The University Of Sheffield.

Mini-project report

Optimum salinity conditions for producing lipids from *Dunaliella salina* for biofuels production

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Abstract

The manipulation of microalgae species for commercial products is a rapidly expanding area of research. In particular, some species have been identified as promising producers of useful lipids for biofuels production. *Dunaliella salina* is one such species, which shows lipid accumulation in response to high environmental salinities. This study measured the accumulation of algal biomass and lipid content of cultures grown in salinities ranging from 0.4 to 3.5M NaCl. The highest biomass contents per volume of solution and the highest overall lipid content per ml were found at cultures grown in 2M NaCl. The highest lipid percentage content of biomass was found at 3.5M NaCl. This research demonstrates that although salinity can increase the species lipid production, the most important factor in accumulating lipid content was overall biomass productivity. Therefore, the main hurdle to overcome in this area of research is the compromise between cell health and lipid productivity. This may only be overcome with synthetic biology techniques.

Introduction

The need for algal biofuels: Fossil fuels are starting to reveal their limitations as an energy source; both literally, as resources are depleted, and through their contributions to climate change. The result of this revelation is that the search for clean renewable fuels has been gathering momentum, in a bid to continue to meet our huge population's energy demands in the future. Although there are many ways electricity can be generated from renewable sources, biofuels are currently the only option for the replacement of conventional liquid fuels in combustion engine vehicles; most significantly, in cars and aeroplanes. Finding a viable liquid biofuels supply is therefore crucial to the maintenance of the current transport infrastructure.

The viability of some liquid biofuels has already been assessed. Biodiesel production from microalgae has demonstrated many advantages over conventional land-crop biofuels, most notably, a lower demand for land and for intensive agricultural practises; and a higher energy content than bioethanol (Chisti 2008).

Microalgae are very diverse (Harwood and Guschina 2009). They can create a range of useful products, and there is a variety of ways in which they can be cultivated, manipulated, harvested and utilised (Harun, Singh et al. 2010). Most importantly for the interests of this study, they have the ability to produce large amounts of lipids, including triacylglycerides (TAGs), a high energy density storage molecule (Courchesne, Parisien et al. 2009), which can converted into biodiesel via transesterification. Natural oil levels vary between microalgae species; a review of 14 microalgae genera reported oil contents of 15-77% total dry weight (Chisti 2007).

Lipid production can be increased by manipulating the environment of the microalgae, also known as biochemical engineering (Courchesne, Parisien et al. 2009). As lipid content is affected by many environmental factors, there exist a variety of ways of doing this in different algae species, including nitrogen deprivation, temperature and light, pH stress, CO_2 aeration (Chiu, Kao et al. 2009), and osmotic stress (Takagi, Karseno et al. 2006). Some algal species are halotolerant and produce lipids as compatible solutes to cope with high or fluctuating salinities (Edwards 1990). *Dunaliella salina* is one such halotolerant species, and the focus of this study.

A focus on Dunaliella salina: *D. salina* is a unicellular species with no cell wall (Edwards 1990). It lives in areas of fluctuating salinity and can tolerate extreme salinities of between 0.5 and 5M NaCl. It does this by maintaining a steadily low intracellular ion concentration (Fisher 1994), and by forming compatible solutes that maintain the structure and volume of the cell. This ability to adapt, and its high metabolic and physiological versatility, has led to its identification as a high potential for large-scale cultivation of beta carotene (Raja, Hemaiswarya et al. 2007). Unlike *D. tertiolecta*, which demonstrates inhibited cell growth above 1M salinity, *D. salina* can survive in very high saline conditions, making it one of a few suitable candidate species for outdoor open culture systems, as it can dominate in extreme saline environments with little chance of contamination from other species (Garcia-Gonzalez, Moreno et al. 2003; Ami Ben-Amotz 2009).

D. salina is known to increase its lipid production in response to halostress (Alhasan, Ghannoum et al. 1987), but not to nitrogen deprivation – a common way of increasing lipid production in other microalgae species (Griffiths and Harrison 2009). Whilst other species, such as *D. tertiolecta*, have been thoroughly investigated for lipid production under salt stress (Takagi, Karseno et al. 2006), *D. salina* has had little research into its lipid accumulation and content in response to halostress, as most former work in this area has focused on beta-carotene production. The only two existing studies investigating *D. salina*'s lipid production in response to halostress are not informative enough to fully describe the

phenomenon; one analyzed the lipids in the plasma membrane only (Peeler, Stephenson et al. 1989). The other showed specifically that phosphatidylglycerols increased under halostress conditions, but tested at two salinities only: 2.5% and 20% NaCl (Alhasan, Ghannoum et al. 1987).

D. salina is reported to produce 35% lipid content in non-stressed conditions (Griffiths and Harrison 2009). The aim of the current study is to investigate whether applying halostress to *D. salina* cultures can increase this natural lipid content, and to identify the optimum conditions for lipid accumulation and harvesting for use in biofuels production. The following methods to measure biomass accumulation, lipid content, chlorophyll content, cell count, and for identification of polar lipids using Nile Red staining and fluorescence microscopy, were employed for this investigation.

Methodology

Medium production, sub-culturing, and incubation: Dunaliella Growth Medium was prepared at salinities 0.4, 1, 1.5, 2, 3 and 3.5M NaCl. From an original culture of *D. salina* grown in 2.0M NaCl Dunaliella Growth Medium, we took subcultures using medium at each of the different salinities. The cultures were grown in 250ml conical flasks, sealed with a sponge bung. Aseptic techniques were used to transfer 100ml Dunaliella growth medium and 5ml of algal culture into fresh flasks. The flasks were then kept in an incubation room at 25 degrees centigrade, with 24 hour light at light intensity between 52.3 and 121.2 µmol/m_2/second.

Gravimetric measurement of lipid content of algal cells: Biomass per ml culture and lipid mass per ml culture were determined using the gravimetric measurement of lipid content described in Chiu *et al.* (2009), and presented in Table 1.

Cell counting: 20 µl of 9:1 algal culture: grams iodine solution was placed on a Helber slide counting chamber and viewed at x40 magnification. Cell counts were taken 5 times and the average counts are presented in Table 1.

Nile red staining: Nile Red staining and fluorescent microscopy were carried out to identify the non-polar lipids in *D. salina,* following the methods of Cooksey *et al.* (1987) and Elsey *et al.* (2007). 10mg of Nile Red dye were dissolved in 10ml acetone to give a 1 mg/ml solution. 2.5ml of this stock solution was diluted with 7.5ml acetone. The optical density of the algae sample was measured at 720nm using a spectrophotometer, and then the sample was diluted to give OD_{720} of approximately 0.5. 3ml diluted algal cells were added to 10ml of diluted Nile Red solution and vortexed. The solution was then studied and photographed using a florescence microscope (Figures 1-4).

Cholorophyll measurement: The chlorophyll contents of the samples were taken to check that the cell content was high enough to take biomass and lipid measurements from a 15ml sample. 5ml algae culture was centrifuged at 6000rpm for 10 minutes. The supernatant was drained off and the sample was re-suspended in 1ml distilled water and 4ml acetone, and centrifuged at 6000rpm for 5 minutes. The optical density was measured at 645nm and 663nm. The following equations were used to determine chlorophyll content:

$OD_{645} \times 202 = y$ $OD_{663} \times 80.2 = x$ $(y+x)/10 = \mu g$ chlorophyll ml^{-1}

Results

Effect of salinity on biomass production: During the growth phase, defined in this experiment as up to 14 days, the highest biomass measurement was found at 2M salinity (Figure 5, Appendix).

After the growth phase was complete - defined in this experiment as after 14 days, and sampled in this instance at 26 or 27 days - the highest biomass production was found at 0.4M salinity (Figure 8, Appendix).

The cell count was measured only after the growth phase was complete, at 26 or 27 days. The highest cell count was found at 2M salinity. The lowest cell count was found at 3.5M salinity.

Table 1 | Summary of results from gravimetric measurement of lipid content, determination of chlorophyll content, and cell counts. (The results in bold were affected by biomass loss during the freeze drying process).

Salinity of medium (M)	Sample age (days)	Chlorophyll measurement (µg mL-1)	Biomass (µg mL-1)	Lipid content (µg mL-1)	Lipid per biomass (%)	Cell number (per ml)	Lipid content per cell (pg)	Lipid/ chlorophyll ratio
0.4	7	12.75	131.67	78.33	59.49%	n/a	n/a	6.15
1	7	6.3	138.33	75	54.22%	n/a	n/a	11.9
1.5	7	5.26	121.67	70	57.53%	n/a	n/a	13.3
2	7	n/a	236.67	86.67	36.62%	n/a	n/a	n/a
2	9	7.07	208.33	85	40.80%	n/a	n/a	12.02
3	12	4.7	141.67	73.33	51.76%	n/a	n/a	15.59
3.5	**9**	**2.36**	**51.25**	**45**	**87.80%**	**n/a**	**n/a**	**19.03**
2	**21**	**7.66**	**271.67**	**81.67**	**30.06%**	**n/a**	**n/a**	**10.67**
0.4	27	16.44	515	123.33	23.95%	1.604×10^7	7.69	7.5
1	27	18.35	416.67	123.33	29.60%	1.173×10^7	10.51	6.72
1.5	27	17.06	401.67	135	33.61%	1.560×10^7	8.65	7.91
2	26	13.42	446.67	117.78	26.37%	2.088×10^7	5.64	8.78
3	27	12.78	293.33	106.67	36.36%	1.222×10^7	8.73	8.35
3.5	26	9.72	271.67	100	36.81%	8.533×10^6	11.72	10.29
2	42	20.75	540	101.67	18.83%	n/a	n/a	4.9

These results indicate that 2M is the best salinity for creating biomass and for cell division. This is the salinity that *D. salina* is best adapted to for cell and biomass productivity, and it represents the average natural environment in which these halophiles are found.

Effect of salinity on lipid production: During the growth phase, the highest lipid content per ml was found at 2M (Figure 6, Appendix). After the growth phase had completed the highest lipid content per ml was found at 1.5M (Figure 9, Appendix). However, this is largely due to the large biomass; when analysing the lipid content either per cell, or as a percentage of the biomass, both during and after the initial growth phase, the highest lipid contents were found at 3.5M salinity (Figures 7 and 10, Appendix). In fact, after the initial growth phase was complete, the lipid per biomass content dipped slightly at 2M salinity, being higher both at 1.5M and 3M salinities. As this species has high productivity at 2M, the dip in lipid content matches with the general trend that manifests itself: as biomass productivity decreases, the lipid percentage of the biomass increases. This confirms that halostress significantly increases the proportion of lipids in a *D. salina* cell or per biomass measurement, but it also reveals that as the overall biomass productivity of the culture decreases, the overall lipid productivity decreases.

Changes in productivity over time: Analysis of 2M cultures sampled at different ages between 7 days and 42 days show that as the age of the culture increases, the lipid percentage content steadily decreases after an initial peak of 87.8% at 9 days. Conversely, biomass content rises over time, but shows a decrease in productivity rate after 26 days. Lipid content per volume is highest at 26 days and decreases after this period (Figures 11-13, Appendix). There is, therefore, a strong relationship between *D. salina* lipid productivity and time.

Nile Red Analysis: Fluorescent microscopy and Nile Red staining reveal that in 3M NaCl cultures have broken cells and fewer cells overall than 1M NaCl (Figures 1-4). This demonstrates that very high salinities can still cause damage to this halophilic species, despite its halotolerant characteristics, and that it may be unproductive to culture it above its preferred salinity of 2M.

Discussion

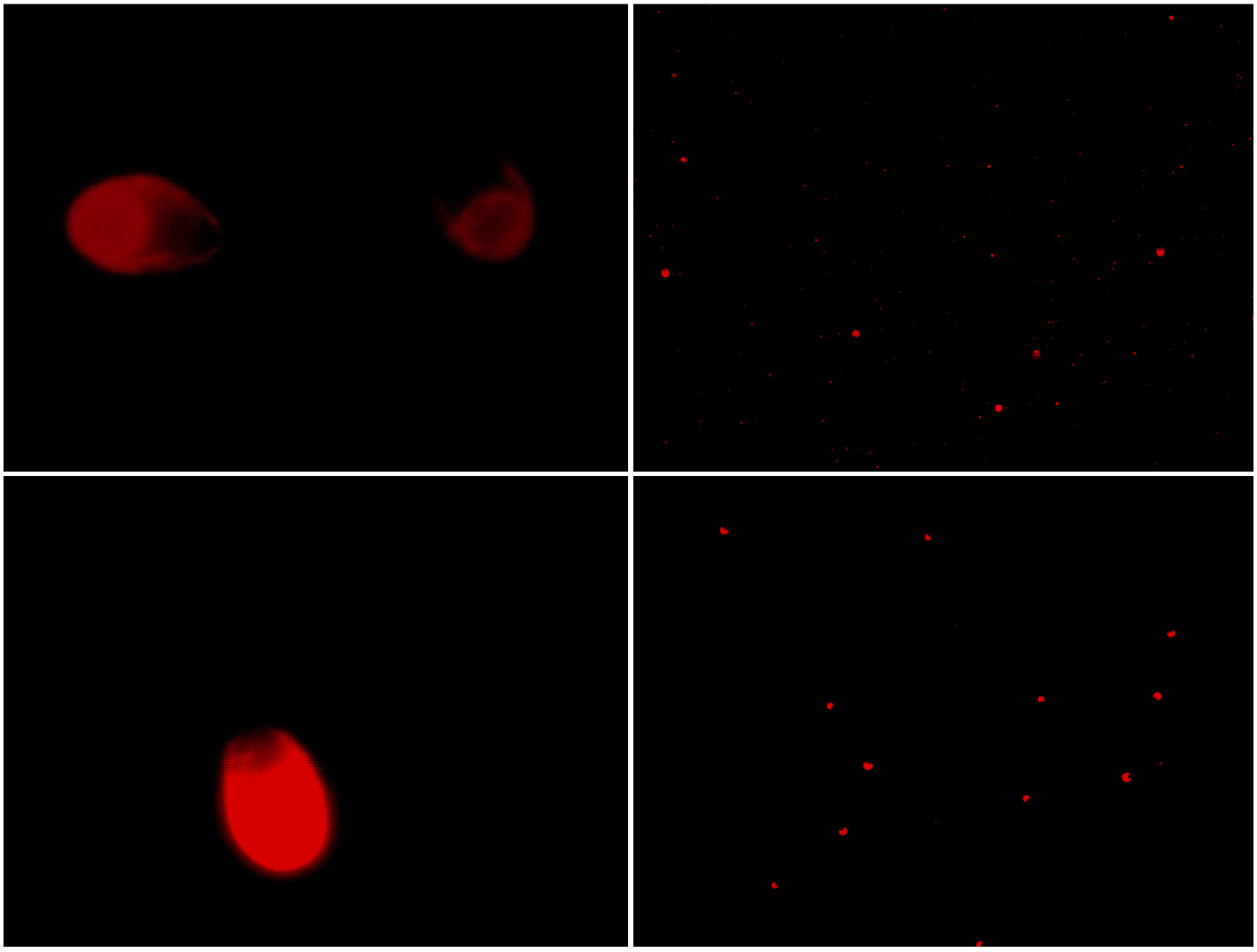
Salt and lipid production: The results yielded from this study show that whilst extremely high salinities induce significantly higher lipid formation within algal cells, this increase is negated overall by the drop in biomass productivity. Large biomass

production and successful culture growth are essential to producing large volumes of lipids overall. Unfortunately, high proportions of lipids and high biomass productivity appear mutually exclusive.

A solution to this problem may be to grow *D. salina* in its preferred salinity of 2M NaCl to stimulate biomass productivity, and then to induce high lipid production in the cells by adding NaCl to increase the solution to 3.5M; this will cause osmotic stress and the production of compatible solutes. The addition of salt may have to be done gradually in a stepwise fashion, so that the cells do not go into osmotic shock and die. This method has been trialled in *D. tertiolecta* (Takagi, Karseno et al. 2006); it successfully resulted in increased lipid and TAG content over time, whilst maintaining a high cell concentration. Whilst this two-stage method has been piloted for increasing beta-carotene production *D. salina* (Tafreshi and Shariati 2006), to date, this method has not been employed for lipid production in *D. Salina.* However, these findings signify that this will be the next important stage in this area of research.

This study has shown that this species achieves relatively high productivities in high salinity conditions, which is vital for producing an outdoor monoculture with low chances of contamination (Garcia-Gonzalez, Moreno et al. 2003). As the ideal culture system is a low energy input open culture such as a race-way pond, this algal species is one of the most suitable known as a biofuels producer on a large scale.

D. salina's suitability in comparison to other species aside, these results also exemplify that lipid production is currently far too low to be viable for biodiesel production on a large scale. The low outputs demonstrate the limitations of biochemical engineering. To be able to rely on *D. salina* as a biodiesel source, the species must undergo some considerable genetic engineering to cause its high lipid production to be expressed whilst also maintaining high biomass productivity.



Figures 1-4 | *Dunaliella salina* cells stained with Nile Red dye. Top left: 3M solution, x100 magnification. Top right: 3M solution, x20 magnification. Bottom left: 1M solution, x100 magnification. Bottom right: 1M solution, x20 magnification.

Future work in biochemical engineering of D. salina: Despite the limitations of biochemical engineering, it will continue to play a vital role in biofuel production research. Using the data in this study, in conjunction with other ways of increasing productivity, may lead to advances in being able to meet the energy production ambitions for biofuels using micro-algae.

Timing of applied environmental conditions and harvesting is a crucial part of maximising lipid production, as demonstrated in this study's results by the decrease in lipid content over time. Other studies have shown that lipid accumulation is associated with growth phases. Chiu *et al*. (2009) found that replacing a culture more quickly was more effective for biomass and lipid accumulation. The optimum time to induce stress and subsequently harvest the algae for their lipids is therefore an important area for future research for this species.

As with all renewable energy research, the life cycle analysis of producing algal biofuels is a significant aspect of assessing the viability of this species for biofuel production (Lardon, Helias et al. 2009). Maintaining low energy inputs to algal biofuels production is imperative. Using waste outputs from other industries may be a way to do this. For example, carbon dioxide from power plant flue gases can be used to encourage productivity (Edwards 1990; Ami Ben-Amotz 2009). Other studies suggest that using waste products from other systems such as phosphorus in waste stabilization ponds (Powell, Shilton et al. 2008), or nutrients found in waste water (Clarens (in press)) can help increase productivity without increasing the energy inputs to the system.

Conclusions

D. salina has demonstrated an ability to produce large percentage content of lipids when under halostress, and this study has added data to the ever expanding pool of knowledge in this area of research. This species will require larger amount of salt as an input than other species, making it possibly a higher energy input system, but it has the advantage over other species of dominating in an outdoor open culture. The decreased productivity of the species in high salinity shows that there is a trade off between biomass productivity and lipid content, and therefore the timing of osmotic stress appliance is crucial. The relationship between culture age, timing of osmotic stress appliance, and culture productivity should be the next step in researching this species as a potential biofuels producer. Genetic engineering will also be crucial to increasing the lipid production to a high enough level to be able to supplement the fuel demand, and current research suggests that biochemical engineering alone will not be enough to make algae a viable biodiesel producer.

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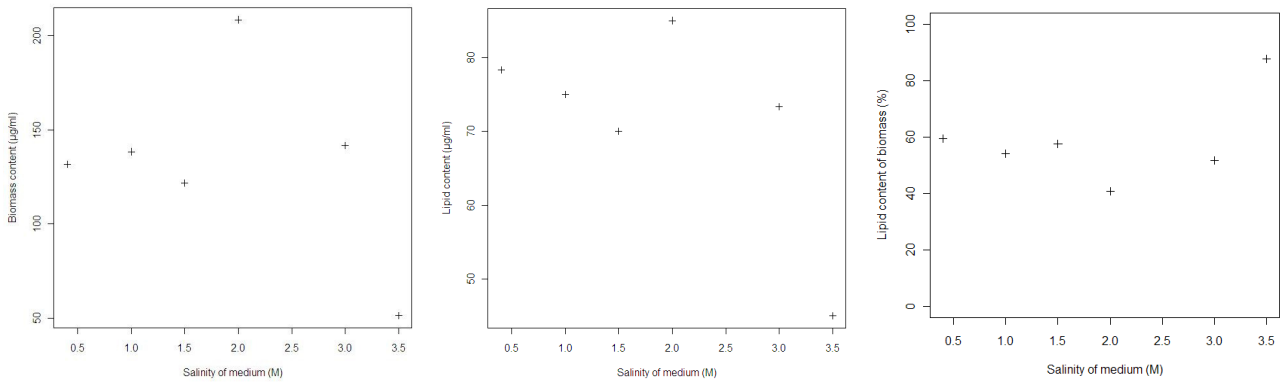
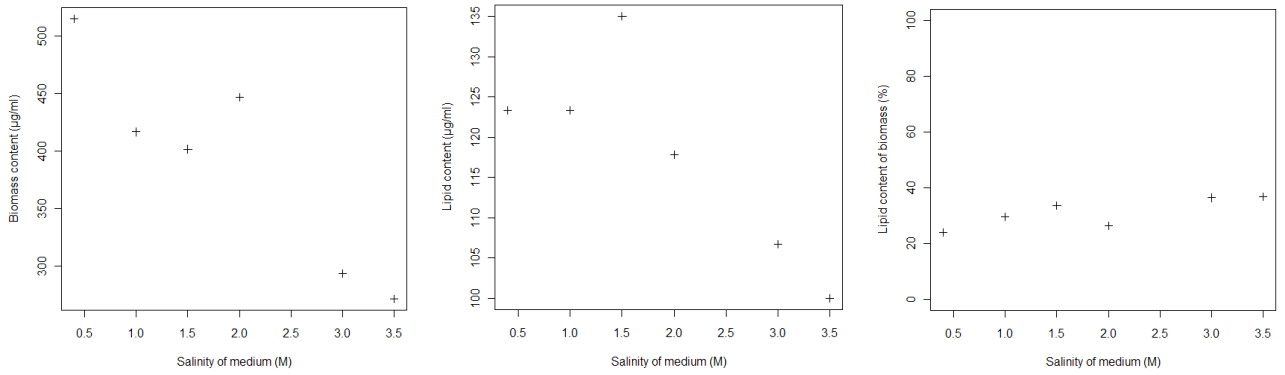
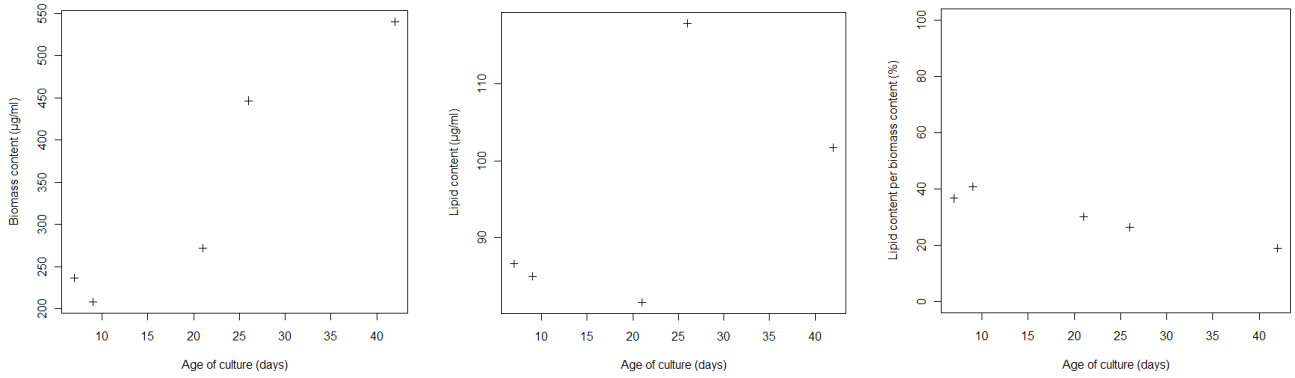
Appendix



Figures 5-7 | Responses of biomass productivity, lipid productivity and lipid percentage content to different salinity growth media in cultures grown to the age 7-12 days.



Figures 8-10 | Responses of biomass productivity, lipid productivity and lipid percentage content to different salinity growth media in cultures grown to the age 26 or 27 days.



Figures 11-13 | Changes in biomass productivity, lipid productivity and lipid percentage content over time, in cultures grown in 2M growth medium, over an age range of 7-42 days.