



DOC dynamics and bacterial community succession during long-term degradation of *Ulva prolifera* and their implications for the legacy effect of green tides on refractory DOC pool in seawater

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ABSTRACT

Under climate warming and coastal eutrophication, outbreaks of green tides have increased in recent decades; e.g., the world's largest green tide caused by *Ulva prolifera* has occurred in the Yellow Sea for 13 consecutive years. The massive assemblage of macroalgae absorbs large amounts of atmospheric CO₂ and converts it into biomass. After the green tide, millions of tons of the macroalgal biomass sink to the seabed to be degraded eventually; this inevitably has a significant impact on the coastal organic carbon pool and microbial community. However, this impact is poorly understood. Here, the degradation of *Ulva prolifera* over 520 days revealed that relatively sufficient degradation of the macroalgae occurred at ca. 7 months. The rapid release of dissolved organic carbon (DOC) mainly occurred in the first week, which not only increased the size and diversity of the DOC pool in a short time but also promoted the rapid growth of bacteria and led to hypoxia and acidification of the seawater. After that, the labile portion of DOC was gradually used up by bacteria within one month, while the degradation of semi-labile or semi-refractory DOC occurred in half a year. The remaining DOC existed in the form of refractory DOC (RDOC), resisting bacterial consumption and remaining stable for 10 months. During the long-term degradation process, bacterial community structure and metabolic function showed obvious successional characteristics, driving the gradual transformation of DOC from labile to refractory through the microbial carbon pump mechanism. After the long-term degradation, the remaining RDOC accounted for approximately 1.6% of the macroalgal carbon biomass. As RDOC can maintain long-term stability, we propose that the frequent outbreaks of green tides not only affect microbial processes but also may have an important cumulative effect on the coastal RDOC pool.

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1. Introduction

The world's largest green tide caused by the macroalga *Ulva prolifera* has occurred successively for 13 years since 2007 in the

Yellow Sea, China, with the distribution and coverage areas reaching up to 57,384 km² and 2906 km², respectively (Cao et al., 2019). After the green tides, millions of tons of the macroalgal biomass sink to the bottom of the sea. The input of this excessive amount of fresh algal biomass places great pressure on the coastal environment. The decomposition of macroalgae, accompanied by strong microbial respiration, could soon cause coastal hypoxia and acidification (Zhang et al., 2019). Meanwhile, the release of carbon, nitrogen and phosphorus from decaying macroalgae could cause eutrophication over a period of time (Wang et al., 2012; Zhang and Wang, 2017). However, complete degradation of macroalgae usually takes a long time, even a few years (Lavery et al., 2013).

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This means that there will be a long-term interaction between the sunken macroalgae and the microbial decomposer communities. Their long-term interaction will have a long-lasting influence on coastal microbial processes and biogeochemical cycles.

Bacteria are the most abundant decomposers in the ocean, and they can break down dead organic materials and transform them into dissolved organic and inorganic nutrients (Grossart et al., 2006). These nutrients can further support the growth of phytoplankton and microbial communities. The processing of organic matter by bacteria influences food web structure, drives marine biogeochemical cycles and even plays roles in climate change (Hansell et al., 2012; Hill et al., 2015). For example, the ocean acts as a buffer against climate change by absorbing a large amount of atmospheric CO₂. A large fraction of the absorbed CO₂ is transformed into algal biomass. When algae die, the algal biomass can be partially converted by bacteria into refractory dissolved organic carbon (RDOC) (Zhao et al., 2017). The RDOC has the potential to be stored in the ocean's interior for millennia and thereby avoid being released as atmospheric CO₂ (Jiao et al., 2010). The dissolved organic carbon (DOC) released early from dead algae is usually labile DOC (LDOC) (Zheng et al., 2019). Microbial uptake of the LDOC can leave behind the refractory part of the DOC, which can make the DOC pool refractory-rich in the process. Meanwhile, according to the "microbial carbon pump" (MCP) theory, an estimated 5–7% of the microbially produced DOC are recalcitrant and can be stably stored in the oceans (Ogawa et al., 2001; Jiao et al., 2014).

In the ocean, bacteria have extremely high diversity and various functions. They usually differ greatly in biodegradability, organic nutrition preference and life strategies (r or K strategies) (Teeling et al., 2012). The long-term decomposition of algal biomass is actually a process under actions of various bacterial communities that determines the release and transformation of DOC. In turn, the variation in DOC composition during different decomposition stages can affect the dynamics of bacterial communities. In addition to physical and chemical factors, the long-term interactions between algae and bacterial communities in seawater will determine the legacy of algal biomass and influence ocean biogeochemistry. Due to complex uncontrollable factors, an in situ study on the dynamics of DOC and bacterial communities in coastal oceans during the long-term degradation of macroalgae is hard to perform. Therefore, algae degradation experiments have mostly been carried out under laboratory conditions, despite the reality that the laboratory degradation process is different from that in the ocean. So far, most degradation experiments have been performed over a short time (from weeks to months) (Castaldelli et al., 2003; Zhang and Wang, 2017). Indeed, as mentioned above, the complete degradation of algal biomass usually takes months to years, but very little is currently known about this long-term process and its impact.

In recent decades, algal bloom outbreaks have been increasing in coastal ecosystems (Smetacek and Zingone, 2013). After the blooms, the enormous amount of macroalgal biomass is one of the important DOC sources in coastal regions (Ortega et al., 2019). A twenty-day period of microbial degradation of the green tide macroalga *U. prolifera* was recently studied in the laboratory, revealing the rapid release of a large proportion of dissolved organic matter (DOM) (ca. 43% of the C and 63% of the N). During the short-term degradation process, bacteria played important roles in regulating the chemical composition of DOM (Zhang and Wang, 2017); however, it is unclear which bacterial communities are mainly involved in the degradation process. In addition, the degradation of *U. prolifera* in 20 days is far from complete; we do not know what carbon legacy these macroalgae will leave behind if the time is extended to ensure sufficient degradation. Addressing

these questions will help unveil the potential long-term impact of large-scale green tides on microbial processes and marine carbon pools. We speculate that the macroalgae and bacterial communities will undergo a series of dynamic changes in the long-term interaction process, and eventually a part of the macroalgae-derived carbon will be converted into RDOC by bacteria to be permanently stored in the ocean.

2. Material and methods

2.1. Sample collection and pretreatment

Surface seawater and fresh fronds of the macroalga *U. prolifera* were collected at the late stage of a green tide along the coast of Qingdao (a major city on the western coast of the Yellow Sea) (120.25°E; 35.96°N), China, in 2016. Once the samples were transported to the laboratory, seawater was immediately prefiltered through 20-μm bolting silk and subsequently filtered through a 0.8-μm-pore polycarbonate filter (47-mm diameter, Millipore, Germany) to exclude the interference of picophytoplankton, protozoa and larger non-living particulates to the macroalgal degradation system (Rochelle-Newall et al., 2004; Kragh et al., 2008; Tessarolli et al., 2018). Meanwhile, *U. prolifera* fronds were rinsed several times by autoclave-sterilized seawater (120 °C, 20 min) to remove any sediment particles attached to the surface of the algae (Zhang and Wang, 2017).

2.2. Experimental design

At room temperature of 20–25 °C and under dark conditions, 180 ± 0.2 g (fresh weight) of the macroalga *U. prolifera* and 18 L of the filtered seawater containing bacterial communities were mixed in an acid-cleaned high-density polyethylene carboy to conduct a long-term (520 days) degradation experiment as a treatment group. The purpose of setting this concentration of algal biomass, i.e., ~10 g L⁻¹ (fresh weight), is to simulate the real situation as best as possible, and the purpose of the use of dark incubation condition is to prevent the possible growth of picophytoplankton that may be present in the filtered seawater and interfere with the experiment. Simultaneously, the long-term degradation of the filtered seawater without *U. prolifera* was set as the control group. Both the two groups had three replicates, and all carboys were covered loosely to control contact with the atmosphere. By collecting water samples at different time points during the 520-day degradation process, the dynamic changes in planktonic bacterial community structure in seawater as well as the concentration and composition of DOC were analyzed.

The samples for analyzing the concentration and composition (using the fluorescent dissolved organic matter [FDOM] component as a proxy) of DOC and bacterial abundance were collected on days 0.5, 2, 4, 8, 11, 20, 30, 33, 36, 39, 43, 47, 51, 57, 61, 68, 74, 81, 113, 224, 251, 284, 315, 348, and 520. Meanwhile, the samples for analyzing the molecular fingerprint of DOC were collected on the 8th and 520th days (T0 and T8) in the treatment groups and the initial day (C0) and 520th day (C520) in the control groups. Moreover, the samples for analyzing the bacterial community structure were collected on days 0.5, 2, 4, 11, 20, 30, 68, 113, 224, 251, and 520. During all samplings in the experiments, the water samples were prefiltered through 3-μm-pore sterile mesh to exclude *U. prolifera* fronds. The samples for DOC analysis were then refiltered with 0.7-μm-pore Whatman™ Glass Fiber Filters (GF/F) (pretreated by 450 °C for 5 h) and stored in 20 mL (for later analysis of the DOC concentration and FDOM components) and 500 mL (for later analysis of the DOC molecular fingerprint) brown glass bottles. Additionally, 1.8 mL of the samples was collected for measuring bacterial abundance (BA). Moreover, after prefiltration through 3-μm-

pore filters, the bacterial communities in the 500-mL water samples for analyzing the community structure were collected using 0.2- μm polycarbonate membranes. All glassware used in this study was acid-cleaned, rinsed with Milli-Q water, and precombusted at 450 °C for 5 h.

2.3. Sample analysis

Concentrations of DOC: The DOC concentrations were measured by a TOC-L analyzer with an ASI-V autosampler (Shimadzu, TOC-L CPH, Japan) through high-temperature catalytic oxidation (HTCO) (Benner and Strom, 1993). Seawater DOC standards, including low-carbon water (1–2 μM) and deep seawater (41–44 μM), produced in Hansell's laboratory at the University of Miami were analyzed to maintain data quality control (Dai et al., 2012). The blank was deducted using Milli-Q water analysis before every five samples, and all samples (including the blank) were acidified to pH=2 by 6 M HCl until the analysis. The average blanks associated with the DOC measurement were approximately 5 μM , and the analytic precision of the triplicate injections was $\pm 3\%$.

Main components of FDOM: The main components of FDOM were analyzed by fluorescence spectroscopy technique coupled with parallel factor (PARAFAC) analysis (Li et al., 2018). A fluorescence spectrophotometer (Hitachi F-4600) with a scanning speed of 1200 nm/min was employed to measure the excitation-emission matrix (EEM). During the experiments, a total of 126 EEMs spectra were obtained for PARAFAC analysis. MATLAB (MathWorks, Natick, MA) together with the DOM Fluor toolbox (<http://www.models.life.ku.dk/>) were employed for data analysis (Coble 1996; Coble et al., 1998). More details on the PARAFAC-EEM analysis of FDOM are described in the supplementary materials.

During the analysis of the main components of FDOM, the characteristic parameter of H/P was employed to estimate the bioavailability of FDOM. H/P is the ratio of the sum of the fluorescence intensities of humic-like components to that of protein-like components. Hence, high values of H/P denote the dominance of humic-like components in the EEM array and a high extent of humification (Kowalczyk et al., 2013).

Molecular fingerprint of DOC: The molecular-level changes in DOC during degradation were analyzed by Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR MS) using a Bruker Apex-ultra FT-ICR mass spectrometer equipped with a 9.4 T superconducting magnet and Apollo II electrospray ionization source (Shi et al., 2013). Before the FT-ICR MS analysis, DOC was first solid-phase extracted, and then the extracted DOC (SPE-DOC) was eluted according to the method described in a previous study (Dittmar et al., 2008). The methodologies for FT-ICR MS calibration, data acquisition, and processing have been described previously (Shi et al., 2013). The data analyses were performed by using custom software. The assigned formulas were limited to the following elemental composition: $^{12}\text{C}_{0-100}$, $^1\text{H}_{0-200}$, $^{14}\text{N}_{0-3}$, $^{16}\text{O}_{0-30}$ and $^{32}\text{S}_{0-2}$. All assigned formulas had to meet the following basic chemical criteria: (1) the number of H atoms could not exceed $2\text{C} + \text{N} + 2$; (2) the sum of H and N atoms had to be even (the "nitrogen rule"); and (3) the H/C and O/C value was restricted less than 3 and 1.5, respectively (Flerus et al., 2012). More details on the FT-ICR-MS technology are provided in the supplementary materials.

Moreover, during macroalgal degradation, to estimate the refractory degree of DOC released from *U. prolifera*, a modified aromaticity index (AI_{mod}) was calculated for all assigned formulas via FT-ICR MS (Schmidt et al., 2011). The index was calculated according to the following equation:

$$AI_{mod} = (1 + c - 0.5o - s - 0.5h)/(c - 0.5o - s - n) \quad (1)$$

where c, h, n, o and s are the numbers of carbon, hydrogen, nitrogen, oxygen and sulfur atoms in a molecular formula, respectively. Moreover, the AI_{mod} value was selected to group all DOC molecules into different species of compounds. Generally, the $AI_{mod} > 0.66$ is an unambiguous criterion for polycyclic aromatic structures, and compounds having an $0.66 \geq AI_{mod} > 0.50$ are aromatics with high aromaticity. $AI_{mod} \leq 0.50$ and $\text{H/C} < 1.5$ indicate highly unsaturated compounds; $AI_{mod} \leq 0.5$, $2.0 > \text{H/C} \geq 1.5$ and $\text{N} = 0$ (Nitrogen atom) indicate nonnitrogenous unsaturated aliphatic compounds; $AI_{mod} \leq 0.5$, $2.0 > \text{H/C} \geq 1.5$ and $\text{N} > 0$ indicate nitrogenous unsaturated aliphatic compounds; and $AI_{mod} \leq 0.5$ and $\text{H/C} \geq 2.0$ indicate saturated fatty and sulfonic acids.

Bacterial abundance (BA): Samples for BA were preserved with a final concentration of 0.5% glutaraldehyde and stored at -80°C . BA was measured using an Accuri II flow cytometer (BD Biosciences, USA) by staining with SYBR Green I. Bacteria were enumerated according to a previously described method (Liang et al., 2017b).

Bacterial community structure analysis: Three parallel samples at each sampling time point in the treatment and control groups were combined before DNA isolation according to Zhao et al. (2019). Bacterial DNA extraction was performed following the phenol-chloroform-isoamyl alcohol method (Harder et al., 2003). The final DNA concentration and purity were determined by a NanoDrop device (Thermo-Fisher, United States). The 16S rRNA gene was amplified with primers targeting the V4-V5 hypervariable regions: 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 907R (5'-CCGTCATTCMTTTRAGTTT-3'). Amplicons were sequenced on an Illumina MiSeq platform using $2 \times 300\text{-bp}$ paired-end sequencing (Illumina, San Diego, USA) by Majorbio Co. Ltd. (Shanghai, China). The raw reads were deposited into the National Genomics Data Center (NGDC) Genome Sequence Archive database (Accession Number: CRA002511). Sequencing data were demultiplexed, quality-filtered using QIIME (version 1.9.1) (Caporaso et al., 2010) and merged using FLASH (version 1.2.11). Chimeric sequences were identified and removed using USEARCH (version 7.0) (Edgar, 2013). RDP Classifier (version 2.11) was used to analyze the taxonomy of each 16S rRNA gene sequence with the Silva database (release 132), and operational taxonomic units (OTUs) were generated by clustering sequences with a 97% similarity threshold (Quast et al., 2013).

Functional prediction of bacterial communities: The functional profiles of bacterial communities were predicted using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States 2 (PICRUSt 2) software. We focused on the predicted abundances of KEGG orthologs (KOs) related to 'glycolysis, sulfur, glycan degradation and polycyclic aromatic hydrocarbon degradation' metabolism (Douglas et al., 2019).

2.4. Statistical analysis

To test for changes in bacterial community composition during macroalgal degradation, β -diversity was calculated using Bray-Curtis distances based on relative abundance matrices of OTUs. Nonmetric multidimensional scaling (NMDS) and hierarchical cluster analysis (HCA) were carried out based on the Bray-Curtis distances. Analysis of similarity (ANOSIM) was applied to test the differences in bacterial communities among groups (Clarke, 1993). Moreover, significant differences in the relative abundance of bacterial taxa were determined using one-way ANOVA. Assumptions of homogeneity of variances and normality in the ANOVAs were assessed by scatterplots of residuals and normality curves of residuals, respectively (Holm-Sidak test), and the significance level was $P = 0.05$ for the tests.

3. Results

3.1. Dynamic changes in DOC concentration and bacterial abundance during long-term macroalgal degradation

The dynamic changes in DOC concentration and bacterial abundance showed different characteristics among stages of the long-term degradation process. In the first week, DOC was released rapidly from the algal biomass, resulting in a sharp increase in the DOC concentration to the peak of 6244 μM (Fig. 1). Along with this process, bacterial abundance also increased rapidly from 1.9×10^6 to 1.22×10^7 cells mL^{-1} . Here, the first week was tentatively named the DOC rising (DR) stage. After that, the DOC concentration began to decline, but the bacterial abundance still increased rapidly, reaching the maximum (i.e., 1.26×10^7 cells mL^{-1}) at 3 days, and then began to decline until the 224th day, i.e., 7.5 months during degradation. We temporarily named this period the DOC decline (DD) stage, which lasted ca. 7 months. In fact, during the first 4 weeks of the DD stage, the DOC concentration decreased sharply from 6244 to 797 μM . The bacterial abundance also decreased to 5.8×10^6 cells mL^{-1} . This period was named the rapid DOC decline (r-DD) substage, representing a rapid DOC consumption phase. After the r-DD substage, the declines in DOC concentration and bacterial abundance became relatively slow until the end of the DD stage. This period was called the slow DOC decline (s-DD) substage. At the end of the s-DD substage, DOC concentration and bacterial abundance decreased to 427 μM and 2.6×10^5 cells mL^{-1} , respectively. After that, the DOC concentration and bacterial abundance remained stable for 10 months until the end of degradation. This long-term stable period was called the DOC stabilization (DS) stage. By contrast, in the control group, no significant fluctuations in DOC concentration or bacterial abundance were observed. Meanwhile, the DOC concentration and bacterial abundance in the control group were much lower than those in the treatment group at the end of degradation.

3.2. Dynamic changes in DOC composition in different stages

Here, we used two methods to track the changes in DOC composition. One method was to take FDOM as a proxy of DOC and analyze its dynamic changes; the other was to use advanced FT-ICR MS technology to analyze the variation in DOC molecular composition.

Four distinct FDOM components (i.e., C1–C4) were identified in this study, the ex|em wavelengths of which were 275|320 nm,

225 (270)|300 nm, 240 (315)|390 nm and 270 (355)|460 nm, respectively (Table S1). Based on their ex|em wavelengths, C1 and C2 represented protein-like fractions, which were the amino acid tryptophan and tyrosine-like components, respectively; C3 and C4 represented different humic-like fluorophores. During the degradation process, the dynamic changes in fluorescence intensity of both protein-like components exhibited two stages (Fig. 2). During the first three weeks, their fluorescence intensities increased rapidly to their maximum values. Then, they began to decline until the end of long-term degradation. Interestingly, the change in the humic-like components showed an opposite trend compared with that of the protein-like components. The two humic-like components increased gradually throughout the whole degradation process. Their final fluorescence intensity increased over 5-fold by the end of degradation. The H/P index showed a substantial increase during the degradation process (Fig. S1). In contrast, the four fluorescence components (i.e., C1–C4) in the control group exhibited no significant changes except for a slight increase in the initial stage of degradation.

FT-ICR MS analysis better revealed the changes in DOC composition at a molecular level during different stages. Throughout the entire degradation process, a total of 5136 different molecular formulas (MFs) covering a mass range of 200–670 Da (excluding ^{13}C isotopologs) were identified. During the DR stage, the normalized peak intensity of 2640 MFs increased obviously, which was reflected by the comparison of the DOC fingerprint at the 8th day with that at the beginning (i.e., 0th day) during the degradation process (Fig. 3a). Among them, 1167 MF species were newly generated during the DR stage (Fig. 4a). However, in the subsequent DD and DS stages, the normalized peak intensity of 2102 MFs clearly decreased, as reflected by the differences in the DOC molecular fingerprint at the 8th and 520th day during degradation (Fig. 3b). Among them, 1292 MF species disappeared. Meanwhile, 508 MF species were newly generated during the long-term degradation process (Fig. 4a). Compared with that of the treatment group, the DOC composition in the control group also changed obviously after 520 days. With the disappearance of 1000 MF species, 713 MF species were newly generated during the long-term degradation process (Fig. 4b).

To further compare the DOC composition changes during different stages, the MFs were classified into four groups based on their broad elemental compositions, i.e., CHO-, CHNO-, CHOS-, and CHNOS-containing MFs (Table 1). During the macroalgal degradation process, the number of CHO- and CHNO-containing MFs decreased gradually, with final declines of 29% and 31%, respectively. However, the changes in CHOS- and CHNOS-containing MFs showed an increasing trend with final increases of 114% and 197%, respectively. Meanwhile, in the control group, the changes in CHO-, CHNO-, CHOS-, and CHNOS-containing MFs were relatively small.

Table 1

The main groups and numbers of molecular formulas (MFs) contained in the DOC at different sampling time points during the 520-day degradation process.

	C0	C520	T8	T520
Total formula	3805	3515	4343	3559
CHO	1517	1322	1411	1075
CHON	1724	1235	1705	1183
CHOS	448	755	899	957
CHONS	116	203	328	344

Notes: T8 and T520 represent the samples collected at the end of the DOC rising (DR) and stabilization (DS) stages (i.e., the 8th and 520th day) during the degradation of *U. prolifera*, respectively. C0 and C520 represent the samples collected at the beginning and end of degradation in the natural seawater, respectively.

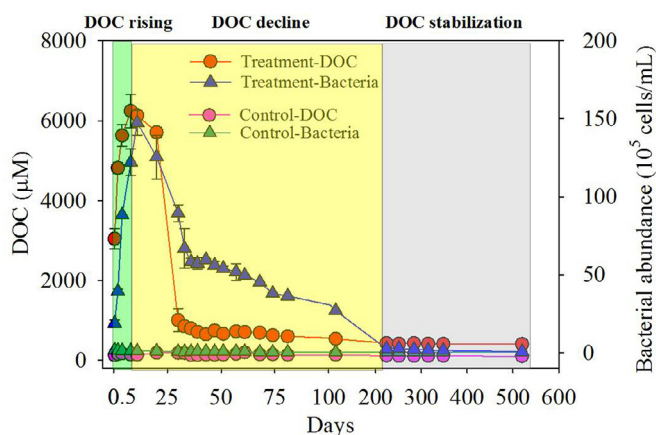


Fig. 1. Dynamic changes in DOC concentration and bacterial abundance (BA) during the 520-day degradation of *U. prolifera* (the treatment groups) and in natural seawater (the control groups).

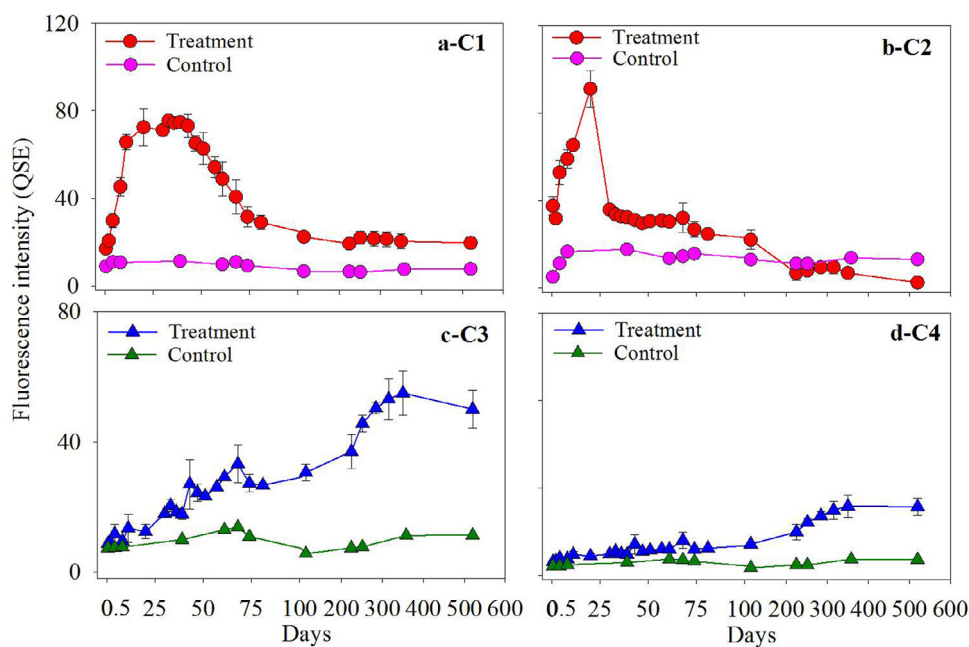


Fig. 2. Dynamic changes in the main components (i.e., C1, C2, C3 and C4) of fluorescent dissolved organic matter (FDOM) during the 520-day degradation of *U. prolifera* (the treatment groups) and in natural seawater (the control groups).

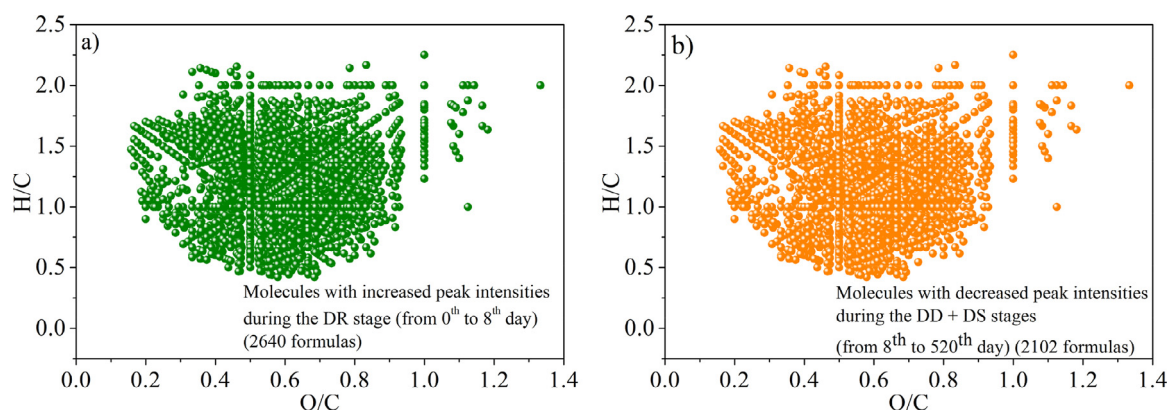


Fig. 3. Variation in the peak intensity of molecular formulas (MFs) contained in DOC during different stages of the 520-day macroalgal degradation process. a) the MFs with increased peak intensities during the DOC rising (DR) stage and b) the MFs with decreased peak intensities during the DOC decline (DD) and DOC stabilization (DS) stages shown by using a van Krevelen diagram.

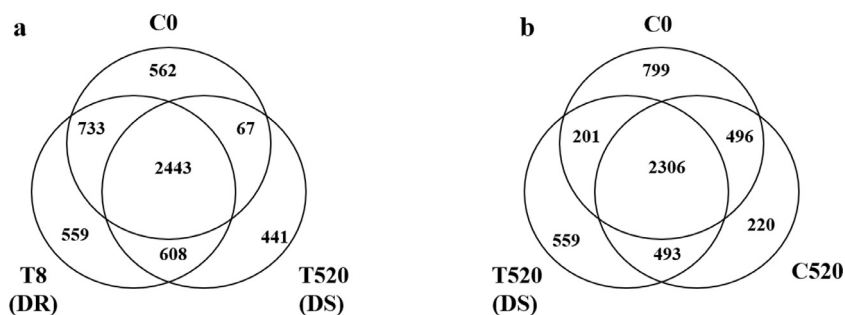


Fig. 4. The numbers of molecular formulas (MFs) contained in the DOC and their comparison in different stages during the 520-day macroalgal degradation process. T8 and T520 represent the samples collected at the end of the DOC rising (DR) and stabilization (DS) stages (i.e., the 8th and 520th day) during the degradation of *U. prolifera*, respectively. C0 and C520 represent the samples collected at the beginning and end of degradation in the natural seawater, respectively. (a) comparison of the MFs contained in the DOC of the C0, T8 and T520 samples; (b) comparison of the MFs contained in the DOC of the C0, C520 and T520 samples.

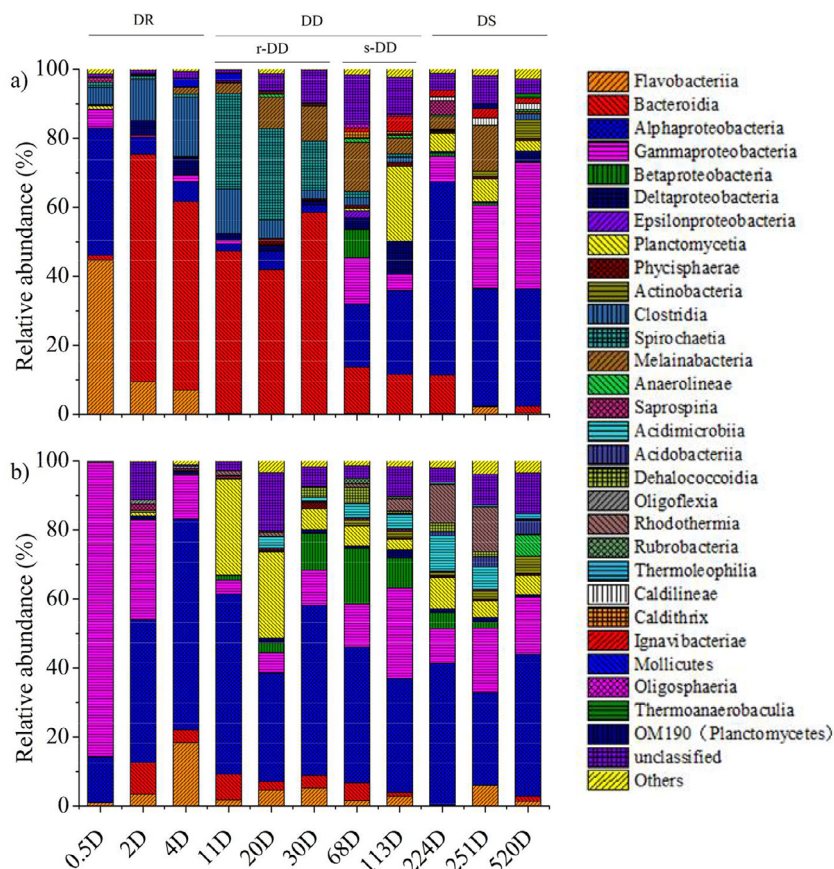


Fig. 5. Variation in the bacterial communities at the class level during the 520-day degradation of the macroalga *U. prolifera* (a) and in natural seawater (b). Others: sum of taxa with a relative abundance below 1%. The abbreviations of DR, DD, DS, r-DD and s-DD represent DOC rising, DOC decline, DOC stabilization, rapid DOC decline and slow DOC decline stages, respectively.

3.3. Succession of bacterial community structure during macroalgal degradation

In total, 995,065 clean 16S rRNA gene sequences and 2688 OTUs were obtained throughout macroalgal degradation. These OTUs (at a 3% dissimilarity threshold) were classified into 43 phyla, 87 classes, 229 orders, 405 families and 762 genera. The addition of macroalgae significantly changed the dominant components of the bacterial community compared with those in the control group (Fig. 5). Except for the eleven bacterial classes existing in both the macroalgal degradation group and the control group, i.e., *Flavobacteriia*, *Bacteroidia*, *Alphaproteobacteria*, *Gammaproteobacteria*, *Betaproteobacteria*, *Deltaproteobacteria*, *Planctomycetacia*, *Phycisphaerae*, *Actinobacteria*, *Anaerolineae* and *Saprospiria*, the dominant bacterial classes in the two groups were quite different. In the macroalgal degradation group, they were *Spirochaetia*, *Clostridia*, *Melainabacteria* and *Ignavibacteriae*, among others, whereas in the control group, they were *Acidimicrobiia*, *Rhodothermia*, *Thermoleophilia*, and *Dehalococcoidia*, among others. At the OTU classification level, only 36% of the total OTUs were shared between the macroalgal degradation group and the control group (Fig. S2). Here, it should be mentioned that the initial bacterial community employed to degrade macroalgae were filtered through the membrane with a pore size of 0.8 μm , which would bring some deviation to the bacterial community, as usually only about 80% of bacterial cells can pass through the similar pore-size membrane (Girollo et al., 2007; Wyatt et al., 2014).

Furthermore, HCA and NMDS analysis were employed to reveal the dynamic changes in bacterial community throughout the degradation process. The bacterial communities at different sam-

pling time points were clearly divided into three clusters (Fig. 6). Interestingly, the three clusters corresponded exactly to the three stages of DOC changes (i.e., the DR, DD and DS stages). Cluster I included all of the bacterial community profiles during the DR stage (i.e., at the 0.5th, 2nd and 4th days), cluster II contained all those during the DD stage (i.e., at the 11th, 20th, 30th, 68th, and 113th days), and cluster III comprised the bacterial community profiles during the DS stage (i.e., at the 224th, 251st, and 520th days). Cluster II could be further separated into two subgroups: the bacterial community profiles during the r-DD (i.e., at the 11th, 20th, and 30th days) and s-DD substage (i.e., at the 68th and 113th days). The bacterial community profiles in the three clusters showed significant differences ($P < 0.05$, one-way analysis of similarity, i.e., ANOSIM) (Table S2), indicating significant succession of the bacterial community during the long-term degradation process. Meanwhile, in the control group, the bacterial communities showed quite different variation characteristics (Fig. S3 and S4).

At the beginning of the DR stage, the addition of macroalgae led to the highest relative abundance of class *Flavobacteriia* on the 0.5th day (Fig. 5). *Flavobacteriaceae*, affiliated with class *Flavobacteriia*, was the most dominant bacterial family (Fig. S3). Meanwhile, only one day later, *Flavobacteriia* declined rapidly, but *Bacteroidia* and *Clostridia* increased and became the two most dominant bacterial classes. The most dominant lineages during this period were also replaced by unclassified *Bacteroidales* and *Prolixibacteraceae* belonging to class *Bacteroidia*. After entering the r-DD substage, the relative abundance of class *Clostridia* decreased gradually, while *Spirochaetia* grew into the second largest class after *Bacteroidia*. Concurrently, *Melainabacteria* increased to be the third largest class. At the family level, *Spirochaetaceae* of class

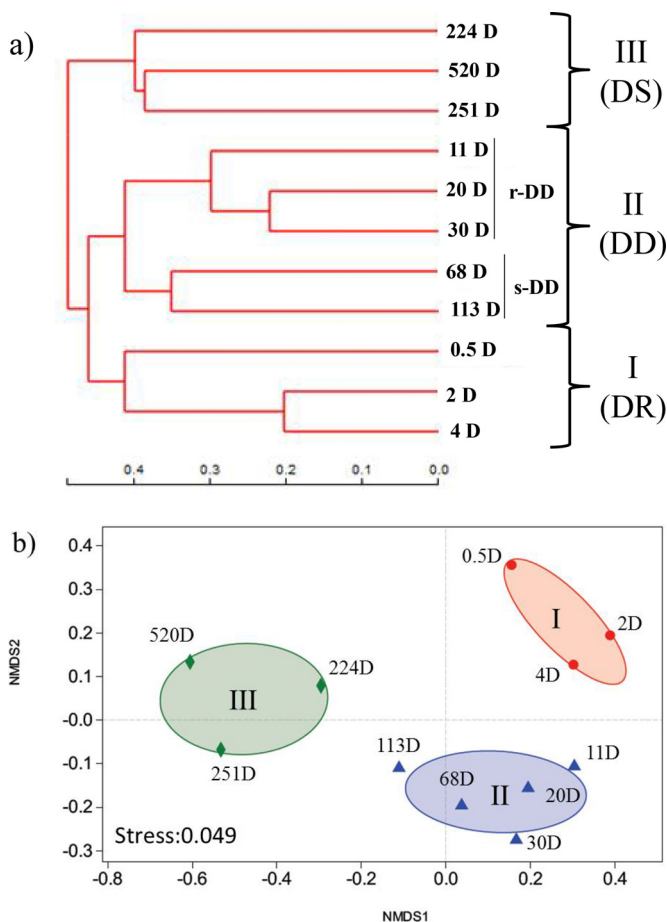


Fig. 6. Hierarchical cluster analysis (a) and nonmetric multidimensional scaling ordination (b) of the bacterial communities sampled at different time points during the 520-day macroalgal degradation process. The abbreviations of DR, DD, DS, r-DD and s-DD represent DOC rising, DOC decline, DOC stabilization, rapid DOC decline and slow DOC decline stages, respectively.

Spirochaetia and *Marinifilaceae* of class *Bacteroidia* increased significantly ($P < 0.05$, one-way ANOVA) and successively became the dominant lineages. During the s-DD substage, the relative abundance of *Bacteroidia* declined; meanwhile, that of *Alphaproteobacteria*, *Gammaproteobacteria*, *Deltaproteobacteria*, *Planctomycetacia* and *Melainabacteria* increased obviously. The dominant classes in the s-DD stage became more balanced compared with that in the earlier stages of degradation. At the family level, *Burkholderiaceae* (belonging to class *Betaproteobacteria*) and *Planctomycetaceae* (belonging to class *Planctomycetacia*) emerged with a high relative abundance on the 68th and 113th days in the s-DD stage, respectively. During the DS stage, *Actinobacteria* increased to be the third largest bacterial class after *Alphaproteobacteria* and *Gammaproteobacteria*, and the dominant bacterial families ultimately became *Rhodospirillaceae* and *Sphingomonadaceae* (both belonging to *Alphaproteobacteria*) and *Lyso bacteraceae* and *Salinisphaeraceae* (both belonging to *Gammaproteobacteria*). Altogether, throughout the whole process of macroalgal degradation, bacterial community structure changed dramatically and showed significant successional characteristics (Fig. S3).

Moreover, the shared and unique OTUs during different stages (Fig. S5) were identified. Only 72 OTUs were stable and existed commonly throughout the entire degradation process, whereas 299, 53, 143 and 650 OTUs were observed only in the DR, r-DD, s-DD and DS stages, respectively. More unique OTUs were observed in the DR and DS stages.

3.4. The potential metabolic functions of the bacterial community in different stages during macroalgal degradation

We predicted 176 functional genes involved in glycolysis, glycan degradation, polycyclic aromatic hydrocarbon degradation and sulfur metabolism based on bacterial community structure by using PICRUSt 2 (Table S3). For simplicity, only the representative genes were selected to reveal the metabolic changes (Fig. S6). In the DR and r-DD stages, abundant genes were involved in glycan degradation, such as the genes *FUCA* (K01206), *lacZ* (K01190), *MAN2C1* (K01191), and *HEXA_B* (K12373) encoding fucosidase, galactosidase, mannosidase, and hexosaminidase, respectively. Meanwhile, the gene *ptsG* (K02779) encoding glucose permease had a higher abundance in the DR stage. This indicates that the degradation of macromolecules (such as glycan) and the transport and utilization of monosaccharides are likely the main metabolic functions of the bacterial community during these stages. Monosaccharides, as a type of DOC with high bioavailability, can promote the rapid growth of bacteria. In addition, there were abundant genes related to sulfur metabolism in the DR stage, such as the *asrA/B/C* (K00385, K16950, and K16951) encoding anaerobic sulfite reductase for the production of sulfide. However, from the s-DD stage to the end of degradation, there were more genes encoding oxygenase, dioxygenase, dehydrogenase, decarboxylase, and reductase, which are involved in the degradation of aromatic or polycyclic aromatic compounds.

4. Discussion

Over the past 13 years, driven by a variety of factors, especially disturbance caused by anthropogenic activities and climate change, the world's largest green tide has occurred every year in the Yellow Sea. After the green tide, up to 2 million tons of the macroalga sinks into deep waters and sediment (Qu et al., 2020). The degradation of such a large amount of macroalgal biomass will inevitably have serious ecological impacts on the coastal ocean, but little is known about these impacts so far. This study conducted a 520-day degradation experiment on the macroalga *U. prolifera* to explore the potential long-term impacts of green tides on the coastal DOC pool and microbial processes.

We found that sufficient degradation of the algal biomass of *U. prolifera* is a long-term process that takes at least 7 months. According to the dynamic changes in DOC concentration, the 520-day degradation process was clearly divided into three stages, i.e., the DOC rising (DR) stage (8 days), DOC decline (DD) stage (216 days), and DOC stabilization (DS) stage (296 days). The DD stage could be further divided into the rapid and slow DOC decline substages (r-DD and s-DD) (Fig. 1). At different stages during the long-term algal degradation, *U. prolifera* fronds changed significantly (Fig. S7). At the beginning of DR stage, *U. prolifera* fronds were slender and green, and floated in the seawater; At the DD stage, the fronds broke into small pieces and sank to the bottom; At the DS stage, no fronds could be directly observed by naked eyes and were all degraded into invisible particles in the seawater. Along with the physical changes of the decaying algal fronds, DOC has undergone drastic changes. The DOC concentration increased sharply and reached its maximum in only one week during the DR stage. Concurrently, the bacterial abundance also increased rapidly. This indicates that the release of DOC from decaying macroalgae is very fast and that algae-released DOC is strongly favored by bacteria (Hansell et al., 2012). During this stage, the abundance of bacteria increased by almost one order of magnitude, indicating that a considerable portion of the released DOC was utilized and transformed into bacterial biomass (Ogawa et al., 2001). After that, the DOC concentration began to decline, entering the DD stage. The decrease in DOC concentration may be due

to the slowdown in DOC release from the macroalgae not keeping up with DOC consumption by the abundant bacteria. The rapid decline in DOC lasted for four weeks. During this period, the DOC concentration decreased by nearly 90%. This showed that most of the DOC released by the macroalgae was labile DOC and very easily utilized by bacteria. In the coastal environment of the Southern Yellow Sea, seawater quality usually deteriorates quickly within 1–2 weeks after green tides. One important reason may be that while utilizing the rich labile DOC released from decaying macroalgae, the heterotrophic bacteria absorb a large amount of oxygen and release a large amount of CO_2 , resulting in hypoxia and acidification of the seawater (Zhang et al., 2019). In this study, we did not measure CO_2 emission, but based on the very low pH value (the lowest value was 6.1) of seawater during degradation (Fig. S8), it indicates that besides being converted into bacterial biomass, a considerable part of the released DOC should be emitted as CO_2 through microbial respiration. After the r-DD stage, the rate of decline of the DOC concentration became very slow, suggesting that most of the labile DOC had been consumed by bacteria. The remaining DOC in the s-DD stage had a much lower bioavailability to bacteria and may have been semi-labile or semi-refractory DOC. Notably, after the bacteria reached their maximum abundance on the 11th day, bacterial abundance decreased gradually in the next 7 months. This may be because the concentration and bioavailability of DOC were significantly reduced and could not continue to meet the needs for bacterial growth, leading to the death of some bacteria; thus, the bacterial abundance decreased gradually. Especially during and after the s-DD stage, the remnants of dead bacteria (i.e., necromass) and bacterial secretions may make an important contribution to the DOC pool. In the subsequent DS stage, although the DOC concentration was significantly higher than that in the control group, both the DOC concentration and bacterial abundance maintained long-term stability until the end of the degradation. This suggests that the DOC remaining after long-term macroalgal degradation is extremely difficult for bacteria to use and represents the refractory DOC (RDOC), and the bacterial community sustained very low metabolic activity.

The changes in DOC composition should be a decisive reason for the transformation of DOC from labile to refractory. First, FDOM was employed as a proxy of DOC to study the dynamic changes in DOC composition. In the early stage of degradation, with the rapid release of DOC, the protein-like fractions of FDOM also increased rapidly, reaching a peak within three weeks, and then declined gradually in the long term to a very low level (Fig. 2). Protein-like substances are usually considered an indicator of biolabile DOC (Yang et al., 2015); the nearly complete depletion of the protein-like fraction indicated that the labile components of DOC were nearly completely consumed by bacteria. Interestingly, the humic-like fraction of FDOM gradually accumulated throughout the degradation process. Humic-like organic matter is often considered as a potential tracer of RDOC (Lønborg et al., 2013). The continuous accumulation of humic-like components hints at why DOC turned increasingly recalcitrant and resisted utilization by bacteria. Moreover, the significant negative correlation (Pearson's correlation, R values: $-0.76 \sim -0.46$, $P < 0.01$) between the protein-like (C1 and C2) and humic-like (C3 and C4) fractions (Table S4) and the substantial increase in the H/P index (Fig. S1) implied a transformation of DOC from labile to refractory during the degradation process. According to the conceptual framework of the microbial carbon pump (MCP), this transformation should be mainly driven by bacterial communities (Jiao et al., 2018). Indeed, similar phenomena have been observed during microbial degradation of phytoplankton and demonstrated the existence of the MCP (Zheng et al., 2019). In the ocean, the RDOC constitutes the main component of the extremely large stable carbon pool, which can sequester carbon

over the long term and plays an important role in climate change (Zhang et al., 2017). A previous study showed that RDOC can be produced by microbial degradation of particulate organic matter or directly from microbial cell materials (such as necromass) (Liang et al., 2017a). Here, we suggest that MCP may play an important role in the production of RDOC during the long-term degradation of *U. prolifera*.

To more clearly explain the variation in DOC composition that determined the conversion of DOC from labile to refractory, the DOC molecular fingerprints in the early and late stages of macroalgal degradation were characterized (Figs. 3 and 4). Compared with that in natural seawater, the early degradation (during the DR stage) of macroalgae introduced a large number (1167) of new molecular formulas (MFs). This indicates that the large amount of macroalgal biomass left in the sea after green tides will soon significantly increase the molecular diversity of the DOC pool in coastal water. Meanwhile, after the long-term degradation process, a large number (1292) of MFs had disappeared, and the CHO- and CHNO-containing molecules decreased significantly. These vanished MFs might represent a portion of the labile molecules that had been consumed by bacteria. However, many (508) new MFs were also generated. We consider that these new MFs might mainly comprise bacterial secretions and necromass of dead bacteria. They were likely the typical molecules of RDOC that maintained long-term stability during the late stage of macroalgal degradation. Meanwhile, we found that the CHOS- and CHNOS-containing molecules increased significantly in the late stage (Table 1), suggesting that diverse sulfocompounds were formed and became the molecular components of RDOC. Sulfur should mainly have originated from *U. prolifera*, as the cell wall of *U. prolifera* contains sulfur-rich ulvan and highly aliphatic lipids (Kendel et al., 2015). In addition, to provide an overview of the likely structures behind the identified MFs, the modified aromaticity index (AI_{mod}) was employed to group the DOM molecules into different classes (Koch and Dittmar, 2006). Polycyclic aromatics ($0.66 < AI_{mod}$) and highly aromatic compounds ($0.5 < AI_{mod} \leq 0.66$) are usually regarded as refractory DOM (Schmidt et al., 2011). During the long-term degradation process, their percentages increased significantly from 3.6% to 7.3% (Fig. S9). Meanwhile, the proportion of some highly unsaturated compounds ($AI_{mod} \leq 0.5$ and $H/C < 1.5$) and saturated fatty and sulfonic acids ($AI_{mod} \leq 0.5$, $H/C \geq 2$), which are usually considered as labile DOM (Cai et al., 2019), decreased dramatically. These changes more strongly manifested the accumulation of RDOC during the long-term degradation of macroalgae.

Indeed, the long-term degradation of macroalgae is a process during which bacterial communities and algae-derived carbon interact with each other. The bacterial community was the driving force for the dynamic changes in DOC composition and vice versa. In response to the changes in DOC, distinct succession of bacterial community structure and the potential metabolic functions at different stages also occurred. The bacterial communities at different sampling time points throughout the degradation process were clearly divided into three clusters corresponding exactly to the three stages of DOC changes. Especially at the OTU level, only a very small proportion of bacteria were commonly present throughout the three stages (Fig. S5). In the DR stage, the addition of macroalgae also likely introduced some associated bacteria (e.g., *Flavobacteriia*) usually inhabiting the phycosphere environment (Ramanan et al., 2015); meanwhile, other bacteria that usually have potential extracellular hydrolase activity increased (Collén et al., 2011). For example, *Bacteroidia* and *Clostridia* became the two most dominant bacterial classes, with relative abundances rising from 1.5% to 27.7% and from 1.5% to 13.2%, respectively; their members can secrete highly active hydrolase, which degrades a variety of macromolecules, such as ulvan and cellulose (Lee et al., 2014; Wang et al., 2015; Joyce et al., 2018). Mean-

while, based on Kyoto Encyclopedia of Genes and Genomes (KEGG) metabolic pathway analysis, the bacterial communities in the DR stage were predicted to have the highest glycan degradation, glucose transport and anaerobic sulfite reductase activities (Fig. S6). These bacteria should greatly promote the conversion of algae-derived carbon from particulate organic carbon (POC) to DOC and the conversion of organic carbon from being large-molecular DOC to small-molecular DOC. The released small-molecular DOC could be easily consumed by bacteria and promoted a rapid increase in bacterial abundance during this stage. *Bacteroidia* and *Clostridia* are typical bacteria that prefer to grow in anaerobic environments (Zarkasi et al., 2016). The predominance of these bacteria indicates that the released DOC promoted a large degree of consumption of oxygen by microbial respiration, transforming the stage into a hypoxic and acidification phase. This also further explained why hypoxia and acidification usually occur soon after green tides in the Yellow Sea. Meanwhile, the anaerobic conditions also promoted the growth of anaerobic sulfate-reducing bacteria, such as *Desulfovibrionaceae* and *Spirochaetaceae*, which are often observed after the massive green tides and are major players in marine sulfur biogeochemical cycling (Qu et al., 2020). At the r-DD stage, bacterial consumption resulted in a decrease in DOC concentration, and bacterial abundance also began to decline after reaching its peak. As mentioned above, since the labile DOC had been gradually used up by bacteria, the remaining DOC became increasingly recalcitrant. Meanwhile, the remaining POC that had not been decomposed may also have become increasingly inert; thus, more diverse bacterial groups, such as *Spirochaetia* and *Melainabacteria* which have a strong organic matter degradation ability (Köchling et al., 2015; Soo et al., 2015), matured and became members of the dominant bacterial groups. At the s-DD stage, the dominant groups in the bacterial community were more diverse and balanced. Their ability to degrade organic matter may also have become stronger, which could be confirmed by the presence of more functional genes involved in the degradation of inert polycyclic aromatic hydrocarbons (Fig. S9). At the DS stage, the remaining DOC concentration and bacterial abundance were very stable within 10 months. Many bacteria that can participate in the degradation of alkanes and aromatics, such as members of the families *Sphingomonadaceae* and *Salinisphaeraceae* (Kertesz and Kawasaki, 2010; Vetriani et al., 2014), became members of the dominant groups. The dominance of these bacteria is also a very clear indicator that the DOC remaining after long-term degradation of the macroalgae was highly recalcitrant. It is worth mentioning that we did not analyze the biofilm bacterial community on the decaying *U. Prolifera* fronds, which is a shortage of this study. Biofilm bacterial communities on the decaying fronds might be quite different from planktonic bacterial communities and also play important roles in the degradation of macroalgae.

Finally, a rough estimate was obtained based on the remaining RDOC concentration after long-term degradation and the amount of macroalgal biomass carbon added initially. In other words, after the long-term degradation process, ca. $1.6 \pm 0.02\%$ of the algal biomass carbon was left in seawater in the form of RDOC. RDOC is highly stable and can be stored and accumulated in seawater for millennia (Lechtenfeld et al., 2014; Lønborg et al., 2018). We propose that the massive *U. prolifera* green tide occurring annually in the Yellow Sea might have a significant long-term cumulative effect on the RDOC pool in the ocean.

Actually, the degradation process in laboratory is quite different from that in the natural ocean environment. Due to the strong water exchange in coastal oceans, the changes in dissolved oxygen, pH, and bacterial community are not as strong as those in the laboratory during long-term degradation of macroalgae. In addition, the temperature change in different seasons in the ocean is more severe than in the laboratory. The bottle effect and the above envi-

ronmental factors will have a significant impact on the macroalgal degradation process, making our experimental results have some deviation from the actual process in coastal oceans. In addition, many organisms in the ocean, such as protozoa and invertebrates, feed on and mineralize algal fronds (Graça, 2001; Sala and Jürgens, 2004; Ribblett et al., 2005). Their feeding activities will also have an important impact on the degradation process of macroalgae. The degradation process of macroalgae in the natural coastal environment is much more complicated than in the laboratory.

5. Conclusion

The present study shows that the degradation of green tide algal biomass is a long-term process that can have long-lasting effects on marine microbial processes and carbon cycling. During the long-term degradation process, the interactions between bacteria and macroalgae-derived carbon resulted in dynamic changes in DOC concentration and composition as well as significant succession of bacterial community structure and function. The successive bacterial communities played an important role in driving the gradual transformation of DOC from labile to refractory and ultimately determined the fate of the carbon biomass of the macroalga *U. prolifera*. It can be inferred from this laboratory study that although *U. prolifera* green tides disappeared in late summer every year, the long-term degradation of massive sank macroalgae would not only have a significant impact on marine microbial processes but also make an important contribution to the growth of the RDOC pool in the coastal ocean.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

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