Viability of combining microalgae and macroalgae cultures for treating anaerobically digested piggery effluent

Ashiwin Vadiveloo¹, Emeka Godfrey Nwoba¹, Navid Reza Moheimani¹,²,⁎

¹. Algae R&D Centre, School of Veterinary and Life Sciences, Murdoch University, Western Australia 6150, Australia E-mail: ashiwin.vadiveloo@murdoch.edu.au
². Centre for Sustainable Aquatic Ecosystems, Harry Butler Institute, Murdoch University, Western Australia 6150, Australia

ARTICLE INFO

Article history:
Received 2 November 2018
Revised 6 March 2019
Accepted 6 March 2019
Available online 18 March 2019

Keywords:
Phytoremediation
Piggery effluent
Co-cultivation
Cladophora
Chlorella sp.
Scenedesmus sp

ABSTRACT

Algal phytoremediation represents a practical green solution for treating anaerobically digested piggery effluent (ADPE). The potential and viability of combining microalgae and macroalgae cultivation for the efficient treatment of ADPE were evaluated in this study. Bioprospecting the ability of different locally isolated macroalgae species illustrated the potential of Cladophora sp. to successfully grow and treat ADPE with up to 150 mg/L NH₄⁺ with a biomass productivity of (0.13 ± 0.02) g/(L·day) and ammonium removal rate of (10.23 ± 0.18) mg/(L·day) NH₄⁺. When grown by itself, the microalgae consortium used in this study consisting of Chlorella sp. and Scenedesmus sp. was found to grow and treat undiluted ADPE (up to 525 mg/L NH₄⁺) with an average ammonium removal rate of (25 mg/(L·day) NH₄⁺) and biomass productivity of (0.012 ± 0.0001) g/(L·day). Nevertheless, when combined together, despite the different cultivation systems (attached and non-attached) evaluated, microalgae and macroalgae were unable to co-exist together and treat ADPE as their respective growth were inversely related to each other due to direct competition for nutrients and available resources as well as the negative physical interaction between both algal groups.

© 2019 The Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences. Published by Elsevier B.V.

Introduction

The vast imbalance between the distribution of freshwater resources and the global human population is significantly expected to widen due to the exponential growth of the population as well as the expansion of the global economy (Häder et al., 1998). Therefore, there is great need for the sustainable and optimized use of available natural resources (i.e., water, food and raw materials) as well as their recovery from waste streams, representing a favorable shift from a traditional linear economy to a circular one (Sharma, 1986). Among the major challenges towards such green environmental initiatives is the efficient recovery and reuse of waste streams generated by a wide range of human activities such as industrial, domestic and agricultural practices. Agricultural wastewater arising from livestock production facilities are among the major contributor of nutrient rich (i.e., nitrogen and phosphorus) wastewater that can be of great concern if not dealt with appropriately (Hoek et al., 1995; Nan and Dong, 2004). Commercial pig production generate a vast amount of

⁎ Corresponding author. E-mail: n.moheimani@murdoch.edu.au. (Navid Reza Moheimani).

https://doi.org/10.1016/j.jes.2019.03.003
1001-0742 © 2019 The Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences. Published by Elsevier B.V.
organic rich waste effluents that can be of great significance, as improper management these waste streams can be detrimental to the environment via the emissions of greenhouse gases (carbon footprint), spread of pathogens, and widespread pollution of soil, surface and ground waters through nutrient enrichment and leaching (Diez et al., 2001; Maraseni and Maroulis, 2008).

Currently, anaerobic digestion (AD) systems are the most common primary treatment method employed for piggery wastewater (Buchanan et al., 2013). AD systems allow for the simultaneous removal of organic carbon and the generation of methane that can be exploited as a source of bioenergy (Buchanan et al., 2013). Nonetheless, anaerobically digested piggery effluent (ADPE) arising from such systems are still restricted by elevated nutrient content that can result in the eutrophication of water bodies if directly released to the environment which in return brings forward an increase to the economic cost of the society (Tucker et al., 2010). Therefore, there is great need for innovative and cost-effective technologies to efficiently treat ADPE for safe environmental discharge or reuse.

Phytoremediation represents a sustainable and energy effective solution that exploits the inherent potential of microalgae and macroalgae for the efficient removal or biotransformation of pollutants (i.e., nutrients) from various wastewater effluents (Phang et al., 2015). The integration of algal cultivation with piggery effluent management systems has been shown to be efficient in removing nutrients from ADPE down to regulatory acceptable limits required for discharge (Ayre et al., 2017; Nwoba et al., 2016a). Moreover, such innovation would also allow for the production of biomass that can be further valorized into multiple end-products from a futile waste stream which would generate additional revenue for farmers and pig producers. On its own, some freshwater microalgae have been shown capable of growth on undiluted ADPE (up to 1600 mg/L of ammonia) (Ayre et al., 2017). However, the commercial cultivation of microalgae is largely restricted due to further downstream processes such harvesting and dewatering which are cost ineffective (de Boer et al., 2012). On the other hand, due to their larger size and inherent characteristics, the harvesting and dewatering of macroalgae have been shown to be favorable and less expensive than microalgae (Nwoba et al., 2016b). Nonetheless, the ability of macroalgae to only grow on rather highly diluted ADPE (up to 8 times dilution required) is unfavorable due to the need of freshwater for dilution (Nwoba et al., 2016b). Therefore, based on their respective characteristics and abilities, an integrated system comprising of both microalgae and macroalgae is expected to increase the overall efficiency of ADPE treatment. In this view, the overarching aim of this study was to develop and evaluate the viability of an integrated and sequential treatment process comprising of both microalgae and macroalgae for treating undiluted ADPE. The main objective of this study was to improve the efficiency and economics of algal bioremediation of ADPE using a combination of both microalgae and macroalgae. Through this proposed system, microalgae would be initially grown on undiluted ADPE to reduce the concentration of nutrients up to an acceptable threshold that is suitable for the cultivation of macroalgae. Subsequently, macroalgae would be introduced into the ADPE-microalgae mix for the further treatment of remaining nutrients. If successful, the combination of microalgae and macroalgae would allow for both groups to absorb and utilize nutrients from ADPE, improving the overall waste stream quality for potential reuse and environmental discharge. Also part of this study, we evaluated the growth, photo-physiology and nutrient removal ability of both microalgae and macroalgae when grown individually as well as the combined cultures of both of them in different cultivation conditions.

1. Materials and methods

1.1. Anaerobic digestate of piggery effluent

The ADPE used in this study was obtained from the Medina Research Station located in Kwinana, Western Australia. The ADPE obtained from site was sand filtered to remove suspended solids and subsequently used for algae cultivation. Physico-chemical properties of the sand-filtered ADPE is summarized in Table 1.

1.2. Microalgae cultures

Microalgae consortium of Chlorella sp. and Scenedesmus sp. used in this study was previously isolated by Ayre et al. (2017) and was found capable of growing on undiluted ADPE up to 1600 mg/L ammonium (NH₄⁺-N). This microalgal consortium was grown and maintained semi-continuously in a 1 m² outdoor raceway pond operated at 20 cm depth and mixed with a single paddle wheel (4 blades = approximately 30 cm/sec mixing velocity) at the Algae R&D Centre of Murdoch University using ADPE as a source of nutrient enrichment (Cuello et al., 2014; Moheimani, 2016). The culture was maintained at exponential growth phase with periodical harvesting of cultures (50%) whenever they reached a pre-determined cellular concentration and was renewed with the required concentration of ADPE.

Microalgae biomass as organic biomass (ash-free dry weight, AFDW) of cultures was evaluated based on the methods of Moheimani et al. (2013). The maximum quantum yield in light (Fₚₐₚ/Fₚₐₚ₋₈) of harvested samples was evaluated and calculated using the saturation light method (=3500 μmol/ (m²·sec)) using a Water PAM fluorometer (Walz GmbH, Germany) as described in Cosgrove and Borowitzka (2006) and Vadiveloo et al. (2016).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonia (mg/L NH₄-N)</td>
<td>480–525</td>
</tr>
<tr>
<td>Nitrate (mg/L NO₃-N)</td>
<td>7.0–7.3</td>
</tr>
<tr>
<td>Nitrite (μg/L NO₂-N)</td>
<td>4.0–4.3</td>
</tr>
<tr>
<td>Magnesium (mg/L Mg)</td>
<td>82.5–87.5</td>
</tr>
<tr>
<td>Potassium (mg/L K)</td>
<td>265–273</td>
</tr>
<tr>
<td>Total iron (mg/L Fe)</td>
<td>4.3–4.8</td>
</tr>
<tr>
<td>Chemical oxygen demand, COD (mg/L)</td>
<td>600–675</td>
</tr>
<tr>
<td>Total phosphate (mg/L PO₄³⁻-P)</td>
<td>12.5–13.3</td>
</tr>
<tr>
<td>Total nitrogen (mg/L N)</td>
<td>525–550</td>
</tr>
</tbody>
</table>

Table 1 – Physico-chemical composition of undiluted and sand-filtered ADPE collected from Medina research station.
1.3. Macroalgae cultures

Three locally isolated species of macroalgae (Spirogyra sp., Rhizoclonium sp. and Cladophora sp.) were collected from different locations of the Canning River, Perth, Western Australia, using fishing nets on multiple visits to the site during different austral seasons. Oedogonium sp., L.1.40 was sourced from Southern Biological Pty Ltd., Australia. All macro-algal samples were initially maintained in aerated Modified Chu 13 medium (KNO₃ was replaced with 10 mg/L NH₄⁺-N of NH₄Cl) and positioned in a (25 ± 2)°C controlled temperature culture room at 120 μmol/(m²·sec) irradiance (Nwoba et al., 2016b).

In order to evaluate the viability of the selected macroalgae for nutrient removal, all four species were initially grown (1.5–2.0 g fresh biomass) in 150 mL of different concentrations of ADPE ranging from 50 to 250 mg/L of ammonium (NH₄⁺) in 250 mL conical flasks. Cultures were subjected to an irradiance of approximately (275 ± 23) μmol/(m²·sec) with a 12 hr:12 hr light dark cycle. The growth and physiological status of each macroalgae in triplicates (n = 3) at the different concentration of ADPE was evaluated through changes in fresh biomass and photosynthetic quantum yield values respectively (Nwoba et al., 2016b).

Biomass productivities of the macroalgae species were determined based on the changes of fresh biomass over time (Nwoba et al., 2016b). Samples collected from each treatment was gently padded down using paper towels until no moisture residues were left and then weighed to obtain values. This procedure for weighing did not appear to have any negative effect on the alga in terms of growth and photosynthesis (Nwoba et al., 2016b). The photosynthetic activity of the macroalgae strains was evaluated via maximum quantum yield measurements of chlorophyll a using a Handy PEA Chlorophyll Fluorimeter (Hansatech, UK) (Nwoba et al., 2016b). The maximum quantum yield in light (Fq’/Fm’) of harvested macroalgae samples were evaluated using the saturation light method (up to 3500 μmol/(m²·sec) at the surface of the sample) (Nwoba et al., 2016b). 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.

1.4. Combined microalgae and macroalgae liquid cultures for the treatment of ADPE

In this study, the selected macroalgae biomass was directly inoculated into 250 mL Erlenmeyer flask either containing a mix of microalgae together with ADPE or just ADPE by itself post the harvest of microalgae biomass at the required concentration.

1.5. Combined microalgae and attached macroalgae scrubbers to treat ADPE

An innovative outdoor algae reactor was also designed and evaluated as part of this study to identify the combined efficiency of microalgae and attached macroalgae scrubbers for the treatment of ADPE. The rationale behind this design was to mimic natural ecosystems in which macroalgae attached to the bottom bed (i.e., pebbles and sand) of various waterbodies was found to be able to co-exist and grow together with free floating microalgae. The cultivation surface of the customized macro-algal reactor was composed of two sheets of PVC Polycarbonate Sheets were glued together using silicone. Walls were constructed along both sides of the cultivation surface using strips of clear Perspex glued onto PVC Polycarbonate sheets sidewalls (Fig. 1a). The cultivation area of the algal reactor was fragmented into four narrow identical compartments to serve as replicates with PVC baffles dividing each of them (Fig. 1a). Each compartment of the reactor was covered entirely with sponge-filter mats for the attachment of the macroalgae (Nwoba et al.,...
2016a). Baffles separating each compartments were made of clear Perspex at a height of 6 cm and had a drilled hole of 1 cm in diameter in its middle (Fig. 1c). This drilled hole in each baffle was to allow ADPE from one compartment to flow into the next, therefore, allowing for the continuous and sequential flow of effluent from the start of the reactor to the end.

A 30-L portable water storage drum containing ADPE with an attached tap was positioned at the start of the reactor (Fig. 1a). The flow rate of ADPE from the storage drum onto the first compartment of the algal inclined reactor was standardized at 0.16 mL/sec. The storage drum was manually refilled with the desired ADPE concentration daily. Treated ADPE that had passed through the algae mats in all four compartments was channeled to a sump collection tank (400 L PVC black tub) at the end of the reactor.

1.6. Nutrient removal rates

Supernatant of the initial and treated ADPE samples at different time intervals were collected to evaluate the utilization of nutrients (i.e., ammonium, phosphate and nitrate) by the algal cultures. Nutrient concentration such as total ammonium, phosphate and nitrate was measured using a Hanna HI 83099 COD and multiparameter photometer (Hanna Instruments, USA) based on standard operating procedures and kits supplied by the manufacturer.

1.7. Statistical analysis

All measurements regarding the growth, photo-physiology and nutrient removal efficiency of both micro- and macroalgae were carried out using a minimum of 3 replicates (n = 3). Repeated Measure (RM) One-Way Analysis of Variance (ANOVA) followed by the post hoc test of Holm–Sidak was used to determine significant differences between the different treatments or parameters. All statistical analysis was performed using SigmaPlot version 12.5 for Windows.

2. Results and discussion

2.1. Microalgae productivity and nutrient removal rates in ADPE

The bioprospecting, establishment and growth of the microalgal consortium (Chlorella sp. and Scenedesmus sp.) used in this study to treat ADPE has been previously described by Ayre et al. (2017) and Nwoba et al. (2016a). The average volumetric biomass productivity of the microalgal consortium cultivated on ADPE and grown in 1 m² raceway pond was (0.012 ± 0.0001) g/(L·day) while the average ammonium removal rates from ADPE was (25.9 ± 8.60) mg/(L·day) NH₄⁻ during the cultivation period of 3 months (Nwoba et al., 2016a).

2.2. Macroalgae growth on ADPE

Among the four macroalgae species obtained, only Rhizoclonium sp. and Cladophora sp. were found capable of growth on ADPE between 50 and 150 mg/L NH₄⁺ (Fig. 2). Spirogyra sp. and Oedogonium sp. declined and did not survive at any of the tested ADPE concentrations (data not shown). Rhizoclonium sp. grew up to a maximum ADPE concentration of 100 mg/L NH₄⁺ while Cladophora sp. successfully grew in ADPE up to 150 mg/L NH₄⁺ (Fig. 2). Any further increase in the concentration of ADPE was found to be detrimental and resulted in the death of Rhizoclonium sp. and Cladophora sp. (Fig. 2). The significant reduction in Fₚ'/Fₚ₀ values to zero in both cultures were also recorded when ADPE nitrogen concentration was higher than their respective thresholds for both macroalgal species (Fig. 2).

The highest growth was observed at 150 mg/L NH₄ and 100 mg/L NH₄ for Cladophora sp. and Rhizoclonium sp., respectively (Fig. 2). However, Fₚ'/Fₚ₀ values of both macroalgae species trended significantly higher when grown at 50 mg/L NH₄ than the higher concentrations of ADPE (Fig. 2). The variation in Fₚ'/Fₚ₀ values highlights the instant physiological condition of a particular organism and represents the effectiveness of the open PSII reaction centres in capturing excitation energy (Genty et al., 1989). The higher Fₚ'/Fₚ₀ values at 50 mg/L NH₄ indicate that cultures were experiencing lower physiological stress when compared to the other concentrations of ADPE.

The biomass productivity and nutrient removal rates of Cladophora sp. and Rhizoclonium sp. when grown at the various ammonium concentrations are summarized in Table 2. The highest biomass productivity of Cladophora sp. was achieved when grown using ADPE at the concentration of 150 mg/L NH₄⁺ while no significant differences in terms of biomass productivity was observed for Rhizoclonium sp. when grown in ADPE with concentrations of 50 and 100 mg/L NH₄⁺ of (Table 2). In terms of ammonium removal rates, Cladophora sp. grown in ADPE with 150 mg/L NH₄⁺ was found to be the most efficient. In the case of Rhizoclonium sp., the highest ammonium removal rate was found when grown using ADPE at 100 mg/L NH₄⁺ (Table 2). Ammonium removal rates of Cladophora sp. at ADPE 150 mg/L NH₄⁺ was three-folds higher than that of Rhizoclonium sp. at ADPE 100 mg/L NH₄⁺ (Table 2). No significant differences were observed in the removal rates of phosphate for both species of macroalgae when grown at the different concentration of ADPE (Table 2).

2.3. Combined cultivation of liquid microalgae and macroalgae cultures in ADPE

Based on its ability to grow at a higher concentration of ADPE and its enhanced ammonium removal efficiency, Cladophora sp. was selected for the co-cultivation study with the previously established microalgae consortium consisting of Scenedesmus sp. and Chlorella sp. Two experimental conditions were evaluated in this study as summarized in Fig. 3.

At the start of the experiment, the microalgae consortium was initially grown on undiluted ADPE until the ammonium concentration was reduced to approximately 150 mg/L NH₄⁺ (Fig. 3b). In the first scenario, Cladophora sp. (1.5 g of fresh biomass) was directly inoculated into 150 mg/L NH₄⁺ ADPE (150 mL) containing approximately 1 × 10⁷ cells/mL of microalgae (predominantly Chlorella sp.) (macro + micro, Fig. 3d) in 250 mL Erlenmeyer flask. For the post-harvest ADPE without microalgae, microalgal cultures grown in ADPE at 150 mg/L NH₄⁺ were centrifuged at 3000 r/min for 10 min and
subsequently the supernatant was filtered through GF-C microfiber filter papers (Fig. 3c). The filtrate was then used as a growth medium for *Cladophora* sp. That represented the post-harvest treatment (macro–micro, Fig. 3e). Fig. 4 summarizes the changes in the growth and photophysiology of *Cladophora* sp. and *Chlorella* sp./*Scenedesmus* sp. consortium when grown together in ADPE at 150 mg/L NH₄⁺. There was an immediate decrease in the biomass yield and Fₚ'/Fₘ' value of *Cladophora* sp. when grown together with microalgal consortium (macro + micro). This decline continued throughout the co-cultivation period and eventually led to the death of the macroalgae as Fₚ'/Fₘ' values reached to zero (Figs. 4 and 5c). When macroalgae was inoculated to ADPE post the harvest of microalgae (macro–micro), there was an initial increase in *Cladophora* sp. biomass (Fig. 4). However, cultures subsequently deteriorated (biomass and Fₚ'/Fₘ' values) as the concentration of microalgae began to increase naturally (Fig. 4). It is to be noted that even the combination of both centrifugation and filtration procedures did not remove 100% of microalgae from the ADPE. It was found that even a very small initial concentration of microalgae (not detected) present in the ADPE could over grow and dominate the *Cladophora* sp. culture in less than 20 days of combined cultivation (Fig. 4).

In terms of microalgae growth, there was a significant drop in the biomass of the microalgae consortium during the initial period (Day 5) of co-cultivation with *Cladophora* (macro + micro) (Fig. 4). This decrease is believed to be brought forward by the epiphytic-like attachment of microalgal cells onto the fibers/filaments of *Cladophora* sp. (Fig. 5b). Nevertheless, biomass yield of microalgae increased steadily throughout the remaining growth period (Fig. 4). For the post-harvest

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cladophora sp.</th>
<th>Rhizoclonium sp.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Biomass productivity</strong></td>
<td><strong>ADPE 50</strong></td>
<td><strong>ADPE 100</strong></td>
</tr>
<tr>
<td>ADPE 50</td>
<td>0.11 ± 0.02a</td>
<td>0.06 ± 0.03b</td>
</tr>
<tr>
<td>ADPE 100</td>
<td>0.09 ± 0.01d</td>
<td>0.09 ± 0.02d</td>
</tr>
<tr>
<td>ADPE 150</td>
<td>0.09 ± 0.01d</td>
<td>0.09 ± 0.02d</td>
</tr>
<tr>
<td><strong>Ammonium removal rates</strong></td>
<td><strong>ADPE 50</strong></td>
<td><strong>ADPE 100</strong></td>
</tr>
<tr>
<td>ADPE 50</td>
<td>3.89 ± 0.11a</td>
<td>6.80 ± 0.12b</td>
</tr>
<tr>
<td>ADPE 100</td>
<td>2.08 ± 0.28d</td>
<td>3.07 ± 0.25e</td>
</tr>
<tr>
<td>ADPE 150</td>
<td>2.08 ± 0.28d</td>
<td>3.07 ± 0.25e</td>
</tr>
<tr>
<td><strong>Phosphate removal rates</strong></td>
<td><strong>ADPE 50</strong></td>
<td><strong>ADPE 100</strong></td>
</tr>
<tr>
<td>ADPE 50</td>
<td>0.44 ± 0.12a</td>
<td>0.59 ± 0.07a</td>
</tr>
<tr>
<td>ADPE 100</td>
<td>0.28 ± 0.08d</td>
<td>0.20 ± 0.01d</td>
</tr>
<tr>
<td>ADPE 150</td>
<td>0.28 ± 0.08d</td>
<td>0.20 ± 0.01d</td>
</tr>
</tbody>
</table>

The same letters after each value indicates no significant differences (one-way repeated measures ANOVA p > 0.05).
Inoculation of macroalgae (Macro + Micro)

Inoculation of macroalgae (Macro - Micro)

Growth, photophysiology and nutrient removal rate measurements

Centrifugation and filtration to remove microalgae biomass from ADPE

Microalgae at ADPE concentration of ≈ 150 mg/L NH₄⁺

Microalgae inoculated in undiluted ADPE (525 mg/L ammonium, NH₄⁺)

Fig. 3 – Flow chart of the experimental conditions for the combined cultivation of both microalgae and macroalgae. Macro: macroalgae; Micro: microalgae.

Fig. 4 – The growth and photo-physiology of Cladophora sp. and the microalgae consortium during the period of co-cultivation.
ADPE cultures (macro–micro), despite the absence of microalgae cells at the start of the experiment, Cladophora cultures outgrown by the microalgal consortium over time, resulting in the negative decline of Cladophora sp. Overall, the growth of Cladophora sp. was found to be inversely correlated with the growth of the microalgal consortium in both experimental conditions (Fig. 4).

The decline of Cladophora sp. during the co-cultivation with microalgae is believed to be due to a number of factors such as the reduced availability of light (due to the turbidity of ADPE and shading by the microalgal cells), low availability of nutrients (spatial competition with microalgae which are more efficient in utilizing nutrients) and the dominancy (faster growth) of microalgae.

![Fig. 5 – Photomicrographs during the co-cultivation of macroalgae with microalgae: (a) at the start of the experiment, (b) attachment of microalgal cell on the surface of the macroalgae, (c) and (d) attachment of microalgal cells on dead macroalgae fibers/filaments.](image)

![Fig. 6 – Nutrient removal rates of combined cultures of microalgae and Cladophora sp. in ADPE 150. (NO₃⁻).](image)
(microalgal epiphytism) which was seen to be detrimental to the Cladophora culture (Hein et al., 1995; Smith and Horne, 1988; Steneck, 1982).

The ammonium (NH₄⁺) removal rates of Cladophora cultures grown together with the microalgal consortium (macroalgae + microalgae) was significantly higher than those grown in post-harvest ADPE (macroalgae – microalgae) while the contrary was observed for the removal of nitrate (NO₃⁻) (Fig. 6). The higher ammonium removal rates recorded in the macroalgae + microalgae cultures corresponded with the higher concentration of microalgae recorded in the cultures. The microalgal consortium used in this study has been previously shown to have ammonium removal rates of up to (63.7 ± 12.1) mg/(L·day) NH₄-N (Ayre et al., 2017) which is approximately six times higher than that of Cladophora sp. used in this study.

It is also important to note that the removal of nutrients in ADPE can also be significantly influenced by the population and distribution of native nitrifying and denitrifying bacteria that are responsible for the conversion of ammonia to nitrate and the further reduction of nitrate (Bohutskyi et al., 2015). The possible presence of denitrifying bacteria could explain the higher nitrate removal rate of the macro-micro culture when compared to the macro + micro cultures. Weerasekara et al. (2016) evaluated the bacterial community composition of raw ADPE sourced from the same pond used in this study and highlighted the dominancy of Actinobacteria (50.7%), followed by Betaproteobacteria (19.9%) and Erysipelotrichi (8.25%) in this effluent.

Common Betaproteobacteria nitrifying bacteria such Nitromonas and Nitrobacter are responsible for the oxidation of ammonia and nitrite. In such, it is possible that bacterial based nitrification and denitrification could have also played a part in the removal of nutrients (i.e., nitrogen) (Hovanec and DeLong, 1996). Nwoba et al. (2016b) showed that an increase in the ammonium concentration of ADPE was found to reduce the overall bacterial population (up to 82%) when treated with macroalgae. Therefore, the impact of denitrifying and nitrifying bacteria on the removal of nutrient might be restricted at higher concentration of ammonia such as when working with undiluted ADPE. Furthermore, another important removal mechanism that needs to be taken into account when working with high initial ammonium-N concentrations is ammonia volatilization that is typically observed high rate algal ponds (HRAP) (Bohutskyi et al., 2015).

The composition of the microalgal consortium in both co-cultivation scenarios was also evaluated during this study. As illustrated in Fig. 7, the microalgal consortium was predominantly composed of Chlorella sp. at the start of macroalgae + microalgae treatment with almost no cells of Scenedesmus sp. Nonetheless, the concentration of Scenedesmus sp. exponentially increased throughout the remaining period and was found to be significantly higher than Chlorella sp. at the end of the experiment (Fig. 7). When Cladophora was grown on post microalgae harvested ADPE (macroalgae – microalgae), microalgal cells were initially not detectable (with concentration lower than 1 × 10⁵). However, the presence of both microalgal cells was observed after day 5 of the cultivation (Fig. 7). Subsequently, cell density of Scenedesmus was found to increase throughout the study while there was no significant increase in Chlorella sp. cell density (Fig. 7). The establishment of microalgae in the “macroalgae – microalgae” treatment despite prior harvest through centrifugation and filtration could be due to the presence of autospores produced by both Chlorella and Scenedesmus sp. which are much smaller than parent cells (Hoek et al., 1995; Sharma, 1986; Yamamoto et al., 2004).

Overall, the combined cultivation of the Cladophora sp. and the microalgae consortium in a single growth vessel under controlled conditions was found to be not viable as it resulted in the loss of Cladophora over time. Moreover, similar outcome was observed for Cladophora grown in post-harvest ADPE that were initially without any microalgae. Overtime, microalgae outgrew and overtook the cultures resulting in the decline of the macroalgae (Fig. 4).

2.4. Combined cultivation of microalgae and attached macroalgae to treat ADPE

The results of Section 2.3 clearly highlighted the inability of co-cultivating microalgae and non-attached macroalgae due

![Fig. 7 - Cellular concentration of the microalgae consortium co-cultivated with Cladophora sp.](image-url)
to the dominancy of microalgae. Based on this outcome, an outdoor reactor (Fig. 8) was customized to evaluate the potential of immobilized and attached macroalgal cultures as a way of scrubbing remaining nutrients from ADPE post-microalgal treatment. A continuous and constant flow of ADPE (with and without microalgae) was channeled through

Fig. 8 – The customized algal reactor setup for the combined cultivation of attached macro- and micro-algae: (a) compartments to house the macroalgae filter mats and culture, (b) baffles separating each macroalgae compartment and holes in the middle to allow the flow of ADPE, (c) shade cloth used to cover compartments, (d) storage bucket and tap to store the ADPE, (e) macroalgae consortium attached to the filter mats and (f) fully functional inclined reactor system.
the immobilised macroalgal culture (Fig. 8a–c). The aim of this continuous flow-through system was to improve the interaction of both these organisms (e.g., light availability and surface area), reduce contact period between both groups of algae and to maximize the nutrient removal rate of ADPE.

Due to the sheer volume of biomass required to cover each compartment of the algal reactor, fresh wild macroalgal biomass was collected from the original site on March 20, 2018. The biomass obtained mainly consisted of a consortium of *Spirogyra* sp. and *Rhizoclonium* sp. Almost, 800 g wet weight of this macroalgal consortium was attached to the sponge filters of each compartment (Fig. 8e and f). The attached macroalgal culture was set up under outdoor condition. Daily solar radiation of the cultivation period ranged between 2 and 1178.4 W/m² while the daily ambient air temperature was between 5.6 and 33.2°C (Appendix A Fig. S1). The maximum rainfall recorded during the study was 2.75 mm (Appendix A).

Fig. S1). High solar irradiance (up to 1178.4 W/m²) and rainfall recorded during the study was 2.75 mm (Appendix A Fig. S1). High solar irradiance (up to 1178.4 W/m²) and temperature (up to 33.2°C) was unavoidable as this study was carried out during the Austral summer. High incident sunlight illuminating the macroalgae attached to the mats was found to be detrimental to the photophysiology of the macroalgal (data not shown). Thus, to reduce the occurrence of photo-inhibition, shade cloths reducing approximately 50% of incoming solar irradiation was used to cover the macroalgae cultivation compartments (Fig. 8c) (Häder et al., 1998).

The macroalgal consortium was initially grown on ADPE ranging from 10 to 55 mg/L of NH₄⁺ to evaluate its growth and nutrient removal efficiency when grown as a batch for 4 day period (Table 3). After the completion of each batch, the treated effluent was completely drained from all the compartments and the sponge-filters hosting the macroalgal consortium were rinsed with tap water to remove debris and particles. Subsequently, the next concentration of ADPE was introduced to each compartment and was allowed to flow through the reactor before measurements were made over the subsequent 4 days.

For the mixed cultivation study, microalgal cultures grown on ADPE at 55 mg/L of ammonium was introduced at the start of the culture and allowed to flow through the reactor for 4 days. During this period, growth, photophysiology and nutrient removal rate of both the microalga and macroalga were evaluated. Table 3 highlights the nutrient removal rates of the macroalgal consortium under the different ADPE concentrations and also in combination with microalgae. The ammonium removal rates were in the range of (6.5 ± 0.8) to (22.6 ± 1.1) mg/(L·m²) NH₄⁺ for ADPE without any microalgae. The highest ammonium removal rate was found at ADPE concentration of 55 mg/L of NH₄⁺ (Table 3). Therefore, microalgal grown in ADPE at 55 mg/L of NH₄⁺ was selected for the combination study with the attached macroalga. The ammonium removal rates of ADPE treated with microalgae at 55 mg/L of NH₄⁺ was 28% and 51% lower than those recorded for ADPE 40 and ADPE 55 without microalgae (Table 3).

In terms of phosphate removal rates, no significant differences were observed between values recorded for ADPE 55 with and without microalgae (Table 3). Nitrate removal rates increased at higher concentrations of ADPE concentration but no significant differences were observed at ADPE 40 and ADPE 55 (Table 3). However, values were significantly lower when ADPE 55 treated with microalgae was used (Table 3). These results indicates a potential decline in physiological status and nutrient removal efficiency of attached macroalgal consortium when ADPE in combination with microalgae was used.

To identify the interaction between the microalgae and attached macroalgae, the distribution and photo-physiology of the microalgae consortium at different lengths (compartment) of the reactor was evaluated during the combined cultivation period at ADPE 55. As illustrated in Fig. 9, there was a significant decrease in the *Fₚ*/*Fₘ* values of the microalgae cultures as it traveled from the storage container (initial) through the four compartments of the inclined reactor. Initial *Fₚ*/*Fₘ* values of cultures in the storage bucket was (0.51 ± 0.04) while values of cultures exiting the 4th compartment of the reactor was as low as (0.14 ± 0.04) (Fig. 9). The decline in *Fₚ*/*Fₘ* of the macroalgae consortium was in line with the loss of biomass as cultures moved through the inclined reactor (Fig. 9). There was approximately 73.5% reduction in the biomass yield of cultures as it traveled from the storage bucket to the end of the first compartment (Fig. 9). This loss in biomass was observed to be primarily caused by the attachment of microalgae cells to macroalgae filaments and also onto the sponge filter itself. As shown previously, we also observed the attachment of microalgae cell onto macroalgae filaments in the indoor liquid (detached) macroalga-microalga treatment study (Fig. 5). There was approximately (90.4 ± 3.8)% and (93.1 ± 2.1)% reduction in the microalgal cell density of *Chlorella* and *Scenedesmus* sp. respectively between the storage containers from the start to the 4th compartment of the reactor (Fig. 9).

Fig. 10 summarizes the *Fₚ*/*Fₘ* of the macroalgae consortium during cultivation at the different concentrations of ADPE.
ADPE and as well as treating ADPE 55 with microalgae. Despite the continuous increase in ammonium concentration from 10 to 55 mg/L, no significant difference in $F'_q/F'_m$ were seen for the macroalgae consortium when grown by itself (Fig. 10). However, macroalgal $F'_q/F'_m$ was found to be significantly lower when ADPE 55 with microalgae was used. This clearly indicates an immediate decline in the physiological status of the macroalgae under this condition. The decline in macroalgal photo-physiology is believed to be a direct result of shading brought forward by the formation of microalgal layers that covered the surface of the macroalgal filaments and of the reactor (Appendix A Fig. 2S). The presence of microalgal layers above the macroalgal cultures also explains the loss of microalgal biomass as it traveled through the

---

Fig. 9 – The cellular composition, biomass yield and photo-physiology of the microalgal consortium at the different sampling compartment of the inclined algae reactor. The same letters above each column indicates no significant differences (one-way repeated measures ANOVA $p > 0.05$). The capital letters are used to indicate the significant difference between Chlorella sp. cell concentration while the small letters indicate difference in Scenedesmus sp. cell concentration for the last graph.
inclined reactor due to the attachment and entrapment of cells in these biological layers (Fig. 9).

The inverse co-relationship and inability of micro- and macro-algae to grow together is most certainly a result of competition for space, nutrients and availability of light between these algal groups (Armitage et al., 2005; Huisman et al., 1999; Smith and Horne, 1988). This was evident in our study as not only the photo-physiological of both the microalgae and macroalgae were negatively affected during co-cultivation but also ammonium removal rates were found to be significantly lower than that of macroalgae cultures grown by itself in ADPE (Table 3). Moreover, the entrapment of microalgae cells on the macroalgae biomass was also seen to be detrimental to both organisms.

2.5. General discussion

Classical ecology theories on competition predicts that, under idealized conditions, the one species capable to best acquire and use available limiting resources can significantly determine the distribution of natural populations by displacing all other competing species (Roughgarden, 1983; Titman, 1976). Similarly, under our controlled laboratory conditions, such competitive displacement was observed in the co-cultivated algal cultures and is believed to be a result of interactions between both groups of algae. The strong inverse competition for nutrients and available resources significantly favored the smaller unicellular microalgae cells with improved surface area to volume ratios over the larger macroalgae in all our experimental conditions (Grover, 1989; Stolte and Riegman, 1995). Especially when competing for light, these local interactions of both these organism are observed to be inversely correlated as the organisms closest to the light source are seen to be more dominant. This was evident in our studies as the smaller microalgae cells suspended in the ADPE and positioned closer to the light source was able to harvest and utilize incident light more successfully than the “heavier” macroalgae cultures, which sank to the bottom of both the Erlenmeyer flasks and the inclined reactor.

In both cultivation systems, the algal groups were still unable to co-exist for efficient treatment of ADPE due to direct competition for available resources and the negative interaction of both microalgae and macroalgae. In this study, we have also highlighted the detrimental changes that were brought upon when both these algal groups were cultivated together in ADPE due to resource competition and their respective interactions. However, we believe the outcome of competition identified in this study may change according to available resources and algal species.

3. Conclusions

Overall, through this study we have successfully isolated and demonstrated the viability of local microalgae and macroalgae strains that were capable of growth and efficient nutrient removal in various concentrations of ADPE. On its own, the microalgal consortium used in this study was capable of growth in undiluted ADPE with an ammonium removal rate of 25 mg/(L·day) NH₄⁺ while the bioprospecting of macroalgae species identified the ability of Cladophora to grow on diluted ADPE up to 150 mg/L of ammonium with a nutrient removal rate of 10.23 ± 0.18 mg/(L·day) NH₄⁺. Nonetheless, when combined together in different cultivation systems, the cultivation of microalgae and macroalgae was found to be unfeasible due to the negative physical interactions and competition for available resources by both algal groups.

Acknowledgments

This work was supported by the Cooperative Research Centre for High Integrity Australian Pork (Pork CRC) through the grant (Pork CRC 4A-109). We would like to also thank Mr. Jack...
Weatherhead for his assistance in designing and constructing the schematic diagrams of the manuscript.

**Appendix A. Supplementary data**

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jes.2019.03.003.

**REFERENCES**


