<table>
<thead>
<tr>
<th>Title</th>
<th>Culture of Microalgae with Digestate from Methane Fermentation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Author(s)</td>
<td>Thien Khanh, Nguyen Tran</td>
</tr>
<tr>
<td>Editor(s)</td>
<td></td>
</tr>
<tr>
<td>Citation</td>
<td></td>
</tr>
<tr>
<td>Issue Date</td>
<td>2015</td>
</tr>
<tr>
<td>URL</td>
<td><a href="http://hdl.handle.net/10466/14840">http://hdl.handle.net/10466/14840</a></td>
</tr>
<tr>
<td>Rights</td>
<td></td>
</tr>
</tbody>
</table>

http://repository.osakafu-u.ac.jp/dspace/
Culture of Microalgae with Digestate from Methane Fermentation

Nguyen Tran Thien Khanh

2015

Graduate School of Life and Environmental Sciences
Osaka Prefecture University
JAPAN
Culture of Microalgae with Digestate from Methane Fermentation

Nguyen Tran Thien Khanh
2015

Environmental Sciences and Technology
Graduate School of Life and Environmental Sciences
Osaka Prefecture University
JAPAN
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT OF THE THESIS</td>
<td>1</td>
</tr>
<tr>
<td>CHAPTER 1</td>
<td>6</td>
</tr>
<tr>
<td>GENERAL INTRODUCTION</td>
<td>6</td>
</tr>
<tr>
<td>1.1 Microalgae</td>
<td>6</td>
</tr>
<tr>
<td>1.2 Digestate from methane fermentation</td>
<td>7</td>
</tr>
<tr>
<td>CHAPTER 2</td>
<td>9</td>
</tr>
<tr>
<td>EFFECTS OF DEPTHS AND CONCENTRATIONS OF DIGESTATE ON GROWTH OF THREE MICROALGAL SPECIES</td>
<td>9</td>
</tr>
<tr>
<td>2.1 Abstract</td>
<td>9</td>
</tr>
<tr>
<td>2.2 Introduction</td>
<td>10</td>
</tr>
<tr>
<td>2.3 Materials and methods</td>
<td>12</td>
</tr>
<tr>
<td>2.4 Results and discussion</td>
<td>22</td>
</tr>
<tr>
<td>2.5 Conclusion</td>
<td>27</td>
</tr>
<tr>
<td>CHAPTER 3</td>
<td>28</td>
</tr>
<tr>
<td>EFFECTS OF ENVIRONMENTAL CONDITIONS ON GROWTH OF MICROALGAE</td>
<td></td>
</tr>
</tbody>
</table>
CULTURED WITH DIGESTATE ................................................................. 28

3.1 Effects of digestate concentrations and temperature in culture solution on growth of microalgae .................................................................................................................. 28

3.1.1 Abstract ............................................................................................. 28

3.1.2 Introduction ......................................................................................... 28

3.1.3 Materials and methods ......................................................................... 29

3.1.4 Results and discussion ......................................................................... 36

3.1.5 Conclusion ............................................................................................ 37

3.2 Effects of digestate concentration and pH in culture solution on growth of microalgae ................................................................................................................... 38

3.2.1 Abstract ............................................................................................. 38

3.2.2 Introduction ......................................................................................... 38

3.2.3 Materials and methods ......................................................................... 40

3.2.4 Results and discussion ......................................................................... 41

3.2.5 Conclusion ............................................................................................ 46

3.3 Effect of digestate concentrations and light intensities in the culture solution on growth of microalgae ......................................................................................... 47

3.3.1 Abstract ............................................................................................. 47
3.3.2 Introduction .................................................................................................... 47
3.3.3 Materials and methods .................................................................................... 48
3.3.4 Results and discussion .................................................................................... 48
3.3.5 Conclusion ...................................................................................................... 57

CHAPTER 4 ................................................................................................................... 59

LIGHT ENVIRONMENT IN THE CULTURE SOLUTION WITH DIFFERENT DIGESTATE CONCENTRATIONS AND MICROALGAL DENSITIES. .................. 59

4.1 Abstract ................................................................................................................. 59
4.2 Introduction ........................................................................................................... 60
4.3 Materials and methods .......................................................................................... 60
4.3.1 Effects of digestate concentrations ................................................................. 62
4.3.2 Effects of microalgal densities ....................................................................... 62
4.3.3 Effects of microalgal densities and digestate concentrations ......................... 63
4.4 Results and discussion .......................................................................................... 63
4.4.1 Effects of digestate concentrations ................................................................. 63
4.4.2 Effects of microalgal density .......................................................................... 66
4.4.3 Effects of microalgal density and digestate concentration ............................. 70
4.5 Conclusion ............................................................................................................ 71
ABSTRACT OF THE THESIS

Microalgae have been used as sources of human food, animal feed, and pharmaceutical products, because of their marked ability to convert CO$_2$ to biomass and their unique capacity to transform photosynthates to other useful compounds. Microalgae have also recently been used as a fuel source.

Microalgal cell growth rates are affected by combinations of environmental parameters such as light intensity, temperature, pH, and nutrients in culture solutions.

Methane fermentation with organic residues and wastes is one of the most attractive renewable energy production technologies for reducing greenhouse gas emissions and for reducing the load of organic wastes. The resultant digestate contains nutrients for plants and can be utilized as valuable fertilizer, particularly due to its high nitrogen concentration.

The goal of this study was to establish a microalgal culture system combining digestate from an anaerobic methane fermentation system. The objectives of this fundamental study were to investigate the effects of environmental conditions on the growth of microalgae cultured with digestate from methane fermentation, and to assess the light
environment in culture solution with different concentrations of digestate and microalgal densities.

1) In order to clarify problems that needed to be solved for establishing a microalgal culture system with digestate, growth performance of mixtures of microalgal cells, including *Euglena gracilis*, *Chlorella vulgaris*, and *Dunaliella tertiolecta*, cultured in containers containing digestate at 5, 10, 15, 20, and 50% dilutions, was investigated. The volume of the solution was 1 L and the depth was 30 mm. Continuous illumination was provided at a photosynthetic photon flux density (PPFD) of 300 µmol m\(^{-2}\) s\(^{-1}\) at the solution surface at 30°C. Sample algal cells were collected daily from different depths, namely 0-5 mm (the surface layer), 10-15 mm (the middle layer), and 25-30 mm (the bottom layer), for counting cell number. The specific growth rate (\(\mu\)) of each species at each depth was calculated as the cellular multiplication rate from the increment in cell number with time. In all layer, the \(\mu\) of each species was highest in 5% digestate solution and the \(\mu\) of all the microalgal species in total was 0.035 h\(^{-1}\). Maximum microalgal cell density was 30 × 10\(^5\) (cells ml\(^{-1}\)) for *D. tertiolecta*.

2) In order to determine suitable conditions for the culture of microalgae, three
microalgal species were cultured in drops of solution containing diluted digestate at
different combinations of digestate concentration, temperature, pH, and PPFD. The
volume of each drop was 3 µl. Drops of solution were retained on the inner surface of a
translucent plastic vessel. The number of cells was monitored daily and specific growth
rates (µ) were calculated.

*D. tertiolecta* was cultured in solutions containing digestate at concentrations
ranging between 5 and 100% and at four temperatures, 20, 25, 30 or 35°C. The PPFD was 150
µmol m⁻² s⁻¹ with continuous illumination. The highest µ value, 0.05 h⁻¹, was obtained at
30°C in 50% digestate. This result for *D. tertiolecta* and previous reports for *E. gracilis*
and *C. vulgaris* demonstrate that these microalgal species can be cultured at a high growth
rate with diluted digestate at 30°C.

In order to determine the optimum combination of concentrations of digestate and
pH levels for culturing the microalgae, *E. gracilis, C. vulgaris* and *D. tertiolecta* were
cultured in drops of solution containing 5-100% digestate at pH 3.4, 6.8, or 8.7. The pH
of the digestate solution was adjusted with 10% HCl solution. The PPFD was 150 µmol
m⁻² s⁻¹ with continuous illumination at 30°C. In this study, only *E. gracilis* survived in
response to different digestate concentrations at pH 3.4. At the exact same conditions, the
other two microalgal did not grow. The highest µ of *E. gracilis* (0.05 h⁻¹) was obtained at
pH 3.4 in 20-25% digestate. This result demonstrates that *E. gracilis* can be produced at a high growth rate with diluted digestate adjusted to a low pH.

In order to ascertain the suitable combinations of digestate concentration and PPFD for microalgal culture, *E. gracilis*, *C. vulgaris* and *D. tertiolecta* were cultured with digestate at 5-100%, and at PPFDs between 75 and 150 µmol m$^{-2}$ s$^{-1}$ with continuous illumination at 30°C. The maximum µ values were 0.047 h$^{-1}$ in 10% digestate for *E. gracilis*, 0.065 h$^{-1}$ in 20% digestate for *C. vulgaris*, and 0.052 h$^{-1}$ in 50% digestate for *D. tertiolecta*. This result demonstrates that all three species could be cultured at high growth rates with diluted digestate under relatively low PPFD.

3) The light intensity decreases logarithmically in the solution according to Lambert-Beer’s law that is given by equation $P_2 = P_1 \exp(-\beta(Z_2-Z_1)).$ Absorption coefficient ($\beta$) = $(\ln (P_1)-\ln (P_2))/(Z_2-Z_1)$, where $P_1$ and $P_2$ are photosynthetic photon flux densities (PPFDs) at depth $Z_1$ and $Z_2$, respectively, in the culture solution. The light absorption coefficients of culture solutions with different digestate concentrations and different microalgal densities were measured by a spectrophotometer in the wavelength range of photosynthetic active radiation (400-700 nm). A linear regression was obtained between the absorption coefficient (cm$^{-1}$) and digestate concentration (%) expressed as $\beta_{\text{digestate}} = 0.0546 \times \text{"digestate concentration"} + 0.005$. A linear regression was also obtained
between the absorption coefficient and the microalgal density (cells ml$^{-1}$) expressed as $eta_{\text{microalgae}} = 0.0655 \times \text{"microalgal density"} + 0.0402$. The light intensity in the culture solution with different digestate concentrations and microalgae densities is given by: $P_2 = P_1 \exp(-(\beta_{\text{digestate}} + \beta_{\text{microalgae}})(Z_2-Z_1))$. In the preliminary experiment, a microalgal species, *Dunaliella tertiolecta*, showed the highest specific growth rate at a cell density of $30 \times 10^5$ cells ml$^{-1}$. In the result of the simulation conducted with this microalgal density, more than 10% of light was transmitted at the depths shallower than 15 mm, using 20% diluted digestate.

4) Conclusion

In this research, it was confirmed that digestate from methane fermentation was a useful nutrient for the culture of green microalgae (*E. gracilis, C. vulgaris, and D. tertiolecta*), which can survive in high concentrations of ammonia in a solution this diluted digestate. To establish a culture system that can accommodate this digestate solution, the depth of the solution must be designed to maintain high light penetration. A culture system consisting of a thin layer of solution under natural light is proposed. This system contains digestate diluted to 10-20% as the basal culture solution. The depth of the solution can be controlled to maintain optimal PPFD penetration, depending on solar radiation.
CHAPTER 1

GENERAL INTRODUCTION

1.1 Microalgae

Microalgae have been used as sources of human food, animal feed, and pharmaceutical products, because of their marked ability to convert CO$_2$ to biomass and their unique capacity to transform photosynthates to other useful compounds. Microalgae have also recently been used as a source of fuel (Chisti, 2007; Kumar et al., 2010; Moazami et al., 2011).

Microalgal cell growth rates are affected by a combination of environmental parameters such as light intensity, photoperiod, and temperature (Kitaya et al., 2005; Kitaya et al., 2008), nutrient composition (Parmar et al., 2011), and pH (Azov and Goldman, 1982; Golueke and Oswvald, 1959; Weissman et al., 1988) in the culture solution.

*Euglena gracilis, Chlorella vulgaris, and Dunaliella tertiolecta* are unicellular green microalgae which can be utilized for feeding mammals and fish (Nakano et al., 1995b; Nevejan et al., 2003; Wong and Cheung, 1985) and can be applied to biofuel oil production (Collet et al., 2011; Ramachandra et al., 2013; Tang et al., 2011).
1.2 Digestate from methane fermentation

Methane fermentation with organic residues and wastes is one of the most attractive renewable energy production technologies to reduce greenhouse gas emissions and to facilitate sustainable development of energy supplies (Mata-Alvarez et al., 2000) as well as for reducing the load of organic waste. This is termed anaerobic digestion, and involves a series of multi-step biological processes in which organic carbon is converted to CH$_4$ and CO$_2$ under anaerobic conditions (Karellas et al., 2010; Ter Veld, 2012). The resultant digestate contains nutrients for plants and can be utilized as valuable fertilizer, particularly due to its high nitrogen concentration (Gunnarsson et al., 2010; Weiland, 2010). The resultant digestate contains nutrients for plants and can be utilized as a valuable fertilizer, particularly because of its high nitrogen content (Gunnarsson et al., 2010; Weiland, 2010). In addition, the biogas from methane fermentation generally contains 40% CO$_2$ accompanied by about 60% CH$_4$ (Ter Veld, 2012). This CO$_2$ could also be utilized for the photosynthesis of microalgae (Figure 1.1).
However, inorganic nitrogen in digestate is mainly composed of ammonium (NH$_4^+$) (Cheng and Liu, 2002; Noike et al., 2004; Ulundag-Demirer et al., 2008). A high level of NH$_4^+$ retards the growth of microalgae because of the high pH and its uncoupling effect on electron transport in their photosystem (Crofts, 1966). However, some species of microalgae have a greater tolerance than higher plants to these toxic elements.
CHAPTER 2

EFFECTS OF DEPTHS AND CONCENTRATIONS OF DIGESTATE ON GROWTH OF THREE MICROALGAL SPECIES

2.1 Abstract

In order to design a culture system for microalgal biomass production with a low cost and convenient cell collection, growth performance of mixtures of microalgal cells, including *E. gracilis*, *C. vulgaris*, and *D. tertiolecta* cultured in a volume of 1 L were investigated at a PPFD of 300 µmol m$^{-2}$ s$^{-1}$ at the surface of the solution with continuous illumination at 30°C. Each culture container contained diluted digestate at concentrations of 5, 10, 15, 20, and 50%. Sample cells for counting cell number were collected daily at three depths: 0–50 mm (the surface layer), 10–15 mm (the middle layer), and 25–30 mm (the bottom layer). Pseudo-specific growth rates ($\mu_s$) of each species at each depth were calculated as cellular multiplication rates using number of cells per time. In each layer, the average $\mu_s$ of each species was highest in 5% digestate. The average $\mu_s$ of all three microalgal species (0.035 h$^{-1}$) was observed in all layers in 5% digestate solution. The $\mu_s$ of each species was highest in the bottom layer in 5% digestate (0.048 h$^{-1}$, 0.041 h$^{-1}$, and 0.022 h$^{-1}$, respectively for *C. vulgaris*, *E. gracilis*, and *D. tertiolecta*). In conclusion, *E.
gracilis, C. vulgaris, and D. tertiolecta showed the highest specific growth rate in 5% digestate in all layers.

2.2 Introduction

*Euglena* sp. are a group of freshwater motile microalgae. *Euglena* is able to grow in a highly acidic medium (pH 3) in which most other microorganisms can barely survive (Miyatake, 1989). This characteristic offers an exceptional advantage, as it allows for the culture of *Euglena* under unsterilized conditions at low pH levels without the risk of contamination by other microorganisms. In addition, *Euglena* is also tolerant to CO$_2$ concentrations up to 40%, regardless of the pH level (Nakano et al., 1995a).

The algal species *Chlorella* has been widely applied for wastewater treatment and has displayed a proven ability of removing nitrogen, phosphorus, and chemical oxygen demand (COD) with different retention times ranging from 10 h to 42 days, by mixing with bacteria or not, which shows the potential of replacing activated sludge in a secondary or tertiary step to optimize the reduction of nutrients and biomass production (Wang et al., 2010b). The optimum pH for *Chlorella* lies in a 7.0–7.5 range (Yeh et al., 2012). Therefore, *Chlorella* has been used to remove nutrient loads, as well as a heavy
metal detoxifier, for various types of wastewater treatments, including industrial, municipal, and agricultural wastewater (Córdoba et al., 2008).

*Dunaliella* sp. is a group of halotolerant, motile microalgae that survive a wide range of stress factors. *D. tertiolecta*, for example, can survive in a wide range of NaCl concentrations, from 0.05 M to 5.5 M, and of pH values, from 1 to 11, even under intense light and high temperature conditions (Chen et al., 2009; Zhu and Jiang, 2009).

Organic wastes are increasing and their disposal is a serious problem. Methane fermentation using organic waste is one of the most attractive renewable technologies (Mata-Alvarez et al., 2000). Liquid sludge (digestate) produces CH\(_4\) and CO\(_2\) in the fermentation process (Karellas et al., 2010; Ter Veld, 2012). In some reports, the cultivation of algae for biogas production has focused on cold climate conditions (Odlare et al., 2011). Research by (Collet et al., 2011) indicates that the coupled process of algal cultivation and succeeding biogas production is a better option than algal biodiesel production.

Zamalloa et al. (2011) proposed methane gas production from a combined system of an anaerobic digester and microalgae culture, in which the effluent of the digester was used for the culture of microalgae and in which microalgae were reused for anaerobic
digestion as a closed cycle.

In this study, the effects of the depth and concentration of digestate from methane fermentation on the growth of three mixed microalgae *C. vulgaris, E. gracilis*, and *D. tertiolecta* cultured in diluted digestate was investigated to identify suitable culture conditions.

### 2.3 Materials and methods

*C. vulgaris* and *D. tertiolecta* were obtained from the culture collection of the Faculty of Fisheries, Can Tho University, Vietnam, and *E. gracilis* (strain name: Z) was obtained from Osaka Prefecture University, Japan. *C. vulgaris* and *D. tertiolecta* were subcultured in Cramer–Myers (CM) medium (Cramer and Myers, 1952). *E. gracilis* was subcultured CM at a modified pH of 3.5 (Figure 2.1).
Euglena gracilis
Chlorella vulgaris
Figure 2. Image of *E. gracilis*, *C. vulgaris*, and *D. tertiolecta*. 

*Dunaliella tertiolecta*
C. vulgaris, E. gracilis and D. tertiolecta were cultured in open plastic boxes with a volume of 1000 mL (222 mm × 150 mm × 30 mm). Different concentrations of digestate were prepared at 5, 10, 15, 20, 25, and 50% with deionized water. The percent of the digestate concentration means the dilution rate. CM medium was used as the control solution. Samples were collected from different depths of the culture box: 0–50 mm (the surface layer), 10–15 mm (the middle layer), and 25–30 mm (the bottom layer). The digestate, which was collected from a methane fermentation facility in Yagi Biomass Town, Kyoto, Japan, mainly contained cattle manure. The original digestate was centrifuged at 2000 RPM for 10 min to remove large particles. No other sterilization was used in the preparation of the medium. The culture box with different digestate concentrations is shown in Figure 2.2.

Figure 2. 2 Culture box with different digestate concentrations.
The components of the original digestate used in this experiment and the CM solution used as the control medium are listed in Table 2.1. Concentrations of ions in the diluted digestate were inversely proportional to dilution rates.

Table 2.1 Components of the original digestate used in this experiment and the Cramer–Myer (CM) solution used as the control medium.

<table>
<thead>
<tr>
<th>Solution medium</th>
<th>pH</th>
<th>NH$_{4}^+$ (mg L$^{-1}$)</th>
<th>K$^+$ (mg L$^{-1}$)</th>
<th>Na$^+$ (mg L$^{-1}$)</th>
<th>SO$_4^{2-}$ (mg L$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CM</td>
<td>3.5</td>
<td>281</td>
<td>299</td>
<td>244</td>
<td>123</td>
</tr>
<tr>
<td>Digestate</td>
<td>8.4</td>
<td>973</td>
<td>1202</td>
<td>328</td>
<td>60</td>
</tr>
</tbody>
</table>

The initial cell densities of *C. vulgaris*, *E. gracilis* and *D. tertiolecta* were: 7–14 × 10$^{3}$ cells mL$^{-1}$, 4–8 × 10$^{3}$ cells mL$^{-1}$ and *C. vulgaris* were 4–14 × 10$^{3}$ cells mL$^{-1}$, respectively. Kitaya et al. (2005) applied a PPFD of 150 µmol m$^{-2}$ s$^{-1}$ and a droplet method for *E. gracilis*. The volume of each solution drop of the droplet method was 3 µl (Kitaya et al., 2005). The volume of this experiment (1 L) was larger than 3 µl. PPFD at the solution surface was 300 µmol m$^{-2}$ s$^{-1}$ (assessed with a quantum sensor; Li-190, LI-COR, USA) applied as continuous illumination. Fluorescent lamps (FPL55EX-N, Matsushita Electric Co., Osaka, Japan) were used as the light source. Temperature was maintained at 30°C (Figure 2.3).
Figure 2.3 Experimental systems for evaluating the effects of environmental variables on the growth rates of microalgal cells.
The NH$_4^+$, K$^+$, Na$^+$, Cl$^-$, and SO$_4^{2-}$ components of the original digestate were measured with an ion-chromatograph (pump, LC-10ADvp; cation column, Shim-pack IC-SC1; anion column, Shim-pack IC-A3; detector, ECD: Shimadzu Co., Kyoto, Japan). The pH was determined with a pH meter (D-52, Horiba Co., Japan).

The number of microalgal cells in 5, 10, 15, 20 and 25% digestate solutions and CM were counted in three samples by using a counting chamber under a microscope of 70× magnification (Figure 2.4).

![Counting chamber](image)

**Figure 2.4** Image of each digestate solution including cells in counting chambers. Cell number was counted daily with a microscope.
The specific growth rate (μ) was determined in the logarithmic multiplication stage.

The cell number is theoretically given by Eq. (1)

\[ N_t = N_0 \exp(\mu t) \quad (1) \]

where \( N_t \) is the cell number at time \( t \), \( N_0 \) is the initial number of cells, and \( \mu \). Then:

\[ \ln N_t = \ln N_0 + \mu t \quad (2) \]

\( \mu \), is then

\[ \mu = (\ln (N_2) - \ln (N_1))/(T_2 - T_1) \quad (3) \]

where \( N_1 \) and \( N_2 \) are the number of cells at times \( T_1 \) and \( T_2 \), respectively. In this experiment, \( T_1 \) and \( T_2 \) were 21 h and 72 h, respectively. The doubling time or the mean generation time (\( T_d \)) is given by the equation, \( t_d = \ln 2/\mu \). The concept of \( \mu \) is shown in Figure 2.5.
Figure 2.5 The concept of $\mu$ was determined in the logarithmic multiplication stage.

The $\mu$ values in each layer were variable because some microalgal cells moved in the box and the initial time ($T_1$) and the final time ($T_2$) of measurements were not counted for the same cell. Therefore the word “pseudo” was used in this experiment. The number of cells was monitored daily and the pseudo specific growth rates ($\mu_s$) in the surface, middle and bottom were calculated as cellular multiplication rates. Example calculation of $\mu_s$ is shown in Figure 2.6.
Statistical analyses were performed using Analysis of Variance (two way ANOVA) to examine the effects of digestate concentration and depth level on $\mu_s$.

2.4 Results and discussion

The $\mu_s$ of each species was highest in the bottom layer in 5% digestate (0.041 h$^{-1}$, 0.048 h$^{-1}$, and 0.022 h$^{-1}$, respectively for *E. gracilis, C. vulgaris, and D. tertiolecta*) in the bottom layer (Figure 2.7).
Figure 2.7  Effects of digestate concentration on specific growth rates $\mu_s$ of *E. gracilis*, *C. vulgaris* and *D. tertiolecta* cells at the surface, middle and bottom layers, a PPFD of
300 μmol m$^{-2}$ s$^{-1}$ at the solution surface and a temperature of 26°C. Each plot indicates mean ± standard error (n = 3–6).

In each layer, the average $\mu_s$ of each species was highest in 5% digestate. The average $\mu$ of all the microalgal species was 0.035 h$^{-1}$ (0.028 for $E$. gracilis, 0.046 for $C$. vulgaris, 0.019 for $D$. tertiolecta) at all layers in 5% digestate solution. The maximum $\mu$ values of these species were smaller in appropriate concentrations of digestate than in CM medium (Figure 2.8).
Figure 2. 8 Effects of digestate concentration on average specific growth rates ($\mu$) of *E. gracilis*, *C. vulgaris* and *D. tertiolecta* cells, at a PPFD of 300 μmol m$^{-2}$ s$^{-1}$ at the solution surface and a temperature of 26°C. Each plot indicates mean ± standard error (n = 3–6).

In the analysis of variance (ANOVA), solution including 5% digestate, depending on the depth, did not significantly affect the $\mu_s$ of the three microalgal species. However, the interactive effect of digestate concentrations and depth level on $\mu_s$ was significant for *E. gracilis* ($p < 0.05$), *C. vulgaris* ($p < 0.05$) and *D. tertiolecta* ($p < 0.05$).

This study confirmed that the highest values of $\mu_s$ for *E. gracilis*, *C. vulgaris*, and *D. tertiolecta* were 0.041, 0.048, and 0.022 h$^{-1}$, respectively (Figure 2.7), compared with that
of previous estimates of 0.045, 0.024 and 0.023 h⁻¹, respectively (Kim et al., 2012; Kitaya et al., 2005; Wang et al., 2010a). The C. vulgaris $\mu_s$ value was two-fold higher than the previous study because this study applied a mixed culture of E. gracilis, D. tertiolecta and C. vulgaris in digestate solutions using digestate from methane fermentation. An earlier study concluded and this can be a good environment for C. vulgaris growth when added with other species (Nguyen et al., 2013).

Moreover, pH also affects the growth of microalgae significantly. The growth of Chlorella and Chaetoceros sp. was reduced by 22% when pH was increased from 8 to 9 (Park et al., 2010; Rachlin and Grosso, 1991; Weissman et al., 1988). In our culture method, pH 8.2 had no negative effect on the growth of three microalgal species. In terms of pH, these species could be suitably cultured in digestate from methane fermentation (Table 2.1) In general, pH significantly depends on the concentration of ammonia or the ammonium ion. The growth of some algal species may not be significantly inhibited by free ammonia at low pH while considerable inhibition may occur at a pH higher than 9. However, Amphora sp. and Ankistrodesmus sp. are able to grow well at a pH ranging between 9 and 10 (Park et al., 2010; Weissman et al., 1988).
2.5 Conclusion

*C. vulgaris, E. gracilis and D. tertiolecta* showed the highest specific growth rate (μ) at 5% digestate when the PPFD at the solution surface was 300 μ mol m⁻² s⁻¹ and at 30°C. However, under different conditions, such as less or more than 5% digestate, μ decreased. The decrease in μ when digestate concentration is less than 5% may be due to fewer nutrients. The decrease in μ at a digestate concentration greater than 5% is due to lower light intensity. Therefore, in chapter 3, the effects of PPFD on the growth of microalgae cultured with digestate from methane fermentation at different digestate concentrations were investigated. In addition, in chapter 4, the effects of the light environment in culture solution with different digestate concentrations and microalgal densities were considered.
CHAPTER 3

EFFECTS OF ENVIRONMENTAL CONDITIONS ON GROWTH OF MICROALGAE CULTURED WITH DIGESTATE

3.1 Effects of digestate concentrations and temperature in culture solution on growth of microalgae

3.1.1 Abstract

In order to determine the optimum combination of digestate concentration and temperature for culturing microalgae, *D. tertiolecta* was cultured in an aqueous solution containing digestate at 5, 10, 13, 20, 25, 40, 50 and 100% and at 20, 25, 30 or 35°C. The temperature of the digestate was controlled with an IC thermostat in a water bath. PPFD was 150 µmol m$^{-2}$ s$^{-1}$ with continuous illumination. The number of cells was monitored daily and specific growth rate ($\mu$) was calculated. The highest $\mu$ value, 0.05 h$^{-1}$, was obtained at 30°C in 50% digestate. This result demonstrates that *D. tertiolecta* could be cultured at a high growth rate with diluted methane fermentation sludge at 30°C.

3.1.2 Introduction

The multiplication rate of *E. gracilis* cells was highest at 27–31°C (Kitaya et al., 2005). Optimal biomass growth and removal of pollutants by *Chlorella pyrenoidosa* was
achieved at temperatures between 35°C and 38°C in summer (Tan et al., 2014). The optimal growth temperature for \( D. \text{ salina} \) was 22°C at a density of \( 3.06 \times 10^6 \) cells mL\(^{-1}\) (García et al., 2007).

Optimal growth temperature for \( D. \text{ salina} \) was lower than that of \( E. \text{ gracilis} \) and \( C. \text{ pyrenoidosa} \). In this study, the effects of digestate concentration and temperature on the growth of \( D. \text{ tertiolecta} \) were investigated to determine the most suitable culture conditions.

### 3.1.3 Materials and methods

\( D. \text{ tertiolecta} \) was cultured with digestate diluted to 5, 10, 13, 20, 25, 40, 50 and 100%, at 20, 25, 30 or 35°C, and at a PPFD of 150 \( \mu \text{mol m}^{-2} \text{s}^{-1} \). This ideal PPFD was based on Kitaya et al. (2005) (Kitaya et al., 2005). The temperature of the digestate was controlled to 20, 25, 30 or 35°C by using an IC thermostat (Five plan EX-003, GEX Co., Osaka, Japan) in a water bath.

The digestate was collected from a methane fermentation facility in Yagi Biomass Town, Kyoto, Japan and mainly contained cattle manure. The original digestate was filtered through a 25-\( \mu \text{m} \) thick membrane filter (0.2 \( \mu \text{m} \) diameter pores, Advantec, Tokyo, Japan) to remove large particles and bacteria. No other sterilization was used in the
preparation of the medium. The components of the original digestate were measured as follows. The concentrations of ammonium (NH$_4^+$), potassium (K$^+$), sodium (Na$^+$), and sulfate (SO$_4^{2-}$) were determined using an ion-chromatograph (pump, LC-10ADvp; cation column, Shim-pack IC-SC1; anion column, Shim-pack IC-A3; detector, ECD: Shimadzu Co., Kyoto, Japan). The pH was determined with a pH meter (D-52, Horiba Co., Kyoto, Japan). The original digestate was filtered through a membrane filter (0.2-μm pore size, Advantec, Tokyo, Japan) to remove large particles and bacteria. No other sterilization was used in the preparation of the medium. Components of the original digestate used in this experiment are indicated in table 3.1.

Table 3.1 Components of the original digestate used in this experiment.

<table>
<thead>
<tr>
<th>Solution medium</th>
<th>pH</th>
<th>NH$_4^+$ (mg L$^{-1}$)</th>
<th>K$^+$ (mg L$^{-1}$)</th>
<th>Na$^+$ (mg L$^{-1}$)</th>
<th>SO$_4^{2-}$ (mg L$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Digestate</td>
<td>8.4</td>
<td>866</td>
<td>1120</td>
<td>447</td>
<td>332</td>
</tr>
</tbody>
</table>

Microalgal cells were cultured in drops of solution containing diluted digestate. The volume of each drop was 3 μL. Medium drops were retained on the inner surface of a translucent plastic vessel (Falcon polystyrene vented cell culture Flask with screw cap, 300 ml capacity). The number of cells in each drop was counted every day for 4 days (Figure 3.1).
Figure 3. 1 Image of each water droplet including cells. Cells were counted daily with a microscope.

The plastic vessel was submerged in water and maintained at 30°C (Figure 3.2). The water layer above the vessel cut out long-wave radiation from the light source and thus avoided the build-up of a heat load inside the vessel. Wet paper sheets inside the culture vessel maintained relative humidity at almost 100% and thus evaporation from the drops was inhibited. The incident light intensity expressed as PPFD was controlled with shading films on the vessel without altering the spectral property of light for the photosynthesis of microalgae. PPFD was measured with a quantum sensor (Li-190, LI-COR, USA). The vessel had sufficient air volume to maintain CO₂ and O₂ inside the vessel at 0.04% and 21%, respectively, throughout the experimental period. However, the air used was normal
atmospheric air because we intended to extend knowledge derived from this study to actual microalgal culture in an open pond system. The tiny droplet of solution could easily exchange CO₂ and O₂ with the air inside the culture vessel because of the high surface area to volume ratio of each drop. The number of drops of solution for each species and for each concentration of digestate was 28 drops within the same vessel.
Figure 3.2 The experimental culture vessel with droplets of the digestate solution, including microalgal cells.
The experimental setup is shown in Figure 3.3. Fluorescent lamps (FPL55EX-N, Panasonic Co., Osaka, Japan) were used as the light source. Temperature was maintained at 30°C.
Figure 3.3 The experimental setup for evaluating the effects of environmental variables on the growth rate of microalgal cells.
Statistical analyses were performed using two-way ANOVA to examine the effects of digestate concentration and PPFD level on $\mu$.

### 3.1.4 Results and discussion

The ANOVA results showed significant effects on $\mu$ ($p < 0.01$) for each digestate concentration and temperature but there was no interaction between digestate concentration and temperature ($p < 0.05$) (Figure 3.4).

The highest specific growth rate ($\mu$) of *D. tertiolecta* cells at 20, 25, 30 and 35°C was $0.031 \text{ h}^{-1}$ (doubling time of 22 h) with 40% digestate, $0.040 \text{ h}^{-1}$ (doubling time of 17 h) with 40% digestate, $0.052 \text{ h}^{-1}$ (doubling time of 13 h) with 50% digestate and $0.026 \text{ h}^{-1}$ (doubling time of 27 h) with 40% digestate (Figure 3.4).
Figure 3.4 Effects of digestate concentration on specific growth rates of *D. tertiolecta* cells at 20, 25, 30 and 35°C and a PPFD of 150 μmol m⁻² s⁻¹. Each plot indicates mean ± standard error (n = 10).

3.1.5 Conclusion

In conclusion, this study indicated that *D. tertiolecta* can be cultured at a high growth rate with diluted digestate from methane fermentation at 30°C. The highest specific growth rate of *D. tertiolecta* cells was 0.052 h⁻¹ and the doubling time was 13 h in 50% digestate.
3.2 Effects of digestate concentration and pH in culture solution on growth of microalgae

3.2.1 Abstract

In order to determine the most suitable combination of concentrations of digestate from methane fermentation sludge and pH levels for culturing microalgae, *E. gracilis*, *C. vulgaris*, and *D. tertiolecta* were cultured in an aqueous solution containing diluted digestate at 5, 10, 20, 25, 40 and 100% and at pH 3.4. In a second experiment, *E. gracilis* was cultured in an aqueous solution containing digestate at 5, 10, 20, 25, 40 and 100% and at pH 3.4, 6.8, and 8.7. The pH of the aqueous digestate solution was adjusted with 10% HCl. PPFD was 150 µmol m$^{-2}$ s$^{-1}$ with continuous illumination at 30°C. The number of cells was monitored daily and specific growth rate (µ) was calculated. Among the three microalgal species, only *E. gracilis* survived in different digestate concentrations at pH 3.4. The highest µ of *E. gracilis* (0.05 h$^{-1}$) was possible at pH 3.4 in 20–25% digestate. This result demonstrates that *E. gracilis* could be produced at a high growth rate with diluted methane fermentation sludge adjusted to a low pH.

3.2.2 Introduction

*Euglena* sp. are a group of freshwater motile microalgae. *Euglena* is able to grow in a highly acidic medium (pH 3) in which most other microorganisms can barely survive
(Miyatake, 1989). This characteristic offers an exceptional advantage, as it allows for the culture of *Euglena* under unsterilized conditions at low pH levels without the risk of contamination by other microorganisms.

The optimum pH for *Chlorella* was found to be in a range between 7.0–7.5 (Yeh et al., 2012). Therefore, it has been used to remove nutrient loads, as well as a heavy metal detoxifier, for various types of wastewater treatments, including industrial, municipal, and agricultural wastewater (Córdoba et al., 2008).

*Dunaliella* sp. is a group of halotolerant, motile microalgae that survive a wide range of stress factors. *D. tertiolecta*, for example, can survive in a wide range of NaCl concentrations, from 0.05 M to 5.5 M, and of pH values, from 1 to 11, even under intense light and high temperature conditions (Chen et al., 2009; Zhu and Jiang, 2009).

Studies to establish a microalgal culture system combined with digestate from an anaerobic methane fermentation system were initiated. Based on the result of a previous study, it was found that *E. gracilis* could be cultured at a high specific growth rate of 0.047 h$^{-1}$ with diluted digestate at a 10% concentration under a PPFD of 150 μmol m$^{-2}$ s$^{-1}$ (Nguyen et al., 2013). *Euglena* sp. is a freshwater unicellular green microalga, which can be a source of feed for mammals and fish (Nakano et al., 1995b). *Euglena* sp. can
tolyerate CO$_2$ concentrations up to 40% (Nakano et al., 1995a).

In this study, the effects of digestate concentration and pH level on the growth of $E.$ gracilis, $C.$ vulgaris and $D.$ tertiolecta were investigated to determine more suitable conditions for $E.$ gracilis production than in the previous study (Nguyen et al., 2013) by employing digestate from methane fermentation.

### 3.2.3 Materials and methods

$E.$ gracilis, $C.$ vulgaris, and $D.$ tertiolecta were cultured with digestate diluted to 5, 10, 20, 25, and 40% with deionized water, and 100%.

$E.$ gracilis, $C.$ vulgaris, and $D.$ tertiolecta were cultured in an aqueous solution containing diluted digestate at 5, 10, 20, 25, 40 and 100% and at pH 3.4. In a second experiment, $E.$ gracilis was cultured in an aqueous solution containing diluted digestate at 5, 10, 20, 25, 40 and 100% and at pH 3.4, 6.8, and 8.7.

The pH of the original digestate solution was adjusted to 3.4, 6.8 and 8.7 (original solution) with 10% HCl, and then diluted to 5, 10, 20, 25, 40 and 100% by adding deionized water for each pH value. The pH values of the digestate solutions were mostly constant, as digestate concentration decreased from 100% to 5%. The pH was determined
with a pH meter (D-52, Horiba Co., Kyoto, Japan).

Solutions were analyzed after filtration and adjustment of pH. The concentrations of ions in 100% digestate at pH 8.7, 6.8 and 3.4 and also of the modified CM solution are shown in Table 3.2.

Table 3.2 Components of the digestate solutions used in this experiment.

<table>
<thead>
<tr>
<th>Solution medium</th>
<th>pH</th>
<th>NH$_4^+$ (mg L$^{-1}$)</th>
<th>K$^+$ (mg L$^{-1}$)</th>
<th>Na$^+$ (mg L$^{-1}$)</th>
<th>SO$_4^{2-}$ (mg L$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Digestate 100%</td>
<td>8.7</td>
<td>229</td>
<td>2706</td>
<td>1189</td>
<td>142</td>
</tr>
<tr>
<td>Digestate 100%</td>
<td>6.8</td>
<td>268</td>
<td>3117</td>
<td>823</td>
<td>179</td>
</tr>
<tr>
<td>Digestate 100%</td>
<td>3.4</td>
<td>263</td>
<td>2939</td>
<td>754</td>
<td>158</td>
</tr>
<tr>
<td>CM</td>
<td>3.5</td>
<td>260</td>
<td>305</td>
<td>205</td>
<td>76</td>
</tr>
</tbody>
</table>

The relation between specific growth rate and ammonia concentration was changed easily by adjusting the level ammonia on the X-axis of Fig. 3.6 (in parenthesis).

Statistical analyses were performed using Analysis of Variance (two way ANOVA) to examine the effects of digestate concentration and pH level on $\mu$.

### 3.2.4 Results and discussion

In the first experiment, only *E. gracilis* survived in different digestate concentrations at pH 3.4 among the three microalgal species.
In the second experiment, *E. gracilis* was cultured in an aqueous solution containing different concentrations of digestate and pH levels. The ANOVA showed that each digestate concentration and pH level significantly affected $\mu$ ($p < 0.01$). There was no significant interaction ($p > 0.05$) between digestate concentration and pH level. However, $\mu$ values at all pH levels were mostly similar at digestate concentrations in the range of 10–20% (Figure 3.5) and the *E. gracilis* grew well regardless of pH in this range of digestate concentration. The maximum specific growth rate ($\mu_{\text{max}}$) of *E. gracilis* cells at pH 3.4, 6.8 and 8.7 was 0.053 h$^{-1}$ (doubling time of 13 h) with 25% digestate, 0.049 h$^{-1}$ (doubling time of 14 h) with 20% digestate, and 0.047 h$^{-1}$ (doubling time of 15 h) with 20% digestate (Figure 3.5). The $\mu_{\text{max}}$ at pH 3.4 with 25% digestate solution was 1.3-fold higher than the $\mu$ (0.041 h$^{-1}$) in CM solution at pH 3.5. Several studies reported the effect of pH on the growth of *E. gracilis* cultured in CM solution, indicating that it could grow at pH 2.5–3.5 (Nguyen et al., 2015) and at pH 3 (Miyatake, 1989). *E. gracilis* cultured under heterotrophic conditions with glucose and (NH$_4$)$_2$SO$_4$ as the carbon and nitrogen sources, respectively, could grow even at an extremely low pH of 2 (Garcia M et al., 2014). However there are no reports on the effect of pH on the growth of *E. gracilis* cultured in digestate.
Figure 3. 5 Effects of digestate concentration on specific growth rates of *E. gracilis* cells at pH 3.4, 6.8 and 8.7. Each plot indicates mean ± standard error (n = 14).

Z: LSD among different digestate concentrations (P = 0.05).

Y: LSD among different pH levels (P = 0.05).
We focused especially on NH$_4^+$ concentration among the components of the digestate, because NH$_4^+$ is an important ion for algal growth although excess NH$_4^+$ concentration generally has a negative effect on the growth of microalgae because of its uncoupling effect on electron transport in their photosystem (Crofts, 1966). Figure 3.6 shows the effect of NH$_4^+$ concentration of the digestate on the $\mu$ of *E. gracilis* cells at different pHs. The optimum NH$_4^+$ concentration was different at different pH conditions, ranging from 50–100 mg L$^{-1}$ at pH 3.4 and 6.8, and 50 mg L$^{-1}$ at pH 8.7.

![Figure 3.6 Effects of NH$_4^+$ concentrations of digestate on specific growth rates of *E. gracilis* cells at pH 3.4, 6.8, and 8.7. Each plot indicates mean ± standard error (n = 14).](image-url)
In a previous study (Nguyen et al., 2013), using an alkaline solution, the \( \mu \) was 0.026 h\(^{-1}\) in 20% digestate at pH 8.4, although the \( \mu_{\text{max}} \) of \textit{E. gracilis} was 0.047 h\(^{-1}\) in 10% digestate. In contrast, the \( \mu \) was 0.047 h\(^{-1}\) in 20% digestate at pH 8.7 in the present study. The \( \text{NH}_4^+ \) concentration in 10% and 20% digestate solutions in the previous study was 87 mg L\(^{-1}\) and 173 mg L\(^{-1}\), respectively, and 46 mg L\(^{-1}\) in 20% digestate solution in the present study. The difference in preferred digestate concentration between both studies is partly due to the \( \text{NH}_4^+ \) content in the digestate solution.

Ion concentrations in the 25% digestate solution at pH 3.4, which showed the best performance for \textit{E. gracilis} in the present study, were calculated from the values in Table 3.2. \( \text{NH}_4^+ \), \( \text{NO}_3^- \), \( \text{PO}_4^{3-} \), \( \text{Na}^+ \), \( \text{SO}_4^{2-} \), and \( \text{Mg}^{2+} \) concentrations in the solution were 66, 4, 11, 189, 39, and 11 mg L\(^{-1}\), respectively, and were 4, 5, 134, 1, 2, and 2 times lower than those in CM solution. In contrast, the concentration of \( \text{K}^+ \) and \( \text{Ca}^{2+} \) in the 25% concentration digestate was 735 and 18 mg L\(^{-1}\), respectively, and both were mostly two-fold higher than in the CM solution. The \( \mu \) was higher in the 25% digestate solution than in the CM solution in spite of the lower \( \text{NH}_4^+ \) concentration in the former and different concentrations of other ions from those in CM solution. The contribution of \( \text{NH}_4^+ \) to algal growth must be reconsidered in relation to nitrate and nitrite as nutritional nitrogen sources. Nitrate and nitrite, apart from the ammonium ion, are important nutrient ions and
often play key roles for algal growth in general (Bauer et al., 2009; Kitaya et al., 2008; Kwon et al., 2013). Nitrate was mostly insufficient in the 25% digestate solution compared with the CM solution and nitrite could not be detected in the present study. Phosphate, which is also a necessary nutrient ion for algal growth, was insufficient in the 25% digestate solution compared with the CM solution. Since phosphate is also generally a necessary nutrient ion for algae (Bauer et al., 2009; Kwon et al., 2013), algal growth will be promoted by adding phosphate in the digestate. The method for supplying other ions which were less than those in the CM solution must also be investigated to promote the growth of three microalgal.

3.2.5 Conclusion

In conclusion, this study indicated that only *E. gracilis* survived in different digestate concentrations at pH 3.4 among the three microalgal species tested.

*E. gracilis* can be cultured at a high growth rate with diluted methane fermentation-derived digestate that was adjusted to a relatively low pH. The highest specific growth rate of *E. gracilis* cells was 0.053 h\(^{-1}\) and the doubling time was 13 h with 25% digestate at pH 3.4.
3.3 Effect of digestate concentrations and light intensities in the culture solution on growth of microalgae

3.3.1 Abstract

In order to decide suitable combinations of digestate concentrations and PPFDs for culturing microalgae, three microalgal species, *E. gracilis*, *C. vulgaris*, and *D. tertiolecta* were cultured in an aqueous solution containing digestate at concentrations of 5–100% and at PPFDs of 75–150 μmol m$^{-2}$ s$^{-1}$ with continuous illumination at 30°C. The number of cells was monitored daily, and specific growth rates (μ) were calculated as cellular multiplication rates. The maximum μ values were 0.047 h$^{-1}$ in 10% digestate for *E. gracilis*, 0.065 h$^{-1}$ in 20% digestate for *C. vulgaris*, and 0.052 h$^{-1}$ in 50% digestate for *D. tertiolecta* at a PPFD of 150 μmol m$^{-2}$ s$^{-1}$. These results demonstrate that these species can be cultured at high growth rates with diluted methane fermentation digestate under a relatively low PPFD level.

3.3.2 Introduction

Inorganic nitrogen in digestate is mainly composed of ammonia (NH$_4^+$) (Cheng and Liu, 2002; Noike et al., 2004; Ulundag-Demirer et al., 2008), which has a toxic effect on
higher plants. A high level of the resultant NH$_4^+$ also retards the growth of microalgae because of the high pH and the uncoupling effect on electron transport in their photosystem (Crofts, 1966). However, some species of microalgae display a greater tolerance than higher plants to these toxic elements.

The goal of this study was to establish a microalgal culture system that combined digestate from an anaerobic methane fermentation system. The objectives in this study were to investigate the effects of digestate concentrations and light intensities in the culture solution on the growth of microalgae.

### 3.3.3 Materials and methods

In the experiment, different concentrations of digestate, diluted to 5%, 10%, 13%, 20%, 40%, or 50% with deionized water and 100% (original digestate) were used as the microalgae culture medium. The experiment was conducted at PPFDs of 75, 113, and 150 $\mu$mol m$^{-2}$ s$^{-1}$ with continuous illumination.

### 3.3.4 Results and discussion

In this study, the effects of digestate sludge from methane fermentation on the growth of *E. gracilis*, *C. vulgaris*, and *D. tertiolecta* were investigated to identify microalgae
suitable for culturing at higher concentrations of digestate under a relatively low light intensity.

*E. gracilis*, *C. vulgaris*, and *D. tertiolecta* could survive in all of the concentrations of digestate and showed logarithmic growth with time (Figure 3.7 a, b, c). *E. gracilis*, *C. vulgaris*, and *D. tertiolecta* grew fastest in 10%, 20%, and 50% concentration of digestate, respectively, among the dilutions tested.

![Graphs showing cell numbers over time for *E. gracilis* (a), *C. vulgaris* (b), and *D. tertiolecta* (c) in different concentrations of digestate.](image)

Figure 3.7 Time courses of cell numbers of *E. gracilis* (a), *C. vulgaris* (b), and *D. tertiolecta* (C) in different concentrations of digestate. The PPFD was 150 μmol m$^{-2}$ s$^{-1}$ and temperature was 30°C. Each plot indicates mean ± standard deviation (n=10).
Figure 3.8 shows the effect of digestate concentrations on $\mu$ of *E. gracilis*, *C. vulgaris*, and *D. tertiolecta* cells at PPFDs of 75, 113, and 150 $\mu$mol m$^{-2}$ s$^{-1}$. The highest specific growth rates ($\mu_{\text{max}}$) of *E. gracilis*, *C. vulgaris*, and *D. tertiolecta* were obtained in 10%, 20%, and 50% dilutions of digestate, respectively. The $\mu_{\text{max}}$ values of microalgae cultured at appropriate digestate concentrations at PPFDs of 75, 113, and 150 $\mu$mol m$^{-2}$ s$^{-1}$ were as follows: 0.039 h$^{-1}$ (doubling time of 18 h), 0.041 h$^{-1}$ (doubling time of 17 h), and 0.047 h$^{-1}$ (doubling time of 15 h), respectively, for *E. gracilis*; 0.038 h$^{-1}$ (doubling time of 18 h), 0.054 h$^{-1}$ (doubling time of 13 h), and 0.065 h$^{-1}$ (doubling time of 11 h), respectively, for *C. vulgaris*; and 0.044 h$^{-1}$ (doubling time of 16 h), 0.046 h$^{-1}$ (doubling time of 15 h), and 0.052 h$^{-1}$ (doubling time of 13 h), respectively, for *D. tertiolecta*. 
Figure 3. Effects of digestate concentration on specific growth rates of *E. gracilis*, *C. vulgaris*, and *D. tertiolecta* cells at PPFDs of 75, 113, and 150 μmol m$^{-2}$ s$^{-1}$ at 30°C. Each
plot indicates mean ± standard error (n = 10).

PPFD had an effect on specific growth rates of *E. gracilis*, *C. vulgaris*, and *D. tertiolecta* cells cultured in digestate solution at 10%, 20%, and 50% digestate (Figure 3.9).
Figure 3.9 Effects of PPFD on specific growth rates of *E. gracilis*, *C. vulgaris*, and *D. tertiolecta* cells at 10%, 20%, and 50% concentrations of digestate, respectively. The maximum value for three species at 30°C is shown. Each plot indicates mean ± standard error (n=10).
The specific growth rates of *E. gracilis* at 75, 113, and 150 µmol m\(^{-2}\) s\(^{-1}\) were 1.5, 1.4, and 1.5 times, respectively (Figure 3.9).

The specific growth rates of *C. vulgaris* doubled as PPFD increased from 75 to 150 µmol m\(^{-2}\) s\(^{-1}\). The specific growth rates of *Chlorella* at 75, 113, and 150 µmol m\(^{-2}\) s\(^{-1}\) were 3.8, 5.4, and 5.9 times, respectively (Figure 3.9).

The specific growth rates of *D. tertiolecta* increased as PPFD increased from 75 to 150 µmol m\(^{-2}\) s\(^{-1}\). The specific growth rates of *Dunaliella* at 75, 113, and 150 µmol m\(^{-2}\) s\(^{-1}\) were 1.7, 1.8, and 2.0 times, respectively (Figure 3.9).

Digestate concentrations and PPFDs significantly (p < 0.01) affected the \(\mu_{\text{max}}\) of the three microalgal species and interactive effects of digestate concentrations and PPFDs on \(\mu_{\text{max}}\) were significant for *E. gracilis* (p < 0.05) and *C. vulgaris* (p < 0.01) following analysis of variance (ANOVA). The \(\mu_{\text{max}}\) of *E. gracilis* was significantly lower than that of *D. tertiolecta* and *C. vulgaris* in digestate solutions at concentrations more than 50%, but significantly higher than that of *D. tertiolecta* and *C. vulgaris* at concentrations lower than 10%, at 75–150 µmol m\(^{-2}\) s\(^{-1}\). *D. tertiolecta* and *C. vulgaris* showed similar \(\mu\) values at 50–100% digestate and at 113 and 150 µmol m\(^{-2}\) s\(^{-1}\). The \(\mu\) value of *D. tertiolecta* was higher than that of *C. vulgaris* at 50–100% digestate, at 75 µmol m\(^{-2}\) s\(^{-1}\). In 50–100%
digestate solutions, *D. tertiolecta* and *C. vulgaris* grew faster than *E. gracilis* at 75–150 μmol m$^{-2}$ s$^{-1}$, and *D. tertiolecta* grew faster than *C. vulgaris* at 75 μmol m$^{-2}$ s$^{-1}$. On the other hand, *C. vulgaris* grew faster than *D. tertiolecta* in 20% digestate solution at 113–150 μmol m$^{-2}$ s$^{-1}$. In addition, *E. gracilis* grew faster than *D. tertiolecta* and *C. vulgaris* in digestate solutions at concentrations lower than 10% (Figure 3.8).

Wang et al. (2010) reported an inverse linear relationship between the average μ of the first 7 days and the initial turbidities for *Chlorella* sp. (Wang et al., 2010a). As it uses a still solution, hanging-drop culture also has the benefit of avoiding mechanical stress due to turbidity, which could occur when using conventional methods with aeration and/or stirring.

Light intensity plays an important role in the growth of microalgae and the distribution of light intensity varies strongly with the water depth and cell density (Wahidin et al., 2013). A further increase in light intensity from an appropriate light level actually reduces the biomass growth rate. This phenomenon is known as photoinhibition (Chisti, 2007). Cell growth of *E. gracilis* followed the Monod model with a half-saturation constant at a PPFD of 180 μmol m$^{-2}$ s$^{-1}$ under photoautotrophic conditions (Ogbonna and Tanaka, 2000). In the present study, the level of PPFD was not reduced in the droplet, because the
height of the droplet was about 1 mm. All of the microalgae, therefore, received the same intensity of light.

pH also affects microalgal growth significantly. The growth of \textit{Chlorella} and \textit{Chaetoceros} sp. was reduced by 22\% when pH was increased from 8 to 9 (Park et al., 2010; Weissman et al., 1988). In our drop culture method, pH in the range of 8.5 to 8.6 showed no negative effect during the experiment, but in the future, larger scale experiments in which the pH is varied more should be undertaken. In general, pH is strongly dependent on the concentration of ammonia or the ammonium ion. Growth of some algal species might not be significantly inhibited by free ammonia at a low pH, while considerable inhibition may occur at a pH above 9. Some algal species, such as \textit{Amphora} sp. and \textit{Ankistrodesmus} sp. were, however, able to grow well at a pH range of between 9 and 10 (Park et al., 2010; Weissman et al., 1988). In terms of pH, these species would be suitable for culture in digestate from methane fermentation.

Zamalloa et al. (2011) proposed methane gas production from a combined system comprising an anaerobic digester and microalgal culture, in which the effluent of the digester is reused for microalgal culture and microalgae are reused for anaerobic digestion as a closed cycle (Zamalloa et al., 2011). We also intend to establish a similar semi-closed
system as one of the applications of this study, especially to allow the recycling of nutritional inorganic materials. Carbon sources will be supplied from the atmosphere and biogas from methane fermentation.

For waste digestate treatment, a dense digestate solution is desirable in order to reduce the system volume and treatment cost. It is recommended that denser digestates should be treated with microalgae more suitable for highly concentrated digestate in upstream phases, and then more dilute digestate can be treated using other microalgae more suitable for less concentrated digestate solutions in downstream phases, in a cascade system for waste digestate treatment.

*Chlorella* sp. showed the highest growth rate at a dilution multiple of 20, i.e., a 5% digestate concentration (Wang et al., 2010a). Our results showed maximum growth rate at a dilution multiple of 5, i.e., 20% digestate concentration. This indicates that *C. vulgaris* has the potential to grow faster at a higher digestate concentration.

**3.3.5 Conclusion**

In conclusion, the drop culture method provides a low-cost option, can deliver
potentially high growth rates and is easily changed to accommodate variations in many environmental elements. Reused digestate materials from methane fermentation can be useful nutrients for culturing green microalgae that can survive in high concentrations of ammonia, such as *E. gracilis, C. vulgaris*, and *D. tertiolecta*. *C. vulgaris* and *D. tertiolecta* are suitable microalgae for culture in a high concentration of digestate solution. In a cascade digestate treatment system, the upstream-to-downstream order of employment of these microalgae would be *D. tertiolecta, C. vulgaris*, and *E. gracilis*.
4.1 Abstract

The light intensity decreases logarithmically in the solution according to Lambert-Beer’s law that is given by equation $P_2 = P_1 \exp(-\beta(Z_2-Z_1))$. Absorption coefficient ($\beta$) = \frac{(\ln (P_1)-\ln (P_2))/ (Z_2-Z_1)}{Z_2-Z_1}$, where $P_1$ and $P_2$ are photosynthetic photon flux densities (PPFDs) at depth $Z_1$ and $Z_2$, respectively, in the culture solution. The light absorption coefficients of culture solutions with different digestate concentrations and different microalgal densities were measured by a spectrophotometer in the wavelength range of photosynthetic active radiation (400-700 nm). A linear regression was obtained between the absorption coefficient (cm$^{-1}$) and digestate concentration (%) expressed as $\beta_{\text{digestate}} = 0.0546 \times "\text{digestate concentration}" + 0.005$. A linear regression was also obtained between the absorption coefficient and the microalgal density (cells ml$^{-1}$) expressed as $\beta_{\text{microalgae}} = 0.0655 \times "\text{microalgal density}" + 0.0402$. The light intensity in the culture solution with different digestate concentrations and microalgae densities is given by: $P_2 = \ldots$
\[ P_1 \exp(-(\beta_{\text{digestate}} + \beta_{\text{microalgae}})(Z_2-Z_1)) \] In the preliminary experiment, a microalgal species, \textit{D. tertiolecta}, showed the highest specific growth rate at a cell density of \(30 \times 10^5\) cells ml\(^{-1}\). In the result of the simulation conducted with this microalgal density, more than 10\% of light was transmitted at the depths shallower than 15 mm, using 20\% diluted digestate.

### 4.2 Introduction

Light intensity and wavelength are essential parameters for microalgal growth. However, varying illumination intensities in outdoor conditions are likely to inhibit the growth of microalgae because of the shortage of light energy, for example very low light intensity during rainy days or photoinhibition caused by excessive irradiation, or very high light intensities at noon during summer (Tan et al., 2014).

In this study, the effects of digestate concentration (\%), microalgal density (cells ml\(^{-1}\)) and the light environment in the culture solution were investigated.

### 4.3 Materials and methods

The light intensity decreases logarithmically in the solution according to Lambert-Beer’s law which is given by Eq (1). Absorption coefficient (\(\beta\)) is given by Eq (2).
\[ P_2 = P_1 \exp(-\beta(Z_2-Z_1)) \] (1)

Absorption coefficient \((\beta) = (\ln (P_1)-\ln (P_2))/(Z_2-Z_1)\) (2)

where \(P_1\) and \(P_2\) are the distribution of PPFDs at depth \(Z_1\) and \(Z_2\), respectively in the culture solution. The concept of the light environment in the culture solution is explained in Figure 4.1.

Figure 4. 1 The concept of light environment in the culture solution.
4.3.1 Effects of digestate concentrations

Digestate was diluted to 5%, 15%, and 25% with deionized water. The original digestate was centrifuged at 2000 rpm for 10 min to remove large particles. The light spectral properties of 5%, 15%, and 25% digestate were determined by a spectrophotometer (UV1240, Shimadzu Co., Kyoto, Japan).

Light environment in the culture solution with different digestate concentrations (%) was simulated with the following equation:

\[ P_2 = P_1 \exp(-\beta_{\text{digestate}}(Z_2-Z_1)) \]

where \( \beta_{\text{digestate}} \) is the absorption coefficient of the digestate.

4.3.2 Effects of microalgal densities

\textit{E. gracilis} was used as the green microalga in this experiment. Microalgae were cultured at \( 162 \times 10^5, 325 \times 10^5, \text{ and } 488 \times 10^5 \) cells ml\(^{-1}\) with CM solution at a ratio of 1:2:3 with CM solution. Absorbance of the CM solution was \( \approx 0 \). The light spectral properties of microalgae at different densities (cells ml\(^{-1}\)) were determined by a spectrophotometer.

Light environment in the culture solution with different microalgal densities (cells ml\(^{-1}\)) was simulated with the following equation:
\[ P_2 = P_1 \exp(- (\beta_{\text{microalgae}})(Z_2-Z_1)) \]

where \( \beta_{\text{microalgae}} \) is the absorption coefficient of microalgae.

4.3.3 Effects of microalgal densities and digestate concentrations

Light environment in the culture solution with different digestate concentrations (%) and microalgae densities (cells ml\(^{-1}\)) was simulated with the following equation:

\[ P_2 = P_1 \exp(- (\beta_{\text{digestate}} + \beta_{\text{microalgae}})(Z_2-Z_1)) \]

where \( \beta_{\text{digestate}} \) is the absorption coefficient of digestate; \( \beta_{\text{microalgae}} \) is the absorption coefficient of microalgae.

4.4 Results and discussion

4.4.1 Effects of digestate concentrations

The absorbance of 5%, 10%, and 15% digestates were higher at the longer wavelength region of the range of PPFDs (Figure 4.2). All digestates absorbed across the entire visible spectrum, with a stronger absorption intensity over the lower half in the lower wavelength region (Crofts, 1966).
Figure 4.2 The absorbance of 5%, 15%, and 25% digestate.

A linear regression was obtained between the absorption coefficient and the digestate concentration, and expressed as:

Absorption coefficient (cm$^{-1}$) = 0.0546 × digestate concentration (%) + 0.005 (as shown in Figure 4.3).
As shown in Figure 4.4, light intensity was affected by depth and digestate concentration of the solution. The greater depth and higher digestate concentration decreased PPFD. At 20% digestate, the depth of the solution must be less than 5, 10, 15, 20, 25, 30, 35, 40, 45, or 50 mm to sustain the preferred PPFD penetration at more than 58, 34, 19, 11, 7, 4, 2, 1, 1, and 0%, respectively, in the digestate solution. A logarithmic relationship between intensity and distance would suggest a more rapid decline in intensity with increasing distance initially, resulting in an increased potential for a relatively shallower depth of cure at shorter distances. (Aravamudhan et al., 2006; Felix and Price, 2003; Meyer et al., 2002; Pires et al., 1993; Prati et al., 1999)
Figure 4.4 Light intensity is affected by depth and digestate concentration.

4.4.2 Effects of microalgal density

The absorbance of microalgal solutions at $162 \times 10^5$, $325 \times 10^5$, and $488 \times 10^5$ cells ml$^{-1}$ are shown in Figure 4.5.
A linear regression was obtained between the absorption coefficient and microalgal density, expressed as: $\text{Absorption coefficient (cm}^{-1}) = 0.0655 \times \text{microalgal (cells ml}^{-1}) \text{ concentration (%) + 0.0402 (Figure 4.6).}$
Light intensity as affected by solution depth and microalgal density (cells ml\(^{-1}\)) is shown in Figure 4.7. More than 81, 65, 52, 42, 34, 27, 22, 18, 14, and 12% of light transmission was obtained at depths shallower than 5, 10, 15, 20, 25, 30, 35, 40, 45, and 50 mm, respectively, in CM solution with a microalgal density of \(30 \times 10^5\) cells ml\(^{-1}\). To estimate and predict algal growth under various levels of light intensity, it is also essential to formulate the relationship between growth and light intensity. In several harmful algae, such as *Chattonella marina/C. ovata* (Yamaguchi et al., 2010; Yamaguchi et al., 1991), *Karenia mikimotoi* (Yamaguchi and Honjo, 1989), and *Gambierdiscus* species (Kibler et al., 2012), such relationships were established using the following model equations: Michaelis–Menten (MM) (Yamaguchi and Honjo, 1989), modified MM (mMM) (Yamaguchi et al., 2010; Yamaguchi et al., 1991) and Gaussian/Lorentzian model (probability density function of the normal distribution) (Kibler et al., 2012).
them, the MM and mMM model equations are incapable of displaying algal growth inhibition at intense levels of light intensity, due to the appearance of a saturated growth rate at infinite light intensity. In contrast, the Gaussian/Lorentzian model equation is capable of displaying algal growth inhibition as well as promotion as light intensity increases when the growth-light intensity curve resembles a normal distribution; however, such curve-forms are infrequently observed in dinoflagellates (Kibler et al., 2012; Morton et al., 1992). Growth-light intensity relationships can be estimated quantitatively by formulae. Importantly, none of these model equations are capable of displaying algal growth inhibition and promotion with varying light intensity or determining the threshold of light intensity required for algal growth.
4.4.3 Effects of microalgal density and digestate concentration

In chapter 2, *D. tertiolecta* showed the highest specific growth rate in 5% digestate at a cell density of $30 \times 10^5$ cells ml$^{-1}$. This microalgal density was selected for this experiment (Figure 4.8).

The light environment in the culture solution with difference digestate concentrations at a microalgal density of $30 \times 10^5$ cells ml$^{-1}$ is shown in Figure 4.8. More than 20% of light transmission was obtained at a depth shallower than 5, 10, 15, and 20 mm at a microalgal density of $30 \times 10^5$ cells ml$^{-1}$ at 47, 22, 10, and 5% digestate concentration, respectively.
Figure 4. Light intensity is affected by depth and digestate concentration (%) at a microalgal density of $30 \times 10^5 \text{cells ml}^{-1}$.

4.5 Conclusion

More than 10% of light transmission was possible at depths shallower than 15 mm at a microalgal density of $30 \times 10^5 \text{cells ml}^{-1}$ at 20% digestate concentration.
CHAPTER 5

GENERAL DISCUSSION

Results from chapter 2 indicate that at more than 5% digestate, the $\mu$ decreases significantly, which implies that more than 5% of digestate is not appropriate for all three microalgae growth. A low light intensity in the solution is necessary. PPFD distribution is affected by depth and 10% digestate showed very low PPFD compared with 5% digestate at a microalgal density of $30 \times 10^5$ (cells ml$^{-1}$) for all three microalgae growth (Figure 5.1).
Figure 5.1 Effects of digestate concentration on average specific growth rates (μ) of *E. gracilis*, *C. vulgaris* and *D. tertiolecta* cells, at a PPFD of 300 μmol m$^{-2}$ s$^{-1}$ at the solution surface and a temperature of 26°C. Each plot indicates mean ± standard error (n = 3–6).

10% digestate showed very low PPFD compared with 5% digestate at a microalgal density of 30 × 10$^5$ (cells ml$^{-1}$) (Figure 5.2).

![Figure 5.2 Light intensity is affected by depth and digestate concentration (%)](image)

Figure 5.2 Light intensity is affected by depth and digestate concentration (%), a PPFD of 300 μmol m$^{-2}$ s$^{-1}$ at the solution surface, and a microalgal density of 30 × 10$^5$ cells ml$^{-1}$. 

1.
In chapter 3, the highest $\mu$ of *E. gracilis* was observed to be 10% digestate, for *C. vulgaris* 20% digestate, and for *D. tertiolecta* 50% digestate.

Culture of *E. gracilis* with 10% digestate at a microalgal density of $30 \times 10^5$ cells ml$^{-1}$, the depth of the solution must be less than 23 mm to maintain the preferred PPFD penetration more than 10% in the solution (Figure 5.3).

Culture of *C. vulgaris* with 20% digestate at a microalgal density of $30 \times 10^5$ cells ml$^{-1}$, the depth of the solution must be less than 15 mm to maintain the preferred PPFD penetration more than 10% in the solution (Figure 5.3).

Culture of *D. tertiolecta* with 20% digestate at a microalgal density of $30 \times 10^5$ cells ml$^{-1}$, the depth of the solution must be less than 7 mm to maintain the preferred PPFD penetration more than 10% in the solution (Figure 5.3).
Figure 5.3 Light intensity is affected by depth and digestate concentration (%) at a microalgal density of $30 \times 10^3$ cells ml$^{-1}$. 
CHAPTER 6

CONCLUDING REMARKS

E. gracilis, C. vulgaris and D. tertiolecta showed the highest specific growth rate (\(\mu\)) at 5% digestate, at a PPFD of 300 \(\mu\)mol m\(^{-2}\) s\(^{-1}\) at the solution surface and at 30°C.

D. tertiolecta can be cultured at a high growth rate with diluted methane fermentation at 30°C. The highest specific growth rate of D. tertiolecta cells was 0.052 h\(^{-1}\) and the doubling time was 13 h in 50% digestate at 30°C.

Only E. gracilis survived in different digestate concentrations of pH 3.4 among the three microalgal species. E. gracilis can be cultured at a high growth rate with diluted methane fermentation-derived digestate that was adjusted to a relatively low pH. The highest specific growth rate of E. gracilis cells was 0.053 h\(^{-1}\) and the doubling time was 13 h with 25% digestate at pH 3.4.

Reused digestate materials from methane fermentation can be useful nutrients for culturing green microalgae that will survive in high concentrations of ammonia, such as E. gracilis, C. vulgaris, and D. tertiolecta, which are also suitable microalgae for culture in a high concentration of digestate solution. The order of employment of these
microalgae would be *D. tertiolecta*, *C. vulgaris*, and *E. gracilis* in an upstream-to-downstream cascade digestate treatment system.

This research is fundamental for the culture of microalgae with digestate from methane fermentation. In future research, I will investigate a larger scale microalgal culture system combined with digestate from a methane fermentation system.

I am also intending to establish a system for one of the applications of this study, namely the recycling of nutritional inorganic materials. Carbon sources will be supplied from the atmosphere and biogas from methane fermentation (Figure 6.1).

<table>
<thead>
<tr>
<th>Biogas</th>
<th>CH$_4$</th>
<th>50~70 %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CO$_2$</td>
<td>50~30 %</td>
</tr>
</tbody>
</table>

![Figure 6.1 A system as one potential application of this study.](image)
When digestate is added to the tanks in which microalgae are cultured, it supplies nutrients for the growth of microalgae under sunlight. When microalgae grow, sediment forms at the bottom of the tank. Microalgae are collected from the bottom of the tank and directed to a membrane filter by a pump allowing microalgae to be collected from the filter. The levels of nutrition before and after microalgal growth, contains low nutrition, and can be discharged from the tank (Figure 6.2).

Figure 6.2 Large-scale culture and collection of microalgae with digestate.
This culture system allows light penetration in the culture solution to be controlled: high solar light intensity by a smaller angle, and low solar light intensity by a larger angle (Figure 6.3). This culture system was connected to a rotor and solar panel. When solar intensity is high, the solar panel receives light energy and replaces the electric current. Electricity is needed to run the rotor and to decrease the wing of the culture system, making the angle smaller and increasing the depth of the culture solution. When solar intensity and electricity are low, the culture system will open again and decrease the depth of the culture solution.

Figure 6.3 A culture system that can control light penetration in the culture solution.
ACKNOWLEDGEMENTS

I would specially like to express my deep and sincere to my supervisor, Professor Yoshiaki Kitaya, for his generous and kind guidance throughout my study, for sharing his broad knowledge on environmental and culture of microalgae with digestate from methane fermentation, for supporting and encouraging me to become an independent researcher. He is not only my teacher in science but also in life. It is great honor to be his student. All your supports make my work success and my life better.

I would like to express my warm and sincere to Professor Yasuaki Maeda, who introduced me to the field of renewable energy production technologies. He always encourages and supports; gives me innovative ideas during my research. His extensive discussions and interesting explorations have been helpful for me in this study.

I would like to thank to Prof. Haruhiko Horino and Prof. Hiroyuki Yamada for their detail review, wonderful advice and valuable comments to improve this thesis.

I am greatly appreciate to Associate Professor Toshio Shibuya, Dr. Ryosuke Endo and Dr. Liya Xiao at Osaka Prefecture University, for their fruitful, excellent advice and revising the manuscripts of this study.

I am also thankful to Dr. Masami Nakazawa, Osaka Prefecture University, Dr
Vu Ngoc Ut Can Tho University, Viet Nam for supplying Microalgae and Dr. Shinji Sakurai, Osaka Prefecture University for supporting the measurement of culture solution components. Many thanks to all students of Laboratory of Environmental control in Biology for their friendly and kindly helping me in my research; especially,

My sincere thanks to Dr Ken Phillips, Mr Pham Quoc Thai, Mr. Truong Dang Quang, and the members of Faculty of Science, Technology and Environment, An Giang University, Viet Nam to support and encourage me during my study in Japan.

I would like express my heartiest gratitude to my family for their love, care and supporting throughout my life. Especially, I am indebted to my father for his teaching and love.

I would like to express huge thanks to my wife, Tuyet Trang, my daughter Thien Kim and Thien An for their love, understanding and supporting for my mind during my study and daily life.

Finally, Thanks to the Vietnam Government support scholarship for the PhD course. Thanks to Osaka Prefecture University for providing me the necessary facilities to complete my study.

Nguyen Tran Thien Khanh
REFERENCES


Golueke, C.G. and Oswvald, W.J., 1959: Biological conversion of light energy to the


Pires, J., Cvitko, E., Denehy, G. and Swift, E., 1993: Effects of curing tip distance on light


Zamalloa, C., Vulsteke, E., Albrecht, J. and Verstraete, W., 2011: The techno-economic
potential of renewable energy through the anaerobic digestion of microalgae.


Zhu, Y.H. and Jiang, J.G., 2009: Combined toxic effects of typical mutagens - dimethylphenol, tribromomethane and dinitroaniline, on unicellular green algae

# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1. 1 Methane fermentation technology</td>
<td>8</td>
</tr>
<tr>
<td>Figure 2. 1 Image of <em>E. gracilis</em>, <em>C. vulgaris</em>, and <em>D. tertiolecta</em></td>
<td>15</td>
</tr>
<tr>
<td>Figure 2. 2 Culture box with different digestate concentrations.</td>
<td>16</td>
</tr>
<tr>
<td>Figure 2. 3 Experimental systems for evaluating the effects of environmental variables on the growth rates of microalgal cells.</td>
<td>18</td>
</tr>
<tr>
<td>Figure 2. 4 Image of each digestate solution including cells in counting chambers. Cell number was counted daily with a microscope.</td>
<td>19</td>
</tr>
<tr>
<td>Figure 2. 5 The concept of $\mu$ was determined in the logarithmic multiplication stage.</td>
<td>21</td>
</tr>
<tr>
<td>Figure 2. 6 Example $\mu_s = 0.0501 \text{ h}^{-1}$ was determined in the logarithmic multiplication stage.</td>
<td>22</td>
</tr>
<tr>
<td>Figure 2. 7 Effects of digestate concentration on specific growth rates $\mu_s$ of <em>E. gracilis</em>, <em>C. vulgaris</em> and <em>D. tertiolecta</em> cells at the surface, middle and bottom layers, a PPFD of 300 $\mu$mol m$^{-2}$ s$^{-1}$ at the solution surface and a temperature of 26°C. Each plot indicates mean ± standard error ($n = 3–6$).</td>
<td>23</td>
</tr>
</tbody>
</table>
Figure 2. Effects of digestate concentration on average specific growth rates (μ) of *E. gracilis*, *C. vulgaris* and *D. tertiolecta* cells, at a PPFD of 300 μmol m\(^{-2}\) s\(^{-1}\) at the solution surface and a temperature of 26°C. Each plot indicates mean ± standard error (n = 3–6). ................................................................. 25

Figure 3. 1 Image of each water droplet including cells. Cells were counted daily with a microscope. ................................................................. 31

Figure 3. 2 The experimental culture vessel with droplets of the digestate solution, including microalgal cells......................................................... 33

Figure 3. 3 The experimental setup for evaluating the effects of environmental variables on the growth rate of microalgal cells................................. 35

Figure 3. 4 Effects of digestate concentration on specific growth rates of *D. tertiolecta* cells at 20, 25, 30 and 35°C and a PPFD of 150 μmol m\(^{-2}\) s\(^{-1}\). Each plot indicates mean ± standard error (n = 10). .................................................. 37

Figure 3. 5 Effects of digestate concentration on specific growth rates of *E. gracilis* cells at pH 3.4, 6.8 and 8.7. Each plot indicates mean ± standard error (n = 14). ........................................................................................................ 43

Figure 3. 6 Effects of NH\(_4^+\) concentrations of digestate on specific growth rates of *E.*
gracilis cells at pH 3.4, 6.8, and 8.7. Each plot indicates mean ± standard error (n = 14). ............................................................................................................ 44

Figure 3. 7 Time courses of cell numbers of E. gracilis (a), C. vulgaris (b), and D. tertiolecta (C) in different concentrations of digestate. The PPFD was 150 μmol m$^{-2}$ s$^{-1}$ and temperature was 30°C. Each plot indicates mean ± standard deviation (n=10). .............................................................................................................. 49

Figure 3. 8 Effects of digestate concentration on specific growth rates of E. gracilis, C. vulgaris, and D. tertiolecta cells at PPFDs of 75, 113, and 150 μmol m$^{-2}$ s$^{-1}$ at 30°C. Each plot indicates mean ± standard error (n = 10). ......................... 51

Figure 3. 9 Effects of PPFD on specific growth rates of E. gracilis, C. vulgaris, and D. tertiolecta cells at 10%, 20%, and 50% concentrations of digestate, respectively. The maximum value for three species at 30°C is shown. Each plot indicates mean ± standard error (n=10). ........................................................... 53

Figure 4. 1 The concept of light environment in the culture solution. .................. 61

Figure 4. 2 The absorbance of 5%, 15%, and 25% digestate. ......................... 64

Figure 4. 3 The relationship between digestate concentration (%) and absorption coefficient (cm$^{-1}$). ........................................................................................................... 65
Figure 4. 4 Light intensity is affected by depth and digestate concentration. ........ 66

Figure 4. 5 The absorbance of solutions with different microalgal densities (cells ml\(^{-1}\)). ........................................................................................................................................ 67

Figure 4. 6 The relationship between microalgal density (cells ml\(^{-1}\)) and absorption coefficient (cm\(^{-1}\)). ...................................................................................................................... 67

Figure 4. 7 Light intensity is affected by depth and microalgal density (cells ml\(^{-1}\)). ........................................................................................................................................ 70

Figure 4. 8 Light intensity is affected by depth and digestate concentration (%) at a microalgal density of 30 × 10\(^5\) cells ml\(^{-1}\). .......................................................................................................................... 71

Figure 5.1 Effects of digestate concentration on average specific growth rates (\(\mu\)) of *E. gracilis, C. vulgaris* and *D. tertiolecta* cells, at a PPFD of 300 \(\mu\)mol m\(^{-2}\) s\(^{-1}\) at the solution surface and a temperature of 26°C. Each plot indicates mean ± standard error (n = 3–6). ........................................................................................................ 73

Figure 5.2 Light intensity is affected by depth and digestate concentration (%), a PPFD of 300 \(\mu\)mol m\(^{-2}\) s\(^{-1}\) at the solution surface, and a microalgal density of 30 × 10\(^5\) cells ml\(^{-1}\). .......................................................................................................................... 73

Figure 5.3 Light intensity is affected by depth and digestate concentration (%) at a
microalgal density of $30 \times 10^5$ cells ml$^{-1}$. ................................................................. 75

Figure 6.1 A system as one potential application of this study. ............................... 77

Figure 6.2 Large-scale culture and collection of microalgae with digestate. ......... 78

Figure 6.3 A culture system that can control light penetration in the culture solution.
........................................................................................................................................ 79
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 2. 1 Components of the original digestate used in this experiment and the Cramer–Myer (CM) solution used as the control medium.</td>
<td>17</td>
</tr>
<tr>
<td>Table 3. 1 Components of the original digestate used in this experiment.</td>
<td>30</td>
</tr>
<tr>
<td>Table 3. 2 Components of the digestate solutions used in this experiment.</td>
<td>41</td>
</tr>
</tbody>
</table>