# Microalgae cultivation using undiluted anaerobic digestate by introducing aerobic nitrification–desulfurization treatment

Mutsumi Sekine, Akari Yoshida, Shinichi Akizuki, Masatoshi Kishi and Tatsuki Toda

## ABSTRACT

A novel coupling process using an aerobic bacterial reactor with nitrification and sulfur–oxidization functions followed by a microalgal reactor was proposed for simultaneous biogas desulfurization and anaerobic digestion effluent (ADE) treatment. ADE nitrified by bacteria has a potential to be directly used as a culture medium for microalgae because ammonium nitrogen, including inhibitory free ammonia ($NH_3$), has been converted to harmless $NO_3^-$. To demonstrate this hypothesis, *Chlorella sorokiniana* NIES-2173, which has ordinary $NH_3$ tolerance; that is, 1.6 mM of $EC_{50}$ compared with other species, was cultivated using untreated/treated ADE. Compared with the use of a synthetic medium, when using ADE with 1–10-fold dilutions, the specific growth rate and growth yield maximally decreased by 44% and 88%, respectively. In contrast, the algal growth using undiluted ADE treated by nitrification–desulfurization was almost the same as with using synthetic medium. It was also revealed that 50% of $PO_4^{3-}$ and most metal concentrations of ADE decreased following nitrification–desulfurization treatment. Moreover, upon NaOH addition for pH adjustment, the salinity increased to 0.66%. The decrease in metals mitigates the bioconcentration of toxic heavy metals from wastewater in microalgal biomass. Meanwhile, salt stress in microalgae and limiting nutrient supplementation, particularly for continuous cultivation, should be of concern.

**Key words** | aerobic sludge, anaerobic digestion effluent, *Chlorella sorokiniana*, free ammonia inhibition, post anaerobic digestion treatment, simultaneous bacterial process

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## INTRODUCTION

Anaerobic digestion (AD), which generates biogas by anaerobic microbes, is an economical and environmentally friendly method for the treatment of organic waste/wastewater. However, generated biogas contains hydrogen sulfide ($H_2S$), which is both highly toxic and corrosive, and biogas desulfurization treatment by physicochemical or biological methods is necessary. AD effluent (ADE), which contains a high concentration of nutrients, is generally treated using the nitrification–denitrification process, which involves mechanical aeration and a supply of organic carbon (Fux & Siegrist 2004). The cost and environmental loads of these post-treatments have been a bottleneck for the further spread of AD technology. Mass cultivation of microalgae using ADE has been intensively studied as a possible alternative to conventional ADE treatments (de Godos *et al.* 2009; Kumar *et al.* 2010; Wang *et al.* 2010; Lee *et al.* 2015; González-Camejo *et al.* 2018). This is because the commercialization of microalgal products for purposes such as animal feeds, dietary supplements, and cosmetics is increasing, and microalgae production has the potential to be highly profitable. Both pilot- and full-scale microalgal cultivation facilities using ADE have already been applied for various ADE treatments. For example, *Arthrospira* sp. was cultivated with pig-waste ADE in a 23.6-$m^2$ raceway pond with 84%–91% $NH_4^+$-N and 84%–87% $PO_4^{3-}$-P removal (Olguín *et al.* 2003). ADE from sago starch factory



doi: 10.2166/wst.2020.153

wastewater was treated in a 0.71-$m^2$ high-rate algal pond (HRAP), and *Arthrospira platensis* was produced with 99% $NH_4^+$-N removal (Phang *et al.* 2000). Park & Craggs (2011) treated ADE derived from a wastewater sludge treatment facility with a microalgal–bacterial consortium using HRAP with a supply of $CO_2$. They achieved 24.7 g VSS $m^{-2}$ $d^{-1}$ of high biomass productivity and a maximum removal of 84.5% $NH_4^+$, and then it was scaled up to a 5-ha full-scale plant in New Zealand (Craggs *et al.* 2014). However, with ADE treatment using microalgae, the need to dilute the substrate with a large amount of freshwater has been a serious issue. ADE usually contains 1,000–3,000 mg N $L^{-1}$ of total ammonium nitrogen (TAN) (Xia & Murphy 2016), which can take two forms, free ammonia ($NH_3$) and ammonium ions ($NH_4^+$), depending on the pH, as follows:

$$NH_3 + H_2O \rightleftarrows NH_4^+ + OH^- \quad (pKa = 9.25). \tag{1}$$

Since $NH_3$ severely inhibits microalgae growth, most high efficiency treatments of ADE, including the ones mentioned above, have been achieved by using a 2- to 50-fold dilution of the substrate (Olguín *et al.* 2003; de Godos *et al.* 2009; Kumar *et al.* 2010; Wang *et al.* 2010; Park & Craggs 2011; Lee *et al.* 2015; Xia & Murphy 2016), requiring large amounts of freshwater to be consumed. As an alternative, although we can use other water sources, such as seawater (Sepúlveda *et al.* 2015) and other wastewater with low TAN concentration (e.g. domestic wastewater (Dickinson *et al.* 2015) and secondary effluent (Bohutskyi *et al.* 2016)), the areas near other wastewater treatment facilities were limited and the composition of wastewater must be carefully considered to avoid harmful contamination, particularly for advanced use of microalgae. Using seawater is suitable for seawater species but limits the application of freshwater species.

TAN can be oxidized via nitrification by nitrifying bacteria, producing $NO_3^-$, which is harmless to microalgae. It is expected that the conversion of TAN to $NO_3^-$ via biological nitrification would enable the direct use of ADE in microalgal cultivation without requiring dilution. Furthermore, the optimal growth conditions for nitrifying bacteria; that is, a temperature of less than 30 °C, neutral pH, and aerobic conditions, are similar to those for sulfur-oxidizing bacteria, which can desulfurize biogas (Antoniou *et al.* 1990; Muñoz *et al.* 2015). Therefore, there is the possibility that ADE nitrification could be simultaneously combined with biogas desulfurization in a single reactor (Figure 1). In this aerobic bacterial reactor, $H_2S$ in biogas and TAN in ADE are oxidized to harmless $SO_4^{2-}$ and $NO_3^-$ by sulfur-oxidizing bacteria and nitrifying bacteria, respectively, using $O_2$ produced in the algal reactor. Thus, in this novel coupling process, microalgae are cultivated using undiluted ADE that has been treated by the nitrification–desulfurization process.

In our previous study, we confirmed in a laboratory-scale experiment that nitrification and desulfurization can be stably performed in a single reactor (Sekine *et al.* 2020). However, microalgae productivity when using ADE treated by nitrification–desulfurization remains unknown. Praveen *et al.* (2018) attempted to cultivate microalgae using nitrified ADE and obtained 97% nitrogen removal efficiency. However, they treated ADE after diluting it 3–20-fold with municipal wastewater; therefore, the suitability of nitrified, undiluted ADE for microalgae cultivation remains to be demonstrated. High concentrations of digestate components other than $NH_3$ can inhibit microalgal growth under conditions without dilution. Moreover, there is the possibility that concentrations of some components associated with microalgal growth change through nitrification–desulfurization, causing inhibition or improvement of microalgal growth. Therefore, to evaluate the suitability of the proposed

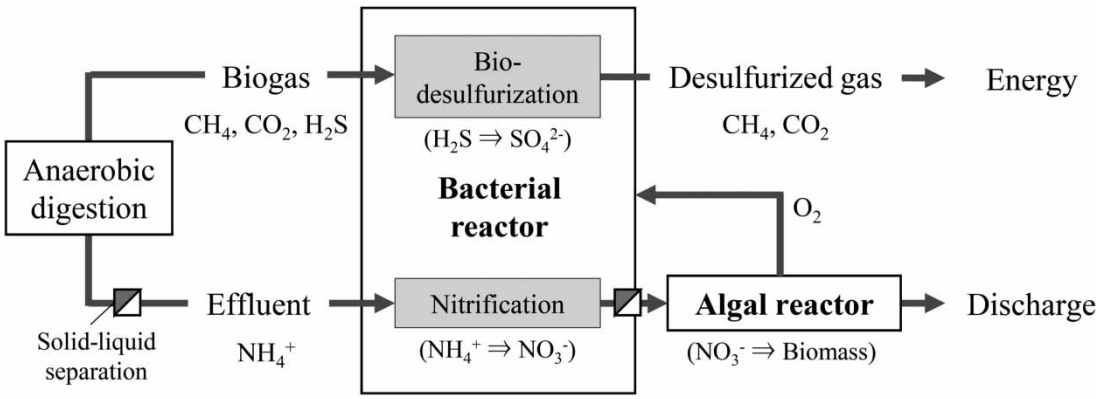


**Figure 1** | A proposed coupling process of biogas desulfurization and effluent nutrients removal after anaerobic digestion using aerobic bacterial reactor and microalgal reactor.

coupling process, the present study was conducted with a focus on microalgae cultivation using ADE treated by nitrification–desulfurization. *Chlorella sorokiniana* NIES-2173 was used as a target species in this research because the genus *Chlorella* is a typical green microalgae that breeds in lakes, rivers, and ponds and has been widely studied and used. First, the $NH_3$ tolerance of the microalgae *Chlorella sorokiniana* NIES-2173 was evaluated as a preparatory experiment to understand the nitrogen utilization characteristics of this species. Second, a comprehensive characterization of ADE treated by nitrification–desulfurization was conducted. Third, the productivity of *C. sorokiniana* using ADE treated by nitrification–desulfurization was investigated compared with using untreated ADE with/without dilution.

## METHODS

### Microorganism and preculture condition

The green algae *C. sorokiniana* NIES-2173 was obtained from the National Institute for Environmental Studies, Tsukuba, Japan, and pre-cultivated in C-medium with 0.6-g C $L^{-1}$ of $NaHCO_3$ at 25 °C under 150 μmol photons $m^{-2}$ $s^{-1}$ continuous light illumination. The composition of C-medium (per liter) was as follows (Ichimura 1971): 500-mg Tris (hydroxymethyl) aminomethane, 150-mg $Ca(NO_3)_2{\cdot}4H_2O$, 100-mg $KNO_3$, 50-mg $\beta$-$Na_2$ glycerophosphate·$5H_2O$, 40-mg $MgSO_4{\cdot}7H_2O$, 100-μg vitamin $B_{12}$, 100-μg biotin, 10-mg thiamine HCl, and 3-mL PIV trace metals solution (Provasoli & Pintner 1959; per liter): 1-g $Na_2$ EDTA·$2H_2O$, 200-mg $FeCl_3{\cdot}6H_2O$, 36-mg $MnCl_2{\cdot}4H_2O$, 10.4-mg $ZnCl_2$, 4-mg $CoCl_2{\cdot}6H_2O$, 2.5-mg $NaMoO_4{\cdot}2H_2O$. The concentrations of $NO_3^-$, $PO_4^{3-}$, and $SO_4^{2-}$ calculated based on the composition of this medium were 32 mg N $L^{-1}$, 7 mg P $L^{-1}$, and 5 mg S $L^{-1}$, respectively, and the N/P molar ratio was 10. Before and after the addition of $NaHCO_3$, the salinity was 0.02% and 0.22%, respectively.

### $NH_3$ tolerance of *Chlorella sorokiniana*

*C. sorokiniana* was cultivated under different $NH_3$ concentrations (0–3.6 mM). Cells in the exponential growth stage were used as the inoculum. C-medium was used after the addition of 0.6-g C $L^{-1}$ $NaHCO_3$ and 941-mg N $L^{-1}$ (67 mM) nitrogen with different ratios of $KNO_3$ and $NH_4Cl$ ($NO_3^-$-N/$NH_4^+$-N: 67/0, 50/17, 34/34, 17/50, and 0/67 mM). The pH was adjusted to 7.99 ± 0.09 by adding 4.8 g $L^{-1}$ 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and NaOH solution. These TAN concentrations and pH values resulted in $NH_3$ concentrations of 0, 0.9, 1.8, 2.7, and 3.6 mM (a total of five conditions, with three to four replicates). After the pH was adjusted, each medium was sterilized by filtration using a 0.22-μm pore size syringe-driven filter unit (Millex GV; Merck Millipore, USA). Twelve-well microplates were used as the cultivation vessels, with 2 mL medium. The initial optical density at 750 nm ($OD_{750}$) was adjusted to 0.021 ± 0.001. The temperature was maintained at 25 °C ± 1 °C and the illumination was set to 150-μmol photons $m^{-2}$ $s^{-1}$. During the experimental period, the $OD_{750}$ was periodically measured after mixing the culture by pipetting. The pH value and the concentrations of TAN, $NO_3^-$, and $NO_2^-$ were measured before and after cultivation.

### Nitrification–desulfurization treatment of ADE

The centrifuged liquid fraction of ADE and nitrifying sludge were obtained from a mesophilic AD reactor treating sewage sludge and an anaerobic–anoxic–aerobic ($A_2O$) bioreactor treating ADE, respectively, in the Hokubu Sludge Treatment Center, Yokohama, Japan. A sequencing batch reactor with an effective volume of 2.1 L was used as the nitrification–desulfurization reactor for the nitrifying sludge. The reactor was operated with a 24-h cycle consisting of a filling and reaction phase (23.5 h), a sedimentation phase (20 min), and a decanting phase (10 min). The hydraulic retention time was adjusted to 3 days. ADE was supplied with NaHS solution to imitate biogas desulfurization. The TAN concentration of the influent was 870 mg N $L^{-1}$. $S^{2-}$ concentration was adjusted to 384 mg S $L^{-1}$, after increasing in a stepwise manner from 0 to 96, and 192 mg S $L^{-1}$ to allow the microbes to acclimatize to $S^{2-}$ based on the assumption of 1:70 of ADE and biogas production volume ratio in AD and 3,000 ppm of $H_2S$ concentration in biogas. The pH was adjusted to 7.5 by the addition of NaOH using a process controller (EYALA EPC-2000; Tokyo Rika, Japan). The temperature and agitation rate were maintained at 30 °C ± 1 °C and 200 rpm, respectively, using a water bath and a magnetic stirrer. The dissolved oxygen concentration was maintained at 1.5–4.0 mg $O_2$ $L^{-1}$ by aeration through two air stones. As a result of sequential batch operation, 170 ± 26 days of long sludge retention time were maintained throughout the operation period. Further details of the reactor operation were previously described in Sekine *et al.* (2020). In the 384 mg S $L^{-1}$ of $S^{2-}$ concentration phase, the influent and effluent of the

reactor were filtered through a 0.45-μm pore size glass fiber filter (GC-50; Advantec, Japan) and stored in a freezer at −30 °C until use for the composition analysis and the next experiment (microalgal cultivation). For the composition analysis of the influent and effluent, the concentrations of phosphate and dissolved metals and the salinity were determined. Additionally, previously reported values for nitrogen, sulfur, and dissolved organic compound (DOC) concentrations in the influent and effluent (Sekine *et al.* 2020) were used for analysis.

### Microalgae cultivation using ADE treated by nitrification–desulfurization

*C. sorokiniana* was cultivated using different media: C-medium, untreated ADE with a 1-, 3-, 6-, or 10-fold dilution, and ADE treated by nitrification–desulfurization (a total of six conditions performed in triplicate). Cells in the exponential growth stage were used as the inoculum. The influent and the effluent of the nitrification–desulfurization reactor were used as the medium for the conditions of untreated ADE and ADE treated by nitrification–desulfurization, respectively, after addition of $MgSO_4{\cdot}7H_2O$ and PIV metals solution adjusted to the same concentration with C-medium. Each medium was supplemented with 0.6-g C $L^{-1}$ $NaHCO_3$ and the pH was adjusted to 7.5 by adding HCl solution; the solutions were then filtered using a 0.22-μm pore size syringe-driven filter unit (Millex GV; Merck Millipore, USA). For microalgae cultivation, 10-mL glass vials with silicone stoppers were used with 5 mL medium. Temperature and light intensity were maintained at 25 °C ± 1 °C and 150 μmol photons $m^{-2}$ $s^{-1}$, respectively. The cultures were mixed thoroughly by inversion every 12 h, and the $OD_{750}$ was measured each time. The silicone stopper was removed every 24 h to allow air exchange. The pH value and nutrient (TAN, $NO_3^-$, $NO_2^-$, $PO_4^{3-}$) concentrations were determined before and after cultivation.

### Measurements and calculations

The $OD_{750}$ was measured using a microplate reader (EPOCH 2; BioTek, USA) and a spectrophotometer (DR2800; HACH, USA) for the $NH_3$ tolerance test and the other experiments, respectively. The pH was measured using a compact pH meter (LAQUAtwin-pH-22B; Horiba, Japan). Nutrient (TAN, $NO_3^-$, $NO_2^-$, and $PO_4^{3-}$) concentrations were measured using a high-performance liquid chromatography system with a conductometric detector (CDD-10$A_{VP}$; Shimadzu, Japan) and two types of columns (an IC YS-50 column for cation analysis and an IC I-524A column for anion analysis; Showa Denko, Japan). Dissolved metals and salinity were determined by inductively coupled plasma spectroscopy (ICPS-$7000_{ver2.1}$; Shimadzu, Japan) and a digital salt meter (Conductivity Method, ES-421; Aatago, Japan), respectively.

The specific growth rate of *C. sorokiniana* was calculated using Equation (2).

$$\mu = \frac{\ln\left(\frac{x_1}{x_2}\right)}{t_2 - t_1} \tag{2}$$

where $\mu$ is specific growth rate ($d^{-1}$) and $x_1$ and $x_2$ are the $OD_{750}$ at time $t_1$ and $t_2$ (d), respectively. The $NH_3$ ratio in TAN (%) was calculated using Equation (3) (Anthonisen *et al.* 1976):

$$NH_3(\%) = \frac{10^{pH}}{e^{\left(\frac{6344}{T}\right)} + 10^{pH}} \times 100 \tag{3}$$

where $T$ is the temperature (K). The half-maximal effective concentration ($EC_{50}$) of $NH_3$ on microalgae was determined by fitting microalgal specific growth rates at the different $NH_3$ concentrations to the following four-parameter logistic curve-regression using SigmaPlot 11.0 software (Systat Software, UK):

$$y = \min + \frac{(\max - \min)}{1 + \left(\frac{x}{EC_{50}}\right)^{-\text{Hillslope}}} \times 100 \tag{4}$$

where *min* is the bottom of curve, *max* is the top of curve, and *Hillslope* is the slope of the curve at its midpoint.

## RESULTS AND DISCUSSION

### $NH_3$ tolerance of *Chlorella sorokiniana*

After 56-h cultivation, the $OD_{750}$ of the conditions with 0-, 0.9-, 1.8-, 2.7-, and 3.6-mM initial $NH_3$ concentration conditions tended to be lower with higher initial $NH_3$ concentration conditions, from 0.097 ± 0.018 to 0.030 ± 0.021 (Figure 2, Table 1). The pH increased to a maximum of 9.47 based on the level of microalgal growth with inorganic carbon consumption. The TAN concentrations at the end of the cultivation were decreased by 21%–78% in the 0.9-, 1.8-, 2.7-, and 3.6-mM initial $NH_3$ concentration

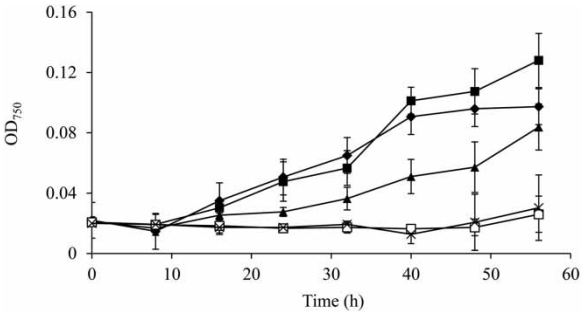


**Figure 2** | Time course of the optical density at 750 nm ($OD_{750}$) under different initial $NH_3$ concentration: 0 mM (♦), 0.9 mM (■), 1.8 mM (▲), 2.7 mM (□), and 3.6 mM (×).

conditions. The main reason for TAN reduction was considered to be $NH_3$ volatilization because the $NO_3^-$ concentration was almost unchanged in all conditions, including the condition using only $NO_3^-$ where we obtained the highest microalgal growth, indicating that no nitrification had occurred and the added nitrogen was almost not assimilated into microalgal biomass. The specific growth rates calculated from the $OD_{750}$ between 8 and 24 h of the logarithmic growth period were $1.85 \pm 0.22$, $1.32 \pm 0.53$, $0.77 \pm 0.37$, $-0.14 \pm 0.37$, and $-0.16 \pm 0.28$ $d^{-1}$ with the initial $NH_3$ concentrations of 0, 0.9, 1.8, 2.7, and 3.6 mM, respectively (Table 1); that is, there was a decreasing trend with increasing initial $NH_3$ concentration. $NH_3$ strongly inhibits the growth of microalgae via two main mechanisms that lead to the breakdown of photosystem II (PSII) function: (1) by diminishing the proton gradient across the thylakoid membrane and suppressing adenosine triphosphate (ATP) production, which is necessary for repairing photodamaged PSII (McCarty 1969; Gutierrez *et al.* 2016); and (2) by ligation to the organo-metallic reactor core in the D1 subunit of PSII, which is essential for its correct functioning (Britt *et al.* 2004; Gutierrez *et al.* 2016). These inhibitory mechanisms associated with $NH_3$ might have led to the decreased growth of *C. sorokiniana* NIES-2173 we observed.

The correct functioning of PSII tends to be maintained until the $NH_3$ concentration exceeds a certain concentration and the above-mentioned mechanisms take effect, therefore the $NH_3$ inhibition level can be easily fitted to a sigmoid regression curve to obtain the $EC_{50}$ (Azov & Goldman 1982). Based on this fitting regression, the $EC_{50}$ of $NH_3$ for the *C. sorokiniana* NIES-2173 used in the present study was 1.6 mM ($p < 0.01$, Figure S1, Supplementary Material). This value falls within the range of $EC_{50}$ (from 0.003 to 3.3 mM) for other species, both reported by Collos & Harrison (2014) and calculated from other studies (Figure S1; Azov & Goldman 1982; Belkin & Boussiba 1991; Tan *et al.* 2016). Therefore, it is reasonable to use *C. sorokiniana* NIES-2173 as a representative species for the validation of nitrification–desulfurization treatments to avoid $NH_3$ inhibition.

## Composition of ADE treated by nitrification–desulfurization

Table 2 shows the composition of the influent and effluent of the nitrification–desulfurization reactor under a supply of 384 mg S $L^{-1}$ of $S^{2-}$. TAN was completely converted to $NO_3^-$; $S^{2-}$ was removed at 100% efficiency, 40% of which was converted to $SO_4^{2-}$, leading to an increase in $SO_4^{2-}$ concentration from 25 mg S $L^{-1}$ to 183 mg S $L^{-1}$. $H_2S$ was not detected from the exhaust gas, and white precipitates were observed attached to the reactor; therefore, a part of the removed $S^{2-}$ was probably converted to elemental sulfur (Sekine *et al.* 2020). The concentration of $SO_4^{2-}$ tends to be between 0.3 and 1.6 mg S $L^{-1}$ in the soil/freshwater environments where microalgae grow (Bochenek *et al.* 2013), and between 3 and 160 mg S $L^{-1}$ in microalgal synthetic medium (Mera *et al.* 2016), therefore the $SO_4^{2-}$ concentrations of both influent and effluent were probably sufficient for microalgal cultivation. $SO_4^{2-}$ does not inhibit the freshwater microalgae *Chlamydomonas moewusii*, even at concentrations of more than 600 mg S $L^{-1}$ (Mera *et al.* 2016). The phosphate concentration of the effluent

**Table 1** | Specific growth rate and final optical density ($OD_{750}$) of *Chlorella sorokiniana* NIES-2173 and cultivation conditions using different $NH_3$ concentrations

| Initial $NH_3$ [TAN] concentration (mM) | Specific growth rate[a] ($d^{-1}$) | Final $OD_{750}$ | Final pH | TAN reduction (%) | Final TAN concentration (mM) |
|---|---|---|---|---|---|
| 0 [0] | $1.85 \pm 0.22$ | $0.10 \pm 0.02$ | 9.47 | – | – |
| 0.9 [17] | $1.32 \pm 0.53$ | $0.13 \pm 0.02$ | 9.21 | 78 | $3.7 \pm 1.2$ |
| 1.8 [34] | $0.77 \pm 0.37$ | $0.08 \pm 0.02$ | 8.63 | 69 | $10.5 \pm 3.6$ |
| 2.7 [50] | $-0.14 \pm 0.37$ | $0.03 \pm 0.01$ | 8.01 | 44 | $28.2 \pm 3.5$ |
| 3.6 [67] | $-0.16 \pm 0.28$ | $0.03 \pm 0.02$ | 7.93 | 21 | $53.2 \pm 1.2$ |

[a]Calculated from the $OD_{750}$ between 8 and 24 hours of the logarithmic growth period.

**Table 2** | Nitrogen, sulfur, and other compounds' concentrations of anaerobic digestate before and after desulfurization-nitrification treatment

| Elements | Influent | Effluent |
|---|---|---|
| TAN-N (mg $L^{-1}$) | 856 ± 12 | n.d. |
| $NO_2^-$-N (mg $L^{-1}$) | n.d. | n.d. |
| $NO_3^-$-N (mg $L^{-1}$) | n.d. | 847 ± 54 |
| $S^{2-}$-S (mg $L^{-1}$) | 384[a] | n.d. |
| $S_2O_3^{2-}$-S (mg $L^{-1}$) | n.d. | n.d. |
| $SO_4^{2-}$-S (mg $L^{-1}$) | 25 ± 1 | 183 ± 34[b] |
| $PO_4^{3-}$-P (mg $L^{-1}$) | 119 ± 11 | 58 ± 38[b] |
| DOC (mg $L^{-1}$) | 63 ± 1 | 81 ± 1[d] |
| Salinity (%) | 0.35 ± 0.06 | 0.66 ± 0.04[c] |

TAN, Total ammonium nitrogen; DOC, Dissolved organic carbon; n.d., not detected.
[a]Calculated from supplied solution concentration. Student's t-test was conducted on $SO_4^{2-}$, $PO_4^{3-}$ and DOC concentrations and salinity of influent and effluent ($n \geq 3$).
[b]$p < 0.05$.
[c]$p < 0.005$.
[d]$p < 0.001$.

fluctuated mainly in the range of 15 to 101 mg P $L^{-1}$, with an average of 58 mg $L^{-1}$, and tended to be lower than the average influent concentration of 119 ± 11 mg P $L^{-1}$. Microbes need to assimilate phosphorus into their cells to grow. Moreover, phosphate can easily precipitate in the presence of some components that exist in ADE, such as TAN, Mg, Al, Ca, and Fe, particularly under alkaline pH conditions. Al, Ca, and Fe were also removed with more than 50% efficiency in this experiment, as described below. The precipitation with these components possibly contributed to the reduction in the phosphate content of the effluent. In particular, Ca, which does not precipitate with sulfide, probably affected $PO_4^{3-}$ precipitation. The molar ratio of N/P in the treated ADE was 53 ± 42, which was higher than the ratio in the untreated ADE (15 ± 0.7). Microalgae can live in freshwater environments with a wide range of N/P molar ratios, from 8 to 45, by changing their cellular composition according to the surrounding nutrients in their environment, but a high enough level of phosphorus (N/P < 22) is required to maintain a high growth rate (Hecky *et al.* 1993; Beuckels *et al.* 2015). There is a possibility that the treatment under pH lower than 7.5 can suppress $PO_4^{3-}$ precipitation; however, lower pH can promote sulfide inhibition in nitrification because the portion of $H_2S$ inhibiting microbes more than $HS^-$ or $S^{2-}$ increased under lower pH. Therefore, especially with continuous algal cultivation, it may be necessary to add phosphate to ADE that has been treated with nitrification–desulfurization to adjust the N/P molar ratio to <22.

The concentration of most metals decreased in the nitrification–desulfurization reactor (Table 3). Aerobic sludge, such as activated sludge and nitrifying sludge, has the potential to remove metals both through its assimilation into cells and by sorption to flocs (Üstün 2009). Additionally, several metals (Al, Mn, Zn, Fe, Ni, Cd, Sn, Pb, Cu, Hg, Ag, Pt, and Au) can easily precipitate in the presence of $S^{2-}$ (Lewis 2010). Precipitation with phosphorous was also considered because $PO_4^{3-}$ concentration decreased at the same time. Therefore, these metals were probably taken into bacteria and precipitated with $S^{2-}$ and $PO_4^{3-}$. A nitrification–desulfurization reactor can decrease the effects of inhibitory metals (Al, Co, Ni, Cu, Cd, and Pb) in microalgal cultivation, although the addition of some essential metals (Mg, K, Ca, Fe, Mn, and Zn) may be necessary if they are present in insufficient quantities. Furthermore, from a commercial perspective, the accumulation of heavy metals (which are toxic to humans) in microalgae cultivated using ADE should be avoided to realize the utilization of microalgae to high-added-value products, such as feed and food supplements

**Table 3** | Metals concentrations of anaerobic digestate before and after desulfurization-nitrification treatment

| Essential metals for microalgae | | | | Inhibitory metals for microalgae | | | |
|---|---|---|---|---|---|---|---|
| | Concentration (mg $L^{-1}$) | | | | Concentration (mg $L^{-1}$) | | |
| Metal | Influent | Effluent | Change (%) | Metal | Influent | Effluent | Change (%) |
| Mg | 3.2 | 2.9 | −10.5 | Al | 0.35 | 0.18 | −50.1 |
| K | 31.2 | 39.5 | 26.6 | Co | 0.16 | 0.04 | −73.8 |
| Ca | 19.4 | 5.3 | −72.5 | Ni | 0.29 | 0.19 | −34.4 |
| Fe | 0.34 | 0.10 | −70.6 | Cu | 0.44 | 0.13 | −70.7 |
| Mn[a] | 0.08 | 0.02 | −71.4 | Cd | 0.19 | 0.07 | −66.3 |
| Zn[a] | 0.19 | 0.06 | −65.4 | Pb | 0.75 | 0.15 | −80.5 |

[a]Metals which inhibit microalgae under high concentration.

(Chamorro-Cevallos & Barrón 2007). There is a report of toxic heavy metals being detected in *Arthrospira platensis*, at a higher concentration than the upper limit of the acceptable range for human intake, following the contamination of a cultivation pond with environmental pollutants (Boudene *et al.* 1975). Removal of these toxic metals by a nitrification–desulfurization reactor may mitigate the accumulation of these metals in microalgae.

The salinity of ADE increased from 0.35% to 0.66% following nitrification–desulfurization, because NaOH was added to increase the reactor pH as it was decreased by nitrification and desulfurization. It has been reported that the final biomass concentration of *Chlorella vulgaris* decreased by approximately 30%, even under 0.3% salinity, in batch cultivation for 24 days (Kebeish *et al.* 2014). This increase in salinity may negatively affect microalgal growth depending on the salinity tolerance of the algal strains used.

## Microalgae cultivation using ADE treated by nitrification–desulfurization

Batch cultivation of *C. sorokiniana* NIES-2173 was conducted using C-medium, untreated ADE with 1-, 3-, 6- and 10-fold dilutions, and ADE treated by nitrification–desulfurization (Figure 3). The specific growth rates within the first two days were very similar (0.43–0.57 $d^{-1}$) for all conditions except untreated ADE without dilution (0.32 $d^{-1}$) (Table 4). The highest initial $NH_3$ concentration (0.91 mM) of untreated ADE without dilution decreased the specific growth rate by 44% compared with the C-medium. Therefore, ADE treated using nitrification–desulfurization is effective for use in microalgal cultivation without dilution. In this connection, although TAN generally improves microalgal growth rate more than $NO_3^-$ because energy requirement for TAN assimilation is lower than for $NO_3^-$ assimilation (Sanz-Luque *et al.* 2015), it appeared that the specific growth rate was not affected by differences in nitrogen sources (TAN of untreated and diluted ADE and $NO_3^-$ of C-medium and treated ADE). There is a possibility that increasing contaminant concentrations (e.g. DOC, $SO_4^{2-}$) or decreasing toxicity of metals via nitrification–desulfurization treatment improved microalgal growth, apart from the suppression of $NH_3$ inhibition. However, the observed negligible effect of salinity might differ if other microalgal species were to be used. The reported salinity tolerance of *Chlorella* spp. and *Chlorococcum* spp. that are commonly found in terrestrial habitats ($EC_{50}$: 0.9%–5%) is higher than that of other freshwater microalgae ($EC_{50}$: 0.3%–1.8%) (Kim *et al.* 2016; von Alvensleben *et al.* 2016; Figler *et al.* 2019). Therefore, for microalgal cultivation using ADE treated by nitrification–desulfurization without dilution, it may be necessary to select species which exhibit a high salinity tolerance.

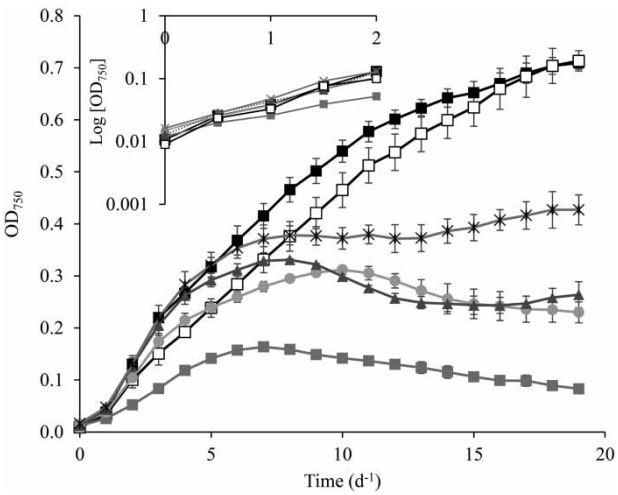


**Figure 3** | Time course of the optical density at 750 nm ($OD_{750}$) under using C-medium (■), 1- (without), 3-, 6-, or 10-fold diluted untreated anaerobic digestion effluent (ADE) (■, •, ▲, ×), and ADE treated by desulfurization-nitrification (□).

In all conditions using untreated ADE, the microalgal growth rate, which was initially almost the same as with the other medium conditions, decreased gradually throughout the cultivation period, with a final $OD_{750}$ that was 40% to 88% lower than that in conditions that used C-medium (Figure 3). During the cultivation period, the pH increased to 8.9–11.2 in all conditions (Table 4). This increase in pH increased the $NH_3$ concentration of untreated ADE from 0.08–0.85 mM to 2.12–13.2 mM (Table 4) and inhibited microalgal growth. However, in the condition using ADE treated using nitrification–desulfurization, the same final $OD_{750}$ of that in C-medium condition was achieved.

At the end of the experiment, both nitrogen and phosphate remained, in all conditions, more than 20.0-mg N $L^{-1}$ and 3.5-mg P $L^{-1}$, respectively (Figure 4). The growth of *C. sorokiniana* in conditions using C-medium and treated ADE was probably suppressed by light limitation, increased pH, and/or lack of dissolved inorganic carbon, trace metals, and other micronutrients, rather than by nitrogen or phosphate limitation. In this batch cultivation, TAN volatilization can be reduced by using a glass tube with a silicone stopper as a cultivation vessel compared with the $NH_3$ tolerance assay using microplates, which resulted in a loss of 21%–78% TAN. However, in the case of conventional microalgal cultivation using untreated ADE, TAN would

**Table 4** | Specific growth rate and final optical density ($OD_{750}$) of *Chlorella sorokiniana* NIES-2173 and cultivation conditions using different mediums

| Medium | | Specific growth rate ($d^{-1}$) | Final $OD_{750}$ | Final pH | $NH_3$ concentration (mM) | |
|---|---|---|---|---|---|---|
| | | | | | Initial | Final |
| C-medium | | 0.57 ± 0.16 | 0.71 ± 0.01 | 10.5 | 0 | 0 |
| Untreated anaerobic digestion effluent (ADE) | Without dilution | 0.32 ± 0.04[a] | 0.08 ± 0.01[b] | 8.9 | 0.85 | 13.2 |
| | 3-fold dilution | 0.43 ± 0.05 | 0.23 ± 0.02[b] | 9.3 | 0.35 | 7.15 |
| | 6-fold dilution | 0.45 ± 0.11 | 0.26 ± 0.03[b] | 9.6 | 0.16 | 3.40 |
| | 10-fold dilution | 0.47 ± 0.04 | 0.43 ± 0.03[b] | 10.1 | 0.08 | 2.12 |
| Treated ADE | | 0.54 ± 0.09 | 0.71 ± 0.02 | 11.2 | 0 | 0 |

Dunnett's test was conducted on specific growth rate and final $OD_{750}$ values ($n = 3$) assuming C-medium condition was the control.
[a]Significantly different at the 5% level.
[b]Significantly different at the 1% level.

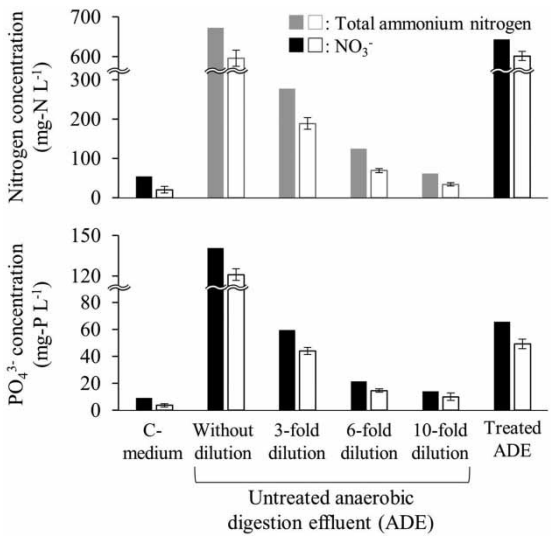


**Figure 4** | Nitrogen compounds and phosphate concentrations of medium before (filled bar) and after (hollow bar) *Chlorella sorokiniana* NIES-2173 cultivation.

easily volatilize by culture mixing, gas exchange, and/or increases in pH. The prevention of nitrogen loss in a large-scale reactor should be an advantage of ADE nitrification treatment because of the conversion of TAN to $NO_3^-$.

## The proposed post-AD process and further prospects

In microalgal cultivation using ADE, in addition to the dilution of ADE, sustaining the pH at less than 7.5 or controlling the concentration of TAN inside the reactor are also ways to avoid inhibition of growth by $NH_3$. However, to maintain uniform pH control requires complete agitation, which is difficult to achieve in a large full-scale reactor (Eroglu *et al.* 2014), and the TAN removal rate in a reactor fluctuates widely depending on the algal growth rate, particularly with outdoor cultivation (González-Camejo *et al.* 2018). In the present study, it was confirmed that microalgae grow well in ADE that has been treated by nitrification–desulfurization, as well as in a synthetic medium even without dilution. It is considered that the proposed process can be an effective option to recover nutrients from the ADE as a variable microalgal biomass that will contribute to the further spread of the AD process. Moreover, the present study also highlighted some problems; for example, a higher N/P molar ratio and salinity of treated ADE than is appropriate for microalgal cultivation. The need to add $PO_4^{3-}$ is probably unavoidable, especially for continuous microalgal cultivation. The high salinity can limit various algal species that can be cultured in this process.

For the real application, first, we note that all ADE composition analysis and microalgal cultivation in this research was conducted using ADE after freezing and thawing to unify the analysis and cultivation conditions. Therefore, this freezing and thawing step might have changed the treated/untreated ADE composition; that is, by precipitation of dissolved contaminates and decomposition of organic compounds, and affected the microalgal growth. The effect of this experimental step required further confirmation. Furthermore, the handling of gas phases (biogas, $O_2$) in each bacterial/algal reactor will be crucial to achieve effective and stable operation of the proposed system. For instance, (1) an appropriate method for $O_2$ recovery from a microalgal reactor and (2) for further biogas utilization, effective supplementation of biogas and $O_2$ in a nitrification–desulfurization reactor to mitigate $O_2$ contamination into biogas should be established. Biological reactor operation and monitoring technologies, which are developing nowadays, should be appropriately applied.

## CONCLUSION

It was confirmed that *C. sorokiniana* NIES-2173 has an average $NH_3$ tolerance of 1.6 mM $EC_{50}$. As a result of cultivating this species using different growth media, the final biomass concentration ($OD_{750}$) decreased by 40% or more in every condition using untreated ADE even after 3-, 6-, and 10-fold dilution compared with the final biomass concentration obtained in the synthetic medium condition. In contrast, high productivity of *C. sorokiniana* was achieved without dilution by using ADE that had been treated by nitrification–desulfurization, the same as was seen with synthetic medium, indicating that this novel ADE treatment, without dilution, is useful for microalgal cultivation using ADE with suppression of the amount of freshwater consumption.

## ACKNOWLEDGEMENTS

We are grateful to the Hokubu Sludge Treatment Center in Yokohama, Japan, for providing the nitrifying sludge and anaerobic digestion effluent. This research was supported by (1) the Japan Science and Technology Agency (JST)/Japan International Cooperation Agency (JICA), Science and Technology Research Partnership for Sustainable Development (SATREPS) and (2) Japan Society for the Promotion of Science (JSPS) KAKENHI, Japan, Grant Number JP19J15095.

## CONFLICTS OF INTEREST

M. Sekine, S. Akizuki, T. Toda and M. Kishi are inventors on Japanese Patent Application No. 2019-35294 (Treatment method of anaerobic digestion products).

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this paper is available online at https://dx.doi.org/10.2166/wst.2020.153.

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First received 30 December 2019; accepted in revised form 22 March 2020. Available online 3 April 2020