Enhancement of microalgal production through bacterial mineralization of ethylene glycol

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Authors Info

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Abstract

Aim: In this study, ethylene glycol was supplied to an algal-bacterial consortium to investigate the effect of bacterial mineralization of ethylene glycol on algal growth.

Methodology: Batch cultures of bacteria and *Chlorella fusca* monocultures, as well as algal-bacterial consortium were fed with 14,000 mg l^{-1} of ethylene glycol and incubated in one liter glass bottles at 25°C with light intensity of 300 µmol m^{-2} s^{-1} in a 12 hr-light:12 hr-dark cycle. A monoculture of *C. fusca* without ethylene glycol was also cultured under similar conditions to test algal growth inhibition.

Results: In the consortium, there was more than 2-fold increase in the maximum algal specific growth rate and a 1.3-fold increase in the maximum bacterial specific growth rate. Furthermore, the maximum ethylene glycol removal efficiency by consortium was 89%, while those by bacteria monoculture was 31%. No apparent removal of ethylene glycol by the *C. fusca* monoculture was observed.

Interpretation: The enhancement of microbial growth rates and ethylene glycol degradation in consortium compared to monocultures indicates that symbiotic relationship between algae and bacteria enhanced oxygen and CO_2 exchange between them, as a result promoting growth and ethylene glycol degradation. This study demonstrated the possibility of an algal-bacterial system for the production of microalgal biomass by feeding organic wastes, such as ethylene glycol-containing wastewater.

Enhanced growth

Biomass

Sunlight

Algae

CO_2

O_2

Bacteria

Organic substrate
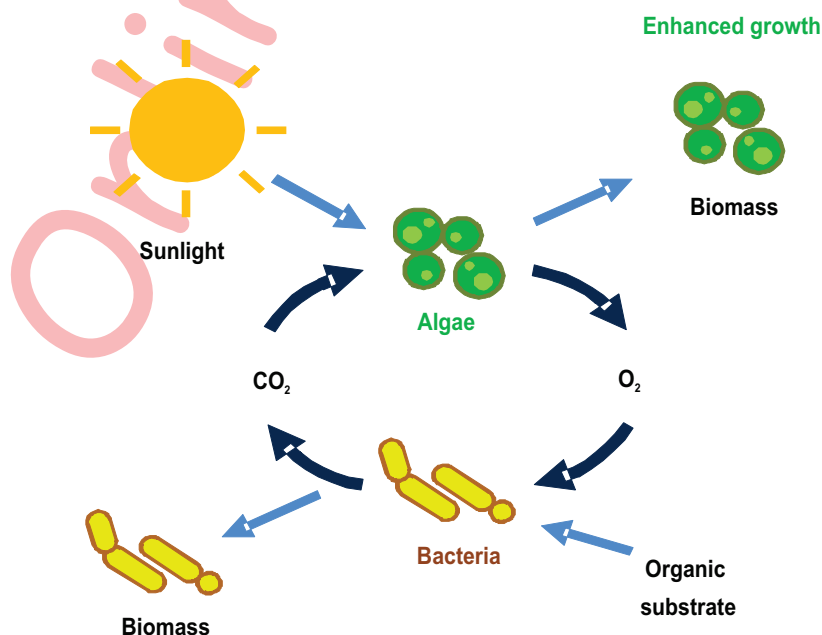
Biomass

Introduction

Microalgal wastewater treatment can convert waste into useful biomass that can be used for various purposes including bioenergy production, and their application to anaerobic digestion effluent, manure, and municipal wastewater (González *et al.*, 2008; Wang *et al.*, 2010; Zhou *et al.*, 2012; Ruiz-Martinez *et al.*, 2012). In addition to the conventional organic wastewaters mentioned above, chemical-based organic wastewater has been problematic around the world. There is a strong need to investigate the applicability of microalgal processes to a wide range of wastewater types.

A large quantity of ethylene glycol (EG; $C_2H_6O_2$) has been produced as a raw material for polyester resins, antifreeze/deicing fluids, cosmetics and surface coatings (ATSDR, 2010). For example, in the United States, more than 3.2 megatons of EG is produced annually (ATSDR, 2010). Wastewaters containing EG are globally discharged from plastic factories, airports and automobile maintenance factories (CERI and NITE, 2007). Since EG exhibits a high chemical oxygen demand (Switzenbaum *et al.*, 2001; Staples and Gulledge, 2006), the effect of its wastewater onto the water environment cannot be neglected.

As a cost-effective wastewater treatment alternative, an algal-bacterial consortium has been applied to wastewaters containing various hazardous chemicals, including salicylate, acetonitrile and bisphenol A (Guieysse *et al.*, 2002; Muñoz *et al.*, 2005; Eio *et al.*, 2014, 2015). The algal-bacterial system utilizes a symbiotic relationship between algae and bacteria, where bacteria use photosynthetically generated oxygen to degrade organic waste. Meanwhile, algae utilize carbon dioxide and nutrients produced through bacterial mineralization of the waste (Oswald *et al.*, 1953). Consequently, with an algal-bacterial system, microalgae biomass is produced through organic waste treatment that uses less energy for aeration.

Previous studies have shown that some microalgae such as *Chlorella protothecoides* (Kishi *et al.*, 2015) and *Chlorella regularis* (Endo *et al.*, 1974) can heterotrophically utilize EG for growth, but not all (Kishi *et al.*, 2015). Contrarily, an algal-bacterial system can be constructed with virtually any photosynthetic microalgae with the aid of bacterial decomposition of the organic fraction.

In the view of microalgal biomass production, bacterial symbiosis may not only support CO_2 and nutrients delivery, but may also reduce the cost and energy required to maintain the system. In order to evaluate the algal-bacterial system as a microalgal biomass production using wastewater, it is necessary to investigate the effects of bacterial co-culture onto the biomass productivity and wastewater treatment efficiency. Therefore, in this study microalgal productivity and EG removal rate of algal-bacterial system with algae-only and bacteria-only cultures was compared.

Materials and Methods

Microorganisms and pre-culture: *Chlorella fusca*, which has been applied to the treatment of chemical wastewaters (Wehrheim and Wettern, 1994; Tsuji *et al.*, 2003; Hirooka *et al.*, 2005), was in this experiment. *C. fusca* NIES-2151 was obtained from the National Institute of Environmental Studies (Tsukuba, Japan). Bacterial inoculum free of bacterivorous or algivorous protists was obtained by filtering the activated sludge (Kitano Sewage Treatment Plant, Tokyo, Japan) through GF/C filter (Whatman, UK). Both organisms were individually incubated with mineral salt medium (MSM) (Muñoz *et al.*, 2004) incorporated with 14,000 mg l^{-1} of ethylene glycol (EG) as pre-culture.

Experimental setup : In order to compare the EG utilization characteristics by each microorganisms, three different treatments were prepared: (1) *C. fusca,* (2) bacteria, and (3) algal-bacterial consortium. Additionally, (4) *C. fusca* without EG (*C. fusca*-EG) was prepared to identify EG inhibitive effect on algal growth. In total four treatments were conducted in triplicates. Mineral salt medium (MSM) was used as culture medium and all, except *C. fusca*-EG, received 14,000 mg l^{-1} of EG in the medium. Assuming the complete mineralization of EG in the medium with algal-bacterial consortium, the C/N ratio of EG and medium nitrate was calculated to be approximately 36.5. Pre-cultures were centrifuged and washed with EG-free MSM, and inoculated as indicated in Table 1. The initial concentration of algae was adjusted to 35 mg d. wt. l^{-1}, and bacterial initial dry weight was set at 5 mg l^{-1}.

Culture medium was injected into one liter cylindrical glass bottles (Fig. 1). Head spaces were continuously replaced with aseptic air through 0.2 µm air filters (Millipore, Aervent-50, USA) at 0.5 l min^{-1} to simulate the air condition of *in situ* algal-bacterial reactors. The cultures were mixed with magnetic stirrers, approximately, at 150 rpm. The culture bottles were incubated at 25°C with light intensity of 300 µmol m^{-2} s^{-1} (fluorescent lamp) in a 12 hr-light:12 hr-dart cycle for 14.5 days. Samples were taken every 12 hr for the first 2 days (day 0, 0.5, 1, 1.5, 2), immediately after every light period from day 2.5 to day 7.5 (day 2.5, 3.5, 4.5, 5.5, 6.5, 7.5), and after every other light period from day 8.5 to the end of the experiment (day 8.5, 10.5, 12.5, 14.5).

Table 1 : Experimental setup

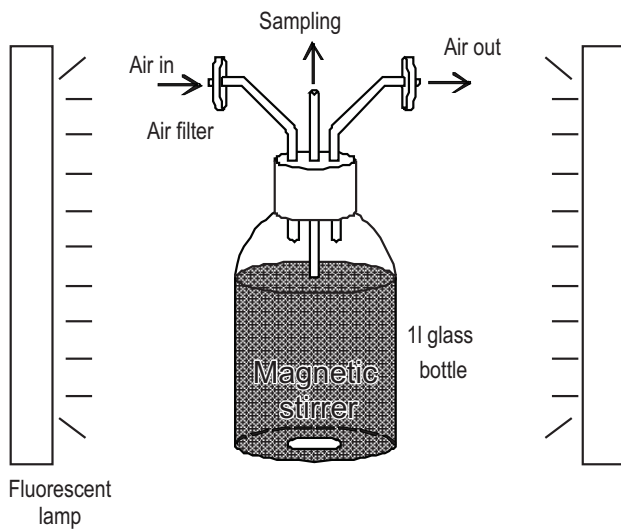| Run	Organisms	Ethylene glycol (mg l^{-1})
Consortium	*C. fusca* + Bacteria	14,000
Bacteria	Bacteria	14,000
C. fusca	*C. fusca*	14,000
C. fusca -EG	*C. fusca*	0

Fig. 1 : Schematic diagram of experimental apparatus

Analytical procedures : Ethylene glycol concentration was determined using a gas chromatograph equipped with a packed column (Shincarbon A) and flame ionization detector (Shimadzu, GC-9A, Japan). The column was maintained at 140°C, and both injector and detector temperatures were 280°C. The carrier gas was helium and was introduced at 40 ml min⁻¹. Dissolved oxygen (DO) was measured using a portable DO meter (Mettler Toledo AG, SG68, Switzerland), and pH was measured with a portable pH meter (Shindengen, KS723, Japan). Algal cells and bacterial cells were counted with a fluorescent microscope (Zeiss, Germany) using epifluorescence and a fluorescent dye (SYBR Gold, Thermo Fisher, USA) as previously reported (Shibata *et al.*, 2006), respectively. To separate algal-bacterial flocs, samples were sonicated for about 5 mins before measurements. Nutrients were analyzed with a nutrient analyzer (SWAAT, BL-TEC, Japan). Specific growth rate (μ; day⁻¹) was calculated using the following equation:

$$\mu = \frac{\ln (x_2 / x_1)}{t_2 - t_1} \quad (1)$$

where, x_i is cell count (cells ml⁻¹) at time t_i (d).

Results and Discussion

pH, DO and algal/bacterial growth : The variations of pH in *C. fusca* and *C. fusca*-EG were relatively small, and it increased slightly from 6.8 to 7.2 during the experimental period in both cultures (Fig. 2a). On the other hand, the variation was much larger in bacteria and consortium. The pH in these cultures initially rose up to over 8, probably owing to the rapid consumption of nitrate (Fig. 3a), and then dropped later on, especially in the consortium. The cause of pH reduction could be CO_2 production with a large amount of EG decomposition (Fig. 4a).

Dissolved oxygen (DO) in *C. fusca* and *C. fusca*-EG remained near or over saturation throughout the experimental period (Fig. 2b). On the other hand, DO was rapidly consumed in consortium and bacterial cultures; on Day 1.5, DO dropped to less than 0.1 mg l⁻¹ and remained near 0 mg l⁻¹ till the end of the experiment. Even in the consortium, despite the photosynthetic oxygen supply, bacteria consumed most of the oxygen, implying that oxygen was a limiting agent in decomposition of EG.

Algal cell counts of *C. fusca* and *C. fusca*-EG were similar (Fig. 2c), and there was no significant difference between the two (Student's t-test; $p > 0.05$), except for Day 14.5 ($p = 0.04$). Almost no inhibitive effect of EG onto algal growth was observed as both *C. fusca* and *C. fusca*-EG showed similar values in pH, DO and algal cell counts (Fig. 2a,b,c). Algal cell count in algal-bacterial consortium was 3.5 and 4.2 times higher than *C. fusca* on Day 2.5 and 6.5, respectively (Fig. 2c). Similarly, bacterial cell count was 2 to 5 times higher than bacteria-only culture after Day 2.5 (Fig. 2d).

Nitrate (NO_3^-) and nitrite (NO_2^-) concentrations reached near depletion both in bacteria and consortium culture on Day 6.5 (Fig. 3a,b). In these cultures, NO_3^- seemed to have been firstly reduced into NO_2^- for the utilization by bacteria and microalgae. In the algae-only cultures, NO_2^- increased towards the end of the experiment (Fig. 3b), probably due to the reduction of NO_3^-. A slight decrease of NO_3^- could have been the sum of the reduction into NO_2^- and assimilation by the algae. Phosphate exhibited less variation likely owing to its less requirement compared to nitrogen (Fig. 3c).

After the depletion of NO_3^- and NO_2^-, the algal growth almost ceased in consortium after Day 6.5, probably owing to the depletion of nutrients (Fig. 3) or oxygen (Fig. 2b). The bacterial growth rate also reduced after Day 6.5, similarly to the algal growth.

In this study, both algal and bacterial cell growth were enhanced in algal-bacterial consortium compared to algae-only and bacteria-only cultures (Fig. 2c,d). The maximum algal specific growth rates were 0.46 and 0.96 d⁻¹ for *C. fusca* and consortium, respectively, exhibiting more than 2-fold increase. The maximum bacterial specific growth rates were 1.45 and 1.94 d⁻¹ for bacteria and consortium, respectively, demonstrating bacterial growth promotion in the consortium. In an open pond system without vigorous aeration, inorganic carbon supply often limits the growth of microalgae (Campbell *et al.*, 2011), as surface atmospheric CO_2 gas transfer does not often meet the requirement. In this study, the growth of algae-only cultures could have been limited by inorganic carbon availability. Contrarily, in the algae-bacterial consortium, the bacterial decomposition of EG must have likely produced CO_2 as byproduct, which raised the limitation and enhanced the algal growth rate. In turn, bacterial growth was likely limited by oxygen availability in bacteria-only culture, but photosynthetic oxygen supply enhanced the bacterial activity in the consortium. Therefore, as previous studies have
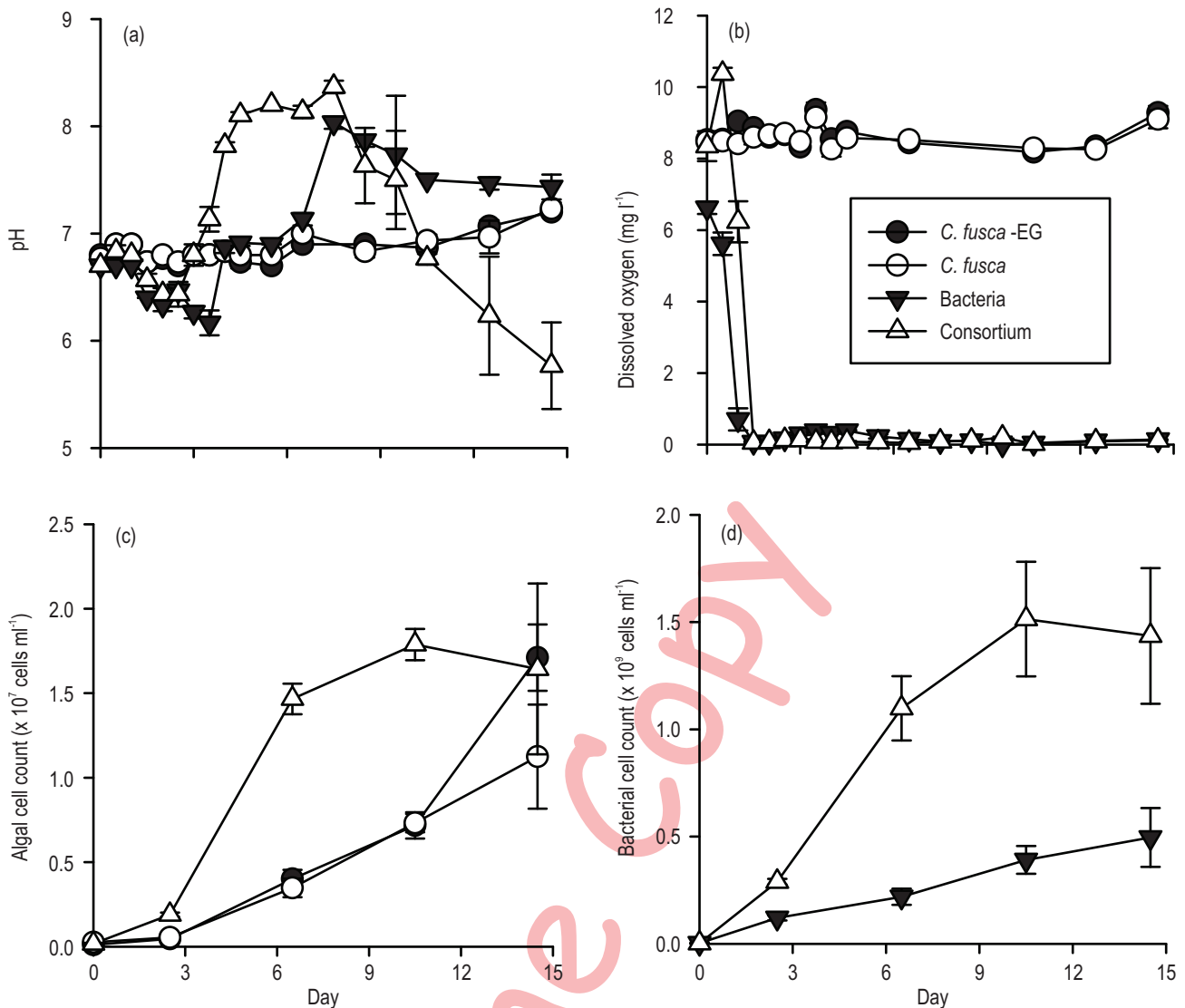
Fig. 2 : Environmental and biological parameters studied during the experiment: (a) pH, (b) dissolved oxygen, (c) algal cell count and (d) bacterial cell count

suggested (Oswald *et al.*, 1953; Bell, 1980; Imase *et al.*, 2008), this study reconfirmed that microalgae and bacteria can create a symbiotic relation, in which growth is enhanced through the accelerated exchange of oxygen and CO_2 within the small community of microorganisms. It was shown in this study that, by providing a carbon source into an algal-bacterial system, biomass production can be greatly enhanced in an open pond without vigorous aeration.

Ethylene glycol removal : Ethylene glycol removal efficiency was highest in consortium; the removal efficiency was 89% with the final concentration of 1.56 g l^{-1} (Fig. 5). The EG removal efficiency in bacterial culture remained 31%, and no apparent decrease of EG was observed in *C. fusca* culture. Since *C. fusca*

did not seem to decompose EG, the degradation in the consortium culture should have been mostly achieved by bacterial activity.

Consortium decomposed three times more EG than bacteria-only culture, probably owing to the enhanced bacterial activity induced by photosynthetic oxygen supply (Imase *et al.*, 2008). The decomposed EG was likely decomposed into CO_2, which should have been eventually assimilated into algal biomass. Through the algal-bacterial system, the organic compounds were converted into algal biomass through bacterial mineralization.

This study also demonstrated that algal-bacterial system can be applied to a medium with high chemical oxygen demand
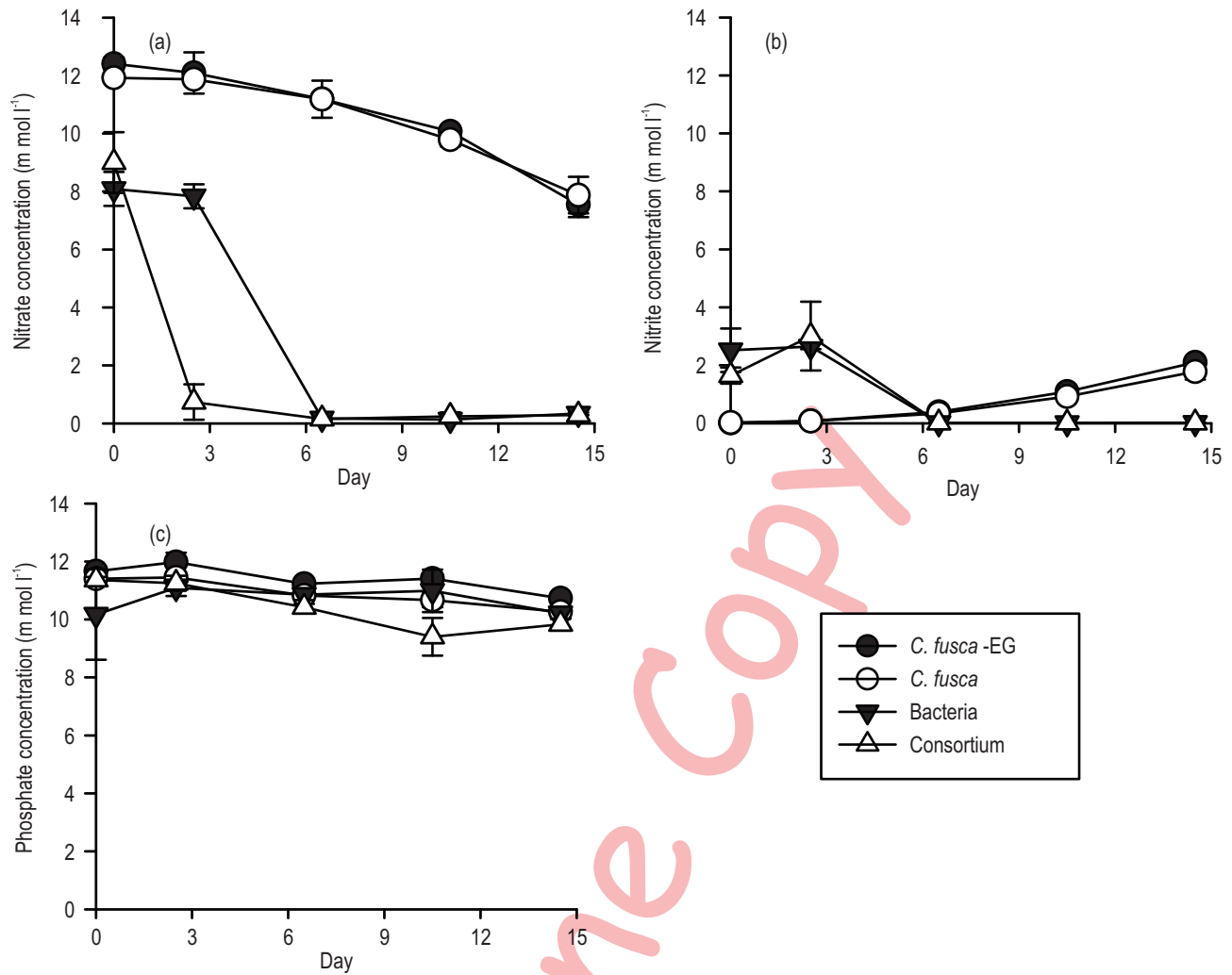
Fig. 3 : Nutrients concentration: (a) nitrate (NO_3^-), (b) nitrite (NO_2^-), (c) phosphate (PO_4^-)

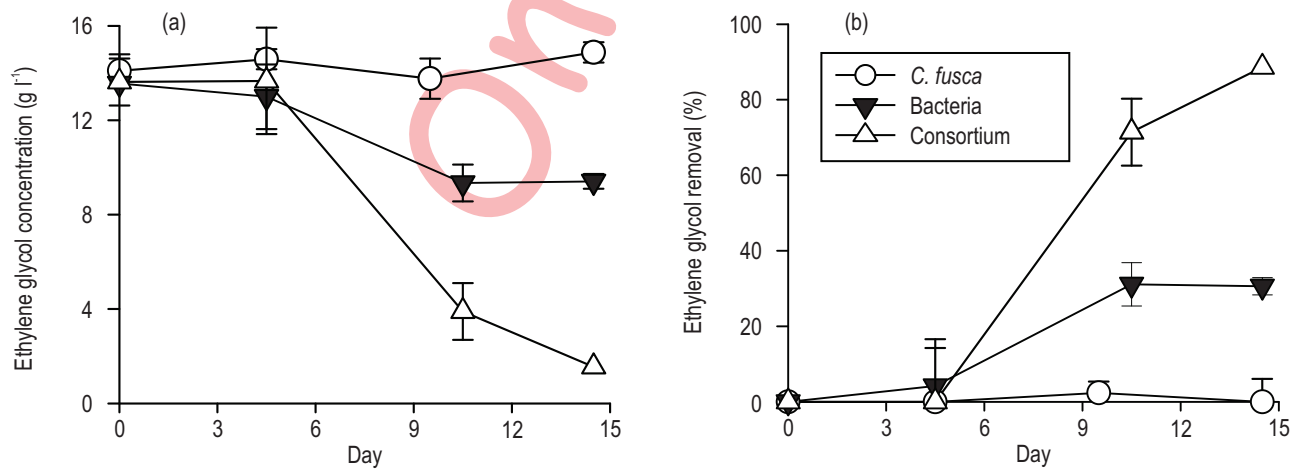**Fig. 4 :** Ethylene glycol: (a) concentration and (b) removal rate

(COD). In this study, estimated initial COD was 18 g l⁻¹, which was much higher than other algal-bacterial studies, ranging from several hundred milligram per litre (Picot *et al.*, 1991; García *et al.*, 2006), several gram per litre (Muñoz *et al.*, 2005; Riaño e*t al.*, 2011), up to 4.3 g l⁻¹ (de Godos *et al.*, 2009). In this study, It was found that as long as there was enough lighting and nutrients, an algal-bacterial system could convert a large portion of organic materials into algal and bacterial biomass.

The low DO (Fig. 3), however, indicated that oxygen limitation certainly occurred during the treatment process with algal-bacterial consortium. The cause of this oxygen limitation could be ceasing of algal growth owing to the depletion of nutrients. Since the C/N ratio of the current study (approximately 36.5) was much higher than an ordinary algal C/N ratio of 7 (Geider and Roche, 2002), nitrogen supply was much lower than the requirement. The NO_3^- and NO_2^- in the medium was, therefore, quickly utilized by the microorganisms at the beginning of the experiment (Fig. 3a,b). For the latter days of experiment, autophagous decomposition of chlorophyll *a* may have occurred in reaction to N limitation (Ito *et al.*, 2013; Ren *et al.*, 2014), as a change in color from deep green into brown was observed in all three bottles of the consortium. For future study, addition of a sufficient amount of nitrogen may further enhance biomass production and EG removal efficiencies.

This study compared algal-bacterial treatment of ethylene glycol with treatment by bacteria and algae monoculture. The growth of microorganisms was greatly enhanced in the consortium, probably owing to the production of oxygen by algae and utilization of CO_2 generated from oxidation of organic compounds. This study demonstrated that bioconversion of ethylene glycol into microalgal biomass is possible with an algal-bacterial system.

Acknowledgments

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