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Original article

# Vetiver and *Dictyosphaerium sp.* co-culture for the removal of nutrients and ecological inactivation of pathogens in swine wastewater

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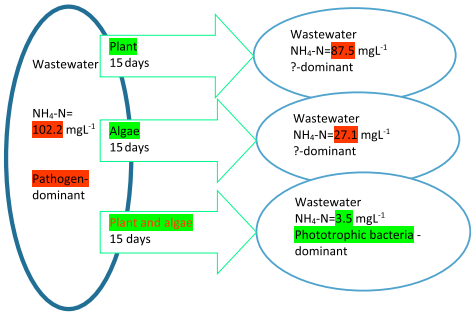
## HIGHLIGHTS

- The wastewater was significantly acidified by plant root respiration.
- Algal culture alleviated hypoxia stress and bicarbonate toxicity for the plants.
- Oxygen from algal photosynthesis could enhance nutrient removal in the wastewater.
- The co-culture significantly increased rapidity in the wastewater treatment.
- Pathogens could be ecologically inactivated in the co-culture.

## GRAPHICAL ABSTRACT

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## ABSTRACT

Swine wastewater poses chemical and biological risks because it contains high concentrations of ammonia and diverse species of pathogens. Herein, a vetiver-*Dictyosphaerium sp.* co-culture for the rapid removal of ammonia and the effective inactivation of pathogens was developed. Plants and microalgae benefited mutually and co-utilized the nutrients in the wastewater in the co-culture. The pathogens were inactivated by reactive oxygen species that were released by the microalgae as well as the supersaturated concentrations of dissolved oxygen in the enclosed bioreactor. In a greenhouse experiment, the time required for wastewater $NH_4$-N to decrease from 102 mg $L^{-1}$ to 5 mg $L^{-1}$ was 65.5 days, 34.2 days, and 13.3 days in the plant culture, the algal culture, and the plant-algal co-culture, respectively. Among the 35 detected genera of bacteria, the operational taxonomic units for 31 tended to decrease with culture time in the plant-algal co-culture. Additionally, certain bacteria (e.g., *Escherichia spp.*) were completely removed by day 9 or 15, and the aerobic phototrophic bacterium *Erythromicrobium spp.* became most abundant on day 15 in the plant-algal co-culture. Important positive interactions that were observed between plants and microalgae included co-utilization of the nutrients, wastewater acidification through plant root respiration and algal growth with reduced ammonia toxicity, algal depletion of bicarbonate and alleviation of bicarbonate toxicity to plants, and release of oxygen from algal photosynthesis and plant growth with reduced hypoxic stress.


## Introduction

In response to the serious swine wastewater problem in China, the government has implemented environmental regulations

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targeting the swine industry [1]. The high concentrations of nutrients (e.g., $NH_4$-N and P) [1,2] in swine wastewater pose a particular challenge for treatment using current technologies. The physical and chemical processes for treating wastewater containing high concentrations of nutrients are very expensive. In addition, widely used microbial processes are not suitable for such wastewater and require high energy inputs (e.g., aerobic digestion, nitrification, and denitrification) [3]. These processes can also produce substantial amounts of sludge. Accordingly, nutrient recovery from wastewater has received increasing attention in the field of wastewater treatment [4–6]. It has been shown that plants and microalgae are able to efficiently recover nitrogen (N), phosphorus (P), and heavy metals from a wide variety of wastewater types [7–9]. Nutrients in wastewater are absorbed and degraded by plants or microalgae and microorganisms, and are recovered as biomass at harvesting. The harvested plants and microalgae can then be utilized as value-added byproducts such as biofuels [10]. Plant and microalgal cultures are sustainable, low-cost, and do not produce sludge. In particular, bicarbonate-rich wastewater is well-suited for culturing certain algal species [11]. However, the slow nutrient removal by plants and algae can hinder the wastewater treatment process [4]. Additionally, various wastewater components are inhibitory for plant and algal growth (e.g., bicarbonate for plants and ammonia for microalgae). Zhang et al. [12] recently developed a plant-microalgal co-cultivation strategy in which plants and microalgae benefit mutually by co-utilizing nutrients in the solution, while additional mutual benefits have been further reported [13,14]. This strategy could substantially enhance the rate of nutrient removal if applied to wastewater treatment, thereby increasing the suitability of plants and microalgae for use in engineered wastewater treatment systems. However, the interactions between plants and microalgae need to be studied further under wastewater conditions.

Pathogen removal is another challenge regarding swine wastewater treatment. Multiple studies have identified *Salmonella*, *Escherichia coli*, *Porcine circovirus type 2* (*PCV2*), and many other microorganisms in swine wastewater, even after it was subjected to conventional biological treatments, such as anaerobic digestion [15–17]. Chemical sanitizers are typically used for pathogen inactivation in water treatment, but bacteria tend to develop chemical resistance to different sanitizers [18]. The chlorination of drinking water, which represents one of the greatest achievements in public health, can lead to the unintended generation of disinfection byproducts (DBPs) associated with an increased risk of bladder cancer [19]. Alternative methods of drinking water treatment such as ozonation are expensive, while treatment with ultraviolet (UV) light is not effective against swine wastewater because the organic materials and suspended solids present in the effluent inhibit the ability of UV light to penetrate the liquid [20]. Reactive oxygen species (ROS) have been shown to possess antibacterial effects [21], and microalgae generate ROS during their life cycle [22,23]. If an algal species capable of releasing a large quantity of ROS is cultured in wastewater, the pathogens could be inactivated. Therefore, an ecological strategy of culturing algae in wastewater is proposed for the inactivation of pathogens in wastewater. This ecological strategy requires only organisms and sunlight, thus eliminating the use of chemicals and need for extra energy while avoiding side effects. Water scarcity is expected to become more widespread in the coming years, and eliminating wastewater discharges is critical for water preservation [24]. As one of the most cost-effective and beneficial uses for algal biomass is returning it to local land [25], ecologically-remediated wastewater could be utilized as both irrigation water and soil amendment, thus eliminating wastewater discharge. The present study aimed to develop a plant-microalgal co-culture strategy for increasing the suitability of plants and microalgae for use in engineered wastewater treatment systems.

## Material and methods

### *Culture experiments*

A batch of culture experiments was conducted in a greenhouse under ambient air conditions from September through November 2016 at the Zhejiang University Experimental Farm, Hangzhou, China. Natural light conditions were maintained in the greenhouse throughout the entire culture period, the temperature was controlled with respective daily minimum and maximum values of 20.2 °C and 36.5 °C, and the average daily relative humidity (RH) ranged from 36.7% to 85.2%. The swine wastewater used in this study was anaerobically digested effluent from a local swine farm in Tonglu County, Hangzhou, China. The wastewater was diluted (1:3) with water for use in the culture experiments. Three treatments were conducted in this study: a plant culture (PC), an algal culture (AC), and a plant-algae co-culture (PACC). A completely randomized experimental design with three replicates was used. The culture containers consisted of 23 L transparent plastic bottles filled with 21 L of working solution.

The green alga *Dictyosphaerium sp*. that was used in the cultures was isolated from wastewater originating from an experimental farm at Zhejiang University and then cultured in BG11 medium [11]. Microalgal seeds were added to the AC and PACC wastewater, and the initial optical density (OD) values were adjusted to an absorbance of 0.05–0.07 at a wavelength of 680 nm ($OD_{680}$).

*Vetiveria zizanioides* plants were obtained from a local farm in Hangzhou, China. The plants that were used in the PC and PACC treatments were fixed to the bottle mouth with a sponge strip. The average plant height when installing the treatments was 191.3 ± 11.8 cm ($n = 6$), and the average root length was 41.2 ± 5.6 cm ($n = 6$).

To validate the relationship between water ROS and algal biomass, a batch of algal culture experiments was conducted as described by Cheng et al. [11]. On day 13 during the exponential growth stage, samples from each medium were collected to measure the algal biomass and the ROS concentration. The algal biomass in each medium was also determined as described by Cheng et al. [11]. The samples from each medium were first centrifuged at 7000 rpm for 2 min, after which the collected supernatant was filtered through a 0.45 μm cellulose membrane. The supernatant was then used to determine the ROS content of the water, based on the method of Xiao et al. [26].

### *Wastewater properties*

Measurements of hydrogen ion concentration (pH), electrical conductivity (EC), and dissolved oxygen (DO) were taken daily between 12:00 pm and 12:30 pm with a PHB-4 pH meter (INESA CO., Shanghai, China), a DDB-303A EC meter (INESA CO., Shanghai, China), and a JPB-607A dissolved oxygen meter (INESA CO., Shanghai, China), respectively. Furthermore, samples from the wastewater were analyzed for bicarbonate ($HCO_3^-$), $NH_4$-N, $PO_4$-P, and ROS concentrations. Each wastewater sample was centrifuged at 7000 rpm for 2 min, after which the supernatant was collected and filtered through a 0.45 μm cellulose membrane. The filtrate was then analyzed for $HCO_3^-$ (using the methods described by Kozaki et al. [27]) via ion chromatography using a Dionex ICS-1500 Ion Chromatography System with an IonPac AS11-HC 4 × 50 mm column (SpectraLab Scientific Inc., Markham, Ontario, Canada); $NH_4$-N (Nash-reagent spectrophotometric method); $NO_3$-N (phenoldisulfonic acid method); and $PO_4$-P (molybdenum–antimony anti-spectrophotometric method). Furthermore, wastewater ROS were determined according to the method described by Xiao et al. [26].

*Algal growth*

Algal cell growth in the solutions was determined by measuring the $OD_{680}$ on selected dates with a spectrophotometer (722S, Leng Guang Tech., Shanghai, China). The algal dry mass was subsequently estimated from the fitted relationship between $OD_{680}$ and algal dry weight biomass. The algal growth rate was calculated as follows:

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

where $x_1$ and $x_2$ denote the absorbance values at time intervals $t_1$ and $t_2$, respectively, and $\mu$ represents the specific growth rate.

Algal biomasses harvested on culture day 9 and day 15 were oven-dried at 60 °C, and the dried samples were milled and passed through a 0.425 mm sieve. Carbon (C) and N contents were then determined using a Flash EA 1112 analyzer (ThermoFinnigan Italia, Milan, Italy).

*High-throughput sequencing*

On culture days 0, 9, and 15, 100 mL wastewater sample was collected from the PACC and submitted for bacterial 16S ribosomal ribonucleic acid (rRNA) gene amplification and sequencing. The analysis was performed at the Beijing Nuo He Zhi Yuan Science and Technology Co. (Beijing, China). Polymerase chain reaction (PCR) amplification of the V4 region of bacterial 16S rRNA was performed using the universal primers 515F 50-GTGCCAGCMGCCGCGGTAA-30 and 806R 50-GGACTACHVGGGTWTCTAAT-30. All of the PCR products were sequenced using an Illumina Miseq Sequencing platform following standard protocols. High-quality sequences were assigned to samples based on barcodes. Chimeric sequences were identified and removed using UCHIME. The operational taxonomic units (OTUs) were clustered with a 97% similarity cutoff using Usearch (http://www.drive5.com/usearch/).

Taxonomic classifications were assigned to OTUs with a Ribosomal Database Project (RDP) Classifier (http://rdp.cme.msu.edu/) and confidence threshold of 80%, as well as the Nucleotide Basic Local Alignment Search Tool (BLASTN) program of the National Center for Biotechnology Information (NCBI) with an output of >90% sequence identity over 90% coverage.

*Plant growth*

Plant fresh weight was determined after transplanting on culture day 15 and at the end of the culture period. At the end of the culture period, plants were divided into root and shoot parts, oven-dried at 70 °C to a consistent weight, and then weighed to determine the dry mass. The plant growth rate was calculated as:

$$Vp = (Mp_2 - Mp_1)/(t_2 - t_1), \quad (2)$$

where $Mp_1$ and $Mp_2$ denote plant fresh biomass (g) at time (d) intervals $t_1$ and $t_2$, respectively, and $Vp$ (g FW·$d^{-1}$) represents the growth rate.

*Root respiration rate*

Respiration rates were measured on 0.5 g of fresh roots using a portable infrared gas analyzer (LI-COR 6400, LI-COR, Lincoln, NE, USA). The root respiration rate was expressed as μmol $CO_2$·kg $FW^{-1}$·$s^{-1}$.

*Statistical analysis*

Means and standard deviations of the dataset were calculated using Microsoft Excel (Microsoft Corporation, Albuquerque, NM, USA). The student's $t$ test and one-way analysis of variance (ANOVA, post-hoc Tukey's tests) were conducted using SPSS version 16.0 (SPSS Inc., Chicago, IL, USA) to compare the two means and the three means of the measured variables, respectively. Statistically significant results were determined at the 0.05 confidence level ($P < 0.05$).

## Results and discussion

*Wastewater properties*

The wastewater contained high concentrations of bicarbonate and nitrogen as well as low to medium levels of other essential nutrients including P, potassium (K), calcium (Ca), magnesium (Mg), iron (Fe), and zinc (Zn) (Table 1). While nitrogen and other nutrients are suitable for culturing both plants and microalgae, the bicarbonate required for algal growth could be stressful to plants as excessive amounts inhibit plant growth and stimulate physiological processes [28,29]. The salinity (EC = 4.89 mS/cm) of the wastewater was too high for use in irrigation as the World Health Organization (WHO) recommends that the total dissolved solids (TDS) in irrigation water should not exceed a value of 450 mg·$L^{-1}$, corresponding to an EC value of 0.95 mS/cm.

Thirty-five dominant genera of bacteria with OTUs ranging from 0 to 5138 were detected in the wastewater, and the pathogenic bacteria *Clostridium spp.* [30] and *Arcobacter spp.* [31] were dominant in the original wastewater and on day 9 in the PACC, respectively (Table 2). Other pathogens, including *Escherichia spp.*, *Chryseobacterium spp.* [32], and *Pseudomonas spp.* [33], were also abundant in the wastewater (Table 2). The dominant pathogens detected in this study were different from those identified in previous studies [15–17].

Swine wastewater containing pathogens and high levels of ammonia and salts can cause substantial soil salinization, and soil and groundwater pollution. Therefore, this wastewater is not suitable for use as irrigation water.

*Validation of the relationship between algal biomass and water ROS level*

As shown in Fig. 1, there was a significant ($P < 0.01$) positive correlation between the algal biomass and water ROS level. Furthermore, the water ROS level was zero when no algae were

**Table 1**
The properties of the wastewater used in this study. EC-electrical conductivity.

| pH<br>7.15 | Bicarbonate<br>$gL^{-1}$ | DO<br>$mgL^{-1}$ | $NH_4$-N<br>$mgL^{-1}$ | $NO_3$-N<br>$mgL^{-1}$ | $PO_4$-P<br>$mgL^{-1}$ | K<br>$mgL^{-1}$ | Ca<br>$mgL^{-1}$ | Mg<br>$mgL^{-1}$ |
|---|---|---|---|---|---|---|---|---|
| 7.45 | 1.53 | 0 | 306.68 | 1.52 | 36.64 | 12.02 | 8.85 | 3.40 |
| Mn<br>$mgL^{-1}$ | Fe<br>$mgL^{-1}$ | Cu<br>$mgL^{-1}$ | Zn<br>$mgL^{-1}$ | C<br>$\mu gL^{-1}$ | Ni<br>$\mu gL^{-1}$ | Pb<br>$\mu gL^{-1}$ | Cd<br>$\mu gL^{-1}$ | EC<br>mS/cm |
| 1.14 | 5.77 | 2.39 | 2.83 | 29.9 | 6.3 | 15.8 | 4.3 | 4.89 |

**Table 2**
The operational taxonomic units (OTUs) for the bacteria in the wastewater in plant-algae co-culture (PACC) at different culture time (day). Data represent means ± SD ($n$ = 3).

| Culture time (days) | 0 | 9 | 15 |
|---|---|---|---|
| **Pathogens** | | | |
| *Escherichia* | 279 ± 56 | 1 ± 1 | 0 ± 0 |
| *Arcobacter* | 570 ± 53 | 4701 ± 378 | 228 ± 52 |
| *Clostridium* | 2437 ± 1150 | 231 ± 6 | 143 ± 34 |
| *Chryseobacterium* | 234 ± 12 | 1958 ± 1391 | 112 ± 11 |
| *Pseudomonas* | 182 ± 249 | 362 ± 230 | 19 ± 25 |
| **phototrophic bacteria and Rhizobacteria** | | | |
| *Roseococcus* | 120 ± 61 | 109 ± 13 | 496 ± 164 |
| *Rhodobacter* | 216 ± 157 | 319 ± 67 | 785 ± 64 |
| *Erythromicrobium* | 441 ± 258 | 494 ± 11 | 3676 ± 46 |
| *Rhodospirillum* | 902 ± 247 | 186 ± 12 | 97 ± 32 |
| *Agrobacterium* | 1 ± 1 | 5 ± 5 | 232 ± 57 |
| **Other bacteria** | | | |
| *Mycoplana* | 342 ± 174 | 2299 ± 97 | 331 ± 112 |
| *Aquamicrobium* | 323 ± 179 | 1836 ± 147 | 250 ± 56 |
| *Methanosaeta* | 906 ± 783 | 0 ± 0 | 0 ± 0 |
| *Parvibaculum* | 197 ± 113 | 1379 ± 163 | 179 ± 55 |
| *Luteolibacter* | 27 ± 14 | 1059 ± 310 | 13 ± 15 |
| *Sedimentibacter* | 1025 ± 160 | 147 ± 7 | 79 ± 26 |
| *Aequorivita* | 120 ± 46 | 912 ± 186 | 45 ± 13 |
| *Marinobacter* | 351 ± 605 | 61 ± 49 | 4 ± 8 |
| *Syntrophomonas* | 863 ± 204 | 127 ± 21 | 67 ± 30 |
| *Anaerovorax* | 864 ± 107 | 137 ± 15 | 97 ± 24 |
| *Devosia* | 283 ± 197 | 622 ± 310 | 80 ± 68 |
| *Tissierella_Soehngenia* | 869 ± 88 | 175 ± 5 | 83 ± 25 |
| *Paenibacillus* | 266 ± 445 | 12 ± 13 | 2 ± 3 |
| *Acinetobacter* | 275 ± 388 | 28 ± 4 | 14 ± 9 |
| *T78* | 384 ± 291 | 14 ± 6 | 6 ± 5 |
| *Rhodococcus* | 470 ± 228 | 529 ± 191 | 1 ± 1 |
| *Desulfobulbus* | 574 ± 55 | 147 ± 17 | 71 ± 16 |
| *heteroC45_4W* | 68 ± 31 | 411 ± 129 | 56 ± 10 |
| *Ralstonia* | 170 ± 288 | 21 ± 10 | 1 ± 1 |
| *Citrobacter* | 240 ± 215 | 7 ± 10 | 1 ± 1 |
| *Stenotrophomonas* | 186 ± 199 | 62 ± 82 | 7 ± 2 |
| *Allochromatium* | 301 ± 80 | 84 ± 16 | 35 ± 14 |
| *Halomonas* | 272 ± 130 | 343 ± 44 | 5 ± 2 |
| *Rhodanobacter* | 123 ± 209 | 92 ± 157 | 4 ± 7 |
| *Anaerospora* | 169 ± 81 | 285 ± 66 | 83 ± 14 |

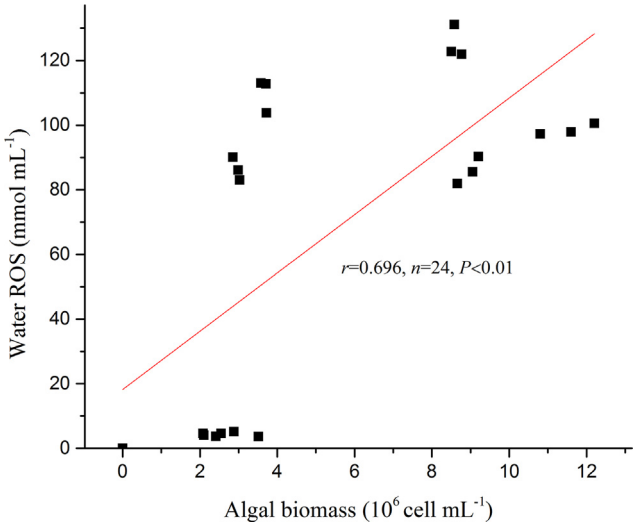


**Fig. 1.** Relationship between the water reactive oxygen species (ROS) level and algal biomass in algal cultures of different media.

present in the medium. These results confirm that the water ROS were produced by the algae.

The water ROS concentration was high (>90 nmol·mL$^{-1}$) in both the AC and PACC but the concentration did not differ significantly

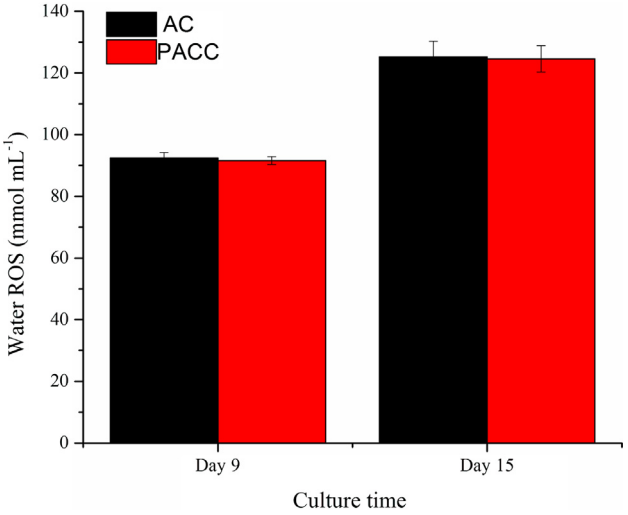


**Fig. 2.** The water reactive oxygen species (ROS) level in the algal culture (AC) and plant-algae co-culture (PACC) at different times. Data represent the mean ± SD ($n$ = 3).

($P$ > 0.05) between the two treatments (Fig. 2). These results indicate that *Dictyosphaerium sp.* is capable of releasing large quantities of ROS.

### *Plant growth*

The plant growth rate in the PACC treatment on day 15 of the culture period was 12.8 ± 1.2 ($n$ = 3) g FW·d$^{-1}$, which is significantly ($P$ < 0.01) higher than what was measured in the PC treatment (1.5 ± 1.0 g FW·d$^{-1}$, $n$ = 3). In addition, the root dry weight at the end of the culture period was 33.2 ± 7.6 ($n$ = 3) g·plant$^{-1}$ and 57.4 ± 14.5 ($n$ = 3) g·plant$^{-1}$ in the PC and PACC treatments, respectively. In summary, plant growth rates were inhibited in the PC culture, but were rather high for the PACC plants.

Throughout the culture period, the water DO in the PC treatment was approximately 0 mg·L$^{-1}$, which could be the result of oxygen being depleted from the anaerobic wastewater by root respiration. In contrast, the DO in the water of the AC and PACC treatments was supersaturated (>10 mg·L$^{-1}$) after day 3 of the experiment (Fig. 3) due to oxygen generation by algal photosynthesis [12,13]. Consistently, the root respiration rates on day 9 were 8.1 ± 0.2 ($n$ = 3) μmol $CO_2$·kg FW$^{-1}$·s$^{-1}$ and 30.1 ± 0.2 ($n$ = 3) μmol $CO_2$·kg FW$^{-1}$·s$^{-1}$ in the PC and PACC treatments, respectively. Therefore, hypoxic stress could be an important factor leading to slow plant growth in the AC.

As shown in Fig. 4, the wastewater bicarbonate concentration in the PC remained nearly unchanged throughout the experiment but decreased rapidly in the AC and PACC since *Dictyosphaerium sp.* is capable of depleting bicarbonate quickly [11]. The water bicarbonate was more rapidly depleted in the AC as compared to the PACC, which is likely because of a greater carbon dioxide ($CO_2$) supply generated from root respiration in the PACC [12]. The high bicarbonate concentration in the PC could inhibit plant growth as bicarbonate can induce or aggravate Fe deficiency [28] and Zn deficiency [29], while the depletion of bicarbonate by algae in the PACC could alleviate bicarbonate stress on plant growth.

Additionally, microalgae are able to produce and excrete hormones (auxins and cytokinins) [34,35] and biostimulants [13,14] into the growing substrate, which could also enhance plant growth.

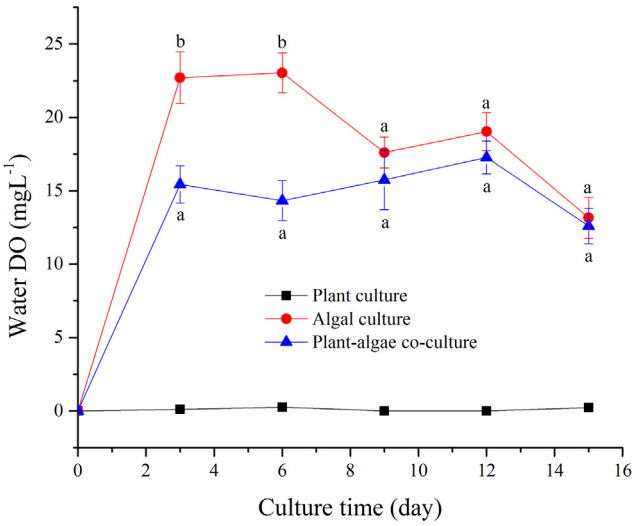


**Fig. 3.** Time series of water dissolved oxygen (DO) level in the different cultures. Data represent the mean ± SD ($n$ = 3). The different letters indicate significant differences at the 0.05 level between the plant culture and the plant-algae co-culture.

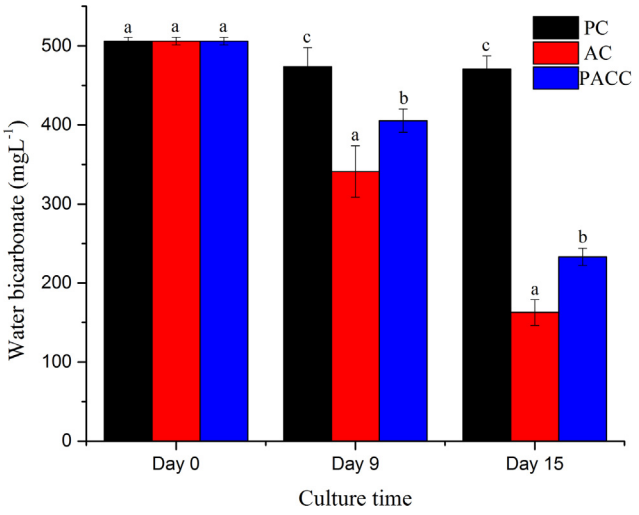


**Fig. 4.** The water bicarbonate level in the plant culture (PC), algal culture (AC), and plant-algae co-culture (PACC) at different culture times. Data represent the mean ± SD ($n$ = 3). The different letters denote significant differences between the treatments at the 0.05 level.

### *Microalgal growth*

After day 3, the algal biomass was significantly higher in the PACC than in the AC (Fig. 5). Consequently, the relative growth rate was also significantly higher in the PACC (0.168 ± 0.009, $n$ = 3) than in the AC (0.151 ± 0.007, $n$ = 3).

The initial $NH_4$-N concentration was above 100 mg·L$^{-1}$, which can inhibit algal growth. The inhibitory effects of $NH_4$-N on microalgae have been widely reported in digestate treatment [36] where growth inhibition has been observed at $NH_4$-N > 100 mg·L$^{-1}$ due to the presence of free ammonia [37]. To alleviate ammonia toxicity, Praveen et al. [37] developed a culture strategy where nitrification is applied as a pretreatment. Ammonia toxicity is closely related to the pH of the medium as free ammonia increases with pH. Microalgae growth is usually associated with an increase in medium pH, which often leads to higher ammonia concentrations and enhanced toxicity [38].

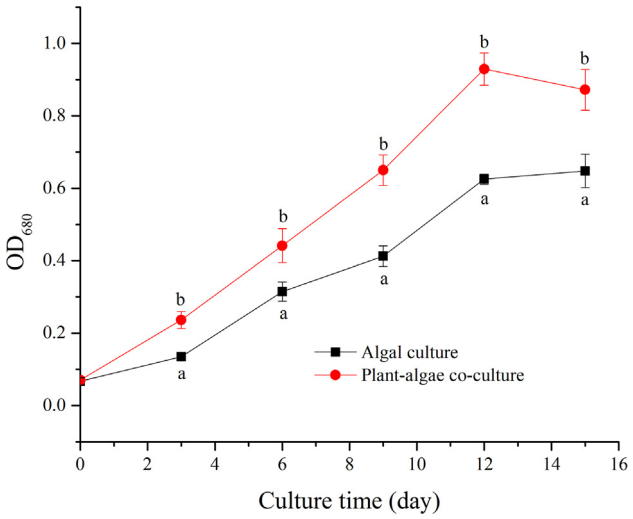


**Fig. 5.** The algal growth curve in the algal culture and plant-algae co-culture. The different letters indicate significant differences between cultures at the 0.05 level.

Interestingly, the $CO_2$ from root respiration significantly acidified the wastewater. The water pH tended to decrease with culture time and fell below 6.2 on day 15 in both the PC and PACC (Fig. 6). In the PACC, the increase in pH from day 0 to day 3 likely occurred because during this early stage, the amount of $CO_2$ derived from root respiration was less than that depleted by algal photosynthesis. In contrast, the water pH in the AC increased with culture time, reaching 8.92 on day 15 (Fig. 6), which is in agreement with previous studies [38]. The wastewater pH in the PACC was 0.15 and 2.68 units lower than in the AC on day 3 and day 15, respectively. The acidic pH could result in the hydrogenation and solubilization of ammonia [37], thus alleviating the ammonia toxicity for the microalgae in the PACC. Ammonia toxicity can also be alleviated through the uptake of $NH_4$-N by plants. Therefore, the plants in the PACC could significantly reduce ammonia toxicity that inhibits algal growth.

The DO was consistently lower in the PACC than in the AC (Fig. 3), which is attributed to the consumption of oxygen by plant root respiration in the PACC. Since dissolved oxygen supersaturation in enclosed photoreactors can be as high as 400% [39], oxygen

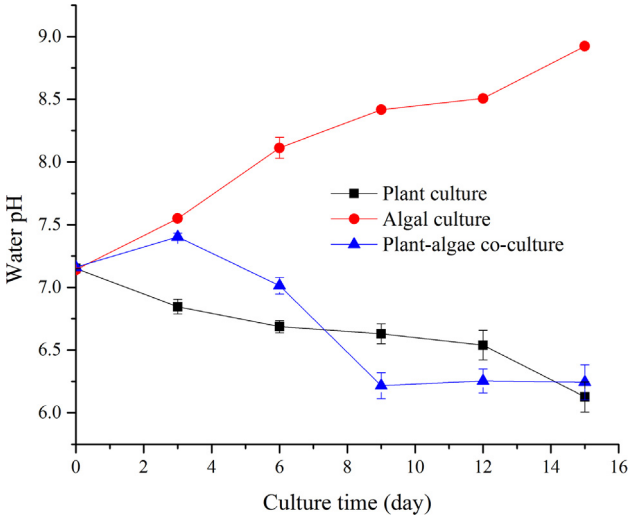


**Fig. 6.** Time series of water pH in the different cultures. Data represent the mean ± SD ($n$ = 3).

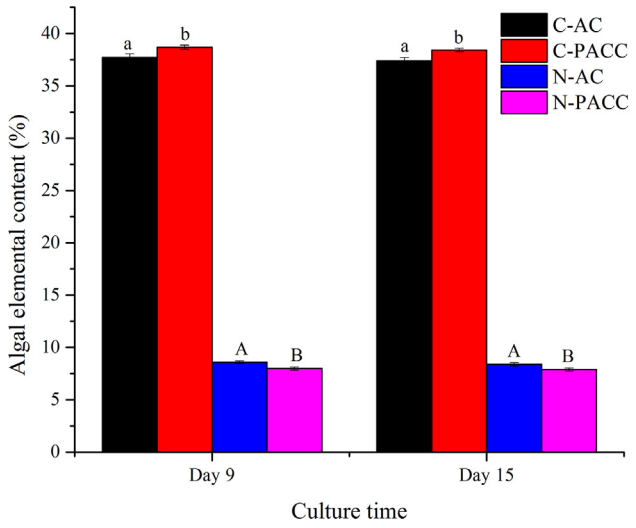


**Fig. 7.** Carbon (C) and nitrogen (N) mass fractions of the dry algal biomass in the algal culture (AC) and plant-algae co-culture (PACC) at different culture times. Data represent the mean ± SD ($n$ = 3). The different letters denote significant differences between the treatments at the 0.05 level.

consumption by root respiration could enhance algal photosynthesis in the PACC by removing the inhibitory effects of oxygen.

The algal C content was significantly lower in the AC than in the PACC. In contrast, the algal nitrogen content was significantly higher in the AC than in the PACC (Fig. 7). Most likely, root respiration in the PACC increased the available carbon for algal growth while the competition for nitrogen by plants reduced the available nitrogen for algal growth. Carbon deficiency is an important limiting factor in algal cultures [11,12]. Therefore, the increased availability of carbon from root respiration could enhance algal growth in the PACC.

### *Bacterial community shift*

Interestingly, the OTUs for 31 of the bacterial genera that were detected in the wastewater tended to decrease with culture time in the PACC, while the OTUs of three phototrophic bacteria and one rhizobacteria increased with culture time (Table 2). In particular, the OTUs of *Methanosaeta*, *Escherichia*, *Paenibacillus*, *Rhodococcus*, *Ralstonia*, and *Citrobacter* decreased to zero or near zero, indicating that they were completely removed by the PACC on day 9 or day 15. Importantly, *Erythromicrobium* belonging to aerobic anoxygenic phototrophic bacteria [40], became most dominant on day 15, which could be attributed to the light and oxygen conditions being more favorable for this bacterium in the PACC. When compared to the original wastewater, the OTUs for all of the pathogens became significantly ($P$ < 0.01) lower in the PACC on day 15. In particular, *Escherichia spp.* was completely removed from the PACC on day 9. Consequently, the bacterial community in the PACC shifted from being pathogen-dominant as in the original wastewater on day 9, to being photobacteria-dominant on day 15. These results indicate that pathogens could be effectively inactivated in the PACC. Interactions between plants and bacteria and between autotrophic algae and heterotrophic bacteria can be cooperative or competitive [41]. Plant and microalgal exudates could serve as an endogenous source of growth substrates for bacteria [42]. However, plant and algal growth could also inhibit bacterial activity by releasing toxic metabolites and maintaining high oxygen levels through algal photosynthesis [43]. In this study, with the exception of the phototrophic bacteria and rhizobacteria, all of the bacteria were inactivated in the PACC. This is likely attributed to the large quantity of ROS released by *Dictyosphaerium sp.* (Fig. 2) and the supersaturation of dissolved oxygen (Fig. 3) in the PACC.

### *Nutrient removal*

Changing water $NH_4$-N and phosphorus concentrations with culture time in each treatment are presented in Figs. 8 and 9, respectively. The relationship between the water nutrients and culture time is best described using a linear or exponential equation (Table 3). The amount of time needed for $NH_4$-N to decrease

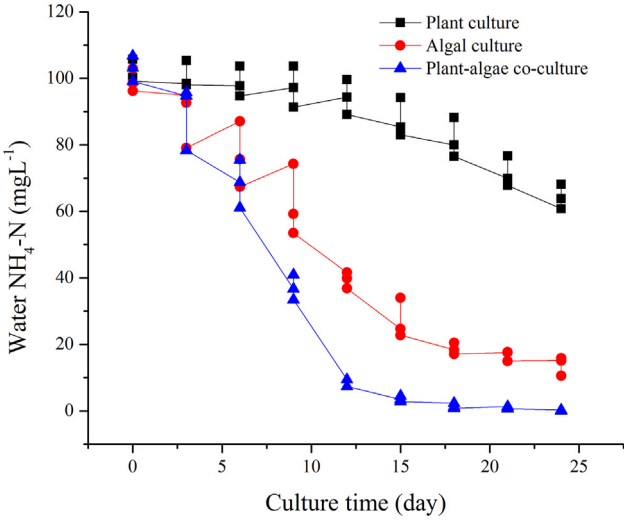


**Fig. 8.** Time series of water ammonia ($NH_4$-N) in the different cultures.

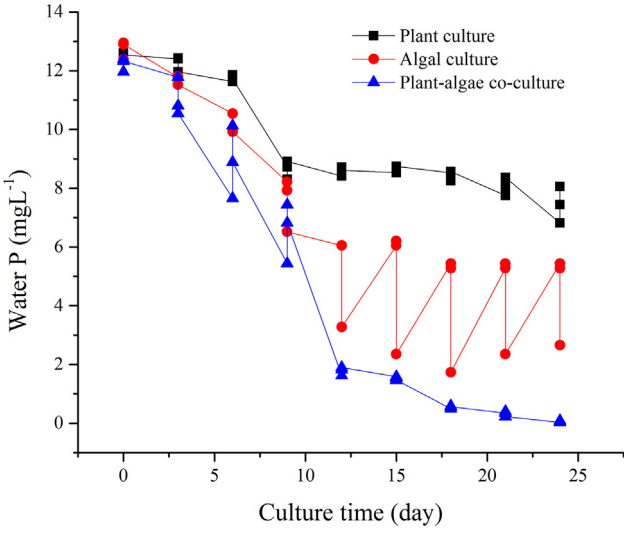


**Fig. 9.** Time series of water phosphorus (P) in the different cultures.

**Table 3**
Equations best describing the relationships between the water $NH_4$-N (Y1, $mgL^{-1}$) or P (Y2, $mgL^{-1}$) and culture time (X, day).

| | |
|---|---|
| Plant culture | Y1 = −1.5634X + 107.37, $r$ = 0.9043, $n$ = 27, $P$ < 0.01Y2 = 12.331$e^{-0.022X}$, $r$ = 0.9113, $n$ = 27, $P$ < 0.01 |
| Algal culture | Y1 = 116.46$e^{-0.295X}$, $r$ = 0.9750, $n$ = 27, $P$ < 0.01<br>Y2 = −0.3903X + 11.94, $r$ = 0.8736, $n$ = 27, $P$ < 0.01 |
| Plant-algae co-culture | Y1 = 251.88$e^{-0.295X}$, $r$ = 0.9478, $n$ = 27, $P$ < 0.01<br>Y2 = 24.118$e^{-0.215X}$, $r$ = 0.9600, $n$ = 27, $P$ < 0.01 |

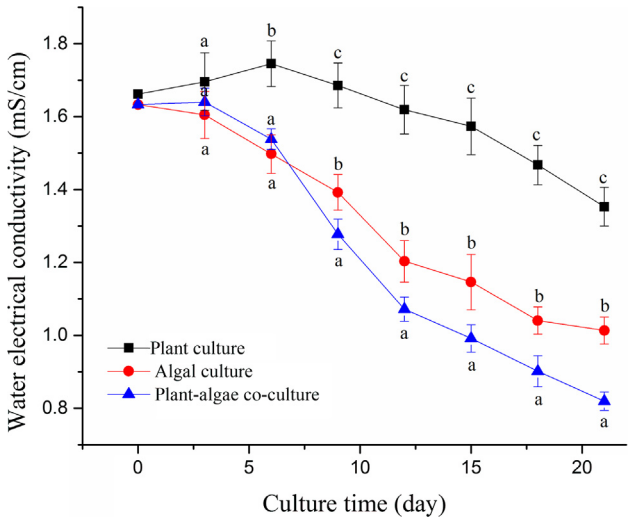


**Fig. 10.** Time series of water electrical conductivity (EC) in the different cultures. Data represent the mean ± SD ($n = 3$). The different letters indicate significant differences between cultures at the 0.05 level.

to 5 mg·L$^{-1}$ in the PC, AC, and PACC was calculated at 65.5 days, 34.2 days, and 13.3 days, respectively, while the amount of time needed for phosphorus to decrease to 2 mg·L$^{-1}$ was 82.7 days, 25.5 days, and 11.6 days, respectively. These results indicate that the removal of $NH_4$-N and phosphorus was very rapid in the PACC relative to the AC or PC. As shown in Fig. 10, since day 9, the water EC was consistently and significantly lower in the PACC relative to the AC and PC, and decreased from an initial value of 1.63 mS·cm$^{-1}$ to 0.82 mS·cm$^{-1}$ at the end of the culture period. The rapid removal of nutrients in the PACC is promising because 30 days and 40 days were required respectively for $NH_4$-N to decrease from 137 mg·L$^{-1}$ to 5 mg·L$^{-1}$ and for phosphorus to decrease from 25 mg·L$^{-1}$ to 5 mg·L$^{-1}$ in an optimized microalgae-based membrane photobioreactor (MPBR) [37]. These results demonstrate that the PACC could be used in engineered wastewater treatment systems. The improved nutrient removal efficiency in the PACC is mainly attributed to the enhanced growth and nutrient uptake of both plants and microalgae since there was poor growth of plants in the PC and of microalgae in the AC. In the PACC, both plants and microalgae grew rapidly, allowing of the rapid uptake of essential nutrients (e.g., C, N, P, sulfur (S), K, and Fe) [12,44]. The continuous oxygen supply by the microalgae could influence the activity and metabolism of microorganism, and thus enhance nutrient removal in the PACC as Chen et al. [45] reported that increasing the aeration rate or moderately lengthening the aeration time could achieve good removal efficiency of nitrogen and phosphorus in treating the wastewater. Particularly, the growth of the phototrophic bacterium (see Table 2) could enhance nutrient removal since phototrophic bacteria require nutrients (e.g., N and P) for growth [46].

The only inputs required for the PACC treatment were the organisms and solar energy (i.e., sunlight). The use of transparent containers made sunlight available to both the plants and microalgae. Since the multispecies interactions occurred continuously throughout the entire culture, little maintenance was required to operate this system. Furthermore, the simple infrastructure and operation make the PACC system suitable for local wastewater treatment, which is in agreement with the 'decentralized recovery' strategy [47]. Irrigation that leads to soil salinity, chemical pollution, and pathogen loading is problematic and precludes wastewater reuse. The PACC-treated wastewater that attained low salinity, chemical pollution, and pathogen risk could be suitable for irrigation. In addition, the PACC system would allow for plants and microalgae to be produced at an extremely low cost. In this study, both the plants and microalgae that were used are marketable. Vetiver plants can be used as animal feed, feedstock for the refinement of essential oils and plants for water conservation engineering, whereas the high protein green algae can be used as bio-fertilizers and animal feed.

## Conclusions

A vetiver and *Dictyosphaerium sp.* co-culture was developed for the rapid removal of nutrients and ecological inactivation of pathogens in swine wastewater. The most dominant pathogens in the wastewater were *Clostridium spp.* and *Arcobacter spp.*, and the bacterial community shifted from pathogen-dominant in the original wastewater to photobacteria-dominant on day 15 of the culture period. In 15 days, the PACC decreased $NH_4$-N and phosphorus levels below acceptable limits, significantly decreased the salinity, and inactivated pathogens in the wastewater. Additional important interactions between plants and microalgae (e.g., water acidification and alleviation of ammonia toxicity by root respiration, and alleviation of bicarbonate stress by microalgae) were also identified in the PACC.

## Conflict of interest

*The authors have declared no conflict of interest.*

## Compliance with Ethics Requirements

*This article does not contain any studies with human or animal subjects.*

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