BIO-PROSPECTING AND GROWTH OF MACROALGAE ON ANAEROBIC DIGESTION PIGGERY EFFLUENT (ADPE).

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Executive Summary

Anaerobic digestion piggery effluent (ADPE) has high ammonium content (toxic to most organisms) and is very turbid. The environmental consequences of high productivity piggeries is significant and can result in negative environmental impacts, hence bioremediation techniques (in particular using macroalgae) are therefore of great interest. In this study, we evaluated the growth potential of several locally isolated macroalgae in ADPE under outdoor climatic conditions and investigated their nutrient removal rates and biochemical composition. A consortium of two macroalgae, *Rhizoclonium* sp. and *Ulothrix* sp. was isolated and could efficiently grow in the ADPE with concentration of up to 248.4 mg NH_3. $N L^{-1}$. Macroalgal consortium growth could not be maintained at higher ADPE concentration. Maximum ammonium removal rate (30.6 ± 6.50 mg NH_4^+-$NL^{-1}d^{-1}$) was achieved at ADPE concentration equivalent to 248.4 mgNH_4^+-NL^{-1}. Mean biomass productivity of 31.1 ± 1.14 g AFDW $m^{-2}d^{-1}$ was attained. Total carbohydrate and protein contents ranged from between 42.8-54.8 and 43.4-45.0% (ash-free dry weight), respectively, while total lipid content was very low. Our findings highlight the potential use and promise of *Rhizoclonium* and *Ulothrix* sp. consortium for the bioremediation of ADPE and biomass production. To the best of author's knowledge, this is the first study evaluating the potential of using macroalgae to treat ADPE. While there is a need for further optimisation, successful macro algae growth on ADPE indicates the potential of using these organisms for not only treating ADPE but also as a potential source of animal feed or bioenergy production.

Table of Contents

1. Introduction

Global rates of biologically available nitrogen and phosphorous entering the terrestrial biosphere have more than doubled since the 1950's (Galloway et al., 2004), with significant nutrient loads derived from crop farming and animal husbandry accumulating in watersheds and sewage systems (Schoumans et al., 2014), soils and tile drains (Schelde et al., 2006) or released as gaseous emissions (Hristov et al., 2011; Philippe et al., 2011). These nutrients enter into freshwater streams (Allen & Mallarino, 2008), which are ultimately exported by rivers to coastal ecosystems (Seitzinger et al., 2010). From Australia's intensive agriculture, an estimated 19,000 and 141,000 tonnes of phosphorous and nitrogen are transferred down river systems (NLWRA, 2001). Likewise in the United Kingdom, around 70% of nitrogen and 40% of phosphorous pollution of inland waterways was derived from agriculture (DEFRA, 2006). Nutrient pollution from agriculture is also acknowledged as one of the major sources of eutrophication in the United States of America (Morgan & Owens, 2001; Ribaudo et al., 2001; Sharpley et al., 2008). Such increases in nutrient inputs to waterways and coastal ecosystems contribute to various environmental impacts (Redfield, 1958; Vollenweider, 1968). Controlling the flow of nutrients from farming operations into the surrounding environment poses both technical and economic challenges that must be overcome to reduce such effects.

Although management strategies exist in preventing sources of nutrients entering ground and surface waters, considerably more work is required to reduce nutrient loads from intensive farming practices. One such method of reducing these high nutrient loads is phycoremediation, otherwise known as industrial ecology. This

involves the cultivation of macroalgae or microalgae for the removal or biotransformation of pollutants, including excess nutrients derived from agricultural sources (Olguín, 2003). Phycoremediation can be applied to point sources such as animal husbandry facilities including dairies (Wilkie & Mulbry, 2002) and piggeries (Kebede-Westhead et al., 2006), and to non-point source nutrient runoff from soil improvement practices such as fertilizer application (Adey et al., 2011). Macroalgae have previously been cultivated as a food source for humans or livestock (Machado et al., 2015; McHugh, 2003), as an aquaculture feed source (Azim et al., 2003; Viera et al., 2005), as a slow-release fertilizer (Mulbry et al., 2005), used as a bio-filtration medium for aquaculture effluent (Marinho-Soriano et al., 2009), and for the production of by-products such as bioethanol (Yun et al., 2015). Macroalgae has also been studied as a potential source of pharmaceutical compounds (Mulbry et al., 2008a). However, many of these studies have focused on marine species and cultivation techniques. Cultivation in freshwater environments offers many opportunities for the development of phycoremediation that have yet to be explored. Here we summarise the current technologies and some of the future research implications of freshwater phycoremediation with macroalgae, focusing on how this may apply to remediating point-source agricultural nutrients.

Macroalgae and Eutrophication

To further understand why phycoremediation with macroalgae is so applicable in treating waterborne nutrients, the natural behaviour of these organisms in waterways should be further examined. Eutrophication in freshwater systems, estuaries and coastal lagoons can induce shifts in biodiversity (Smith et al., 1999), from slow-growing autotrophic species like aquatic plants and seagrasses, towards blooms of

faster-growing algae, as nutrient conditions favor the establishment of *r*-strategist growth (Patrıcio et al., 2004). In most cases, phosphorous sources determine the extent of primary productivity in freshwater systems, whereas in marine systems, nitrogen sources are the limiting nutrients (Anderson et al., 2002). At peak production, algal blooms establish dominance over slow-growing species, severely reducing plant and seagrass growth and reducing system diversity (Valiela et al., 1997). This continues until nutrients become limited, at which point a crash in primary productivity may occur, causing pollution from anoxia, toxin release and/or promoting potentially harmful bacterial outbreaks (Jaworski, 1981). However if nutrients are continually supplied, such crashes can be avoided.

When comparing macroalgal and microalgal blooms, macroalgae tend to be different to microalgae in that they lack direct toxicity, therefore offering a safer alternative in phycoremediation. Furthermore, macroalgal blooms are more persistent when faced with nutrient-limited conditions (Hay & Fenical, 1988). Examples of this are *Cladophora* blooms in the Peel-Harvey Estuary in Western Australia (Gordon & McComb, 1989) and a mixed bloom of *Cladophora* and *Gracilaria* in Waquoit Bay, Massachusetts (Valiela et al., 1992). In both cases, these blooms lasted for more than a decade, utilizing the existing nutrient runoff entering the waterways.

Nutrient requirements

Often ratios of phosphorous (P) and nitrogen (N) are used to establish which nutrients may limit the growth of primary producers in aquatic ecosystems (Elser et al., 2007; Hecky et al., 1993). In addition, Ca, Mg, S and a variety of trace elements including Fe, Cu, Zn and others required in lower amounts (Wang et al., 2010). Thus, it is possible that controlling the export of nutrients with cultures of high-density

macroalgae on the farm site can elicit a positive effect on the downstream aquatic ecology (Schoumans et al., 2014; Seitzinger et al., 2005). Algae require nutrients such as N and P in large quantities (both of which are usually in abundance in wastewaters), although the amounts required are highly variable between species. The Redfield C:N:P ratio of 106:16:1, which is used to determine the amounts typically required by photosynthetic organisms, only applies to macroalgae under conditions of maximum growth (Goldman et al., 1979). This is due both to variation in the composition of the ultrastructure and the elemental composition operating on a continuum under nutrient-limiting conditions (Hecky et al., 1993; Townsend et al., 2008). Atkinson and Smith (1983) presented a table of C:N:P ratios for many marine macroalgae and seagrasses, which showed that required ratios are indeed highly variable, with the typical requirements for carbon (C) being far larger in proportion to N and P in the Redfield ratio.

Nitrogen

Macroalgae can typically use nitrate, urea and ammonium as nitrogen sources. However, some macroalgae can consume organic nitrogen sources. Although it may appear a simple proposition to balance the supply of elements, tolerances to certain molecular species (e.g. NH_3/NH_4^+) of nitrogen can have a dramatic effect on macroalgal growth and survival. Ammonia (NH_3) especially at high concentration is toxic to algae, whereas ammonium (NH_4^+) can be consumed in large quantities and NH_4^+ can also be toxic at very high concentrations. It is to be noted that the mechanisms of this toxicity are poorly understood (Britto & Kronzucker, 2002). Where ammonia is present, speciation of ammonia to ammonium in water is required. The reaction of ammonia to ammonium is temperature and pH dependent:

$$NH_3(aq) + H_2O \leftrightarrow NH_4+(aq) + OH^-(aq) \; pKa \; (25^oC) = 9.25$$
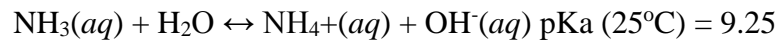
Tolerances to NH_3, NH_4^+ are specific to each macroalgal species; however these tolerances have rarely been explored in freshwater macroalgae. Several studies have investigated various nutrient sources and their general applicability to freshwater macroalgal cultivation (Table 1). These studies included raw sewage, anaerobically digested effluent (ADE) from various post-production processes and sources of nonpoint wastewater. One such study examined the growth of three *Stigeoclonium* species and one *Oedogonium* species on NH_4^+ gradients (1-100 mg L^{-1}) in cultivation ditches using synthetic N, sewage treatment plant effluent and raw pig farm effluent (Francke & Den Oude, 1983). Maximum growth rates varied, but 'ideal' NH_4^+ concentrations were found between 5-50 mg L^{-1}, depending upon species. A similar study examined nine *Stigeoclonium spp.* cultured in synthetic medium with various concentrations of NH_3, NH_4^+, NO_3^-, NH_4NO_3 and $CO(NH_2)_2$ (de Vries & Kamphof, 1984). Concentrations of NO_3^- and $CO(NH_2)_2$ between 12-5 and 25 mg N L^{-1} appeared to have little adverse effect on growth between species and strains; however cultures were inhibited at ammonia and ammonium concentrations beyond 5 and 12.5 mg N L^{-1} respectively. Growth rates obtained with NH_4NO_3 showed intermediate values when compared to NO_3 and NH_4 alone (de Vries & Kamphof, 1984). Whatever the case may be, there is a paucity of research that must be addressed to determine the physiological effect of high ammonium concentration on the growth of macroalgae, especially if wastewater treatment is the sole purpose of the application.

Phosphorous

Unlike nitrogen, macroalgae are much more tolerant to high P concentrations (Chen et al., 2012); however high P concentrations can still be toxic to most macroalgae, with tolerance levels being very species-specific (Rodhe, 1948). High pH can also lead to the precipitation of various phosphate salts (reducing macroalgal growth). Phosphorous gradients examined in Francke & Denoude (1983) in the range of 0.1-15 mg L^{-1} determined that certain *Stigeoclonium* species could maintain peak growth rates at the maximum concentration, with peak growth in other species tested in concentrations of 1.5 mg L^{-1}. Phosphate levels of point-sources reported in the literature are highly variable, being in the range of 100 - 620 mg L^{-1}. Furthermore, high P loading also appears to significantly contribute to the turbidity of the effluent (Ong et al., 2006). However, there is limited information on the turbidity of point-sources, in particular anaerobically digested sources. Two reports in particular found untreated piggery wastewater to be in the range of 712 - 2,946 nephelometric turbidity units (NTU) (Mean of 1,910) from piggery wastewater settlement in Mexico (de Victorica-Almeida et al., 2008) and 976 NTU from piggery lagoon wastewater in China (Liu et al., 2013).

Carbon

Carbon sources are an often overlooked component of nutrient requirements, because they rarely become limiting. However, some recent research has focused on how carbon sources influence the bio-recovery and power generation capabilities of point source agricultural wastes (Buchanan et al., 2013). This includes heterotrophic production of biofuels from manures using anaerobic digesters for the production of fuels such as methane, ethanol and hydrogen gas (Holm-Nielsen et al., 2009).

Subsequently, these fuels have been used in site-based power generation plants and incinerators to produce electricity and heat for farming operations. From these systems, CO_2 emissions and high levels of nutrient effluent are derived. Phycoremediation of CO_2 derived from power stations has been studied in the past, with much of this research focused on microalgae (Moheimani, 2005). Little attention has been paid to macroalgae, due to the bulk of current cultivation practices being focused on marine offshore and nearshore systems. Of the effluent sources derived from both raw effluent and anaerobic digestion, significant proportions of carbon are released. Key carbon substrates such as volatile fatty acids (VFAs) are used to balance pH in anaerobic digesters, substantially adding to both the internal biomass of the anaerobic digester and to substrate effluent. The use of carbon substrate through heterotrophic processes by macroalgae is seemingly absent from the literature though, leaving a gap in knowledge that would aid in both the refinement of cultivation practices from anaerobically digested effluent (ADE), and the bio-recovery of potentially valuable chemicals. Only one single study on the use and comparison of VFAs with free fatty acids (FFAs) by *Scenedesmus sp.* and *Chlorella sp.* (Mohan & Devi, 2012) from acidogenic biohydrogen production has been done, which found that FFAs from the ADE performed better than VFAs as a substrate. However, this study also found that growth and lipid productivity of both microalgae was greatly enhanced, with VFAs pushing fatty acid composition towards saturated fatty acids suitable for biofuel production.

Table 1. Studies conducted on the freshwater cultivation of macroalgae in different nutrient sources. Key nutrients in each of these studies were derived from either raw effluent or anaerobically digested effluent (ADE). Nitrogen and phosphorous concentrations are given in mg L^{-1} for ammonium (NH_4-N), total nitrogen (TN) and total phosphorous (TP).

Study	Nutrient Source	Type	NH4-N	TN	TP	Algae Species
Mulbry & Wilkie (2001)	Dairy	Raw	306	1210	303	*Ulothrix sp., Oedogonium sp, Rhizoclonium sp.* Consortia
	Dairy	Digested	1620	2371	240	
	Dairy	Digested	178	225	24.7	
Pizarro et al. (2006)	Dairy	Digested	5 to 80		1 to 20	*Ulothrix sp., Oedogonium sp, Rhizoclonium sp.* Consortia
Kebede-Westhead (2006)	Dairy	ADE	233	412	64.5	*Microspora sp., U. ozonata, R. hieroglyphicum, Oedogonium sp.*
Mulbry et al.(2005)	Dairy	ADE		2760	332	*Oedogonium sp.*
Mulbury et al. (2008b)	Dairy	ADE		500 to 2300	85 to 300	*Rhizoclonium, Microspora, Ulothrix, Oedogonium sp.* Consortia
Francke & Denoude (1983)	Swine	ADE & Raw	1 to 100		0.1 to 15	*Stigeoclonium sp., Oedogonium sp.*
Mulbury et al. (2008a)	Swine	ADE & Raw		1.5 to 9.76		*Rhizoclonium hieroglyphicum*
Kebede-Westhead et al. (2006)	Swine	ADE	220 to 1130	270 to 1390	80 to 420	*Microspora sp., U. ozonata, R. hieroglyphicum, Oedogonium sp.*
Cole et al.(2015)	Aquaculture	Raw		1.49 to 3.75	0.50 to 0.62	*Oedogonium sp.*
Neveux et al. (2016)	Municipal	Raw		27.2	5.04	*Oedogonium sp.*
Cole et al. (2014)	Synthetic		0.067 hr^{-1}			*Oedogonium sp.*
Francke & Denoude (1983)	Synthetic		1 to 100		0.1 to 15	*Stigeoclonium sp., Oedogonium sp.*
De Vries & Kamphof (1984)	Synthetic		0.1 to 30			*Stigeoclonium sp.*
Lawton et al. (2013)	Synthetic			13.4	1.4	*Cladophora sp., Spirogyra sp., Oedogonium sp.*
Lawton et al. (2014)	Synthetic			12.3	1.12	*Oedogonium sp.*

Current algal production practices

Typically, land-based large-scale commercial microalgae cultivation uses open culture systems, in particular raceway ponds. This is fundamentally due to economic issues that constrain scaling up of closed systems like photo-bioreactors (Borowitzka, 1992; Borowitzka, 1999). These systems are rarely suitable for the cultivation of macroalgae. Open culture systems and techniques that offer the most potential for land-based macroalgal cultivation can be classified in the following systems, as adapted from Borowitzka and Moheimani (2013) and Aitken (2014):

- Shallow lagoons
- Ponds
- Bottom planting
- High Rate Algal Ponds (HRAPs)
- Algal Turf Scrubber (ATS)
- Long line cultivation

Some species of macroalgae can be cultivated in a similar way to microalgae using tanks and ponds as they are free-floating. Below is a summary of some of these cultivation techniques.

Shallow Lagoons

Wastewater treatment ponds or lagoons are an extensive cultivation method in which algae is used to treat wastewater. They are typically constructed as shallow earthen basins ranging from 2-5 m in depth, under low/no water exchange (Metcalf, 2003). Both shallow and deep algae lagoons have been used also as simple wastewater treatment systems for thousands of years, but they are inefficient. Algal production is low in these systems, with other processes such as vermiculture used to produce activated sludge for subsequent removal. As a consequence, these systems would be more suited to remediation of low-level eutrophication only.

Ponds

Land-based algal pond systems, both as independent farms and as effluent management systems in aquaculture have also been used for cultivation of macroalgae (Friedlander, 2008). An example of this is the remediation of waste from salmonid cultivation, where crops of the macroalgae *Porphyra* spp*., Saccharina latissima*, and *Nereocystis luetkeana* were cultivated (Burton et al., 2009). Furthermore, Marinho-Soriano et al. (2009) examined the use of *Gracilaria birdiae* in remediating wastewater from white shrimp (*L. vannamei*) farms and found that nutrient concentrations of PO_4^+, NH_4^+ and NO_3^- decreased by 93.5%, 34% and 100% after a four week experimental period. The advantages of the land-based ponds are the levels of control over the growing environment and the ease of access to crops (Chynoweth, 2002). Much of the pond culture of macroalgae is currently focused on high-value seaweed products; however adaption to freshwater cultivation would take very little effort in terms of techniques and technology.

Bottom planting

Bottom planting is a method used in the cultivation of marine macroalgae in extensive systems that have applicability in ponds and raceways. Macroalgae thalli can either be planted directly into pond sediments or fixed to removable structures to aide in its subsequent harvest (Buschmann et al., 1995). Although seeding cultivation beds requires significant labour input initially, planting may only be required once every two or more years, with several crops produced per year (Buschmann et al., 1995). Using this method in ponds and raceways allows for significant control over the growth environment. Bottom planting is used for *Gracilaria chilensis* cultivation in Chile for the production of agar and is one of the successful industries in this field (Halling et al., 2005).

High Rate Algal Ponds

High Rate Algal Ponds (HRAPs) are shallow ponds used for the growth of suspended microalgae (Craggs et al., 2011). These designs have been thoroughly researched; however modification to the mixing systems in these ponds may provide opportunities for macroalgae cultivation (Hoffmann, 1998). These systems are often mixed with paddlewheel(s), thus are not suitable for macroalgae as macroalgae can foul on paddlewheels. HRAPs are used to remove nutrients from anaerobic digestate effluents, municipal wastewater and agricultural wastewaters (Craggs et al., 2011). HRAPs can provide reductions in biological oxygen demand (BOD), total suspended solids (TSS), N, P, and metals (Hoffmann, 1998). Wastewater treatment HRAPs are normally part of an Advanced Pond System, which consist of multiple different ponds in a series (Craggs et al., 2011).

Algal Turf Scrubbers

The genesis of this system came from research into benthic algal mats, resulting in the Algal Turf Scrubber system. These benthic algal mats were designed and tested to reduce P from both freshwater and marine ecosystems (Adey et al., 1993; Marinho-Soriano et al., 2009). These have been used in the Great Wicomico River for the sequestration of both P and N from agricultural runoff (Adey et al., 2013). This production system utilises mixed microalgal and cyanobacterial communities on mesh substrates, removing large quantities of P and N. Briefly, a community of algae and bacteria (a periphyton) are grown on artificial substrates in a flow-way. Nutrients are delivered down the flow-way in a series of pulses, with nutrients cycled through biological processes down a spatial gradient (Craggs et al., 1996). Succession and dominance of photoautotrophs that develops along these systems changes, depending on flow rates, nutrient changes and growth conditions. Although attempts have been made

3

to engineer the communities in these systems, invariably they become naturally seeded

by the algae/bacteria in the wastewater itself, due to the lack of bio control and seasonal

changes that occur naturally.
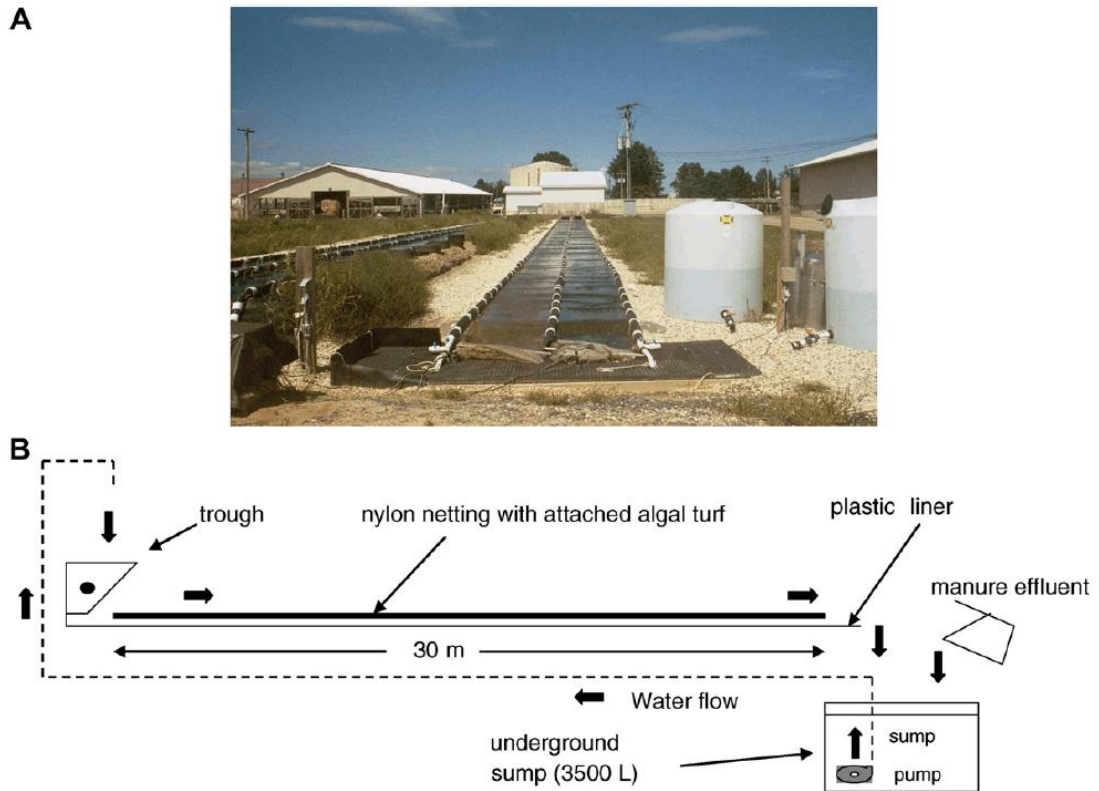
Figure 1. Photograph (panel A) and schematic drawing (panel B) of pilot-scale raceways at the Dairy Research Unit in Beltsville, Maryland. Each raceway is 1 m in width, 30 m in length, and has a water depth of 1–3 cm. Two raceways (left of center in panel A) were constructed at a 1% slope and two raceways (center in panel A) were constructed at a 2% slope. Raceway effluents (approximately 3500 L for each raceway) are contained in four separate underground concrete tanks covered by plastic grating (foreground in panel A) and are continuously recycled from the sumps to the top of the raceways using four separate sump pumps at a flow rate of 93 L min^{-1}. A trough at the top of each raceway fills and tips over, releasing pulses of effluent that wash over the attached algal turf every 8–15 s before draining into the concrete sump at the base of the raceway adapted from Mulbry et al. (2008b).

Algal turf systems were originally designed to remediate nonpoint source pollution

from upstream effluent before being discharged into downstream waterways.

However, preliminary studies have shown that they have a potential to remediate

point-source pollution as well. Anaerobic digestion effluent from dairy manure was

4

treated in a marine algal turf scrubber system at five different concentrations of N from 5 to 80 mg NH_4–N L^{-1} (Adey & Hackney, 1989). The algal nutrient removal rates in this study were 3.68 ± 2.55 µmol min^{-1} g^{-1} DW for NH_4^+–N and 0.40 ± 0.08 µmol min^{-1} g^{-1} DW for PO_4^{3+}-P for a 4% manure dilution. One of the key findings in this study however, was that pH during the incubation phase should be maintained between 7 and 7.5, as pH greater than 8.5 resulted in loss of algal culture as high pH caused a) the formation of NH_3 from NH_4^+, and b) precipitation of P, subsequently reducing algal growth. Dried algal yield from this system was approximately 5 g m^{-2} day^{-1}, with the biomass containing 15–20 mg/g P and 50–70 mg/g N. In a similar study, Mulbry and Wilkie (2001) studied the production of benthic freshwater algae on dairy anaerobic digestion effluent. Using TN loading rates of 0.64 to 1.03 g m^{-2} d^{-1}, dried algal yields were 5.3 to 5.5 g m^{-2} d^{-1}, with yields containing 15 - 21 mg/g P and 49 – 71 mg/g N.

Despite these systems appearing to be effective at reducing nutrient loads with microalgae, significant economic constraints have inhibited their use for commercial production. High costs associated with harvesting and processing, particularly dewatering, often prevent the commercialisation of microalgae production systems (Uduman et al., 2010). However this is not the case with macroalgae production, as post-production processes such as anaerobic digestion, fermentation, and hydrothermal liquefaction require little dewatering for processing (Roesijadi et al., 2010). In addition, algal harvest and water/algal separation can be easily accomplished by mechanical or manual scraping and vacuuming of the substrate once water flow is removed (Adey et al., 2013). Overall, algal turf systems offer the

greatest potential in the cultivation of macroalgae in land-based systems, due to their ease of use and high biomass production.

Long-line cultivation

The long-line cultivation method is commonly used for macroalgal production. Macroalgal spores or thalli are attached to ropes and are subsequently placed in water (Figure 2). The cultivation method is generally batch and can take six to nine months. Long-line cultivation can be used for a wide range of macroalgae, but it is mostly used for mass cultivation of brown seaweed (e.g. *Macrocystis, Laminaria*, and *Saccharina*).
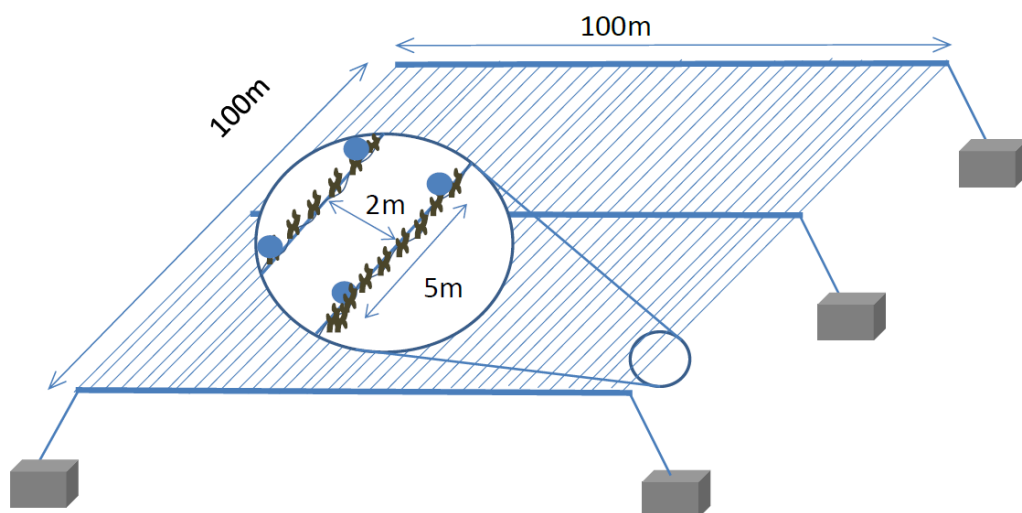

Figure 2. Typical cultivation set-up for 1 hectare of long-line macroalgae cultivation (Aitken 2014).

Macroalgae biomass production

In general, marine macroalgae have higher biomass productivity than freshwater macroalgae (Neveux et al., 2014); however four genera of freshwater macroalgae including *Oedogonium*, *Cladophora*, *Stigeoclonium* and *Spirogyra,* have all shown promise in biomass applications. When nutrient enrichment and increased solar

intensity is available productivities in algal consortia have been known to increase to between 25 - 45 g m^2 d^{-1} in algal turf systems (Adey et al., 2011; Mulbry et al., 2008b). Cole et al. (2014) examined the minimum nutrient requirements of *Oedogonium* to maintain growth at 16 – 17 g DW m^{-2} day^{-1} and found that a nitrogen flux of 1.45 g m^{-2} day^{-1} and a phosphorous flux of 0.6 g m^{-2} day^{-1} were required. Following this study, Cole et al (2015) investigated the biomass productivity in *Oedogonium*, cultured in intensive freshwater fish farm waste. The dry weight (DW) productivity ranged between 23.9 and 35.7 g m^{-2} day^{-1}, with an ash-free dry weight between 17.1 and 23.6 g m^{-2} day^{-1}, when supplied with a mean concentration of nitrogen between 1.49 (±0.13) and 3.75 (±1.39) mg L^{-1}, and phosphorous between 0.50 (±0.04) and 0.62 (±0.07) mg L^{-1}.

Oedogonium, *Cladophora* and *Spirogyra* have been examined for biomass production in mono and mixed cultures in Townsville, Queensland (Lawton et al., 2013). Of these species, monocultures of *Oedogonium* had the highest productivity (8.0 g ash-free dry weight m^2), lowest ash content (3–8%), highest carbon content (45%) and highest bioenergy potential (20 MJ kg^{-1}) of each of the three genera, when under high aeration with added CO_2. These trends continued, albeit with lower biomass output, when aeration and/or CO_2 was reduced or removed. Co-cultures of *Oedogonium* with *Cladophora* and/or *Spirogyra* were also examined over a range of stocking densities in this study. After three weeks of culture, *Oedogonium* coverage had increased to between 82 – 96 % in all treatments.

Kebede Westhead et al. (2006) also examined a macroalgae consortia comprised of *Microspora willeana*, *Ulothrix ozonata*, *Rhizoclonium hieroglyphicum* and *Oedogonium sp*. These trials examined dairy manure effluent at loading rates of 0.24,

0.40, 0.62 and 1.23 L m^{-2} d^{-1}, corresponding to daily total nitrogen and total phosphorous loading rates of 0.3–1.4 g and 0.08–0.42 g respectively. Mean algal productivity values increased from 7.1 g DW m^{-2} d^{-1} at the lowest loading rate to 9.4 g DW m^{-2} d^{-1} at the second lowest loading rate. Above this, algal productivity did not increase further, becoming highly variable at the highest loading rate (1.23 L m^{-2} d^{-1}). Under all loading rates, ash-free dry weight remained at 91 ± 2.3 % of DW, with moisture content at 10.2 ± 1.5 %.

Potential biochemical products

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. (2014; 2015) 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%). In general 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. Freshwater macroalgae also had a higher calorific value (15.8–16.4 MJ kg^{-1}) than marine (10.3 - 12.7 MJ kg^{-1}), which can be due to the lower ash content. In terms of the theoretical biocrude yields of each species, net productivity appeared to be the predominant influence on yield. All marine species had higher biomass productivity and biocrude yields than freshwater species. However, both marine and freshwater macroalgae lipid production is very low to be considered for biocrude production. Algae with lipid yield below 40% are unlikely to be financially or energetically worth processing (yield and extraction efficiencies) for bio-diesel

production (Sialve et al., 2009). However such biomass can be used for anaerobic digestion or bioethanol production.

Project rationale:

The quest for efficient treatment of wastewaters from piggery operations, which cannot be sewered to a centralised 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 used worldwide for the treatment of these wastewaters. This is mainly due to the overall inefficiency and the unfavourable operation cost associated with aerobic and physico-chemical-based technologies. Some major advantages associated with AD 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. Anaerobic digestion piggery effluent, while constituting a treated effluent, does not 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 3630 ± 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) have been reported in ADPE. This is because currently available technologies for wastewater treatments are not able to ameliorate the large increase in nutrient concentrations post-anaerobic digestion (Nwoba *et al*., 2016; Ogbonna *et al*., 2000). Continual discharge of these

9

highly concentrated treated effluents can result in eutrophication of aquatic environments (Carpenter & 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 because of their natural ability to strip away nutrients from wastewaters without the need for an additional carbon source (Neori *et al.*, 2004; Pulz, 2001). 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_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 requires substantial amounts 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 our knowledge, no peer-reviewed information is available regarding the treatment of ADPE using macroalgae.

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

2. Methodology

Collection of samples

Five local species of macroalgae (*Spirogyra* sp., *Rhizoclonium* sp., *Ulothrix* sp., *Gayralia* sp. and *Cladophora* sp., see Figure 3a to 3e) were collected from three different locations of the Canning River (Figure 4), Western Australia, using a sponge-like water filter mat (Figure 5) during the austral winter (August 2015). Upstream from the Canning River weir is 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 under natural temperature and solar radiation in Modified Chu 13 medium (KNO$_3$ replaced by NH$_4$Cl, 27.5 mg NH$_4^+$-N L^{-1}) (Yamaguchi *et al*., 1987). Only two strains, *Rhizoclonium* sp. and *Ulothrix* sp. (Figure 3b, c), survived and successfully grew as a

consortium in the artificial culture medium for more than one month and these were
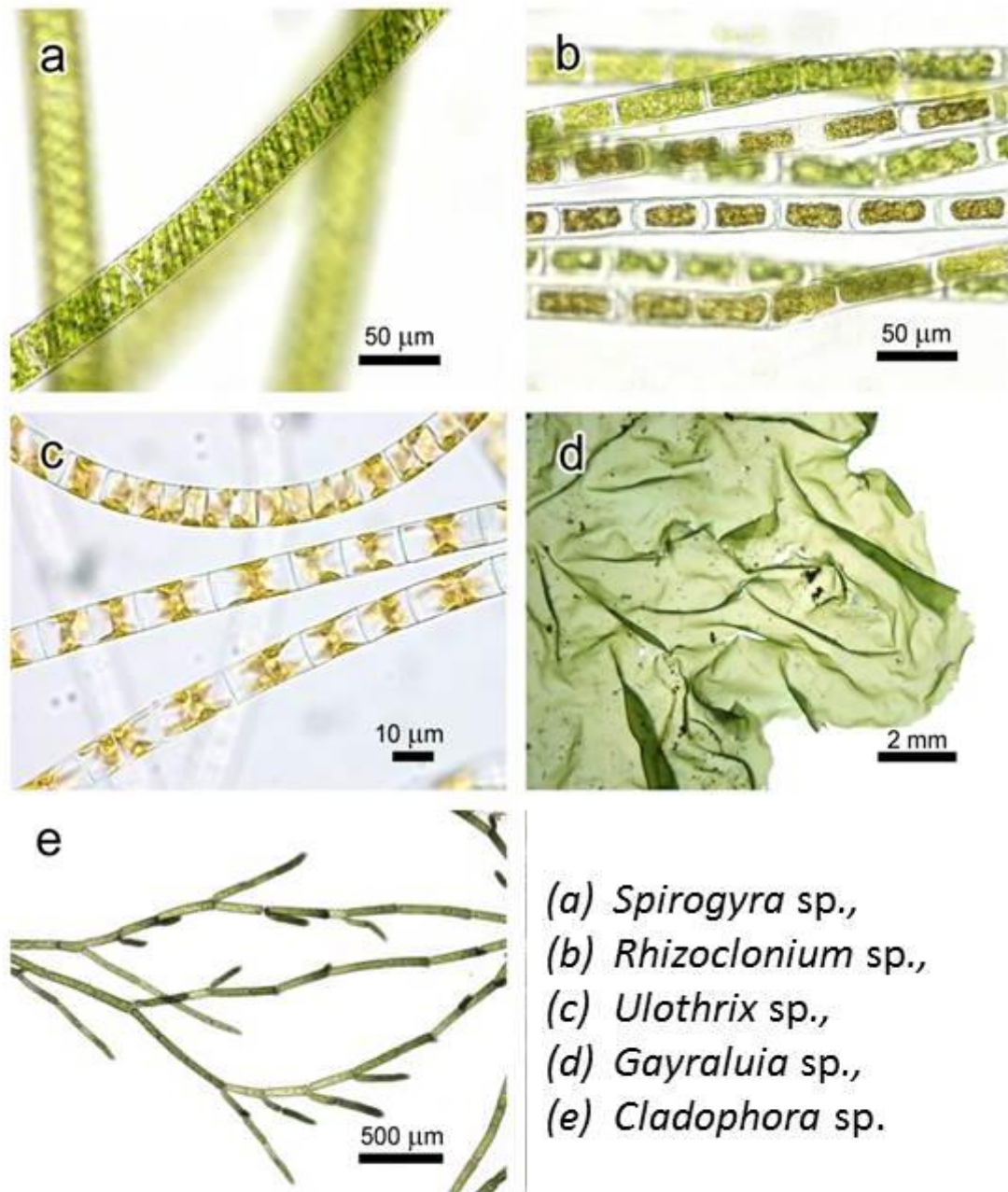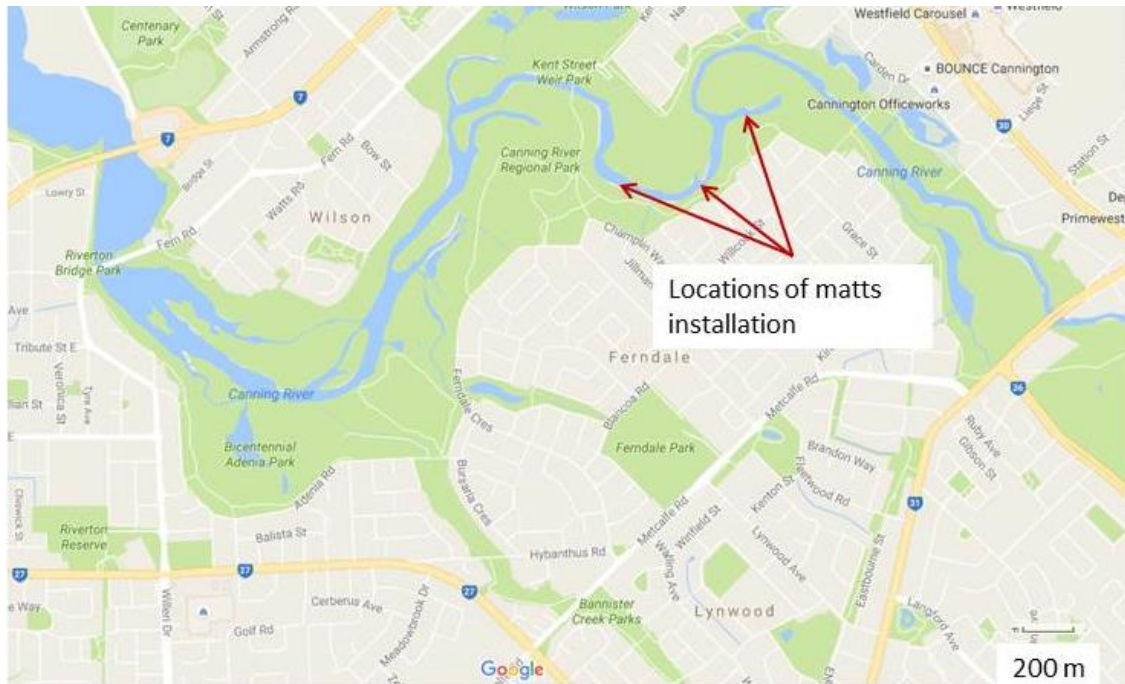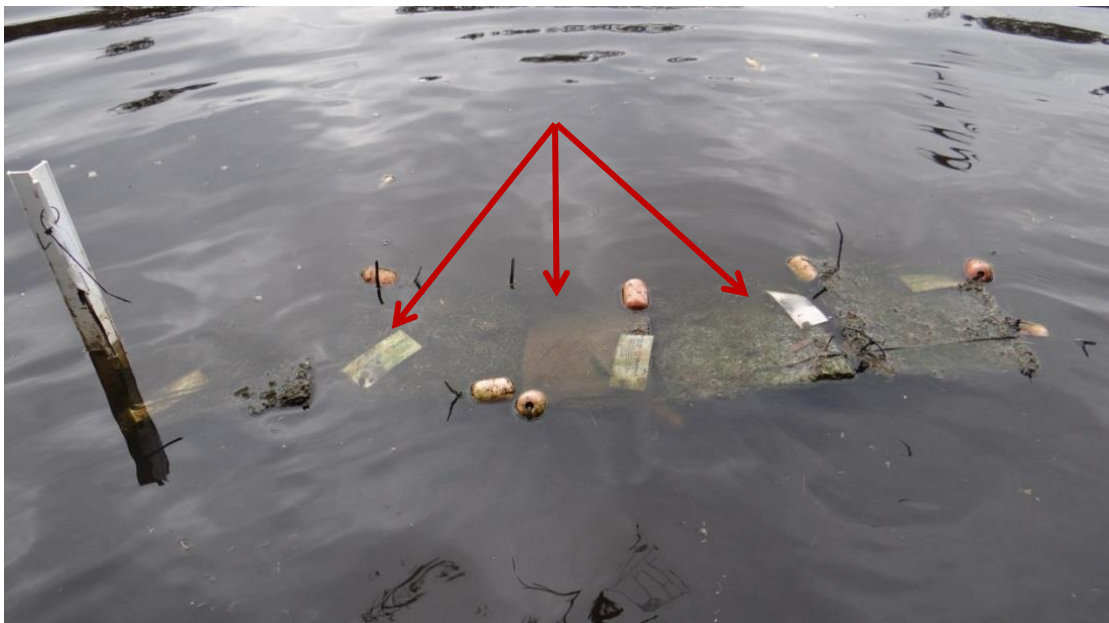used for further studies.

Figure 3. Photomicrographs of macroalgae.

Figure 4. Locations of mats installed at the Canning River (32°01'28.4''S 115°55'21.1''E, 32°01'30.2''S 115°55'32.8''E and 32°01'20.6''S 115°55'40.0''E).

Figure 5. Photograph of the sponge-filter mat installed in Canning River

Anaerobic digestion piggery effluent

The ADPE used for the study was collected from Medina Research Station located in

Kwinana, Western Australia (Nwoba *et al*., 2016). The research facility employs a

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 our study was sourced from the covered anaerobic digestion pond (Figure 6). The ADPE was sand-filtered (Figure 7) and used for cultivation of macroalgae without any further pre-treatment (Nwoba *et al.*, 2016). Physico-chemical properties of the sand-filtered ADPE were characterised using standard protocols (Table 1).

Figure 6. Photograph of the covered anaerobic digestion pond at Medina Research Station (photographed by Ayre).

Figure 7. The setup of the sand filter. The materials layered into the drum from bottom to top include: a) Perforated pipes, b) Coarse Gravel, c) Medium Gravel, d) Coarse Sand, e) Non-absorbent cotton wool placed as the top layer in the drum (Ayre, 2013).

Table 2. Chemical composition of untreated and undiluted ADPE* used for the growth of the macroalgae

Parameter	Value
Ammonia (mg L^{-1} NH_4^+-N)	1315.17±40.48
Total Phosphate, (mg L^{-1} PO_4-P)	34.55±3.75
Nitrite (µg L^{-1} NO_2-N)	10.53±2.15
Magnesium (Mg L^{-1} mg)	224
Potassium (mg L^{-1} K)	700
Total Iron (mg L^{-1} Fe)	12.4
Total alkalinity (or acid capacity) (mmol L^{-1} OH)	129
Nitrate (mg L^{-1} NO_3-N)	18.70±2.96
Chemical Oxygen Demand, COD (mg L^{-1})	1585.50±122.50
Total nitrogen (mg L^{-1} N)	1430
pH	8.2±0.09

* Chu Media used in these studies were prepared by diluting ADPE to desired ammonia concentration.

Bioprospecting

Sponge-like water filters (25 cm x 25 cm, Figure 5) were positioned at five locations,

1km apart upstream from Canning River weir (Figure 4). The filters were collected

from the river after three weeks and transported in river water to the laboratory. The morphological structures of the collected macroalgae species found to attach on to the filters were observed under light microscope (Figure 3 a-e). 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 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 NH_4^+-N L^{-1} with the ammonium concentration increased by a factor of 13.75 mg L^{-1} upon establishment of growth, until 55 mg NH_4^+-N L^{-1} (denoted in this study as Modified Chu 13, Table 3). At this stage, the algae were finally switched to ADPE-based medium starting with ADPE concentration equivalent to 27.5 mg NH_4^+-N L^{-1} and gradually increased until the breaking point ($\approx$ 260 mg NH_4^+-N L^{-1}) of the culture (i.e. not able to tolerate more ammonium concentration).

Table 3. Modified Chu-13 Medium (Chu, 1942).

Stock Solution	Amount (g.L^{-1})	Volume Added (mL.L^{-1})	Final concentration (mg.L^{-1})
KNO$_3$	400	1	400
K$_2$HPO$_4$	80	1	80
CaCl$_2$ dihydrate	107	1	107
MgSO$_4$ heptahydrate	200	1	200
Ferric Citrate	20	1	20
Trace elements:		1	
CoCl$_2$	0.02		0.02
H$_3$BO$_3$	5.72		5.72
MnCl$_2$	3.67		3.67
ZnSO$_4$ heptahydrate	0.44		0.44
CuSO$_4$ pentahydrate	0.16		0.16
Na$_2$MoO$_4$ dihydrate	0.084		0.084

* All stock solutions were added to the required amount of deionised water, pH was adjusted to 7.5 using 1M KOH and for solid media agar was added at a concentration of 10 g.L^{-1}.

Experimental set-up

To test the suitability of macroalgae isolates for nutrient removal from ADPE, the consortium was trialed for feasibility of growth and nutrient removal efficiency from ADPE. The consortium was first grown in Modified Chu 13 medium (with initial concentration of 27.5 mg NH_4^+-N L^{-1}) using a tipping bucket system (as per the design depicted in Figure 8) and acclimated to outdoor meteorological conditions (as described above).

Figure 8. Schematic of the tipping bucket system used for the cultivation of the macroalgae.

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 8). The sponge-filters were arranged in a 2x2 matrix design inside the upper tubs. An

adjustable submersible centrifugal pump (PU4500, PondMax, 4500L hr^{-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 lower tubs by gravity at a constant flow rate through a manifold. All experiments were run simultaneously in separate tubs for a six (6) day interval before medium renewal, with controls consisting of no alga in ADPE (negative control) and alga in Modified 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 as ash-free dry weight (AFDW), 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,

$$Prodctivity, P \ (g \ m^{-2} day^{-1} AFDW) = (FW_t - FW_i)\{T(FW:AFDW)x \ A\}^{-1},$$

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 (m^2).

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 florescence 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 (LEDs) that provided the non-actinic measuring light (spectral peak wavelength of 650nm). The maximum quantum yields in light (F_q'/F_m') 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.

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[TM] Petrifilm[TM] *Enterobacteriaceae* Count Plates Kit (Silbernagel & Lindberg, 2003). The 3M[TM] Petrifilm[TM] *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:

$[(initial\ bacterial\ count - final\ bacterial\ count)/initial\ count]\ x\ 100\%.$

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. Outcomes

Bioprospecting

Five macroalgal species (Figure 3a-e) were observed to attach to the filters, two of which were found to efficiently grow in both Modified 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. (Figure 3b, 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.

Culture conditions

The average daily solar radiation (Figure 9) ranged from 91.2 to 486.3 W m^{-2} (Mean, 341.7 ± 6.43 W m^{-2}) with nearly all days sunlight 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 9) 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.
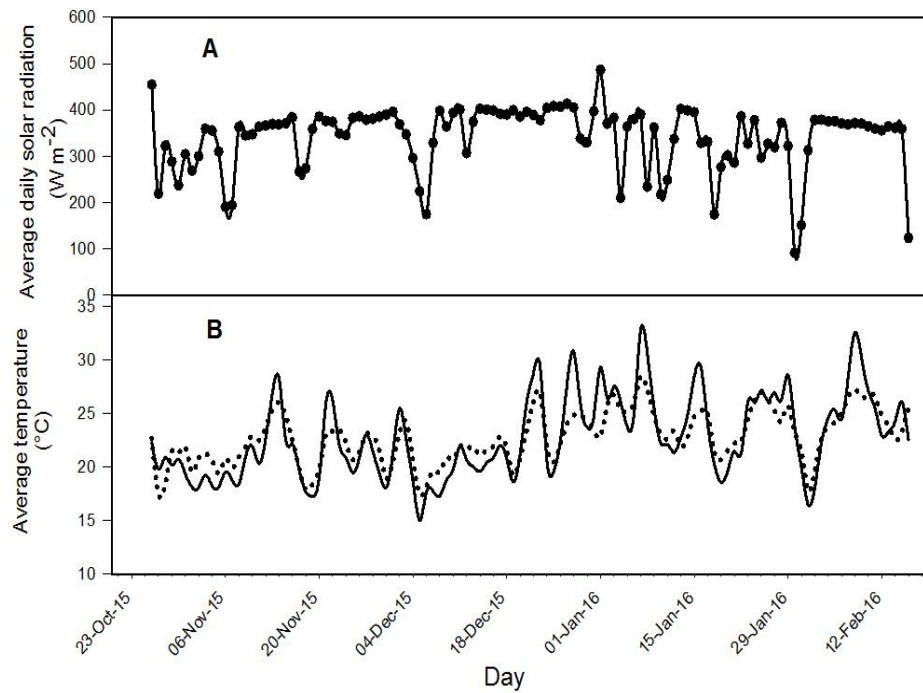
Figure 9. Panel A, average solar radiation, panel B, average culture (dotted line) and air (solid line) temperatures variation during growth of macroalgae consortium over the experimental period.

The dissolved oxygen (DO) concentration (Figure 10) in the cultures showed no fluctuations and the value was 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 10) 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 10).
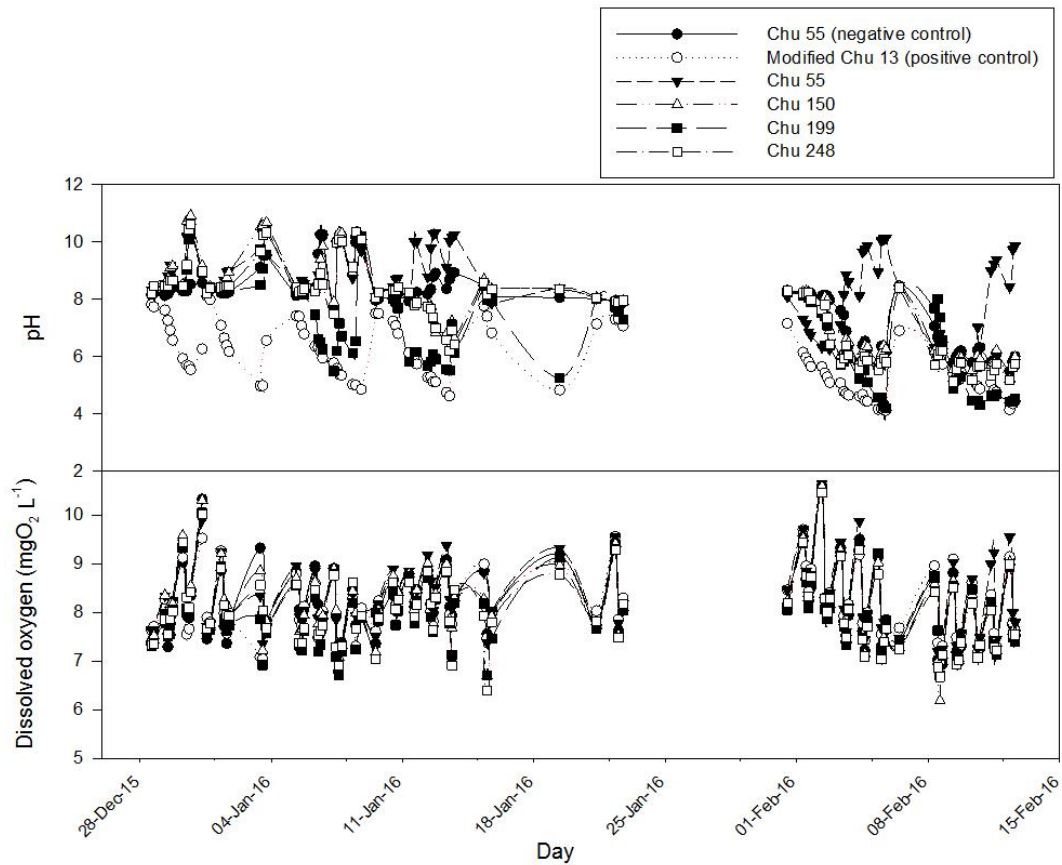
Figure 10. Changes in pH and DO of the various treatments throughout the experimental period.

Ammonium removal rates

Table 4 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 (data are 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 55 mg NH_4^+-N L^{-1} ADPE (Chu 55) and positive control (Modified 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 150, 199, and 248 mg NH_4^+-N L^{-1}

25

(denoted as Chu 150, 199, and 248 respectively) were not significantly different from each other (Duncan test, p = 0.291). The highest ammonium removal rate (30.6 $\pm$ 6.50 mg NH_4^+-N L^{-1}) was achieved in the treatment with 248 mg NH_4^+-NL^{-1}. 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 = 248.4) 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 (248.4 mg NH_4^+-N L^{-1}), 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 4).

Table 4. Ammonium removal rates, biochemical composition and chlorophyll _a_ content of macroalgae consortium treated with different ammonium concentration in ADPE-based medium

Ammonium concentration (mg NH_4^+-N L^{-1})	Protein (%AFDW)	Carbohydrate (%AFDW)	Lipids (% AFDW)	Ammonia removal rates	Chlorophyll content (μg mL^{-1})		Biomass Productivity (gAFDWm^{-2}d^{-1})	Bacterial load reduction (%)
					Chl-a	Chl-b		
Modified Chu 13	44.63±0.967[a]	42.82±2.197[b]	4.72±0.206[a]	1.96±0.70[b]	14.34±3.238[b]	7.01±1.894[c]	30.17±0.760[ab]	NA
Chu 55	43.84±1.919[a]	48.13±0.327[ab]	5.57±0.173[a]	3.80±1.16[b]	17.84±1.411[b]	7.85±0.989[bc]	31.19±1.540[ab]	48.31±0.22
Chu 150	43.39±0.476[a]	43.54±2.003[b]	4.98±0.251[a]	27.34±6.18[a]	26.38±0.272[a]	15.93±1.030[a]	33.73±1.259[a]	66.84±5.97
Chu 199	44.99±1.607[a]	51.62±3.534[a]	3.57±0.212[b]	23.70±2.82[a]	25.61±0.489[a]	13.95±1.263[ab]	30.99±1.165[ab]	73.02±3.71
Chu 248	44.45±2.889[a]	54.79±1.264[a]	3.07±0.189[b]	30.62±6.50[a]	26.26±0.580[a]	18.11±3.773[a]	29.56±0.584[b]	81.73±4.43

The ammonium removal rates of the consortium of algae trialed shows that they are a potential sink for ammonia in ADPE and excellent candidates for integrated pork farming. This is because of their 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. We did not study the exact role of alternative routes for ammonia removal. Volatilisation, 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 3 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 optimised 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 oxidised 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^+. Based on the removal rate from the

highest ammonium concentration tolerated by the consortium in this study, our 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}, which again are 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, our 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 4).

Integrating algal 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 NH4$^+$-N L^{-1}. Conversely, Nwoba et al. (2016) successfully grew

a microalgae consortium (*Chlorella* sp, *Scenedesmus* sp. and pennate diatom) in undiluted ADPE under outdoor condition. A promising option would be a two-stage sequential technology that would involve first treating the undiluted ADPE with the microalgae consortium to reduce the ammonia content to a level that the macroalgae consortium can be used to polish the effluent.

Biomass productivity

The biomass productivity of the consortium in the different ADPE-based medium is shown in Table 4. The biomass productivity obtained from the treatment in ADPE concentration at Chu 150 (33.7 ± 1.26 g AFDW m^{-2} d^{-1}) was 1.14 times higher than when Chu 248 was used (Table 4). However, no difference was found between the macroalgal productivities at ADPE concentrations of Modified Chu 13 medium, Chu 55, Chu 150 and Chu 199 (Table 4). Moreover, at ADPE concentration equivalent to Chu 248.44, the biomass productivity (29.6 ± 0.58 g AFDW m^{-2} d^{-1}) was not significantly different from that obtained with Modified Chu 13 medium (30.2 ± 0.76 g AFDW m^{-2} d^{-1}). 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}. We also found a similar outcome in this study, since the biomass productivity at ADPE concentrations equivalent to 199 and 248 mg NH_4^+-NL^{-1} 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 DWm^{-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 macroalgal biomass

produced by this consortium under the meteorological conditions of our current experiment are similar to other studies and shows the possibility of obtaining higher economic revenue during ecotechnological application of these algae. However, further studies on the reliability of mass cultivation and larger scale production is necessary prior to any commercialisation.

Biochemical composition of biomass

Typical biomass after each harvest is shown in Figure 11. The variation of the biochemical contents (total protein, carbohydrates, and lipids) of the consortium biomass is shown in Table 4. 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 Modified 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 & Cheung, 2000). Furthermore, our 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 (248.4 mg 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 4). The total carbohydrate content of the Modified Chu 13 medium (55 mg NH_4^+-N L^{-1}) was similar to the ADPE concentrations equivalent to Chu 55 and 150, but significantly ($p < 0.05$) lower than

31

the ADPE concentrations equivalent to Chu 199 and Chu 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.

Figure 11. a and b) using a commercial Ryobi 1250W, 20L Wet and Dry Vacuum cleaner for harvesting macroalgae biomass (image curtsey of channel 9 news). c) method for Harvested macroalgal biomass.

Chlorophyll content of the consortium grown in ADPE

Here, chlorophyll *a* and *b* contents were found to increase with increasing ADPE concentration (Table 4). Correlation indicated a significantly positive association (r = 0.889, p = 0.044, Pearson product moment) between initial ammonium concentrations and chlorophyll *a* content of the biomass from the different treatments. Comparisons of treatments, Modified Chu 13 Medium and ADPE concentration equivalent to Chu 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* content of ADPE concentrations equivalent to Chu 150, 199, and 248 media. However, the chlorophyll *a* content found in ADPE

concentrations equivalent to Chu 150, 199 and 248 systems were significantly ($p<0.05$) different from the ADPE concentration equivalent to the Chu 55 and Modified Chu 13 media. Similar results and relation was found in the chlorophyll *b* content of the treatments (Table 4).

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 the dark colour of the effluent which significantly reduces 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 chlorophyll *a* and *b* content 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 increases in their cellular nitrogen (Fogg & Thake, 1987). The pigment content of macroalgae can decrease because of growth and insufficient availability of nitrogen for sustained

33

biosynthesis (Kim *et al*., 2007). Considering that in the high ammonium treatments (e.g. 150 – 248 mg NH_4^+-NL^{-1}) of our study, ammonium was not exhausted, coupled with the high protein content irrespective of ammonium concentration applied, nitrogen availability was not a limiting factor. We also found a similar trend in chlorophyll *a* content, ammonium removal rates and protein content of the consortium biomass grown in high ammonium (i.e. ADPE concentrations equivalent to Chu 150, 199, and 248 media), where an increase in ammonium concentration yielded no further increase in the parameters.

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, we measured the maximum quantum yield (Fv/Fm) as a sensitive indicator of the algae photosynthetic performance. The Fv/Fm 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 Fv/Fm for all the treatments remained high, ranging between 0.42 ± 0.011 and 0.66 ± 0.006 at pre-dawn. The Fv/Fm 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 (Figure 12). Under light adaptation, the effective quantum yield (Vadiveloo et al., 2016), Fq′/Fm′ 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 (Figure 12). However, values were found to recover to be highest at pre-dusk when solar irradiance and subsequently temperature decreased (Figure 12). The rapid decrease in

Fv/Fm during the midday solar irradiance indicated a high degree of photoinhibition. In other words, the decrease in Fv/Fm at midday was 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 Fv/Fm obtained at pre-dusk mean that the photosynthetic machinery was able to recover from solar-induced photodamage by ultra violet (UV) radiation. These results indicate that we most likely need to shade the culture to reduce the overall light received at the surface of the culture. That can also be an indicative that this methodology can potentially be more efficient if used in places with lower irradiance. Wavelengths in the UV range of the electromagnetic spectrum have been found to be lethal to photosynthetic processes because of their ability (due to high energy content) to destabilise 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). Our data reveals that this consortium is robust and tolerant to the confounding variables of high temperature, solar radiation and ammonium concentration, with the Fv/Fm inversely proportional to the available solar radiation.
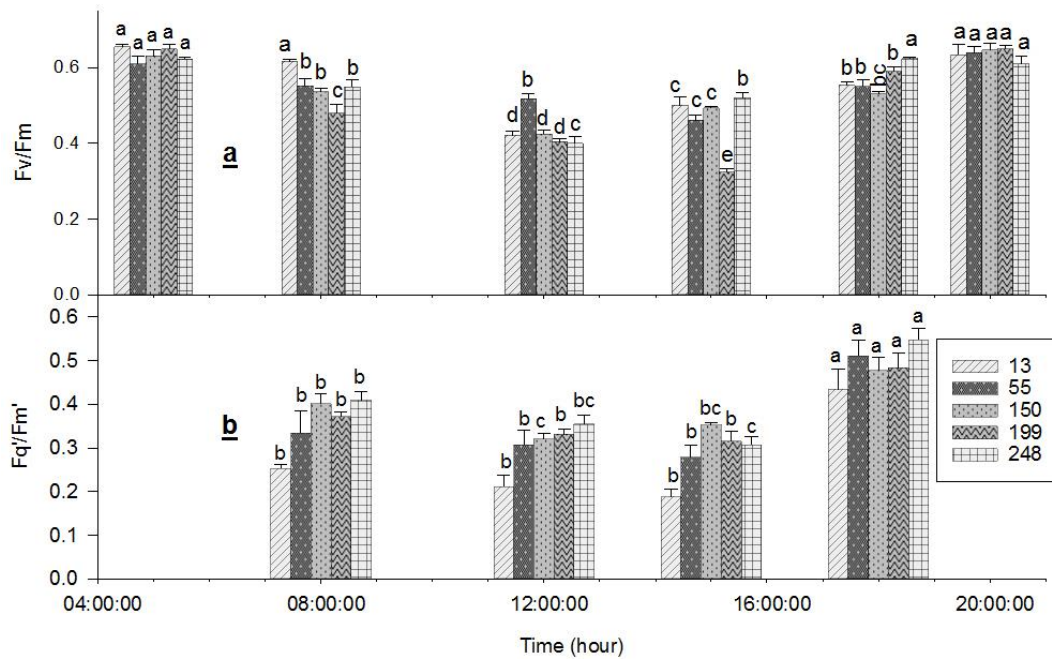
Figure 12. Diurnal changes in the photosynthetic response of the macroalgae consortium under light (panel a) and dark (panel b) adaptations. Bars with the same letter across groups are not significantly different (p>0.05).

Effect of CO₂ addition on biomass productivity and ammonia removal rates

An additional experiment was conducted using the same macroalgal consortium (*Rhizoclonium* sp. and *Ulothrix* sp.) in September 2016. Similar outdoor systems were used, which held 90 L of ADPE medium composed of ADPE diluted with tap water to yield a 27 mg L^{-1} concentration of ammoniacal-nitrogen (NH_3-N). Three replicates received no additional inputs, while three replicated had CO_2 injected into the system to maintain pH between seven and eight using a custom-made pH controller (TPS, Australia). All replicates were exposed to natural conditions of temperature, irradiation and rainfall. The ADPE media was replaced every seven days, and the systems were topped up with tap water before each measurement to make up for evaporation.
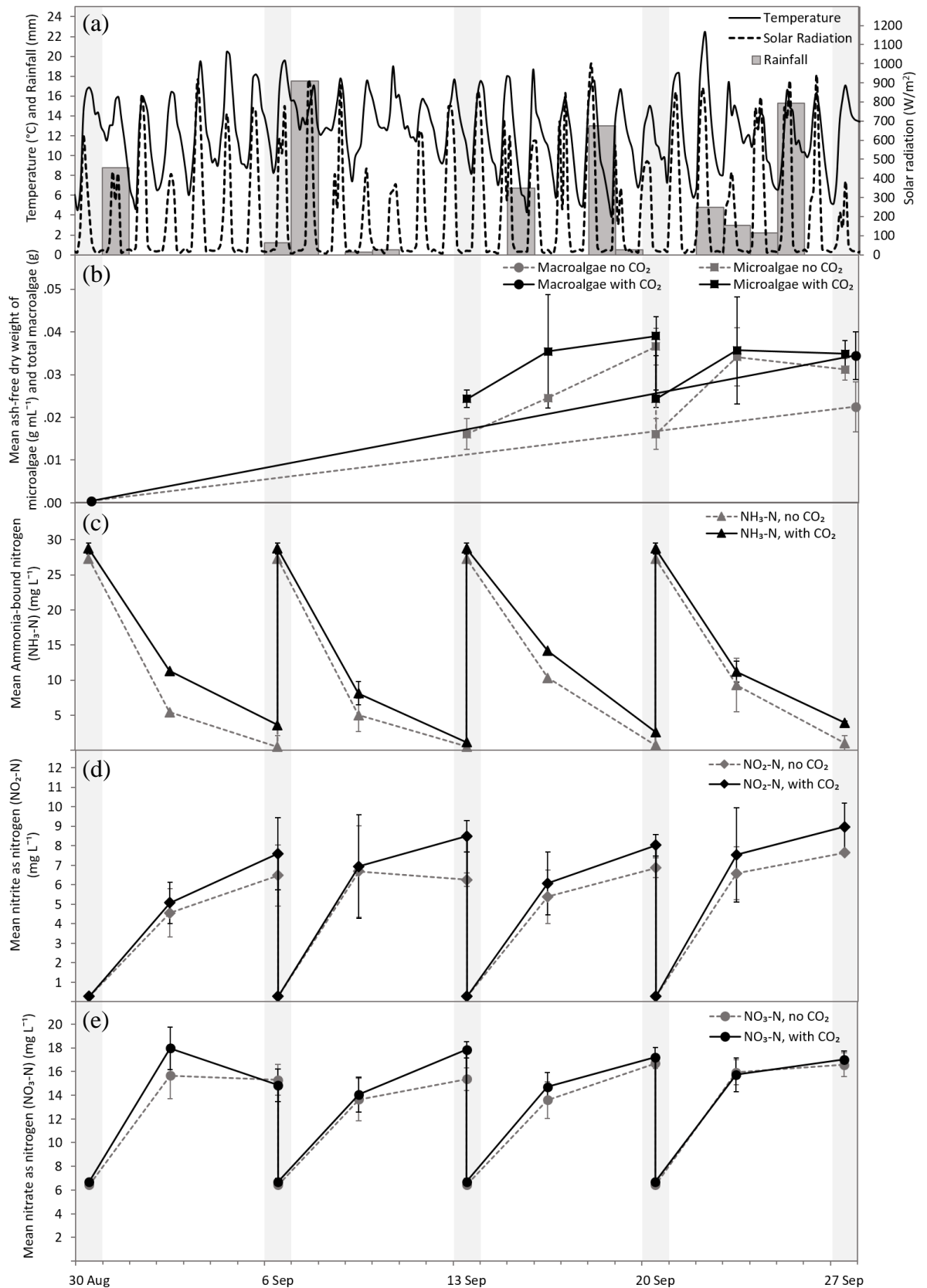
Ambient temperatures over the study period ranged from four to 22 °C, with an average daily maximum temperature of 18 °C (Figure 13a). It rained on 12 days in total, or on 43% of days of the experiment (Figure 13a). Irradiation was reduced on many days due to cloud cover (Figure 13a), with the average daily solar radiation ranging from 79.0 to 281.2 W m^{-2} (mean, 201.2 ± 29.6 W m^{-2}). For the samples treated with CO_2 input, the pH of the medium ranged from 6.63 to 7.97 over the experiment, while for those without CO_2 input pH ranged from 7.89 to 8.79.

All macroalgal biomass created over the one month period was collected at the end of the experiment, and microalgal concentration was measured bi-weekly throughout the experiment. Mean macroalgal production (ash-free dry weight) over the experiment for the replicates without CO_2 input was only 0.24 ± 0.06 g, while for replicates with CO_2 input a mean of 0.36 ± 0.06 g was produced (Figure 13b). Microalgal concentrations were also greater for the CO_2-treated replicates than those without CO_2 treatment, although the difference was mostly within the inter-replicate variation. Microalgal concentrations increased over each week (most microalgae were removed during weekly the media change process), reaching a mean highest concentration of 0.030 g mL^{-1} (ash-free dry weight) for untreated samples and 0.032 g mL^{-1} for CO_2-treated samples (Figure 13b).

Almost all ammonia (NH_3) was removed from all replicates over each week of the study period (Figure 13c). It appears that more ammonia was removed by untreated systems (mean NH_3-N reduction of 98.8% ± 0.4) than by CO_2-treated systems (91.4% ± 2.2). However, due to varied starting concentrations, slightly more ammonia was removed by untreated systems (mean reduction of 26.6 ± 0.1 mg L^{-1} NH_3-N) than CO_2-treated systems (mean reduction of 25.9 ± 0.6 mg L^{-1} NH_3-N).

Figure 13. (a) Air temperature, solar radiation and daily rainfall over the study period; (b) Mean total macroalgal biomass produced over the experiment and mean microalgal concentrations present, only measured in weeks 3 and 4; (c), (d) and (e) respectively show mean ammoniacal-nitrogen (NH_3-N), nitrite-nitrogen (NO_2-N) and nitrate-nitrogen (NO_3-N) measured in the ADPE medium throughout the experimental period. The ADPE medium was replaced once per week, on days indicated by bars of shading.

The rate of ammonia removal was greater during the first half of each week than during the second half (Figure 13c). For the untreated samples an average of 6.6 ± 1.7 mg L^{-1} NH$_3$-N was removed per day in the first half of each week compared to only 0.4 ± 0.3 mg L^{-1} each day for the second half of each week. A similar trend was observed for CO$_2$-treated samples, with a mean of 5.8 ± 2.1 mg L^{-1} NH$_3$-N removed per day for the first half of the week and 0.4 ± 0.7 mg L^{-1} per day for the second half. This reduced daily removal was most likely due to the available ammonia being already significantly reduced by the middle of each week (an average reduction of $73.5\% \pm 5.0$ for uncontrolled samples and $61.8\% \pm 4.3$ for CO$_2$-controlled samples.

Concurrently with reductions in ammonia, nitrite (NO$_2$) and nitrate (NO$_3$) were observed to increase over each week (Figure 13d,e). A similar trend to ammonia removal is apparent for increases in both nitrite and nitrate, in that there was a greater production rate for both for the first half of each week as compared to the second half of each week. Overall production of NO$_2$ and NO$_3$ were slightly greater for CO$_2$-treated samples than untreated samples. A mean total of 6.5 ± 0.3 mg L^{-1} NO$_2$-N and 11.1 ± 1.8 mg L^{-1} NO$_3$-N was produced per week for untreated samples, compared to 8.0 ± 0.3 mg L^{-1} NO$_2$-N and 11.7 ± 1.9 mg L^{-1} NO$_3$-N for CO$_2$-treated samples.

The concurrent increases in NO$_2$ and NO$_3$ with decreases in NH$_3$ indicate that most of the NH$_3$ was being converted to NO$_2$ and then NO$_3$, rather than being lost from the system in some other way (such as being exsolved from the system). However, if this pathway was the only factor, it would be expected that greater loss of NH$_3$ would be mirrored by greater gain in NO$_2$ and NO$_3$, but this was not the case here (CO$_2$-treated samples showed a greater decrease in NH$_3$, but a lesser increase in NO$_2$ and NO$_3$).

Biomass production of macroalgae observed in this experiment in both CO2-treated and untreated systems was very limited and significantly less than that seen in previous studies using the same consortium. Macroalgae production for this experiment was less than 0.5% of previous the experiment. The most likely factor involved in this significant reduction is the changed weather, as the previous experiment was conducted over summer, while this one was conducted in winter-spring. The mean daily maximum temperature over this study was only 18 °C compared to 23 °C for the previous experiment, and mean daily solar radiation was only 201 W m^{-2} compared to 341 W m^{-2} for the previous experiment. This indicates that biomass productivity for this macroalgae consortium is strongly seasonal and significantly reduced during the non-optimal months (summer).

Despite extremely low biomass productivity, this experiment has shown very good removal of NH_3 and increased production of safer NO_2 and NO_3. This suggests that this consortium of macroalgae could be used throughout the year to significantly diminish the ammonia concentration of high-ammonia ADPE and recover the quality of the wastewater.

While algal growth and NH_3 removal were greater for replicates which had their pH controlled by addition of CO_2, these differences were not large when compared to uncontrolled replicates. Especially when coupled with the increased cost of a pH control system, these results suggest that further consideration of pH control for this type of system would not be recommended.

4. Application of Research

Anaerobic digestion (AD) is a waste treatment methodology which has been adopted by many piggeries worldwide. The large amount of effluent produced from AD has been one of the most important environmental problems to be urgently solved in many countries including Australia. To remove fertilisers from ADPE, several treatment strategies have been proposed, including land application and activated sludge process. However, these methods are costly and not very efficient for nitrogen (N) and phosphorus (P) removal.

In recent years, macroalgae have been considered as a promising potential solution for wastewater management. Macroalgae have the ability to deplete inorganic nutrients (N and P) from a wide range of wastewater.

Current worldwide seaweed production is around 16 million wet tonnes (~1 million tonnes dry) per year and growing, and around 90% of current production is harvested from near-shore mariculture farms (Roesijadi et al., 2008). Seaweed can also be grown in several aquaculture polycultures to prevent high-nutrient effluent release with no net loss of productivity from primary species (Borines et al., 2011; Mai et al., 2010; Stickney & McVey, 2002). As such, macroalgae are a potentially cost-effective biomass feedstock (~US$7.5 – 1.5 t^{-1}). It should be noted that the bulk of macroalgae grown worldwide are seawater based. There is very little known on the potential of freshwater macroalgae for wastewater treatment and biomass (feed, food and energy) production. Here we clearly demonstrated the potential of using a consortium of macroalgae capable of treating anaerobic digestion of piggery effluent.

5. Conclusion

Our current study indicates the ability of *Rhizoclonium* sp. and *Ulothrix* sp. to significantly diminish the ammonium concentration of high ammonia ADPE and recover the quality of the water. The result of our 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 seasonally generating a significant amount of biomass that could be suitable for animal feed or bioenergy.

6. Acknowledgment

7. Limitations/Risks

The main limitations to this study were:

1) We had limited time to conduct the trials. Therefore, the outdoor cultivation was conducted in just a few months. There is a need for a long term trial for identifying the reliability of the process.

2) The trial conducted was small scale and using just one type of cultivation system (tipping bucket). This resulted in almost no collection of the macroalgae for further analyses.

3) We could only grow the isolated macroalgal consortium on the 248 mg NH_3. N. L^{-1}. This means that production would require either dilution or a preliminary treatment before macroalgae cultures can be applied.

The major potential risk:

1) While the overall bacterial contamination was significantly reduced after the macroalgal treatment (Table 4), there is also the need to assess the quality of the produced biomass.

8. Recommendations

Our main recommendations to the industry are:

1) Further bioprospecting especially aimed at isolating macroalgal species capable of tolerating a higher concentration of ammonium.
2) Developing other cultivation methods for mass macroalgal growth on ADPE.
3) Further long term cultivation studies.
4) Conducting pilot studies.
5) Testing the quality of the produced biomass as a feed.
6) Conducting detailed feeding trials.
7) Conducting detailed techno-economics and life cycle assessment of the whole process.

9. Acknowledgment

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Appendix 1 - Notes

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If a Final Report contains Confidential Information:

- NA

Deficient Report

If the Pork CRC reasonably forms the view that the Final Report does not adequately set out matters referred to, it must notify the Researcher of the extent to which it believes the Final Report is deficient.

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Appendices

Appendix 1: