LIMNOLOGY and OCEANOGRAPHY

ASLO

Limnol. Oceanogr. 67, 2022, 2360–2373

doi: 10.1002/lno.12206

Increased microbial and substrate complexity result in higher molecular diversity of the dissolved organic matter pool

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Abstract

Marine dissolved organic matter (DOM) represents one of the largest reduced carbon pools on Earth. Diverse microbial metabolic activities play important roles in shaping this pool. However, the links between changing DOM substrates and microbial communities and its influence on the microbial produced DOM remain poorly understood. In this study, we investigated how the addition of substrates with different complexity (glucose, laminarin, and *Synechococcus*-derived DOM) impacted the production of new DOM by an individual opportunist *Alteromonas* strain and a coastal natural microbial community. Our experiments reveal that the microbial complexity was more important than the substrate in shaping the molecular composition of DOM. Furthermore, our results showed that the DOM composition played a larger role in shaping the microbial compositions than the concentrations of DOM and inorganic nutrients. Combined our network analysis revealed that complex substrates resulted in higher molecular diversity of the microbial produced DOM, sustained a higher microbial diversity, and maintained a more diverse association between microbial communities and DOM molecules. Overall, the present study indicate that the microbial diversity contributes to DOM molecular diversity and vice versa, highlighting the importance of microbial–substrate interactions in shaping the marine DOM pool.

Dissolved organic matter (DOM) in the ocean, represents one of the largest reduced carbon pools on Earth (Hansell et al. 2012). In the surface ocean phytoplankton-derived organic matter is the primary source of labile organic substrates of which the majority is consumed by heterotrophic microbes within minutes/hours to days (Carlson and Hansell 2015). Heterotrophic bacteria metabolize these compounds and convert part of them into less bioavailable refractory DOM (RDOM), which is sequestered in the ocean at a rate of approximate 86 Tg C yr^{-1} (Hansell et al. 2009; Koch et al. 2014; Jiao et al. 2018). Therefore, the interactions between microbes and DOM play an important role in the global carbon cycling and regulating climate changes (Jiao et al. 2010; Lønborg et al. 2020).

Polysaccharides are among the most abundant molecules synthesized by phytoplankton and macroalgae (Becker et al. 2020; Arnosti et al. 2021) and they constitute important substrates for heterotrophic bacteria (Mühlenbruch et al. 2018; Koch et al. 2019). Previous DOM-amended (mainly adding phytoplankton-derived organic matter) experiments, have frequently found that members of the Alteromonadales group respond quickly to these additions, in terms of their cell abundance and activity (Mou et al. 2008; McCarren et al. 2010; Xie et al. 2020). A single *Alteromonas* strain has furthermore been found capable of utilizing a broad range of carbohydrates and consuming comparable amounts of DOC over short time scales (5 d) as the natural microbial communities (Pedler et al. 2014, 2015). In addition, the genus, *Alteromonas*, can contribute up to 30% of the total active bacterial community during

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

Author Contribution Statement: Q.Z., C.L., and N.J. conceived and designed the experiments. Q.C., Q.Z., Y.L., R.C., J.C., C.H., and Q.S. conducted the experiments. Q.C., Y.W., Y.L., F.C., R.C., M.G., C.H., and Q.S. analyzed the data. All of the authors assisted in writing the manuscript, discussed the results, and commented on the manuscript.

phytoplankton bloom conditions (Tada et al. 2011). These findings indicate that single opportunists, such as *Alteromonas*, likely have important roles in determining DOC and nutrient fluxes in the marine environment (Pedler et al. 2014).

Previous studies have primarily focused on the bacterial utilization and responses to the addition of different DOM substrates (Sarmento et al. 2016; Xie et al. 2020). Recently, the chemical composition of DOM produced by single bacterium and natural microbial community have also been investigated. For example, two *Roseobacter* strains have been shown to generate diverse molecules by metabolizing simple substrates (e.g., glutamic acid), thereby contributing to the high DOM chemodiversity and stability (Noriega-Ortega et al. 2019). An *Alteromonas* strain was furthermore shown to produce specific DOM compounds (e.g., pyrroloquinoline quinone) when growing on different carbohydrates (Pedler et al. 2015; Koch et al. 2019). For natural heterotrophic microbial communities, studies have shown rapid utilization of simple organic substrates and production of compounds with similar chemical composition and structural complexity to naturally seawater DOM (Lechtenfeld et al. 2015). However, the microbial produced DOM varies when different substrates (glucose and agal exudates) were amended (Koch et al. 2014). DOM are dominating substrates for microbes in the ocean but microbial metabolism can also impact the DOM composition (Kujawinski 2011), and the detailed microbe–substrate interactions have been investigated in both field (Osterholz et al. 2016; Osterholz et al. 2018) and experimental studies (Zhao et al. 2019). Whereas, no study, to our knowledge, so far has compared how single bacterium vs. natural communities interact with DOM substrates of different complexity and shape the produced DOM pool.

In this study, we determined how distinct substrates (from "simple" to "complex") impact the production and composition of DOM released by an individual heterotrophic bacterial strain and a natural microbial community. This was conducted by growing marine opportunistic copiotroph strain *Alteromonas macleodii* and a natural coastal microbial community on three different organic substrates (glucose, laminarin, and *Synechococcus*-derived DOM [*Syn*-DOM]). Overall, our aims were to (1) determine how a single bacterial strain compared with a natural microbial community utilize and transform different substrates; (2) show how organic substrates (from "simple" to "complex") shape the chemodiversity and microbial communities; and (3) reveal how the (re)processing of phytoplankton-derived metabolites by heterotrophic bacteria contributes to the marine DOM pool.

Materials and methods

Experimental setup

Artificial seawater (ASW) was modified from Kester et al. (1967), containing NaCl (24 g L^{-1}), KCl (0.67 g L^{-1}), KBr (0.1 g L^{-1}), NaF (0.04 g L^{-1}), H_3BO_3 (0.03 g L^{-1}), $CaCl_2$ (0.05 g L^{-1}), $NaHCO_3$ (0.2 g L^{-1}), $MgSO_4 \cdot 7H_2O$ (5 g L^{-1}), $NaNO_3$ (2 mg L^{-1}), NH_4Cl (1.25 mg L^{-1}), and KH_2PO_4 (0.45 mg L^{-1}) dissolved in Milli-Q water. The 1st four salts were precombusted (450°C, 6 h) before use. For the *Syn*-DOM preparation, cells were harvested by centrifuged during the exponential growth phase, washed, and resuspended in Milli-Q water, subsequently disrupted by freeze–thaw and using a high-pressure cracker (Constant, TS 0.75 kw, UK). Lysate were thereafter filtered through a precombusted (450°C, 6 h) GF/F (nominal pore size 0.7 μm, Whatman) filter and subsequently used as experimental substrate for the natural microbial community. Surface seawater was collected at a station close to Xiamen Island, China (~24°N, ~118°E; March 2019) and filtered through a precombusted GF/F filter to remove particulate organic matter and plankton, especially predators of bacteria, and stored in the dark for 3 d as the "natural" microbial community, and added in a ratio of 1 part of microbial culture to 49 parts of ASW as described before (Ogawa et al. 2001). Members of the Alteromonadales group contribute up to 6.3% of the total microbial community in our sampling area for one and half year observation and their relative abundance were 4.7% before the dilution step (data from Chen et al. 2021). Three different substrates (glucose, laminarin, and *Syn*-DOM) were prepared in triplicate at final concentration of ~ 265 μmol C L^{-1} and duplicated controls (ASW only) received microbial inoculum without amended substrate. The amended substrate concentrations were comparable to DOC levels found during coastal bloom periods (Billen et al. 1987; Oh et al. 2018). In total for the natural experiment, 11 20-L polycarbonate bottles (acid washed and rinsed with Milli-Q water) were incubated for 180 d in the dark at 26°C. Results are presented as averages of three replicates, except in the control experiments (only two replicates).

Type strain *A. macleodii* ATCC 27126 (GenBank: GCA_000172635.2) was grown initially on the glucose medium, and bacterial cells were pelleted and washed three times with ASW before use. Three different substrates, glucose, laminarin, and extracted *Syn*-DOM were added at final concentration of ~400 μmol C L^{-1} except for the extracted *Syn*-DOM which was added at final concentration ~700 μmol C L^{-1} in order to maintain similar maximum bacterial abundance. Control group was only ASW inoculated with *Alteromonas*. For the *Alteromonas* experiments, 12 1-L glass bottles (acid washed, rinsed with Milli-Q water, and precombusted) were incubated for 60 d in the dark at 26°C. All results are presented as averages of three replicates. It should be noted that the natural microbial community and *Alteromonas* incubations are two batch of experiments, and incubation time (180-d vs. 60-d), volume (20 L vs. 1 L), and substrate concentration were different and could potentially affect the final molecular composition of the bacterial produced organic matter. However, we mainly focused on how distinct substrates (from simple to complex) impact the composition of DOM released by the natural microbial community and microbial–DOM molecular interactions (Figs. 2–4), and the single-strain *Alteromonas* was primarily used for comparison (Figs. 1, 5).

In order to compare the chemical composition of the produced organic molecules with natural DOM, the molecular compositions were compared with data from 33 samples collected around Xiamen Island, China (~24°N, ~118°E) during December 2017 and July 2019 (Chen et al. 2021; Supporting Information Fig. S1).

Sample collection

In the natural microbial experiments, each bottle was subsampled at eight time points (Days 0, 1, 3, 7, 16, 110, and 180) for bacterial abundance, DOC, FDOM, microbial community composition, inorganic nutrients, and DOM extraction for chemical analysis. In the *Alteromonas* experiment, each bottle was subsampled for bacterial abundance, DOC, and FDOM at seven time points (Days 0, 2, 4, 7, 15, 30, and 60), while DOM extraction for chemical analysis was performed on Days 0, 4, and 60. Briefly, unfiltered 2 mL samples for bacterial abundance were collected and fixed with glutaraldehyde (1% v/v) and kept at −80°C until analysis. For DOM analysis, 200 mL of sample water from the *Alteromonas* experiments were filtered through a 0.3-μm GF 75 filter (precombusted, Whatman), while in the natural microbial experiments 700 mL of sample water was filtered through a 0.22-μm polycarbonate filter (prewashed with 1 L Milli-Q water, Millipore). The filters from the natural microbial experiments were preserved at −20°C and subsequently used for microbial community analysis. Subsequently, these filtered water samples (20 mL) were stored in 40-mL glass vials at −20°C until analysis. For DOM extraction, 100 mL or 500 mL filtrate was acidified to a pH of 2 in a 250-mL or 1-L glass bottle for the *Alteromonas* and natural microbial experiments, respectively. In addition, 40 mL of filtrate from the natural microbial experiments was transferred to duplicate 50-mL clean tubes and stored at −20°C for inorganic nutrient analysis. All glassware was acid washed, rinsed with Milli-Q water, and precombusted before use.

Inorganic nutrient and DOM analysis

Inorganic nutrient concentrations (phosphate, nitrate, and nitrite) were measured using a PowerMon Kolorimeter AA3 (Bran+Luebbe, Co.), following the methods previously described (Knap et al. 1996), while ammonium was measured using a fluorometric method (Holmes et al. 1999). The DOC concentrations were measured by high-temperature (680°C) catalytic oxidation using a Shimadzu TOC-VCPH analyzer. Samples were defrosted and acidified to a pH of 2 with phosphoric acid. Concentrations were determined by subtracting a Milli-Q water blank and dividing by the slope of a standard curve made from potassium hydrogen phthalate standards. The consistency between runs was verified by comparing peak areas of "standard solutions" (0.2-μm filtered coastal seawater) with no large deviations found. In addition, Milli-Q water blanks inserted every 6th sample in order to correct for variations during the sample runs. The difference between the initial and final DOC concentrations was in this study defined as the bioavailable DOC.

Excitation (Ex)–emission (Em) matrices (EEMs) were generated using the Horiba Aqualog system. Samples were defrosted and allowed to reach room temperature and then scanned from 240 to 600 nm at 5 nm intervals using a 1-cm path length quartz cuvette. Emission scans ranged from 248 to 829 nm with 2.33 nm intervals at a 2-s integration time. Parallel factor analysis (PARAFAC) was performed to analyze data into individual fluorescent components in Matlab 2018 (Murphy et al. 2013). All fluorescence intensities of the samples were normalized to Raman units (RU) by Milli-Q (excitation at 350 nm, emission from 371 to 428 nm) (Lawaetz and Stedmon 2009).

For the solid-phase extraction (SPE) of DOM, 100 or 500 mL of filtered and acidified (pH 2) water sample were extracted using cartridges (200 mg, Agilent Bond Elut PPL) as described previously (Dittmar et al. 2008). The estimated SPE extraction efficiency of DOM was approximately 30–50% in the latter period of incubations. The Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR MS) analysis was performed using a 9.4 T Bruker Apex Ultra with an Apollo II electrospray ion source operated in negative mode as described previously (He et al. 2020). Briefly, the operating settings for the negative-ion ESI analysis include spray shield voltage (4.0 kV), capillary column introduced voltage (4.5 kV), and capillary column end voltage (320 V). Ions accumulated in the collision cell for 0.2 s before being transferred into the ICR cell with a time-of-flight of 1.1 ms. The mass range is between 200 and 800 Da. Samples dissolved in methanol were injected into the electrospray source at an infusion flow rate of 250 μL h^{-1} and 128 single scans were added for each mass spectrum. Subsequently, a high-quality mass peak (m/z 371.0620 corresponding to $[C_{15}H_{16}O_{11}-H]^-$) at the center of the mass range was selected as a reference peak. The resolution power of this peak was higher than 280,000. Two sets of mass peaks near the most abundant ones in the spectrum were selected to evaluate the relative abundance distribution. For calibration, the mass spectrometer was initially calibrated with sodium formate and recalibrated with a known mass series of the Suwannee River fulvic acids, which provides a mass accuracy of 0.2 ppm or higher for the mass range of interest. The mass peaks with signal-to-noise (s/n) ratio greater than 4 were exported for the formula assignment and s/n ratio greater than 6 were selected for further analysis. Matched formulas consist of the elemental combinations of $^{12}C_{1-60}$, $^{1}H_{1-120}$, $^{14}N_{0-3}$, $^{16}O_{0-30}$, and $^{32}S_{0-1}$. The relative abundance of identified formulas (CHO, CHON, CHOS, and CHONS), average intensity-weighted m/z (mass-to-charge ratio), and ratios of hydrogen to carbon (H/C) and oxygen to carbon (O/C) were then calculated for each sample (Sleighter et al. 2009). Double-bond equivalents (DBE) and modified aromaticity index (AI_{mod}) of the identified molecular formulas were calculated by the number of element atoms as previously described (Koch and Dittmar 2006; He et al. 2019).

Bacterial abundance measurement and the microbial community

Samples for bacterial abundance was measured on thawed samples by staining with the nucleic acid-specific dye SYBR Green I (Invitrogen) (Marie et al. 1997) for 15 min in the dark and analyzed using a flow cytometry (BD Accuri C6). The cell counts were measured based on the targeted detector (FL1, 530 ± 30 nm) and two additional detectors, next to forward (FSC) and side (SSC) scatter.

In the natural experiment, the total microbial DNA was extracted using the phenol-chloroform-isoamyl alcohol method as described by Wang et al. (Wang et al. 2017). To determine the microbial community structure, the V4-V5 region (forward primers 515F 5′-GTGCCAGCMGCCGCGGTAA-3′ and reverse primers 907R 5′-CCGTCAATTCMTTTRAGTTT-3′) of the bacterial 16S rRNA gene of the DNA samples were amplified using the PCR procedure (Flores et al. 2011). Quantified amplicons were sequenced using the Illumina MiSeq platform (Illumina, details see Supporting Information).

Statistical analysis

The significance between the two groups data comparison was tested using TTEST. The principal component scores and molecular characteristics difference were compared by ANOVA and PERMANOVA using the package "multcomp." Alpha-diversity was determined by the Shannon and richness indices (Faith 2006) using the package "picante." Principal component analysis (PCA) was used to investigate the compositional differences of molecular characteristics of the *Alteromonas* and natural microbial community processing the three substrates. Redundancy analysis (RDA) was applied to access the microbial population successions (normalized with "Hellinger" method) in the natural community experiments with different DOM and inorganic nutrients levels (log transformed). The PCA and RDA were performed using the package "vegan". The associations between the individual amplicon sequence variants (ASVs) and molecular formulas (relative abundance/intensity defined > 0.01%) are presented in the co-occurrence network based on Spearmen's rank correlation coefficient of $r^2 \geq 0.95$, $p < 0.01$. Corresponding R codes were derived from GitHub (https://github.com/ryanjw/co-occurrence) (Williams et al. 2014). The multiple testing of the correlations was corrected (Q-value < 0.05) using the package "fdrtool." All of the statistical analyses were performed in R 3.6.1 (www.R-project.org), and network visualizations were conducted in Cytoscape V3.7.2.

Results

Variation of microbial abundances and nutrients

In the *Alteromonas* experiments, bacterial abundance increased rapidly from initial levels of $\sim 2.9 \pm 0.5 \times 10^5$ cells mL^{-1} to $\sim 1.6 \pm 0.3 \times 10^7$ cells mL^{-1} on Day 2, and decreased to $\sim 2.8 \pm 0.5 \times 10^6$ cells mL^{-1} at the end of the incubations. The DOC bioavailabilities were 86.2% ± 0.7% in the glucose addition, 80.7% ± 0.4% in the laminarin addition and 56.4% ± 2.2% in the *Syn*-DOM addition experiments, respectively (Supporting Information Fig. S2a,b).

When incubated with natural microbial community, the bacterial abundance increased from $\sim 1.4 \pm 0.6 \times 10^5$ cells mL^{-1} to $\sim 1.1 \pm 0.1 \times 10^7$ cells mL^{-1} on Day 3, thereafter decreased to $\sim 3.5 \pm 0.5 \times 10^6$ cells mL^{-1} by the end of the incubations. The bioavailable parts of DOC were 92.0% ± 0.1% in the glucose, 89.7% ± 0.1% in the laminarin and 82.6% ± 0.1% in the *Syn*-DOM experiments, respectively (Supporting Information Fig. S2c,d).

In the natural microbial community experiments, the initial decrease in DOC concentrations generally were followed by a clear initial decline in nutrient concentrations (except nitrate and nitrite) which was followed by a gradual increase until the end of the incubations (Supporting Information Fig. S2c–f). However, the concentration of nitrate and nitrite remained stable except a slight decrease in the glucose experiment (Supporting Information Fig. S2g).

Fluorescent DOM utilization and production

Three fluorescent components were identified using PARAFAC analysis (Supporting Information Fig. S3). It should be noted that we compared the fluorescence intensity dynamics of thawed samples in our incubations. Component C1 (Ex/Em wavelengths: 270/314 nm) was defined as protein-like FDOM which is associated with bacterial metabolic activities and belongs to autochthonous components (Goncalves-Araujo et al. 2015). The fluorescence intensities of component C1 slightly increased in the glucose and laminarin experiments while it decreased in the *Syn*-DOM in both the *Alteromonas* and natural microbial experiments (Supporting Information Fig. S3d,g). Component C2 (Ex/Em wavelengths: [< 245], 360/456 nm) was similar to humic-like FDOM which is related to biopolymers (Soto Cárdenas et al. 2017). The fluorescence intensities of C2 increased in both glucose and laminarin additions in both the *Alteromonas* and natural microbial experiments (Supporting Information Fig. S3). In the *Syn*-DOM, C2 remained stable in the *Alteromonas* but decreased in the natural microbial experiments after Day 3 (Supporting Information Fig. S3e,h). The other humic-like component C3 (Ex/Em wavelengths: [< 240], 315/385 nm), which has a microbial/marine humic-like signature (Goto et al. 2017), accumulated in all treatments (Supporting Information Fig. S3f,i). According to their bioavailability and changes in fluorescence intensities over incubations, C2 and C3 could be classified into semi-labile and recalcitrant DOM components, respectively.

Molecular composition and chemodiversity

A summary of molecular characteristics of the identified DOM compositions for all experiments are shown in Supporting Information Table S1. A PCA was conducted to investigate the molecular compositional differences of the *Alteromonas* and natural microbial experiments when processing different substrates (Fig. 1a). The 1st principal component PC1 explained 54.5% of the variability in the DOM compositions showing that the chemical composition was significantly different between the experiments with single bacterial species and natural microbial communities (ANOVA, $p < 0.001$). The 2nd principal component (PC2) explained 22.5% of the variability in the dataset, suggesting a clear impact of different substrates on the composition of the produced DOM (ANOVA, $p < 0.001$) (Fig. 1b). The PERMANOVA test demonstrated that the microbial composition had significant influence ($R^2 = 0.52$, $p = 0.001$) on the molecular characteristics, while substrate showed less of an impact ($R^2 = 0.22$, $p = 0.034$) on the molecular characteristics. Combined this also suggests that the composition of the microbial communities was more important in shaping the DOM molecular compositions than the different substrates. In addition, the 1st axis is positively associated with richness, O/C, *m/z*, CHONS, and DBE, but negatively associated with H/C, suggesting that the natural microbial community produced more oxidized molecular formulas with high-molecular weight containing CHONS atoms, as well as, more unsaturated molecular formulas. Contrary, the *Alteromonas* experiments contained more hydrogenated molecular formulas. Along the axis of the PC2, there are positive association with CHON and CHOS, which are negatively associated with CHO and AI_{mod}. This indicates that the *Syn*-DOM experiment contained N- and S-rich molecular formulas. While the simple compounds (glucose or laminarin) resulted in more aromatic molecular formulas with CHO atoms.

At the end of the incubations, the number of identified molecular formulas in all three substrates was significantly higher in the natural microbial (3272 ± 386) compared to the *Alteromonas* (1257 ± 764) experiments (TTEST, $p < 0.05$) (Supporting Information Fig. S4). Assigned molecular formulas between the natural microbial community and *Alteromonas* experiments were higher in the *Syn*-DOM (2910 ± 1091) compared to the simple compounds (both glucose and laminarin, 1942 ± 1305) (TTEST, $p < 0.5$). Based on the presence and absence of molecular formulas, the mutual molecular formulas

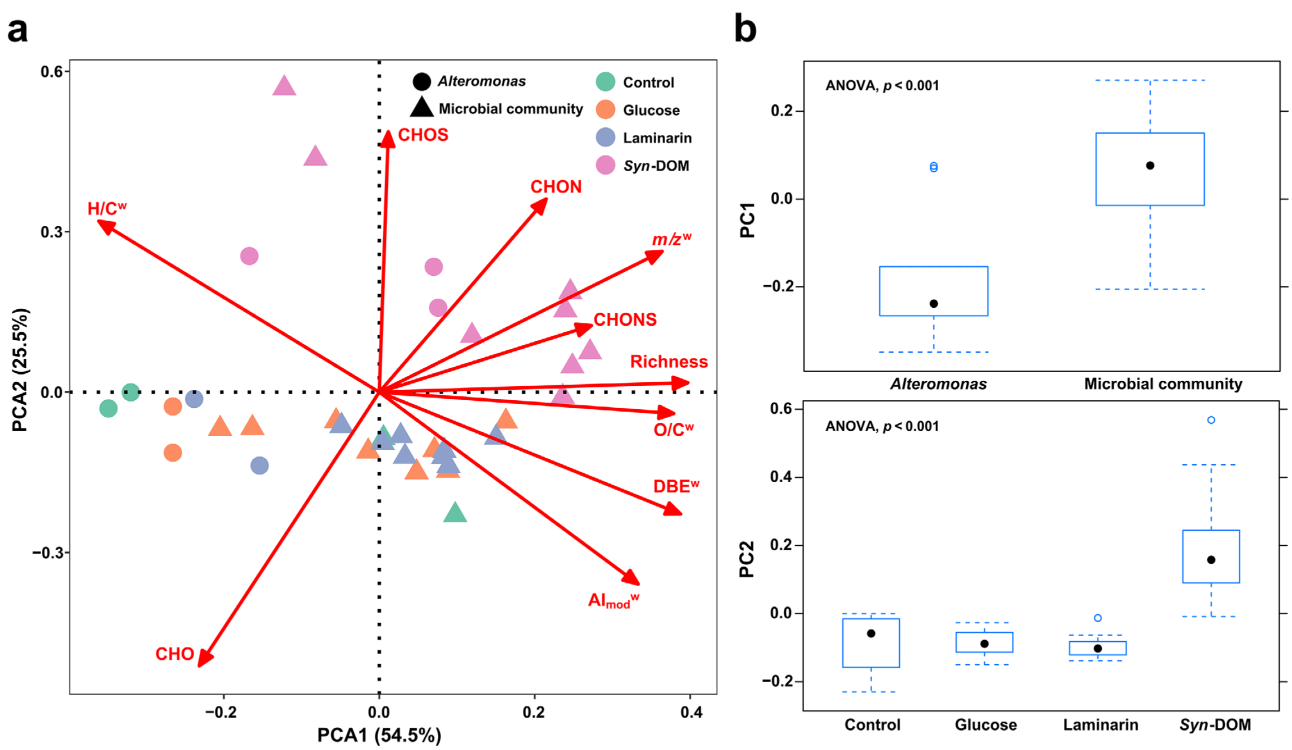

Fig. 1. PCA plots derived from organic molecular characteristics (molecular richness, relative abundance of identified formulas [CHO, CHON, CHOS, and CHONS], average intensity-weighted *m/z* [mass-to-charge ratio], ratios of hydrogen to carbon [H/C] and oxygen to carbon [O/C], DBE, and AI_{mod}) in different amended substrates (control, glucose, laminarin, and *Syn*-DOM) of the *Alteromonas* and natural microbial community experiments (**a**). The significance of groups comparison for microbial compositions of the 1st principal component (PC1) and substrate compositions of the 2nd principal component (PC2) is displayed on the right side, respectively (**b**).

between our *Alteromonas* and natural microbial experiments with natural coastal DOM were higher in the *Syn*-DOM (2727 ± 990) compared to the simple compounds (1841 ± 1246) DOM (TTEST, $p < 0.5$). The number of mutual molecular formulas between microbial produced and environmental DOM were significantly higher in the natural microbial community experiments (3086 ± 326) compared to the *Alteromonas* (1186 ± 729) experiments (TTEST, $p < 0.05$).

Microbial successions and alpha-diversity in the natural microbial incubations

A total number of 667 ASVs were identified based on the 16S rRNA gene sequences recovered from the natural microbial community incubated with the three substrates. The major bacterial groups, Alphaproteobacteria, Gammaproteobacteria, and Bacteroidetes comprised 95.8% of total microbial community (Fig. 2a). The contribution of Alteromonadales accounted for 27.6% of the total microbial community during the experiments. In the simple compound (glucose or laminarin) experiments, the relative abundance of Gammaproteobacteria increased rapidly on the 3rd day, and then decreased until the end of the incubations. Contrary, the relative abundance of Alphaproteobacteria and Bacteroidetes decreased until Day 3, and thereafter increased their overall contribution to the community. In the *Syn*-DOM experiment, Gammaproteobacteria was dominant during the 1st 3 d, and then decreased until reaching a steady level at Day 7. Bacteroidetes reached the highest contribution on Day 7, and then slowly decreased. Similar trends were observed in the calibrated bacterial abundances, obtained by multiplying the bacterial abundance by their relative contribution, with Gammaproteobacteria showing a quick growth during the 1st 3 d where after it declined, while the growth of Bacteroidetes and Alphaproteobacteria was slower (Fig. 2b).

The analyzed alpha-diversities (mean species diversity) of the microbial communities in the simple compound (glucose or laminarin) experiments decreased during the 1st 3 d, and increased thereafter (Fig. 2c,d). However, the alpha-diversity in the *Syn*-DOM experiment increased gradually throughout the experimental period. In addition, alpha-diversity (both richness and Shannon index) was significantly higher in the *Syn*-DOM (115.3 ± 8.1 and 3.6 ± 0.2) compared to the simple compounds (both glucose and laminarin, 67.5 ± 5.8 and 2.3 ± 0.6) at the end of the incubations ($\text{TTEST}_{\text{richness}}$, $p < 0.001$, $\text{TTEST}_{\text{Shannon}}$, $p < 0.05$). This suggests that the microbial community with *Syn*-DOM maintained a higher microbial diversity compared to the simple compound experiments.

Linking DOM, inorganic nutrients, and microbial groups

A RDA was used to gain insights into the microbial successions when the natural microbial community was incubated

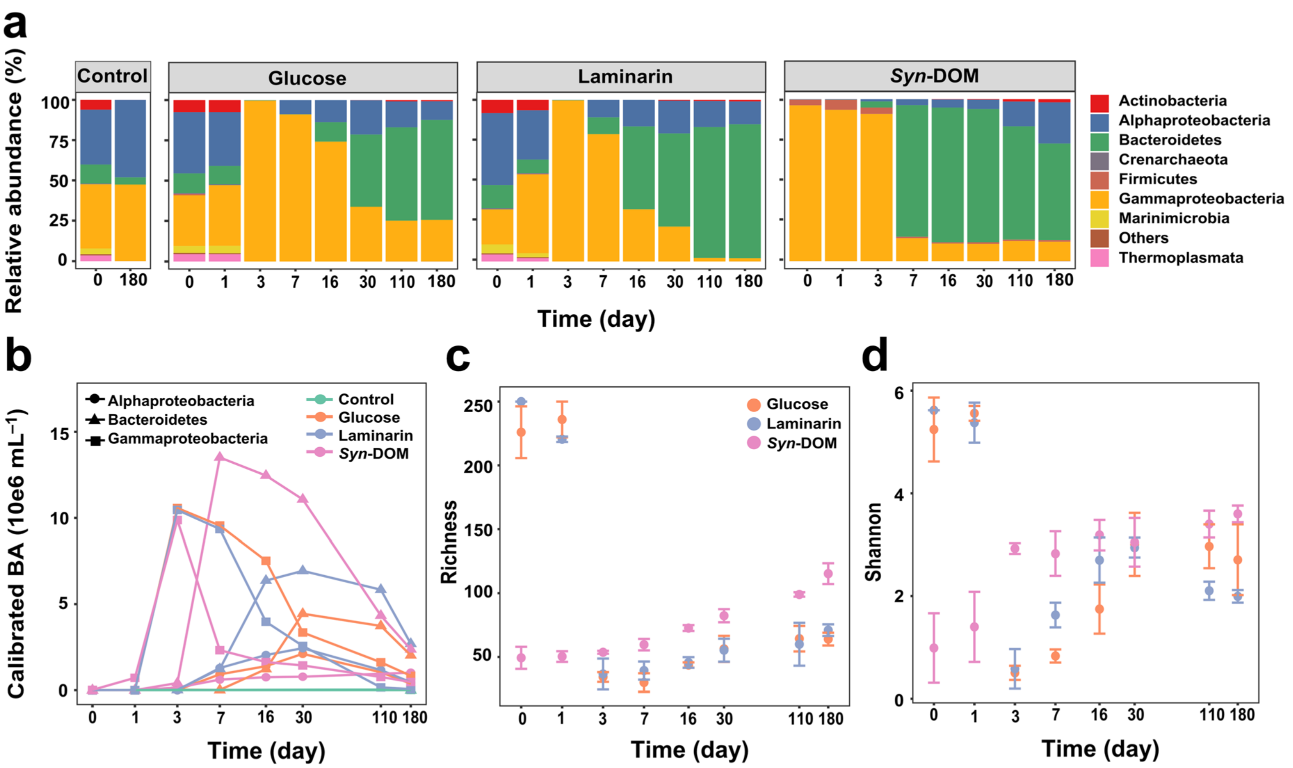

Fig. 2. Changes in the relative abundance of microbial groups in the control and three addition experiments (glucose, laminarin, and *Syn*-DOM) (**a**). Calibrated bacterial abundance of the dominant groups based on individual relative abundance multiplied by total bacterial abundance (**b**). Alpha diversity (richness and Shannon, respectively) during the incubation period (**c,d**). Note that the *x*-axis (time) is log-transformed.

with different substrates (Fig. 3a). The weights explained for the 1st axis (61.6%) and the 2nd axis (17.9%) accounted for 79.5% of microbial compositional changes. Furthermore, partial constrained factors analysis showed that the DOM composition (51.6%) was the most important factor followed by the inorganic nutrients (12.1%) and DOC concentration (1.0%) in determining the microbial community compositions (Fig. 3b). The results also revealed that Alphaproteobacteria was positively correlated with CHO, Gammaproteobacteria was positively correlated with CHOS and H/C, and Bacteroidetes was positively correlated with CHON, CHONS, O/C, *m/z*, DBE, and AI_{mod}.

Co-occurrence network between the microbial community and DOM compositions

To further investigate potential links between the microbial community and DOM molecular compositions, network analysis based on Spearmen's rank correlation coefficient was applied to the individual ASV and molecular composition data (Fig. 4). The number of ASVs and molecular formulas generally increased from glucose (44 and 55) to laminarin (116 and 104) and *Syn*-DOM (73 and 405) experiments, and the degree (number of formulas connected to an ASV) also rose from glucose to laminarin and *Syn*-DOM (168, 286, and 1465, respectively). This suggested a much closer and more complex relationship between microbes and DOM composition with increasing substrate complexity and the complex *Syn*-DOM substrate strengthen the microbial–DOM interactions compared to the simple compounds (both glucose and laminarin). In addition, subnetworks demonstrated that Alteromonadales ASVs mostly were associated with CHO (19), followed by CHON (14) and CHOS (5) containing formulas, and that most Alteromonadales ASVs were included in the laminarin network.

Discussion

In this study, we investigated the combined effects of substrate (glucose, laminarin, and *Syn*-DOM) and microbial complexity (*Alteromonas* culture and natural community) on the DOM chemical composition and microbial–substrate interactions (Fig. 5). The ASW incubation system, excluding effects from in situ background DOM, provided intuitively occurring interactions between microbial and organic molecular compositions. Our study demonstrates positive relationships between chemical and microbial diversity, and that successive microbial populations continuously contribute to the formation of new DOM molecules.

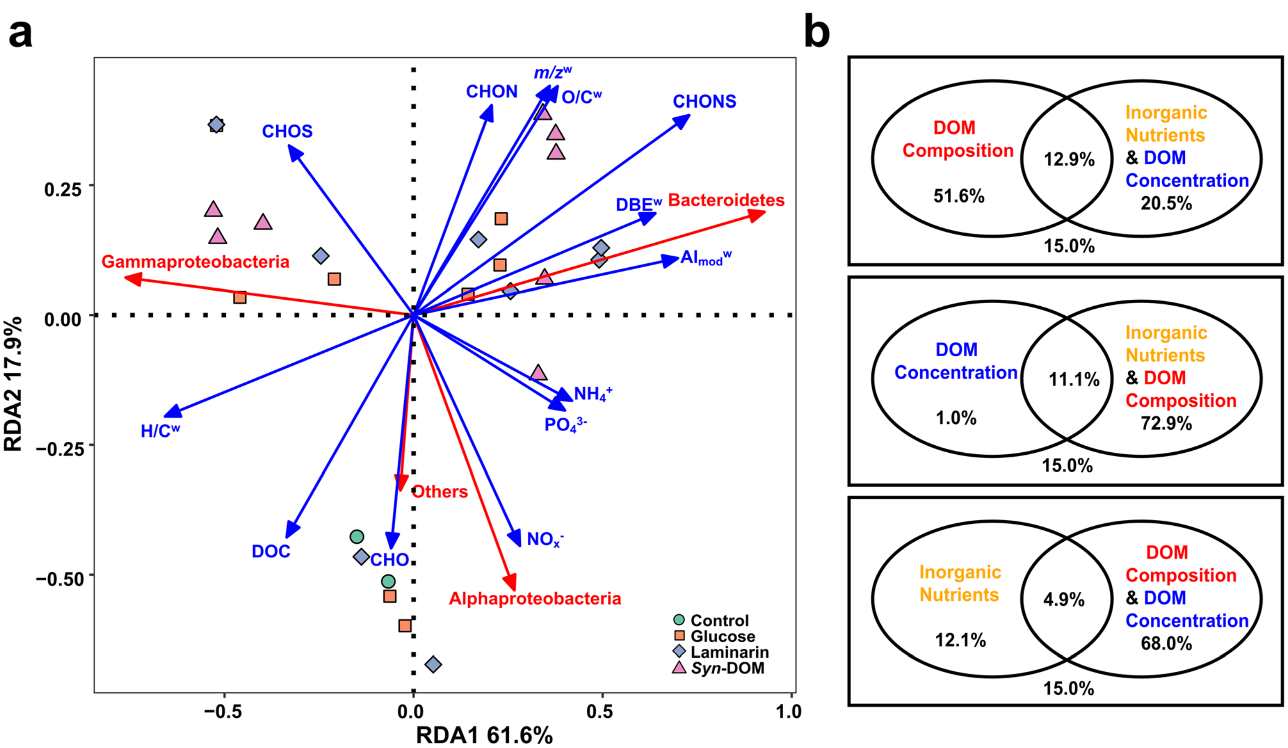

Fig. 3. RDA plots derived from microbial populations, DOM concentration and DOM composition (molecular richness, relative abundance of identified formulas [CHO, CHON, CHOS, and CHONS], average intensity-weighted *m/z* [mass-to-charge ratio], ratios of hydrogen to carbon [H/C] and oxygen to carbon [O/C], DBE, and AI_{mod}), and inorganic nutrient concentrations (phosphate, nitrate and nitrite, and ammonium) during the experiments (**a**). Partial constrained factors analysis of DOM concentration and DOM composition, and inorganic nutrients contributed to the shift of microbial community compositions (**b**).

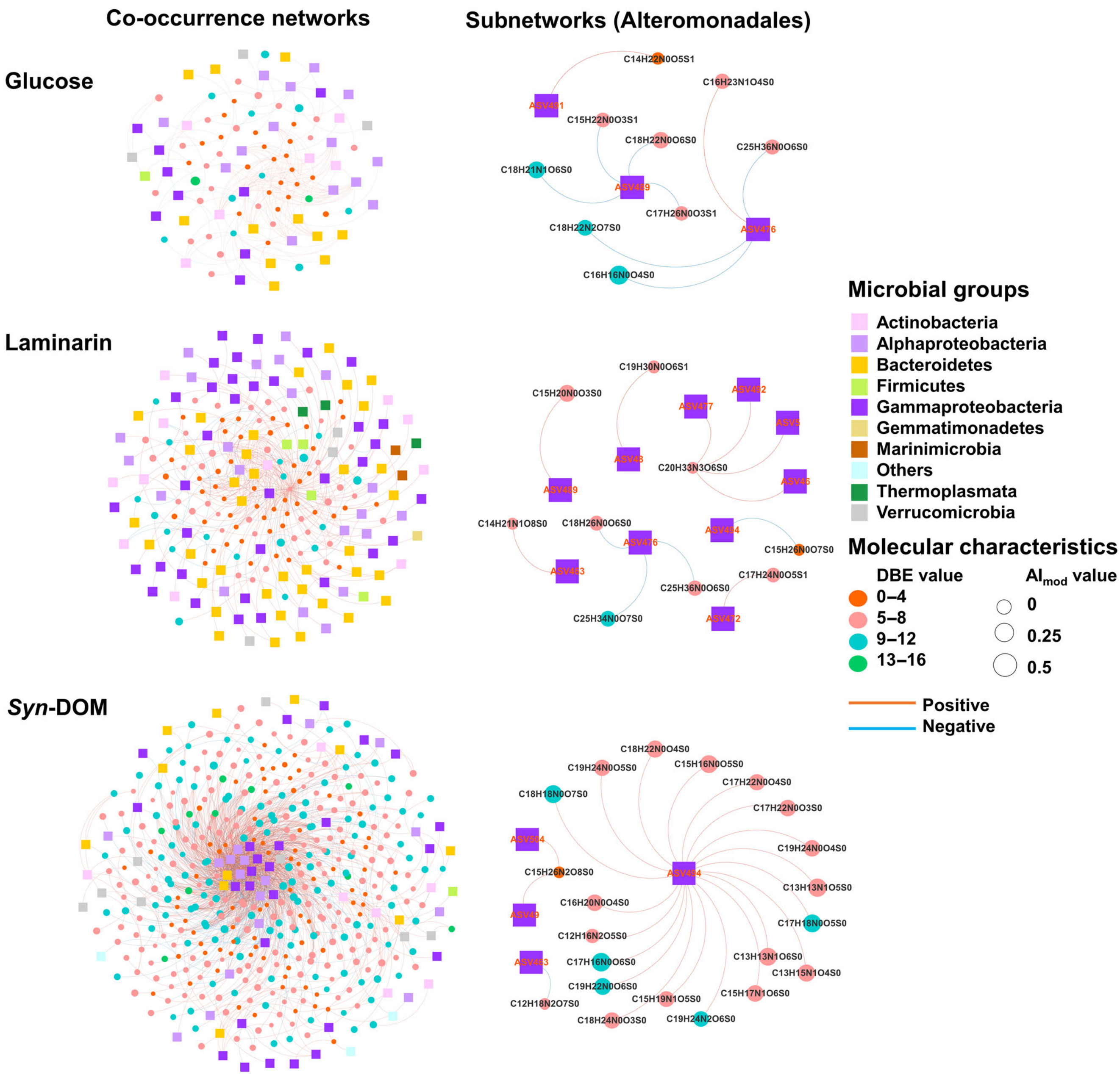

Fig. 4. The co-occurrence network based on Spearman's correlation between the microbial ASVs (square nodes) and the DOM molecular formulas (circle nodes) in the three addition experiments (glucose, laminarin, and *Syn*-DOM). Spearmen's rank correlation coefficient is strong when $r^2 \geq 0.95$, $p < 0.01$, *Q*-value < 0.05. ASVs and molecular formulas with a > 0.01% relative abundance/intensity were included in the network analysis. Subnetworks show correlations between Alteromonadales ASVs and molecular formulas in the three addition experiments, respectively. The color bars represent the different microbial taxonomy and molecular characteristics (DBE and AI_{mod}). Red lines and blue lines represent significant positive or negative correlations.

In our experiments, the utilization efficiency of all three substrates, at the end of the incubations was lower in the *Alteromonas* experiment compared to the natural microbial community. Furthermore, the intensities of the C2 component remained the same in the *Alteromonas* culture with *Syn*-DOM substrate over the entire incubation period, but greatly decreased in the natural microbial community with the same substrate. An earlier study has demonstrated that *Alteromonas* have a limited ability to degrade DOM compared to natural microbial communities over longer terms (Pedler et al. 2014). We also found a difference in DOC utilization between *Alteromonas* and natural microbial community, which could be due to that natural populations undergo succession which increase the niche breadths and DOM degradation (Bunse and

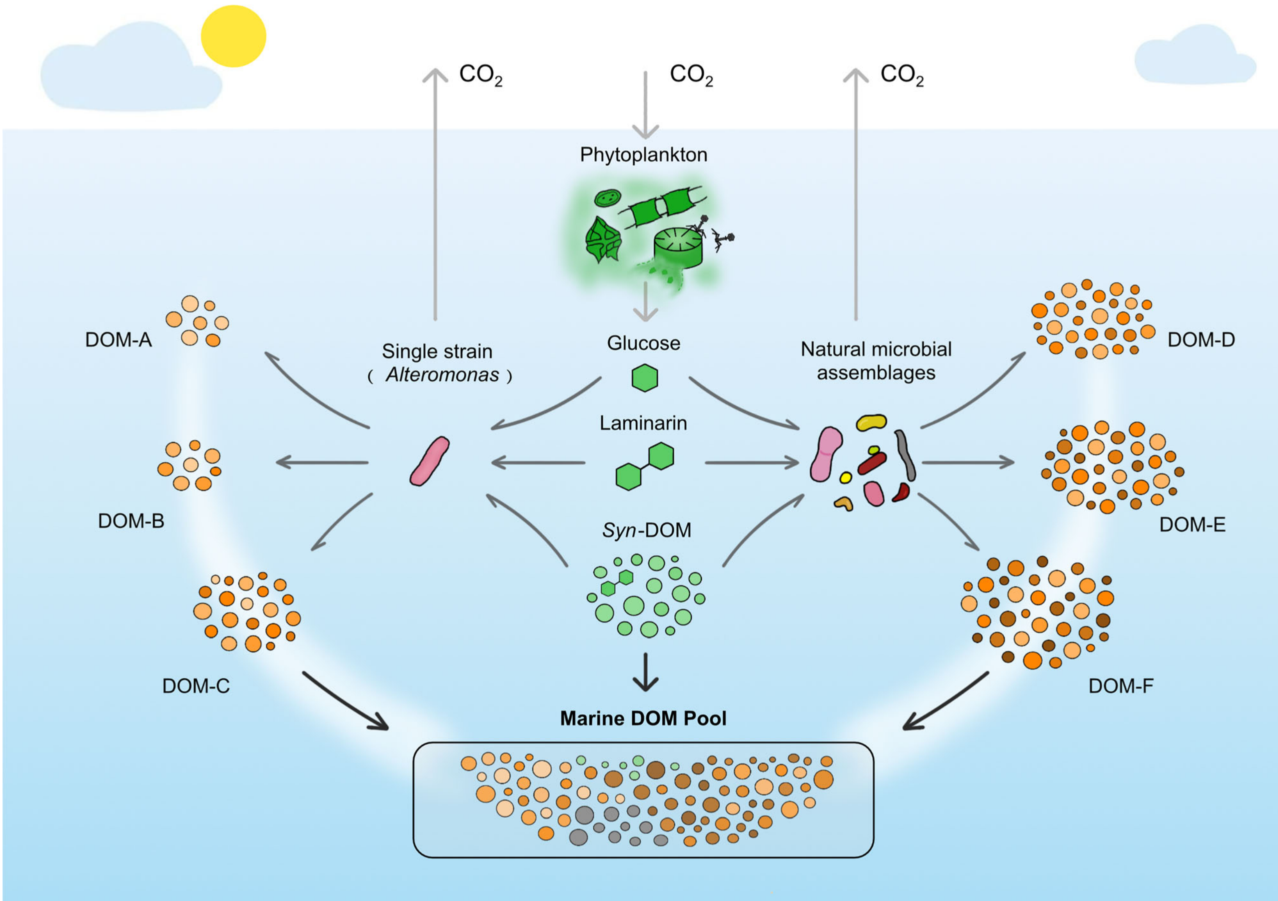

Fig. 5. The diagram of microbial (*Alteromonas* vs. natural microbial community) produced organic molecules contributing to the marine DOM pool when growing on three substrates with different complexity (glucose, laminarin, and *Syn*-DOM). Both single strain (*Alteromonas* sp.) and a natural microbial community could produce more diverse DOM molecules when growing on more complex substrates. DOM-A, -B, and -C represent produced organic molecules by the *Alteromonas* using glucose laminarin and *Syn*-DOM, respectively. DOM-D, -E, and -F represent produced organic molecules by the natural microbial community using glucose, laminarin, and *Syn*-DOM, respectively.

Pinhassi 2017; Ortega-Retuerta et al. 2021). We further assessed the extent to which DOM or microbial composition is potentially responsible for this divergence.

During our experiments more diverse compounds were produced which require a larger collection of enzymatic processes and/or transport systems to be degraded. This production of new compounds supports specialists with specific metabolic capabilities (Zhou et al. 2021). An example of this is the growth of Bacteroidetes which contains diverse hydrolytic enzymes (Bauer et al. 2006; Zheng et al. 2018) allowing them to utilize the DOM produced by the initially growing Gammaproteobacteria. It has been reported that Flavobacteria are a major contributor for the utilization of an *Alteromonas* produced exopolysaccharide (Zhang et al. 2015). The minor increase in Alphaproteobacteria during the incubation is likely due to their preference for small organic compounds and high competitiveness under resource-limited and aromatics-rich conditions (Zheng et al. 2018; Zhou et al. 2021). The successional changes in the microbial taxa slightly differed from an earlier study of a natural spring bloom, where Bacteroidetes were the 1st to respond followed by Gammaproteobacteria and Alphaproteobacteria (Teeling et al. 2012). Members of the Gammaproteobacteria (e.g., Alteromonadales) group could likely in this experiment have benefited from the so called "bottle effect" as has been observed in other studies (Stewart et al. 2012; Maas et al. 2020; Xie et al. 2020). The addition of labile organic matter as done in this experiment compared with a slow release of organic matter from phytoplankton may also cause different microbial responses. Furthermore, the rapid response of Gammaproteobacteria in our experiments could also be due to their ability to take up both low- and high-molecular-weight substrates so they could have outcompeted the other groups (Teeling et al. 2012; Francis et al. 2021). Our finding of an increased microbial alpha-diversity at the end of the incubations when moving from simple compounds to *Syn*-DOM also suggests that more complex substrates sustain a higher microbial diversity. Moreover, *Syn*-DOM contained more hydrogenated molecular formulas with higher molecular weight containing CHON, CHOS, or CHONS atoms (Zhao et al. 2019; Zheng et al. 2020). These heteroatom contents have also previously been found in phytoplankton-derived DOM with relative high bioavailability for heterotrophic bacteria, which could potentially increase microbial diversity (Vorobev et al. 2018; Zhao et al. 2019; Zheng et al. 2019). Combined this demonstrates that complex microbial communities can more fully degrade DOM compared to pure cultures, and that more complex substrates sustain a higher microbial diversity.

The DOM compounds produced by a natural microbial community consisted of more oxidized molecular formulas with unsaturated aromaticity compared to those in the *Alteromonas* experiments. This is in line with previous studies demonstrating that the DOM produced by a natural coastal microbial community is characterized by a relatively high abundance and broad spectrum of unsaturated aromatic compounds (Lechtenfeld et al. 2015). On the contrary, the composition of DOM produced by *Alteromonas* on polysaccharides has previously been shown to display a decreasing relative fraction of oxygen (Koch et al. 2019). Our network analysis showed that Alteromonadales ASVs in the natural community were mostly associated with CHO formulas, which is consistent with the pure culture experiment. Furthermore, more Alteromonadales ASVs were found in the laminarin subnetworks, which might be due to substrate prioritization, as previously demonstrated for *A. macleodii*, which showed a preference for laminarin over other polysaccharides (Koch et al. 2019). In addition, Bacteroidetes and Alphaproteobacteria were positively correlated with more unsaturated aromatic molecular characteristics, while Gammaproteobacteria showed the opposite. This finding is well in line with recent findings in both field and laboratory studies suggesting that Gammaproteobacteria are related with less aromatic molecules (Chen et al. 2021; Zhou et al. 2021). Such microbe–molecule correlations can potentially infer microbial affinity for DOM compounds (Chen et al. 2021) and suggests that higher DOM molecular diversity will also lead to greater microbial diversity (Finke and Snyder 2008), as also suggested by our finding of increased alpha-diversity during the incubation period. Moreover, such partitioning can increase resource exploitation (Finke and Snyder 2008), leading to the formation of a more complex and potentially more recalcitrant DOM pool, which is due to the successive metabolism and transformation by the microbial community (Jiao et al. 2018; Dittmar et al. 2021).

In both the *Alteromonas* and natural community experiments, parts of the DOC persisted at the end of the incubations, with a larger fraction in the *Syn*-DOM experiment. Approximately, 8–19% and 17–44% of the initial organic carbon was transformed into nonlabile DOC in both the two-simple-substrate and *Syn*-DOM experiments, respectively. This suggests a larger portion of nonlabile DOM in the *Syn*-DOM experiment which is likely linked with the structural complexity (Koch et al. 2014) and/or that more nonlabile DOM was generated during these experiments. A direct support for the last point is that relatively more "recalcitrant" humic-like C3 component accumulated in the *Syn*-DOM compared to simple compounds experiments. Component C3 represents a typical and recalcitrant DOM component that is produced as a byproduct of the microbial turnover of organic matter (Jørgensen et al. 2011; Goto et al. 2017). The loss of tyrosine-like FDOM component corresponding to the increase of humic-like C3 suggested that bacteria mediated the transformation from labile to relative recalcitrant DOM compounds (Jiao et al. 2010; Zheng et al. 2019). However, the specific mechanism behind this is still unresolved as one study suggests that microbes are inefficient at producing recalcitrant compounds (Osterholz et al. 2015).

Recently a study has revealed that natural microorganisms can utilize and transform four different substrates with different complexities (L-alanine, trehalose, sediment DOM extract, and diatom lysate) into a DOM pool with a similar composition (Lian et al. 2021). However, in that study the large background DOM signal hindered further identification of newly produced organic molecules under different substrate conditions. In this study, we overcame this limitation by using ASW with low DOM background levels. We demonstrate that the molecular richness increased from simple compounds to *Syn*-DOM in both the *Alteromonas* and natural community experiments, demonstrating that more complex substrate results in higher molecular diversity of the microbial produced DOM. Similar higher molecular diversity with increasing substrate complexity have also previously been found when comparing microbial degradation of algal exudates and glucose (Koch et al. 2014). In addition, our results also revealed, in accordance with other studies, the interdependency of marine bacteria and labile organic compounds in shaping the DOM pool by supplying compound with different complexities (e.g., monomers and polymers) (Pontiller et al. 2020). Heterotrophic bacterial growth in the ocean is partly limited by the DOM bioavailability in terms of concentration, organic molecular composition and structure (Jiao et al. 2014, 2015; Arrieta et al. 2015). Our results show that the DOM composition was more important in shaping the microbial diversity (microbial succession) than the DOC and inorganic nutrients concentrations. This further indicates that DOM diversity potentially could impact the trade-offs under the complex interactions between microbial communities and DOM (Ferenci 2016). These interactive effects depend on the initial added organic matter which impact the subsequent microbial successive metabolic activities (Quigley et al. 2019), and the DOM chemical diversity. In addition to the complexities of substrates, the DOM concentration may also play an important role in shaping microbial community composition as well as their products (Sarmento et al. 2016), but whether this is indeed the case needs further study.

In a recently published study, both production efficiency and composition of fluorescent DOM differed among three bacterial strains (Goto et al. 2020). Furthermore, different bacterial strains, growth phases and metabolized substrates can also affect the chemical composition of the microbial exometabolomes (Noriega-Ortega et al. 2019). Hence, it should be noted that we in this study compared one bacterial strain with a complex microbial community, as well as only three different substrates. Therefore, results from our experiments might not necessarily be applicable and transferable to all environmental settings.

Conclusion

Our study revealed that a natural microbial community compared to a single bacterial culture is capable of degrading DOM more efficiently and producing more unsaturated aromatic organic molecules, as well as, increase molecular richness. In contrast to simple compounds, the complex substrate contributed to higher molecular diversity of the microbial produced DOM, increased microbial diversity, and sustained more linkages between the microbial communities and DOM molecules. Overall, our findings suggest that complex substrates and microbial communities contribute to the diversity of the environmental DOM pool and strengthen microbe–DOM interactions. The interaction between microbes and DOM at the molecular level is therefore key to our understanding of microbial transformation of DOM in the ocean.

Data Availability Statement

Sequence data were deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive with BioProject PRJNA752505. FT-ICR MS data were deposited on figshare (https://doi.org/10.6084/m9.figshare.19433018).

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Acknowledgments

This work was supported by the National Key Research Programs (2018YFA0605800), the Senior User Project of RV KEXUE (KEXUE2019GZ03), the National Natural Science Foundation of China (41776145, 41876150, 91751207 and 41861144018), and the Fundamental Research Funds for the Central Universities (20720190095).

Conflict of interest

None declared.

Submitted 02 November 2021
Revised 04 April 2022
Accepted 05 August 2022

Editor-in-Chief: K. David Hambright