Growth of microalgae on undiluted anaerobic digestate of piggery effluent with high ammonium concentrations

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Abstract

Anaerobic digestate of piggery effluent (ADPE) is extremely high in ammonia toxic to many microorganisms. Bioprospecting and nutrient enrichment of several freshwater and wastewater samples combined and further acclimation resulted in a mixed culture containing at least three microalgae species capable of growing on undiluted ADPE. Outdoor growth of the mixed culture using raceway ponds showed potential for up to 63.7 ± 12.1 mg N-NH_4^+ L^{-1} d^{-1} ammonium removal from the ADPE. The microalgal consortium was dominated by *Chlorella* sp. and was stable at between 800 and 1600 mg N-NH_4^+ L^{-1}. Regulation of CO_2 addition to the ponds to maintain a pH of 8 increased chlorophyll content of the microalgal consortium. Average microalgal biomass productivity of 800 mg N-NH_4^+ L^{-1} culture conditions during five weeks semicontinuous growth was 18.5 mg ash-free dry weight L^{-1} d^{-1}. Doubling the ammonium concentration from 800 to 1600 mg N-NH_4^+ L^{-1} resulted in a 21% reduction of productivity, however the culture grown at 1600 mg N-NH_4^+

L^{-1} with the addition of CO_2 by keeping pH at pH =8 led to a 17% increase in biomass productivity.
Keywords:
Biomass, Wastewater, Open pond, Ammonia, Bioenergy

1. Introduction

A well-managed piggery should seek to handle and reuse wastewater appropriately, maintain control of odour emissions and aim to minimise its output of greenhouse emissions [1]. There is potential for improvement in the management and reuse of piggery wastewater. Piggery wastewater is very high in ammoniacal nitrogen and phosphorous as well as having significant chemical and biological oxygen demands [2]. These pollutants however can serve as beneficial nutrient sources for the growth of some microalgae. Microalgae produced in the context of pig production may provide income from the algal biomass produced as a source of animal or aquaculture feed [3], plant fertiliser [4] or biofuel [5].

Due to potential benefits of microalgae production incorporated into piggery systems, studies into the use of microalgae culture as a treatment for piggery wastewater have been ongoing for several decades [6, 7, 8]. So far however, results have failed to bring about widespread applications for the industry primarily due to concerns regarding the economic and

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environmental sustainability associated with pretreatment or dilution of the waste before growth of microalgae.

In the context of intensive pig production in places such as Australia, Europe and the USA, anaerobic digestion is a common treatment system [9, 10, 11]. Anaerobic digestion systems in Australian piggeries typically consist of covered outdoor ponds enabling the capture of biogas and output of partially treated water in the form of anaerobic digestate slurry. The adoption of this management approach for piggeries leads to the current scenario whereby anaerobic digestate of piggery effluent (ADPE) is now attractive as a microalgae growth medium. Current barriers to the adoption of microalgae culture for ADPE treatment include very high ammonia levels [12], high pH [13] and high turbidity (dark colour) [14]. The combination of high ammonia (around 1000 to 2000 mg N-NH_4^+ L^{-1}) and basic pH (above 8) in ADPE shifts the chemical equilibrium from NH_4^+ to NH_3 which is toxic to most organisms (microorganisms, aquatic ecosystems and terrestrial life such as vertebrates) [15, 16, 17]. NH_3 toxicity in microalgae has also been well documented, although the mechanisms for this are not well understood [13, 18]. The effect of the toxicity at high pH is compounded as microalgae take up CO_2 during photosynthesis leading to a net increase in pH [19].

Although there are reports of raw piggery wastewater and ADPE use as microalgae growth medium, the majority of examples found in the current literature report the need for significant dilution for adequate microalgal growth [6, 20, 21, 22]. The dilution of piggery wastewater for microalgae growth is not considered to be a viable option in most places with

limitations of fresh water supply and potential problems with the disposal of this larger volume of water. Here we attempt to overcome some of these limitations by bioprospecting microalgae capable of growing well in undiluted ADPE.

We also examined the long term reliability of the growth of microalgae in diluted and undiluted ADPE. Testing reliability of any microalgae cultivation is necessary to indicate the potential practical application of the process [19]. Inorganic carbon is one of the main limits to the growth of microalgae [23]. The high pH of piggery anaerobic digestate results in lowering the amount of CO_2 available for microalgae growth. Therefore, we also tested cultivation of isolated microalgae with and without CO_2 addition to emulate growth as might be expected with the addition of a CO_2 source such as flue gas (as might be obtained through anaerobic digestion process and methane combustion). This CO_2 addition should also serve to improve the otherwise poor ratio of C:N which is likely to be another growth limiting factor [24]. Microalgae growth experiments were carried out in a series of four outdoor raceway ponds with a variety of synthetically increased ammonia concentrations to test the tolerance of the mixed microalgae consortium to high ammonia growth conditions as might be found in an ADPE based growth medium.

2. Materials and Methods

2.1. Source and pre-treatment of ADPE

Samples of anaerobic digestate of piggery effluent (ADPE) from the Medina Research Station, Kwinana, Western Australia were collected and transported in 30 L plastic drums and used as the source of culture media for the duration of cultivation with no dilution. For the initial bioprospecting stage charcoal filtration [14] was used to remove some solids and reduce turbidity from the ADPE. For the remaining enrichment and cultivation experiments a slow feed sand filtration system was used to remove suspended solids and reduce the turbidity. A trickling speed of around 80 mL.min^{-1} was maintained through the filter which was made from a 30L drum consisting of layered perforated PVC pipes, gravel and sand. Where required, ammonium chloride was added to the sand-filtered ADPE to allow for testing of higher ammonia concentrations. At the time of each ammonium chloride addition, pH was adjusted to pH= 9 using potassium hydroxide. Ammonia, total phosphorous, non-purgeable dissolved organic carbon (NPDOC) and non-purgeable total organic carbon (NPTOC) of ADPE were measured by the Marine and Freshwater Research Laboratory at Murdoch University. Methods used were: 2000 (Ammonia), 4700 (Total-P) 6000 (NPDOC) and 6000 (NPTOC) of 'Standard Methods for the Examination of Water and Wastewater' [25]. The effluent had an ammonium content of 240 - 690 mg N-NH_4^+ L^{-1}, total phosphorous of 33 - 43 mg P L^{-1}, NPDOC of 69 - 97 mg C.L^{-1}, NPTOC of 97 - 220 mg C.L^{-1}.

2.2. Bioprospecting and enrichment

Bioprospecting and enrichment for isolating suitable microalgae included sampling from water sources such as outdoor raceway ponds, an animal drinking trough in a university paddock, a waste processing facility and a secondary evaporation pond from a research piggery. Microalgae samples were then gradually scaled up in volume and concurrently the strength of ADPE and concentration of ammonia was increased in a stepwise manner to select for strains suitable for growth on undiluted ADPE. The enrichment cultures were carried out in aerated 2L aquaria using approximately 200mL of the freshwater source and made up to 1L volume using charcoal filtered ADPE. Initial cultures were conducted indoors using fluorescent light at 440 µmol photons $m^{-2}s^{-1}$ in a controlled temperature room at 25 ± 3 °C with a 12:12 day:night cycle. Cultures were also grown outdoors using natural light between 215 and 700 µmol photons $m^{-2}s^{-1}$ during day time with variable temperatures ranging from 2 °C overnight up to a maximum of 47 °C during the day.

2.3. Outdoor trials for examining microalgae culture reliability

Larger scale outdoor cultivations were also conducted to determine reliability of the microalgae cultures outdoors as well as the effects of different ammonium concentrations and CO_2 addition. Cultivation at a large scale by increasing the depth also provides benefits of greater aerial productivity as well as maximising nutrient removal rates.

Using a mixed microalgae culture obtained during bioprospecting

screening and culture enrichment, outdoor cultivation was carried out using 1 m^2 fibreglass paddle-wheel driven raceway ponds during the winter months of 2013 at Murdoch University [26]. In order to provide a smooth transition to large scale cultivation the culture depth was gradually increased rather than immediately brought to it's maximum final capacity. The combination of initially using charcoal filtered ADPE before transitioning to sand-filtered ADPE along with the gradual increase in depth was expected to reduce the risk of culture collapse and allow for further adaptation and acclimation to outdoor culture conditions and much larger volumes. Once the depth and volume of the culture were operating at full capacity, the ammonium concentration was increased gradually to also provide opportunity for adaptation and selection of the most fit algal strains present in the mixed culture.

To this end, a volume of the combined bioprospecting cultures grown at the lab totalling 3.5L was introduced to an empty raceway pond with around 15L of charcoal filtered ADPE (pond 'g' in Figure 1). The following day a further volume of charcoal filtered ADPE was added to the pond to make up a total of 50L pond medium (around 5cm depth). Over the course of two months the pond volume was gradually increased to 150L total pond volume (15cm depth) by the addition of fresh sand-filtered ADPE, and also a second pond (pond 'e' in Figure 1) was established from the same culture. Fresh tap-water was also used to top-up any losses due to evaporation. From this point on, pond depth was maintained at 15-18 cm with variation due to rainfall and evaporation. The flow rate in the ponds was 20 cm s^{-1}.

From 5 June to 9 July two more ponds were added to the experiment (ponds 'd' and 'f' in Figure 1) making a total of four ponds in use for cultivation. As the ammonium concentration had dropped during the previous cultivation period it was necessary to gradually increase the ammonium concentration to 800 mg N-NH_4^+ L^{-1} by the addition of ammonium chloride on average around every three days (see section 2.1). At this time the other pond (pond 'd' in Figure 1) was a control culture with no addition of ammonium. From 10th of July to 18th of August 2013 four 1 m^2 raceway ponds were operated in batch growth mode. In two of the ponds ammonium concentrations were maintained at 800 mg N-NH_4^+ L^{-1} (ponds 'd' and 'e' in Figure 1). In the other two ponds ammonium concentrations were increased from 800 mg N-NH_4^+ L^{-1} to 1600 mg N-NH_4^+ L^{-1} stepwise over a 3 week period (ponds 'f' and 'g' in Figure 1). One of the 800 mg N-NH_4^+ L^{-1} and one of the 1600 mg N-NH_4^+ L^{-1} ponds were supplemented with CO_2 using a pH-stat system set at pH =8 [27] (ponds 'e' and 'g' in Figure 1).

From 19 August to 25 September all ponds were operated in a semicontinuous culture mode with. Again one of the 800 mg N-NH_4^+ L^{-1} and one of the 1600 mg N-NH_4^+ L^{-1} ponds was supplemented with CO_2 using a pH-stat system set at pH =8 continuing on from the previous experiment. Air temperature, irradiance, humidity and rainfall data were obtained from the Murdoch University weather station (http://wwwmet.murdoch.edu.au).

2.4. Biochemical analyses

Culture temperature, pH and dissolved oxygen were measured using in situ YSI sonde probes (600R and 600XL models). Cell counts were measured using a Neubauer haemocytometer. Organic weight of the biomass measurement was performed in triplicate as per the method of Zhu and Lee [28]. Chlorophyll extraction and measurement was carried out in triplicate using the method of Jeffrey and Humphrey [29].

A Hanna Instruments HI733 Ammonia High Range Checker test kit was used for regular measurements of ammonium concentration in the ponds. Concentrations of ionised and unionised ammonia were calculated using pH and temperature according to Emerson [30].

Estimations of the total nitrogen removed by microalgal biomass were made using the nitrogen ratio figures published by Ketchum and Redfield [31]. Due to the predominance of *Chlorella* observed in the mixed microalgae culture used in these experiments, the *Chlorella* C:N:P ratio values were used from this source.

2.5. Statistical Analysis

Statistical analysis performed on the chlorophyll and cell density were repeated measures one-way analysis of variance (ANOVA) with *post-hoc* multiple comparisons using Holm-Sidak method using Sigmaplot (v 12.5, Systat Software Inc.). All data were checked for normality using the Shapiro-Wilk test. For all statistical tests, the significance level was set at

0.05. Standard error calculations for the biomass data were performed with Excel (v 15.11.2, Microsoft Corp.).

3. Results

3.1. Weather and environment

The outdoor experiments were carried out between June (winter) and September (spring) 2013. There was intermittent rainfall with a mean of 4.05 mm d^{-1} during the cultivation period. The solar radiation mean was 155 W m^{-2} for the period and peaked at a maximum of 1335 W m^{-2} during September (Figure 1). Overnight (minimum) air temperature mean (recorded by the campus weather station) was 9.33 °C (ranged between 1.04 - 16.57 °C). Direct recording of the pond temperature resulted in pond minimum temperature mean of 9.26 °C (ranged between -0.24 - 16.87 °C). Daytime (maximum) air temperature mean (recorded by the campus weather station) was 19.64 °C (ranged between 14.27 - 25.64 °C). Direct recording of pond temperature gave a maximum pond temperature mean of 18.61 °C (ranged between 12.81 - 22.99 °C).

3.2. Batch growth phase

3.2.1. Culture enrichment

During the gradual enrichment of the mixed culture from early June to 9 July 2013 two of the ponds were maintained at 800 mg N-NH_4^+ L^{-1} (ponds 'e' and 'g' in Figure 1) while the other pond was used as a control (pond

'd' in Figure 1). By the end of this phase cell counts reached up to around 3.2×10^7 cells mL^{-1} in the ponds. Microalgae species observed in this mixed culture were primarily *Chlorella* spp. with low levels of *Scenedesmus* spp. at around 3% of the total cell count figures. The species composition remained stable throughout this period.

3.2.2. Culture acclimatisation

The pond cultures were merged together, mixed and redistributed between the four ponds at the start of the culture acclimatisation stage to establish consistent diversity of species and homogenise cell counts across the ponds. The cultures were then operated in batch mode with different ammonium concentration in four raceway ponds between 10 July and 18 August as shown in Figure 1. *Chlorella* remained the most dominant species at all ammonium concentrations. For this five week period (not including figures from 10 July) the mean (n=56) cell density was 1.1×10^7 cells ml^{-1} with a range from 0.3×10^7 - 2.7×10^7. After several weeks of acclimatisation to the different ammonium and CO_2 treatments, some divergence in the different pond cultures was observed in the cell-count data (Figure 3). For the last 11 days in this period the mean (n=20) combined species cell density was 1.0×10^7 cells ml^{-1} with a range from 0.3×10^7 - 1.8×10^7. Combined species cell-count data from this set passed the normality test with F=14.595. When no CO_2 was added, the mean difference in algal cell density between 800 mg N-NH_4^+ L^{-1} and 1600 mg N-NH_4^+ L^{-1} conditions was less than 2% and was not statistically significant (n=5, repeated measures one-way ANOVA, P= 0.880, t= 0.154).

When CO_2 was added to the ponds, the culture at 1600 mg N-NH_4^+ L^{-1} compared with 800 mg N-NH_4^+ L^{-1} showed a significantly higher mean cell density with an increase of 38.2% (n=5, repeated measures one-way ANOVA, P= 0.007, t= 4.029). The influence of CO_2 addition at 800 mg N-NH_4^+ L^{-1} gave an increased mean cell density of 18.1% however this was found to be not statistically significant (n=5, repeated measures one-way ANOVA, P=0.246, t= 1.618). The influence of CO_2 addition at 1600 mg N-NH_4^+ L^{-1} showed a statistically significant mean cell density increase of 66.2% (n=5, repeated measures one-way ANOVA, P>0.001, t= 5.802).

For the last 11 days of the acclimatisation stage, the proportion of *Scenedesmus* in the cell-counts were significantly higher in the CO_2 treatment ponds with a mean proportion of the culture 7.1% compared to 5.6% in the ponds without CO_2 treatment (repeated measures one-way ANOVA, P>0.001, t= 11.085 F=122.881). (Figure 3)

3.3. Semicontinuous growth phase

Batch mode is not the most efficient method for microalgae biomass production when compared to semicontinuous mode (see discussion section for more details). Therefore, at the end of the batch mode, the cultures were operated semicontinuously. A semicontinuous phase of growth was undertaken from August 19, 2013 to September 25, 2013 with pond conditions leading on immediately from the batch phase (Figure 1).

Culture cell densities were maintained at 1×10^7 cells mL^{-1} during this period with variations in culture growth performance due to

environmental conditions and the variable nutrient composition of the ADPE. Harvesting of the culture took place with an average of 5 days between harvests and 33% of the pond volume removed each time. After harvest the pond volume was topped up with ADPE and the ammonium N and pH was adjusted to maintain the desired test conditions.

During the semicontinuous phase, the dominant species were *Chlorella* and *Scenedesmus* (the same as batch growth phase). However, toward the end of the semicontinuous growth phase a pennate diatom was also seen in the culture (Figure 4). Pennate cell density remained at relatively low background levels, peaking at around 1 x 10^6 cells per mL in the 800 mg N-NH_4^+ L^{-1} ammonium concentration with no CO_2 addition to the pond. This species was first observed in the ponds without CO_2 addition but gradually established in all of the ponds by the end of the experiment at an average around 3 x 10^5 cells per mL (Figure 4).

3.3.1. Culture productivity

Biomass productivity during the semicontinuous growth period was mean (n=56) 17.4 mg L^{-1} d^{-1} (range from -40.0 - 94.9 mg L^{-1} d^{-1}) (see also Figure 1). Doubling the ammonium concentration from 800 mg N-NH_4^+ L^{-1} to 1600 mg N-NH_4^+ L^{-1} without CO_2 addition resulted in a mean 20.9% decrease in biomass productivity (Figure 1, n=14). The addition of CO_2 keeping the pH at pH =8 for the 1600 mg N-NH_4^+ L^{-1} pond conditions increased mean biomass productivity by 17.1% (Figure 1, n=14). The mean productivity of the 800 mg N-NH_4^+ L^{-1} pond culture without CO_2 addition was 18.5 mg L^{-1} d^{-1}, whereas the mean

productivity of the 1600 mg N-NH_4^+ L^{-1} pond culture with CO_2 addition was 17.1 mg L^{-1} d^{-1} (Figure 1, n=14). The highest productivity was attained at 800 mg N-NH_4^+ L^{-1} with CO_2 addition with a mean of 19.5 mg L^{-1} d^{-1} (Figure 1, n=14).

The nitrogen content gained in microalgae biomass during growth did not fully account for the overall loss of ammonium from the growth media. However, the nitrogen values were regularly topped up to the desired level using ammonium chloride to maintain experimental conditions (See Figure 2). Table 1 shows the calculated ammonium removal rate from the pond determined by ammonium measurements compared to ammonium removal rate by biomass productivity based on the redfield ratio. Towards the end of the semicontinuous period a maximum conversion of just over 10 % ammonium nitrogen to biomass is observed.

3.3.2. Chlorophyll content

Increasing ammonium concentration from 800 mg N-NH_4^+ L^{-1} to 1600 mg N-NH_4^+ L^{-1} resulted in significantly less chlorophylls *a* (73.1%) and *b* (62.2%) when no CO_2 treatment was applied (Repeated measures one-way ANOVA, n=16: $P<0.001$, $t= 5.843$, $F=34.141$; $P<0.001$, $t= 4.237$, $F=17.954$ respectively) (Figure 5).

With the same change in ammonium concentration, a smaller yet still statistically significant decrease was also found under the influence of CO_2 addition for chlorophyll *a* (Repeated measures one-way ANOVA, n=16: $P=0.033$, $t=2.372$ $F=5.627$) corresponding to a mean decrease of 13.1%.

Chlorophyll b under the same conditions followed the same pattern however the statistical support for this was inconclusive (Repeated measures one-way ANOVA, n=16, differences in the means may be due to sampling variability).

When the algae were grown at 800 mg N-NH_4^+ L^{-1}, addition of CO_2 resulted in an increase for chlorophyll a of 28.4% and chlorophyll b of 67.6%, however the statistical support was inconclusive for each of these (Repeated measures one-way ANOVA, n=16: differences in the means may be due to sampling variability and normality test failed). At 1600 mg N-NH_4^+ L^{-1}, chlorophyll increases under CO_2 addition were 315.4% and 231.2% for chlorophyll a and b respectively with inconclusive statistical support for chlorophyll a (Repeated measures one-way ANOVA, n=16: normality test failed) and a statistically significant finding for chlorophyll b (Repeated measures one-way ANOVA, n=16: P=0.007, t= 3.138, F=9.847).

Due to the mixed species nature of the culture and diverse range of bioprospecting sources, chlorophyll c analysis was also performed in case the presence of organisms using these pigments might be detected. Chlorophyll c_1+c_2 figures remained very low across all treatments and pond conditions (at around 5% of the total chlorophyll content). These data were assumed to not provide sufficient accuracy for meaningful statistical analysis.

4. Discussion

To the best of the authors knowledge, this is the first time that several microalgae (*Chlorella*, *Scenedesmus*, and a pennate diatom) were isolated that are capable of growing on undiluted anaerobic digestate of piggery effluent with the only pre-treatment being sand filtration. This is also the first time that these cultures were grown reliably under outdoor conditions for a long term period at high levels of ammonium Nitrogen up to 1600 mg N-NH_4^+ L^{-1} using paddle wheel driven raceway ponds. *Chlorella* sp was found to the be the most dominant species throughout the bioprospecting, batch phase and semicontinuous growth.

In a typical *Chlorella* cultivation productivity expected might be around 20 - 25 g $m^{-2}d^{-1}$ [32] however our average productivity was around 1/7 of this. Our semicontinuous growth was during winter months for only 5 weeks duration and beyond this pilot-scale trial has potential to be optimised further. Although measurements of cell sizes were not recorded in this experiment, a diverse range of sizes were observed under the microscope which corresponds with observations elsewhere in the literature [31] and might explain some of the divergence between culture productivity and cell count figures as seen in Figure 1 'd' - 'g'.

A range of microalgae sources were sampled in order to isolate the most suitable algal culture capable of being conditioned and acclimatised to the harsh growth conditions characteristic of piggery effluent such as high ammonia, pH and turbidity. The resulting culture was grown under semicontinuous conditions outdoors directly on sand-filtered, undiluted

piggery anaerobic digestate effluent and species were subsequently isolated onto synthetic media under laboratory conditions. The dominant strain in the culture was a *Chlorella* species however a *Scenedesmus* species and a pennate diatom were also found to grow in the culture successfully.

Previous studies targeting growth of microalgae on piggery anaerobic digestate effluent have often relied upon dilution or pre-treatments such as centrifugation or sterilisation [8, 21]. A recent study achieved growth of mutated *Chlorella* under laboratory conditions on media up to 1093 mg N-NH_3 L^{-1} [33]. In the example we report here we have focused not on optimisation of the culture but rather testing on conditions that might be reasonable to expect in a media consisting of minimally pre-treated (sand filtered only), undiluted and unsterilised piggery digestion effluent which tends to have a variable nutrient composition. For instance, the ammonia concentrations reported in the literature for anaerobic digestate from actual pig production ranges from around 1200 to around 1600 mg ammoniacal N per litre [12, 34], although some studies report much higher concentrations up to around 2400 to 3600 mg ammoniacal N per litre from raw wastewater or fermented manure [35, 36]. We also managed to grow the selected species under outdoor conditions. During the conditioning and acclimatisation stages of the experiment the culture was found to grow very well at up to 1600 mg N-NH_4^+ L^{-1} and up to pH 9. No treatments were made to modify the turbidity of the media apart from sand-filtration of the incoming effluent which only removed the solid particles. It is worth noting, however that a more concentrated digestate might have higher turbidity levels and would likely present more of a challenge to culture

growth than the conditions tested here. By moving the culture to a semicontinuous growth phase and maintaining a relatively steady culture density over more than five weeks, the groundwork for optimisation of the culture appears to be set allowing further adjustment of the growth conditions with a focus on improving productivity. There appears to be much room for improvement in this area with Cheng at al. reporting up to 601.2 mg L^{-1} d^{-1} growth rates under controlled laboratory conditions [33].

Comparisons of ammonia loss from the culture media against algal biomass production as shown in Table 1 indicate a significant amount of nitrogen is being lost from the system without being assimilated into algal growth. This is consistent with previous findings that a large part of the ammonia is lost either to the atmosphere or is utilised in nitrification/denitrification conversion pathways [37, 38, 39]. This study focused primarily on bioprospecting and growth of the acclimatised culture, however it would be informative for future studies to further investigate these nitrogen conversions and the microbiota involved.

The ammonium concentration tested in this experiment was much higher than what was found in our samples of raw anaerobic digestate obtained from the piggery. However this adjusted level of ammonium is within the range typical of many piggeries using water flushing to maintain a satisfactory level of cleanliness in the piggery and appears representative of international examples cited in the literature. Even higher ammonium concentrations in the effluent media might be manageable for an ADPE based algal cultivation system dependant on both the rate at which fresh media is introduced to the pond and the rate at which ammonium

conversion and removal occurs from the pond. Further advances in anaerobic treatments could also change these digestate ammonium concentrations if the technology adopted by piggeries changes in future.

Carbon dioxide addition keeping the pH at pH =8 for the higher ammonium concentration abated the majority of the decrease in productivity due to the toxicity of the extreme concentration. This strategy appears useful to maximise productivity under high ammonium conditions which otherwise inhibit the microalgae growth. Figure 2 shows the concentration of the toxic unionised ammonia (NH_3) concentration as it relates to pond media harvesting and top-up, and illustrates the rapid reduction of toxicity due to the addition of CO_2 and the influence of the pH control. Interestingly the reduction in NH_3 concentration also occurs in the ponds with no CO_2 addition, however takes a much longer time (roughly one day) to return to pre-harvest concentrations. Depending on the duration between pond harvest and top-up events it seems that NH_3 levels might remain much lower than the non-toxic NH_4^+ proportion. Reports from the literature indicating strong inhibition of microalgal growth using ADPE growth media might be due to the influence of pH or other properties of ADPE from different sources which was not seen in this experiment. It might also be possible that the lengthy enrichment and acclimatisation stages of this experiment might have allowed for development of a more resilient culture less influenced by the toxicity of the high ammonia concentrations. The low productivity observed in this study also did not induce a significant increase in pH which can occur during photosynthesis and would have increased the toxicity. Also during

this experiment the addition of KOH to the pond media might have lead to the formation of a chemical buffer which could have kept the pH low.

The significant difference found in chlorophyll *a* and *b* values across the different ammonium concentrations while no CO_2 addition is used indicates chlorophyll production also inhibited by the increased ammonia concentration. The application of CO_2 to the growth conditions allowed for much higher chlorophyll production indicating that either the increase in CO_2, the decrease in pH or a combination of both allows for some tolerance to the toxic effect of the high ammonia concentration.

Addition of CO_2 may also bring the C:N ratio of the growth media closer to the ideal ratio of around 7:1 C:N [31]. Although specific measurements of alkalinity and carbon availability were not included in this preliminary study, further investigation of the impacts on improving the C:N ratio for the growth of a mixed culture on ADPE based media would be helpful to understand the response of chlorophyll to higher carbon concentrations and also whether increases in lipid concentration or other carbon rich cellular components (ie. cell wall and overall increase in cell size) may result from increased carbon availability.

Yet another interesting aspect of the chlorophyll response is found when CO_2 addition to both high and lower ammonium conditions results in a significant increase in chlorophyll *b* content per cell. The chlorophyll *b* per cell is slightly increased at the higher ammonium concentration with CO_2 pH control is used even when compared with the moderate ammonium no CO_2 addition pond. The same effect is difficult to assess with certainty for

chlorophyll *a* as the statistical support was inconclusive. As the role of chlorophyll *b* is associated with improved photosynthetic antenna and greater light harvesting [40, 41] this chlorophyll production response by the microalgae culture might indicate the higher CO_2 availability and/or the associated pH change can allow the microalgae to acclimatise further to the lower light conditions and high turbidity of the growth media [42]. In terms of culture optimisation, the results of the chlorophyll analysis appear to indicate that a CO_2 addition or a pH control strategy might lead to more consistent and improved growth performance under conditions of high ammonium concentration and high culture turbidity. This also correlates with results reported elsewhere in the literature regarding pH control of microalgae culture using CO_2 [43].

In order to reduce the risk of pollution due to ammonia loss to the atmosphere and to more efficiently utilise the nitrogen content of the anaerobic digestion effluent it seems reasonable for an optimisation strategy to maximise conversion of nitrogen to algal biomass. This might be partially achieved through maintaining low pH in the culture media along with use of a well acclimatised mixed microalgal culture, although there are clearly more measures to be taken to fine-tune this process.

Another interesting observation highlighted during the course of the experiment was that the outdoor mixed culture system did not exclude the possibility of new previously unobserved species appearing in the culture. The appearance of the pennate diatom towards the end of the long-term growth period indicates that even though the culture had been established for many weeks this new species was able to gain a reasonably

successful level of growth and establish at low background levels in each of the ponds. It is difficult to know whether further acclimatisation and/or adaption of the *Scenedesmus*, pennate diatom or other strains not observed during this study might be possible. Undesirable contamination of the culture even at the very high ammonium levels used in this study might also be possible during long-term growth. Given the outcome of this study, we have no doubt that more optimisation is required to improve the productivity of microalgae on undiluted ADPE. Nevertheless, the outcome is very promising for using microalgae to treat ADPE. Since the completion of the experiments reported here further research performed by Nwoba et al. has already pointed towards optimisations such as changes to reactor design which can reduce the impact of high media turbidity (ie. using closed biocoil photobioreactors) [44].

In conclusion, The work presented here demonstrates the that microalgae can be grown on undiluted, sand-filtered piggery anaerobic digestion effluent outdoors at a very high ammonium concentration range between 800 - 1600 mg N-NH_4^+ L^{-1}.

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Table 1: Conversion of ammoniacal nitrogen from the pond water into biomass nitrogen content. The calculation for proportion of nitrogen in the algal biomass is taken from the average figures of the *Chlorella* species as reported by Ketchum and Redfield [31]. Data presented here are the means ± standard error.

Pond ammonium (mg N-NH_4^+ L^{-1})	ADPE ammonium removal rate (mg N-NH_4^+ L^{-1} d^{-1})	Ammonium uptake to biomass (mg N-biomass L^{-1} d^{-1})
800	40.9 ± 9.5	0.9 ± 0.3
800 *	33.2 ± 6.5	1.1 ± 0.4
1600	63.7 ± 12.1	0.8 ± 0.3
1600 *	51.6 ± 10.8	1.1 ± 0.5

* with CO_2

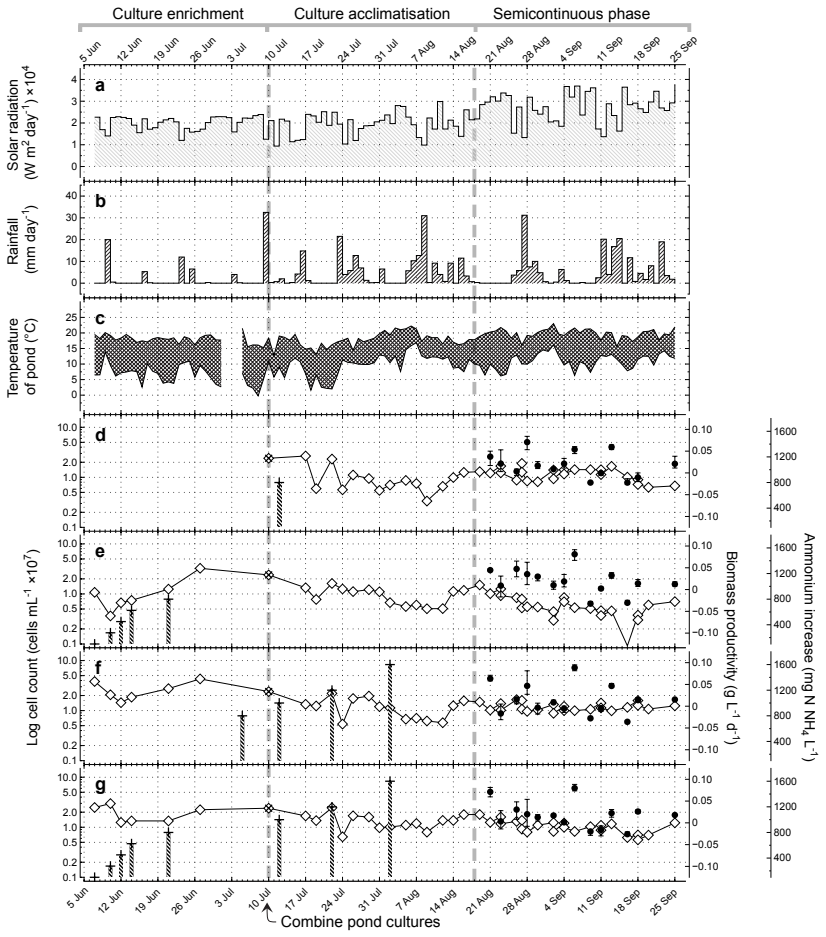

Figure 1: Weather condition and temperature data graphs (a - c) and outdoor raceway pond measurement graphs (d - g) including total cell-counts (⋄), biomass productivities (•) and ammonium increases (vertical bars marked with +). Biomass productivity data are presented as means ± range, n=3. Ponds (e) and (g) included the adjustment of pH using CO_2. The arrow at 10th July (also marked with × on the plot line) indicates merging the pond cultures at the end of culture enrichment and start of the acclimatisation stage of batch phase growth. Ponds (d) and (e) were maintained at 800 mg N-NH_4^+ L^{-1} for the acclimatisation and semicontinuous growth phase while ponds (f) and (g) were increased to 1600 mg N-NH_4^+ L^{-1} during the acclimatisation phase and maintained at those concentrations during semicontinuous growth.

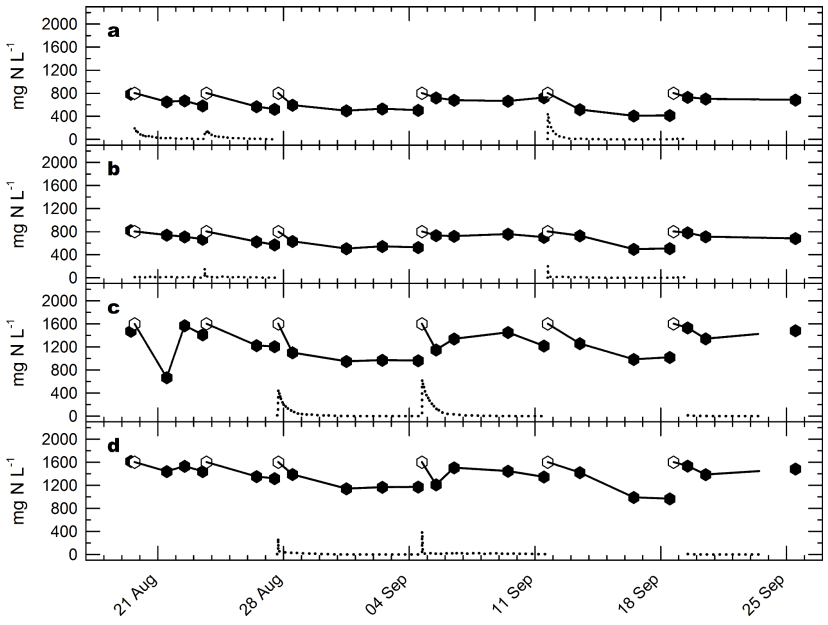

Figure 2: Ammonium concentrations of the four pond conditions over the course of the semicontinuous growth. (a) and (b) are boosted to 800 mg N-NH_4^+ L^{-1}; (c) and (d) are boosted to 1600 mg N-NH_4^+ L^{-1}; (b) and (d) include pH control via addition of CO_2 to the raceway ponds. White symbols indicate concentrations due to the top-up of ammonium chloride to the pond water. Black symbols indicate the subsequently measured ammonium concentrations before and after top-ups. Solid lines represent the concentration of ionised ammonium (mg N-NH_4^+ L^{-1}). Dotted lines represent the concentration of unionised ammonia (mg N-NH_3 L^{-1}).

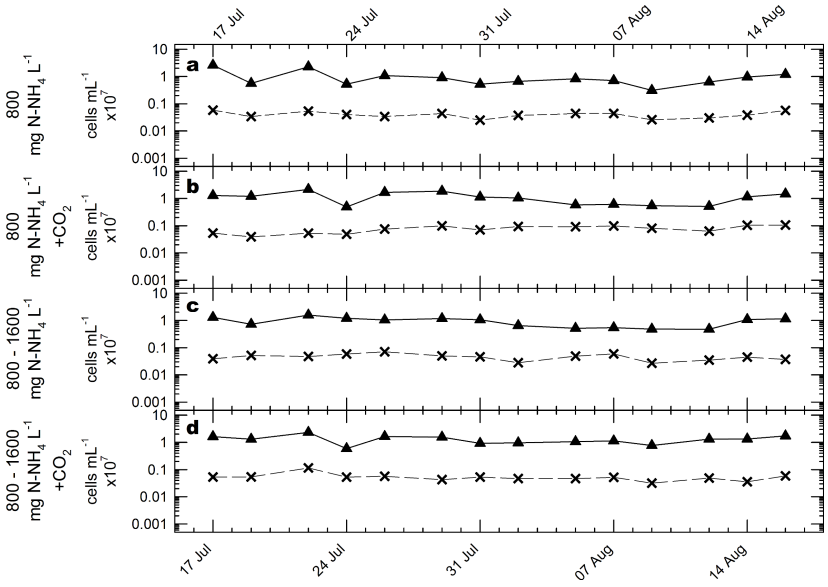

Figure 3: Cell count observations of *Chlorella* cells (▲) and *Scenedesmus* cells (×) during the acclimatisation stage of the experiment. Graph (a) pond conditions were 800 mg N-NH_4^+ L^{-1}, (b) 800 mg N-NH_4^+ L^{-1} with CO_2 addition, (c) 800 boosted to 1600 mg N-NH_4^+ L^{-1} and (d) 800 boosted to 1600 mg N-NH_4^+ L^{-1} with CO_2 addition.

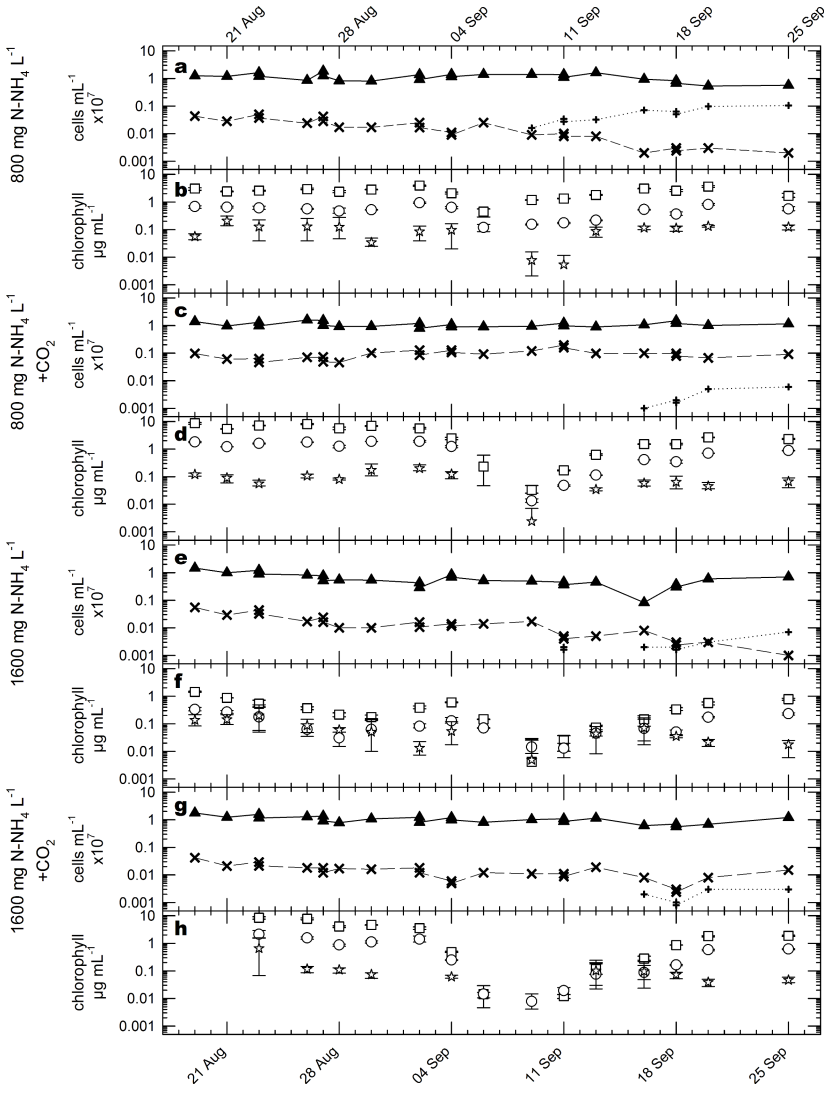

Figure 4: Cell count graphs (a, c, e and g) include data for observations of *Chlorella* cells (black ▲), *Scenedesmus* cells (black ×) and pennate diatoms (black +) during the semicontinuous phase of the experiment. Chlorophyll graphs (b, d, f and h) includes calculations for chlorophyll quantities: chlorophyll *a* (white □), chlorophyll *b* (white ○) and chlorophyll c_1+c_2 (white ⋆) Graphs (a) and (b) represent pond conditions with 800 mg N-NH_4^+ L^{-1}, (c) and (d) 800 mg N-NH_4^+ L^{-1} with CO_2 addition, (e) and (f) 1600 mg N-NH_4^+ L^{-1} and g and h 1600 mg N-NH_4^+ L^{-1} with CO_2 addition. Chlorophyll data are presented as means ± range, (n=3).

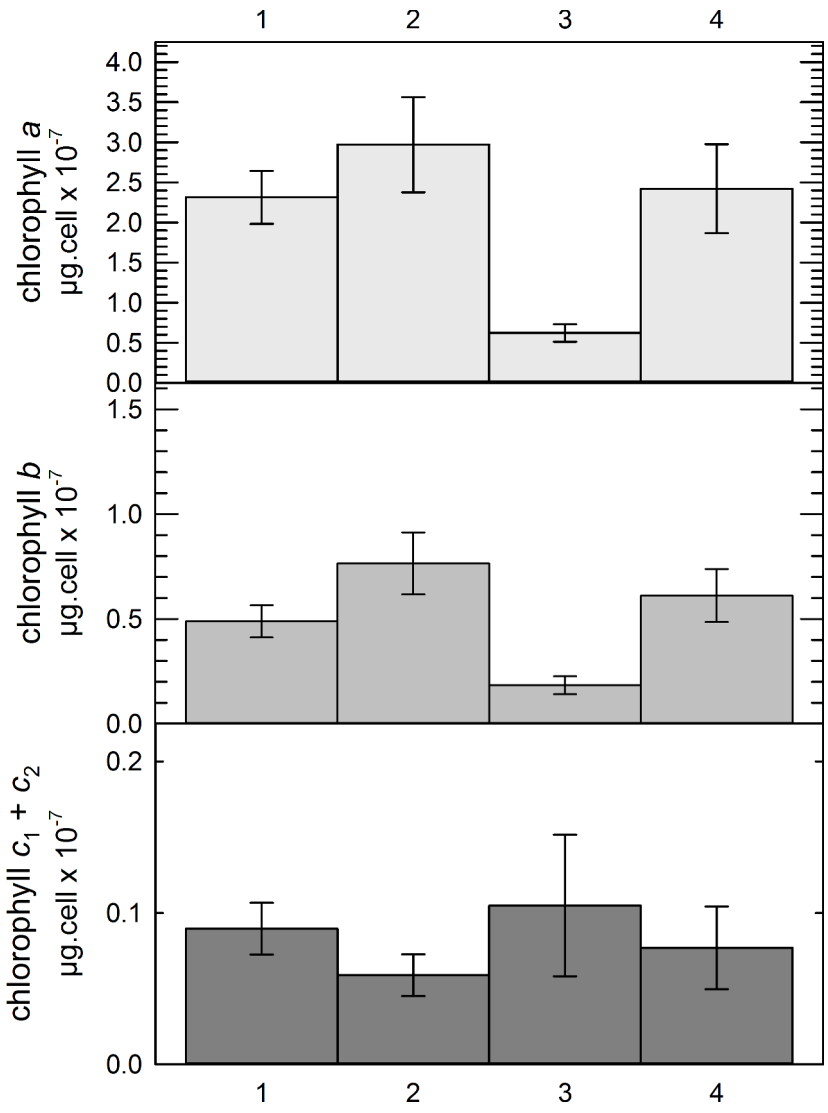

Figure 5: Chlorophyll content per cell. Bar graphs 1 and 2 represent ammonium concentration of 800 mg N-NH_4^+ L^{-1}, 3 and 4 represent 1600 mg N-NH_4^+ L^{-1} and graphs 2 and 4 include CO_2 addition for pH control. Chlorophyll data are presented as means with error bars showing standard error (n=16).