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Mechanism of recalcitrant dissolved organic matter production revealed by model marine bacterial strains

(モデル海洋細菌単離株による
難分解性溶存有機物の生成機構の解明)

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Abstract

Marine dissolved organic matter (DOM) constitutes one of the largest reduced organic carbon pools on the earth's surface. The recalcitrant DOM (RDOM) has been considered to be a major fraction of marine DOM and plays an important role as a slow cycling carbon reservoir. However, source and production mechanism of marine RDOM have not been fully understood. Bacteria have been considered to be major producers of marine RDOM, and bacterial production of RDOM has recently been proposed as a carbon sequestration process. The concept of this process called microbial carbon pump (MCP) was derived from the previous studies of *in vitro* culture experiments, in particular using natural microbial community. Since it would be difficult to identify that the specific species as well as key physiology that contribute to MCP from incubation experiments with natural microbial community, it is still unclear whether differences in bacterial species and/or physiology can affect RDOM production. In this study, to compare mechanisms of RDOM production among bacterial species, I conducted batch culture experiments in which glucose was added as sole carbon source using three model marine bacterial strains, namely *Alteromonas macleodii* (*A. macleodii*) and *Vibrio splendidus* (*V. splendidus*) and *Phaeobacter gallaeciensis* (*P. gallaeciensis*). Furthermore, incubation experiments of natural bacterial community with DOM produced by a bacterial strain were carried out to evaluate degradability of bacterial DOM. Dissolved organic carbon (DOC) concentrations were drastically decreased during the exponential growth phases and relatively stable during the stationary phases, irrespective of differences in bacterial strains. While, bacterially derived DOM from

glucose, namely residual DOC concentration at the end of incubation was considerably high for *V. splendidus* compared with the other two strains. Excitation-emission matrix (EEM) during/after incubations showed that all bacterial strains used in this study produced fluorescent DOM (FDOM) including refractory humic-like FDOM. Interestingly, the EEM patterns of bacterially derived FDOM were different among the three strains. Furthermore, four production processes of humic-like FDOM by bacteria were suggested; (1) production during active growing of bacteria, (2) production with reutilization of bacterial DOM, (3) production with consumption of reserves in bacterial body, and (4) release from the lysis of bacteria. The efficiency of humic-like FDOM production through mineralization of organic matter was different among bacterial species as well as production processes, namely bacterial physiology. Although a part of the DOM derived from bacterial strains were degraded, major part of the DOM (60–80%) was not degraded during incubation using the natural bacterial community. The results of two types of incubation experiments indicated that the efficiency of RDOM production depended on bacterial species and suggested that composition of bacterial species in natural community regulates MCP. All humic-like FDOM derived from bacterial strains was recalcitrant to natural bacterial community. Furthermore, spectral characteristics of the humic-like FDOM were similar to those derived from bacterial strains, suggesting the presence of precursor of humic-like FDOM in DOM derived from bacterial strains.

Contents

Chapter 1. General Introduction

1-1 Marine dissolved organic matter in the ocean.....	1
1-2 Recalcitrant dissolved organic matter from marine microbes.....	2
1-3 Advantages of incubation with bacterial isolates to evaluate RDOM production.....	4
1-4 The objectives of this study.....	7

Chapter 2. Establishment of incubation methods of model marine bacterial strains

2-1 Introduction.....	10
2-2 Materials and methods.....	11
2-2-1 Bacterial strain.....	11
2-2-2 Experiment setup.....	12
2-2-3 Bacterial abundance.....	13
2-2-4 DOC concentration and DOM optical properties.....	13
2-3 Results.....	15
2-3-1 Growth of <i>A.macleodii</i> and Change in DOC concentration.....	15
2-3-2 FDOM derived from <i>A.macleodii</i>	17
2-3-3 Changes in fluorescence intensity of individual peaks during <i>A.macleodii</i> incubation.....	18

2-4 Discussion.....	21
2-4-1 <i>Changes in DOM quantity and quality with A.macleodii growth.....</i>	<i>21</i>
2-4-2 <i>Humic-like fluorophores produced by A.macleodii.....</i>	<i>24</i>
2-4-3 <i>Potential relationship between bacterial growth and the production of humic- ike fluorophore.....</i>	<i>26</i>
2-5 Conclusion.....	29
Tables and Figures of chapter 2.....	31

Chapter 3. Comparison of production of dissolved organic matter by model marine bacterial strains

3-1 Introduction.....	38
3-2 Materials and methods.....	40
3-2-1 <i>Bacterial strain.....</i>	<i>40</i>
3-2-2 <i>Experiment setup.....</i>	<i>41</i>
3-2-3 <i>Bacterial abundance.....</i>	<i>41</i>
3-2-4 <i>Organic carbon concentrations and DOM optical properties.....</i>	<i>42</i>
3-3 Results.....	43
3-3-1 <i>Growth of bacteria and change in organic carbon concentration.....</i>	<i>43</i>
3-3-1-1 <i>Alt treatment.....</i>	<i>44</i>
3-3-1-2 <i>Vib treatment.....</i>	<i>46</i>
3-3-1-3 <i>Pha treatment.....</i>	<i>48</i>
3-3-1-4 <i>Control treatments without inoculum.....</i>	<i>51</i>
3-3-2 <i>Spectral characteristics of FDOM produced by bacterial strains.....</i>	<i>52</i>

3-3-3 Changes in fluorescence intensity of individual FDOM produced by bacterial strains.....	54
3-3-3-1 Protein-like FDOM.....	54
3-3-3-2 Humic-like FDOM.....	55
3-3-4 Regression analyses against humic-like FDOM.....	56
3-4 Discussion.....	57
3-4-1 Bacterial production of FDOM with consumption of labile substrate during the exponential growth phase.....	57
3-4-2 Bacterial production of FDOM during the stationary phase.....	60
3-4-2-1 Humic-like FDOM production with reutilization of bacterial DOM...61	
3-4-2-2 Humic-like FDOM releasing with lysis of bacteria.....	62
3-4-2-3 Humic-like FDOM production with consumption of reserves in biomass.....	65
3-4-3 Efficiency of bacterial DOM production based on organic carbon concentration.....	67
3-5 Conclusion.....	70
Tables and Figures in chapter 3.....	73

Chapter 4. Estimation of degradability of dissolved organic matter produced by bacterial strains with natural bacterial community

4-1 Introduction.....	99
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4-2 Materials and methods.....	99
4-2-1 Preparation of stable DOM produced by bacterial strains.....	99
4-2-2 Experiment setup.....	100
4-2-3 Bacterial abundance.....	101
4-2-4 DOC concentration and DOM optical properties.....	101
4-3 Results.....	102
4-3-1 Growth of bacteria in natural community and change in DOC concentration.....	102
4-3-1-1 Alt-DOM treatment.....	102
4-3-1-2 Vib-DOM treatment.....	103
4-3-1-3 Pha-DOM treatment.....	104
4-3-1-3 Control treatment.....	104
4-3-2 Spectral characteristics of FDOM during incubation.....	105
4-3-3 Changes in fluorescence intensity of strain-DOM during incubation of natural bacterial community.....	106
4-3-3-1 Protein-like FDOM.....	106
4-3-3-2 Humic-like FDOM.....	107
4-4 Discussion.....	107
4-4-1 Degradability of bacterial DOM with natural bacterial community.....	107
4-4-2 Humic-like FDOM production from strain-DOM.....	110
4-5 Conclusion.....	112
Tables and Figures in chapter 4.....	114

Chapter 5. General conclusions

5-1 Bacterial production of RDOM in terms of carbon.....	122
5-2 Bacterial production of recalcitrant humic-like FDOM.....	123
5-3 Limitations and implications.....	125
Tables and Figures in chapter 4.....	129

Acknowledgements.....	132
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Reference.....	134
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Chapter 1.

General introduction

1-1 Marine dissolved organic matter in the ocean

Marine dissolved organic matter (DOM) is one of the largest reduced organic carbon pools on the earth's surface, indicating that it plays an important role in the total carbon pool in the ocean (Hedges, 1992; Hansell and Carlson, 1998; Hansell et al., 2009). The average residence time of bulk DOM in the ocean has been estimated at approximately 4000–6000 years by ^{14}C dating analysis (Bauer et al., 1992; Druffel et al., 1992; Beupré, 2015). Observations of the global distribution of dissolved organic carbon (DOC) concentration with a coupled physical/biogeochemical model also showed that a major fraction of marine DOM is recalcitrant to microbial degradation with a time scale of more than a century (Hansell et al., 2009). Some constituents of marine DOM, e.g., humic-like fluorescent DOM (FDOM) (Yamashita and Tanoue, 2008; Catalá et al., 2015), carboxyl-rich aliphatic materials (Hertkorn et al., 2006), and polyaromatic compounds (Dittmar and Paeng, 2009), have been considered to be recalcitrant DOM (RDOM). However, the chemical characteristics of RDOM have not been fully clarified. Such molecularly uncharacterizable features of RDOM preclude a

comprehensive understanding of the source and production mechanism of marine RDOM.

1-2 Recalcitrant dissolved organic matter from marine microbes

Although sources of marine RDOM have not been elucidated, marine microbes have been considered as a major source of RDOM. The microbial production of RDOM has been proposed as microbial carbon pump (MCP) playing a role in a carbon sequestration (Jiao et al., 2010). The MCP concept was derived from the results of microbial incubation studies (Ogawa et al., 2001; Kramer and Herndl, 2004; Kawasaki and Benner, 2006; Lønborg et al., 2009; Shimotori et al., 2009; Koch et al., 2014). These studies observed that semi-refractory DOM, which was distinct from labile substrates (such as glucose and glutamate) for microbes, was present in 20-day to 2-year incubations of microbial communities obtained from seawater. The residual DOM has been considered to be microbially derived DOM, which could not be utilized by heterotrophic bacteria during incubation. Ogawa et al. (2001) found that bacterially derived DOM after *in vitro* incubations were similar to marine DOM in terms of C/N ratio, composition of amino acids and amino sugars, and proportion of uncharacterized fraction of DOM.

Ultrahigh resolution Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS) has recently been applied to determine the molecular composition of DOM obtained from *in vitro* incubations of marine microbial community. The results showed that experimentally obtained microbial DOM was similar to marine RDOM in terms of the molecular composition (Koch et al., 2014; Lechtenfeld et al., 2015). More recently, the results obtained from incubation experiments of two bacterial strains belonging to *Roseobacter* clade demonstrated that a part of DOM derived from the two bacterial strains was consistent with RDOM from deep ocean (Noriega-Ortega et al., 2019). However, it has also been reported that most microbial DOM is distinct from marine RDOM (Osterholz et al., 2014).

The products of MCP have also been traced using fluorescence techniques, e.g., excitation-emission matrices (EEMs), during *in vitro* incubation of marine microbial communities with various substrates, e.g., simple substrates, such as glucose (Kramer and Herndl, 2004; Lønborg et al., 2009; Shimotori et al., 2009), marine DOM (Jørgensen et al., 2014), and humic substances (Aparicio et al., 2015). These studies found that humic-like fluorophores were produced by marine microbes, and microbial humic-like fluorophores were usually not degraded during incubation. Shimotori et al. (2009) conducted a 90-day incubation experiment using a coastal microbial community

with glucose as a substrate, and reported that the coastal bacterial community generated a humic-like fluorophore that was similar to marine RDOM in terms of fluorescence characteristics, molecular size, and photo-degradability. Zhao et al. (2017) recently reported that strains of cyanobacteria, namely *Synechococcus* and *Prochlorococcus*, produced similar humic-like FDOM to marine recalcitrant humic-like FDOM. Recent studies also suggest that production rates of humic-like fluorophore by marine microbial communities depended on the quality of substrates (Jørgensen et al., 2014; Aparicio et al., 2015). Such substrate dependency of humic-like fluorophore production by microbial communities is possibly due to changes in bacterial physiology and/or species responded to different substrates.

1-3 Advantages of incubation with bacterial isolates to evaluate RDOM production

It has been suggested that the microbial RDOM is produced through bacterial exudation (Jiao et al., 2010; Jiao et al., 2014), indicating a potential relationship between bacterial physiology and RDOM production. However, the relationship of RDOM production with bacterial physiology has scarcely been discussed because incubation experiments for evaluating MCP have usually been conducted with natural microbial communities, which include a wide variety of microbes with various physiological conditions. The

incubation of a bacterial isolate could allow the evaluation of bacterial physiological states, including bacterial growth phase; yet, only few studies have been conducted on this topic. The FT-ICR-MS-based exometabolomic analyses of bacterial strain incubation showed that the composition of bacterial DOM was affected by the growth phase (Romano et al., 2014; Wienhausen et al., 2017; Noriega-Ortega et al., 2019). Gruber et al. (2006) suggested that *Pseudomonas chlororaphis* produced persistent DOM, mainly during the exponential growth phase. In contrast, Eichinger et al. (2009) reported that DOC accumulated during the stationary phase in the incubation of *Alteromonas infernus*. Zhao et al. (2017) reported that strains of cyanobacteria, produced humic-like FDOM during viral lysis of those bacteria. As such, at present, only a few studies have assessed the microbial production of RDOM using marine bacterial isolates. Therefore, knowledge on the relationship between RDOM production and bacterial physiology is still limited, even though it is possibly one of the key parameters shaping the size and composition of the marine DOM pool.

It has been reported that chemical composition of bacterially derived DOM is related to bacterial community composition (Lucas et al. 2016; Osterholz et al. 2016; Tada et al., 2016; Tada et al., 2017). A strong linkage between specific bacterial taxa and particular DOM molecules was found from the monitoring of DOM composition

determined by FT-ICR-MS and bacterial community structure determined by 16S rRNA gene sequencing during phytoplankton bloom in the North Sea (Lucas et al., 2016). However, it is difficult to identify the key bacterial species contributing to the RDOM production from the observation or in *vitro* experiments with bacterial community. Two previous studies incubated two bacterial strains in *Roseobacter* group, *Phaeobacter inhibens* and *Dinoroseobacter shibae*, with simple substrates (glucose, glutamate, acetate or succinate) as sole carbon source, and found that chemical composition of exometabolites were clearly different between the two bacterial strains (Wienhausen et al., 2017; Noriega-Ortega et al., 2019). The results of these previous studies suggest that capability of DOM production is different among bacterial species and that key players contributing to RDOM production existed in the ocean. Thus, comparison of the efficiency as well as process of RDOM production among bacterial strains can provide new insights into mechanisms of RDOM production and of MCP.

Incubation experiments of bacterial strains or bacterial community with labile DOM (LDOM) may ensure that remnant DOM at the end of incubations for sufficient periods are not available for the microorganisms. However, the remnant DOM may be degraded by the other bacterial species which are absent in the incubation, especially in the case of bacterial strain incubation. A previous study reported that

exopolysaccharides derived from bacterial isolate of *Alteromonas* sp. were degraded by natural microbial community (Zhang et al. 2016). Recently, Shen and Benner (2018) found that RDOM in the Atlantic Ocean was partly degraded by a coastal microbial community during one year incubation, and pointed out that changes in metabolic diversity of microbial community induced degradation of RDOM. Therefore, it is important to evaluate the degradability of bacterial DOM derived from incubations of bacterial strains to elucidate the recalcitrant fraction of strain-derived DOM for broader bacterial species.

1-4 The objectives of this study

The goals of this study are obtaining the knowledges regarding with mechanisms/processes of bacterial RDOM production with *in vitro* incubations of bacterial strains to a better understanding of MCP. The RDOM produced by bacterial strains was quantitatively and qualitatively determined by organic carbon analysis and EEM technique, respectively.

In Chapter 2, to establish the methodology about incubations of bacterial strains to determine quantity and quality of bacterial DOM in relation to DOM with bacterial physiology, the *in vitro* incubation of a model bacterial strain with glucose as a labile

substrate was carried out. *Alteromonas macleodii*, a ubiquitous gammaproteobacterium from the surface to the deep layer of tropical and temperate oceans (López-Pérez et al., 2012), was used as a model marine bacterial isolate.

In Chapter 3, to investigate the differences in quantity and quality of bacterial DOM among bacterial species and the key physiological processes for bacterial DOM production, the incubation experiments established in Chapter 2 were applied for three bacterial strains. In addition to *Alteromonas macleodii*, *Vibrio splendidus*, a ubiquitous free-living gammaproteobacterium in culturable marine bacterial community in the ocean (Urakawa et al., 1999; Radjasa et al., 2001), and *Phaeobacter gallaeciensis*, an attached alphaproteobacterium categorized into *Roseobacter* clade (Freese et al., 2017), were used as representative marine bacterial strains.

In Chapter 4, the degradability of bacterial DOM derived from bacterial strains was estimated by incubation experiments of natural microbial community with bacterial strain-derived DOM. Surface sea water from the subtropical North Pacific Ocean was used as the water contained natural free-living bacterial community in the open ocean.

In Chapter 5, the mechanisms of bacterial RDOM production revealed by two types of incubation experiments carried out in the present study are comprehensively discussed to draw the conclusions, and then, limitations and implications of the present

study were discussed.

Chapter 2.

Establishment of incubation methods of model marine bacterial strains

2-1 Introduction

The objective of this chapter is to establish incubation methods of model marine bacterial strains. Incubation of model bacterial strains is useful to investigate the relationship between physiology in terms of growth phase and RDOM production.

Alteromonas macleodii, a ubiquitous gammaproteobacterium from the surface to the deep layer of tropical and temperate oceans (López-Pérez et al., 2012), was used as a model marine bacterial isolate in this study. *Alteromonas* was reported to grow predominantly during diatom blooms in the western North Pacific Ocean (Tada et al., 2011). An *Alteromonas* sp., designated strain AltSIO, was isolated by the Scripps Institution of Oceanography from coastal seawater and found to be identified with ~99% 16S ribosomal RNA gene from *A. macleodii* (Pedler et al., 2014; Pedler Sherwood et al., 2015). Isolated AltSIO alone consumed the entire pool of labile DOC, defined as the quantity consumed by coastal microbial assemblages within 5 days (Pedler et al., 2014), and had the capability to significantly alter marine DOM

composition (Pedler Sherwood et al., 2015). These studies suggested that *A. macleodii* also might contribute to high consumption and alteration of labile DOM in the ocean surface. In this study, the humic-like fluorophores determined by EEM were used to evaluate bacterial RDOM, while protein-like fluorophores in EEM were used to monitor the reuse of the bacterial exopolymer.

2-2 Materials and method

2-2-1 Bacterial strain

The strain *A. macleodii* ATCC 27126 was obtained from the Japan Collection of Microorganisms, RIKEN Bio Resource Center (Tsukuba, Ibaraki, Japan), and was used as a model marine bacterial isolate.

Prior to the present experimental setup, 100 μ L frozen stock of *A. macleodii* was inoculated onto 100 mL artificial seawater-based Aquil medium (Price et al., 1989) supplemented with glucose (1 mmol C L⁻¹) at 25°C for 24 hours. Then, the medium was transferred onto new Aquil medium and incubated under the same conditions as the pre-culture medium. After 24 hours of incubation, the culture was used as inoculum for the present experiment.

2-2-2 Experiment setup

A. macleodii was cultivated in modified organic carbon-free Aquil medium (Price et al., 1989) (Table 2-1). Anhydrous salts used for artificial seawater (NaCl, Na₂SO₄, KCl, KBr) were combusted at 450°C for 4 hours. NaH₂PO₄·H₂O (10 µmol P L⁻¹) and NaNO₃ (161 µmol N L⁻¹) were added as major nutrients. The media for control and experimental treatments were prepared without and with 1 mmol C L⁻¹ glucose (#G8270, Sigma-Aldrich) in each triplicate, respectively. The *A. macleodii* inoculum was added to the medium of each treatment condition at a 1:1000 dilution. Then, each treatment condition with *A. macleodii* inoculum was dispensed into acid-washed (1 M HCl) 250 mL polyethylene terephthalate bottles. The bottle was filled with 100 mL of culture medium, and thus, gas phase was maintained in the bottles to keep oxygen at a high enough level to catabolize all of the added glucose. Incubations of the experimental and control treatments were conducted in the dark and in an incubator (CN-40, Mitsubishi-Engineering Co.) in which the temperature was maintained at 25°C. To determine the bacterial abundance, DOC concentration and DOM optical property, triplicate bottles were sampled at 0, 6, 12, 18 24, 72, 120, and 168 hours in the experimental treatment and at 0 and 168 hours in the control treatment.

2-2-3 Bacterial abundance

Bacterial cell density was measured with an EPICS flow cytometer (XL ADC system, Beckman Coulter) equipped with a 15 mW air-cooled laser exciting at 488 nm, according to the protocol of Tada and Suzuki (2016). Samples were fixed in paraformaldehyde (2% (vol/vol) final concentration) and preserved at -25°C. Just before analysis, samples were stained with SYBR Gold (SYBR Gold Nucleic Acid Gel Stain, Life Technologies) at a final concentration of 10^{-4} commercial stock solution for at least 15 minutes. To calculate the flow rate, 2 μm fluorescent beads (Fluoresbrite YG Carboxylate Microspheres 2.00 μm , Polysciences, Inc.) were added to the flow samples. We used a low flow rate mode and analyzed the samples until twenty thousand particles were counted or the measurement time reached 5 minutes. The measured values were corrected by blank value subtraction; the blank was measured with artificial seawater filtered with 0.2- μm pore size cellulose membrane filters (DISMIC-25AS 0.20 μm , ADVANTEC).

2-2-4 DOC concentrations and DOM optical properties

Samples for DOM analyses were filtered through pre-combusted (450°C, 3 hours) glass fiber filters with a nominal pore size of 0.3 μm (GF75, ADVANTEC) under gentle

vacuum (< 0.02 MPa) at each incubation time to remove particles, including *A. macleodii*. The filtrate was collected into a pre-combusted (450°C , 3 hours) glass vial with teflon-lined cap and was preserved at -25°C until analysis.

The DOC concentration was determined by high-temperature catalytic oxidation with a total organic carbon analyzer (TOC-V CSH, Shimadzu). The DOC concentrations were calculated using the standard curve of potassium hydrogen phthalate solution, which was determined daily. The accuracy and consistency of the measured DOC concentrations were checked by a deep seawater reference sample (Hansell laboratory, University of Miami), which was assessed daily.

EEM was measured using a fluorometer (FluoroMax-4, Horiba) according to the procedure of Tanaka et al. (2014). Samples were allowed to reach near room temperature before the EEM measurements were undertaken. Forty-one emission scans from 290 to 600 nm taken at 2-nm intervals were acquired for the excitation wavelengths between 250 and 450 nm at 5-nm intervals. The bandpass was set to 5 nm for both excitation and emission. The fluorescence spectra were scanned with a 0.25 s integration time and acquired in the S/R mode. Several post-acquisition steps were involved in the correction of the fluorescence spectra, including instrumental bias correction and corrections of inner filter effect using absorbance. Following this, the

EEM of Milli-Q water was subtracted from that of the sample, and fluorescence intensity in EEM was converted to Raman Units (RU) with the peak area of Raman scatter at 350 nm excitation (Lawaetz and Stedmon, 2009). RU can be converted to quinine sulfate units (QSU) by using the equation $QSU = RU / 0.0767$ (Lawaetz and Stedmon, 2009). The absorbance spectrum of each sample for correction of inner filter effect was measured with a Shimadzu UV-1800 spectrophotometer in a 1-cm quartz cuvette according to Yamashita et al. (2013). Because nitrate has relatively high absorbance in the UV-B region (Catalá et al., 2016) at high concentrations (e.g., $161 \mu\text{mol N L}^{-1}$ in the medium), investigations of changes in the absorption spectrum associated with *A. macleodii* incubation were abandoned. The contour of EEMs was plotted by R (version 3.2.3) (R Development Core Team, 2015).

2-3 Results

2-3-1 Growth of A. macleodii and change in DOC concentration

The abundance of *A. macleodii* increased exponentially during the first 24 hours in the experimental treatment (Figure 2-1A). The cell density was 4.1×10^4 cells mL^{-1} at the initiation of incubation ($t = 0$) and increased up to $3.7 \pm 1.8 \times 10^6$ cells mL^{-1} after 24 hours. The cell density then remained stable on a logarithmic scale until the end of the

experiment (Figure 2-1A). Therefore, the periods of 0–24 hours and 24–168 hours were defined as the exponential growth and stationary phases, respectively. The average specific growth rate was $0.19 \pm 0.10 \text{ h}^{-1}$ during the exponential growth phase. In the control treatment, cell density increased approximately twofold during the incubation (from $3.4 \times 10^4 \text{ cells mL}^{-1}$ at the initiation of the incubation to $8.0 \pm 0.5 \times 10^4 \text{ cells mL}^{-1}$ at the end of the incubation), indicating that bacterial growth caused by the medium substrate and carbon storage in the cells of the inoculum was much lower than that caused by glucose in the experimental treatment.

The DOC concentration gradually decreased during the early part of the exponential growth phase (first 18-hours) from $995 \mu\text{mol C L}^{-1}$ to $830 \pm 28 \mu\text{mol C L}^{-1}$ (Figure 2-1B) and then drastically decreased to $58 \pm 15 \mu\text{mol C L}^{-1}$ in the latter part of the exponential growth phase (18–24 hours). There was a larger relative decrease in DOC concentration normalized by bacterial cell density between 18 and 24 hours ($0.29 \text{ mmol C cell}^{-1}$) than between 0 and 18 hours ($0.17 \text{ mmol C cell}^{-1}$), thus suggesting a substantial increase in *A. macleodii* cell volume between 18 and 24 hours. The DOC concentration was almost constant (from $51 \pm 4 \mu\text{mol C L}^{-1}$ to $89 \pm 29 \mu\text{mol C L}^{-1}$) over the stationary phase and was $51 \pm 4 \mu\text{mol C L}^{-1}$ at the end of the experimental incubations (168 hours).

The DOC concentrations in the control treatment were 1–2 orders of magnitude lower than those in the experimental treatment and were $7.6 \mu\text{mol C L}^{-1}$ and $9.6 \pm 1.5 \mu\text{mol C L}^{-1}$ at the beginning and end of the incubation, respectively. The DOC concentrations detected in the control experiment were possibly derived from the medium and bacterial inoculum.

2-3-2 FDOM derived from *A. macleodii*

Fluorescent peaks found in EEMs at the 24-hour time point and the end of the experimental treatment are shown in Figure 2-2B and 2-2D, respectively. Because EEMs at the beginning of the experimental treatment (Figure 2-2A) and at the end of the control treatment (Figure 2-2E) did not show distinct fluorescent peaks, it can be concluded that fluorescent peaks found for the experimental treatment were derived from *A. macleodii* and not from the incubation medium or contamination during incubation.

Five fluorescent peaks were defined from EEMs obtained at the end of the experimental treatment (Figure 2-2D; Table 2-2). Two peaks were characterized as protein-like fluorescent peaks similar to the aromatic amino acids tyrosine and tryptophan (Coble, 1996; Mayer et al., 1999). From EEMs observed during the

experimental treatment (Figures 2-2B, 2-2C and 2-2D), the excitation and emission wavelength (Ex/Em) of tyrosine-like and tryptophan-like peaks were defined to be 275/300 nm and 275/330 nm, respectively. Three peaks could be categorized to humic-like fluorophores. Two of them had emission maximum longer than 500 nm (H1: Ex/Em = 270/520 nm, H3: Ex/Em = 425/520 nm) and have usually been defined to be terrestrial humic-like FDOM in coastal environments (Stedmon and Markager, 2005). The combined fluorescence characteristics of H1 and H3 were similar to that found in the most abundant component in the humic acid fraction extracted from sediments/soils (He et al., 2006; Santín et al., 2009). The other humic-like fluorophore (H2: Ex/Em = 315/400 nm), which had a shorter emission wavelength than H1 and H3, was similar to traditionally defined marine/microbial humic-like FDOM (Coble, 1996; 2007) and was also similar to a fluorophore excreted by cultured phytoplankton (Romera-Castillo et al., 2010).

2-3-3 Changes in fluorescence intensity of individual peaks during *A. macleodii*

incubation

The fluorescence intensities of two protein-like peaks, namely tyrosine-like and tryptophan-like peaks, increased during the exponential growth phase, particularly

during the period of 18–24 hours (Figure 2-3), in which the DOC concentration drastically decreased (Figure 2-1). Tyrosine-like fluorescence intensity increased during the early part of the stationary phase (24–72 hours, Figure 2-3; Student's *t*-test, $p < 0.05$) and was tended to decrease during the latter part of the stationary phase (72–168 hours; Student's *t*-test, $p > 0.05$). Tryptophan-like fluorescence intensity decreased significantly during the latter part of the stationary phase (Figure 2-3; Student's *t*-test, $p < 0.05$).

Tyrosine-like and tryptophan-like fluorophores exhibited fluorescence peaks with an excitation wavelength of 275 nm (Coble, 2007). It is difficult to quantitatively evaluate changes in protein-like fluorescence intensities, because the emission spectra of tyrosine and tryptophan molecules overlap (Mayer et al., 1999; Lakowicz, 2006). Thus, in this study, the emission spectrum at an excitation wavelength of 275 nm was used to evaluate the dominant protein-like fluorophore during *A. macleodii* incubation (Figure 2-4). The emission spectra were almost the same during 0–18 hours, but the fluorescence intensities at wavelengths corresponding to protein-like fluorescence (approximately 290–370 nm) were slightly increased with incubation time (Figures 2-3 and 4). A single fluorophore peaked at 330 nm appeared in 24 hours, indicating that tryptophan-like fluorophore was mainly produced during the exponential growth phase (Figures 2-2B and 2-4). It seemed that two fluorophores peaked at 330 nm and 300 nm

were the main components of the spectrum at 72 hours, indicating that both tryptophan-like and tyrosine-like fluorophores were produced during the early part of the stationary phase (Figures 2-2C and 2-4). Although fluorescence intensities corresponding to protein-like fluorophores decreased entirely during 72–168 hours, the decrease in the tryptophan-like fluorophore (peak at 330 nm) was greater than that of the tyrosine-like fluorophore (peak at 300 nm) (Figures 2-2D and 2-4). Furthermore, the tyrosine-like fluorophore became a major peak at 168 hours. The fluorescence intensity at 300 nm should be composed of the peak maximum of tyrosine-like fluorophore and the peak edge of tryptophan-like fluorophore. Thus, changes in the shape of emission spectrum observed during 72–168 hours imply that the tryptophan-like fluorophore decreased but the tyrosine-like fluorophore was relatively stable during the latter part of the stationary phase.

The fluorescence intensities of all humic-like fluorophores increased during the exponential growth phase (Figure 2-5). The changes in fluorescence intensity during the stationary phase varied among the humic-like fluorophores (Figure 2-5). Two humic-like fluorophores with emission maxima at longer wavelengths (H1 and H3) continuously increased throughout the stationary phase. In contrast, the other humic-like fluorophore (H2) was relatively stable during the stationary phase (Student's *t*-test,

$p>0.05$).

Fluorescence intensities of all fluorophores were comparable in the control and experimental treatments at the initiation of these treatments (Figures 2-3 and 2-5). In the control treatment, the fluorescence intensities of all humic-like fluorophores remained unchanged during the incubation (Figure 2-5). Although the fluorescence intensities of protein-like fluorophores in the control treatment increased several fold during the 168-hour incubation, these intensities were 1 order of magnitude lower than those observed for the experimental treatment at the end of the incubation (Figure 2-3).

2-4 Discussion

2-4-1 Changes in DOM quantity and quality with *A. macleodii* growth

A. macleodii has been reported to be a ubiquitous gammaproteobacterium in temperate oceans (López-Pérez et al., 2012). The possibility that *A. macleodii* substantially contributes to the consumption and alteration of labile DOM in surface waters of marine environments has also been considered (Tada et al., 2011; Pedler et al., 2014; Pedler Sherwood et al., 2015). Glucose was used as a labile substrate for *A. macleodii* incubation in this study. Previous studies demonstrated that coastal microbial communities completely consumed glucose within 2 days of incubation (Ogawa et al.,

2001; Kawasaki and Benner, 2006). The decrease in the DOC concentration was coupled with the increase in *A. macleodii* abundance, indicating that the decrease in the DOC concentration during the exponential growth phase should be due to the consumption of glucose by the bacterial strain. Assuming that the glucose was completely consumed within several days of incubation, residual DOC was produced by *A. macleodii*.

Protein molecules that contain both tryptophan and tyrosine molecules usually show only tryptophan fluorescence because of energy transfer (Lakowicz, 2006). A previous study analyzed the fluorescence properties of surface DOM in the Sagami Bay and showed that only tryptophan-like fluorescence was evident in high-molecular-weight fractions, whereas tyrosine-like fluorescence was dominant in low-molecular-weight fractions (Yamashita and Tanoue, 2004). Thus, changes in the composition of protein-like fluorophores with *A. macleodii* growth possibly reflected the changes in the relative molecular weight of peptides/proteins, which were released and reused by *A. macleodii*. Tryptophan-like fluorophore clearly appeared at 24 hours in our experiment (Figure 2-4), implying that large polymers, such as protein molecules, were predominantly released during the exponential growth phase. The incubations contained not only tryptophan-like fluorophore but also tyrosine-like fluorophore, which both

increased during 24–72 hours (Figure 2-4). This result suggested that large polymers were still released but that part of these exopolymers were degraded to small peptides. The decrease in tryptophan-like fluorophore was predominant, but tyrosine-like fluorophore was relatively stable at the latter part of the stationary phase (72–168 hours), suggesting that the production of small peptides due to the degradation of large exopolymers was correlated with the reutilization of tyrosine-like fluorophores.

The reutilization of exopolymer by *A. macleodii* was suggested in the stationary phase while two humic-like fluorophores continued to accumulate, implying that unavailable DOM were produced from available substrate. The DOC concentrations were relatively stable during the stationary phase, as mentioned above. These results suggested that major portions of DOM produced by *A. macleodii* are unavailable to these bacteria, although a minor fraction of this DOM is reusable by the strain. The remaining DOC concentration produced by *A. macleodii* was estimated to be $41 \pm 4 \mu\text{M}$ C by subtracting the DOC concentration at the end of control treatment ($9.6 \pm 1.5 \mu\text{M}$ C; Figure 2-1) from that of experimental treatment ($51 \pm 4 \mu\text{M}$ C; Figure 2-1). This remnant DOC concentration corresponded to approximately 4% of the initial glucose used for the *A. macleodii* incubation. Notably, this estimation is comparable to the results obtained by previous studies that conducted incubations of bacterial isolates or

microbial communities with glucose. Gruber et al. (2006) showed that 3% of initial glucose was converted to residual DOC by the bacterial isolate *P. chlororaphis* during 36 days of incubation. In addition, 3–6% of initial glucose was found to convert to molecularly uncharacterizable DOM in marine microbial communities, and occurred after 90-day to 2-year periods of incubation (Ogawa et al., 2001; Shimotori et al., 2009; Koch et al., 2014). Such consistency in the production efficiency of residual DOC (i.e., 3–6% of initial substrate) suggests that neither microbial community structure nor microbial growth affected the efficiency of MCP.

2-4-2 Humic-like fluorophores produced by A. macleodii

The production of humic-like fluorophores has been observed in incubation experiments using marine microbial communities (Kramer and Herndl, 2004; Lønborg et al., 2009; Shimotori et al., 2009). This study demonstrated that humic-like fluorophores can be generated not only by microbial communities but also by single bacterial strains using simple substrates, such as glucose. This finding is consistent with those of Shimotori et al. (2012), who found that humic-like fluorophores were generated during incubations of several bacterial isolates in Marine Broth culture medium.

A humic-like fluorophore (H2) produced by *A. macleodii* was similar to a

previously defined marine microbial humic-like peak M (Coble, 1996; 2007). This fluorophore also corresponded to a fluorophore that was found to be microbial recalcitrant FDOM with the time scale of the thermohaline circulation (Yamashita and Tanoue, 2008). Fluorescent components similar to H2 were obtained through parallel factor analysis (PARAFAC) of FDOM from the open ocean and were found to be related to apparent oxygen utilization in the deep ocean (Jørgensen et al., 2011; Tanaka et al. 2014), implying that H2 produced by *A. macleodii* is possibly recalcitrant DOM. On the other hand, peak M was produced by phytoplankton isolates (Romera-Castilo et al., 2010) and consumed partially by bacterial communities (Romera-Castilo et al., 2011). These results suggest that the reactivity of the peak M-type fluorophore was dependent on its source; specifically, the fluorophore produced by heterotrophic bacteria is possibly recalcitrant, while that produced by phytoplankton includes a labile fraction.

The other two humic-like fluorophores (H1 and H3), which peaked at longer emission wavelengths than that of H2, continued to be produced by *A. macleodii* during both the stationary phase and the exponential growth phase. Shimotori et al. (2009) also observed a fluorophore peaked at >500 nm emission during the incubation of coastal microbial communities with glucose. On the other hand, there was a high presence of

PARAFAC components that were similar to a combination of H1 and H3 in humic acid fractions extracted from soils and sediments (He et al., 2006; Santín et al., 2009). These PARAFAC components were also considered to be terrigenous FDOM (Stedmon and Markager, 2005; Singh et al., 2013). These results suggest that the fluorophore that peaked at a longer emission wavelength (>500 nm) might be ubiquitously produced by heterotrophic bacteria but is enriched in humic acid fractions from soils and sediments. Notably, in Shimotori's incubation studies (Shimotori et al., 2009), this fluorophore appeared in EEMs during the period of increasing bacterial abundance but disappeared from EEMs during the period of decreasing bacterial abundance. In the open ocean, this fluorophore is generally not present (Jørgensen et al., 2011; Tanaka et al., 2014). Thus, *A. macleodii* may be one of the key species that produces this fluorophore, and this fluorophore might be consumed by other microbes in natural environments.

2-4-3 Potential relationship between bacterial growth and the production of humic-like fluorophore

The production rates of microbial humic-like fluorophore differ among substrates used in the incubation of marine bacterial communities (Jørgensen et al., 2014; Aparicio et al., 2015). For example, Aparicio et al. (2015) found that the production rates of marine

humic-like fluorophore during the incubation of marine bacterial communities with Suwannee River humic acids were higher than those with glucose and acetate. Using different substrates for microbial community incubation reportedly cause changes in microbial community structure and/or bacterial physiology (Gómez-Consarnau et al., 2012). Therefore, in studies of bacterial communities, it is difficult to evaluate whether the factor regulating the production of humic-like fluorophores is bacterial species or physiology; in contrast, incubations using a bacterial isolate allow insight into the relationship between bacterial physiology and RDOM production.

The bacterial physiology was evaluated in terms of growth phases in this study. The quantity or quality of substrate is one of the critical factors in controlling bacterial growth (Jannasch and Egli, 1993). Therefore, the physiology of *A. macleodii* was likely altered by the change in substrate availability. The exponential growth phase of *A. macleodii* can be characterized as the period that the strain consumed glucose and produced various extracellular compounds, e.g., three humic-like fluorophores as well as a tryptophan-like fluorophore. The physiology of *A. macleodii* shifted to the stationary phase after the complete consumption of glucose, and the tryptophan-like fluorophore was consumed, but two humic-like fluorophores were produced. The results showed that the production mechanisms of humic-like fluorophores, which cannot be

reused by the strain, are correlated with the physiology of *A. macleodii*. The humic-like fluorophore (H2) that is similar to recalcitrant microbial humic-like FDOM (Yamashita and Tanoue, 2008; Catalá et al., 2015) is only produced in the exponential phase, but other fluorophores (H1 and H3), which might be consumed by other microbes (Shimotori et al., 2009), are produced during both the exponential and the stationary phases of *A. macleodii*.

The relationship between bacterial growth and RDOM production that is apparent with *A. macleodii* was not consistent with the previous studies conducted by Gruber et al. (2006) and Eichinger et al. (2009). *A. macleodii* was found to produce extracellular humic-like fluorophores, which were unavailable to the strain during the exponential growth and the stationary phases. However, DOC produced by a different *Alteromonas* strain (*A. infernus*) was accumulated under conditions of carbon starvation of the isolate (i.e., the stationary phase in this study) during the incubation in which pyruvate was added every 48-hours as labile substrate (Eichinger et al., 2009). Specific compounds detected by mass spectrometry with electrospray ionization were produced in the exponential growth phase and remained at the end of *P. chlororaphis* incubation with glucose for 36 days, suggesting that recalcitrant compounds, which cannot be reused by the strain, were possibly produced during the exponential growth phase (Gruber et al.,

2006). Although incubation conditions, such as temperature, the quality and quantity of substrate, and the composition of artificial seawater, varied among the studies, the results obtained by this study and the previous studies suggest that the physiology related to RDOM production might differ between bacterial species.

2-5 Conclusion

The present study first evaluated the relationship between bacterial growth and the production of humic-like fluorophores during the incubation of a model bacterial isolate, *A. macleodii*, with glucose as a substrate. The results of the experiment showed that *A. macleodii* produced three humic-like fluorophores; one is optically similar to recalcitrant microbial humic-like FDOM, while the other two might be consumed by other species of marine bacteria. The compositions of humic-like fluorophores produced by *A. macleodii* differed between the growth phases of the isolate, which might have been affected by the changes in available substrate from glucose to extracellular compounds (such as tryptophan-like fluorophore) released by the isolate.

On the basis of prior studies, variable recalcitrant characteristics are suggested by the humic-like fluorophores produced by *A. macleodii*, although these humic-like fluorophores appeared to be unavailable to the strain. To confirm the recalcitrant

characteristics of DOM produced by bacterial isolates (e.g., humic-like fluorophores), the incubation of bacterial DOM with marine microbial communities is necessary. Isolate culture experiments with different incubation conditions (e.g., temperature, substrate quality and quantity) may provide a better understanding of the effect of bacterial physiology on RDOM production. Incubation studies using several species of bacterial isolates with the same conditions are also necessary to confirm whether the relationship between bacterial physiology and RDOM production differs among bacterial species. The cumulative results of such in vitro experiments will provide better insight into the relationship between bacterial physiology and RDOM production, and thus, such experiments will contribute to a better understanding of the factors that regulate the efficiency of MCP, which potentially shapes the pool size and composition of marine DOM.

Table 2-1 Composition of modified Aquil medium

	Compound	Final concentration (mol L ⁻¹)
Salts	NaCl	4.20×10 ⁻¹
	Na ₂ SO ₄	2.88×10 ⁻²
	KCl	9.39×10 ⁻³
	NaHCO ₃	2.38×10 ⁻³
	KBr	8.40×10 ⁻⁴
	H ₂ BO ₃	4.85×10 ⁻⁵
	NaF	7.15×10 ⁻⁵
	MgCl ₂ · 6H ₂ O	5.46×10 ⁻²
	CaCl ₂ · 2H ₂ O	1.05×10 ⁻²
	SrCl ₂ · 6H ₂ O	6.38×10 ⁻⁵
Trace metal	FeCl ₂ · 6H ₂ O	1.00×10 ⁻⁶
	ZnSO ₄ · 7H ₂ O	7.97×10 ⁻⁸
	MnCl ₂ · 4H ₂ O	1.21×10 ⁻⁷
	CoCl ₂ · 6H ₂ O	5.03×10 ⁻⁸
	CuSO ₄ · 5H ₂ O	1.96×10 ⁻⁸
	Na ₂ MoO ₄ · 2H ₂ O	1.00×10 ⁻⁷
	Na ₂ SeO ₃	1.00×10 ⁻⁸
Major nutrients	NaHPO ₄ · H ₂ O	1.00×10 ⁻⁵
	NaNO ₃	1.61×10 ⁻⁴

Table 2-2 Characteristics of fluorescent peaks produced by *A. macleodii*

Excitation wavelength (nm)	Emission wavelength (nm)	Peak (abbreviation)	Description of previous study
275	300	Tyrosine-like (B)	Tyrosine ¹
275	330	Tryptophan-like (T)	Low molecular weight peptide ² Tryptophan ¹
270	520	Humic-like 1 (H1)	High molecular weight peptide, protein molecule ² Enriched in humic acid fraction ^{3,4}
315	400	Humic-like 2 (H2)	Marine autochthonic substance ⁵
425	520	Humic-like 3 (H3)	Enriched in humic acid fraction ^{3,4}

1, Mayer et al. (1999); 2, Yamashita and Tanoue (2004); 3, He et al. (2006); 4, Santín et al. (2009); 5, Coble (1996)

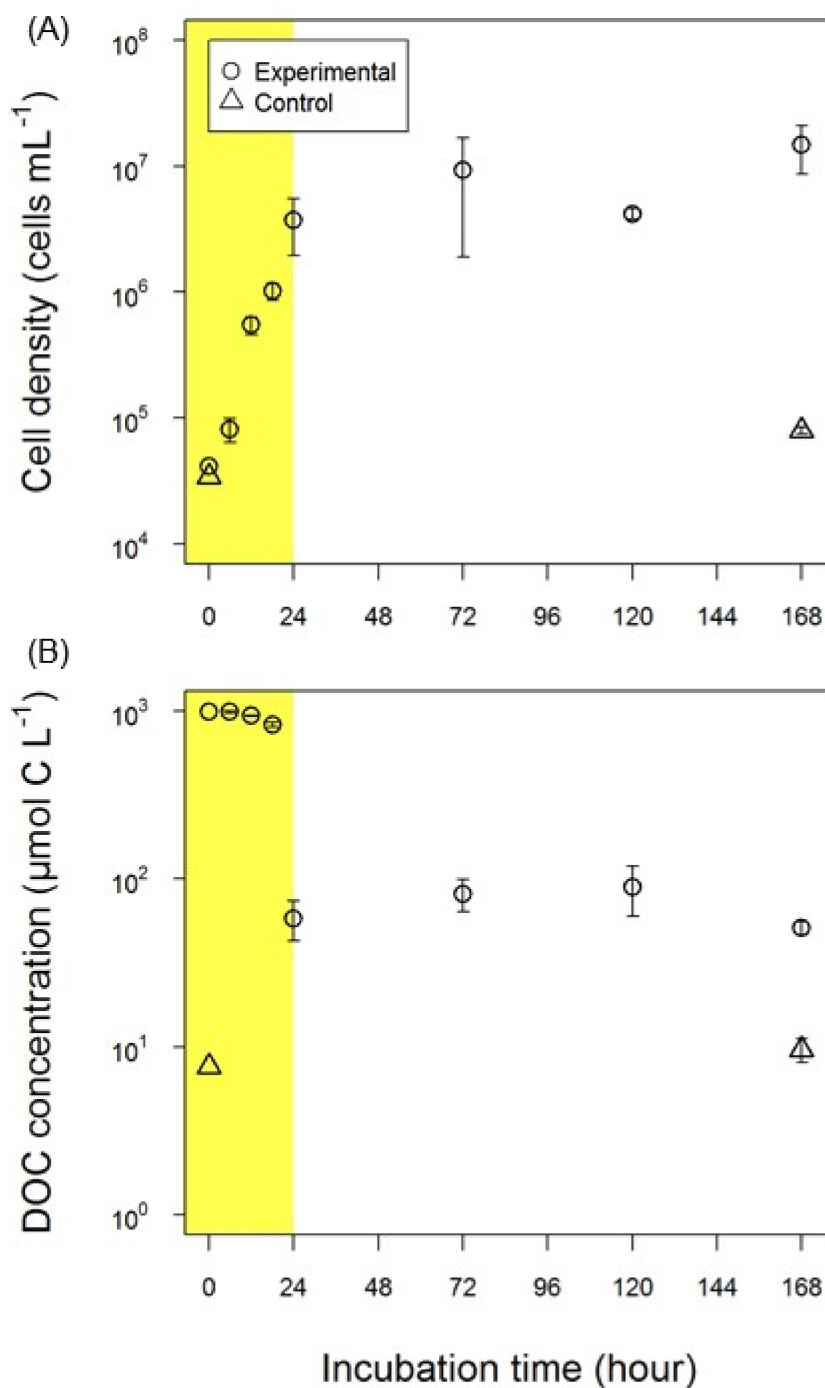


Figure 2-1 Changes in (A) the abundance of *Alteromonas macleodii* (*A. macleodii*) and (B) the concentration of dissolved organic carbon (DOC). Circles and triangles represent the experimental and control treatments, respectively. Yellow shading indicates the exponential growth phase.

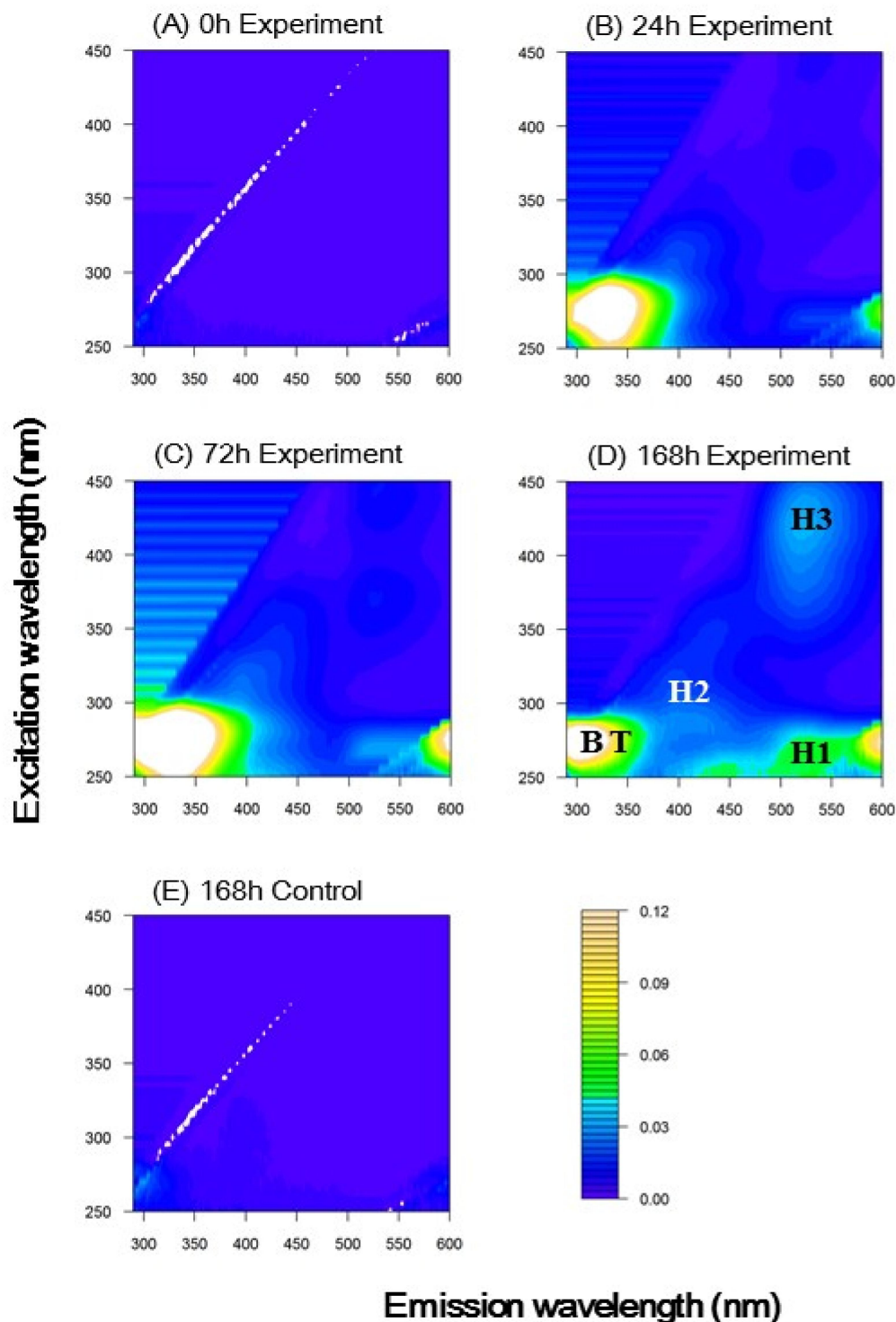


Figure 2-2 Excitation-emission matrix (EEMs) based on the average of triplicate samples at (A) 0, (B) 24, (C) 72 and (D) 168 hours in the experimental treatment, and at (E) 168 hours in the control treatment. Peaks generated by *A. macleodii* were indicated with abbreviations: Tyrosine-like (B, Ex/Em = 275/300 nm); Tryptophan-like (T, Ex/Em = 275/330 nm); Humic-like 1 (H1, Ex/Em = 270/520 nm); Humic-like 2 (H2, Ex/Em = 315/400 nm); Humic-like 3 (H3, Ex/Em = 425/520 nm).

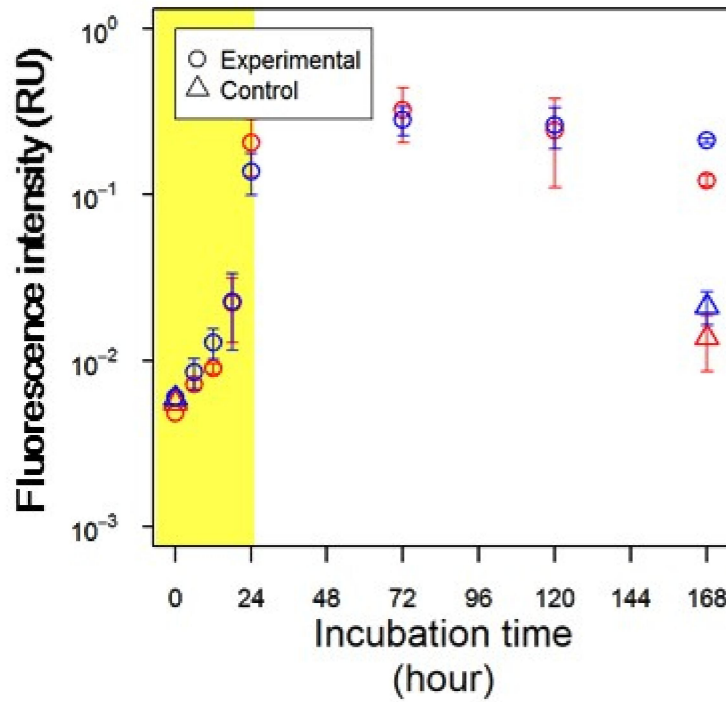


Figure 2-3 Changes in fluorescence intensities of protein-like peak. Red and blue symbols indicate tyrosine-like peak ($\text{Ex/Em} = 275/300 \text{ nm}$) and tryptophan-like peak ($\text{Ex/Em} = 275/330 \text{ nm}$), respectively. Circles and triangles represent the experimental and control treatments, respectively. Yellow shading indicates the exponential growth phase.

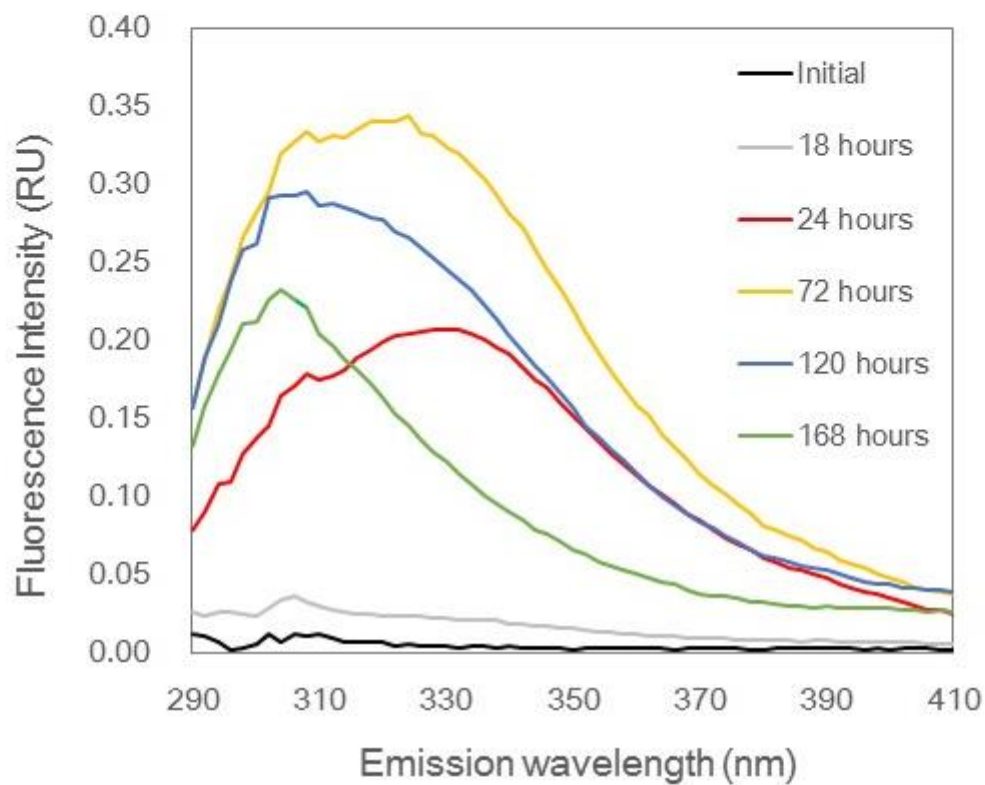


Figure 2-4 Emission spectra at 275 nm excitation in the experimental treatment based on the average of triplicate samples. These spectra indicate changes of protein-like fluorophore. The color of each spectrum represents each incubation time.

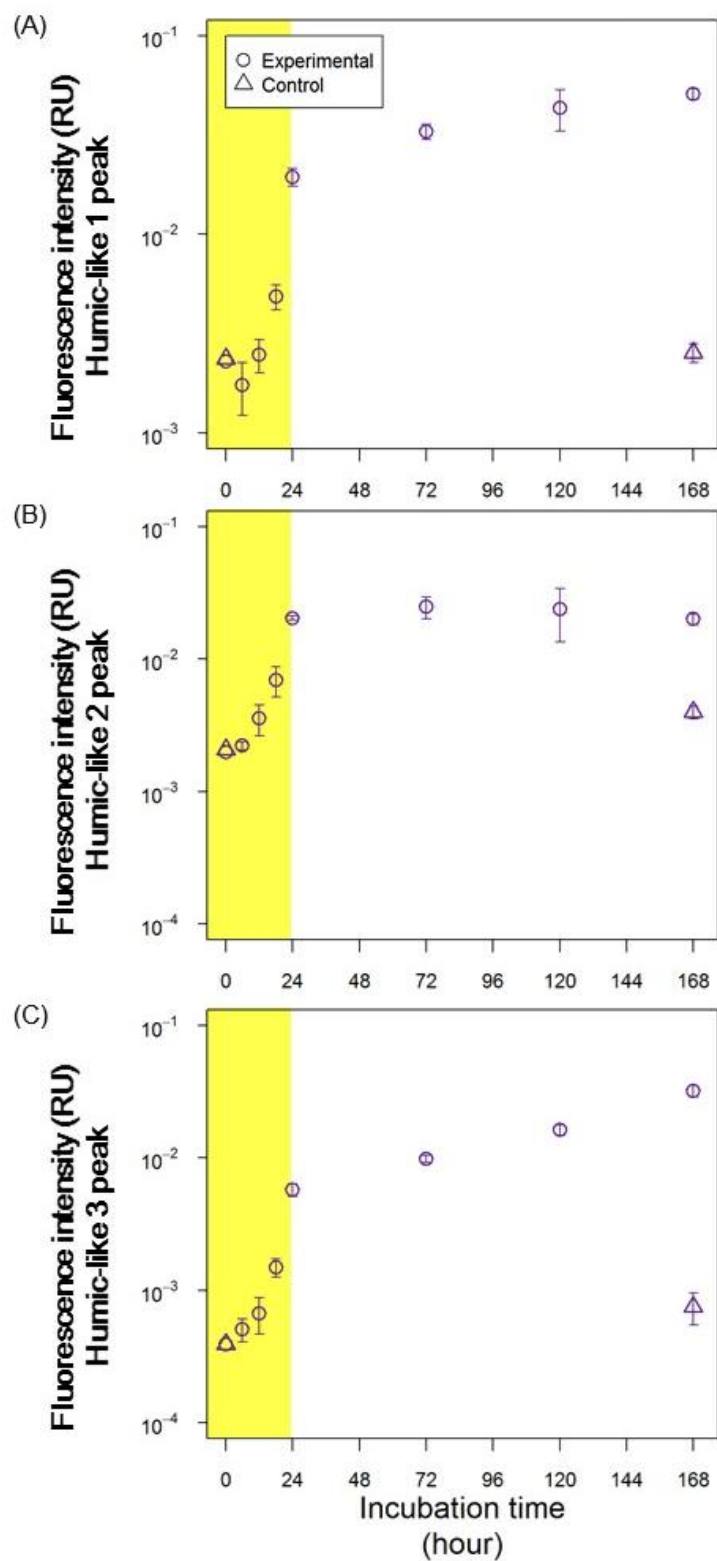


Figure 2-5 Changes in fluorescence intensities of (A) humic-like 1 peak (Ex/Em = 270/520 nm), (B) humic-like 2 peak (Ex/Em = 315/400 nm) and (C) humic-like 3 peak (Ex/Em = 425/520 nm). Circles and triangles represent the experimental and control treatments, respectively. Yellow shading indicates the exponential growth phase.

Chapter 3.

Comparison of production of dissolved organic matter by model marine bacterial strains

3-1 Introduction

In Chapter 2, a method of bacterial strain incubation was established to evaluate DOM production by the bacteria with consumption of labile substrate. In this chapter, DOM production of three marine bacterial strains were compared to understand differences in physiological process and efficiencies of bacterial DOM production among bacterial species.

The features in *Alteromonas macleodii* (Alt) as a model marine bacterial strain were mentioned in Chapter 2. Another model marine bacterial strain belonging to Gammaproteobacteria, namely *Vibrio splendidus* (Vib), was used in this study. *Vibrio splendidus* has been reported to be common heterotrophic free-living bacteria in culturable bacterial community in the coastal and open ocean (Urakawa et al., 1999; Radjasa et al., 2001). The population of *Vibrio splendidus* in coastal region has been reported to comprise variable phylogenetic groups adapted to ecological condition, namely seasons and habitats categorized with size fractions of samples (Hunt et al.,

2008). These studies suggested that the strain can be a model marine bacterial strain for free-living and for responding to supply of labile DOM. *Phaeobacter gallaeciensis* (Pha) was used as the third model marine bacterial strain in this study. The genus *Phaeobacter*, belonged to the *Roseobacter* clade, are predominant frequently in phytoplankton blooms and have versatile metabolism (Pommier et al., 2005; Newton et al., 2010). *Phaeobacter gallaeciensis* has been reported that they attached to zooplankton or coccolithophore (Freese et al., 2017; Sesedsayamdost et al., 2011). Therefore, the Pha strain can be considered as a model bacterial species of attached bacteria belonging to *Roseobacter* clade.

It has been reported that chemical composition of bacterially derived DOM is related to bacterial community composition (Lucas et al. 2016; Osterholz et al. 2016; Tada et al., 2016; Tada et al., 2017). However, it is difficult to identify the key bacterial species contributing to the RDOM production from the observation or *in vitro* experiments with bacterial community. Previous studies of incubation experiments comparing DOM production between two bacterial strains indicated that compositions of DOM derived from the bacteria were clearly different from each other (Wienhausen et al., 2017; Noriega-Ortega et al., 2019). However, information on difference in efficiency and physiological process of RDOM production among bacterial species has

been limited, even though it is possibly one of the key parameters shaping the size and composition of the marine DOM pool. In the present study, comparison of the efficiency as well as process of RDOM production among the three model bacterial strains can provide new insights into mechanisms of RDOM production.

In this chapter, to investigate the differences in quantity and quality of bacterial DOM among bacterial species and the key physiological processes for bacterial DOM production, the incubation experiments were applied for the three model bacterial strains.

3-2 Materials and methods

3-2-1 Bacterial strains

Alteromonas macleodii ATCC 27126 (Alt), *Vibrio splendidus* ATCC 25914 (Vib), and *Phaeobacter gallaeciensis* ATCC 700781 (Pha) were obtained from the Japan Collection of Microorganisms, RIKEN Bio Resource Center (Tukuba, Ibaraki, Japan). These three strains were used as model marine bacterial isolates in the present study.

Prior to the experimental setup, 100 μ L frozen stock of each strain was inoculated onto 100 mL artificial seawater-based Aquil medium (Price et al., 1989) supplemented with glucose (1 mmol C L⁻¹) at 25°C. The incubation periods were determined based on

the length of the exponential growth phases of three strains (Alt, 24h; Vib, 24h; Pha, 36h). Then, each medium was transferred onto new Aquil media and incubated under the same condition for the same period with pre-incubation. After the incubation, the culture was used as inoculum for the experiments described below.

3-2-2 Experiment setup

Experimental design was basically the same with that described in Chapter 2 (see 2-2-2 for details). However, nitrogen source and incubation periods were optimized for each bacterial strain. While nitrogen source was nitrate for the Alt incubation (Chapter 2), ammonium chloride ($161 \mu\text{mol N L}^{-1}$) was used for incubations of Vib and Pha because these two strains cannot use nitrate as nitrogen source. Incubation periods and sampling time points were summarized in Table 3-1.

3-2-3 Bacterial abundance

In the Alt and Vib experiments, the bacterial cell density measurement was followed the procedure described in detail in Chapter 2.

The bacterial abundance of the Pha experiment was measured with epifluorescence microscope (BZ-9000, KEYENCE). The samples were fixed by

paraformaldehyde (2% (vol/vol) final concentration) and preserved at -80°C. The fixed samples were filtered onto 0.2 µm pore size polycarbonate membrane filters (WHA-110656, Whatman, GE Healthcare) and strained with a DAPI mixture. The DAPI mixture constituted of 5.5 parts (by volume) Citifluor (Citifluor Ltd.), 1 parts Vectachield (Vector Labs) and 0.5 parts PBS with 4',6-diamidino-2-phenylindole at a final concentration of 2 µg mL⁻¹. Filters were prepared for a microscope with glass slides and cover glass.

Fifteen images of fluorescence microscope were stored as TIFF files per sample and analyzed using ImageJ software (ver.1.49, Wayne Rasband, National institutes of Health). The analysis procedures included the Laplace filter (5 × 5 kernel), Gaussian filter (radius 3) and median filter (rank 3), for enhancing cell boundaries and removing noise (Fazi et al., 2008). After the procedures, particles in each image were counted with the software.

3-2-4 Organic carbon concentrations and DOM optical properties

The procedure of measurement in DOC and total organic carbon (TOC) concentration were the same with the DOC measurement described in Chapter 2 (see section 2-2-4 for details). The samples for DOC analyses were filtered with a nominal pore size of 0.3

µm (GF75, ADVANTEC) to exclude particles, mainly bacterial cells, while the samples for TOC analyses were not filtered and included DOM and bacterial cells. Therefore, particulate organic carbon (POC) concentration, corresponding to bacterial biomass possibly including the dead cells during the stationary and death phases, was calculated by subtracting DOC from TOC.

Optical analyses for DOM were also described in detail Chapter 2 (see section 2-2-4).

3-3 Results

3-3-1 Growth of bacteria and change in organic carbon concentration

The bacterial abundance of the three model strains in the experimental treatments increased exponentially with decreases in the DOC concentrations during early phase of the incubations although those changes and timing were different among the three strains. After the early phase of the incubations, patterns of changes in the bacterial abundance, DOC and POC concentrations with incubation time were also different among each strain.

3-3-1-1 Alt treatment

The abundance of Alt in the experimental treatment increased exponentially during the period of 0–24 h, and reached $3.7 \pm 1.8 \times 10^6$ cells mL⁻¹ at 24 h. From 24 h to the end of incubation, the cell density was stable (Chapter 2, Figure 3-1a). The cell density in the control treatment (i.e., glucose-free treatment with inoculum) increased slightly from 3.4×10^4 cells mL⁻¹ at initiation of the incubation to $8.0 \pm 0.5 \times 10^4$ cells mL⁻¹ at the end of the incubation, indicating that bacterial growth caused by the medium substrate of pre-incubation and carbon storage in the cells of the inoculum was much lower than that caused by glucose in the experimental treatment (Chapter 2, Fig. 3-1a).

The DOC concentration gradually decreased during the period of 0-18 h, and then drastically decreased from 18 h to 24 h of incubation time point (Chapter 2, Figure 3-2a). The DOC concentration decreased from 995 $\mu\text{mol C L}^{-1}$ at initiation of the incubation to 830 ± 28 $\mu\text{mol C L}^{-1}$ at 18 h and 58 ± 15 $\mu\text{mol C L}^{-1}$ at 24 h, respectively. Change in the DOC concentration fell within the narrow range relative to initial DOC concentration during the period of 24-168 h. Those DOC concentrations were 82 ± 18 $\mu\text{mol C L}^{-1}$, 89 ± 29 $\mu\text{mol C L}^{-1}$, and 51 ± 5 $\mu\text{mol C L}^{-1}$, at 72, 120, and 168 h, respectively. The DOC concentration in the control treatment were 7.6 $\mu\text{mol C L}^{-1}$ and 9.6 ± 1.5 $\mu\text{mol C L}^{-1}$ at the beginning and end of the incubation, respectively.

The TOC concentration was comparable to the DOC concentration until 18 h, and

then drastically decreased from 18 h to 24 h (Chapter 2, Figure 3-3a). The TOC concentration decreased from $831 \pm 15 \mu\text{mol C L}^{-1}$ at 18 h to $296 \pm 2 \mu\text{mol C L}^{-1}$ at 24 h. The TOC concentration after 24 h remained higher level than DOC concentration in each time point, and slightly decreased until the end of the incubation. The TOC concentration in the control treatment were $7.3 \mu\text{mol C L}^{-1}$ and $15 \pm 11 \mu\text{mol C L}^{-1}$ at the beginning and end of the incubation, respectively.

The POC concentration could not be determined during the period of 0–24 h in the experimental treatment and throughout the incubation in the control treatment since the concentration was equivalent to the analytical errors of DOC and TOC (Figure 3-4a). The POC concentration was firstly able to detect at 24 h and was the highest concentration, i.e., $237 \pm 17 \mu\text{mol C L}^{-1}$, during the incubation. The concentrations were $192 \pm 21 \mu\text{mol C L}^{-1}$, $165 \pm 23 \mu\text{mol C L}^{-1}$, and $188 \pm 8 \mu\text{mol C L}^{-1}$ at 72, 120, and 168 h, respectively. Because POC was equivalent to bacterial biomass, the change in POC concentration indicated that the biomass of Alt was fluctuated during the period of 24–168 h.

The period of 0–18 h was defined as the exponential growth phase, because the cell density did not reach the highest level and the labile DOC concentration which was consumed later was remained during the period. The period of 24–168 h was defined as

stationary phase due to stability of the cell density (Chapter 2, Figures 3-1a and 3-2a).

3-3-1-2 Vib treatment

Figure 3-1b shows changes in the cell density of Vib experiment. The abundance of Vib in the experimental treatment increased drastically just after the start of incubation and reached the highest cell density at 72 h. The cell density was 6.5×10^4 cells mL⁻¹ at the initiation of incubation, and reached $1.5 \pm 0.4 \times 10^6$ cells mL⁻¹ at 72 h. Then, the abundance of Vib decreased until the end of incubation in the experimental treatment. The cell densities were $1.3 \pm 0.4 \times 10^5$ cells mL⁻¹, $6.2 \pm 0.6 \times 10^5$ cells mL⁻¹, and $7.0 \pm 1.3 \times 10^4$ cells mL⁻¹ at 120, 168, 600 h, respectively. In the control treatment, the abundance of Vib increased from initiation of incubation (1.3×10^4 cells mL⁻¹) to other time points (168h; $1.6 \pm 2.2 \times 10^5$ cells mL⁻¹; 600h; $9.1 \pm 2.5 \times 10^4$ cells mL⁻¹). The growth of the control treatment caused by the medium substrate and carbon storage in the cells of pre-incubation was lower than the experimental treatment at 168h, although the cell density was comparable between the two treatments at 600 h because of decreases in the cell abundance of experimental treatment.

The DOC concentration in the experimental treatment slightly decreased from the initial of experiment ($1046 \mu\text{mol C L}^{-1}$) to 12 h ($1022 \pm 8 \mu\text{mol C L}^{-1}$) and largely

decreased from 12 h to 72 h ($173 \pm 38 \mu\text{mol C L}^{-1}$) (Figure 3-2b). From 72 h to the end of incubation, the DOC concentration remained almost constant; the DOC concentrations at 120, 168, and 600 h were $202 \pm 39 \mu\text{mol C L}^{-1}$, $205 \pm 11 \mu\text{mol C L}^{-1}$, and $218 \pm 8 \mu\text{mol C L}^{-1}$, respectively. The DOC concentration in the control treatment was stable with relatively low concentration compared to the experimental treatment; $11 \mu\text{mol C L}^{-1}$, $8 \pm 1 \mu\text{mol C L}^{-1}$, and $11 \pm 1 \mu\text{mol C L}^{-1}$ at initiation of incubation, 168 h, and 600 h, respectively. The stability of DOC concentration in the control treatment suggested that the increases in cell density did not induce changes in DOC concentration.

The TOC concentration was comparable to that of DOC from the initial until 12 h in the experimental treatment and decreased drastically from 12 h to 72 h (Figure 3-3b). The TOC concentration was $994 \mu\text{mol C L}^{-1}$ at initiation of incubation, and then went down to $441 \mu\text{mol C L}^{-1}$ and $412 \mu\text{mol C L}^{-1}$ for the duplicate at 72 h (a value of the other duplicate bottle at 72 h, which was $3849 \mu\text{mol C L}^{-1}$ of TOC concentration was omitted from the data analyses due to apparent contamination). The TOC concentration from 72 h to the end of incubation was relatively stable ($398 \pm 19 \mu\text{mol C L}^{-1}$, $381 \pm 4 \mu\text{mol C L}^{-1}$, and $387 \pm 13 \mu\text{mol C L}^{-1}$ at 120, 168, 600 h, respectively) and exceeded corresponding DOC concentration due to increase in biomass of *Vib* as POC. The TOC

concentrations in the control treatment were $7 \mu\text{mol C L}^{-1}$ and $14 \pm 2 \mu\text{mol C L}^{-1}$ at the initiation and end of incubation, respectively.

The POC concentration in the experimental treatment was firstly detected at 72 h as $276 \mu\text{mol C L}^{-1}$ ($292 \mu\text{mol C L}^{-1}$ and $259 \mu\text{mol C L}^{-1}$ for the duplicate) (Figure 3-4b), since the TOC concentration were not different to DOC concentration significantly from the initial until 24 h of the incubation (Figures 3-2b and 3-3b). As like, the POC concentration could not determine throughout the control treatment. The POC concentration continued to decrease after 72 h of incubation in the experimental treatment and was $168 \pm 6 \mu\text{mol C L}^{-1}$ at the end (600 h).

The period of 0–24 h was defined as the exponential growth phase, while the period of 72-600 h was defined as the stationary phase (including a part of the death phase) from the changes over time in cell density and DOC concentration in the Vib experimental treatment.

3-3-1-2 Pha treatment

Figure 3-1c shows changes in the cell density in the Pha experiment. The abundance of Pha in the experimental treatment increased exponentially from initiation of incubation to 48 h and remained the higher level in cell density from 96 h to the end of incubation.

The cell density was 3.8×10^5 cells mL⁻¹ at initiation of incubation and measured as $3.8 \pm 0.5 \times 10^7$ cells mL⁻¹, $3.8 \pm 0.8 \times 10^7$ cells mL⁻¹ and $4.0 \pm 0.5 \times 10^7$ cells mL⁻¹ at 96, 168, and 360 h, respectively. The cell concentrations in the control treatment were 3.4×10^5 cells mL⁻¹, $6.3 \pm 0.8 \times 10^5$ cells mL⁻¹ at the initiation and end of the incubation, respectively.

The DOC concentration in the experimental treatment decreased gradually from the initiation of incubation to 27 h, and then went down drastically to 96 h (Figure 3-2c). The DOC concentration of $985 \mu\text{mol C L}^{-1}$ at the initial decreased to $646 \pm 32 \mu\text{mol C L}^{-1}$ at 36 h, and the concentrations were $219 \pm 70 \mu\text{mol C L}^{-1}$ and $63 \pm 1 \mu\text{mol C L}^{-1}$ at 48 and 96 h, respectively. From 96 h to the end of incubation, the DOC concentration remained relatively low; $66 \pm 4 \mu\text{mol C L}^{-1}$ and $75 \pm 8 \mu\text{mol C L}^{-1}$ at 168 h and 600 h, respectively. The DOC concentrations in the control treatment remained much lower than those in the experimental treatment throughout the incubation. Those DOC concentrations were $11 \mu\text{mol C L}^{-1}$, $13 \pm 4 \mu\text{mol C L}^{-1}$, and $19 \pm 2 \mu\text{mol C L}^{-1}$ at the initiation of incubation, 168 h, and 360 h, respectively.

Changes over time in the TOC concentration were similar with those in the DOC concentration from the initial of the incubation until 48 h for the experimental treatments (Figure 3-4c). The TOC concentration at the initial of incubation ($991 \mu\text{mol}$

C L⁻¹) decreased to $359 \pm 50 \mu\text{mol C L}^{-1}$ at 48 h of incubation period in the experiential treatment. From 96 h to the end of incubation, the TOC concentration decreased from $147 \pm 7 \mu\text{mol C L}^{-1}$ to $105 \pm 7 \mu\text{mol C L}^{-1}$, which was different from stability in the DOC concentration. The TOC concentration was not changed largely during the incubation in the control treatment; $8 \mu\text{mol C L}^{-1}$, $8 \pm 1 \mu\text{mol C L}^{-1}$ and $10 \pm 1 \mu\text{mol C L}^{-1}$ at the initiation, 168 h, and 600 h, respectively.

Figure 3-4c shows changes in the POC concentrations in Pha experiment. The significant difference between TOC and DOC concentrations in the experimental treatment was detected firstly at 27 h. The POC concentration of $36 \pm 1 \mu\text{mol C L}^{-1}$ at 27 h increased until 48 h of $141 \pm 22 \mu\text{mol C L}^{-1}$. The POC concentration, namely biomass of Pha, decreased from 48 h to the end of incubation. The POC concentrations were $84 \pm 7 \mu\text{mol C L}^{-1}$, $41 \pm 8 \mu\text{mol C L}^{-1}$, and $30 \pm 1 \mu\text{mol C L}^{-1}$ at 96, 168, and 360 h, respectively. The POC concentration in the control treatment could not be determined, since the significant differences were not evident between TOC and DOC concentrations throughout the incubation.

It is grounds of defining period of 0–48 h as the exponential growth phase that the cell abundance increased on the period of 27–48 h. The cell abundance and DOC concentration were rather stable in period of 96–360 h, and thus, the period was defined

as the stationary phase. It is interesting to note that both TOC and POC concentrations decreased, while DOC concentration did not change with incubation time during the stationary phase.

3-3-1-4 Control treatments without inoculum

Table 3-2 summarizes the results of two other control experiments, namely the glucose-free treatment without inoculum and the glucose additive treatment without inoculum, for three strains. The results of the control treatments without inoculum were not shown in figures in this chapter for better visualization. The cell concentrations in those control treatments without inoculum were lower than the limitation of quantification. The organic carbon concentrations (DOC and TOC concentrations) in the control treatment without inoculum and glucose remained lower level than those in the experimental treatments for all strains. While DOC and TOC concentrations in the control treatment of glucose addition without inoculum were rather stable compared with those in the experimental treatments for all strains. These results of control experiments confirmed that contaminations of bacteria or organic carbon during the incubations were not the factor causing the changes in the cell density, DOC and TOC concentrations for the experimental treatments of all strains.

3-3-2 Spectral characteristics of FDOM produced by bacterial strains

EEMs of DOM samples at the end of incubations of all strains experimental treatments showed distinct fluorescent peaks (Figures 3-5c, 3-6c and 3-7c), while those fluorescent peaks did not appear at the beginning of the experimental treatments (Figures 3-5a, 3-6a and 3-7a) and any control treatments (i.e., glucose-free treatment with inoculum (Figures 3-5d, 3-6d and 3-7d), glucose-free treatment without inoculum, glucose additive treatment without inoculum) for three bacterial strains. Therefore, it can be concluded that FDOM was produced by the three bacterial strains during the incubations instead of the contamination during incubation or the background of medium.

The peak positions of fluorescent peaks of FDOM produced by three bacterial strains are summarized in Table 3-3. The fluorescent peaks were firstly grouped protein-like FDOM and humic-like FDOM from their peak positions. Furthermore, the protein-like FDOM found in each strain treatment were separated to tyrosine-like FDOM which had emission maxima at around 300 nm and tryptophan-like FDOM which had emission maxima around 330 nm (Mayer et al., 1999; Yamashita and Tanoue, 2003).

Two types of protein-like FDOM (AP1: Ex/Em = 275/300 nm and AP2: Ex/Em =

275/330 nm) were determined for the Alt treatment, while one type of protein-like FDOM was found for the Vib and Pha treatments. The protein-like FDOM in the Vib treatment was defined as tryptophan-like FDOM which had emission maximum at 340 nm (VP: Ex/Em = 275/340 nm). In the Pha treatment, the protein-like FDOM was defined as tyrosine-like FDOM (PP: Ex/Em = 280/300 nm).

Humic-like FDOM was also found in EEMs in all strain treatments. Three humic-like FDOM patterns were found in each strain treatment. The humic-like FDOM was further separated into three groups from comparisons with spectral characteristics of humic-like FDOM reported in previous studies (Coble, 1996; 2007; Stedmon and Markager, 2005; Yamashita et al., 2010; Romera-Castillo et al., 2011; Tanaka et al., 2014; Catalá et al., 2015). The humic-like FDOM characterized by emission maxima at longer than 500 nm has usually been defined to be terrestrial humic-like FDOM in coastal environments (Stedmon and Markager, 2005), and therefore, defined as terrestrial-like group in the present study. Two humic-like FDOM patterns in the Alt treatment (AH1: Ex/Em = 270/520 nm, AH3: Ex/Em = 425/520 nm) and the Vib treatment (VH2: Ex/Em = 370/520 nm, VH3: Ex/Em = 440/530 nm) were categorized to the terrestrial-like group.

Humic-like FDOM which has emission maxima around 400 nm has been

traditionally reported to be derived from marine microbes (Coble, 1996; 2007). Those humic-like FDOM hence defined as a marine origin-like group. One humic-like FDOM in the Alt treatment (AH2: Ex/Em = 315/400 nm) and the Pha treatment (PH2: Ex/Em = 320/390 nm) were categorized into the marine origin-like group.

Third humic-like FDOM group, characterized by emission maxima around 450 nm, has been traditionally considered as a terrigenous origin (Coble, 1996), while it has been recently reported that the humic-like FDOM was also produced by marine microbes (Yamashita et al., 2010; Romera-Castillo et al., 2011; Tanaka et al., 2014; Catalá et al., 2015). Accordingly, those humic-like FDOM was named as multiple origin-like group in the present study. One humic-FDOM in the Vib treatment (VH1: Ex/Em = 360/450 nm) and two humic-like FDOM in the Pha treatment (PH1: Ex/Em = 265/450 nm, PH3: Ex/Em = 370/450 nm) were categorized into the multiple origin-like group. It should be noted that all humic-like FDOM patterns, even in terrestrial-like group, were produced by the bacterial strains used in the present study.

3-3-3 Changes in fluorescence intensity of individual FDOM produced by bacterial strains

3-3-3-1 Protein-like FDOM

In the experimental treatments of three bacterial strains, protein-like FDOM increased during the exponential growth phases, especially in the later of the phases (Figures 3-8). Then, the fluorescence intensities of protein-like FDOM increased during early part of the stationary phases for three bacterial strains. Two protein-like FDOM patterns in the Alt treatment tended to decrease from 72 h to the end of incubation (Chapter 2, Fig. 3-8a and b). The other protein-like FDOM patterns from the Vib and Pha treatments, which were categorized as tryptophan-like peak and tyrosine-like peak, respectively, tended to increase throughout the stationary phases. The fluorescence intensities of those protein-like FDOM in the control treatment (i.e., glucose-free treatment with inoculum) were relatively stable in the experimental treatments of the three strains.

3-3-3-2 Humic-like FDOM

The fluorescence intensities of all humic-like FDOM increased during the exponential growth phases in the experimental treatments of three bacterial strains (Figures 3-9, 3-10 and 3-11). All of them excluding the AH2 continued to increase during the stationary phases. The AH2 was relatively stable during the stationary phase of the Alt treatment. The fluorescence intensities of those humic-like FDOM in the control treatments were relatively stable in the experimental treatments of the three strains.

3-3-4 Regression analyses against humic-like FDOM

The results of regression analyses between humic-like FDOM and the other parameters (i.e., DOC, POC, and TOC) for the exponential growth and stationary phases were summarized in Table 3-4. All data (not averaged data) from triplicate bottles in each time point were used for the regression analyses. The negative linear relations were evident between DOC concentrations and fluorescence intensities of all humic-like FDOM during exponential growth phase in each strain incubation (Table 3-4, Figure 3-12). The positive linear relations of humic-like FDOM to DOC concentration were significant during stationary phase in the Vib and Pha treatments while those relations were not significant in the Alt treatment (Table 3-4, Figure 3-13).

The negative linear relations to TOC concentration in all humic-like FDOM were significant during exponential growth phase in the Alt and Pha treatments but not in the Vib treatment (Table 3-4, Figure 3-14). Some of those significant relationships of humic-like FDOM to TOC concentrations (i.e., AH1, AH3, PH1, and PH2) were also evident for the stationary phase (Table 3-4, Figure 3-15).

The positive linear relations of humic-like FDOM to POC concentration were significant during stationary phase in the Vib and Pha treatments, while those relations

were not significant in the Alt treatment (Table 3-4, Figure 3-16).

The positive linear relations were significant between protein-like FDOM and humic-like FDOM during the exponential growth phase in the Alt, Vib and Pha treatments (Table 3-4, Figure 3-17, 3-19). The positive linear relations were significant between protein-like FDOM and humic-FDOM during the stationary phase in the Vib and Pha treatments (Table 3-4, Figure 3-18, 3-20), while the relationships were not significant during the stationary phase in part of humic-like FDOM of the Alt treatments. These slopes of the regression line were different between the growth phase (Table 3-4, Figures 3-17, 3-18, 3-19, 3-20).

3-4 Discussion

3-4-1 Bacterial production of FDOM with consumption of labile substrate during the exponential growth phase

In this study, three marine bacterial strains belong to different classes or orders were used for better understanding of DOM production mechanisms by bacterial species.

Glucose was used as sole carbon source in this study. Many studies used glucose as sole carbon source in bacterial strain or community incubation and indicated that glucose was consumed completely within several days (Ogawa et al., 2001; Kawasaki and

Benner, 2006; Winehousesen et al., 2015). The bacterial abundances increased with decreases in the DOC concentrations during exponential growth phase in the three strains experiments, indicating that those bacteria grew up mainly consuming glucose and completely consumed the glucose during exponential growth phase.

The three bacterial strains produced FDOM, which contained protein-like and humic-like substances, during exponential growth phase, suggesting that the bacteria released FDOM actively to extracellular during growing phase in which the bacteria consumed labile substrate. Since the fluorescence intensities of humic-like FDOM did not decrease during not only the exponential growth phase but also the stationary phase (as discussed below), it was suggested that the heterotrophic bacteria produced humic-like FDOM using simple substrate such as glucose with their active growth which can be considered to be recalcitrant during the exponential growth phase.

The production of humic-like FDOM considered as RDOM during the exponential growth phase has also been reported by previous incubation experiments of natural microbial community (Kramer and Herndl, 2004; Lønborg et al., 2009; Shimotori et al., 2009; Arai et al., 2018). In contrast, some previous studies showed ability of single bacteria to produce humic-like FDOM. Shimotori et al. (2012) showed that ten heterotrophic bacterial strains produced humic-like FDOM through incubations

using Marine Broth medium. Incubation experiments of cyanobacterial strains showed that *Synechococcus* and *Prochlorococcus* strains produced humic-like FDOM similar to deep-sea FDOM, which can be considered as RDOM (Zhao et al., 2017). The authors pointed out that the humic-like FDOM was possibly originated from degradation products of the fluorescent cyanobacterial pigments. The results of these previous studies as well as the present study suggested that broad heterotrophic bacterial species produce humic-like FDOM during the isolate incubation. However, the efficiency of humic-like FDOM production with consumption of labile substrate has never been compared among bacterial species.

A regression analysis between DOC concentrations and fluorescence intensities of humic-like FDOM was examined during exponential growth phase of each strain (Table 3-4, Figures 3-12). Significant negative linear relationships were found for all humic-like FDOM against DOC concentrations, suggesting that production rate of humic-like FDOM to consumption of glucose was relatively constant during exponential growth phase. Therefore, the slope of the regression indicates efficiency of humic-like FDOM production with consumption of labile substrate. Interestingly, the slopes were not different largely among each humic-like FDOM produced by the single strain (Table 3-4). On the other hand, the slopes were largely different among three bacterial strains,

namely the slope was largest in the Pha treatment but was smallest in the Vib treatment (Table 3-4). Such difference in efficiency of humic-like FDOM production during the exponential phase suggested that particular bacterial species predominantly produce humic-like FDOM, although a variety of bacteria can produce humic-like FDOM, when labile DOM is supplied. Therefore, it can be concluded that the rate of bacterial RDOM production is regulated by bacterial community structure in the ocean.

3-4-2 Bacterial production of FDOM during the stationary phase

In the present study, DOC concentrations were relatively constant during the stationary phases of three bacterial strains indicating that consumption and production of DOM were relatively balanced after complete consumption of labile substrate during exponential growth phase. However, except the humic-like FDOM produced by the Alt, fluorescence intensities of humic-like FDOM continued to increase during the stationary phase. Such changes in humic-like FDOM during the incubations imply that recalcitrant humic-like FDOM produced by three bacterial strains were accumulated during the incubations. It should be noted that patterns of the change in humic-like FDOM during the stationary phase were different among the three bacterial strains possibly due to different production mechanisms of recalcitrant humic-like FDOM

accompanied with their physiology. Each production mechanism was evaluated from the linear regression analysis between humic-like FDOM against DOC concentration (Figures 3-13), TOC concentration (Figures 3-15) and POC concentration (Figures 3-16) during the stationary phase (Table 3-4).

3-4-2-1 Humic- like FDOM production with reutilization of bacterial DOM

In the Alt treatment, the humic-like FDOM in terrestrial-like group (AH-1 and AH-3) increased, while the protein-like FDOM tended to decrease during the stationary phase, suggesting that humic-like FDOM was produced with recycling of ambient DOM derived from Alt (Figures 3-8 and 3-9). The fluorescence intensities of AH-1 and AH-3 were not significantly related with DOC concentrations in the Alt treatment, because the changes in the two parameters along with incubation were different (Figures 3-2a and 3-9). In contrast, the significant negative linear relations were evident between TOC concentration and fluorescence intensities of AH1 and AH3, suggesting that Alt produced the humic-like FDOM in terrestrial-like group with mineralization of organic matter (Table 3-4, Figures 3-15). However, the other humic-like FDOM produced by Alt, namely AH2 belonging to the marine origin-group, which has been considered to be recalcitrant in the ocean (Yamashita and Tanoue, 2008; Catalá et al., 2015), were not

produced during the stationary phase.

All three types of humic-like FDOM were produced by Alt during the exponential phase, but only the two out of the three were produced during the stationary phase, indicating that composition of humic-like FDOM derived from Alt was different between the exponential growth phase and the stationary phase. The difference in the composition indicated that bacterial physiology can regulate production of RDOM, such as humic-like FDOM. The physiology of Alt could be controlled by difference in substrate, such as glucose or ambient DOM derived from Alt. As mentioned above, the slopes of liner relationship between decreasing in organic carbon concentration and increasing in fluorescence intensity of humic-like FDOM represented efficiency of humic-like FDOM production against consumption of organic carbon. The slopes of regressions between TOC concentration and fluorescence intensity of humic-like FDOM in terrestrial-like group during the stationary phase were larger than those during the exponential growth phase (Table 3-4, Figures 3-14 and 3-15), implying that efficiency of humic-like FDOM production against organic carbon mineralization under a poor condition in terms of labile substrate is higher than that labile substrate is fully available.

3-4-2-2 Humic-like FDOM releasing with lysis of bacteria

In the Vib experiment, all humic-like FDOM (i.e., VH1, VH2, and VH3) increased with decreases in bacterial abundance and POC concentration, equivalent to bacterial biomass, during the stationary phase including a part of the death phase (Figures 3-4b, 3-10), suggesting that humic-like FDOM was produced with decreasing biomass. Those relationships between POC concentration and humic-like FDOM were confirmed by regression analysis between the two parameters (Table 3-4, Figures 3-16). In the incubation experiment without predators, the decrease in bacterial biomass should be caused two mechanisms, which are lysis of bacteria (Riemann and Middelboe, 2002) and reserve consumption in bacterial body (Eichinger et al., 2009). The decrease in biomass of Vib during the stationary phase (including a part of the death phase) was likely caused by bacterial lysis due to simultaneous decreasing in bacterial abundance. There is no significant relation between TOC concentration and fluorescence intensity of humic-like FDOM (Fig. 3-15). Such a relationship suggested that humic-like FDOM was not produced associating with bacterial mineralization, including a consumption of reserve in biomass, but was released from the bacteria with lysis. The positive linear relationships were evident between humic-like FDOM and DOC concentration (Table 3-4, Figures 3-13), suggested that not only humic-like FDOM but also all DOM derived

from Vib by lysis was accumulated throughout the incubations.

It is interesting to note that all FDOM, including the protein-like FDOM, increased throughout the incubation of the Vib treatment. Slopes of liner regressions between protein-like FDOM and humic-like FDOM during the exponential growth phase showed higher values than those during stationary phase (Table 3-4, Figures 3-17 and 3-18). The differences in slopes indicated that FDOM composition, namely ratio of protein-like FDOM to humic-like DOM, was different due to different mechanisms of DOM release. That is, protein-like FDOM-poor DOM was released with active extracellular excretion during the exponential growth phase while protein-like FDOM-rich DOM was passively released by lysis of bacteria during the stationary phase including a part of the death phase. Previous studies reported that viral lysis of bacteria induced release of proteins (Riemann and Middelboe, 2002) and were consistent with the results of FDOM composition during the stationary phase in the Vib treatment. In contrast, Zhao et al. (2017) pointed out that cyanobacteria released humic-like FDOM due to viral lysis rather than active excretion during the exponential growth. Such experimental evidences suggest that viral lysis is an important process for releasing the protein-like and humic-like FDOM, and therefore, that bacterial physiology is strongly related to DOM composition releasing to the surrounding environments.

3-4-2-3 Humic-like FDOM production with consumption of reserves in biomass

The humic-like FDOM in the Pha treatment increased with a decrease in the POC concentration during the stationary phase, although the bacterial abundance did not decrease (Figures 3-1c and 3-4c). TOC concentration had a significantly negative relationship to two humic-like FDOM (PH1 and PH3) in the multiple origin-like group during the stationary phase (Table 3-4, Figures 3-15), suggesting that a part of humic-like FDOM was produced with mineralization of organic matter. The relationships between increasing in humic-like FDOM with the biomass decreasing and mineralization indicated that humic-like FDOM was produced with consumption of cellular materials. The decreasing in biomass with releasing DOM was found during an incubation of a bacterial strain, *Alteromonas infernus* (Eichinger et al., 2009). DOC concentration was positively related significantly with humic-like FDOM (Table 3-4, Figure 3-13), suggesting that all DOM produced by Pha during the stationary phase was accumulated throughout the incubation in the same manner with humic-like FDOM.

The slopes of negative liner regressions between TOC concentration and two humic-like FDOM in multiple origin-like group during the stationary phase were larger than those during the exponential growth phase in the Pha treatment (Table 3-4, Figures

3-14 and 3-15), suggesting that efficiency of humic-like FDOM production against remineralization of organic matter was higher during the stationary phase compared with the exponential growth phase. The result was the same with that obtained from the Alt treatment (see 3-4-2-1). The high efficiency against respiration during the stationary phase implied that the poorer on labile substrate condition possibly induced higher production rate of humic-like FDOM.

All FDOM, including protein-like FDOM, in the Pha treatment also increased throughout the incubation as same as in the Vib treatment (Figures 3-8d and 3-11). Differences in the slopes of positive linear regressions between protein-like FDOM and humic-like FDOM (Table 3-4, Figures 3-19 and 3-20) indicated that FDOM produced by Pha during the stationary phase showed higher relative abundance of humic-like FDOM than those during the exponential growth phase. Such differences suggested that Pha excreted protein-rich DOM during the consumption of labile substrate, but produced protein-poor DOM during the consumption of cellular materials. Furthermore, the difference in composition of bacterial FDOM (ratio of protein-like FDOM to humic-like FDOM) between Pha and Vib during the stationary phases (Table 3-4, Figures 3-18, 3-20) were likely caused by different mechanisms of DOM releasing, that is, more humic rich DOM is produced during the consumption of cellular materials compared

with during cell lysis.

3-4-3 Efficiency of bacterial DOM production based on organic carbon concentration

Bacterial physiology was estimated through the incubation of bacterial strains in this study. The DOC concentrations at the initiation of the three bacterial strain incubations were mainly consumed during the exponential growth phases while the DOC concentrations were stable relatively to the residual DOC concentrations during the stationary phases, indicating that the residual DOC concentrations were mainly determined during the exponential growth phase, namely the period of releasing directly with active bacterial growth using LDOM. The reason for the determination of the residual DOC concentration during the exponential growth phase could be a non-labile fraction of extracellular DOM from marine bacterial strain during active growing phase, and it was hard to be mineralized although those compositions were altered by bacterial production or/and consumption of DOM. It was consistent with studies of incubation using bacterial community, which suggested that DOM compositions were changed during period in which stable DOC concentrations were detected (Shimotori et al., 2009). It was also suggested that the biological processes with a short time scale like as degradation of labile DOM were likely important for determination of RDOM amount

in terms of carbon.

Furthermore, the bacterial physiological processes of DOM production were considered to differ among the three bacterial strains during stationary phase. Especially, the bacterial abundance of Vib strain decreased during the incubation, suggested that the bacterial cells were degraded, like as lysis of bacteria, and released contents of the cells. The FDOM compositions of the Vib treatment during the stationary phase were characterized by higher relative abundance of protein-like FDOM to humic-like FDOM than those during the exponential growth phase, supporting the cell lysis in the Vib treatment during stationary phase. The DOC concentrations in the Vib treatment during stationary phase were relatively stable in spite of the cell lysis, suggesting that bacterial DOM derived from lysis of the bacteria had a small contribution to the residual DOC concentrations, and it was possibly mineralized like a labile fraction, although lysis of bacteria altered the DOM composition. In the previous study which added a labile substrate to a coastal microbial community, it was indicated that DOC concentration derived from sloppy feeding of bacterial body by nanoflagellate grazers was consumed during the incubation for 30 days (Lechtenfeld et al., 2015), suggesting that main part of bacterial DOM derived from cells were labile.

The POC contributed to 30–80% in the TOC at the end of incubations, although

the POC concentrations were different among the three strains. The POC at the end of incubation was considered to include the living bacteria and detritus. Previous studies conducting incubation experiments of marine bacterial community with labile substrates for 1–2 years reported that POC concentration became below the detection limit while DOC concentration was detectable and relatively stable at the end of incubation (Ogawa et al., 2001; Koch et al., 2014). These results derived from long-period incubations suggest that the remnant POC observed at the end of incubations of the bacterial strains would be disappeared after the long-term incubations due to decrease in the biomass and possibly remineralization of its detritus.

The remnant DOC concentrations at the end of incubation were considered as unavailable for each strain, because DOC concentrations were relatively stable during the stationary phase of each strain. The DOC concentrations derived from bacterial strains could be calculated by subtracting the DOC concentration at the end of control treatment from that at the end of experimental treatments in each strain incubation. The efficiency of DOM production (%) from labile substrates of bacterial strain was calculated by the ratio of DOC concentration derived from bacteria at the end of the incubation to initial substrate concentration of the incubation, and the values obtained were different among bacterial strains. The results are summarized in Table 3-4. The

efficiency of DOM production in the Vib treatment (20%) was the highest among the three strains and was about four times higher than those in the Alt and Pha treatments (4% and 6%, respectively). The efficiencies of bacterial DOM production were estimated to 3–7 % of initial glucose in previous studies using microbial communities (Ogawa et al., 2001; Shimotori et al., 2009; Koch et al., 2014; Lechtenfeld et al., 2015) or bacterial strains (Gruber et al., 2006) and were comparable with those in the Alt and Pha treatments. Therefore, Vib is likely a key species to produce bacterial DOM efficiently and that difference in bacterial community composition affects the efficiency of bacterial DOM production from simple labile substrates. However, the remnant DOM in bacterial strains can be consumed by other bacteria, as discussed below (Chapter 4).

3-5 Conclusion

The efficiencies of DOM production of three bacterial strains were estimated in this chapter (i.e., Alt, 4%; Vib, 20%; Pha, 6%). These results suggested that bacterial DOM production efficiencies from simple substrate were different among marine bacterial species. Especially, Vib produced DOM efficiently compared with the other strains, implying that specific bacterial species can produce DOM efficiently from LDOM.

This chapter investigated the effect of differences in bacterial species and

physiology on DOM production. Humic-like FDOM, which can be considered as RDOM, were produced by three bacterial strains. The results of the present study and previous studies consistently indicated that production of humic-like FDOM by bacteria was a general phenomenon, irrespective of bacterial species. However, the present study firstly found that the efficiency of humic-like FDOM production against consumption of labile substrate during exponential growth phase was different among bacterial species and suggested the presence of key species which has high efficiency of humic-like FDOM production in the ocean.

Four mechanisms of humic-like FDOM production were suggested by each strain incubation. (1) In all strain treatments, humic-like FDOM was actively excreted with consumption of labile substrate during the exponential growth phases. (2) The humic-like FDOM as a terrestrial-like group was produced with consumption of extracellular compounds released by the strain during the stationary phase of the Alt treatment. (3) The humic-like FDOM in terrestrial-like and multiple origin-like group was released by lysis of bacteria during the stationary phase (including a part of the death phase) of the Vib treatment. (4) The humic-like FDOM in a multiple origin-like group was produced with consumption of cellular materials during the stationary phase of the Pha treatment. Two production mechanisms of humic-like FDOM during the stationary phase, i.e., (2)

and (4), showed higher efficiency against the remineralization of organic matter compared with the exponential growth phase. The results presented in this study implies that efficiency of humic-like FDOM production depended on bacterial species as well as bacterial physiology, and thus, suggested that changes in bacterial species or physiology of microbial community are key factors controlling the RDOM production in the ocean.

Protein-like FDOM production was also confirmed in the three bacterial strain experiments. All protein-like FDOM was produced during exponential growth phase, suggesting the production of protein-like FDOM with active bacterial growing. Protein-like FDOM tended to decrease during stationary phase in the Alt treatment, suggesting that labile protein-like FDOM was consumed by Alt. In contrast, protein-like FDOM was not decreased during the Vib and Pha treatments, implying that protein-like FDOM production exceeded consumption during incubation. Furthermore, proportion of protein-like FDOM in released whole FDOM in the stationary phase including a part of the death phase were higher than that in the exponential growth phase in Vib treatment, suggesting that lysis of bacterial body was an efficient process of protein-like FDOM production.

Table 3-1 Treatments and sampling time points of incubation experiments of three bacterial strains

Strain	Treatment	Inoculum	Glucose	Sampling time points (h)
Alt	Experiment	+	+	0, 6, 12, 18, 24, 72, 120, 168
	No glucose	+	-	0, 24, 168
	No inoculum	-	+	0, 24, 168
	Nothing	-	-	0, 24, 168
Vib	Experiment	+	+	0, 4, 8, 12, 24, 72, 120, 168, 600
	No glucose	+	-	0, 168, 600
	No inoculum	-	+	0, 168, 600
	Nothing	-	-	0, 168, 600
	Experiment	+	+	0, 9, 18, 36, 48, 96, 168, 360
Pha	No glucose	+	-	0, 48, 168, 360
	No inoculum	-	+	0, 48, 168, 360
	Nothing	-	-	0, 48, 168, 360

Table 3-2 Summary of the results in organic carbon concentration of two control treatments without inoculums

Strain	Treatment	Sampling point (hour)	DOC ($\mu\text{mol C L}^{-1}$)	TOC ($\mu\text{mol C L}^{-1}$)
Alt	Nothing	0	7.5	7.0
		168	18 ± 10	11 ± 1
	No inoculum	0	988	964
		168	994 ± 10	984 ± 4
Vib	Nothing	0	11	13
		600	11 ± 1	19 ± 4
	No inoculum	0	1045	1013
		600	1027 ± 2	1037 ± 12
Pha	Nothing	0	10	8
		360	24 ± 4	15 ± 3
	No inoculum	0	983	986
		360	985 ± 8	992 ± 2

Table 3-3 Summary of peak positions and groups of fluorescent dissolved organic matter (FDOM) produced during incubations of three bacterial strains

Strain	Excitation (nm)	Emission (nm)	Abbreviation	Group of FDOM	Reference
Alt	275	300	AP1	Tryosine-like group	Mayer et al. (1999)
	275	330	AP2	Tryptophan-like group	Mayer et al. (1999)
	270	520	AH1	Terrestrial-like group	Stedmon and Markager (2005)
	315	400	AH2	Marine origin-like group	Coble (1996)
	425	520	AH3	Terrestrial-like group	Stedmon and Markager (2005)
Vib	275	340	VP	Tryptophan-like group	Mayer et al. (1999)
	360	450	VH1	Multiple origin-like group	Yamashita et al. (2010)
	370	520	VH2	Terrestrial-like group	Stedmon and Markager (2005)
	440	530	VH3	Terrestrial-like group	Stedmon and Markager (2005)
	280	300	PP	Tryosine-like group	Mayer et al. (1999)
Pha	265	450	PH1	Multiple origin-like group	Yamashita et al. (2010)
	320	390	PH2	Marine origin-like group	Coble (1996)
	370	450	PH3	Multiple origin-like group	Yamashita et al. (2010)

Table 3-4 Summary of the results in regression analyses between humi-like FDOM and other parameters of the Alt, Vib and Pha treatments

Strain	X	Y	Growth phase	<i>p</i> -value	R ²	Slop
Alt	DOC	AH1	Exponential	$p < 0.01$	0.773	$-1.76 \pm 0.34 \times 10^{-5}$
		AH2	Exponential	$p < 0.01$	0.687	$-2.70 \pm 0.64 \times 10^{-5}$
		AH3	Exponential	$p < 0.01$	0.836	$-6.14 \pm 0.96 \times 10^{-6}$
	TOC	AH1	Exponential	$p < 0.01$	0.926	$-2.07 \pm 0.22 \times 10^{-5}$
		AH2	Exponential	$p < 0.01$	0.857	$-3.13 \pm 0.48 \times 10^{-5}$
		AH3	Exponential	$p < 0.01$	0.900	$-6.57 \pm 0.83 \times 10^{-6}$
		AH1	Stationary	$p < 0.01$	0.843	$-5.21 \pm 0.71 \times 10^{-4}$
		AH3	Stationary	$p < 0.01$	0.768	$-3.96 \pm 0.69 \times 10^{-4}$
Vib	DOC	VH1	Exponential	$p < 0.01$	0.757	$-1.31 \pm 0.28 \times 10^{-5}$
		VH2	Exponential	$p < 0.01$	0.770	$-1.84 \pm 0.36 \times 10^{-5}$
		VH3	Exponential	$p < 0.01$	0.660	$-1.22 \pm 0.31 \times 10^{-5}$
	VP	AH1	Exponential	$p < 0.01$	0.807	0.10 ± 0.02
		AH2	Exponential	$p < 0.01$	0.804	0.10 ± 0.02
		AH3	Exponential	$p < 0.01$	0.602	0.10 ± 0.03
	DOC	VH1	Stationary	$p < 0.05$	0.396	$2.08 \pm 0.81 \times 10^{-4}$
		VH2	Stationary	$p < 0.05$	0.456	$9.91 \pm 3.43 \times 10^{-5}$
		VH3	Stationary	$p < 0.01$	0.597	$5.07 \pm 1.32 \times 10^{-5}$
	POC	VH1	Stationary	$p < 0.01$	0.616	$-1.82 \pm 0.48 \times 10^{-4}$
		VH2	Stationary	$p < 0.01$	0.653	$-8.40 \pm 0.20 \times 10^{-5}$
		VH3	Stationary	$p < 0.01$	0.858	$-4.23 \pm 0.57 \times 10^{-5}$
	VP	VH1	Stationary	$p < 0.01$	0.547	0.038 ± 0.011
		VH2	Stationary	$p < 0.01$	0.590	0.018 ± 0.005
		VH3	Stationary	$p < 0.01$	0.625	0.0081 ± 0.0020

Table 3-4 Summary of the results in regression analyses between humi-like FDOM and other parameters of the Alt, Vib and Pha treatments

Strain	X	Y	Growth phase	<i>p</i> -value	R ²	Slope (SE)
Pha	DOC	PH1	Exponential	$p < 0.01$	0.989	$-3.78 \pm 0.10 \times 10^{-5}$
		PH2	Exponential	$p < 0.01$	0.961	$-6.73 \pm 0.36 \times 10^{-5}$
		PH3	Exponential	$p < 0.01$	0.984	$-3.33 \pm 0.11 \times 10^{-5}$
	TOC	PH1	Exponential	$p < 0.01$	0.974	$-4.34 \pm 0.19 \times 10^{-5}$
		PH2	Exponential	$p < 0.01$	0.962	$-7.79 \pm 0.42 \times 10^{-5}$
		PH3	Exponential	$p < 0.01$	0.971	$-3.83 \pm 0.18 \times 10^{-5}$
	PP	PH1	Exponential	$p < 0.01$	0.987	0.050 ± 0.001
		PH2	Exponential	$p < 0.01$	0.890	0.086 ± 0.008
		PH3	Exponential	$p < 0.01$	0.986	0.044 ± 0.001
	DOC	PH1	Stationary	$p < 0.05$	0.557	$6.63 \pm 2.16 \times 10^{-3}$
		PH2	Stationary	$p < 0.05$	0.512	$1.43 \pm 0.53 \times 10^{-3}$
		PH3	Stationary	$p < 0.05$	0.597	$1.39 \pm 0.43 \times 10^{-3}$
	TOC	PH1	Stationary	$p < 0.01$	0.762	$-1.27 \pm 0.27 \times 10^{-3}$
		PH3	Stationary	$p < 0.01$	0.731	$-5.29 \pm 1.21 \times 10^{-4}$
	POC	PH1	Stationary	$p < 0.01$	0.876	$-1.13 \pm 0.16 \times 10^{-3}$
		PH2	Stationary	$p < 0.05$	0.538	$-4.17 \pm 1.46 \times 10^{-4}$
		PH3	Stationary	$p < 0.01$	0.863	$-4.76 \pm 0.72 \times 10^{-4}$
	PP	PH1	Stationary	$p < 0.01$	0.789	0.43 ± 0.08
		PH2	Stationary	$p < 0.01$	0.830	0.21 ± 0.04
		PH3	Stationary	$p < 0.01$	0.805	0.18 ± 0.03

Table 3-6 Efficiencies of DOM production by the bacterial strains

Strain	Incubation time (hour)	Residual DOC ($\mu\text{mol C L}^{-1}$)	Residual POC ($\mu\text{mol C L}^{-1}$)	DOC (%)	POC (%)
Alt	168	$51 \pm 4.$	$188 \pm 8.$	4.2 ± 0.5	18.9 ± 0.8
Vib	600	$218 \pm 8.$	$168 \pm 6.$	19.8 ± 0.8	16.1 ± 0.6
Pha	360	75 ± 43	30 ± 1	5.7 ± 0.9	3.0 ± 0.1

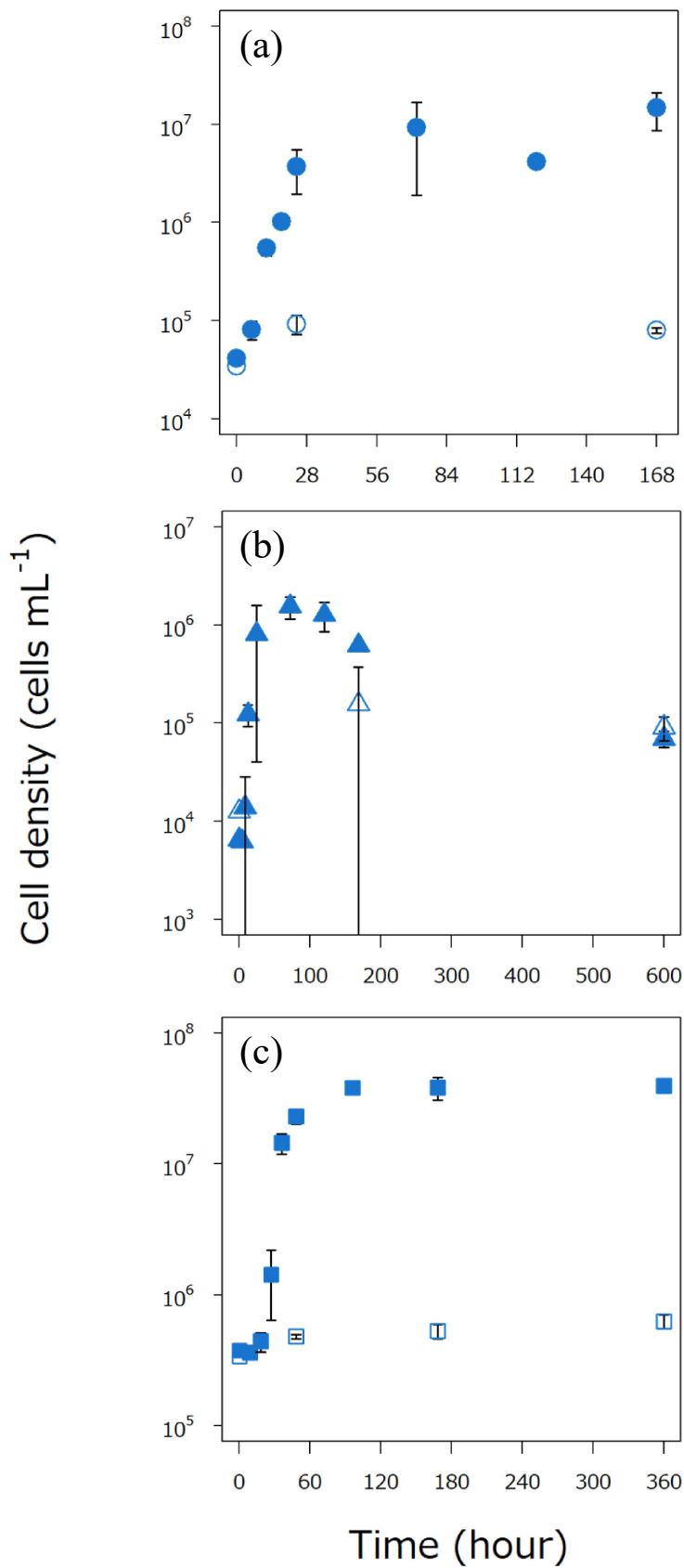


Figure 3-1 Changes in bacterial cell density (cells mL⁻¹) with incubation time in (a) Alt treatment, (b) Vib treatment, and (c) Pha treatment. Closed and opened symbols represented the experimental treatment and no glucose treatment, respectively.

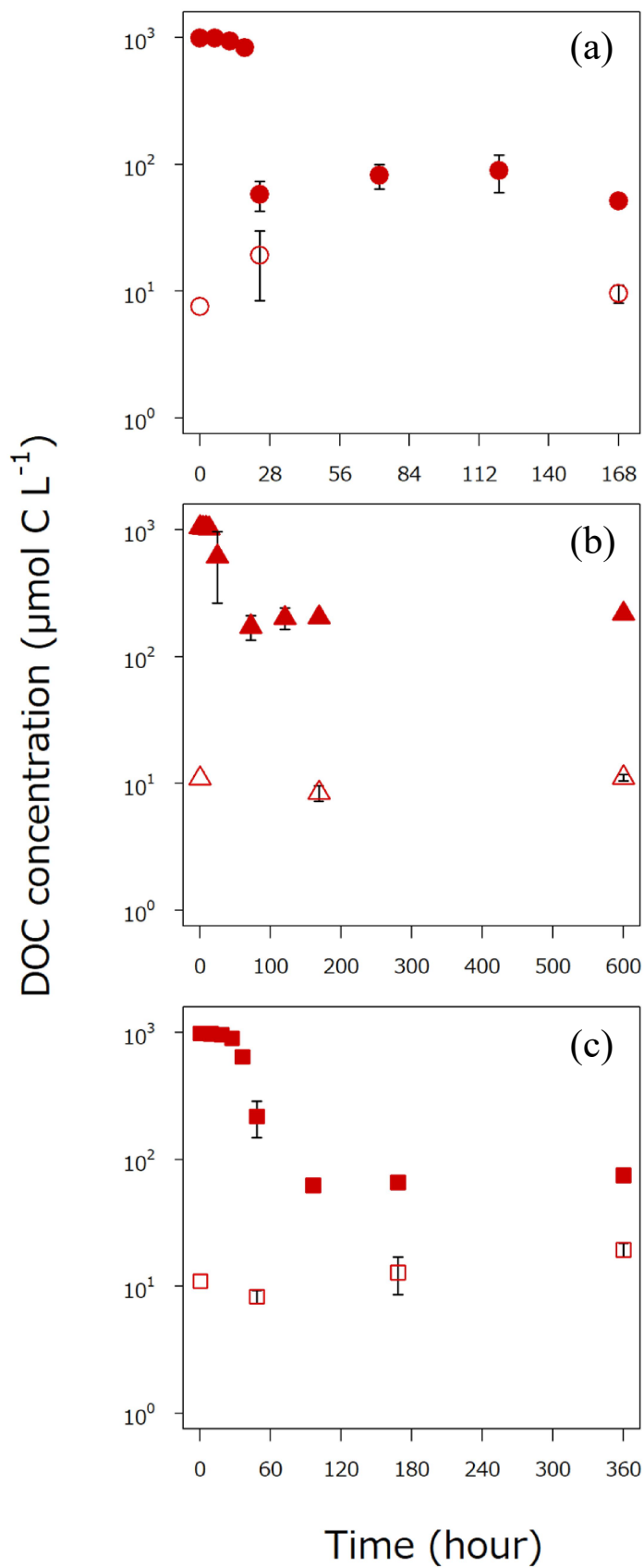


Figure 3-2 Changes in dissolved organic concentration ($\mu\text{mol C L}^{-1}$) with incubation time in (a) Alt treatment, (b) Vib treatment, and (c) Pha treatment. Closed and opened symbols represented the experimental treatment and no glucose treatment, respectively.

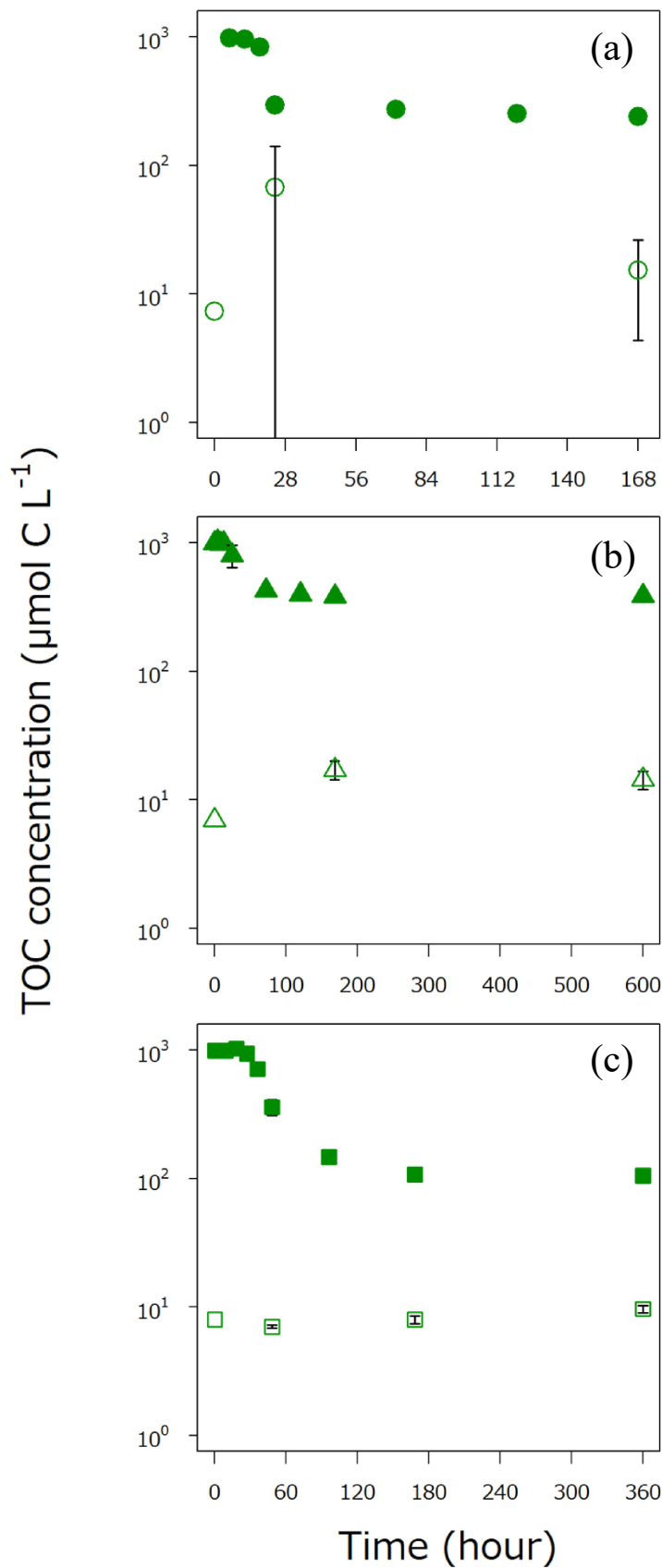


Figure 3-3 Changes in total organic concentration ($\mu\text{mol C L}^{-1}$) with incubation time in (a) Alt treatment, (b) Vib treatment, and (c) Pha treatment. Closed and opened symbols represented the experimental treatment and no glucose treatment, respectively.

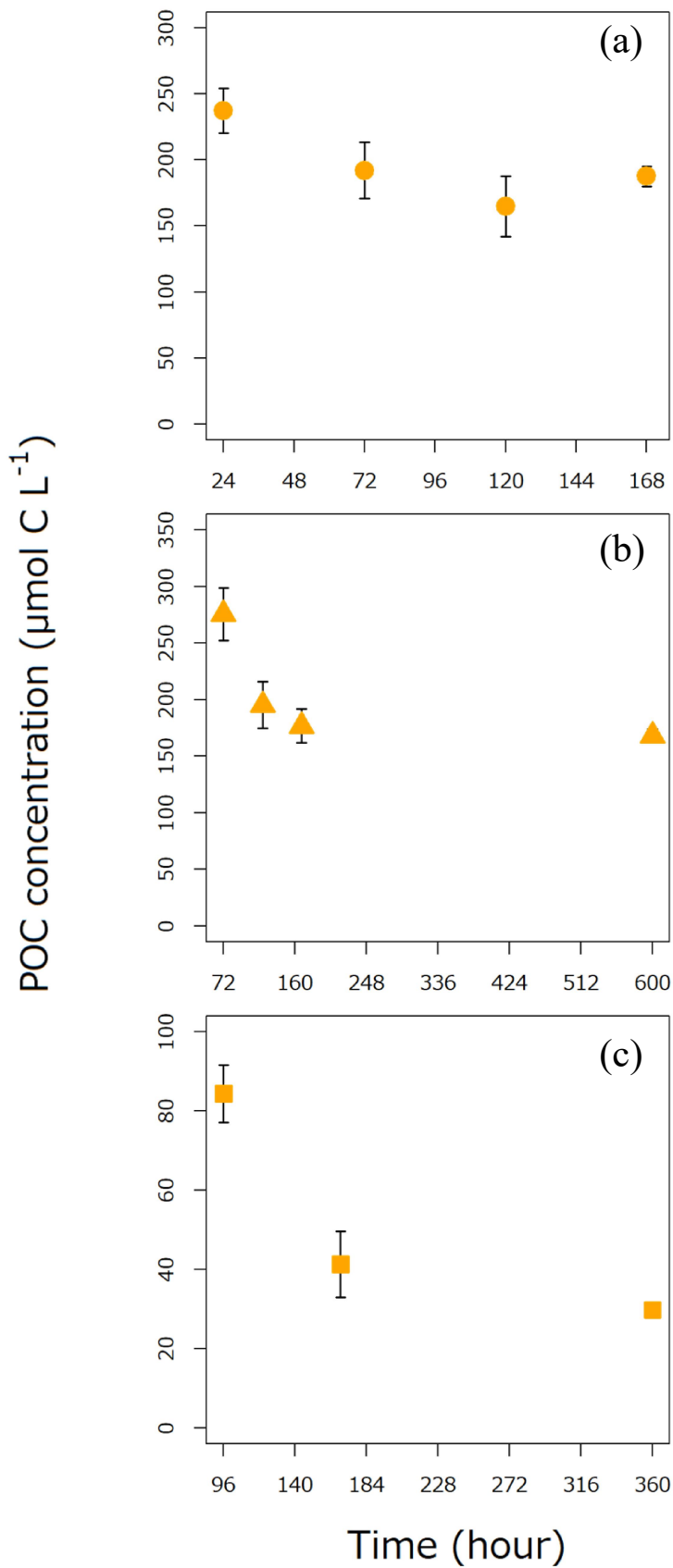


Figure 3-4 Changes in particulate organic concentration ($\mu\text{mol C L}^{-1}$) with incubation in (a) Alt treatment, (b) Vib treatment, and (c) Pha treatment. Closed and opened symbols represented the experimental treatment and no glucose treatment, respectively.

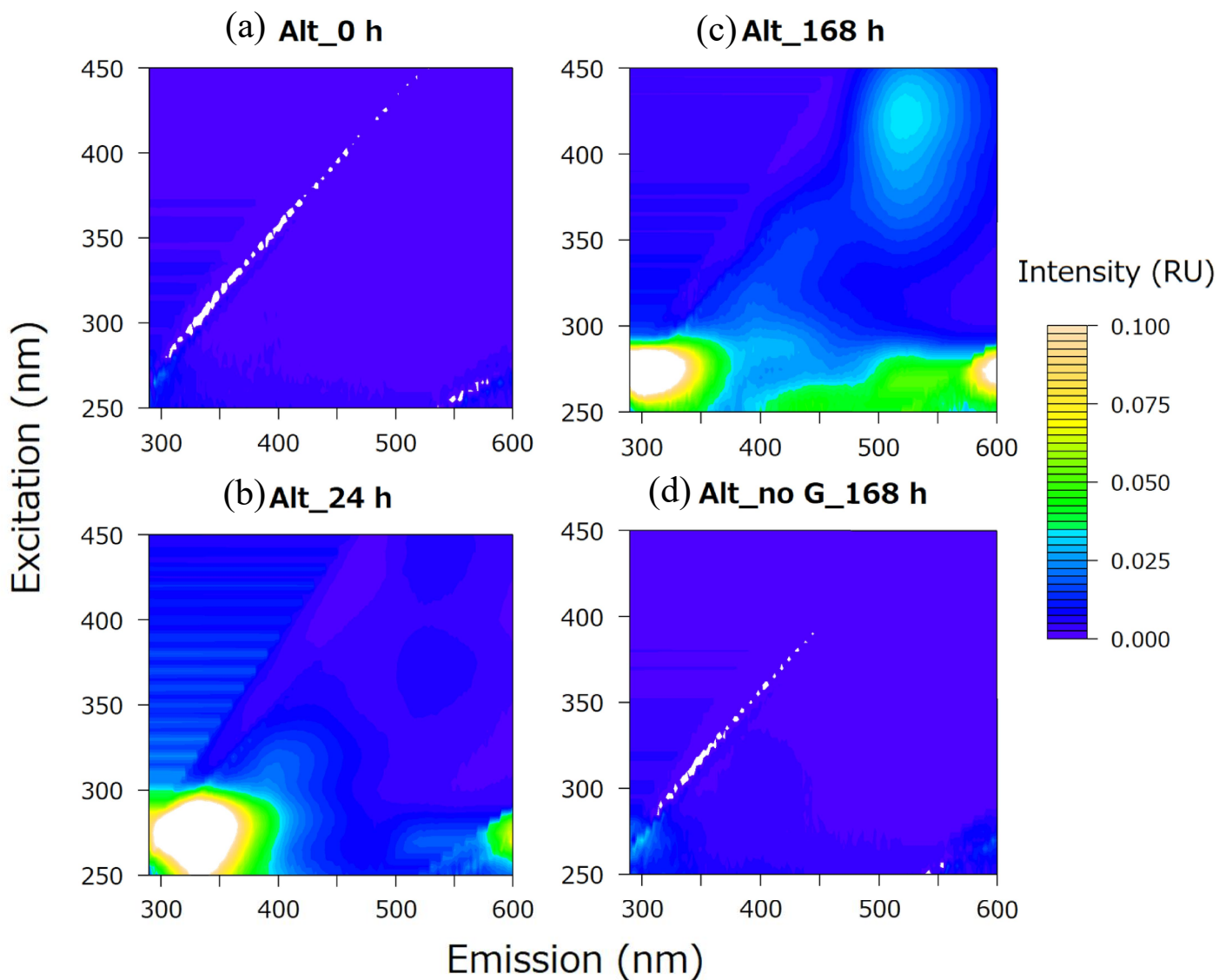


Figure 3-5 Excitation-emission matrix (EEM) of Alt treatment at (a) 0, (b) 24 and (c) 168 hours in the experimental treatment, and at (d) 168 hours in the control treatment. EEMs obtained from triplicate samples were averaged to produce the figures.

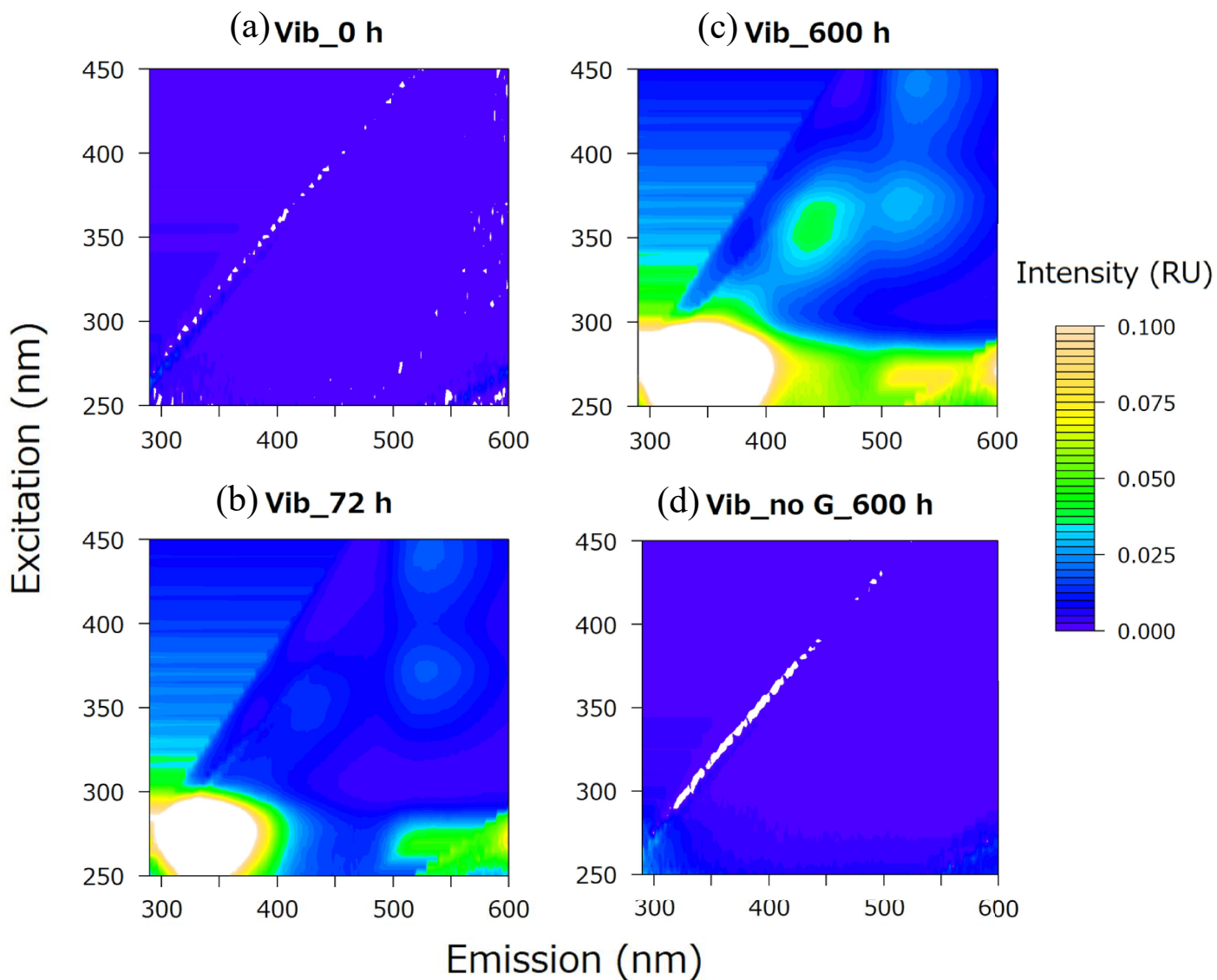


Figure 3-6 Excitation-emission matrix (EEM) of Vib treatment at (a) 0, (b) 72 and (c) 600 hours in the experimental treatment, and at (d) 600 hours in the control treatment. EEMs obtained from triplicate samples were averaged to produce the figures.

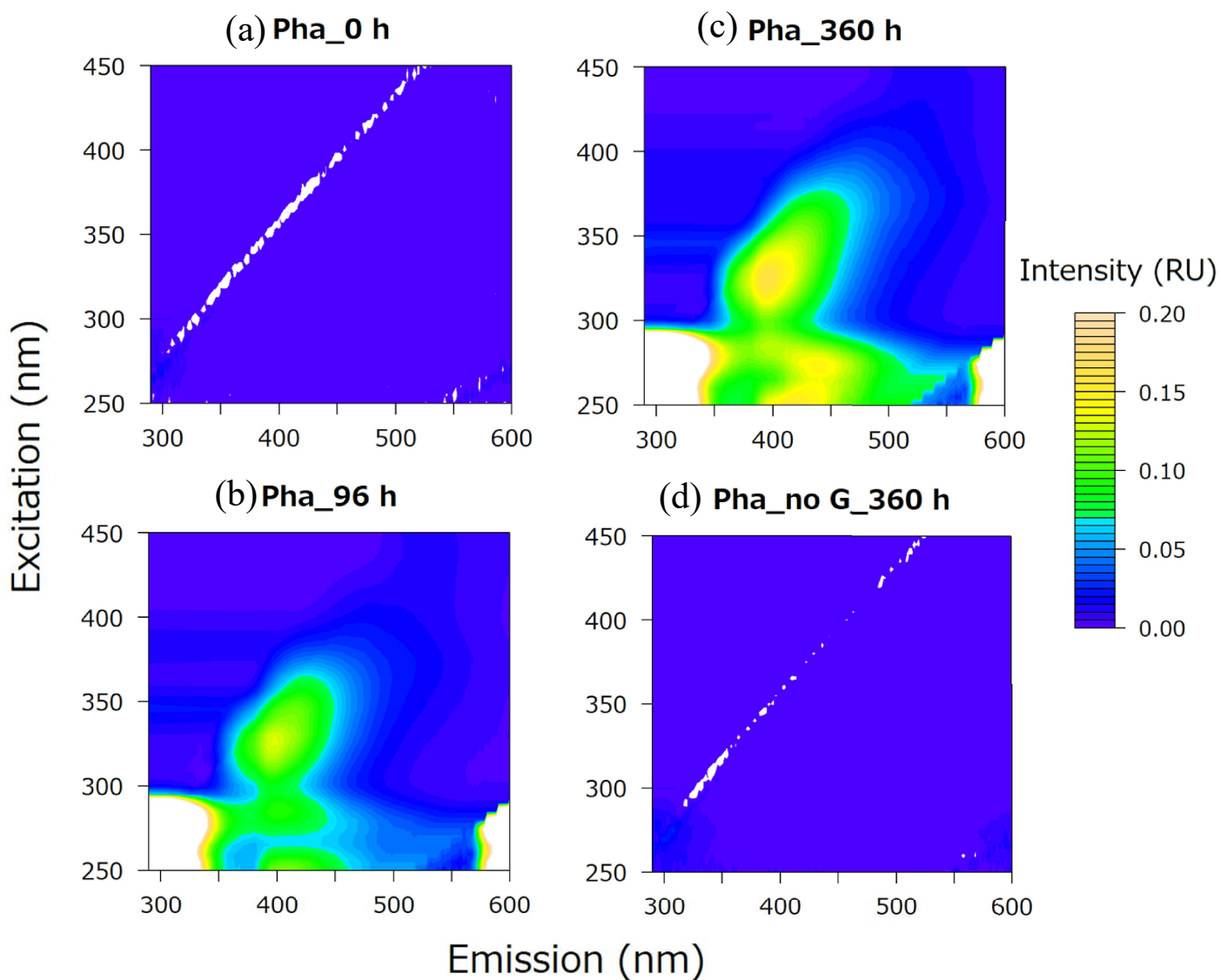


Figure 3-7 Excitation-emission matrix (EEM) of Pha treatment at (a) 0, (b) 96 and (c) 360 hours in the experimental treatment, and at (d) 360 hours in the control treatment. EEMs obtained from triplicate samples were averaged to produce the figures.

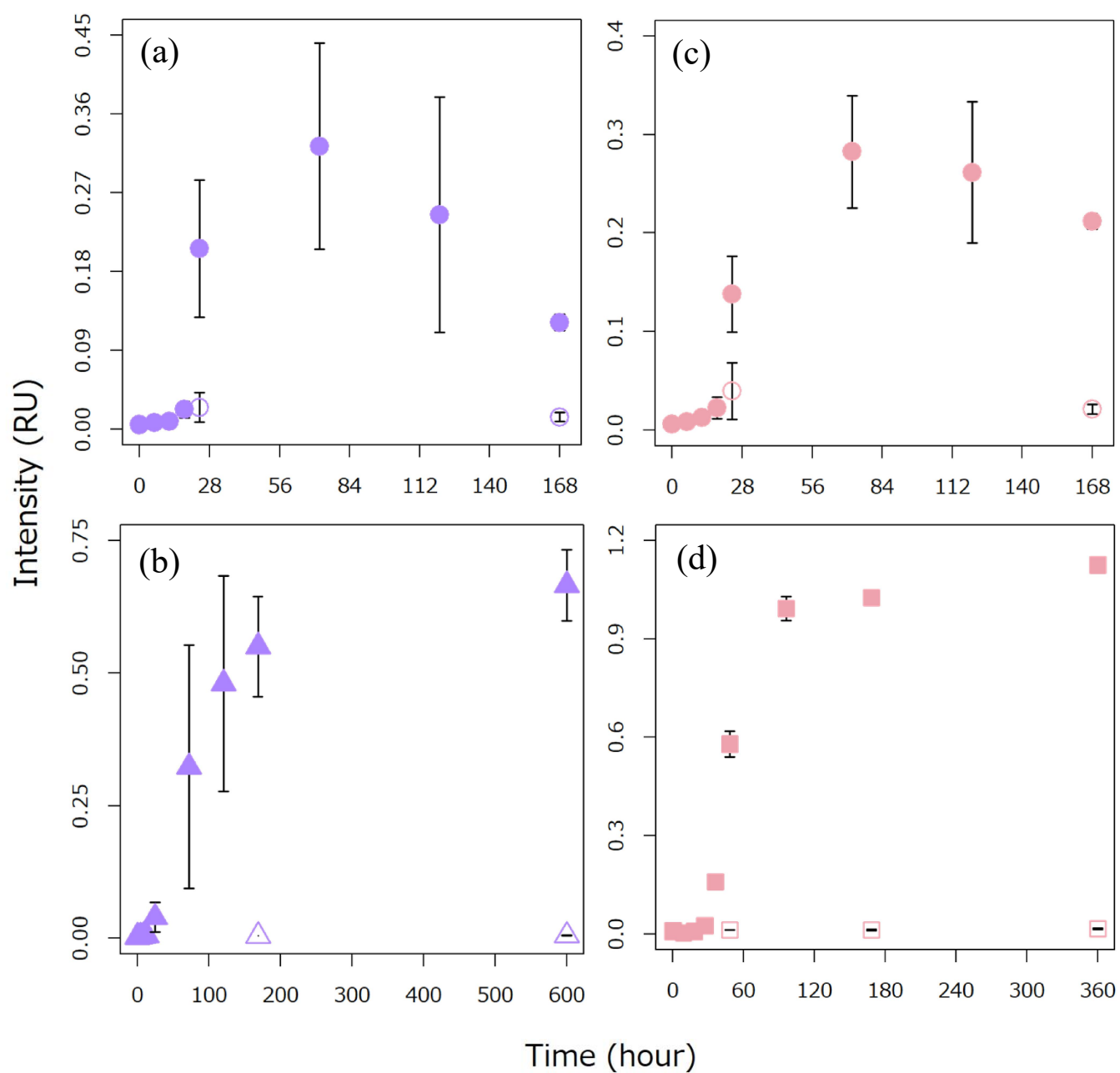


Figure 3-8 Changes in fluorescence intensities of protein-like FDOM in (a, c) Alt treatment, (b) Vib treatment and (d) Pha treatment. Purple and pink symbols indicate tryptophan-like peak and tyrosine-like peak, respectively. Closed and opened symbols represented the experimental treatment and control treatment, respectively.

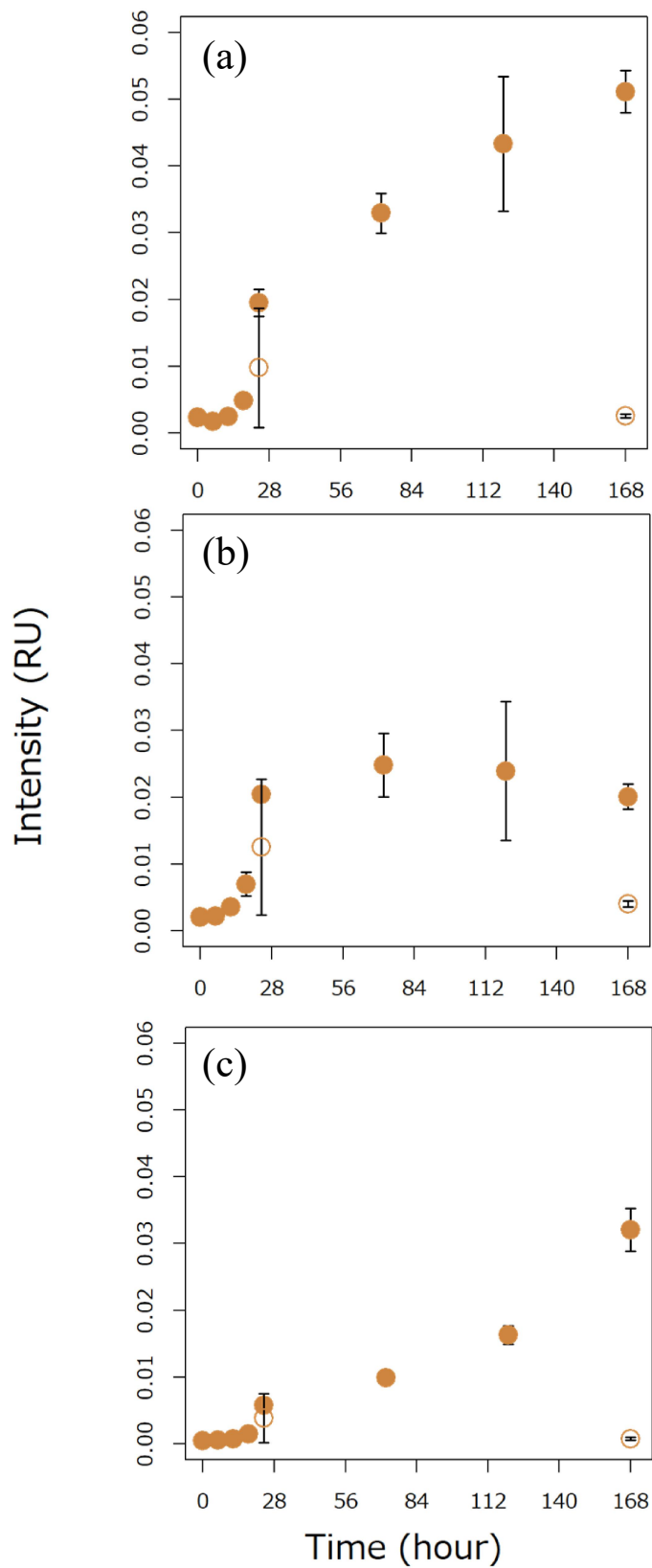


Figure 3-9 Changes in fluorescence intensities of (a) AH1, (b) AH2 and (c) AH3 categorized to humic-like FDOM in Alt treatment. Closed and opened symbols represented the experimental treatment and control treatment, respectively.

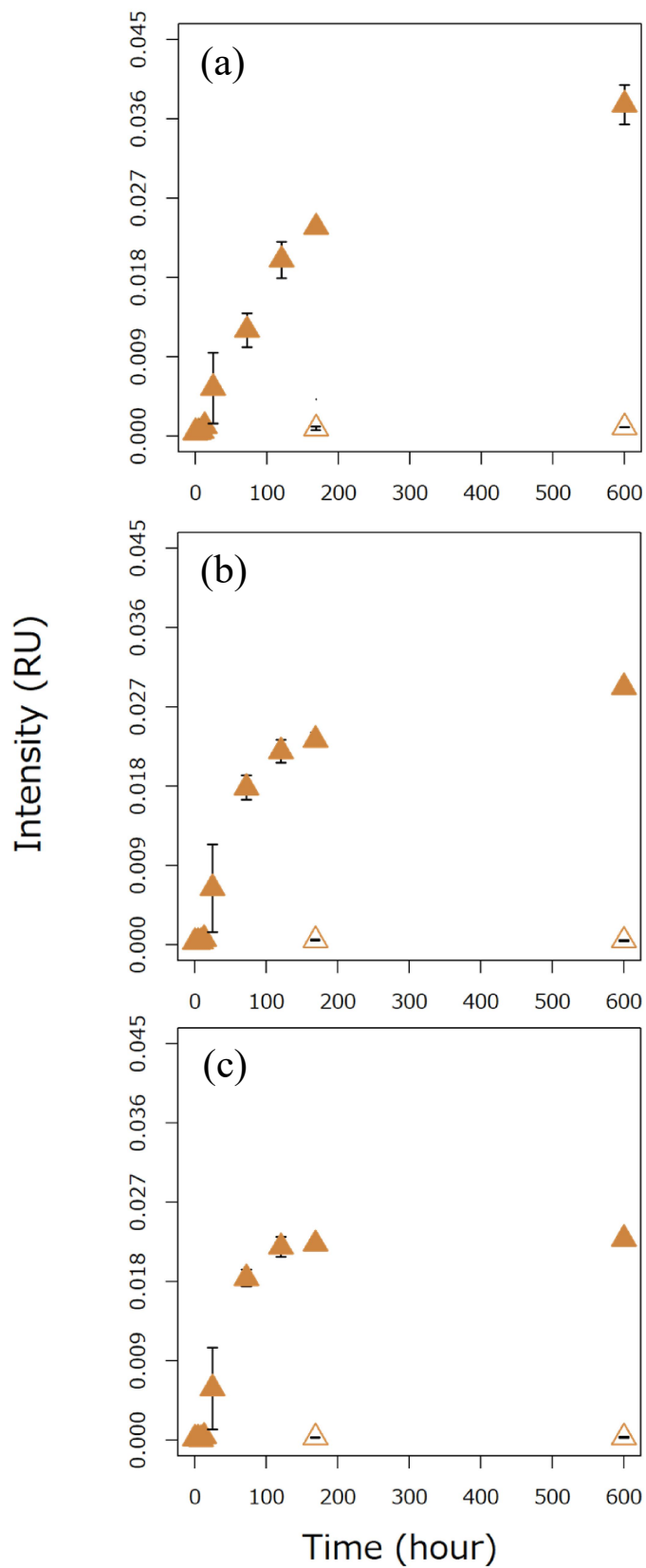


Figure 3-10 Changes in fluorescence intensities of (a) VH1, (b) VH2 and (c) VH3 categorized into humic-like FDOM in Vib treatment. Closed and opened symbols represented the experimental treatment and control treatment, respectively.

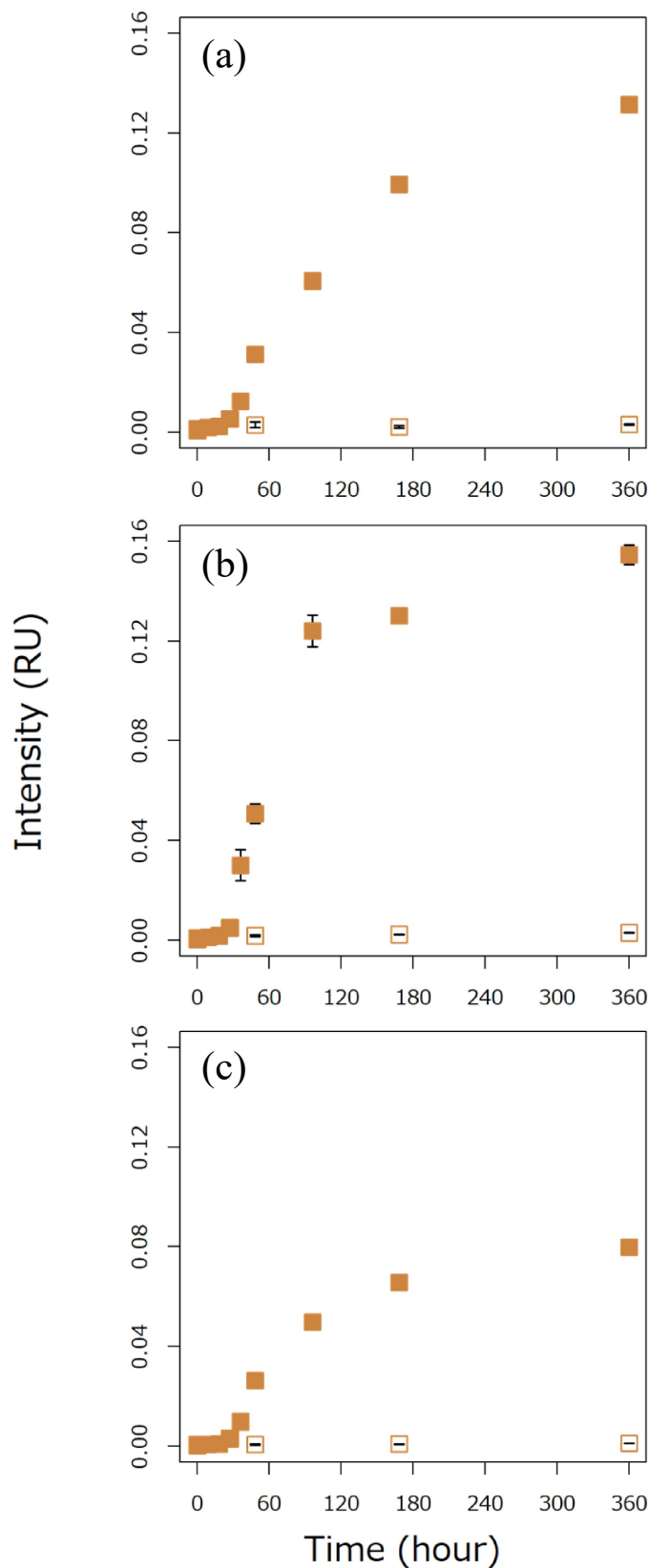


Figure 3-11 Changes in fluorescence intensities of (a) PH1, (b) PH2 and (c) PH3 categorized into humic-like FDOM in Pha treatment. Closed and opened symbols represented the experimental treatment and control treatment, respectively.

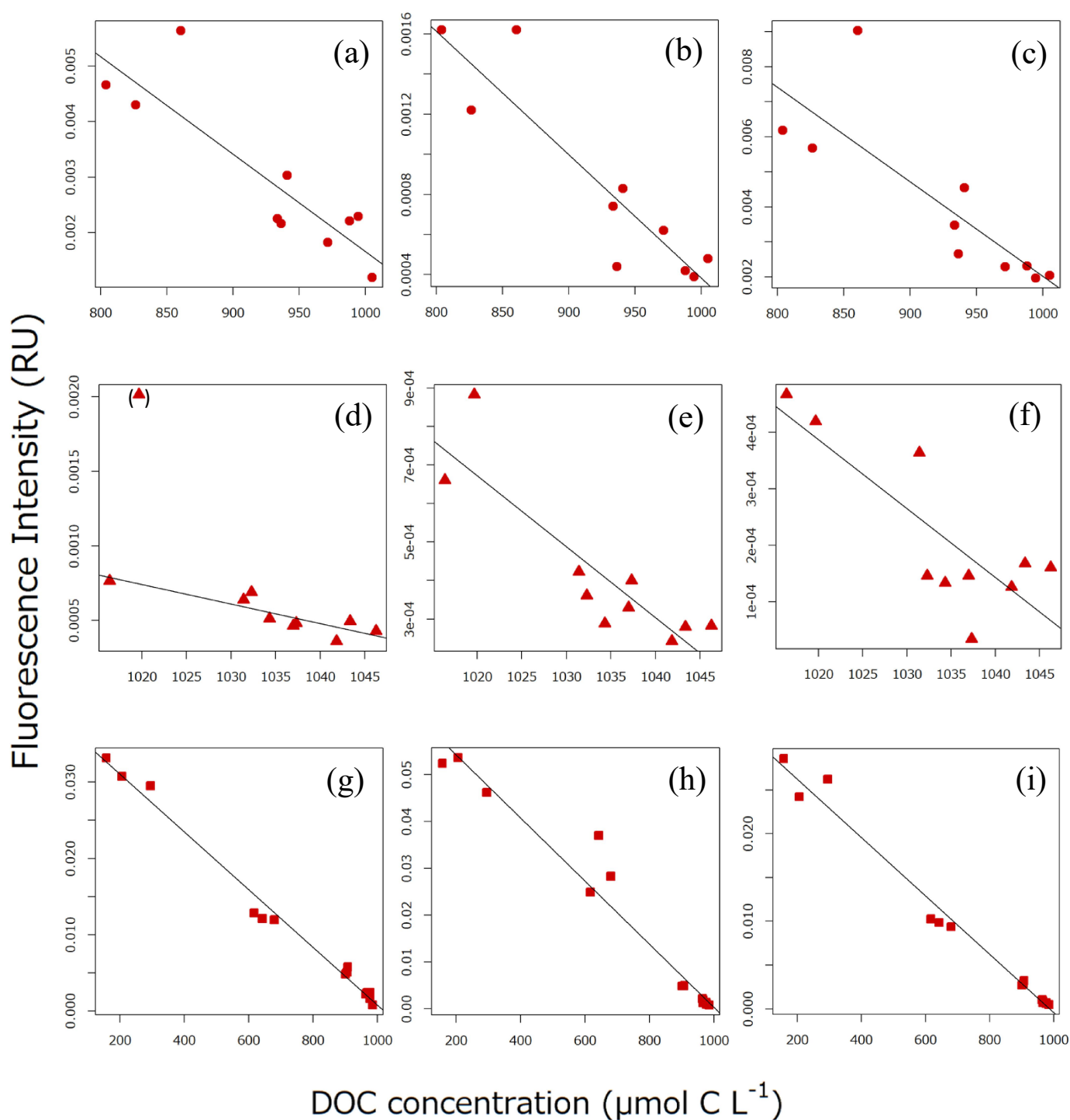


Figure 3-12 Plots between fluorescence intensities of humic-like FDOM and DOC concentrations of (a) AH1, (b) AH2, (c) AH3, (d) VH1, (e) VH2, (f) VH3, (g) PH1, (h) PH2 and (i) PH3 during exponential growth phases. All data from triplicate bottles were used for the figures. Linear lines represent regression lines between two parameters. Symbols in brackets were removed from regression analyses.

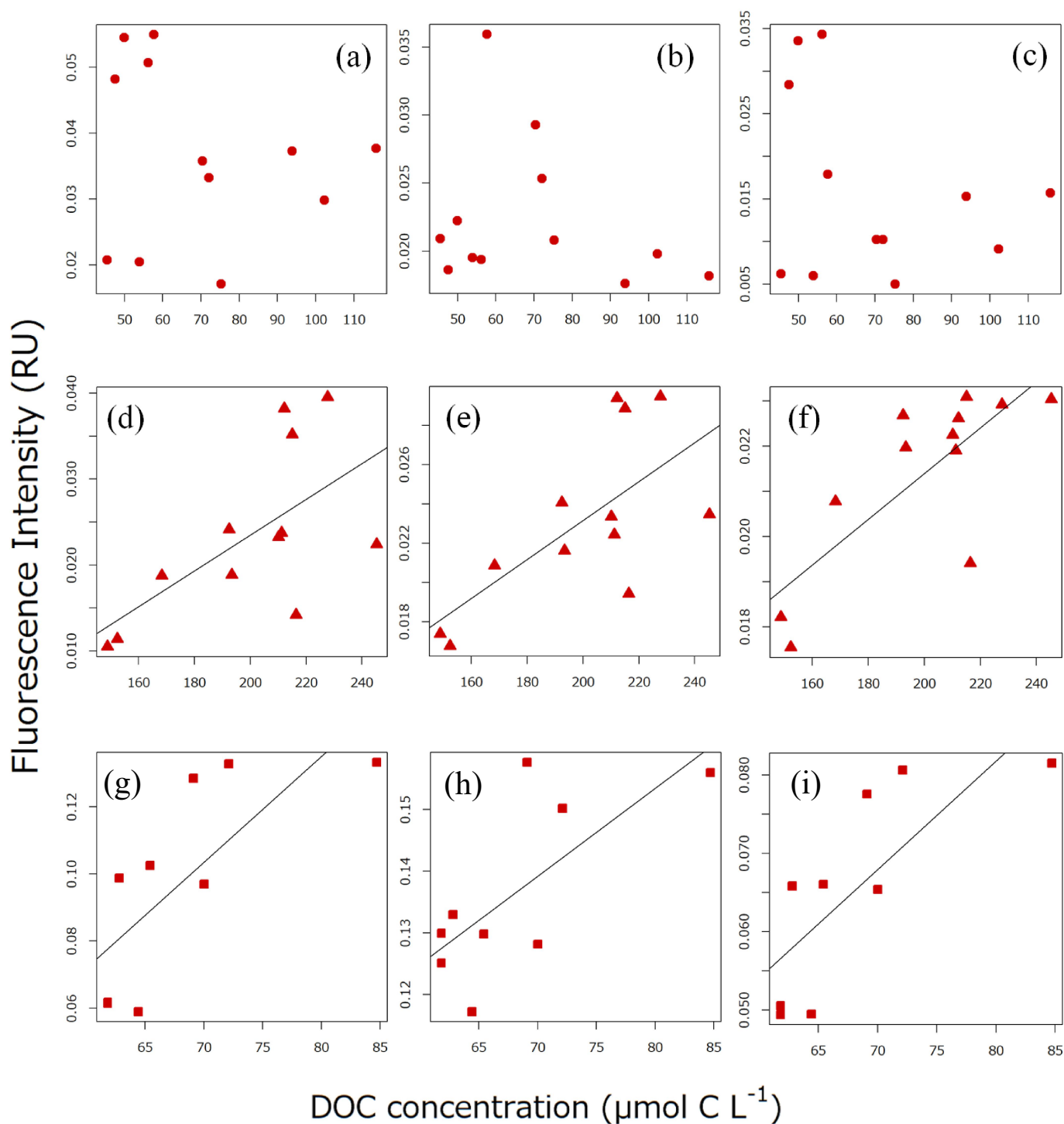


Figure 3-13 Plots between fluorescence intensities of humic-like FDOM and DOC concentrations of (a) AH1, (b) AH2, (c) AH3, (d) VH1, (e) VH2, (f) VH3, (g) PH1, (h) PH2 and (i) PH3 during stationary phases. All data from triplicate bottles were used for the figures. Linear lines represent regression lines between two parameters. Symbols in brackets were removed from regression analyses.

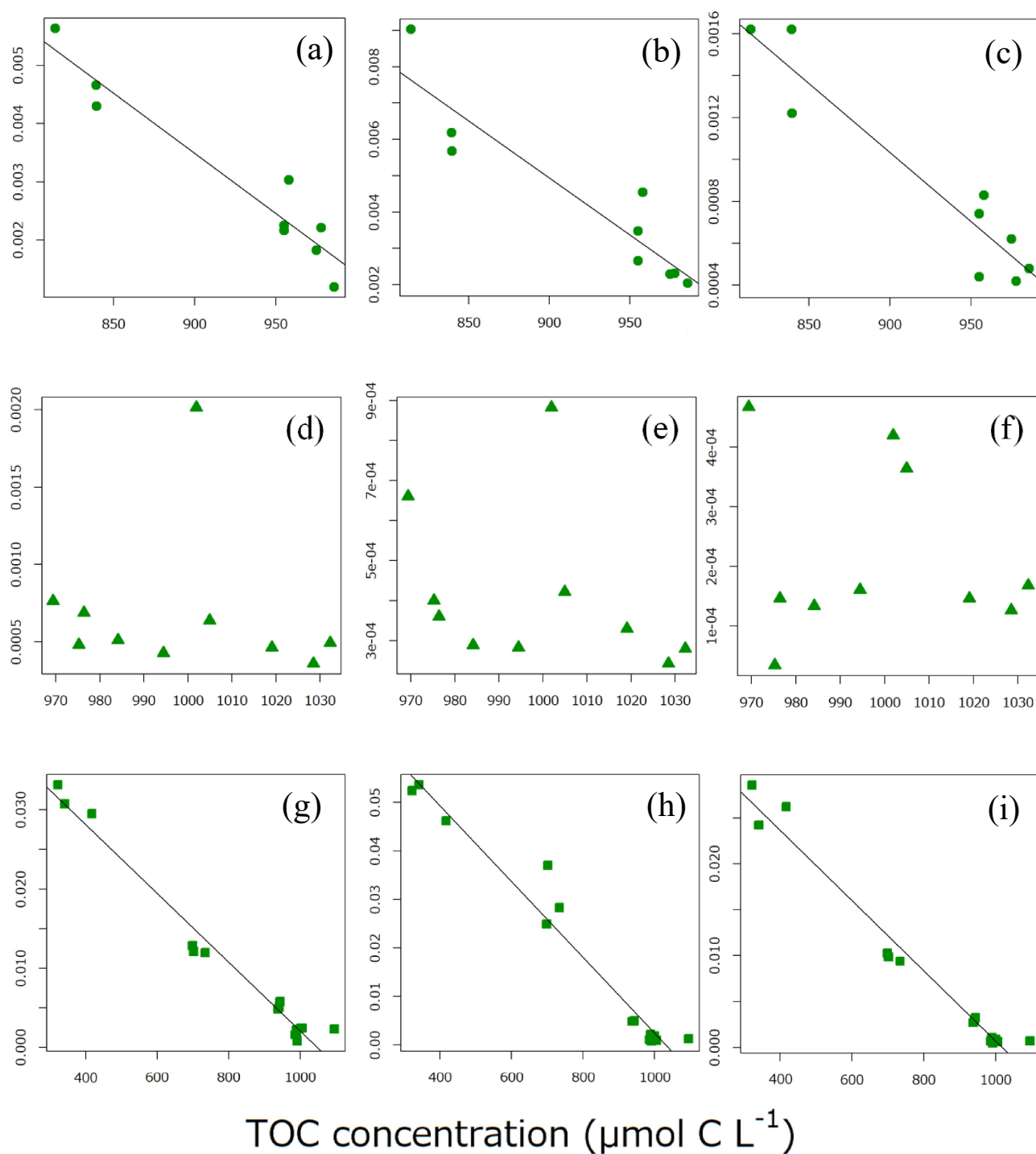


Figure 3-14 Plots between fluorescence intensities of humic-like FDOM and TOC concentrations of (a) AH1, (b) AH2, (c) AH3, (d) VH1, (e) VH2, (f) VH3, (g) PH1, (h) PH2 and (i) PH3 during exponential growth phases. All data from triplicate bottles were used for the figures. Linear lines represent regression lines between two parameters. Symbols in brackets were removed from regression analyses.

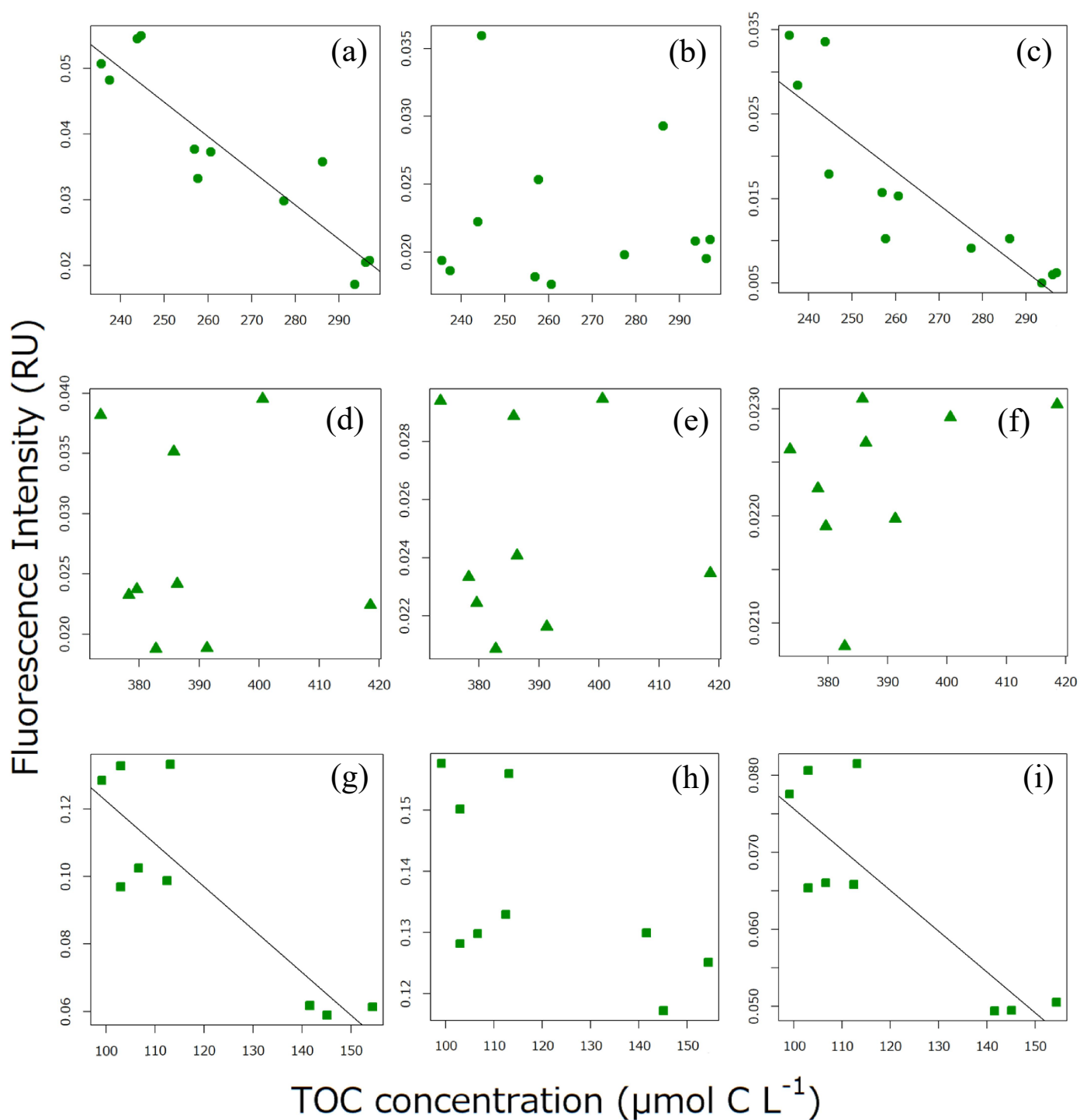


Figure 3-15 Plots between fluorescence intensities of humic-like FDOM and TOC concentrations of (a) AH1, (b) AH2, (c) AH3, (d) VH1, (e) VH2, (f) VH3, (g) PH1, (h) PH2 and (i) PH3 during stationary phases. All data from triplicate bottles were used for the figures. Linear lines represent regression lines between two parameters. Symbols in brackets were removed from regression analyses.

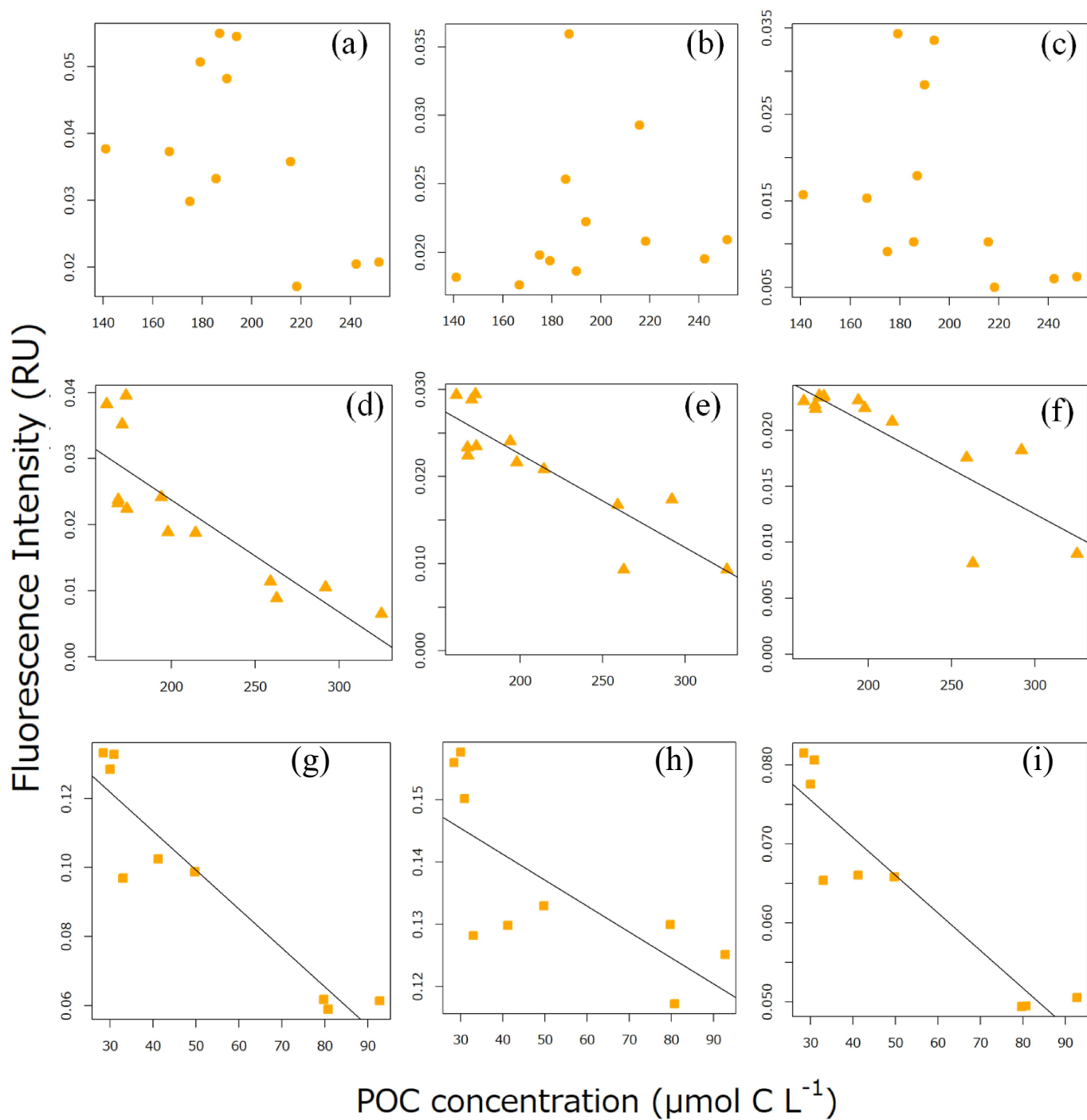


Figure 3-16 Plots between fluorescence intensities of humic-like FDOM and POC concentrations of (a) AH1, (b) AH2, (c) AH3, (d) VH1, (e) VH2, (f) VH3, (g) PH1, (h) PH2 and (i) PH3 during stationary phases. All data from triplicate bottles were used for the figures. Linear lines represent regression lines between two parameters. Symbols in brackets were removed from regression analyses.

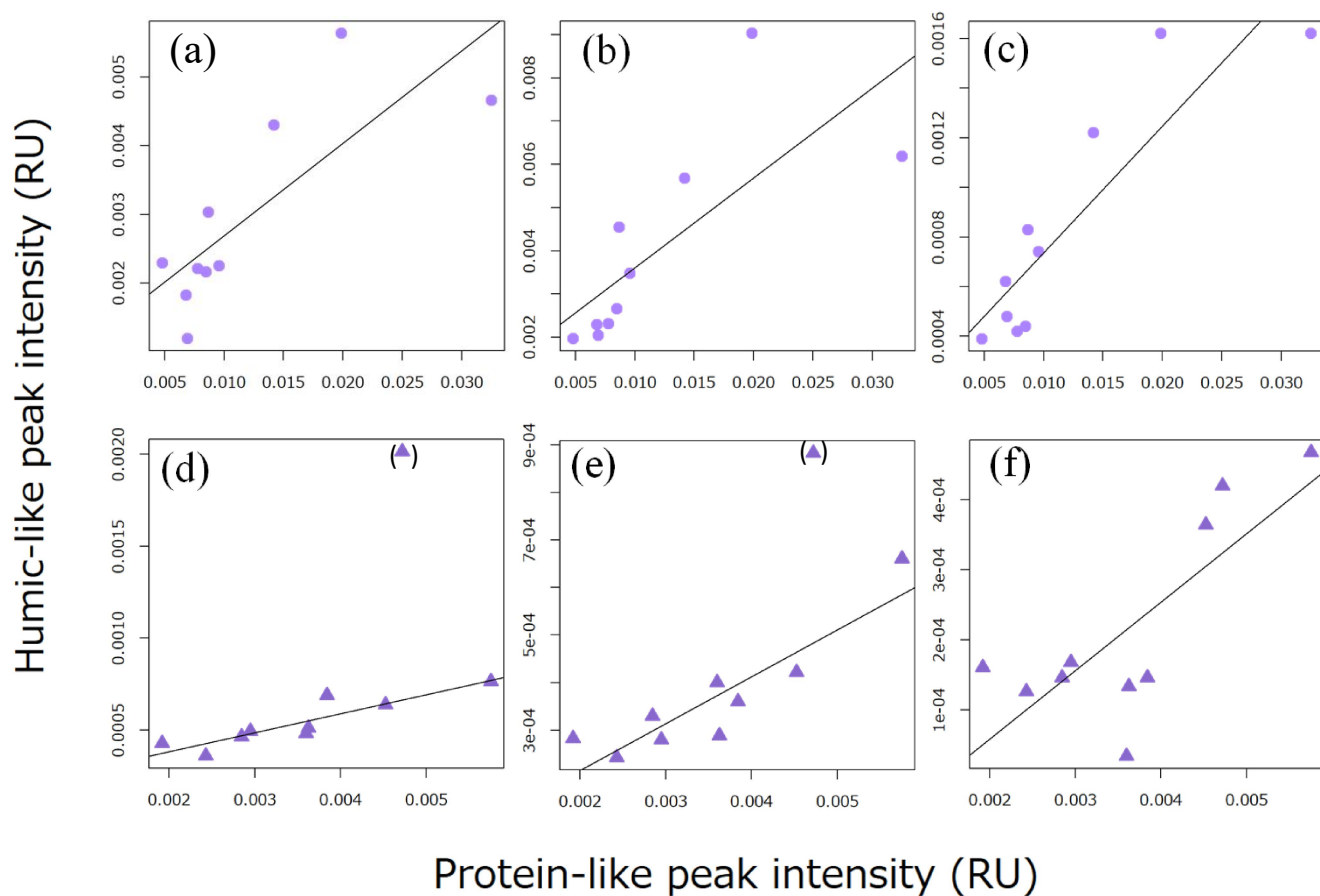


Figure 3-17 Plots between fluorescence intensities of humic-like and tryptophan-like FDOM of (a) AH1, (b) AH2, (c) AH3, (d) VH1, (e) VH2 and (f) VH3 during exponential growth phases. All data from triplicate bottles were used for the figures. Linear lines represent regression lines between two parameters. Symbols in brackets were removed from regression analyses.

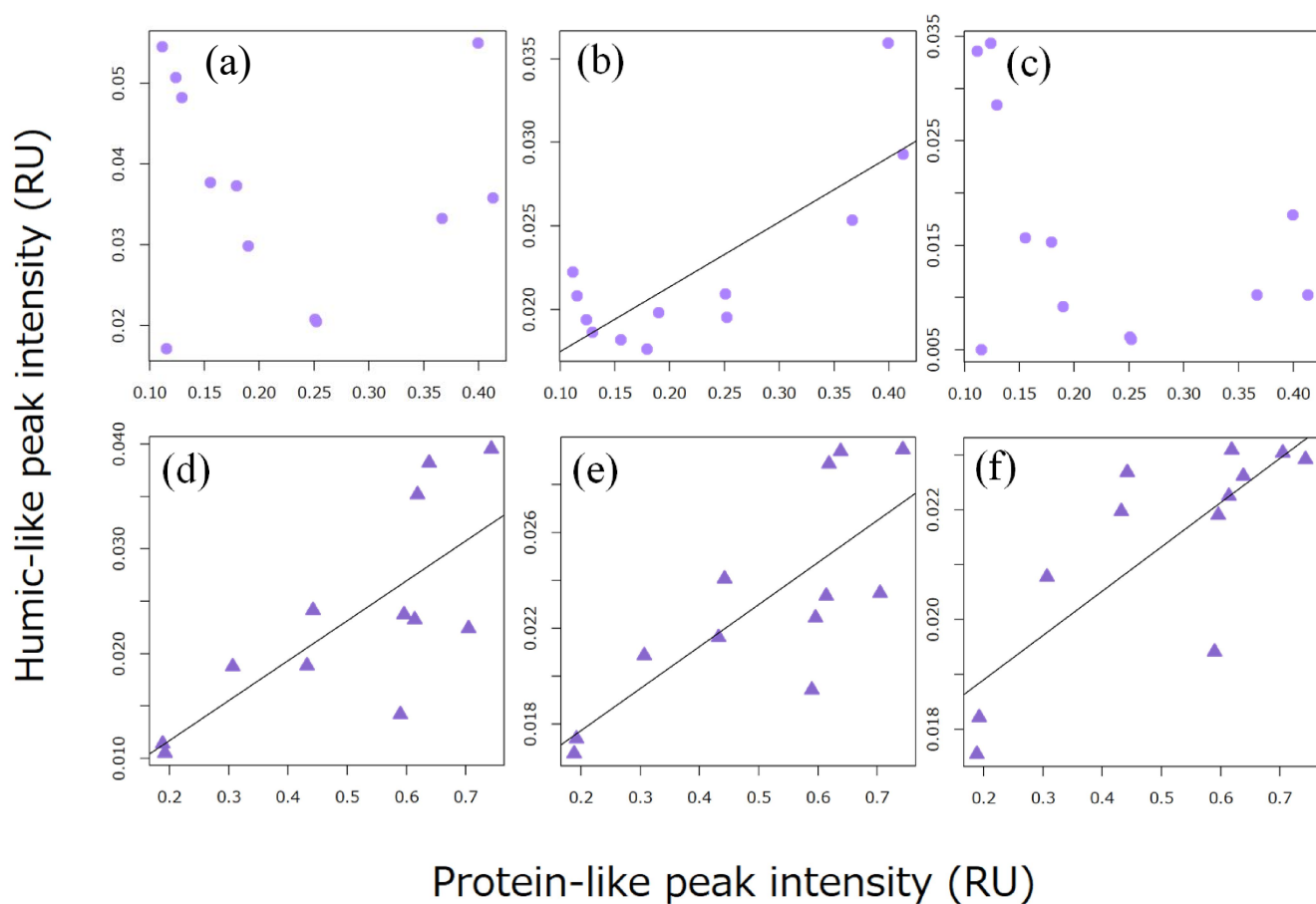


Figure 3-18 Plots between fluorescence intensities of humic-like and tryptophan-like FDOM of (a) AH1, (b) AH2, (c) AH3, (d) VH1, (e) VH2 and (f) VH3 during stationary phases. All data from triplicate bottles were used for the figures. Linear lines represent regression lines between two parameters. Symbols in brackets were removed from regression analyses.

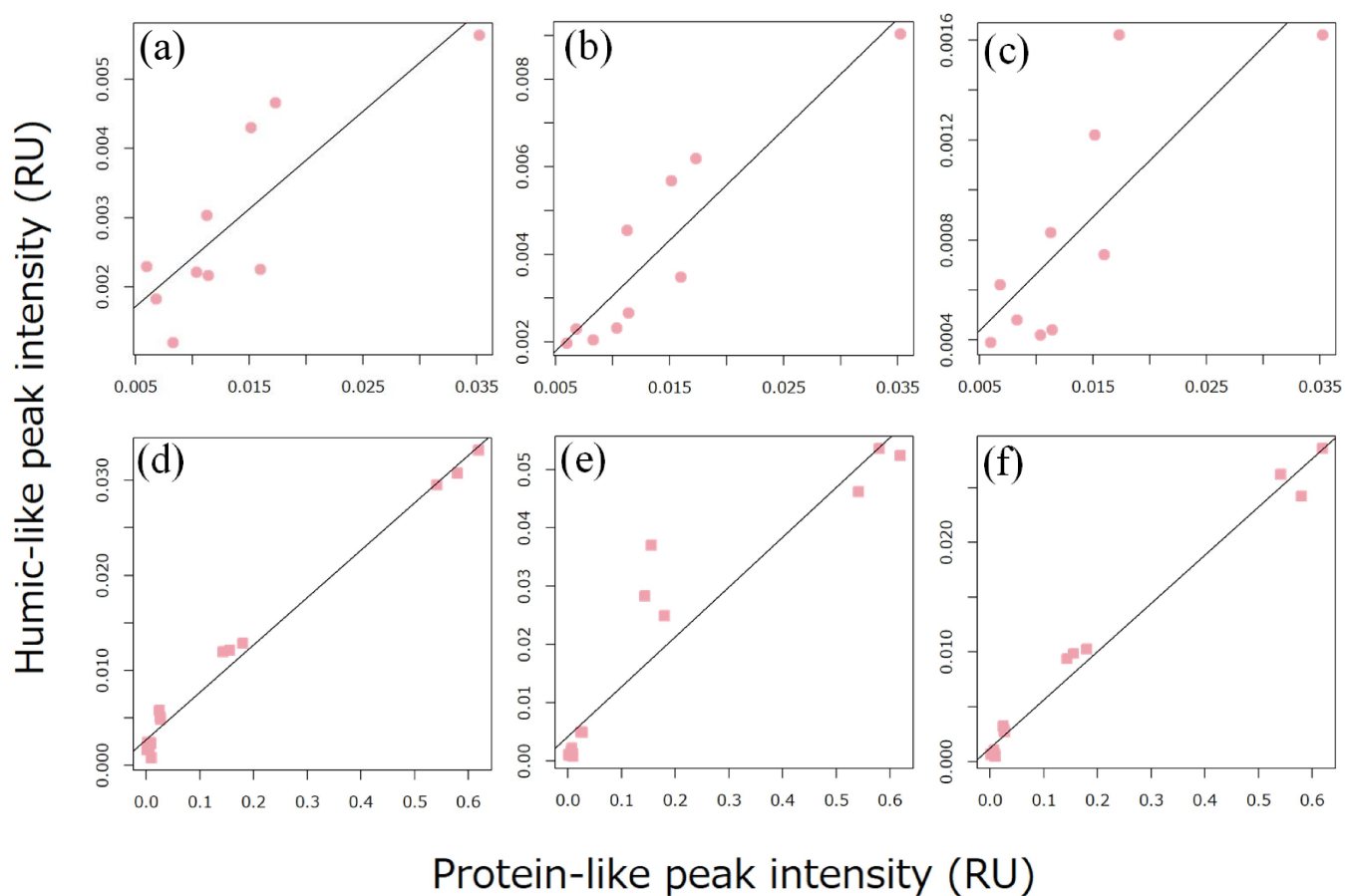


Figure 3-19 Plots between fluorescence intensities of humic-like and tyrosine-like FDOM of (a) AH1, (b) AH2, (c) AH3, (d) PH1, (e) PH2 and (f) PH3 during exponential growth phases. All data from triplicate bottles were used for the figures. Linear lines represent regression lines between two parameters. Symbols in brackets were removed from regression analyses.

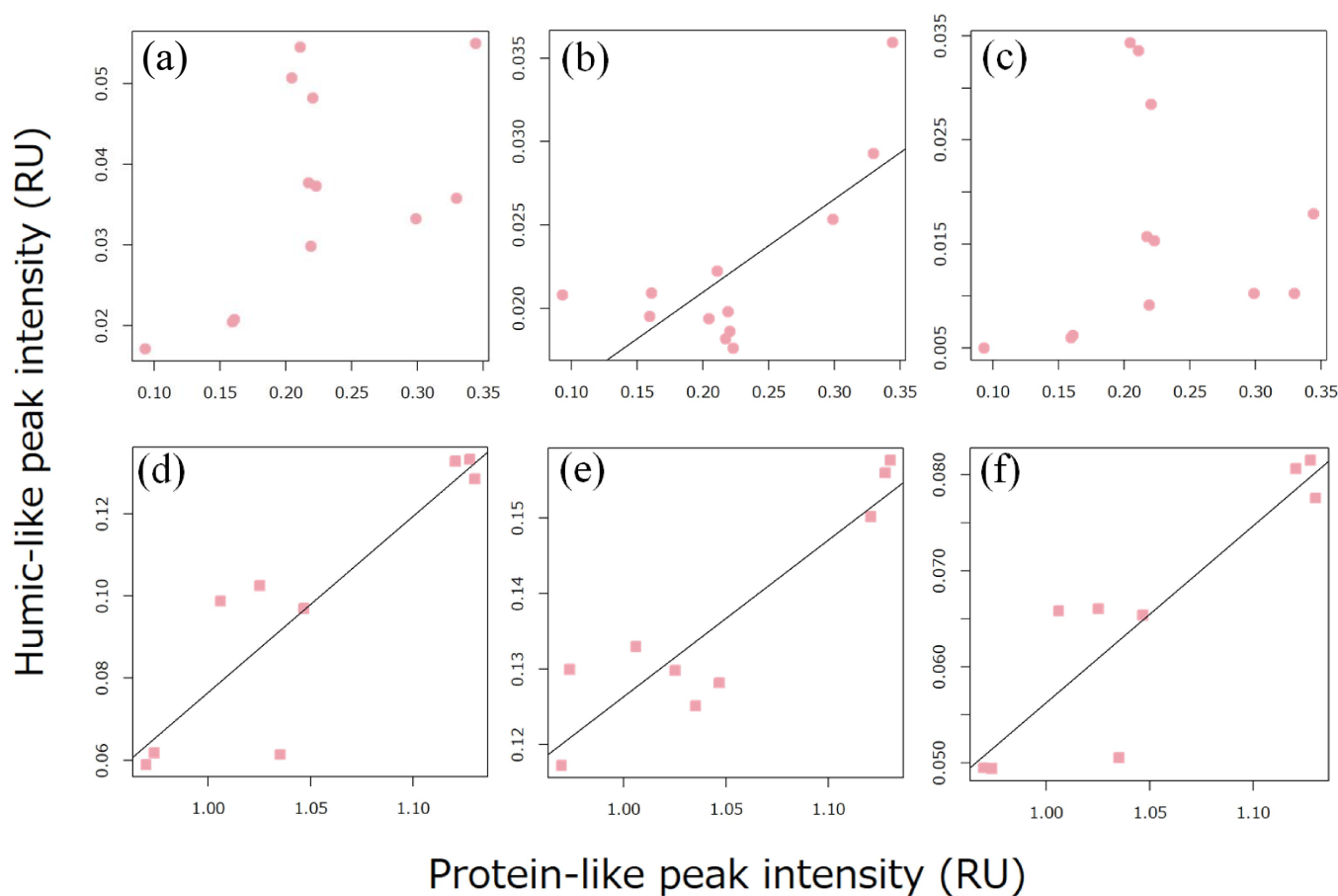


Figure 3-20 Plots between fluorescence intensities of humic-like and tyrosine-like FDOM of (a) AH1, (b) AH2, (c) AH3, (d) PH1, (e) PH2 and (f) PH3 during stationary phases. All data from triplicate bottles were used for the figures. Linear lines represent regression lines between two parameters. Symbols in brackets were removed from regression analyses.

Chapter. 4

Estimation of degradability of dissolved organic matter produced by bacterial strains with natural bacterial community

4-1 Introduction

Three marine model bacterial strains used in the present study produced DOM which is not able to be degraded by each bacterial strain (see Chapter 3). However, the degradability of the bacterial DOM with the other marine bacteria was not elucidated. It has recently been reported that changes in the bacterial community induced RDOM degradation (Shen and Benner, 2018). Therefore, the residual DOM after the end of incubations of each bacterial strain may include a degradable fraction for the other bacterial species. In the Chapter 4, the DOM produced by bacterial strains (strain-DOM) was incubated with natural bacterial community to estimate degradability of strain-DOM for natural bacterial community.

4-2 Materials and methods

4-2-1 Preparation of stable DOM produced by bacterial strains

The incubations of each strain, namely Alt, Vib and Pha, were carried out (referenced to the Chapter 3) for 14 days to obtain strain-DOM. The incubation period of 14 days would be sufficient since the DOC concentrations were already stable on day 14. After the incubation, the medium was filtered to collect DOM included in filtrate by rapid-flow filter unit with 0.2 μm pore size PES membrane filter (567-0020, Thermo Fisher Scientific). The filtrates were preserved in polycarbonate bottle until experiment. The strain-DOM derived from Alt, Vib and Pha were defined as Alt-DOM, Vib-DOM, and Pha-DOM, respectively. Modified Aquil medium (Price et al., 1989) which were described in detail in the Chapter 2 were prepared for a control treatment in the incubation of natural bacterial community.

4-2-2 Experiment setup

A microbial community was collected from the subtropical North Pacific Ocean (23°N, 141°E) on 10 December 2016 during a cruise of the R/V *Hakuho Maru* (KH 16-7). The seawater sample was collected from 5 m depth using an acid-cleaned Teflon-coated Niskin-X bottle. The natural seawater was filtered through pre-combusted (450°C, 3 hours) glass fiber filters with a nominal pore size of 0.7 μm (GF/F, Whatman) under gentle vacuum (< 0.02 MPa) to collect free-living bacterial community without

predators of bacteria. The filtrate was added to the strain-DOM or artificial seawater as inoculum of natural bacterial community with the ratio of 1:9 for inoculum to those media. Then, each medium with inoculum was dispensed into acid-cleaned (1 M HCl) 250 mL polyethylene terephthalate bottles. Each bottle was filled with 100 mL of the samples (seawater medium). Incubations of the experimental and control treatments were conducted in the dark with in an incubator (CN-40, Mitsubishi-Engineering Co.) in which the temperature was maintained at 27°C which was the temperature of seawater sampled. To determine the bacterial abundance, DOC concentration, and DOM optical property, triplicate bottles were sampled on 0, 3, 6, 14, and 40 days in the experimental treatments and on 0, 6, and 40 days in the control treatment.

4-2-3 Bacterial abundance

The procedure of bacterial cell density measurements was described in detailed in Chapter 2.

4-2-4 DOC concentration and DOM optical properties

The procedure of measurement in DOC concentration was the same with the DOC measurements described in section 2-2-4 in Chapter 2. Optical analyses for DOM were

also described in section 2-2-4 in Chapter 2.

4-3 Results

4-3-1 Growth of bacteria in natural community and change in DOC concentration

Figure 4-1 shows the changes in the bacterial abundance in all treatments during incubations. The respective value for each triplicate bottle was plotted in Figure 4-1, because the values were largely different among the triplicate bottle at the same time points. The changes in the DOC concentrations of all treatments are showed in Figure 4-2. The bacterial abundance in all experimental treatments with strain-DOM and the control treatment without the strain-DOM increased during incubations, although the increments of bacterial abundance were different among the treatments. The DOC concentrations of the strain-DOM treatments decreased during incubation, while the DOC concentration of the control treatment was relatively stable compared with the strain-DOM treatments. Furthermore, at the end of the incubations of the experimental treatments, DOC concentrations were different among the three strain-DOM treatments.

4-3-1-1 Alt-DOM treatment

The cell density in the Alt-DOM treatment was 1.3×10^5 cells mL⁻¹ at the initiation (day

0) and was stable until day 3. Then, the cell density increased to $2.6 \pm 1.0 \times 10^5$ cells mL⁻¹, $1.9 \pm 2.5 \times 10^6$ cells mL⁻¹ and $2.0 \pm 1.8 \times 10^6$ cells mL⁻¹ on day 6, 14, and 40, respectively (Figure 4-1a).

The DOC concentrations decreased from 114 $\mu\text{mol C L}^{-1}$ at the initiation of the incubation to 105 ± 4 $\mu\text{mol C L}^{-1}$, 93 ± 2 $\mu\text{mol C L}^{-1}$, 90 ± 5 $\mu\text{mol C L}^{-1}$ and 82 ± 3 $\mu\text{mol C L}^{-1}$ at day 3, 6, 14, and 40, respectively (Figure 4-2a).

4-3-1-2 Vib-DOM treatment

Increment of the bacterial abundance in the Vib-DOM treatment was highest among the all treatments (Figure 4-1b). The cell density at the initial of the Vib-DOM treatment was 8.6×10^5 cells mL⁻¹. The cell density increased to $3.5 \pm 4.2 \times 10^6$ cells mL⁻¹ on day 3, and then, reached to $7.9 \pm 0.4 \times 10^6$ cells mL⁻¹ on day 6. After the day 6, the cell density was stable. The cell density on day 14 and 40 was $6.0 \pm 1.5 \times 10^6$ cells mL⁻¹ and $4.8 \pm 2.2 \times 10^6$ cells mL⁻¹, respectively.

The DOC concentrations of the Vib-DOM treatment decreased clearly until day 6 (Figure 4-2b). The DOC concentration was 178 $\mu\text{mol C L}^{-1}$ at the initiation of the incubation, and then the DOC concentrations decreased to 153 ± 23 $\mu\text{mol C L}^{-1}$ and 115 ± 4 $\mu\text{mol C L}^{-1}$ on day 3 and 6, respectively. The DOC concentration during the period

of day 6-40 was stable; $119 \pm 23 \mu\text{mol C L}^{-1}$ and $121 \pm 13 \mu\text{mol C L}^{-1}$ on day 14 and 40, respectively.

4-3-1-3 Pha-DOM treatment

The bacterial abundance in the Pha-DOM treatment was relatively stable compared with the other experimental treatments (Figure 4-1c). The cell density was 7.5×10^5 cells mL^{-1} at the initiation of the treatment and increased to $1.7 \pm 1.3 \times 10^6$ cells mL^{-1} at the end of the incubation.

The DOC concentration in the Pha-DOM treatment was not changed largely compared with other experimental treatments. The DOC concentrations at the initiation and the end of the treatment were $68 \mu\text{mol C L}^{-1}$ and $57 \pm 6 \mu\text{mol C L}^{-1}$, respectively.

4-3-1-4 Control treatment

The bacterial abundance in the control treatment increased from the initiation to day 6 and 40 of the incubation although the DOC concentration was stable, suggesting that the bacterial growth was not apparently accompanied with the consumption of DOM. The cell density of the control treatment increased from 5.7×10^4 cells mL^{-1} at the initiation of the incubation to $9.5 \pm 2.5 \times 10^5$ cells mL^{-1} at the end of the incubation. The DOC

concentration at the initiation of the control treatment was $14 \mu\text{mol C L}^{-1}$ including DOC of the background in artificial seawater and the inoculum. The DOC concentrations during incubation of the control treatment were $15 \pm 2 \mu\text{mol C L}^{-1}$ and $15 \pm 1 \mu\text{mol C L}^{-1}$ on day 6 and 40, respectively.

4-3-2 Spectral characteristics of FDOM during incubation

Figure 4-3 shows EEMs of DOM in all treatments during the incubation. The EEMs in the control treatment (Figure 4-3d and h) did not indicate that distinctive fluorescent peaks appeared in the EEMs during the incubation, suggesting that fluorophores were not evident in the artificial seawater and were not produced during the incubation of the control treatment. In all experimental treatments, EEM patterns on day 0 (Figure 4-3a, b and c) were almost the same with those produced by each strain as described in Chapter 3 (Figure 3-5, 6 and 7), although the fluorescence intensities of each peak were different among strains. Then, the definitions of protein-like and humic-like FDOM peaks described in Chapter 3 (Table 3-3) were used for the results of the experiments in this chapter. The EEM patterns on day 40 in all experimental treatments (Figure 4-3e, f and g) were likely the same to those on day 0 in each strain-DOM treatment, without one fluorescent peak in the Vib-DOM treatment, although those

fluorescence intensities were changed during incubation. The fluorescent peak newly generated in the Vib-DOM treatment (VH4: Ex/Em = 260/470 nm) was categorized into the multiple origin-like group according to the category in Chapter 3.

4-3-3 Changes in fluorescence intensity of strain-DOM during incubation of natural bacterial community

4-3-3-1 Protein-like FDOM

The tyrosine-like and tryptophan-like FDOM in the Alt-DOM sharply decreased by day 14, and then were stable until the end of the incubation (Figure 4-4a). The tyrosine-like FDOM in Vib-DOM treatments sharply decreased by day 14 like Alt-DOM treatments, and then gradually decreased in the latter of the incubation (Figure 4-4b). Substantial fractions of protein-like FDOM in the Alt-DOM and Vib-DOM treatments were degraded during the incubation, suggesting that a part of protein-like FDOM derived from bacterial strain was degraded by the natural bacterial community, although the other part of the protein-like FDOM were not degraded.

The protein-like FDOM in the Pha-DOM treatment was stable during the incubation (Figure 4-4c), suggesting that the protein-like FDOM produced by this bacterial strain did not contain labile fraction for the natural bacterial community.

4-3-3-2 Humic-like FDOM

The fluorescence intensities of all humic-like FDOM did not decrease in the experimental treatments of three strain-DOM (Figures 4-5, 6 and 7), suggesting that the humic-like FDOM was recalcitrant for the natural bacterial community.

In the Alt-DOM treatment, AH2 increased by day 14, and then, was likely stable due to large deviation. AH1 and AH3 were stable during the incubation. Although VH3 was stable during the incubation of Vib-DOM, the other humic-like FDOM, namely VH1, VH2 and VH4, increased during the incubation. PH1 and PH3 were stable during the incubation in the Pha-DOM treatments, while PH2 tended to increase from day 6 to day 40.

4-4 Discussion

4-4-1 Degradability of bacterial DOM with natural bacterial community

In Chapter 3, I concluded that quantitative and qualitative characteristics of bacterially derived DOM (stain-DOM) were different among bacterial species and physiological states. In this chapter, the strain-DOM produced by bacterial strains were incubated with a natural bacterial community to evaluate the degradability by the other bacterial

species. The decrease in the DOC concentration of the experimental treatments indicated that a part of the DOM produced by one bacterial species were degraded by the other bacterial species in natural bacterial community. Shen and Benner (2018) indicated that a part of recalcitrant DOM occurred in the open ocean was degraded by coastal bacterial community which was different in structure from in situ bacterial community in the open ocean, suggesting that degradability of DOM by marine bacteria depends on bacterial community composition, namely differences in metabolic versatility. As the same manner with these previous studies, the degradation of strain-DOM in the experimental treatments suggested that bacterially derived DOM, which are resisted to consumption by the single bacterial species, was degradable by the different bacterial species in the natural bacterial community possibly due to high metabolic versatility in the community. However, it should be noted that substantial strain-DOM remained after the incubations with natural bacterial community.

The degraded and residual fractions during the strain-DOM incubation were defined as labile and non-labile fraction, respectively, in this study. The amounts of non-labile fraction were different among the three strain-DOM treatments in spite of the same amount of glucose added to the pre-incubation of each bacterial strain (Table 4-2), suggesting that the amount of non-labile strain-DOM was different among bacterial

species. Furthermore, the amount of non-labile strain-DOM was highest in the Vib treatment, like the highest residual DOC concentration among three strains incubations as described in Chapter 3 (Table 3-5), implying that non-labile strain-DOM against the other bacterial species was also produced efficiently by the bacterial species which can produce strain-DOM efficiently from labile substrates such as glucose. It is interesting to note that the proportions of non-labile fraction were similar among the all strain-DOM, although the amounts of labile and non-labile fractions were different among the strain-DOM treatment (Table 4-2). This result suggested that the bacterial species, which can produce strain-DOM efficiently from LDOM, are key players to produce RDOM in the ocean.

The protein-like FDOM (namely tyrosine-like and tryptophan-like FDOM) of the Alt-DOM and Vib-DOM was degraded by the natural bacterial community, suggesting that protein-like FDOM produced by these strains included a labile fraction for the natural bacterial community. In contrast, the tyrosine-like FDOM in the Pha-DOM did not decrease during the incubation, suggesting that the tyrosine-like FDOM derived from Pha was not labile for the natural bacterial community. Thus, the results in the present study showed that the protein-like FDOM produced by marine bacteria contained both labile and non-labile fractions. The previous studies have also reported

that protein-like FDOM as a labile component (Jørgensen et al., 2010; Lønborg et al., 2015) or a non-labile component (Catalá et al. 2015; Arai et al., 2018). These pieces of evidences indicated that the difference in degradability of protein-like FDOM was possibly related to the bacterial species producing them.

All humic-like FDOM of the three strain-DOM did not decrease during the incubation with natural bacterial community, suggesting that the humic-like FDOM produced by the bacterial strains was possibly recalcitrant for the other bacterial species.

4-4-2 Humic-FDOM production from strain-DOM

Some of humic-like FDOM increased in the strain-DOM treatments during the incubation of the natural bacterial community (Figures 4-5, 4-6, and 4-7), implying that humic-like FDOM was also produced by some bacterial species in the natural bacterial community. Figure 4-8 shows changes in EEM spectra during the incubations of strain-DOM with the natural bacterial community. The changes in EEM spectra shows consumption of protein-like FDOM and production of humic-like FDOM during the incubation. Interestingly, the spectral characteristics of humic-like FDOM produced by the natural bacterial community were similar with those of strain-DOM used as substrates for the natural bacterial community, irrespective of differences in strain-DOM

(Figures 4-3 and 4-8). The similarity of humic-like FDOM between products and substrates suggest that substrates (i.e., strain-DOM) control the spectral characteristics of products (i.e., humic-like FDOM produced by the natural bacterial community).

Previous studies conducting laboratory experiments have pointed out that the marine microbes produce humic-like FDOM efficiently under the presence of terrigenous components (Jørgensen et al., 2014; Aparicio et al., 2015). The incubation experiments of natural DOM from the Arctic and Atlantic Oceans indicated that microbial production of humic-like FDOM against respiration in the Arctic seawater was higher than those in the Atlantic seawater, and then suggested that the greater terrigenous component in the Arctic Ocean pronounced microbial production of humic-like FDOM. The study of incubation experiments using bathypelagic microbial community collected from South Atlantic Ocean indicated that the cell-specific production rate of humic-like FDOM in the incubation amended with humic acid from Suwannee River was higher than that with labile DOM, suggesting that precursors of humic-like FDOM occurred in the terrestrial humic substances and that the precursors could be transferred to the humic-like FDOM with marine microbial activity (Aparicio et al., 2015). The previous study of incubation experiments using *Synechococcus* strains suggested that humic-like FDOM derived from the strain was originated from

degradation products of the fluorescent phycobilin pigments (Zhao et al., 2017). The results of the present study suggested that precursors of humic-like FDOM were evident not only in terrigenous humic substances, but also DOM produced by marine bacteria. The hypothesis of humic-like FDOM precursor might provide a reasonable mechanism for distribution of humic-like FDOM in the ocean. In Chapter 3, it was indicated that marine bacteria produce various humic-like FDOM containing terrestrial humic-like FDOM group which has not been reported in the open ocean (Jørgensen et al., 2011; Tanaka et al., 2014). In contrast, the humic-like FDOM in the marine origin-like or multiple origin-like group clearly increased during the incubation of bacterial community in Chapter 4. These results suggested that the precursors derived from marine bacteria were mainly changed to the humic-like FDOM which could be ubiquitous in the ocean.

4-5 Conclusion

In this chapter, degradability of DOM produced by bacterial strains was evaluated by the incubation with natural bacterial community. The DOC concentration of the strain-DOM decreased during the incubation with the natural bacterial community, but it remained substantially at the end of the incubation. The results suggested that bacterial

strain can produce non-labile DOM which resist against degradation by the other bacterial species, although the other fraction of strain-DOM was degradable by the organisms. Furthermore, the residual DOC concentrations in the experimental treatments were different among the strain-DOM, suggesting that occurrence of key bacterial species to produce non-labile DOM for the other bacterial species and to efficiently contribute the microbial carbon pump.

The degradability of the protein-like FDOM during the incubation with natural bacterial community was different among strain-DOM, implying that the protein-like FDOM derived from marine bacteria have variable degradability and that the variability possibly depends on the bacterial species which produced them. The humic-like FDOM in the strain-DOM during the incubation was stable or increased, suggesting that the humic-like FDOM was likely recalcitrant fraction for the natural bacterial community. In addition, the similar EEM patterns of humic-like FDOM in the samples between the initiation and the end of incubation of strain-DOM with natural bacterial community, suggesting that the precursor of humic-like FDOM occurred in the strain-DOM.

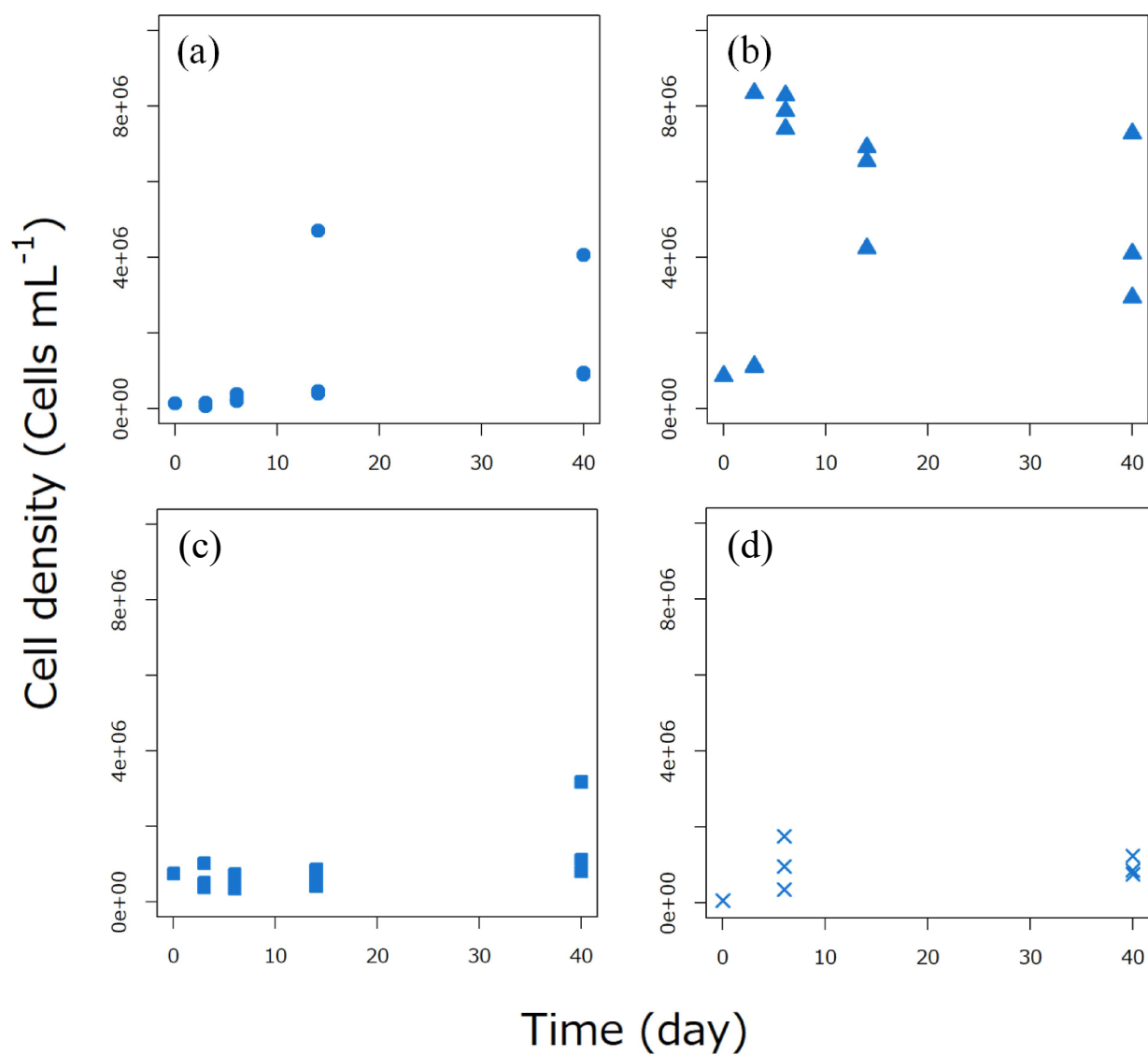


Figure 4-1 Changes in bacterial cell density (cells mL⁻¹) during incubations of in the experimental treatments of (a) Alt-DOM, (b) Vib-DOM and (c) Pha-DOM, and (d) the control treatment. All data from triplicate bottles were used for the figures.

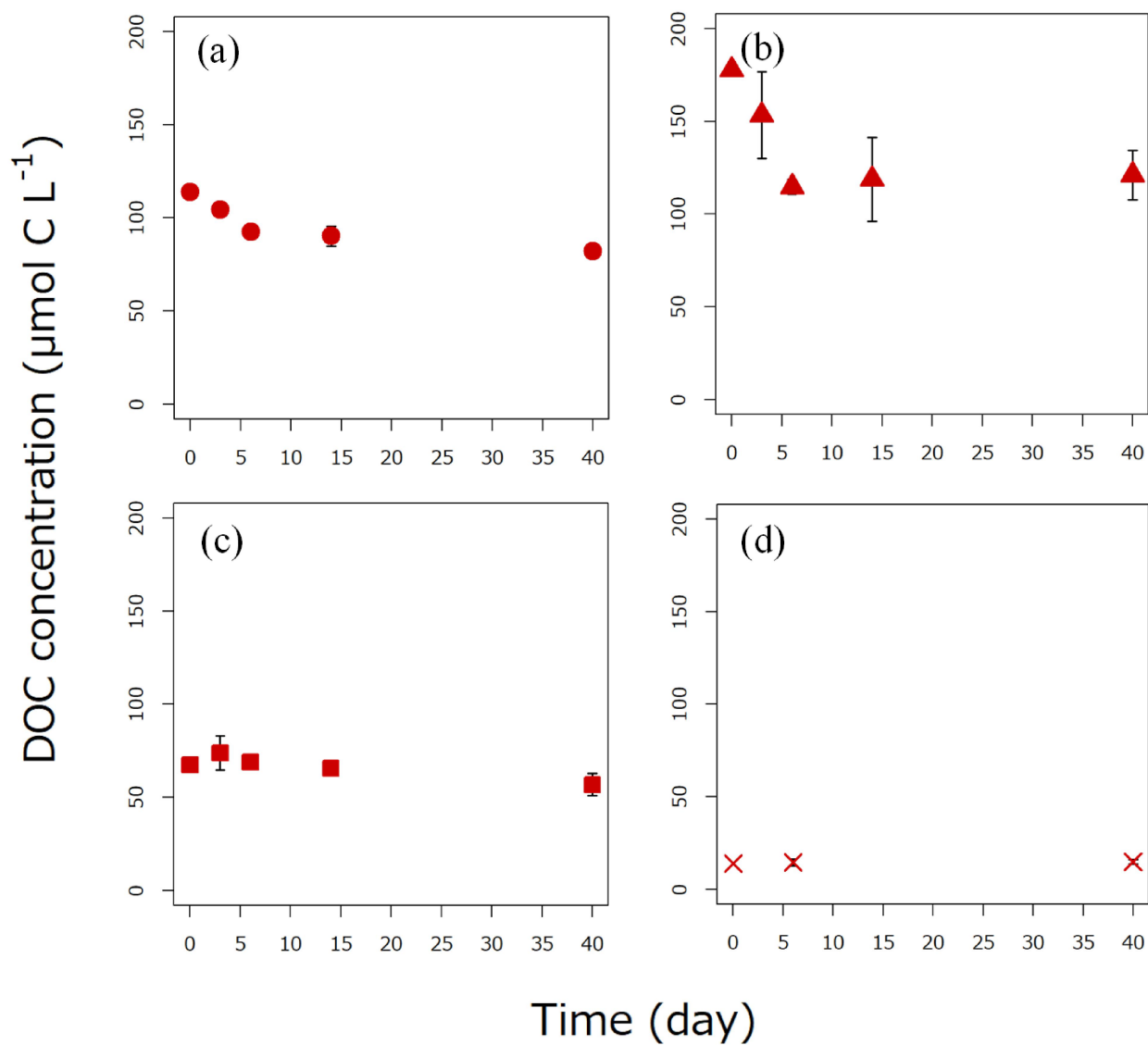


Figure 4-2 Changes in DOC concentration ($\mu\text{mol C L}^{-1}$) during incubations in the experimental treatments of (a) Alt-DOM, (b) Vib-DOM and (c) Pha-DOM, and (d) control treatment.

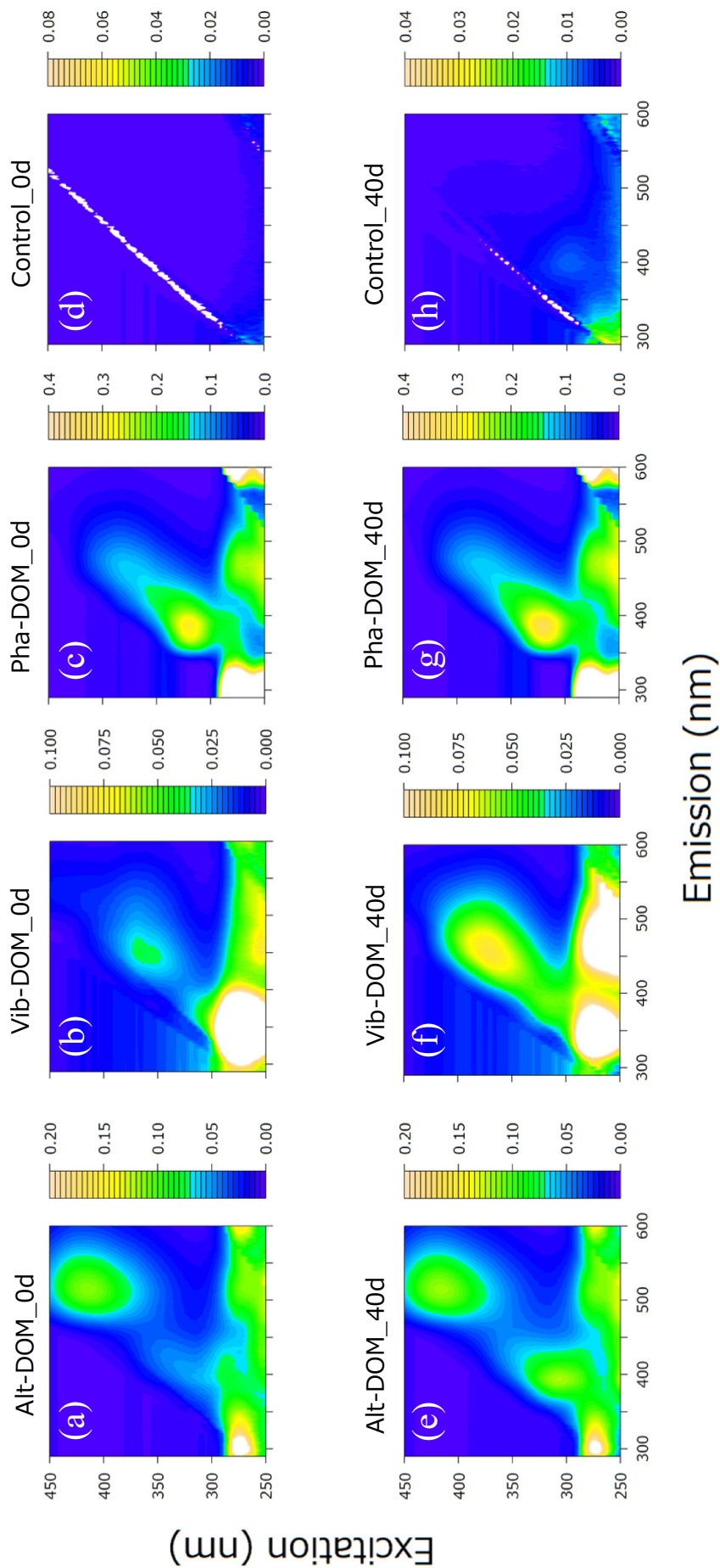
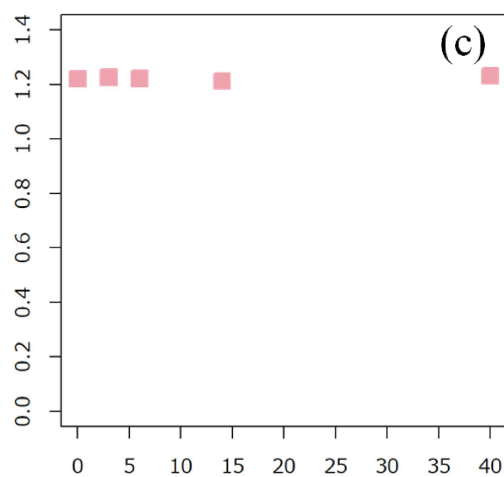
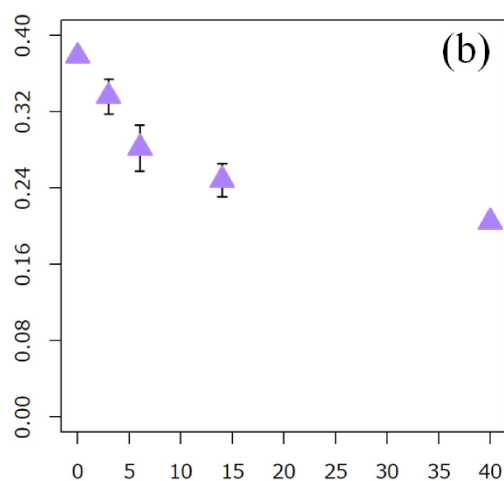
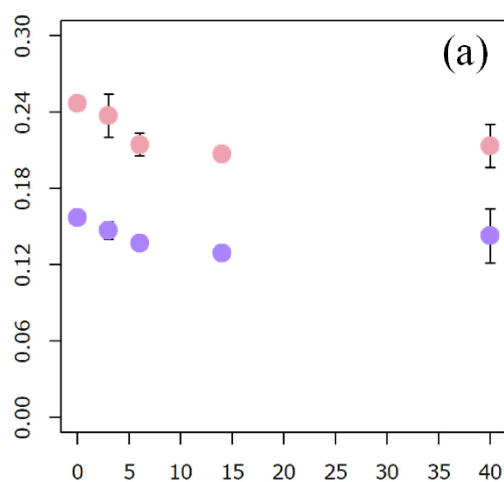


Figure 4-3 Excitation-emission matrix (EEM) of the strain-DOM at the initiation and the end of the incubation of (a), (e) Alt-DOM, (b), (f) Vib-DOM, (c), (g) Pha-DOM and (d), (h) control treatment with natural bacterial community. EEMs obtained from triplicate samples were averaged to produce the figures.

Intensity (RU)



Time (day)

Figure 4-4 Changes in fluorescence intensities of protein-like FDOM in the experimental treatments of (a) Alt-DOM, (b) Vib-DOM and (d) Pha-DOM. Purple and pink symbols indicate tryptophan-like peak and tyrosine-like peak, respectively.

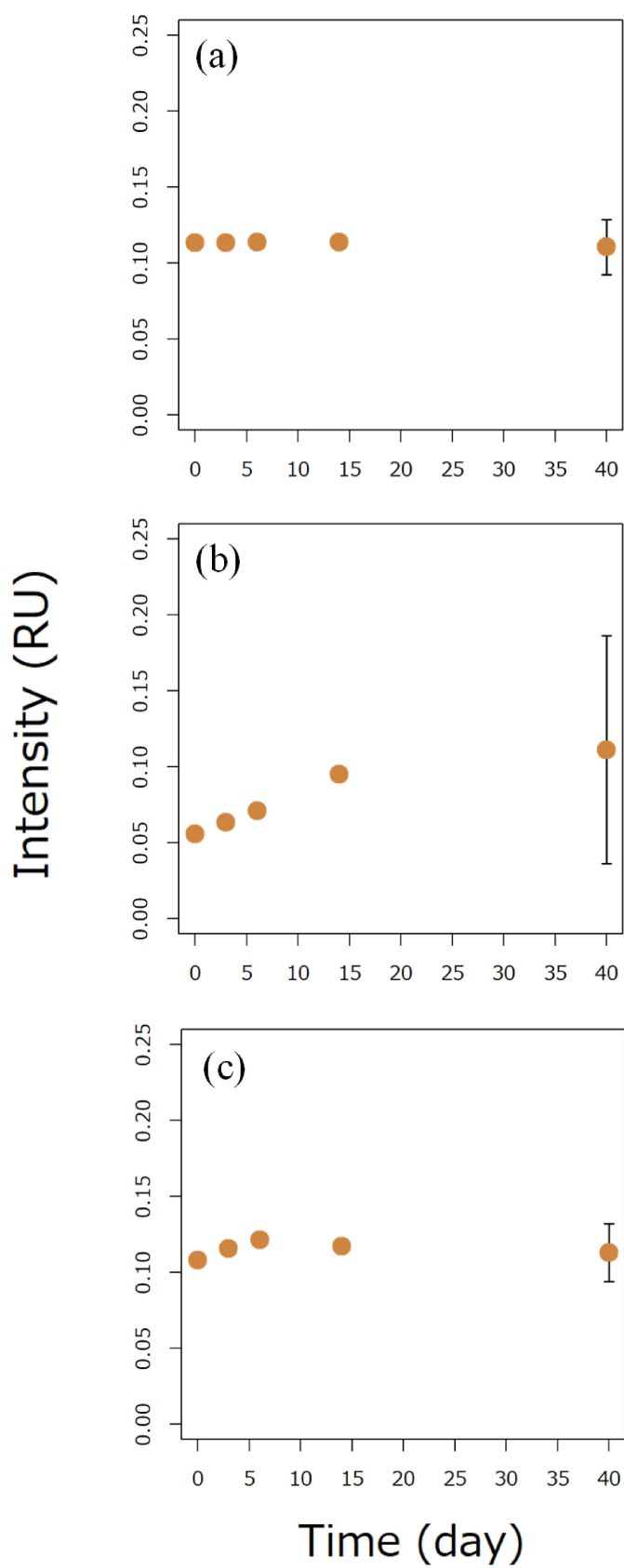


Figure 4-5 Changes in fluorescence intensities of (a) AH1, (b) AH2 and (c) AH3 categorized to humic-like FDOM in the Alt-DOM treatment.

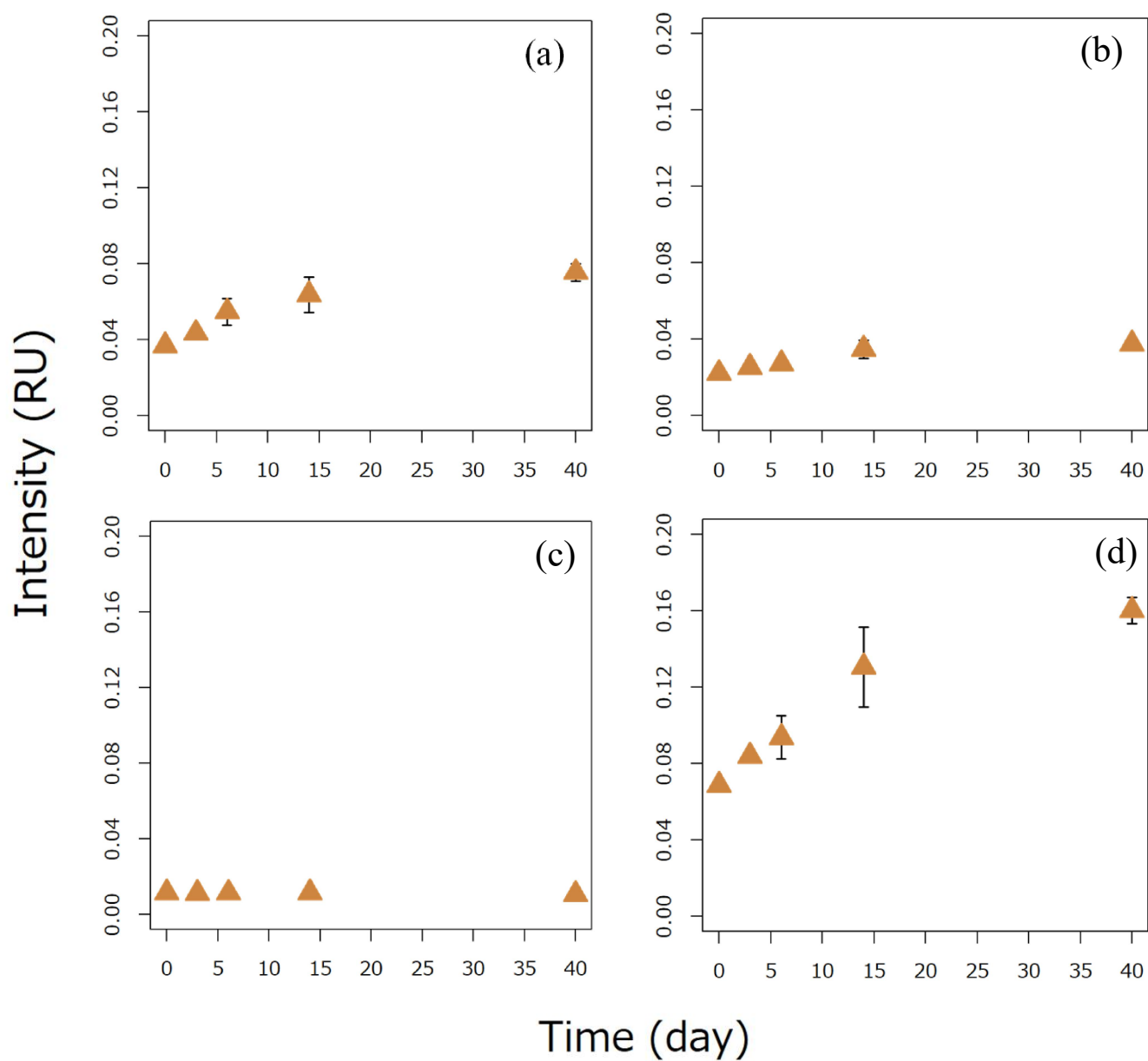


Figure 4-6 Changes in fluorescence intensities of (a) VH1, (b) VH2, (c) VH3 and (d) VH4 categorized to humic-like FDOM in the Vib-DOM treatment.

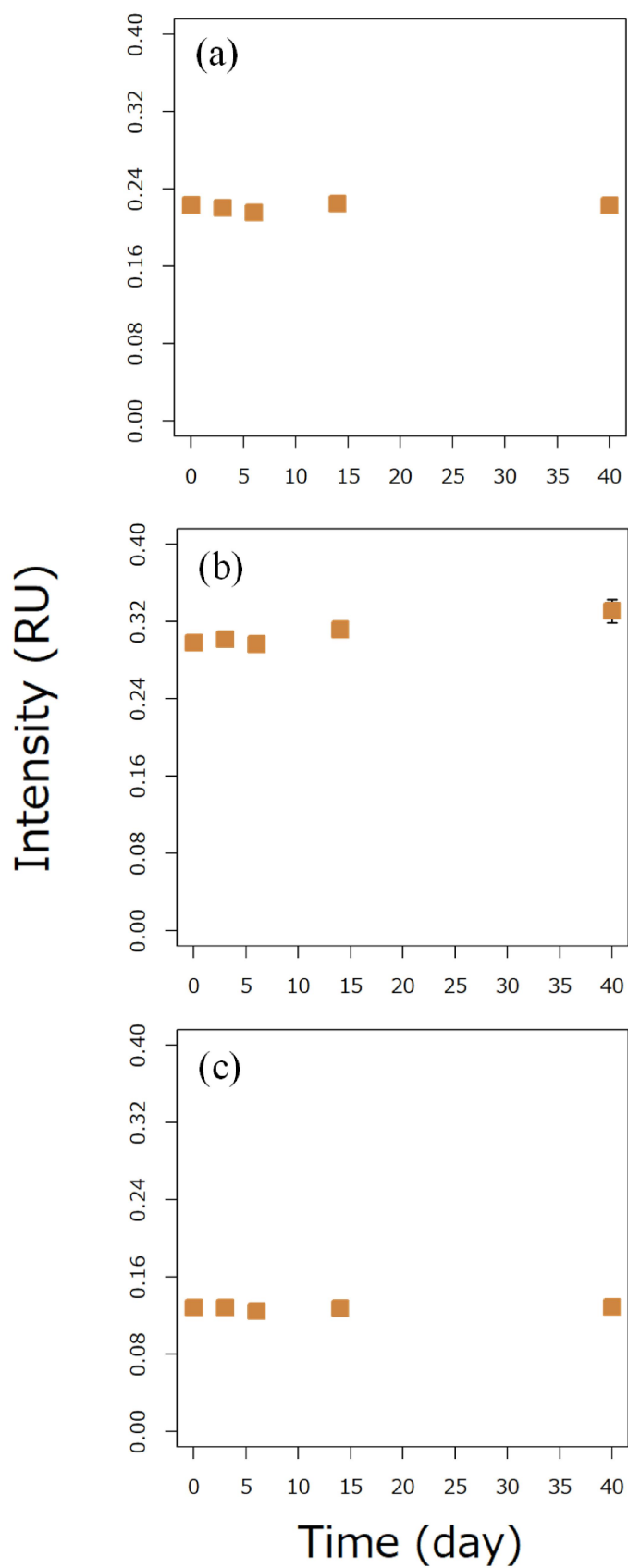


Figure 4-7 Changes in fluorescence intensities of (a) PH1, (b) PH2 and (c) PH3 categorized to humic-like FDOM in the Pha-DOM treatment.

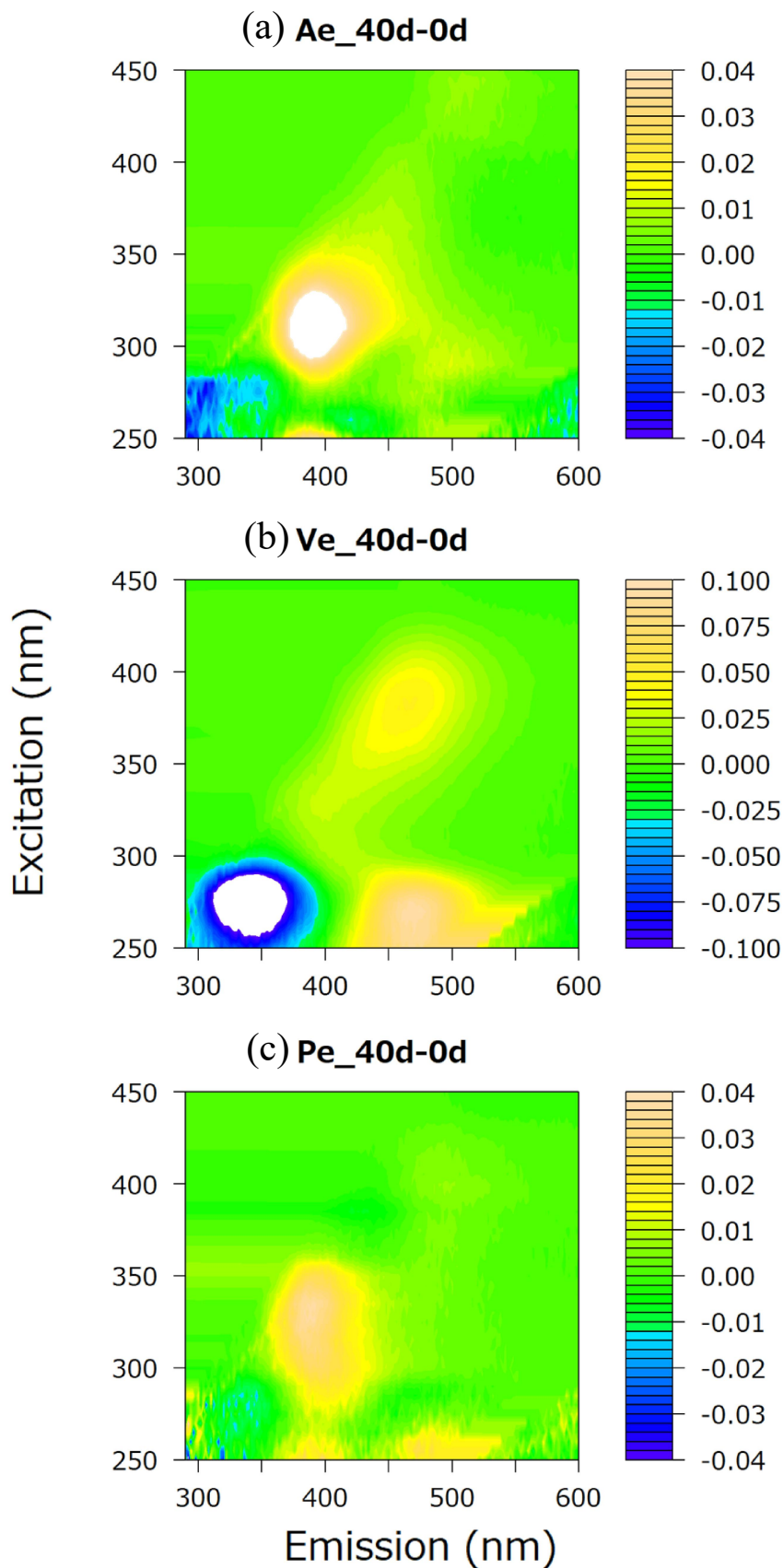


Figure 4-8 Changes in excitation-emission matrix (EEM) during the incubation of strain-DOM with natural bacterial community. The EEM were produced to subtract EEM at the initiation from correspondence at the end of incubations of (a) Alt-DOM, (b) Vib-DOM, and (c) Pha-DOM treatment.

Chapter 5.

General conclusions

5-1 Bacterial production of RDOM in terms of carbon

Estimations of the RDOM production flux by marine bacteria in terms of carbon are important for understanding of its quantitative impact on global carbon cycle. The present study firstly clarified that bacterial DOM production efficiencies from a simple substrate, namely glucose, were different among three model marine bacterial strains (i.e., *Alt*, 4%; *Vib*, 20%; *Pha*, 6%) (Chapter 3, Figure 5-1). *Vib* produced DOM efficiently compared with the other strains, and the efficiency was also higher than those produced by bacterial community previous reported, i.e., 3–7% (Ogawa et al., 2001; Shimotori et al., 2009; Koch et al., 2014; Lechtenfeld et al., 2015). Although specific bacterial species which are quantitatively important as DOM producers has not been identified in the natural seawater, the high efficiency of DOM production by *Vib* found in the present study suggests that specific bacterial species can produce DOM efficiently from labile substrates. Furthermore, Chapter 4 clarified that although the strain-DOM was degraded by the other bacterial species, major fraction (60–80%) of strain-DOM was recalcitrant for the natural bacterial community, possibly other bacterial species.

These results suggest that the bacterial species, which can produce DOM efficiently, such as *Vib* are also important as a RDOM producer and can be a key player in the microbial carbon pump (Figure 5-1).

5-2 Bacterial production of recalcitrant humic-like FDOM

Chapter 3, on the one hand, showed that all three model bacterial strains used in the present study produced humic-like FDOM (Figure 5-2). The results were consistent with previous studies which have reported humic-like FDOM production by marine bacterial strains (Shimotori et al., 2012; Zhao et al., 2017). The results of the present study coupled with previous studies suggest that the broad bacterial species can produce humic-like FDOM. On the other hand, Chapter 3 showed that efficiencies of humic-like FDOM production for LDOM (i.e., glucose) consumption during the exponential growth phase were different among the three bacterial strains. The efficiency by *Pha* was higher than those by the others, indicating that the specific bacterial species can produce humic-like FDOM efficiently, although broad bacterial species can produce it. Furthermore, the humic-like FDOM produced by bacterial strains did not decrease during the incubation of natural bacterial community (Chapter 4), suggesting that bacterial humic-like FDOM was likely recalcitrant.

Chapter 3 clearly showed that recalcitrant humic-like FDOM was produced during the exponential growth phase, the stationary phase and the death phase in the incubation of three bacterial strains. On the one hand, active humic-like FDOM production during the exponential growth phase and stationary phase was accompanied with consumption of LDOM, extracellular DOM released by the strain, or cellular materials (Figure 5-3). Humic-like FDOM release during the death phase possibly with viral lysis was a passive sense (Figure 5-3). Previous studies also suggested that humic-like FDOM was produced with various bacterial physiological states (Jørgensen et al., 2014; Zhao et al., 2017; Arai et al., 2018). Chapter 3 firstly determined the efficiencies of bacterial humic-like FDOM production against mineralization of organic matter and compared them among bacterial species as well as bacterial physiological state. The efficiencies with consumption of LDOM during the exponential growth phase were different among three bacterial strains. The efficiencies of active production with consumption of extracellular DOM released by the strain or cellular materials during the stationary phase were higher than those of active production with consumption of LDOM during the exponential growth phase. These results suggest that efficiency of recalcitrant humic-like FDOM production depended on bacterial species as well as bacterial physiology, and thus, suggest that changes in bacterial species or physiology of

microbial community are key factors controlling the RDOM production in the ocean.

The indirect process of humic-like FDOM production mediated by its precursor (Figure 5-3) was suggested from the similarity of EEM patterns between before and after incubation of the natural bacterial community with strain-DOM (Chapter 4). It was speculated that the precursors produced by the strains were changed to the humic-like FDOM by the other bacterial species during the incubation of bacterial community.

Notably, the recalcitrant humic-like FDOM contained the terrestrial origin-like group which has seldom been reported the presence in the open ocean (Jørgensen et al., 2011; Tanaka et al., 2014). The results suggested that bacterial species composition regulate humic-like FDOM production. Zhao et al. (2017) suggested that humic-like FDOM released by cyanobacteria mainly due to viral lysis was recalcitrant since the produced humic-like FDOM was similar to those in the deep ocean. Furthermore, the humic-like FDOM in the marine origin-like or multiple origin-like group clearly increased during the incubation of bacterial community, suggesting that the precursors were mainly changed to the humic-like FDOM which could be ubiquitous in the ocean.

5-3 Limitations and implications

In terms of carbon, efficiencies of DOM production of the three bacterial strains were

estimated in Chapter 3. In Chapter 4, a part of strain-DOM was possibly degraded by the other bacterial species. These results indicated that estimation of RDOM production efficiency of single bacterial species might be difficult, because bio-degradability of organic matter is dependent on bacterial species composition. The present study was able to estimate the production efficiencies of bacterial DOM which were not available for the natural bacterial community.

The present study indicated that humic-like FDOM produced by broad bacterial species was RDOM for natural microbial community. In addition to humic-like FDOM, the remnant DOM at the end of natural microbial community incubations in Chapter 4 should include the other bio-recalcitrant compounds which have been reported as bacterial RDOM, such as carboxyl-rich alicyclic molecules (CRAM) (Hertkorn et al., 2006; Lechtenfeld et al., 2015), D-enantiomer amino acid (Dittmar et al., 2001) and membrane proteins (Tanoue et al., 1995). For a better understanding of the process of microbial RDOM production, the changes in these recalcitrant compounds during microbial incubation should determine with bacterial physiological states.

In Chapter 4, I suggested that non-fluorescent precursors in bacterial DOM became humic-like FDOM by bacterial activity during incubation of natural bacterial community, while the mechanism could not be determined in the present study.

Extracellular enzymes are well known to be important bacterial substances for microbial consumption of DOM (Arnosti et al., 2014). Thus, extracellular enzymatic processes can be considered as the mechanism of processing of humic-like FDOM from its precursors. It is possible that the enzymatic reaction with the precursor of humic-like FDOM causes its conformational changes and induces fluorescence. The hypothesis should be examined by in vitro experiments using strain-DOM and model extracellular enzymes. Such elucidation of the mechanisms would attribute to a better understanding of the mechanisms of formation of not only recalcitrant humic-like FDOM, but the whole bacterial RDOM.

The present study examined the biotic degradation of strain-DOM by natural bacterial community (Chapter 4). In natural environments of surface ocean, it is well known that organic matter can be photochemically degraded by solar irradiance (Mopper et al., 1991). Examination of degradability and its mechanisms in bacterial DOM against the photochemical process may provide an insight of the fate of bacterial RDOM in the ocean.

In the present study, it was suggested that bacterial RDOM production can be regulated by bacterial species composition and physiological process. These biological parameters are affected by environmental factors (i.e., organic and inorganic substrates,

water temperature, light condition, pH, oxygen concentration, etc.) in the ocean.

Elucidating effects of these factors on the bacterial RDOM production makes a better understanding of the regulating mechanisms of bacterial RDOM production, and thus, of factors regulating the microbial carbon pump.

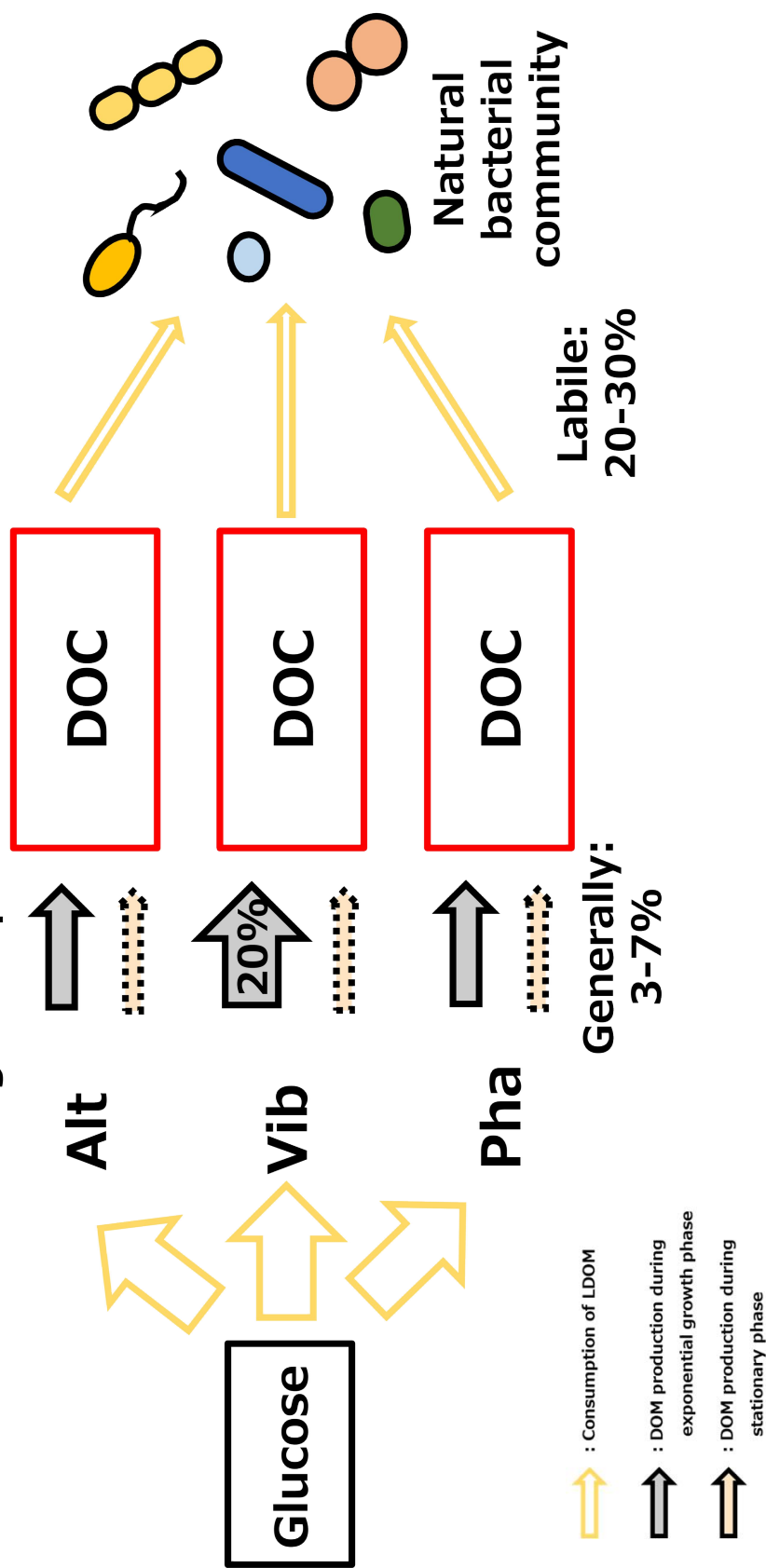


Figure 5-1 Schematic diagram summarizing results of the present study in efficiency of bacterial RDOM production in terms of DOC.

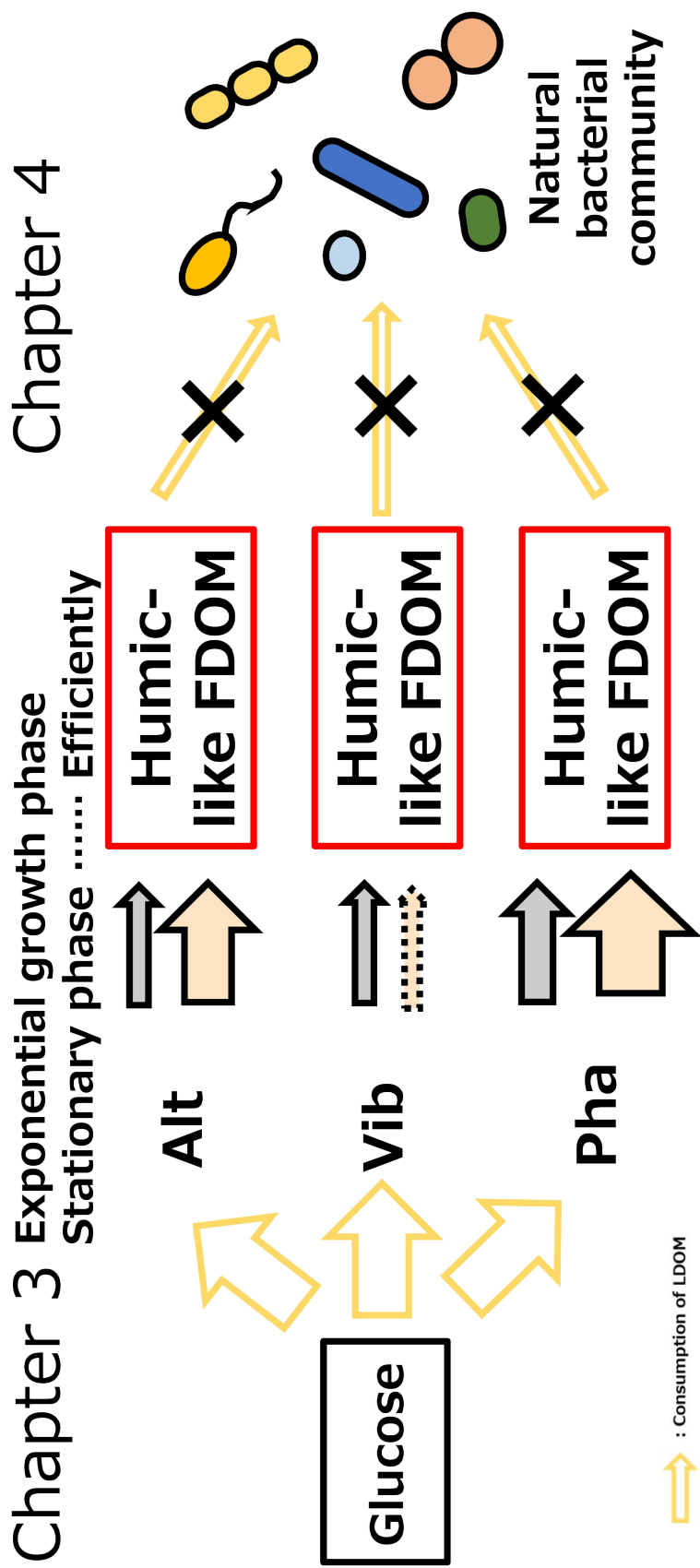


Figure 5-2 Schematic diagram summarizing results of the present study in bacterial RDOM production in terms of humic-like FDOM.

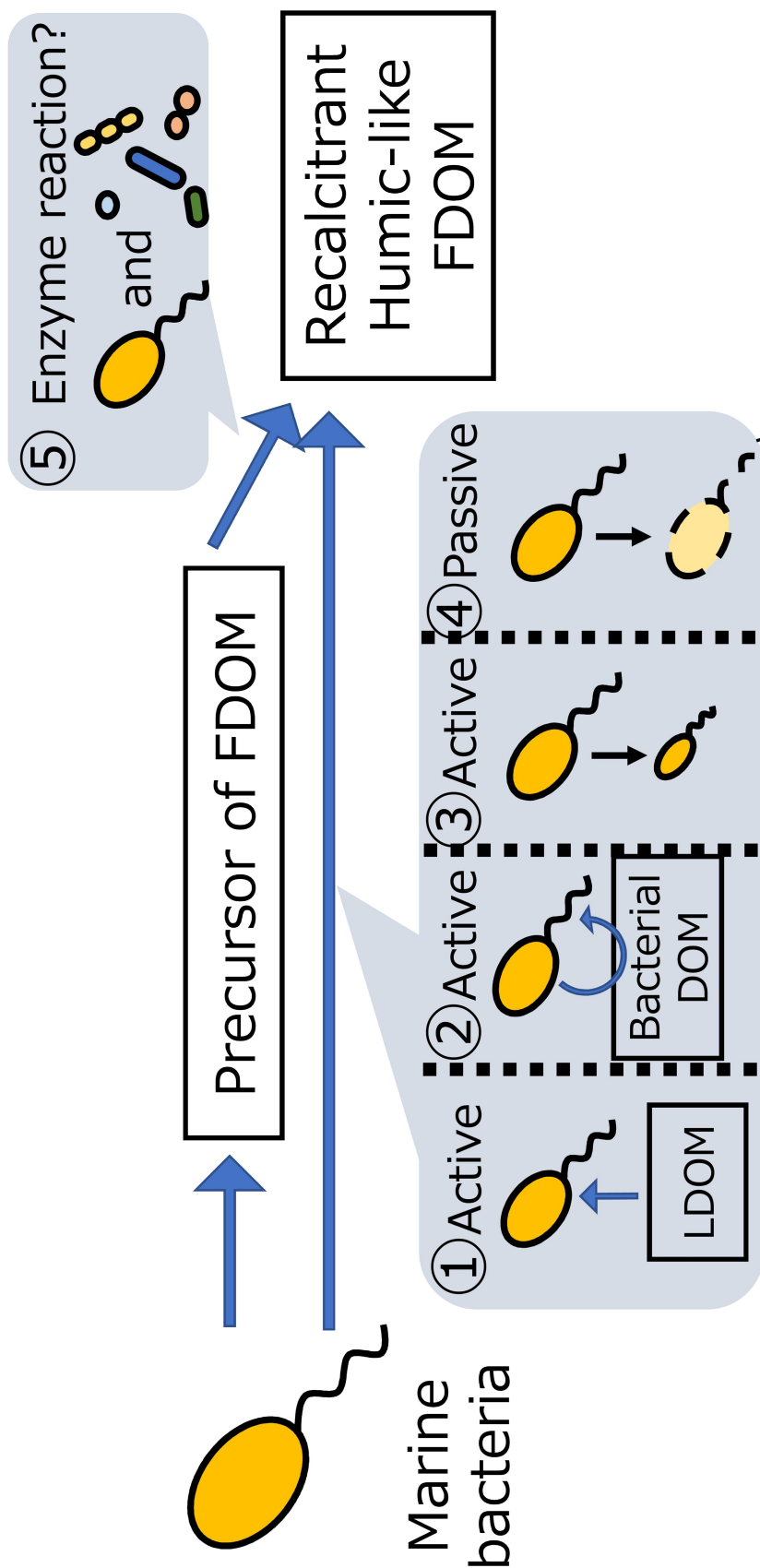


Figure 5-3 Schematic diagram summarizing results of the present study in bacterial production mechanism of recalcitrant humic-like FDOM.

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