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Pilot-scale continuous recycling of growth medium for the mass culture of a halotolerant *Tetraselmis* sp. in raceway ponds under increasing salinity: A novel protocol for commercial microalgal biomass production.

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Abstract

The opportunity to recycle ~~possibility of recycling~~ microalgal culture medium for further cultivation is often hampered by salinity increases from evaporation and fouling by dissolved and ~~suspended substances~~ particulate matter. In this study, the impact of culture re-use after electro-flocculation of seawater-based medium on growth and biomass productivity of a the halotolerant green algal strain ~~of~~ *Tetraselmis* sp., MUR 233, was investigated in

pilot-scale open raceway ponds over five months. Despite a salinity increase from 5.5 to 12% (w/v) NaCl, *Tetraselmis* MUR 233 grown on naturally DOC-enriched recycled medium produced 48 to 160% more ash free dry weight (AFDW) biomass daily per unit pond area than when grown on non-recycled medium. A peak productivity of 37.5 ± 3.1 g AFDW.m^{-2}.d^{-1} was reached in the recycled medium upon transition from ~14 to ~7% NaCl. The combination of high biomass–yielding mixotrophic growth under high salinity has been proven to be a successful sustainable cultivation strategy.

Keywords

Marine microalgae; recycling; electro-flocculation; mixotrophy.

1. Introduction

Currently~~,~~ there is substantial interest in the utilisation of microalgae towards the mitigation of the present and future world crises in food, fuel and environment. Microalgae are very diverse and represent a rich source of phytochemicals which can be used in human food, animal feed, aquaculture, for pharmaceutical, cosmetic and health food products, and conceivably for biofuel and ~~its~~ associated by-products (Brennan & Owende, 2010; Pulz & Gross, 2004; Spolaore et al., 2006). As an alternative form of agriculture, ~~microalgal~~ microalgae cultivation has much commercial appeal because it can potentially yield better biomass productivity rates than land crops over similar land areas (Posten & Schaub, 2009; Schenk et al., 2008). Furthermore, ~~this~~ it offers the possibility to use resources that are otherwise under-utilised (e.g. non-arable land, saline water, wastewater, etc.) or that are accumulating to polluting levels

(e.g. excess nutrients leading to eutrophication of waterbodies, build-up of CO_2 in the atmosphere which causes the greenhouse effect, etc.).

To date, much of the successful mass cultivation of microalgae is limited to the production of high value products, high revenues of which offset the high capital and operational expenditures incurred in generating and processing the biomass. Some of the most cost-prohibitive components of microalgae biomass production are directly associated with the large volume of water, which needs to be processed for cultivation and harvesting (Borowitzka & Moheimani, 2013; Fon Sing et al., 2013; Molina Grima et al., 2003). According to the life-cycle assessments of ~~microalgal~~ microalgae cultivation reported by Clarens et al. (2010), Flesch et al. (2013) and Yang et al. (2011), the cost-effectiveness of production could be substantially improved by minimising the water and nutrient footprint through the continuous recycling of the culture medium and by using non-potable sources of water. However, while culture medium recycling seems to confer certain advantages, ultimately the ~~possibility~~ prospect of culture reuse depends largely on the suitability of the harvested water for further continuous cultivation. This is because as opposed to freshly made medium, the recycled medium, if untreated, potentially carries over and accumulates all of the dissolved chemical compounds and suspended particles remaining after the harvesting process. For instance, cell wall debris, contaminating organisms (e.g. other algal species, bacteria, etc.), dissolved organic compounds and other potentially-growth inhibiting chemicals released from the cells commonly foul the return water. If the water is left untreated prior to returning to the ponds, these chemical compounds and particles can quickly lead to increased bacterial

activity and culture deterioration (Ben-Amotz, 1995; Chini-Zittelli et al., 1999; Rodolfi et al., 2003). In addition to this, the gradual increase in inorganic salts (salinity) due to evaporation must also be considered, especially in open cultivation systems where brackish or saline water is used. This change in salinity can potentially impact on the culture in two ways, namely: by a gradual dominance of more halotolerant species of microalgae, and/or in steady decline in the density of the desired microalga due to its inability to cope with osmotic changes and changing salt ratios. Another potential challenge of recycling medium with increasing salinity is the precipitation of calcium salts, especially in calcium-laden water, thereby causing loss of alkalinity and other minerals such as iron and phosphorus (Shimamatsu, 2004).

Clearly more exhaustive research should be carried out to enhance and optimise current processes used to grow monocultures of microalgae in recycled culture medium. Considering the ~~challenges of medium recycling~~ difficulties of recycling culture media and the need to use non-potable water for improving the economics and sustainability of microalgal biomass production, the real challenge lies in the ability to sustain monocultures of microalgae for as long as possible in a recycled culture medium of potentially increasing salinity without any loss in biomass productivity. ~~and quality~~. This study investigates the effect of culture medium recycling on culture health and biomass productivity of a halotolerant strain of *Tetraselmis* sp. grown at increasing salinity and continuously for long periods in ~~an~~ open raceway ponds under outdoor field conditions. Thus, the proposed protocol is a direct contribution to the

development of cost-efficient and sustainable production of biomass from saline microalgae in the field by a rational recycling of the culture medium.

2. Materials and methods

2.1. Location and microalgal species

The experiment was carried out from August to December 2012 in outdoor open raceway mixed ponds in a remote area in a semi-arid climate at Karratha, Western Australia, Australia (20S 45'47.72'', 116E 44'9.88''). *Tetraselmis* sp. MUR 233, originally ~~sourced~~ from the Murdoch University Algae Culture Collection (Perth, Western Australia) was used as test organism. This alga was maintained in semi-continuous cultivation mode in outdoor raceway ponds at the same location for at least two years prior to this experiment.

2.2. Ponds

Two 2 m^2 (M1 and M2) above-ground fibreglass open raceway ponds (2 x 1 x 0.4 m, L x W x H) and two 25m^2 (P4 and P5) in-ground 40 mm HDPE-lined raceway ponds, the only ponds available at the time of the experiment, were employed in this study. The cultures in M1 and M2 were used as controls, where the harvested portion of the cultures was replaced with fresh medium whereas recycled medium was used in P4 and P5 ponds (experimental treatments) (**Figure 1**). The use of the smaller ponds as controls instead of having a control pond at each scale were dictated by the need for a minimum volume for the downstream processing, and the small ponds alone would not have met this requirement. Based on data gathered from previous long-term

continuous cultivation in both sets of ponds, ~~revealed~~ no significant differences in growth and biomass productivities between the pond sizes were found (Isdepsky, unpublished data) and therefore, it was deemed reasonable to proceed with the current experimental setup.

To limit operational differences between the ponds to the effect of the recycled medium only, all cultures were kept at an operating depth of 20 cm, were mixed with a surface velocity of 20 cm.s^{-1} and were harvested at 50% of the culture volume. Also, the salinity of M1 and M2 ponds was maintained equal to that of P4 and P5 ponds by salt addition. ~~to limit operational differences between the ponds to the effect of the recycled medium only.~~

2.3. Culture conditions

The target starting cell density was ca. 40x10^4 cells.mL^{-1}. The pH of all ponds was maintained at pH 7.2 ± 0.3 with food grade CO_2 using a pH-stat system. The CO_2 supply was switched off between 20:00 and 07:00 to avoid CO_2 loss due to a reduction in water level upon culture harvest~~. The CO_2 supply~~ It was switched back on the following morning after the harvested volume has been replaced. The starting culture medium salinity was set to 5.5% (w/v) NaCl by adding commercial grade pool salt (Lake Deborah Natural Australian Lake Salt) to raw seawater collected locally in Karratha. Nutrients in the form of commercial grade sodium nitrate and potassium di-hydrogen phosphate were added on a daily basis to provide a nominal concentration of 35 mg.L^{-1} of NO_3^- and 2 mg.L^{-1} of PO_4^{3-} in the culture medium. Additional nutrients were added to the recycled medium to maintain the NO_3^- and PO_4^{3-} concentration in the ponds.

Water losses from evaporation and processing (~10% from the centrifugation step) were replenished with unfiltered raw seawater only (~3.5% NaCl (w/v)), as shown in **Figure 1**.

2.4. Harvesting and medium recovery

On harvesting days, 50% of the culture volume from P4 and P5 was pumped to a 3600 L proprietary electro-flocculation unit and processed for a maximum period of 2 h. Floating flocs formed during the electro-flocculation process (see Lee et al. (2013)) were harvested and transferred into a ~~250~~ 900 L conical tank for ~~biomass settling prior to~~ centrifugation using a T10 Evodos centrifuge. Thereafter, the clarified portion (i.e. supernatant) of the water in the electro-flocculation unit was pumped into a 2500 L open-top conical tank, left to stand overnight or longer without any chemical treatment and then pumped back to the ponds on the next harvesting day (**Figure 1**). Any suspended flocs remaining in the supernatant were gravity-settled in conical tanks for further biomass concentration and collection for centrifugation.

2.5. Analytical procedures

Cell counts were performed daily with an improved Neubauer haemocytometer after fixing the cells with Lugol's iodine solution. NO_3^- and PO_4^{3-} concentrations were determined using a DataLine photometer and Aquaspex© reagent kits (Aquaspex, South Australia). A digital hand-held Atago refractometer (model PAL-106S) was used to measure the salinity (% NaCl (w/v)) of the culture medium. Total dissolved organic carbon (DOC) was

147 analysed at the Marine and Freshwater Research Laboratory, Murdoch
148 University, Western Australia. DOC (0.45 µm filtered water samples) was
149 measured as non-purgeable organic carbon (NPOC) following
150 acidification using the high temperature combustion non-dispersive infrared gas
151 analysis method (MAFRL Method 6000) using a Shimadzu Corporation TOC-V-
152 CSH Organic Carbon Analyser. For dry weight and ash free dry weight (AFDW)
153 determinations, culture samples were filtered on GF/C Whatman filter papers
154 pre-combusted at 450°C. Filtered samples were dried at 70°C ~~for 3 h~~ overnight
155 and cooled to room temperature over activated silica gel under vacuum prior to
156 weighing. Dried samples were then combusted at 450°C for 3 h and
157 subsequently cooled to room temperature over activated silica gel under
158 vacuum prior to weighing. The AFDW and ash contents were calculated as a
159 percentage of the dry weight of the filtered samples.

160 **2.6. Statistical analysis**

161 One-Way ANOVA and One-Way repeated measures ANOVA analysis was
162 used to determine significant differences between treatments (α=0.05%). An
163 ANOVA analysis based on ranks was performed whenever the normality
164 Shapiro Wilk test or equal variance tests failed.

165 **3. Results and Discussion**

166 **3.1. Long-term cultivation under increasing salinity**

167 The semi-continuous culturing of *Tetraselmis* MUR 233 in both non-recycled
168 and recycled media lasted for almost five months (127 days) without any major
169 interruptions or culture loss. Throughout this period, the cultures benefited from

abundant sunlight, night and day temperatures above 10 and 28^{0}C respectively, and no rain (**Figure S1** in Supporting Information). For the purpose of the experiment and due to technical constraints that prevented the assessment of the impact of variation in solar intensity and temperature on growth and biomass productivity, any variations in these two parameters were considered as minor compared to the impact of increasing salinity and medium recycling on culture performance.

Evaporation losses were on average 20 L.m^{-2}.d^{-1} in all ponds over the experimental period, which resulted in a salinity increase from 5.5 up to 14.0% NaCl in the recycled medium. For the entire cultivation period, the daily average nutrients remaining in both sets of ponds amounted to 36% of the overall nitrate input and 26% of the overall phosphate input. An average volume of 920 L of water (including seawater makeup for evaporation and process losses) was recirculated on a daily basis to each 25 m^2 pond, which equates to ~115 kL of culture water in each pond over 127 days being recycled instead of being discarded.

Throughout the experiment, minimal contamination by other microorganisms was observed in the culture and the harvested biomass. Diatoms, filamentous cyanobacteria and the ciliated protozoan *Euplotes* sp. (**Figures S2a-c** in Supporting Information) were occasionally detected, but rarely in noticeable quantities (<1x10^4 cells.mL^{-1}) which might significantly impact the overall long-term culture quality. Microscope observation and turbidity measurements of the recycled water indicated that the return water was colourless and clear of any suspended particles. Examination of the

flocculated cells under the microscope revealed that the electro-flocculation process actually resulted in the entrapment of the cells within a gelatinous matrix (**Figures S2di-diii** in Supporting Information) and did not impart any noticeable physical damage or changes in cell shape.

The cultivation periods for M1, M2, P4 and P5 are shown in **Figures 2a, b, c and d** respectively. This was characterised by four stages labeled I, II, III and IV, each of which corresponded with harvesting frequencies of 2, 3, 4 and variable days, and mean salinity ranges of 5-9, 8-12, 9-14 and 6–9% NaCl respectively. ~~It is worth noting that~~ After Stage III, a portion of the culture water was discarded ~~after stage III~~ and substituted with raw seawater to lower the salinity for the last stage of cultivation (i.e. stage IV).

3.2. Analysis of specific growth rate, biomass productivity and AFDW/ ash content

The specific growth rate, the AFDW biomass productivity and the AFDW/ Ash content of the dry biomass obtained for each aforementioned stages in which the long-term cultivation experiment under increasing salinity was carried out are shown in ~~**Figures 3 and 4**~~ **Table 1 and Figure 3**. During the first fifty-three days of culturing (i.e. stage I in **Figure 2**) the cell density in the control ponds M1 and M2 (**Figures 2a and b**) remained relatively stable between $40x10^4$ and $80x10^4$ cells.mL^{-1}. At a harvesting frequency of every two days and salinity range of 5-9% NaCl, the control cultures had an average specific growth rate of 0.35 ± 0.02 d^{-1} and mean biomass productivity of 15.4 ± 0.7 g AFDW.m^{-2}.d^{-1} (stage I in ~~**Figures 3a and b**~~ **Table 1**). In contrast, despite having the same

initial starting cell densities, the cultures receiving the recycled medium, P4 and P5, reached a higher cell density at a faster rate, resulting in twice as many cells as the control ponds by the 38th day of cultivation (stage Ia in **Figures 2c and d**). This boost in the standing biomass concentration correlated with a significantly higher AFDW content ($P<0.001$) (~~**Figures 4c and d**~~ **Figures 3c and d**), a rate of growth 11% faster than in the control ponds and an 11% improvement in the AFDW biomass productivity to yield a mean of 26.9 ± 1.9 g AFDW.m^{-2}.d^{-1} over the initial salinity range. Because the rate of growth of the cells was faster than the harvest frequency, there was a gradual increase in the baseline cell density, which did not match that of the control ponds anymore. Consequently, the cultures in P4 and P5 were harvested consecutively on the 37th and 38th days in an attempt to bring the starting cell density back to 40x10^4 cells.mL^{-1} (stage 1b in **Figures 2c and d**). This was ~~in turn~~ followed in turn by a 26% increase in growth rate (i.e. from 0.39 ± 0.06 d^{-1} to 0.49 ± 0.02 d^{-1}) in the more dilute culture in the recycled medium for the next 15 days (stage Ib in ~~**Figure 3a**~~ **Table 1**) as a result of better light penetration. This spike in growth rate was followed by a decline in AFDW content of the biomass, such that overall, the biomass productivity during that particular period of cultivation slightly declined to 23.8 ± 3.0 g AFDW.m^{-2}.d^{-1} (stage Ib in ~~**Figure 3b**~~ **Table 1**).

In the course of the stage II of this experiment, (i.e. between the 53rd and 78th days), all four cultures were harvested every 3 days. Both sets of cultures maintained a steady growth pattern as the salinity gradually increased from 8 to 12% NaCl. There were no signs of culture deterioration in any of the ponds, and the cultures receiving the recycled medium outperformed the control

cultures by close to 50% in terms of AFDW biomass productivity (stage II in ~~**Figure 3b**~~ **Table 1**). This was reflected in a higher range of cell densities and AFDW content as compared to the previous days of culture. No adverse effect associated with medium recycling on the continuous culturing of *Tetraselmis* MUR 233 could be observed.

For the following thirty-four days of the experiment (i.e. stage III), the limit of salinity tolerance of the cultures was investigated by increasing the residence time between harvests to 4 days, which allowed for a longer evaporation period, and thus higher salinities in the ponds. The cultures in the control ponds M1 and M2 maintained a steady growth pattern until ~12% NaCl (stage III in **Figures 2a and b**), at which point the cultures appeared paler and not as healthy as before. It was therefore decided to maintain those cultures at 11% NaCl for as long as possible. Likewise, there was a gradual decline in cell numbers in experimental ponds P4 and P5 up to a salinity of 14% NaCl, at which point the cell densities were too low to sustain the same harvesting frequency (stage III in **Figures 2c and d**). The salinity was temporarily brought down to ca. 12% NaCl with raw seawater to revive the cultures before again bringing the salinity up to 14% NaCl through evaporation. Once again, the cell densities in the recycled medium gradually dipped. During the period of extreme salinity, the average growth rates in the recycled medium were 24% slower than those in the control ponds (stage III in ~~**Figure 3a**~~ **Table 1**), but because the decline in biomass (i.e. AFDW content (w/v)) was not as significant as for the growth rates ($P<0.001$) (~~**Figures 4a and b**~~ **Figures 3a and b**) and because the average biomass concentration in the experimental ponds was 42% higher than in the control

ponds, the overall biomass productivity remained high at 31.9 ± 2.7 g AFDW.m^{-2}.d^{-1}, which was twice as much as that achieved in the control ponds (~~**Figure 3b**~~ **Table 1**). Therefore, it appears that the limit of salinity tolerance of *Tetraselmis* MUR 233 is close to 12 % NaCl but more importantly, it also seems that the cells are slightly more tolerant to the high salinity when grown in the recycled medium. A likely reason for this difference could be the differences in DOC content between the fresh and recycled ~~medium~~ media (see Section 3.3).

The final stage of the experiment (i.e. stage IV) consisted of returning the culture salinity to ca. 7% NaCl with raw seawater over two harvesting periods in all ponds to determine the rate and extent of culture recovery from the prolonged high salinity treatment. The M1 culture recovered quickly from the high salinity treatment with much higher cell densities than in the previous five days. However, despite the increase in cell numbers, the growth rates and AFDW productivities remained at the same level as those obtained in stage I (~~**Figure 3**~~ **Table 1**). A breakdown in the pH-stat system in M2 resulted in poor culture recovery and as the culture was left undisturbed (i.e no harvest and change of medium), the culture salinity increased due to evaporation and eventually the culture could no longer be maintained. Dilution of the experimental cultures (P4 and P5) with raw seawater to bring the salinity to 7% NaCl resulted in a steady recovery in the cell number and culture appearance. The mean growth rate of 0.34 ± 0.05 d^{-1} of all ponds over this brief period at lower salinity was comparable to that obtained in stage I. In contrast, the AFDW content of the biomass in P4 and P5 increased by 33% in comparison to that obtained in stage I of the experiment. The concomitant increase in cell density

and AFDW content in these two ponds consequently led to a biomass productivity of 37.5 ± 3.1 g AFDW.m^{-2}.d^{-1}, which is the absolute maximum achieved throughout the experiment.

Up until the beginning of stage IV of the experiment, the inorganic portion (ash content) of the biomass in the experimental ponds P4 and P5 was 6% less than that obtained in the control ponds M1 and M2 (~~**Figure 4**~~ **Figure 3**). This difference was small enough to be statistically significant ($P<0.001$), which means that overall, the cells in the recycling culture medium contained slightly more organic carbon. Upon returning the culture salinity to 7% NaCl with raw seawater, the mean differences in declines in ash content was much more significant in the experimental ponds P4 and P5 (from 72.0 % to 61.2 % ash, ($P< 0.001$)) than in the control (from 76.5% to 72.4% ash). It thus appears that the cultures having undergone culture recycling treatment are in a much better condition to grow faster under the return of normal culture conditions.

In ~~the~~ light of the results obtained with respect to salinity tolerance, it can be concluded that ~~One of the most fundamental observations from this study is that~~ *Tetraselmis* sp. MUR 233 demonstrated true halotolerance traits in that it adapted extremely well to both gradual and sudden changes in salinity. This is not atypical of the *Tetraselmis* genus; in fact, there is supporting evidence that certain *Tetraselmis* species and strains are equipped with a highly efficient Na^+ pump (Popova & Balnokin, 2013; Strizh et al., 2004) and a highly adaptable ~~osmolyte regulatory~~ osmoregulatory mechanism to cope with rapid and gradual changes in salinity (Hellebust, 1976; Kirst, 1977; Kirst, 1988). However, it is quite unusual to observe exceptionally good growth and biomass productivities

313 at the high salinities tested in this experiment and ~~to our knowledge,~~ it is
314 believed that this current study is the first ever to report the growth of
315 *Tetraselmis* continuously in actual outdoor conditions ~~in large quantities~~ over a
316 wide salinity range without any significant loss in biomass. It appears that the
317 combination of constant abundant sunlight, high temperatures, adequate supply
318 of inorganic nutrients (CO_2, NO_3^- and PO_4^{3-}) were conducive towards providing
319 adequate energy to the cells to combat and cope with the high salinity ~~stress~~.
320 Using the recycled medium as growth medium seems to have provided with an
321 additional benefit, the exact cause and effect of which is yet to be identified.

322 **3.3. Analysis of dissolved organic carbon**

323 Dissolved organic carbon (DOC) concentration in the raw seawater,
324 recycled medium, and in ponds M2 and P4 was analysed over a period of ten
325 days between the 50th and 59th days of the experiment to investigate the
326 potential influence of culture medium recycling on DOC loads. A cyclic pattern
327 in DOC levels was observed in both ponds whereby a drop in DOC occurred
328 after each harvesting day which was subsequently followed by a gradual
329 increase in DOC up to when the culture was next harvested and refilled with
330 fresh/recycled medium (~~**Figure 5**~~ **Figure 4**). The DOC input from the recycled
331 medium in pond P4 was on average four times more concentrated than that
332 added to pond M2 via the raw seawater. This resulted in an overall increase of
333 27% in DOC in P4 over the ten days, compared to pond M2.

334 DOC of the culture water was also measured on two occasions
335 immediately before and after the electro-flocculation process to determine the

effect of electro-flocculation on DOC concentration in the return water. The results showed that the harvesting process removed on average 25% of the initial DOC from the water so that the residual DOC concentration in the clarified water that was left to stand overnight in the settling conical tanks was less than 7.0 $mg.L^{-1}$. However, by the next harvesting/pond refilling occasion, the DOC would have increased to more than 8.0 $mg.L^{-1}$ (~~**Figure 5**~~ **Figure 4**).

3.4. Analysis of electro-flocculation method

Given the sustained high growth and high AFDW productivities achieved in the recycled medium throughout the cultivation period, it is strongly believed that the electro-flocculation method could have been a major contributing factor to the success of the study. The electro-flocculation process could have conferred two critical advantages which allowed for sustained culture medium re-use: (1) the apparent absence of cell breakage during the harvesting process and (2) the partial reduction in dissolved organic carbon compounds during electro-flocculation. In opposition to other harvesting methods such as centrifugation and filtration which use centrifugal forces and pressure to concentrate the microalgal cells and which general lead to cell damage, electro-flocculation is non-destructive as the cells are simply aggregated and entrapped in growing networks of aluminium polymers after neutralisation of cell-to-cell charge repulsion (Lee et al., 2013; Pearsall et al., 2011). The resultant clarified water is thus much less contaminated with intracellular organic compounds, reducing the risk of fouling by bacteria, cell debris and growth-inhibiting substances, and therefore making the culture medium more amenable for re-use. The successful cultivation of *Tetraselmis* sp. in the flocculant-treated

culture medium as observed in this study is in full agreement with reports by Farid et al. (2013), Wu et al. (2012) and Rwehumbiza et al. (2012), even though different types of flocculants were used in these experiments.

The second advantage of the electro-flocculation step is that it in itself a cleaning process due to its oxidative and flocculating characteristics (Koren & Syversen, 1995; Sasson et al., 2009).The fact that the DOC level in the water declined by over 25% immediately after the electro-flocculation step confirms the sanitising effect of this harvesting method, which could have been a significant contributing factor towards the healthy growth of the *Tetraselmis* MUR 233 cells in the water recycled to the culture. At present, the exact effect of the electrolysis of the culture water is unknown. However, changes in the physicochemical parameters as well as in the water chemistry have been observed and will be reported elsewhere.

3.5. Analysis of AFDW biomass productivity performance of the proposed cultivation method

One of the most important criteria for microalgal cultivation to be economically viable is the ability to maintain high growth rates in the cultures to ensure ~~fast biomass throughput~~ high productivities. ~~Another key performance indicator for the commercial potential of microalgal cultivation is the biomass productivity of the culture system.~~ A specific growth rate of ≦ ≥1 d^{-1} would be ideal to maximise harvesting frequency and biomass output. With the simplest setup and cultivation technology and, in the absence of freshwater input to maintain a constant salinity, we have shown that the growth rates of *Tetraselmis*

MUR 233 in open raceway ponds that can be expected in real conditions are between 0.25 and 0.50 d^{-1}. This is within the normal range of growth rates reported in the literature and summarised by Griffiths & Harrison (2009) and is therefore reassuring that the baseline growth rates can be easily achieved with *Tetraselmis* MUR 233, even at high salinity. Given that the anticipated average areal biomass productivity of an open outdoor pond culture system is ~ 24-27 $g.m^{-2}.day^{-1}$ according to Griffiths & Harrison (2009) and Lee (2001), it was clear that the AFDW biomass productivities achieved with the recycled medium in this study showed a net 19.9% improvement over the expected average biomass productivity. Interestingly, the differences in growth rates between the control and experimental ponds are rather small, compared to the differences in the AFDW biomass productivities. If the cells in the experimental ponds were truly undergoing mixotrophic growth (see below), then it appears that the growth rates in the ponds were not limited by the amount of carbon (inorganic and/or organic) present in the medium, but rather by other factors, for example by light.

In terms of growing *Tetraselmis* MUR 233 in mineral culture medium of increasing salinity, the current study demonstrated an average 15 g $AFDW.m^{-2}.d^{-1}$ (i.e. in the control ponds), which represents 35% less than the average values obtained from other raceway pond data (Griffiths & Harrison, 2009; Lee, 2001), This could indicate that cultivation of *Tetraselmis* MUR 233 in mineral medium of increasing salinity is possible but is not an economical option. It is to be noted, however, that the biomass productivity values obtained in the current study could be excessively higher than the true mean biomass productivities

406 reported in the literature as these are often reported on a dry weight basis,
407 which makes biomass productivities often difficult and misleading.

408 The fact that pond P4 was more productive, received and contained
409 relatively higher amounts of DOC as compared to pond M2 suggests that the
410 culture was growing mixotrophically. ~~Some~~ Several *Tetraselmis* species ~~are~~
411 have been shown to be capable of utilising a wide range of carbon compounds
412 to complement photosynthesis and under such circumstances, it is often
413 reported that this mixotrophic growth results in improved biomass production
414 (Cid et al., 1992; Day & Tsavalos, 1996; Xie et al., 2001) as compared to
415 phototrophic growth (Biller et al., 2012). It certainly appears to have been the
416 case in this study, given the significant improvement in growth rate and biomass
417 productivity in ponds P4 and P5 compared to those in ponds M1 and M2.
418 Furthermore, this improvement occurred in spite of variations in starting cell
419 densities and culture thickness, which under phototrophic growth, would
420 probably have led to irregular or lower culture performance due to inconsistent
421 light penetration through the cultures. The most probable source of organic
422 carbon would have been from the partial decomposition of residual biomass that
423 would have settled at the bottom of the conical tanks prior to returning the
424 clarified water to the ponds. Lysates from the biomass can represent a rich
425 source of highly suitable and assimilable organic carbon compounds that the
426 living cells can readily ~~absorb~~ take up, a fact that Spectrova et al. (1982)
427 successfully ~~embraced~~ used for the cultivation of *Dunaliella tertiolecta*. In
428 addition, the changes in the water chemistry after the electro-flocculation
429 process could have led to a shift in bacterial population towards the elimination

of growth-inhibiting bacteria and/or the increase in growth-promoting bacteria in the culture. This hypothesis, as well as the effects of DOC on growth in *Tetraselmis* MUR 233, is being investigated further.

~~One of the most fundamental observations from this study is that *Tetraselmis* sp. MUR 233 demonstrated true halotolerance traits in that it adapted extremely well to both gradual and sudden changes in salinity. This is not atypical of the *Tetraselmis* genus; in fact, there is supporting evidence that certain *Tetraselmis* species and strains are equipped with a highly efficient Na^+ pump (Popova & Balnokin, 2013; Strizh et al., 2004) and a highly adaptable osmolyte regulatory mechanism to cope with rapid and gradual changes in salinity (Hellebust, 1976; Kirst, 1977; Kirst, 1988). However, it is quite unusual to observe exceptionally good growth and biomass productivities at the high salinities tested in this experiment and to our knowledge, this current study is the first ever to report the growth of *Tetraselmis* continuously in actual outdoor conditions in large quantities over a wide salinity range without any significant loss in biomass. It appears that the combination of constant abundant sunlight, high temperatures, adequate supply of inorganic nutrients (CO_2, NO_3^- and PO_4^{3-}) and the availability of dissolved organic carbon compounds was conducive towards providing adequate energy to the cells to combat and cope with the salinity stress.~~

4. Conclusions

This proof-of-concept study demonstrates that (1) the expected baseline productivity from a halotolerant ~~microalgal~~ microalgae culture grown under

increasing salinity in a semi-arid climate is ~~close to and beyond~~ consistently greater than 15 g AFDW.m^{-2}.d^{-1}, (2) *Tetraselmis* MUR 233 can be grown continuously with minimal freshwater input, and in recycled culture medium without any ~~interruption~~ decline in biomass productivity and, (3) the electro-flocculation harvesting technique circumvents the wastage of water and nutrients and the need for any water treatment for medium sterilisation. This novel integration of cultivation and harvesting processes opens an exciting new avenue in the production of microalgal biomass in saline water.

5. Acknowledgements

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7. Figure captions

Figure 1. Flow diagram for the cultivation of *Tetraselmis* MUR 233 in fresh medium, the harvesting of the biomass through the electro-flocculator and the Evodos centrifuge, and the subsequent return of the clarified supernatant into the experimental cultures.

Figure 2. Cell density (filled circle) and salinity trends (area) in the control treatments M1 (A) and M2 (B), and in the recycled culture medium treatments P4 (C) and P5 (D). The cultures stages I(a, b), II and III represent harvesting frequencies of 2, 3 and 4 days respectively and stage IV represents a period of operation at low salinity with harvesting carried out whenever possible. Thin arrows in graph C and D indicate two consecutive harvesting days (day 37 and 38), and the bold arrow in graph B indicates a breakdown in the pH-stat for the M2 culture.

~~**Figure 3.** Specific growth rates and AFDW biomass productivity of *Tetraselmis* MUR 233 at the different stages of semi-continuous growth in fresh medium (mean od M1 and M2 results) (white bar) and in recycled culture medium (mean of P4 and P5 results) (grey bar). Mean growth rate and AFDW biomass productivity obtained during culture stage Ib are illustrated as filled circles. Bars represent standard errors.~~

~~Figure 4.~~ Figure 3. AFDW (filled circle) and ash content (open circle) on a DW basis of *Tetraselmis* sp. MUR 233, and salinity (area) in the control treatments M1 (A) and M2 (B) and the recycled medium treatments P4 (C) and P5 (D). The culture stages I, II, III and IV are as explained in **Figure 2** caption.

~~Figure 5.~~ Figure 4. DOC concentrations in control pond M2 (filled circle) and experimental pond P4 (open circle) over a ten-day period in September 2012. DOC input from raw seawater into pond M2 and from the recycled medium into pond P4 on harvesting days are indicated. Bars represent standard errors for 3 replicates.

8. Table captions

Table 1. Specific growth rates and AFDW biomass productivity of *Tetraselmis* MUR 233 at the different stages of semi-continuous growth in fresh medium (mean of M1 and M2 results- control, except for stage IV where results from M1 only are shown) and in recycled culture medium (mean of P4 and P5 results-experimental).

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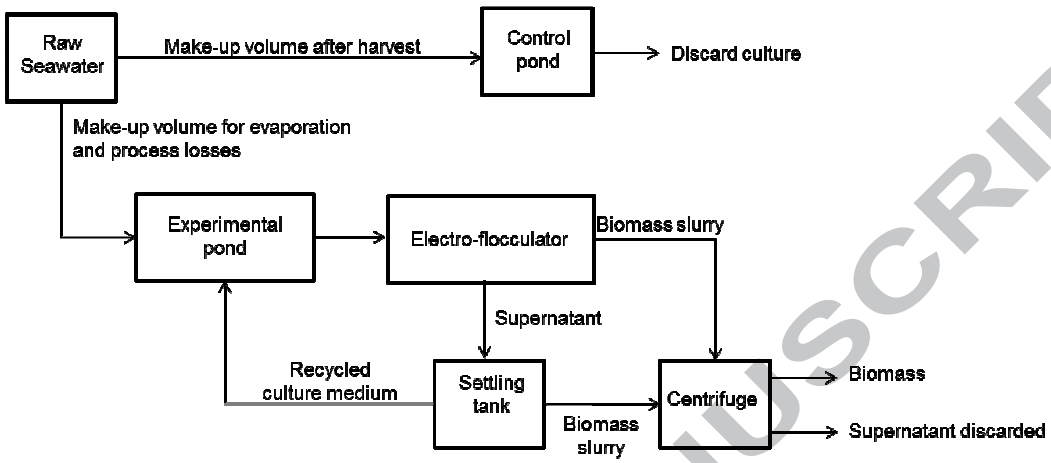

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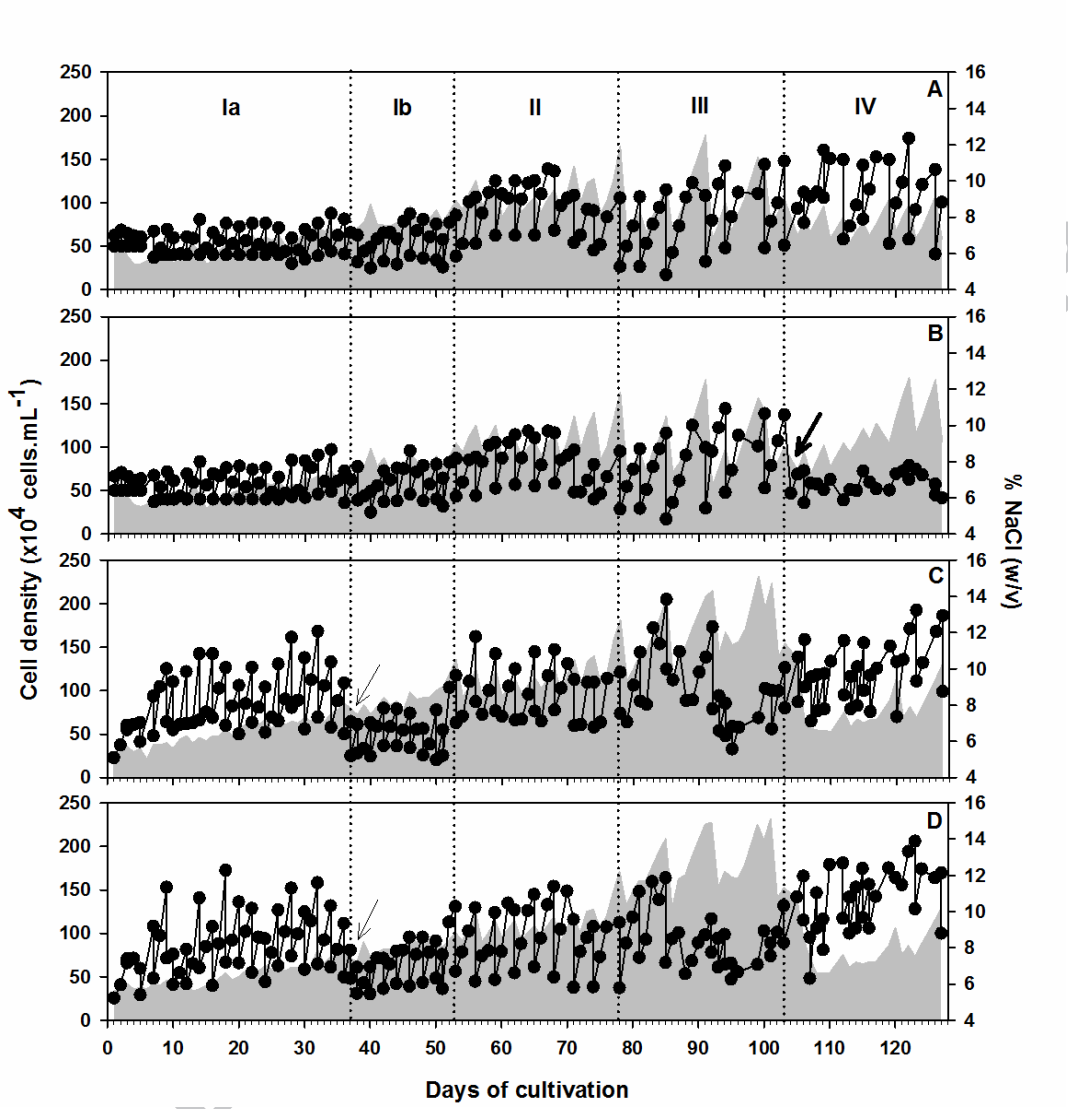
623 **Figure 1.**



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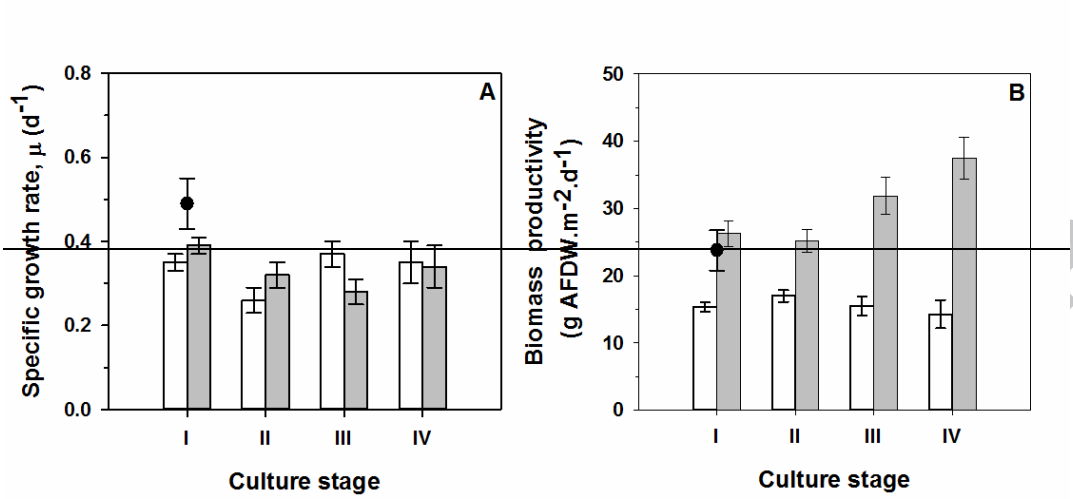
625 **Figure 2.**



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627 **~~Figure 3.~~**

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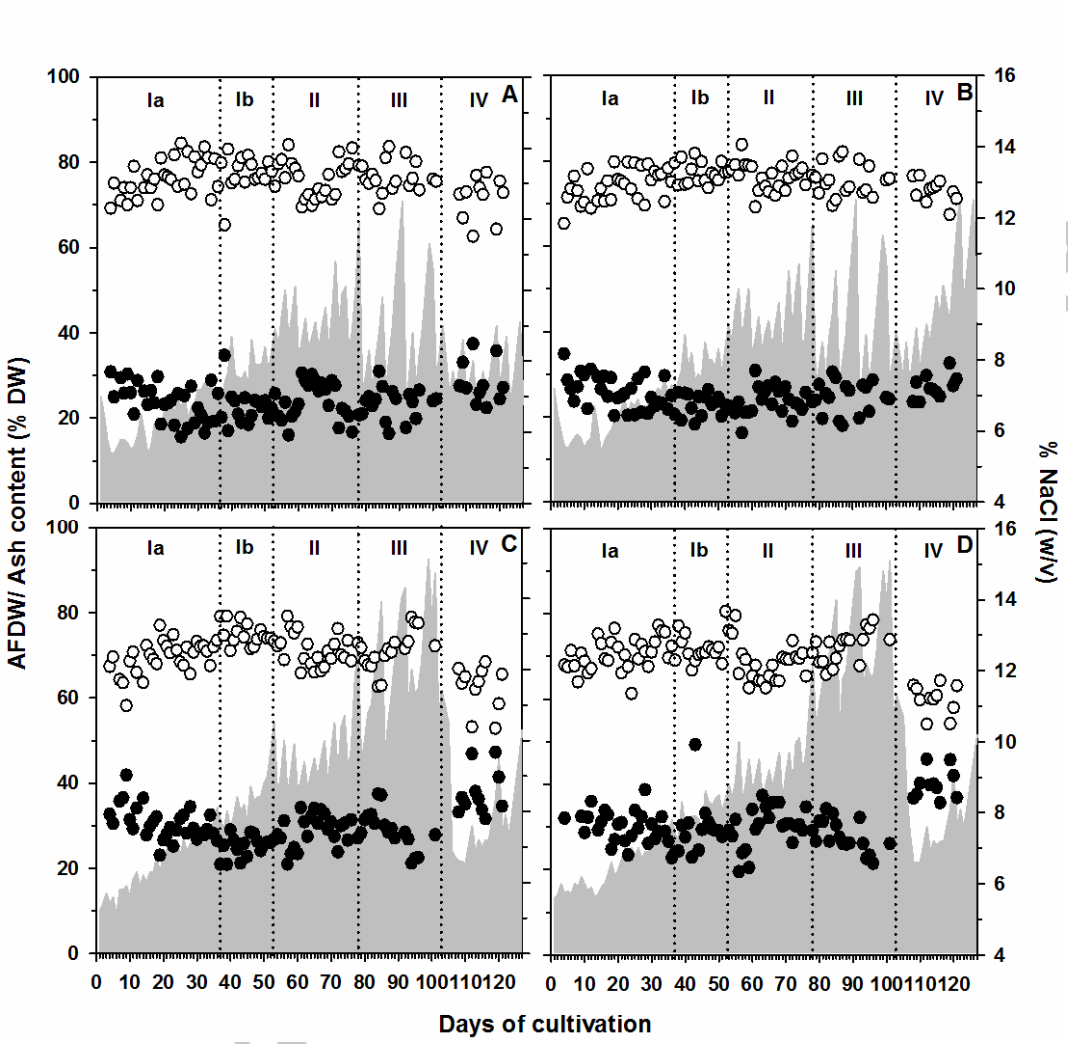

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630 **Figure 3.**

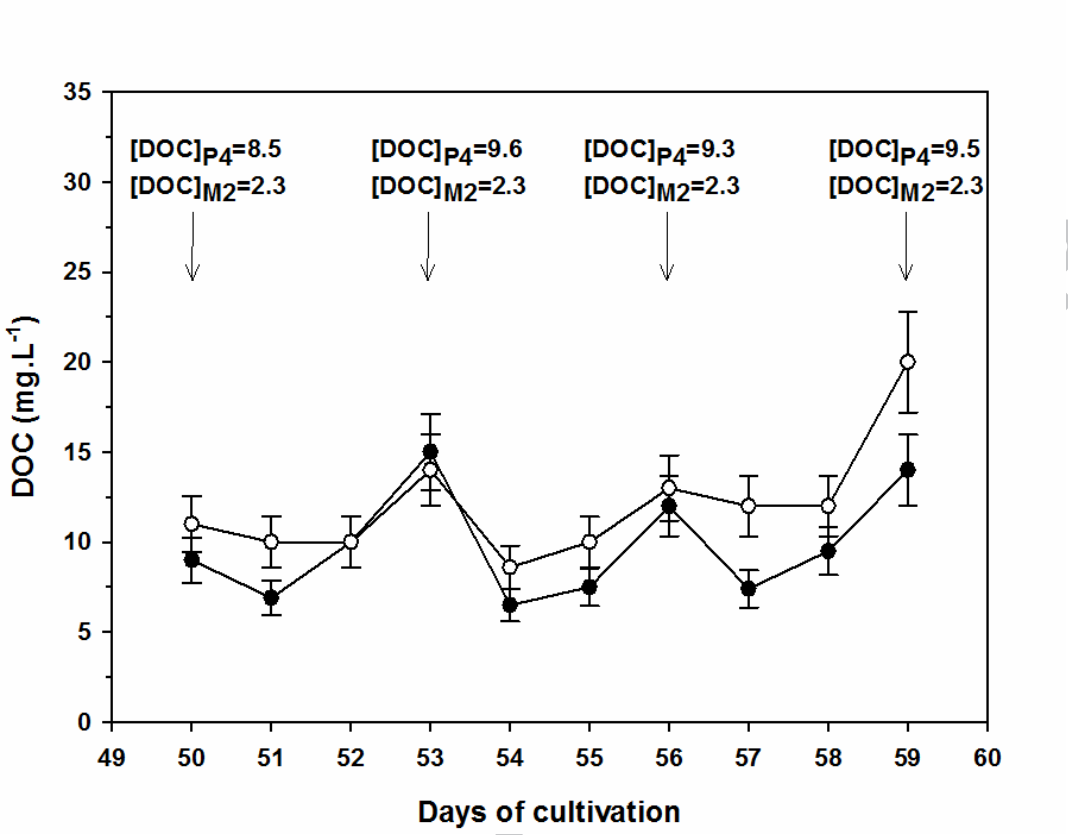

Figure 4.

644 **Table 1.**

Culture stage	Specific growth rate, μ (d^{-1})		Biomass productivity (g AFDW.m^{-2}.d^{-1})	
	Control	Experimental	Control	Experimental
Ia	0.35 ± 0.02	0.39 ± 0.02	15.4 ± 0.7	26.3 ± 1.9
Ib		0.49 ± 0.06		23.8 ± 3.0
II	0.26 ± 0.03	0.32 ± 0.03	17.00 ± 0.9	25.2 ± 1.7
III	0.37 ± 0.03	0.28 ± 0.03	15.50 ± 1.4	31.9 ± 2.7
IV	0.35 ± 0.05	0.34 ± 0.05	14.3 ± 2.1	37.5 ± 3.1

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Pilot-scale continuous recycling of growth medium for the mass culture of a halotolerant *Tetraselmis* sp. in raceway ponds under increasing salinity: A novel protocol for commercial microalgal biomass production.

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673 **Highlights**

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675 ➢ *Tetraselmis* sp. MUR 233 grew in recycled culture medium with
676 increasing salinity
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678 ➢ The salinity of the recycled culture medium ranged between 5 and 12%
679 NaCl (w/v)
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681 ➢ Electro-flocculation was employed as harvesting technique
682
683 ➢ Growth and AFDW biomass productivity were superior in the recycled
684 culture medium

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