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Biomass Production and Nutrient Removal through Cultivation of *Euglena gracilis* in Domestic Wastewater

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Abstract

Laboratory experiments were performed to evaluate the potential of a cultivation of *Euglena gracilis* strain Z, a kind of microalgae, as a method for biomass production and nutrient removal in domestic wastewater. *E. gracilis* was found to be able to almost completely utilize ammonium and phosphate in primary settling tank effluent (PSTE) and final settling tank effluent (FSTE), and could grow efficiently while achieving high biomass productions both in PSTE (6.3×10^3 cells/L) and FSTE (4.5×10^3 cells/L) on the average. The removal of dissolved organic carbon by *E. gracilis* was also observed in PSTE, though the dissolved organic carbon and nitrogen were increased after cultivation in FSTE possibly due to the leakage from *E. gracilis* cells. It was found that removal of microorganisms from PSTE and FSTE by filtration with 1.0 μm- and 0.2 μm-pore size filters lowered the biomass productivity, indicating that the microbes existing in the wastewater samples may have certain positive effects on the growth of *E. gracilis*. The results suggest that it is possible to achieve efficient biomass production using *E. gracilis* in PSTE; this phenomenon is coupled with the co-benefit of nutrient removal.

Keywords: *Euglena gracilis*, biomass production, domestic wastewater, nutrient removal

INTRODUCTION

Recently, microalgal biomass has attracted much attention as a feedstock for use in the production of fuels so-called "third generation biofuel"[1]. Microalgae are small eukaryotic organisms that rapidly grow with sunlight and carbon dioxide as energy and carbon sources, respectively, under photosynthetic condition, storing energy as sugars and/or lipids in the cells. These features enables the eco-friendly biomass production for bioethanol and/or biodiesel. In contrast to the use of terrestrial plants to obtain biofuel, microalgae production does not compete with food crop production for land, which is favorable in the context of global sustainability. Microalgae have been also utilized as a cost-effective option in wastewater treatment[2]. Usually, microalgal wastewater treatment systems are employed to remove nutrients, mainly nitrogen and phosphorous; these are directly taken up from wastewater by the microalgae to synthesize their biomass. Consequently, microalgal cultivation in wastewater is considered a potentially promising system that combines biomass production and wastewater treatment, a combination that features several advantages[3)-4)].

However, studies on microalgal biomass production in wastewater are limited, and little effort has been made to clearly evaluate

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its potential and feasibility. In general, wastewater contains not only nutrients but also a variety of other organic and inorganic compounds, alongside various microbes including protozoa, fungi, and bacteria. It has not been fully investigated whether these components have either positive or negative effects on the growth of microalgae. In particular, there has been very limited research to date into the effects caused by microbes existing in the wastewater. Torihara and Kishimoto[5] reported that a particular type of wastewater can show adverse effects on the growth of a microalgal strain, and that inhibitory effects caused by microbes existing in the wastewater may have contributed significantly to this effect. A similar conclusion was reached in a study dealing with the cultivation of cyanobacteria called "blue-green algae"[6]. Conversely, a study on the treatment of wastewater by a co-culture of a microalga, *Chlorella vulgaris*, and a bacterium, *Pseudomonas putida*, observed that the presence of the bacterium enhanced the nutrient uptake capability of the alga, suggesting a mutualistic relationship[7]. Another study also reported the enhanced growth of *Chlamydomonas reinhardtii*, *C. vulgaris* and *Euglena gracilis* by co-cultivation with indigenous bacteria in secondary effluents of municipal and swine wastewater[4]. Therefore, further studies on microalgal cultivation in wastewater are necessary to establish an effective biomass production system in wastewater, particularly concerning the effects of indigenous microbes.

In this study, *E. gracilis*, a representative microalga useful for biofuel production, was cultivated in both a primary settling tank effluent (PSTE) and a final settling tank effluent (FSTE) from a full-scale domestic wastewater treatment plant, and the microalgal biomass production and nutrient removal were evaluated. In order to investigate the effects of indigenous microbes on the growth of *E. gracilis*, wastewater samples were filtered using a series of filters with different pore sizes prior to the algal cultivation experiments.

MATERIALS AND METHODS

Wastewater samples The PSTE and FSTE used for three independent algal cultivation experiments (Runs 1–3) were collected from a full-scale domestic wastewater treatment plant employing a conventional activated sludge process in Osaka, Japan. The corresponding Run No. of the cultivation experiments, sampling date, temperature, pH, dissolved oxygen (DO), and suspended solid (SS) concentrations of the wastewater samples are shown in Table 1. For the stable cultivation of *E. gracilis*, the PSTE and FSTE were filtrated with a 10 μm-pore size filter to reduce the turbidity and remove SS prior to experimentation, through which a portion of protozoa in the samples might have been removed (this effluent is referred to herein as PSTE-10 and FSTE-10). In order to investigate the effects of indigenous microbes on the algal growth in detail, these samples were then sequentially filtered using filters of pore sizes of 1.0 μm and 0.2 μm to prepare the protozoa/microalgae-free and bacteria-free (sterilized) samples, respectively (PSTE-1.0, PSTE-0.2; FSTE-1.0, FSTE-0.2); these samples were also used for the algal cultivation experiments. By comparing the algal growth in the filtered samples, it was possible to separately evaluate the effects of chemical components, bacteria, and other microbes, such as protozoa and indigenous algae in the wastewater, on the cultivation of *E. gracilis*.

Table 1 Wastewater samples used in this study

	Run No.	Sampling Date	Temp. [℃]	pH	DO [mg/L]	SS [mg/L]
PSTE	1	Oct. 12, 2016	26.3	7.0	1.9	135
	2	Aug. 17, 2017	29.3	7.0	2.9	101
	3	Nov. 2, 2017	23.1	7.1	3.4	55
FSTE	1	Oct. 27, 2016	25.5	6.8	5.4	12
	2	Aug. 17, 2017	29.7	6.7	5.0	53
	3	Nov. 2, 2017	24.7	6.8	5.4	9

Microalgal strain and cultivation The strain of alga used in this study was *E. gracilis* strain Z (NIES-48), which was obtained from the National Institute of Environmental Studies, Japan[8]. *E. gracilis* was cultivated in CYT medium (C medium[9] supplemented with 1 g/L yeast extract and 2 g/L tryptone) to prepare the inoculum for the algal growth experiments in PSTEs and FSTEs. *E. gracilis* was grown in 100 mL of the medium in 300-mL Erlenmeyer flasks with rotation at 110 rpm in a growth chamber (25℃, an irradiance of 80 μmol/m^2/s, and a photoperiod of 16 h/8 h day/night) for 3 days. The cells were recovered by centrifugation (25℃, 4,000×*g*, 2 min), washed once in an 8 g/L NaCl solution, and resuspended in 1 mL of the same solution.

Algal cultivation experiments A suspension of *E. gracilis* was inoculated into 100 mL of each PSTE and FSTE sample in 300-mL Erlenmeyer flasks, to achieve a cell density of 2.0×10^6 cells/L. Each sample was then cultivated at a rotation of 110 rpm in a growth chamber (25℃, an irradiance of 80 μmol/m^2/s, and a photoperiod of 16 h/8 h day/night). *E. gracilis* was also grown in modified C medium ($MgSO_4$, 40 mg/L; *β*-Na_2glycerolphosphate, 16.3 mg/L; NH_4Cl, 76.4 mg/L; vitamin B_{12}, 0.1 μg/L; biotin, 0.1 μg/L; thiamine HCl, 10 μg/L; tris(hydroxymethyl) aminomethane, 500 mg/L; $Na_2EDTA \cdot H_2O$, 30 mg/L; $FeCl_3 \cdot 6H_2O$, 5.88 mg/L; $MnCl_2 \cdot 4H_2O$, 1.08 mg/L; $ZnCl_2$, 312 μg/L; $CoCl_2 \cdot 6H_2O$, 120 μg/L; and $Na_2MoO_4 \cdot 2H_2O$, 750 μg/L). Nitrogen and phosphorus concentrations were adjusted to be almost the same as those of PSTE (total nitrogen of 11 mg-N/L and total phosphorus of 2 mg-P/L) so that the medium could act as a reference medium to evaluate the growth of *E. gracilis* in a synthetic medium. The cell number, dry cell weight (DCW), and total chlorophyll content (chlorophyll *a* + chlorophyll *b*; Chl *a*+*b*) of *E. gracilis* were periodically measured during the cultivation to evaluate algal biomass production. Concentrations of dissolved organic carbon (DOC), total dissolved nitrogen (TDN), total dissolved phosphorus (TDP), ammonium nitrogen (NH_4-N), nitrite nitrogen (NO_2-N), nitrate nitrogen (NO_3-N), and phosphorus as phosphate (PO_4-P) were also measured before and after cultivation to evaluate the wastewater treatment performance of *E. gracilis*. The data of NO_2-N and NO_3-N in Run 1 were missing.

Analytical procedures Temperature and pH were recorded on site using a portable pH meter (TPX-999i; Toko Kagaku Co. Ltd., Tokyo, Japan), and DO concentration was measured on site using a portable galvanic DO sensor (DO-30; Kasahara Chemical Instruments Corp., Saitama, Japan). Water quality, including DOC, TDN, NH_4-N, NO_2-N, NO_3-N, TDP, and PO_4-P were determined after filtration by 0.2 μm-pore size membrane filters (Isopore GTTP, Merck Millipore, Darmstadt, Germany) according to the test methods of the Japan Industrial Standards K0102:2013 with minor modifications. SS was also determined according to the test methods of the Japan Industrial Standards K0102:2013 using 1.0 μm-pore size glass microfiber filters (Whatman GF/B, GE Healthcare, Buckinghamshire, UK). The cell number of *E. gracilis* was monitored using a Countess automated cell counter (Thermo Fisher Scientific Inc., MA, USA). The Countess determines the cell number by taking a photo of the culture in a compartment of defined size. The *E. gracilis* cells were automatically judged by their cell sizes and aspect ratio, and counted. Therefore, the microbial cells other than *E. gracilis* can be theoretically excluded. We also confirmed all analysis by manually observing the photos taken by Countess. According to the observation, the cell number data obtained with Countess would accurately reflect the actual growth of *E. gracilis*. The DCW of *E. gracilis* was obtained by measuring the SS of the samples; therefore, the value may include biomass derived not only from the algae but also from the other microbes like protozoa and bacteria. Chl *a*+*b* was determined spectrophotometrically (UV-1850, Shimadzu, Kyoto, Japan) according to the following method: *E. gracilis* cells were recovered from 1 mL of the culture by centrifugation (4℃, 21,900×*g*, 1 min). They were then soaked in 1 mL of methanol for 10 min at room temperature in the dark to extract the chlorophyll. Chl *a*+*b* was calculated using absorbance at 650 nm (A_{650}) and 665 nm

(A_{665}) using the following equation (1)[10]:

$$\text{Chl } a+b \text{ [μg/L]} = 4 \times A_{665} + 25.5 \times A_{650} \quad (1)$$

RESULTS AND DISCUSSION

Growth of *E. gracilis* in wastewater The growth of *E. gracilis* in the domestic wastewater samples was evaluated using the cell number, DCW, and Chl *a*+*b* variables, and shown in Fig. 1. Fig. 2 shows averages of the maximum values of *E. gracilis* growth in the three independent experiments (Runs 1–3).

PSTE–10 and FSTE–10, which were intended to represent raw wastewater samples that had undergone simple SS removal by 10 μm–pore size filter, were both able to successfully support the growth of *E. gracilis*, indicating that domestic wastewater can be utilized as a medium for algal biomass production. *E. gracilis* showed a higher growth in PSTE–10 than in FSTE–10; 6.3 × 10^3 cells/L, 987 mg–DCW/L, and 21.0 μg/L of Chl *a*+*b* in PSTE–10 and 4.5 × 10^3 cells/L, 809 mg–DCW/L, and 13.0 μg/L of Chl *a*+*b* in FSTE–10, on the average (Fig. 2). The difference is especially prominent in Chl *a*+*b*, which may be attributed to restriction of chlorophyll synthesis by the limited concentration of nitrogen in FSTE[11]. From the results, it appears that complex components, typically degradable organic compounds, in the domestic wastewater do not have inhibitory effects on the growth of *E. gracilis*, and that secondary treatment is not necessary as a pretreatment to utilize domestic wastewater for algal growth. In other words, PSTE is more suitable for the cultivation of *E. gracilis* than FSTE.

The growth of *E. gracilis* in the modified C medium, which contained similar concentrations of nitrogen and phosphorus to those in the PSTE, was also evaluated as shown in Fig. 3. Since the average of maximum values of the cell number, DCW and Chl *a*+*b* of *E. gracilis* cultivated in PSTE–10 and FSTE–10 were higher than those in the modified C medium (5.5 × 10^3 cells/L, 680 mg–DCW/L, and 13.8 μg/L of Chl *a*+*b*), it can be said that domestic wastewater is a more suitable medium than modified C medium for *E. gracilis* culture.

Effects of indigenous microbes on the growth of *E. gracilis* In order to investigate the effects of indigenous microbes on the growth of *E. gracilis*, wastewater samples pretreated by filters with different pore sizes were used for algal cultivation in this study (Figs. 1 and 2). Based on the experimental design, PSTE–0.2 and FSTE–0.2 should not contain microbial cells (with the exception of viruses), while the bacteria should still be present in PSTE–1.0 and FSTE–1.0. PSTE–10 and FSTE–10 may also contain smaller-size protozoa and algae indigenous to the wastewater.

Among PSTE samples, PSTE–10 most effectively supported the growth of *E. gracilis*, and its cell number (6.3 × 10^3 cells/L) was approximately 20% higher than that in PSTE–1.0 (5.3 × 10^3 cells/L) and PSTE–0.2 (5.2 × 10^3 cells/L) on the average, though the difference was not significant when the growth was evaluated by DCW and Chl *a*+*b*, (Fig. 2). Since the cell number is considered to reflect the actual growth of *E. gracilis* more accurately than DCW and Chl *a*+*b*, it was suggested that microbes existing in the PSTE may have certain positive effects on the growth of *E. gracilis*. On the other hand, the algal growth in the FSTE samples filtered by filters with different pore sizes was almost similar (4.5 × 10^3, 4.1 × 10^3, and 3.1 × 10^3 cells/L in FSTE–10, –1.0, and –0.2, respectively).

As a special case, it was demonstrated that the density of *E. gracilis* sharply dropped after maximal growth in FSTE–1.0 in Run 3 (Fig. 1). Optical microscopic analysis after this experiment revealed that the *E. gracilis* cells were burst and surrounded by dark-colored spherical and other small microbes (Fig. 4), suggesting the predation of *E. gracilis* by these indigenous microbes. For practical *E. gracilis* biomass production in domestic wastewater, it should be recognized that such predatory loss can occasionally occur. To stably maximize the biomass production, microbes responsible for predation of *E. gracilis* should be identified, and strategies for their proper control should be proposed.

It has been reported that certain bacteria in the microalgal phycosphere are capable of

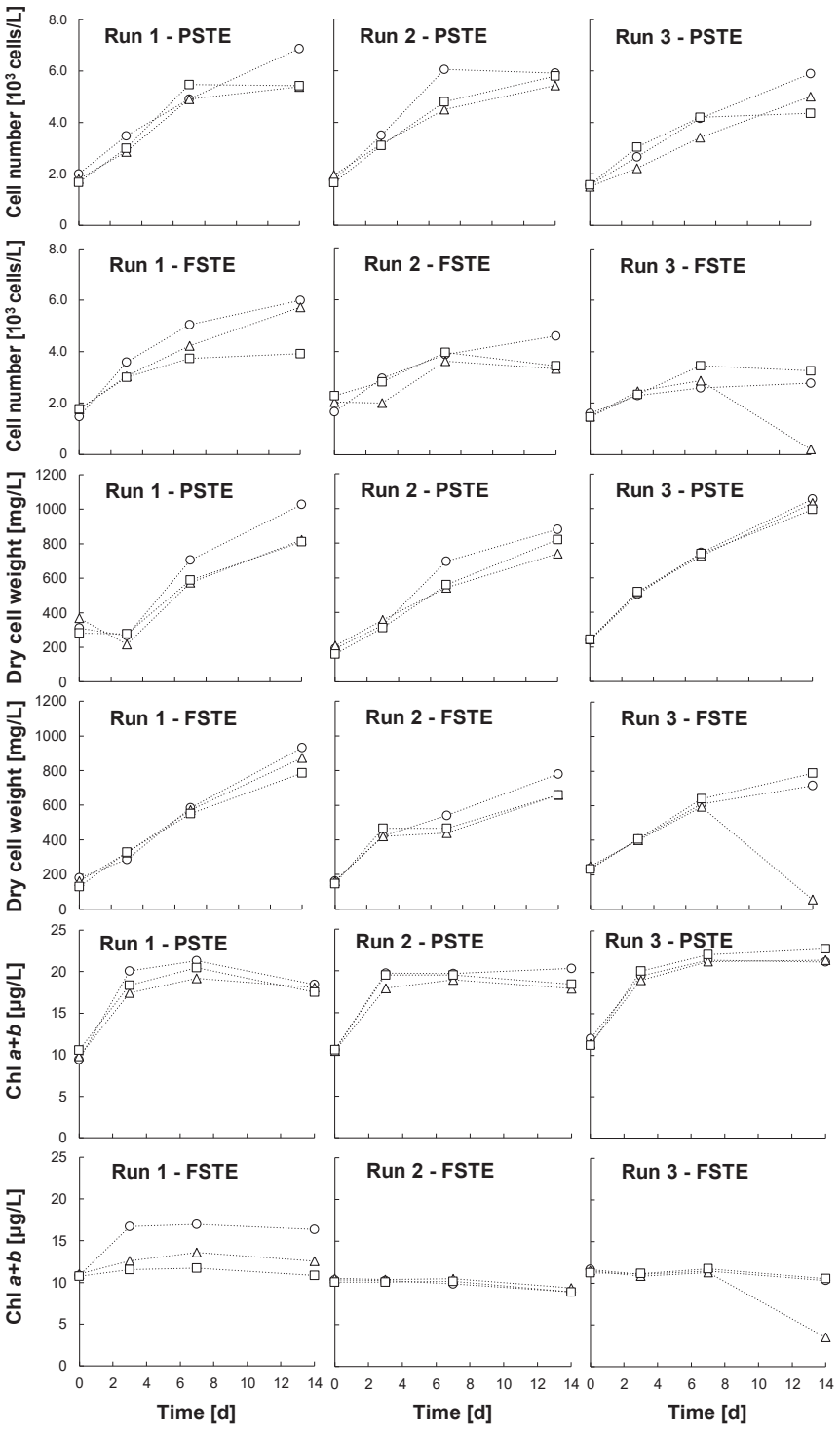

Fig. 1 Time courses of cell number, DCW, and chlorophyll content of *E. gracilis* culture during cultivation in PSTE and FSTE in Runs 1–3. Samples were filtrated with 10 µm– (circles), 1.0 µm– (triangles), and 0.2 µm–pore size filters (squares). It is possible that DCW contained not only algal biomass but also other microbes such as bacteria and protozoa.

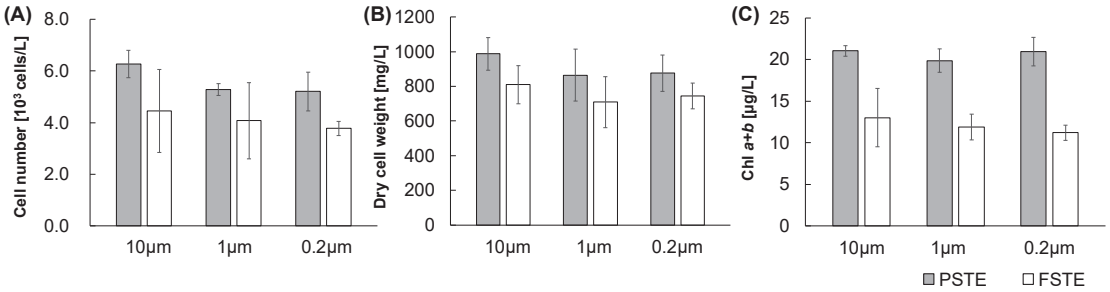

Fig. 2 Comparison of *E. gracilis* growth in PSTE and FSTE. (A) cell number, (B) DCW, (C) chlorophyll content, shown on the average values of the maximum growth in Run 1–3. Bars indicate standard deviation.

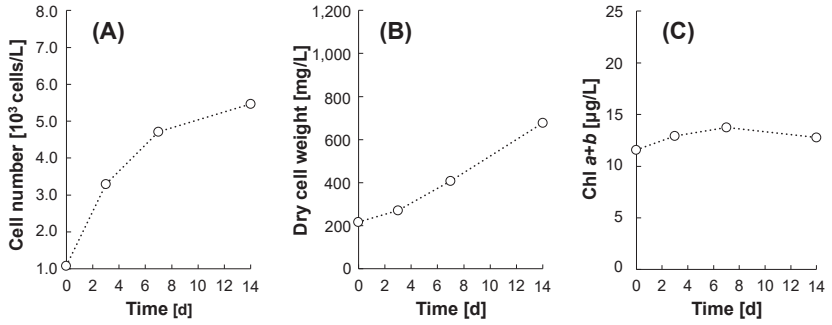

Fig. 3 Growth of *E. gracilis* in modified C-medium. (A) cell number, (B) DCW, (C) chlorophyll content.

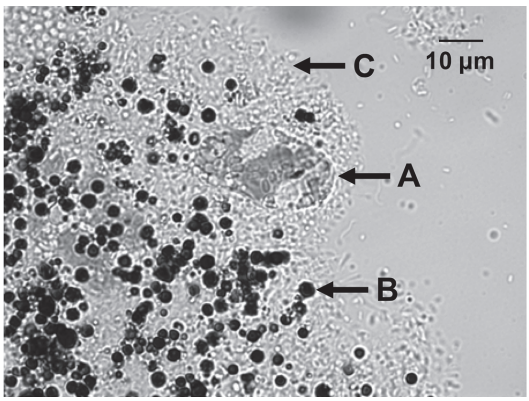

Fig. 4 Optical microscopic image of the culture in FSTE–1.0 of Run 3 (Day 14). A, *E. gracilis* cell; B, dark-colored spherical microbe; C, small-size microbe.

promoting microalgal growth through providing nutrients, vitamins, phytohormones, chelators, or volatile organic compounds[12)–16)], or by creating a favorable microenvironment[17)]. Some previous studies have demonstrated that microalgal growth can be promoted with the help of indigenous bacterial populations in wastewater[4)]. In contrast, other studies have reported that certain indigenous microbes such as viruses, bacteria, other algae, fungi, and protozoa can be detrimental to algal cultivation systems, through parasitism, competition for nutrients, and predation[18)]. In fact, completely opposite results were obtained in previous studies that reported that microbes in wastewater considerably repressed the growth of microalgae[5),6)]. Detailed analysis of the interactions among microalgae and other microbial members in wastewater should be conducted to better understand the microbial effects on algal growth.

Nutrient removal during *E. gracilis* cultivation The water quality parameters (DOC, TDN, NH_4–N, NO_2–N, NO_3–N, TDP, and PO_4–P) before and after the cultivation of *E. gracilis* in PSTE and FSTE are summarized in Fig. 5. These data refer to the wastewater treatment performance

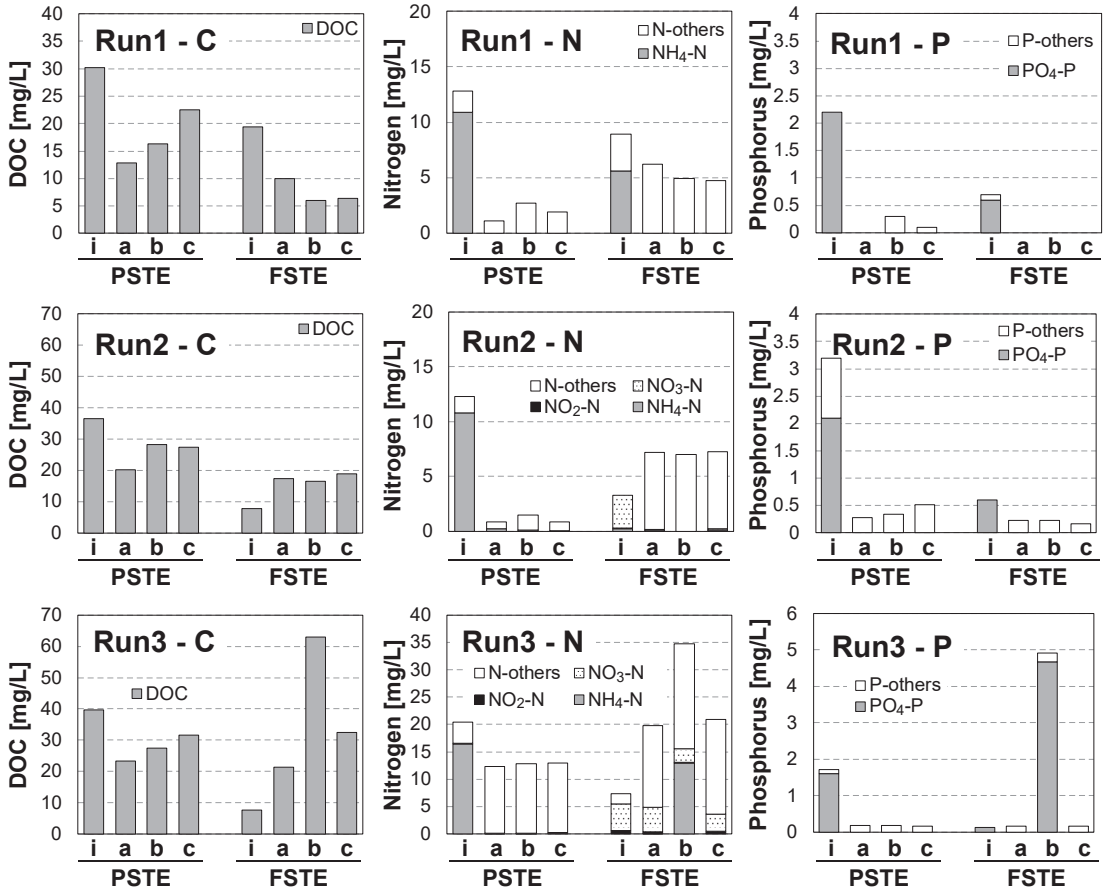

Fig. 5 Wastewater treatment performance of *E. gracilis* cultivation in PSTE and FSTE filtered with different pore sizes. i on the horizontal axes indicates the sample taken at Day 0; a, b, and c on the horizontal axes indicate the samples taken at Day 14 from the *E. gracilis* cultures in wastewater samples filtrated with a 10, 1.0, and 0.2 μm-pore size filters, respectively. N-others and P-others were calculated by the subtractions of inorganic N and P (NH_4-N, NO_2-N, NO_3-N, PO_4-P) from DTN and DTP, respectively.

through the cultivation of *E. gracilis*.

Nitrogen and phosphorous were efficiently removed from PSTE samples by *E. gracilis* regardless of the filtration conditions with exception of Run 3; NH_4-N and PO_4-P were completely removed (≤ 0.2 mg/L), and TDN and TDP concentrations were 0.8-2.7 and ≤ 0.5 mg/L, respectively on day 14. Although NH_4-N, PO_4-P, and TDP were efficiently removed from FSTE samples, TDN remained at a certain high concentration in Run 1, and rather increased in Runs 2 and 3. Especially in FSTE-1.0 in Run 3, where the cell burst of *E. gracilis* occurred as mentioned above, TDN and TDP were increased to very high concentration, 34.7 mg-N/L and 4.9 mg-P/L, respectively. These may be attributed to the leakage of N and P from *E. gracilis* cells.

In addition to nutrients, DOC was substantially removed during the cultivation of *E. gracilis* especially from PSTE samples. DOC removal in the samples without indigenous bacteria (PSTE-0.2) can be attributed to the heterotrophic growing ability of *E. gracilis*. In addition, higher DOC removal in PSTE-10 than PSTE-1.0 and PSTE-0.2 suggests that indigeneous bacteria contributed to the degradation of organic compounds. The increments of DOC in Runs 2 and 3 were also attributed to the leakage of organic substances from *E. gracilis* cells.

Based on these results, it appears that

PSTE is more suitable for the cultivation of *E. gracilis* than FSTE from the both viewpoints of algal biomass production and nutrient removal performance.

CONCLUSIONS

The microalga, *E. gracilis*, was effectively cultivated in PSTE and FSTE collected from a full-scale domestic wastewater treatment plant, suggesting that domestic wastewater treatment processes could be potentially upgraded to algal biomass production processes. TDN and TDP were almost completely removed from PSTE through the cultivation of *E. gracilis*, suggesting that nutrient removal from wastewater can be successfully achieved concomitantly with algal biomass production. DOC was also significantly reduced by culturing *E. gracilis* in PSTE. From these results, PSTE is more suitable than FSTE for algal production. It was found that the presence of microbes in the wastewater had no adverse effects on algal growth in PSTE, indicating that the pretreatment of wastewater to sterilize or deactivate indigenous microbes is not always necessary for algal cultivation. However, as a special case, deterioration of *E. gracilis* growth coincided with the growth of indigenous microbes occurred in FSTE-1.0 in Run 3. Thus, proper control of the indigenous microbial population may be key to achieving more stable algal cultivation in wastewater, which is an interesting subject for future study.

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