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Title: Macroalgae culture to treat anaerobic digestion piggery effluent (ADPE)

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Abstract
Environmental consequences of high productivity piggeries are significant and can result in negative environmental impacts; hence bioremediation techniques (in particular using macroalgae) are therefore of great interest. Here, the growth potential of several freshwater macroalgae in anaerobic digestion piggery effluent (ADPE), their nutrient removal rates and biochemical composition of the biomass were investigated under outdoor climatic conditions. A consortium of two macroalgae, \textit{Rhizoclonium} sp. and \textit{Ulothrix} sp. was isolated and could efficiently grow in the ADPE. Maximum ammonium removal rate (30.6 ± 6.50 mg NH$_4^+$-NL$^{-1}$d$^{-1}$) was achieved at ADPE concentration equivalent to 248 mgNH$_4^+$-NL$^{-1}$. Mean biomass productivity of 31.1 ± 1.14 g ash-free dry weight (AFDW) m$^{-2}$d$^{-1}$ was achieved. Total carbohydrate and protein contents ranged between 42.8-54.8 and 43.4-45.0% AFDW, respectively, while total lipid content was very low. The study indicates the potential use of this macroalgal consortium for treating ADPE as well as source of animal feed production.

Key words: \textit{Rhizoclonium}; \textit{Ulothrix}; Bioenergy; Productivity; Phytoremediation
Highlights

- *Rhizoclonium* sp. and *Ulothrix* sp. were successfully grown in minimally diluted ADPE.
- Highest biomass productivities achieved at ADPE with 199 and 248 mg NH$_4^+$-N L$^{-1}$.
- The Fv/Fm was inversely related to diurnal solar irradiance availability.
- Biomass can be used as an animal feed or as a bioenergy feedstock.

1.0 Introduction

The quest for efficient treatment of wastewaters from piggery operations, which cannot be drained to a centralized wastewater treatment system in a cost-effective manner, is of great interest. Potential technologies for treatment of these wastewaters should be reliable, have low capital cost and low operating cost, and be simple in operation. Anaerobic digestion (AD)-based systems are currently sought after for the treatment of these wastewaters due to the overall inefficiency and the unfavourable operation cost associated with aerobic and physico-chemical based technologies. Some major advantages associated with AD-based systems include the elimination of foul odour, capture of gases, biodegradation of organics and the ability to treat large volume of wastewaters.

Anaerobic digestion piggery effluent (ADPE) is the by-product (liquid digestate) of microbial degradation of organics and pollutants in piggery wastewater performed under anaerobic conditions. ADPE, while constituting a treated effluent, does not however meet ecologically acceptable physical, chemical and biological composition requirements for direct disposal into the environment or water bodies without further treatment. For instance, ammonia concentrations of 3,630 ± 1250 mg NH$_3$-N L$^{-1}$, chemical oxygen demand, COD, 8,933 mg L$^{-1}$ (Hu, 2013), and phosphate levels of 620 mg L$^{-1}$ (Olguín et al., 2003) in ADPE have been reported. This is because currently available technologies for wastewater treatments are not able to ameliorate the large increase in nutrients concentrations post-anerobic digestion (Nwoba *et al*., 2016; Ogbonna *et al*., 2000). Continual discharge of these highly concentrated treated effluents can result in eutrophication of aquatic environments (Carpenter and Bennett, 2011), with severe potential consequences such as modification of habitat, harmful algal blooms, and development of hypoxic and anoxic conditions (Bonsdorff *et al*., 2002; Naylor *et al*., 2000). Thus, there is a need for new engineering efforts to significantly reduce the nutrient load of ADPE in order to limit the negative environmental impacts of excessive nutrients in wastewaters.
Biological organisms have demonstrated great capacity for removing excessive nutrients arising from secondary treatment of wastewaters (Ji et al., 2013). Nutrient recovery, wastewater and biomass reuse are the main drivers for the great interest in the use of biological organisms in water pollution control (i.e. wastewater management). Nevertheless, the use of organisms such as bacteria and fungi would require additional carbon sources (Ji et al., 2013).

Algae (micro- and macro-algae) have been proposed as a practical green solution for wastewater treatment (Neori et al., 2004; Pulz, 2001) because of their natural ability to strip away inorganic nutrients especially nitrogen and phosphorous efficiently from wastewaters. Harvesting of nutrients by algae from wastewater is viewed as a more reliable, responsible, sustainable and less energy intensive strategy for recycling the biologically available nitrogen and phosphorus (Chopin et al., 2012; Neori et al., 2004). Integrating algal cultivation with piggery effluent management plans can moderate the nitrogen and phosphorus loads in effluent before discharge and indirectly improve farm productivity, reducing their eutrophic contribution. Algae require dissolved nutrients such as nitrogen and phosphorus (waste products from piggery operations) for their growth. Milestones recorded so far from research have positioned microalgae as a leader of renewable biological solution to myriads of environmental issues (e.g. biofiltration of nutrients and CO\textsubscript{2} mitigation). Several species of microalgae, including Chlorella sp., Spirulina sp., Chlamydomonas sp., Scenedesmus sp., Selenastrum sp. etc. have shown potential for use in phycoremediation of municipal, industrial, agricultural and animal manure (including ADPE) wastewaters (Ji et al., 2013). It is proposed that the produced microalgal biomass could be used for food, feed, energy or the production of fine chemicals (i.e. creates economic incentives for farmers or to spinoff industries).

Microalgae harvesting require substantial amount of energy contributing to high processing cost. Macroalgae, on the other hand, do not require cost-intensive harvesting procedures as they can be harvested through scraping or straining, depending on whether they are attached or floating in the culture. Several macroalgae including Ulva sp. (Al-Hafedh et al., 2012), Gracilaria sp. (Al-Hafedh et al., 2012), Rhizoclonium sp. (Mulbry et al., 2009), Cladophora sp. (de Paula Silva et al., 2012), and Oedogonium sp. (Saunders et al., 2012) have been successfully used for the treatment of different wastewater sources such as aquaculture effluent, ash dam water, and dairy and swine manure effluents. In order to achieve a significant reduction of nutrients in ADPE through algal biotechnology, careful
selection of macroalgal species is required. Recognition of promising species should be based on high growth rates in such conditions that suggest a high nutrient removal ability (Neori et al., 2004) and a tolerance to broad environmental conditions (de Paula Silva et al., 2012), that would allow year-round cultivation. Other characteristics of the target macroalgae should include large nutrient uptake capability, the ability to outcompete biotic pollutions (epiphytes) and pathogens in open culture systems, the ability to grow attached for ease of harvest, and need for local prevalence and some added (or market) value (Kim et al., 2007; Neori et al., 2004). To the best of authors knowledge, no peer-reviewed information is available regarding the treatment of minimally diluted ADPE using macroalgae.

In this study, local macroalgal species that could efficiently grow in slightly diluted ADPE was bioprospected. In addition, nutrient removal rate, productivity and biochemical composition of biomass of the isolated macroalgae when directly grown in ADPE was investigated under the outdoor climatic conditions of Perth, Western Australia.
2.0 Materials and Methods

2.1 Collection of samples

Five local species of macroalgae (*Spirogyra* sp., *Rhizoclonium* sp., *Ulothrix* sp., *Gayraluia* sp. and *Cladophora* sp., see Figure 1a–e) were collected from five different locations of the Canning River (32°01′41″ S, 115°54′58″E), Western Australia, using a sponge-like water filter mat (Figure 1f) during the austral winter (August 2015). Upstream from the Canning River weir is composed of freshwater and receives wastewaters from nearby industries. As all algae are regarded as protected flora in Western Australia, a collection license was obtained from the Department of Wildlife and Parks. Choice of macroalgae samples collected was restricted to only freshwater species as the targeted ADPE was of freshwater origin. Samples were transported submerged in water obtained from the collection area to the Algae R&D Centre, Murdoch University, Western Australia. The samples were maintained outdoor in Modified Chu 13 medium (KNO$_3$ replaced by NH$_4$Cl, 27.5 mg NH$_4^+$-N L$^{-1}$) (Yamaguchi et al., 1987) under natural temperature and solar radiation. Only two strains, *Rhizoclonium* sp. and *Ulothrix* sp. (Figure 1b, c), survived and successfully grew as a consortium in the artificial culture medium for more than one month and these were used for further studies. The proportion of the *Rhizoclonium* sp. and *Ulothrix* sp. in the consortium was 3:1 based on light microscopy.

2.2 Anaerobic digestion piggery effluent

The ADPE used for the study was collected from Medina Research Station located at Kwinana, Western Australia (Nwoba et al., 2016). The research facility employs biological anaerobic digestion pond to treat its wastewater. Despite the anaerobic treatment process, the ADPE still contained high nutrient (nitrogen) load at the point of discharge to the evaporation pond. The ADPE for this study was sourced from the covered AD pond. The ADPE was sand-filtered and used for cultivation of macroalgae without any further pre-treatment (Nwoba et al., 2016). However, the ADPE was diluted with tap water to reduce the ammonium concentration. Physico-chemical properties of the sand-filtered ADPE were characterised using standard protocols (Table 1).

2.3 Bioprospecting

Sponge-like water filters (25 cm x 25 cm, Fig. 1f) were positioned at five locations, 1 km apart upstream from Canning River weir. The filters were collected from the river after
three weeks and transported in the river water to the laboratory. The morphological structures of the collected macroalgae species (Fig. 1a-e) found to attach on to the filters were observed under light microscope. The macroalgae attached to the filters were first grown in enriched river water medium (i.e. river water supplemented with Modified Chu 13 nutrients, 27.5 mg \(\text{NH}_4^+\)-N L\(^{-1}\)). These algae were grown and established in the medium using a tipping bucket system (see description on the section for experimental set-up below). The algae growing attached to the filters were switched to Modified Chu 13 medium containing 27.5 mg \(\text{NH}_4^+\)-N L\(^{-1}\) (Chu 27.5) with the ammonium concentration increased by a factor of 13.75 mg L\(^{-1}\) upon establishment of growth, until 55 mg \(\text{NH}_4^+\)-N L\(^{-1}\) (denoted in this study as Modified Chu 13). At this stage, the algae were finally switched to ADPE-based medium starting with ADPE concentration equivalent to 27.5 mg \(\text{NH}_4^+\)-N L\(^{-1}\) (ADPE 27.5) and gradually increased until the breaking point (≈ 260 mg \(\text{NH}_4^+\)-N L\(^{-1}\), ADPE 260) of the culture (i.e. not able to tolerate more ammonium concentration).

2.4 Experimental set-up

To test the suitability of macroalgae isolates for nutrient removal from ADPE, the consortium was trialled for feasibility of growth and nutrients removal efficiency from ADPE. The consortium was first grown in Chu 27.5 using a tipping bucket system (as per design depicted in Figure 1g) and acclimated to outdoor meteorological conditions (as described above). The consortium was tested in ADPE concentrations equivalent to 55, 150, 199, 248 mg \(\text{NH}_4^+\)-N L\(^{-1}\), respectively designated as ADPE 55, 150, 199, 248, and compared with Chu 13.

The experimental tipping bucket system was based on a two-level design consisting of rectangular tubs (1040 mm x 570 mm x 170 mm, Length x Width x Height) placed on a table and another set of tubs containing 75 L of the nutrient medium, positioned lower than the first (preferably, on the ground). The upper tubs housed the sponge-filters with the macroalgae consortium attached and received a constant volume of 5 L of nutrient medium from tubs situated on the basement (ground, see Figure 1g). The sponge-filters were arranged in a 2x2 matrix design inside the upper tubs. An adjustable submersible centrifugal pump (PU4500, PondMax, 4500L h\(^{-1}\)) was used to introduce the nutrient medium via a vertical PVC pipe into the filter-containing tubs. The nutrient medium in the algae growth tubs drain to the tubs originating the nutrient by gravity at constant flow rate through a manifold. All experiments were run simultaneously in separate tubs at six (6) days interval before medium renewal, with
controls consisting of no alga in ADPE (negative control) and alga in Chu 13 medium (positive control). The negative control (no macroalga) was used to determine if the consortium was the only sink for ammonium in the culture. Each condition was run in three successive batches with the same initial macroalgal biomass (on wet weight basis). At the completion of each batch, the treated effluent was drained from the tubs and the sponge-filters with the consortium were rinsed with tap water to remove debris and particles. All the tubs were cleaned at the end of each batch.

Evaporative loss in the tubs occurred throughout the duration of the experiment. The evaporation loss was replenished by the daily addition of tap water before sampling. Daily 10-minutes interval recordings of solar irradiance for the period of the experiment (October 2015 – February 2016) were downloaded from Murdoch University Weather Station (http://wwwmet.murdoch.edu.au).

2.5 Analytical methods

Samples were collected for determination of initial and final medium ammonium nitrogen concentration at 10:30 a.m. on the first and last day of the experiment. Macroalgal biomass concentration (AFDW, ash-free dry weight), biochemical composition (total protein, carbohydrate and lipids) and chlorophyll contents of the biomass were assayed in each batch of the experiment during growth in ADPE only. The AFDW was determined according to the method of Moheimani et al. (2013). Wet weight of macroalgal biomass was determined by comparing initial weight of wet sponge-filters (without algae) against wet sponge-filters with algae, the difference representing the wet weight of the macroalgal consortium biomass. An aliquot of the wet biomass was used to determine the dry weight (DW) and AFDW. The procedure for wet weighing did not appear to have a negative effect on the alga in terms of growth and nutrient removal. The biomass productivity was determined according to the method described in de Paula Silva et al. (2012), using the equation,

\[ \text{Biomass productivity, } P \ (g \ m^{-2} \ day^{-1} \ AFDW) = (FW_t - FW_i) [T (FW: AFDW) x A]^{-1}, \]

where \(FW_t\) is the final fresh weight (g), \(FW_i\) is the initial fresh weight (g), \(T\) is the number of days of the cultivation (day), \(A\) is the surface area of the sponge – filter (m²). Due to water loss during measurements, wet biomass measurement in between the experiments was not carried out. There were other indicators of growth such as increase in biomass volume, green colour of the algal tissue and existence of large quantities of air bubbles within the macroalgal biomass. The ammonium removal rate in each treatment was
determined by subtracting the removal rate of the respective negative control (i.e. with no algae) from the removal rates of the treatments.

The relative contents of total lipid, carbohydrate, protein, and chlorophyll were determined according to methods described in Moheimani et al. (2013). The biochemical parameters, carbohydrates, proteins and lipids were analysed and expressed in percent ash-free dry weight (% AFDW).

The photosynthetic activity of the consortium was studied via variable fluorescence cum maximum fluorescence measurements of chlorophyll a using a Handy PEA Chlorophyll Fluorimeter (Hansatech, UK). This fluorimeter consists of a Handy PEA control and sensor units. The sensor unit consisted of an array of three ultra-bright red light emitting diodes (LED’s) that provided the non-actinic measuring light (spectral peak wavelength of 650nm). The maximum quantum yields in light (Fq/Fm') of harvested macroalgae samples were evaluated using the saturation light method (up to 3500 µmol photons m\(^{-2}\) s\(^{-1}\) at the surface of the sample). Samples harvested from treatments were quickly focused and measurements were immediately made. A minimum of three replicates each of fresh samples were used for estimation of the maximum quantum yield.

A diurnal study was carried out by sample measurements at hour 0 (pre-dawn) and hour 13 (pre-dusk) to investigate the photosynthetic response of the macroalgae to the increase in temperature that usually follow high daylight solar irradiance and probable recovery of the photosynthetic apparatus after sunset. A pseudo-replicate that consisted of a minimum three 2 g (wet weight) aliquots of light adapted algae on each sampling time, was dark adapted for 20 minutes (based on preliminary experiment in this study), and the maximum quantum yield (Fv/Fm), which indicates the quantum efficiency of photosystem II (PSII), was measured according to Cosgrove and Borowitzka (2006). The dark adaptation is significant because it enables the oxidation of electron transport chain and cause all non-photochemical quenching processes to relax, allowing maximum chlorophyll fluorescence yield to be measured.

2.6 Operational condition

The sand-filtered effluent was characterised for ammonia, dissolved oxygen, phosphorus, total alkalinity, chemical oxygen demand (COD), biochemical oxygen demand (BOD), pH and selected metals. Temperature in the cultures treating the ADPE was tracked
with an underwater data recorder (Tinytag TG-4100). The culture DO and pH were monitored daily by manual measurements using DO (SevenGo Pro, Metler Toledo) and pH (Aqua-P) meters respectively at 8 am, 12 pm, 3 pm and 6 pm. Measurements of ammonia, phosphorus, total alkalinity, COD, BOD, and metals were carried out using kit methods via a photometer (Spectroquant Move 100).

Bacterial counts were determined at the beginning and end of the experiment using a 3M™ Petrifilm™ Enterobacteriaceae Count Plates Kit (Silbernagel and Lindberg, 2003). The 3M™ Petrifilm™ Enterobacteriaceae Count Plates Method is a simple method for the enumeration of Enterobacteriaceae in products such as foods. The petrifilm consisted of a medium that is optimized for the growth of Enterobacteriaceae but at the same time inhibits the growth of Gram-positive bacteria. This product contained a pH indicator, a dye to improve the visualization of growth, and a cold-water soluble gelling agent enclosed in the plate (http://www.3m.com.au). Samples from the treatments were serially diluted, plated on the petrifilm and incubated at 37°C for 48 hours. The total bacterial colony on the plates were enumerated and the percentage reduction calculated as
\[
\frac{(initial \ bacterial \ count - final \ bacterial \ count)}{Initial \ count} \times 100\%.
\]

2.7 Statistical analysis

The difference between treatments during growth in ADPE was analyzed using a one-way analysis of variance (ANOVA). All measures were expressed in means ± standard error (SE) over the experimental duration and significant differences were declared at 5% probability level. The Duncan’s multiple range test was used for testing significant differences in means.
3.0 Results and Discussion

3.1 Bioprospecting

Five macroalgal species (Figure 1a-e) were observed to attach to the filters, two (2) of which were found to efficiently grow in both Chu 13 and ADPE media while the rest did not survive. These two macroalgal isolates mutually existed together as a consortium and were identified as *Rhizoclonium* sp. and *Ulothrix* sp. (Fig. 1b, c) based on light microscopy. These species were among the macroalgae observed to have attached to the sponge-filters at the beginning of the experiment.

3.2 Culture conditions

The average daily solar radiation (Figure 2a) ranged from 91.2 to 486.3 W m\(^{-2}\) (Mean, 341.7 ± 6.43 W m\(^{-2}\)) with nearly all days sunlit throughout the experiment. Daylight solar intensity in some days was as high as 1551 W m\(^{-2}\). It is necessary to emphasize that the consortium tolerated the high solar radiation, as there was no physical damage or death of the cultures. The other environmental parameters such as culture and air temperatures, did not vary significantly (Wilcoxon Signed Rank Test, N = 115, W = -609.00, p = 0.390) during the entire experiment. The average daily air and culture temperatures (Figure 2b) ranged from 15.0 to 32.8 °C (Mean, 22.8 ± 0.36 °C) and 17.4 to 28.4 °C (Mean, 22.8 ± 0.23 °C) respectively. The dissolved oxygen (DO) concentration (Fig.3b) in the cultures showed no fluctuations and value was usually on average approximately 8 mgO\(_2\) L\(^{-1}\) (range 7.7 to 8.1 mgO\(_2\) L\(^{-1}\)) in all treatments. The average pH values (Figure 3) of treatments (range, 8.6 ± 0.15 - 9.2 ± 0.34) with algae in ADPE were similar to the one with no algae (ADPE only) (8.6 ± 0.20) but was significantly (p<0.05) higher than the value (6.5 ± 0.37) found in the positive control (Modified Chu 13 Medium, 55 mg NH\(_4^+\)-N L\(^{-1}\)). It was observed that pH value of the positive control decreased progressively with time (Figure 3).

3.3 Ammonium removal rates

Table 2 shows the ammonium removal rates of the macroalgal consortium under the different ADPE treatments during the period of the experiment. The variation in ammonium concentration in ADPE-grown algae cultures with time, at different initial concentrations, shows that the final ammonium concentrations decreased after six days of the cultivation (Figure not shown). The ammonium removal rates varied from 2.0 ± 0.70 mg NH\(_4^+\)-N L\(^{-1}\) to 30.6 ± 6.50 mg NH\(_4^+\)-N L\(^{-1}\). Comparing treatments with \(^1\) ADPE 55 and positive control (Chu
13), the ammonium removal rate of the former (3.8 ± 1.60 mg NH$_4^+$-N L$^{-1}$) is statistically insignificant (p = 0.763) to the latter (2.0 ± 0.70 mg NH$_4^+$-N L$^{-1}$). Similarly, removal rates in treatments with ADPE 150, 199, and 248 were not significantly (Duncan test, p = 0.291) different from each other. The highest ammonium removal rate (30.6 ± 6.50 mg NH$_4^+$-N L$^{-1}$) was achieved in the treatment with ADPE 248. Based on the ammonium removal rates from the ADPE, the macroalgae consortium would be ideal for integrated pig farming, with removal rate significantly (p<<0.05) higher in elevated ammonium concentration (initial ammonium concentration = 248) than low ammonium concentration (initial ammonium concentration = 55.6). The final ammonium concentration on the sixth (medium renewal) day appeared to be concentration dependent, since ammonium was almost exhausted in the treatments with low ammonium concentrations. Above the maximum ammonium concentration (ADPE 248), the removal rate decreased with further increase in ammonium concentration, resulting in the death of the alga after 48 hours. The consortium is seen to be unable to tolerate ammonium at concentrations greater than 250 mg NH$_4^+$-N L$^{-1}$. Increasing media ammonium concentration from 55 to 248 mg NH$_4^+$-N L$^{-1}$ resulted in a higher bacterial reduction rate (Table 2).

Ammonium removal rates of the consortium of macroalgae trialled show that they are a potential sink for ammonia in ADPE and excellent candidates for integrated pork farming. This is due to macroalgal capability to survive and efficiently grow, under conditions similar to pond-based piggery wastewater treatment. Removal of ammonium from the growth medium was largely due to nutrient uptake by the macroalgae, considering that the decrease in ammonium level in the negative control was negligibly small (removal rate = 3.21 mg L$^{-1}$d$^{-1}$). However, the negative (no alga) control experiment further reveals that uptake of ammonium by the algae is not the only direct pathway for ammonium removal from ADPE, showing that ammonium removal is not entirely biological. The exact role of alternative routes for ammonia removal was not studied in this experiment. Volatilization, annamox, and denitrification are potential alternative routes for ammonium removal because the receiving vessels for the ADPE medium were unmixed although the DO did not go below 6 mg O$_2$ L$^{-1}$. Besides ammonium uptake by macroalgae, the growth of microalgae was also responsible for ammonium removal due to their dominance in the experimental set-up (including the no alga control) after three days of cultivation. Based on the results, it is reasonable to assume the possibility of achieving even higher ammonium tolerance and removal rates under careful adaptation and optimized conditions. Assimilation of NH$_3$ (and NH$_4^+$) by macroalgae is 2-3
times quicker than NO$_3^-$ (Neori et al., 2004). Ammonia and nitrate are chemically reduced and oxidized compounds respectively. Metabolically, this is an interesting outcome since ammonium can be directly fixed into amino acids of proteins (Ahn et al., 1998). The finding of this study is in agreement with the result of Martínez et al. (2012), who reported a linear increase in ammonium uptake rate (up to 67 mg N gDW$^{-1}$ d$^{-1}$) by Ulva intestinalis with ammonium concentration up to 50 µM NH$_4^+$-N. Based on the removal rate from the highest ammonium concentration tolerated by the consortium in this study, the result compares well (although higher) with the outcome (21.1 mgN gDW$^{-1}$ d$^{-1}$ ammonium removal rate) reported by Sode et al. (2013), who cultivated Ulva lactuca at a maximum of 50 µM NH$_4^+$ concentration of reject water and achieved 94% nitrogen removal. The ammonium removal rates achieved in this experiment were highest at 30 mg NH$_4^+$-N L$^{-1}$d$^{-1}$, which again was similar to the findings described by Msuya and Neori (2008) from fish pond effluents. Looking at the ammonium concentration left in the final effluent on the sixth (renewal) day, this study shows that the concentration in the ADPE should be kept below 150 mg NH$_4^+$-N L$^{-1}$. Nevertheless, to attain higher ammonium removal from ADPE, the concentration should be kept between 150 mg NH$_4^+$-N L$^{-1}$ and 260 mg NH$_4^+$-N L$^{-1}$, where increasing ammonium removal rates correlated with higher biomass productivity (Table 2).

Integrating macroalgal culture to farm management strategies for nutrient removal with methods for biomass removal via controlled harvest could add economic incentives for producers. The harvested biomass could potentially serve commercial functions such as fertilizers, feed, and/or bioenergy feedstock (Cavallo et al., 2006; Nwoba et al., 2016). Hence, for macroalgae to be suitable for an integrated piggery effluent management plan, such algae must be robust to achieve efficient ammonium removal and tolerate the wastewater conditions. In addition, it was observed that the consortium tolerated broad environmental conditions prevalent in the ADPE ponds. In practice, this study shows that growth of the macroalgae consortium in ADPE would require dilution with freshwater (which is increasingly scarce) to reduce the ammonium content, since the algae could not survive ammonium concentration higher than 248 mg NH$_4^+$-N L$^{-1}$. Conversely, Nwoba et al. (2016) successfully grew a microalgal consortium (Chlorella sp., Scenedesmus sp. and pennate diatom) in undiluted ADPE under outdoor condition. Most recently, Wang and colleagues (2016) also found that UV treating can significantly improve microalgal growth on undiluted ADPE and nutrient removal. A promising option would be a two-stage sequential technology that would involve first treating the undiluted ADPE with the microalgal consortium to
reduce the ammonia content to a level that the macroalgae consortium can be used to polish the effluent.

3.4 Biomass productivity

The biomass productivity of the consortium in the different ADPE-based medium is shown in Table 2. The biomass productivity obtained from the treatment in ADPE 150 (33.7 ± 1.26 g AFDW m$^{-2}$ d$^{-1}$) was 1.14 times higher than ADPE 248. (Table 2). However, no difference was found between the macroalgal productivities at ADPE 55, 150, 199 and Chu 55 (Table 2). Moreover, at ADPE 248, the biomass productivity (29.6 ± 0.58 g AFDW m$^{-2}$ d$^{-1}$) is not significantly different from that (30.2 ± 0.76 g AFDW m$^{-2}$ d$^{-1}$) obtained with Chu 55 (Table 2). Nielsen et al. (2012) also observed that increasing the concentration of anaerobic digested pig manure (measured as external ammonium concentration) had a positive impact on the specific growth rate of *Ulva lactuca* at lower concentrations, but stagnated growth rate at concentrations exceeding 0.45 mg NH$_4^+$-N L$^{-1}$. A similar outcome in this study was observed, since the biomass productivity at ADPE 199 and 248 was not significantly different to those obtained at lower ammonium concentration. Furthermore, macroalgal biomass productivity obtained in this study compares well with previous reports, 28.4 – 37.6 g DW m$^{-2}$ d$^{-1}$ by Msuya and Neori (2008), and 25.1 g DW m$^{-2}$ d$^{-1}$ by Bruhn et al. (2011). Excitingly, the high biomass produced by this consortium under the meteorological conditions of the current experiment shows the possibility of obtaining higher economic revenue during ecotechnological application of these algae. Significantly higher macroalgae biomass productivity was achieved at ADPE 150 compared to ADPE 248. However, no significant difference was found between the ammonium removal rates between these treatments.

Generally, biomass is proportional to nutrient removal rate since the nutrient can be uptake by macroalgae biomass for their growth. Such contradicting outcome could indicate two potential scenarios. The first scenario can be higher productivity at ADPE 150 due to less ammonium toxicity when compared to ADPE 248. The alternative scenario can be due to the other potential microbial reactions such as nitrification and de-nitrification during cultivation. It is to be noted that the ADPE tested in this study was not sterilised prior to macroalgal growth. Clearly, there is need for further studies to clarify some of these contradicting outcomes.

3.5 Biochemical composition of biomass
The variation of the biochemical contents (total protein, carbohydrates, and lipids) of the consortium biomass is shown in Table 2. The total protein content (43.4 – 45.0% AFDW) of the consortium grown in ADPE did not vary with ammonium concentration applied. Similarly, the protein content of biomass from the Chu 13 medium was not significantly different (p>0.05) from those grown in ADPE. The protein content of the consortium was within the range of 10–47% dry weight (DW) reported for red and green seaweeds (Wong and Cheung, 2000). Furthermore, the results demonstrate that the protein content of the algae was independent of the concentration of ammonium applied within the experimental conditions. This outcome was contrary to expectation since the highest ammonium concentration (248mg NH$_4^+$-N L$^{-1}$ ADPE) tolerated by the consortium produced similar protein content, revealing that the protein content of the consortium is not directly dependent on the ammonium concentration.

Carbohydrate represented the major biomolecule found in the biomass and ranged between 42.8% AFDW and 54.8% AFDW (Table 2). The total carbohydrate content of the Chu 13 medium (55 mg NH$_4^+$-N L$^{-1}$) was similar to the ADPE 55 and 150, but significantly (p<0.05) lower than the ADPE 199 and 248. The values found in this work, although higher than amounts found in most higher plants, is consistent with results (50.3–55.4 % DW) reported by Wong and Cheung (2000) for red and green seaweeds. Astals and colleagues (2015) found that microalgae biomass can be co-digested with piggery waste to improve the overall methane production. Considering the lower lipid content of macroalgae compared with microalgae, macroalgal generated biomass should also be suitable for generating methane in anaerobic digestion process.

There have been few studies that have examined the potential products available from freshwater macroalgae. Much of what has been studied to date is concerned with the elemental (ultimate) ratios and total fractions of protein, lipid and carbohydrates to determine the greatest yield potential. Studies by Neveux et al. (2015; 2016) focused on four marine and two freshwater macroalgae reported that in general, freshwater algal ash content of dry weight was lower (17.8-20.6%) when compared to marine (25.5-36.6%). Freshwater macroalgae carbohydrate content is very high (41-44.4%) when compared to protein (22.5-26.8%) and lipid (5.3-9.4%) contents. Due to the lower ash content, freshwater macroalgae also had a higher calorific value (15.8– 16.4 MJ kg$^{-1}$) than marine (10.3 - 12.7 MJ kg$^{-1}$). Marine species also showed higher biomass productivity than freshwater species with very low lipid productivity in both marine and freshwater macroalgae species.
3.6 Chlorophylls content of the consortium grown in ADPE

Here, chlorophylls $a$ and $b$ contents were found to increase with increasing ADPE concentration (Table 2). Correlation indicated a significantly positive association ($r = 0.889$, $p = 0.044$, Pearson product moment) between initial ammonium concentrations and chlorophyll $a$ contents of the biomass from the different treatments. Comparisons of treatments, Chu 13 medium and ADPE 55, showed there was no significant ($p>0.05$) difference in the chlorophyll $a$ content. Similarly, there was no significant ($p>0.05$) difference in the chlorophyll $a$ contents of ADPE 150, 199, and 248 media. However, the chlorophyll $a$ contents found in ADPE 150, 199 and 248 systems were significantly ($p<0.05$) different from the ADPE 55 and Chu 13 media. Similar results and relation was found in the chlorophyll $b$ contents of the treatments (Table 2).

Chlorophyll $a$ is one of the light harvesting pigments found in all algae and plays a fundamental role in photochemical energy transformation in photosynthetic organisms. Chlorophyll $b$ is a photosynthetic accessory pigment that participates efficiently in photosynthesis (Kuczynska et al., 2015). Under light limiting conditions (as found in the ADPE treatments due to dark colour of the effluent which significantly reduced light penetration), algae increase the amount or size of their photosynthetic units (PSUs), which are composed of light harvesting molecules (e.g. chlorophyll) (Vadiveloo et al., 2015). A plausible explanation to this phenomenon is that algae increase the size or number of their PSUs in order to compensate for the limiting light through enhanced capturing of the incident natural light and transferring them to the reaction centers (RCs). This invariably means that the maximum rate of photosynthesis will be achieved under limiting light conditions thereby increasing the efficiency of the light harvesting units. Therefore, this serves to explain the higher chlorophylls $a$ and $b$ contents in treatments with ADPE, and clearly shows that the macroalgae have the ability to acclimate to low light levels occasioned by the dark nature of the effluent.

Furthermore, pigments are affected by the nitrogen status of algae. Reports have shown that the chlorophyll $a$ content of algae increases with increase in their cellular nitrogen (Fogg and Thake, 1987). The pigments content of macroalgae can decrease because of growth and insufficient availability of nitrogen for sustained biosynthesis (Kim et al., 2007). Considering that in the high ammonium treatments (e.g. 150 – 248 mg NH$_4^+$-NL$^{-1}$) in this study, ammonium was not exhausted, coupled with the high protein content irrespective of
ammonium concentration applied, nitrogen availability was not a limiting factor. Similar trends in chlorophyll $a$ content, ammonium removal rates and protein content of the consortium biomass grown in high ammonium (i.e. ADPE 150, 199, and 248 media), where an increase in ammonium concentration yielded no further increase in the parameters, were also observed.

3.7 Photosynthetic performance of the consortium

To ascertain the capacity of the photosynthetic apparatus and the photophysiology of the culture under the high solar intensity with increased temperature, the maximum quantum yield ($F_v/F_m$) was measured as a sensitive indicator of the algae photosynthetic performance. The $F_v/F_m$ is an index for the estimation of the maximum quantum yield of photochemistry at PSII and is usually used as a marker of stress (or physical fitness) of plants including algae (Parkhill et al., 2001). The $F_v/F_m$ for all the treatments remained high, ranging between 0.42 ± 0.011 and 0.66 ± 0.006 at pre-dawn. The $F_v/F_m$ values of the dark-adapted samples were highest during the pre-dawn and this was followed by a decrease at hour 06 and further decrease at noon, revealing that the algae started experiencing stress (Fig. 4b). Under light adaptation (Fig. 4a), the effective quantum yield (Vadiveloo et al., 2016), $F_q'/F_m'$ values of the treatments remained low throughout the midday period while the pre-dusk measurement showed that the values were similar to the pre-dawn. However, values were found to recover to be highest at pre-dusk when solar irradiance and subsequently temperature decreased (Figure 4a-b). The rapid decrease in $F_v/F_m$ during the midday solar irradiance indicated a high degree of photoinhibition. In other words, the decrease in $F_v/F_m$ at midday would be probably due to photoinhibition at PSII and regular photoprotective mechanism, but was not due to variations in the nutritional status of the culture. The high values of $F_v/F_m$ obtained at pre-dusk means that the photosynthetic machinery was able to recover from solar-induced photodamage by ultra violet (UV) radiation. Wavelengths in the UV range of electromagnetic spectrum have been found to be lethal to photosynthetic processes because of their ability (due to high energy content) to destabilize molecular bonds and genetic machinery of organisms (Rozema et al., 1997). PSII is the most sensitive photosynthetic apparatus that is prone to damage by elevated temperature and irradiance (Beer et al., 2000). The data reveals that this consortium is robust and tolerant to the confounding variables of high temperature, solar radiation and ammonium concentration, with the $F_v/F_m$ inversely proportional to the available solar radiation.
4. Conclusion

The current study indicates the ability of *Rhizoclonium* sp. and *Ulothrix* sp. to diminish significantly the ammonium concentration in high ammonia ADPE and recover the quality of the water. The result of the design using this consortium reveals that it is possible to develop a better ecotechnologically sound practice that is sustainable for pork production effluent. The consortium showed potential as an efficient ammonium nitrogen pump while at the same time generating significant amount of biomass that could be suitable for animal feed or bioenergy.

Acknowledgements

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References


**Table 1**: Chemical composition of the ADPE used for the growth of the macroalgae

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonia (mg L$^{-1}$ NH$_4^+$-N)</td>
<td>1315.17±40.48</td>
</tr>
<tr>
<td>Total Phosphate, (mg L$^{-1}$ PO$_4$-P)</td>
<td>34.55±3.75</td>
</tr>
<tr>
<td>Nitrite (µg L$^{-1}$ NO$_2$-N)</td>
<td>10.53±2.15</td>
</tr>
<tr>
<td>Magnesium (mg L$^{-1}$ Mg)</td>
<td>224</td>
</tr>
<tr>
<td>Potassium (mg L$^{-1}$ K)</td>
<td>700</td>
</tr>
<tr>
<td>Total Iron (mg L$^{-1}$ Fe)</td>
<td>12.4</td>
</tr>
<tr>
<td>Total alkalinity (or acid capacity) (mmol L$^{-1}$ OH)</td>
<td>129</td>
</tr>
<tr>
<td>Nitrate (mg L$^{-1}$ NO$_3$-N)</td>
<td>18.70±2.96</td>
</tr>
<tr>
<td>Chemical Oxygen Demand, COD (mg L$^{-1}$)</td>
<td>1585.50±122.50</td>
</tr>
<tr>
<td>Total nitrogen (mg L$^{-1}$ N)</td>
<td>1430</td>
</tr>
<tr>
<td>pH</td>
<td>8.2±0.09</td>
</tr>
</tbody>
</table>
Table 2. Ammonium removal rates, biochemical composition and chlorophylls content of macroalgae consortium treated with different ammonium concentration in ADPE-based medium

<table>
<thead>
<tr>
<th>Ammonium concentration (mg NH₄⁺-N L⁻¹)</th>
<th>Protein (%AFDW)</th>
<th>Carbohydrate (%AFDW)</th>
<th>Lipids (% AFDW)</th>
<th>Ammonia removal rates (mg L⁻¹ d⁻¹)</th>
<th>Chlorophyll content (µg g⁻¹)</th>
<th>Biomass Productivity (gAFDWm⁻²d⁻¹)</th>
<th>Bacterial reduction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chu 13</td>
<td>44.63±0.967ᵃ</td>
<td>42.82±2.197ᵇ</td>
<td>4.72±0.206ᵃ</td>
<td>1.96±0.70ᵇ</td>
<td>14.34±3.238ᵇ</td>
<td>7.01±1.894ᵃ</td>
<td>NA</td>
</tr>
<tr>
<td>ADPE 55</td>
<td>43.84±1.919ᵃ</td>
<td>48.13±0.327ᵇ</td>
<td>5.57±0.173ᵃ</td>
<td>3.80±1.16ᵇ</td>
<td>17.84±1.411ᵇ</td>
<td>7.85±0.989ᵇ</td>
<td>31.19±1.540ᵇ</td>
</tr>
<tr>
<td>ADPE 150</td>
<td>43.39±0.476ᵃ</td>
<td>43.54±2.003ᵇ</td>
<td>4.98±0.251ᵃ</td>
<td>27.34±6.18ᵃ</td>
<td>26.38±0.272ᵃ</td>
<td>15.93±1.030ᵃ</td>
<td>33.73±1.259ᵃ</td>
</tr>
<tr>
<td>ADPE 199</td>
<td>44.99±1.607ᵃ</td>
<td>51.62±3.534ᵃ</td>
<td>3.57±0.212ᵇ</td>
<td>23.70±2.82ᵃ</td>
<td>25.61±0.489ᵃ</td>
<td>13.95±1.263ᵇ</td>
<td>30.99±1.165ᵇ</td>
</tr>
<tr>
<td>ADPE 248</td>
<td>44.45±2.889ᵃ</td>
<td>54.79±1.264ᵃ</td>
<td>3.07±0.189ᵇ</td>
<td>30.62±6.50ᵃ</td>
<td>26.26±0.580ᵃ</td>
<td>18.11±3.773ᵃ</td>
<td>29.56±0.584ᵇ</td>
</tr>
</tbody>
</table>

ᵃ,ᵇ: Significant difference at p<0.05; NA, not applicable.
List of Figures:

Figure 1. Photomicrographs of macroalgae, (a) Spirogyra sp., (b) Rhizoclonium sp., (c) Ulothrix sp., (d) Gayraluia sp., (e) Cladophora sp. found attached to the sponge filter upon collection, (f) photograph of the sponge-filter mat installed in Canning River, (g) Schematic of the tipping bucket system used for the cultivation of the macroalgae.

Figure 2. Panel A, average solar radiation, panel B, average culture (dotted line) and air (solid line) temperatures variation during growth of macroalgae consortium over the experimental period.

Figure 3. Changes in pH and DO of the various treatments throughout the experimental period. Negative control Chu 55 (filled circle), positive control, Chu 13 (empty circle), ADPE 55 (filled triangle), ADPE 150 (empty triangle), ADPE 199 (filled square) and ADPE 248 (empty square).

Figure 4. Diurnal changes in the photosynthetic response of the macroalgae consortium under light (panel a) and dark (panel b) adaptations. Bars with the same letter across groups are not significantly different (p>0.05).
Figure 1.
Figure 2.

![Graph showing average daily solar radiation and temperature over time.](image-url)
Figure 3.
Figure 4.