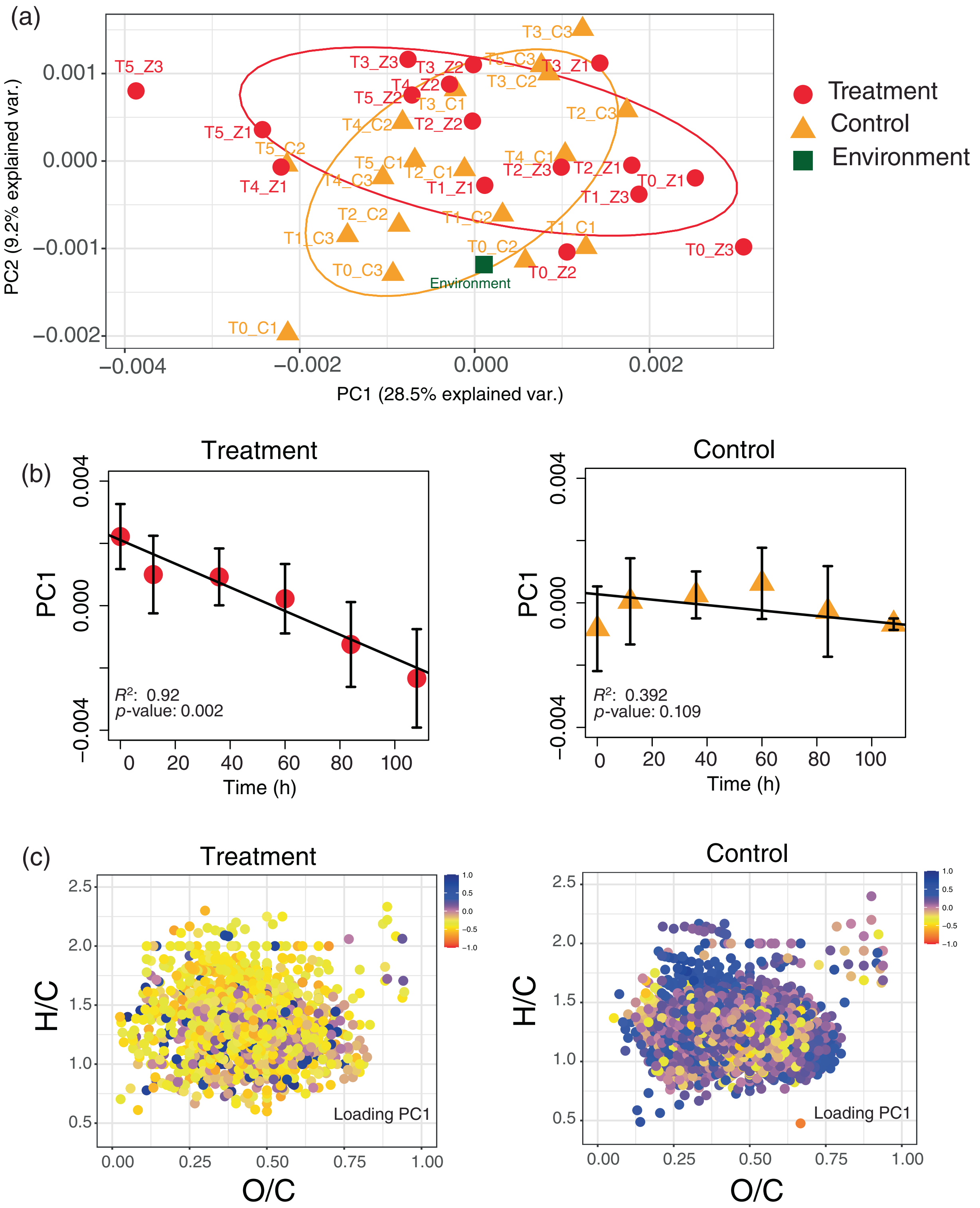

Fig. 2. PCA of the DOM molecular composition obtained with FT-ICR-MS in the DOM-amended treatment (red) and control (yellow) (**a**); variation of the PC1 over the incubation time in the DOM-amended treatment and control (**b**); Van Krevelen diagrams with colors showing the loadings of PC1 calculated from the unique zooplankton molecular formulae detected in the treatment and from the unique molecular formulae detected in the control (**c**).

$p = 0.002$), whereas the PC1 values of the control did not change significantly ($R^2 = 0.392$, $p = 0.109$; Fig. 2b). The Van Krevelen diagrams of the unique zooplankton molecular formulae showed lower (more negative) PC1 loadings in the treatment as compared to the unique molecular formulae detected in the control (Fig. 2c). The DOM-amended

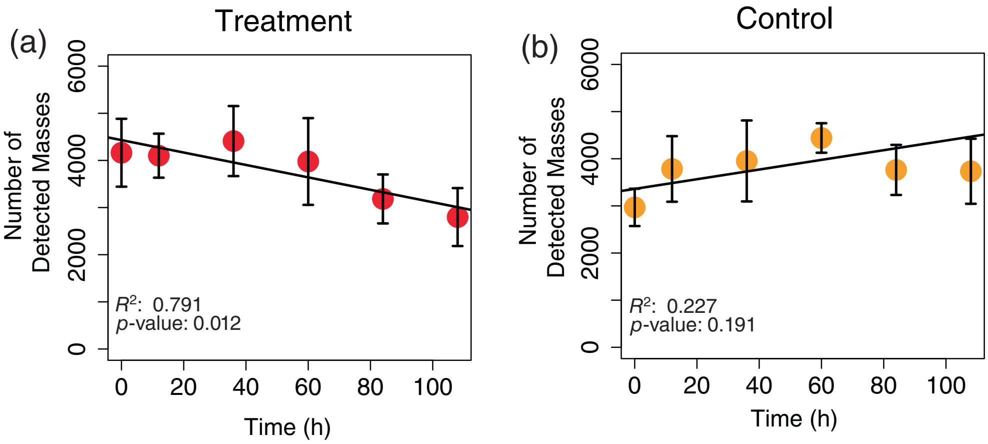

Fig. 3. Variation of the number of molecular formulae along the incubation time in the DOM-amended treatment (**a**) and control (**b**).

treatment and the control shared 2768 (~ 70%) molecular formulae at the beginning of the experiment. This common fraction represents the ambient marine DOM (Supporting Information Fig. S3). The DOM-amended treatment included 1194 (~ 30%) unique molecular formulae, representing the DOM derived from the zooplankton activity (Supporting Information Fig. S3).

To investigate the capability of the prokaryotic community to metabolize freshly released zooplankton DOM and how this affected the molecular diversity of the DOM, the changes in the number of detected molecular formulae were assessed along the incubation time. The total number of molecular formulae significantly declined over time in the samples of the DOM-amended treatment ($R^2 = 0.791$, $p = 0.012$; Fig. 3a), while it was higher at the end than at the beginning of the incubation experiment in the control (Table 2). In addition, we observed a ~ 33% reduction in total number of molecular formulae and a ~ 14% loss in functional diversity (Fig. 3a; Table 2; Supporting Information Fig. S4). Conversely, the number of molecular formulae and the functional diversity in the control did not change significantly over the incubation time (Student's *t*-test, $p > 0.05$) (Table 2; Fig. 3b; Supporting Information Fig. S4).

The DOM-amended treatment contained both zooplankton-derived and ambient DOM. Therefore, to assess the changes experienced only by the zooplankton-derived DOM fraction, the formulae detected with the FT-ICR-MS in the DOM-amended treatment at the beginning of the experiment were split in ambient (formulae shared between DOM-amended treatment and control) and unique molecular formulae (i.e., formulae only in the DOM-amended treatment). The number of ambient molecular formulae did not change over the incubation time ($R^2 = 0.159$, $p = 0.236$), while the number of zooplankton-derived formulae significantly declined by ~ 70% ($R^2 = 0.876$, $p = 0.004$; Fig. 4a,b).

Although the molecular composition of the assigned formulae remained similar in the control over the incubation period, it significantly changed in the DOM-amended samples (Table 2; Fig. 5). Unsaturated compounds were preferentially removed in the DOM-amended treatment, as their relative contribution to the total number of molecular formulae declined along the incubation time. Concurrently, the relative contribution of highly unsaturated compounds increased over time in the DOM-amended treatment (Table 2; Fig. 5).

The total number of nitrogen-containing molecular formulae significantly declined by ~ 37% at the end of the experiment in the DOM-amended treatment ($R^2 = 0.833$, $p = 0.007$) as compared to the control ($R^2 = 0.252$, $p = 0.177$; Supporting Information Fig. S5a,b). In contrast, the total number of nitrogen-containing formulae in the ambient fraction did not change significantly ($R^2 = 0.109$, $p = 0.274$; Supporting Information Fig. S5d) over the time course of the experiment. In addition, the number of nitrogen-containing molecular formulae of the zooplankton-derived DOM significantly declined by ~ 72% during the experiment ($R^2 = 0.918$, $p = 0.002$; Supporting Information Fig. S5e). The steep decline of the molecular formulae containing N in the DOM-amended treatment was also reflected in an increase of the C : N ratio from 27 ± 1 to 31 ± 1 over the course of the experiment ($R^2 = 0.910$, $p = 0.002$; Fig. 6a). This increase was associated to the significant C : N increase in the treatment of the zooplankton-derived DOM (from 21 ± 1 to 27 ± 3; $R^2 = 0.821$, $p = 0.008$) while the C : N of the ambient molecular formulae did not change significantly over time ($R^2 = -0.081$, $p = 0.474$; Fig. 6b).

Discussion

The results obtained from the incubation experiment indicate that the DOM produced by zooplankton activity was composed of sufficiently bioavailable compounds to prompt the exponential growth of the prokaryotic community. These bioavailable compounds were likely generated from the zooplankton excretion of digested phytoplankton food (Steinberg and Landry 2017). The DOM-amended treatment exhibited

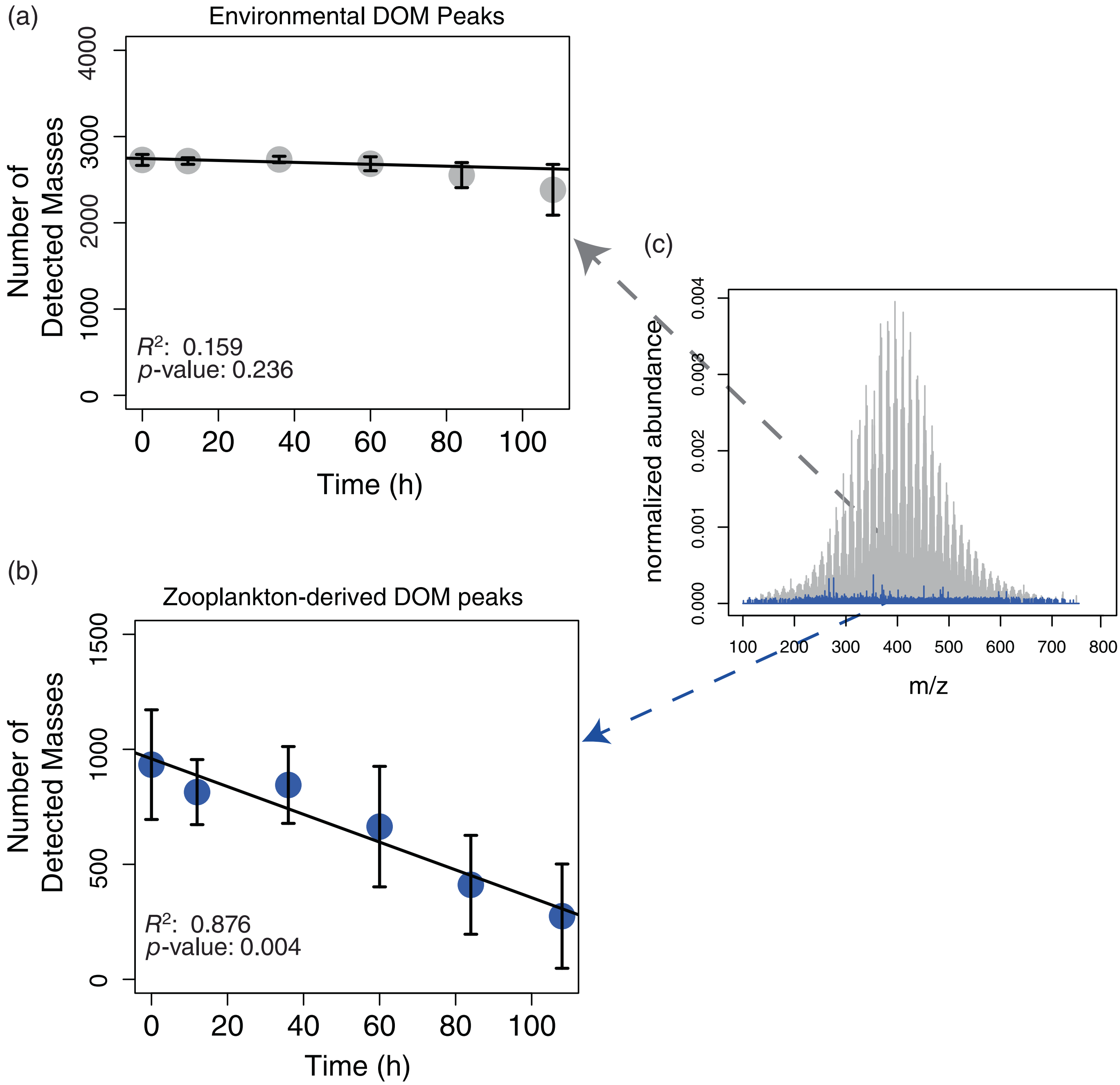

Fig. 4. Temporal dynamics of environmental background (**a**) and zooplankton-derived (**b**) DOM formulae in the amended treatment. Spectra of the normalized signal intensity vs. the mass to charge ratio (m/z) of the environmental background (gray) and zooplankton-derived DOM (blue, **c**).

higher silicate concentrations as compared to the control, suggesting that part of the zooplankton-derived DOM was generated from feeding on phytoplankton that incorporate biogenic silica, such as diatoms. Phytoplankton composition in the ambient water sampled during the experimental approximation was dominated by a bloom of the diatom *Pseudo-nitzschia* ssp. (> 60% in relative abundance, data not shown), which is frequently the dominant phytoplankton at this station and time of the year (Casas et al. 1999). Moreover, the drawdown of N-containing compounds in the DOM-amended treatment suggests their preferential uptake, agreeing with previous studies regarding the importance of zooplankton pellets to provide amino acids and vitamins (Clifford et al. 2017; Maas et al. 2020). Moreover, our results are consistent with the increase of bacterial biomass due to addition of zooplankton-derived DOM reported in other studies (Peduzzi

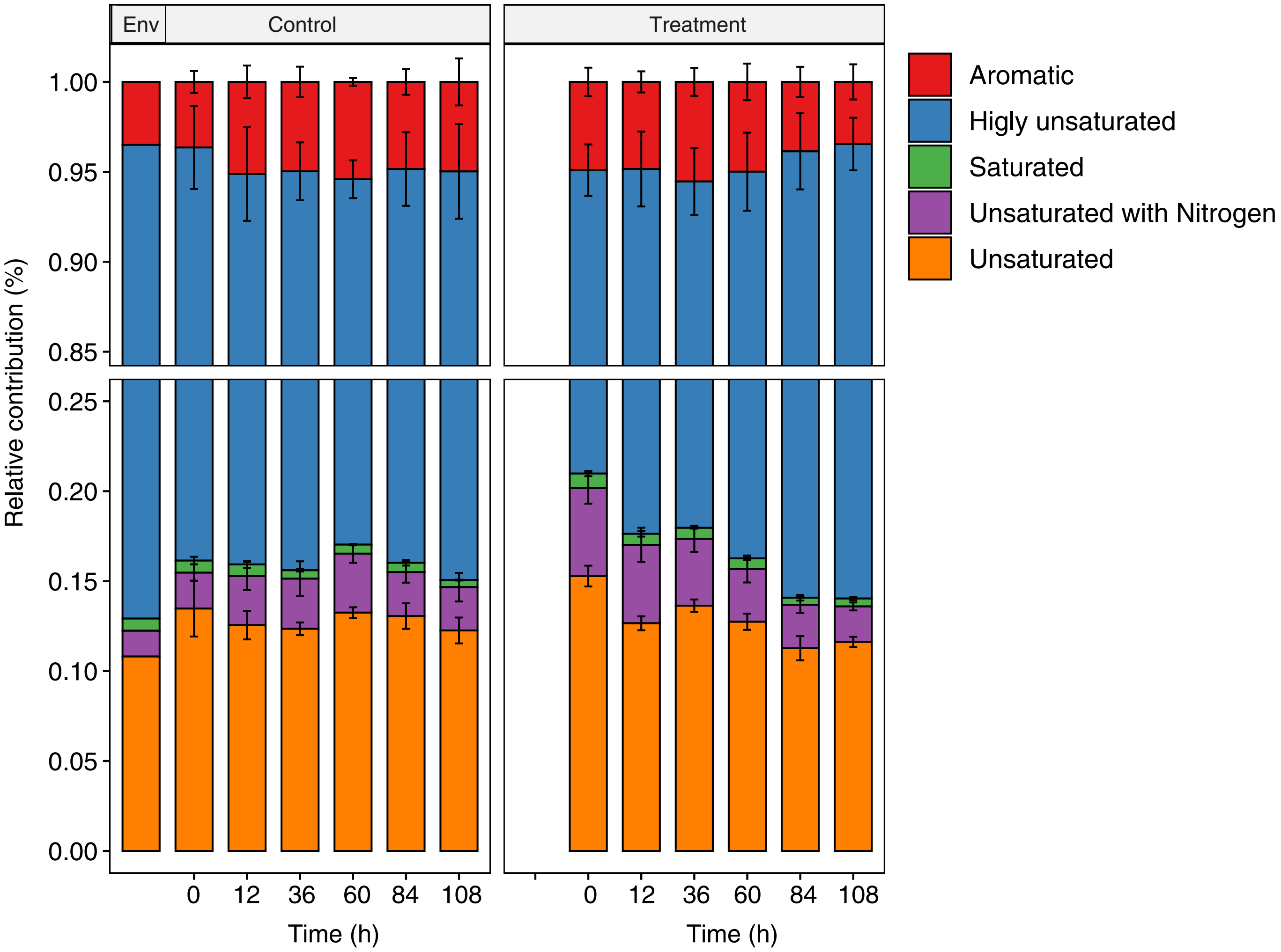

Fig. 5. Relative contribution of aromatic, highly unsaturated, saturated, unsaturated with nitrogen and unsaturated compounds over the 5-d incubation period in the environment, amended treatment and control.

and Herndl 1992; Hygum et al. 1997; Koistinen et al. 1997). Our data showed a DOC drawdown during the first 60 h of incubation, together with significant changes in the DOM molecular composition in the DOM-amended treatment, suggesting that prokaryotes rapidly metabolized the zooplankton-derived DOM (Fig. 2a,b). These findings further support that prokaryotes shape the molecular composition of marine DOM by remineralizing and transforming labile DOM (Ogawa et al. 2001; Lechtenfeld et al. 2015; Osterholz et al. 2015).

The zooplankton-derived SPE-DOM was characterized by 1194 unique molecular formulae that were preferentially metabolized by the prokaryotic community. More than 70% of these characteristic zooplankton-derived DOM compounds were removed by the end of the ~ 5-d incubation experiment, whereas ambient DOM showed lower degradation rate with little changes in the number of detected masses, probably due to relatively low proportion of labile compounds in the complex ambient DOM matrix (Fig. 4b). Although zooplankton are known to contribute to the labile organic matter pool through the release of DOM and POM (Steinberg and Landry 2017), our results show that zooplankton excreta also contribute through releasing secondary metabolites of intermediate to low molecular mass. These freshly released metabolites are a bioavailable source of carbon that can sustain prokaryotic activity and are therefore preferentially removed from the DOM pool.

The bioproduction and degradation indexes in the DOM-amended treatment indicate that the zooplankton-derived DOM was more bioavailable to prokaryotic degradation at the beginning of the incubation (Table 2). The DOM in the amended samples had an average C : N of ~ 4.7, while the unamended control samples had a C : N of ~ 6.7, in the range of typical DOM C : N ratios reported for marine ecosystems (Ogawa et al. 2001; Martiny et al. 2014). The differences in the DOM composition between the DOM-amended treatment

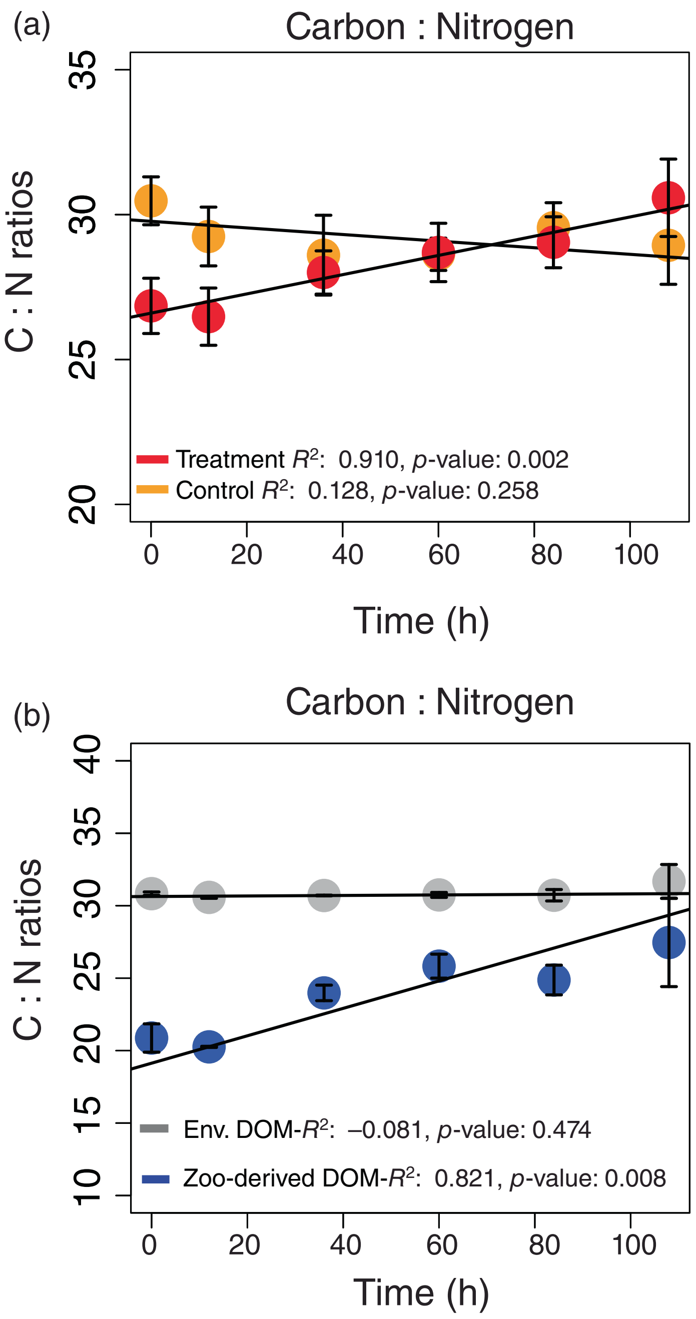

Fig. 6. Carbon to nitrogen ratios (C : N) of molecular formulae in the control and DOM-amended treatment (**a**) over the incubation time. Variation of the C : N ratio in the environmental background and zooplankton-derived DOM (**b**) along the experiment time period.

and the control suggest that the zooplankton excreta likely provided a source of organic nitrogen to the prokaryotic communities, for example, in the form of amino acids, peptides, organic acids, and so on (Clifford et al. 2017, 2019). Furthermore, the steep decline of the of N-containing molecular formulae together with the increase of the C : N ratio of the experimental samples over time (Fig. 6; Supporting Information Fig. S5) suggests that zooplankton not only stimulates inorganic nutrient cycling (Tang et al. 2010) but also provides a bioavailable source of organic nitrogen, a key element for prokaryotic growth in the ocean (Pajares and Ramos 2019).

In addition, unsaturated compounds and those containing nitrogen in the zooplankton-DOM amended samples accounted for $\sim$ 20% of the total detected molecular formulae. However, they exhibited the highest reactivity to prokaryotic degradation in the DOM-amended treatment compared to the control, as they rapidly declined over the incubation period. On the other hand, highly unsaturated compounds, accounting for $\sim$ 74% of the total detected molecular formulae appeared to be not susceptible to prokaryotic degradation, the observed increased in relative contribution along the incubation period in the DOM-amended treatment might at least partly be due to the decrease of unsaturated compounds. These highly unsaturated compounds are found in deep marine DOM that is more biologically recalcitrant (Medeiros et al. 2015). Moreover, the bioproduction and degradation indexes indicated that the zooplankton-derived DOM became less bioavailable over the course of the experiment. Overall, these results suggest that the prokaryotic degradation activity on the zooplankton-derived DOM changed the composition of the detected compounds by quickly removing highly bioavailable compounds and thus decreasing the bioavailability of the remaining DOM.

Previous studies (Møller et al. 2003; Møller 2007) showed that a large fraction of POM is transformed into DOM during zooplankton grazing on phytoplankton. Our results show that this DOM is a bioavailable source of organic matter that is preferentially metabolized by the prokaryotic community. These results suggest that a large fraction of the photosynthetically derived carbon released during the zooplankton excretion phase is shunted directly to the prokaryotic community instead of being transferred to the upper trophic levels, similarly to the viral shunt (Wilhelm and Suttle 1999). We therefore infer that zooplankton can either incorporate photosynthetic-derived carbon and make it available to the upper trophic levels through predation, or release it as DOM, promoting remineralization through the prokaryotic loop.

Our study targeted the fraction of zooplankton-derived DOM that falls into the analytical window of marine SPE-DOM, which is relatively lower in molecular weight and higher in hydrophobicity. Overall, we demonstrate here that zooplankton directly release lower molecular-weight DOM that is preferentially taken up by prokaryotes and assimilated into biomass, likely because the excreta is a source of labile DOC and DON. Prokaryotic processing changed the molecular composition of the zooplankton-derived DOM mainly through removing unsaturated compounds, which resulted in an increase of the relative proportion of highly unsaturated compounds and a decrease of functional diversity over time. It is likely that zooplankton-derived DOM is an important substrate for marine microbe, that has a relatively short turnover time in the ocean.

Data availability statement

All data described are available upon request from Daniele De Corte.

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Conflict of interest

The authors have no conflict of interest to declare.

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