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Research Article

THE GROWTH ANALYSIS OF MICROALGAE IN SUSPENSION CULTURE – *NANNOCHLOROPSIS OCULATA, CHLAMYDOMONAS MOEWUSSI, TETRASELMIS SUECICA, AND CHLORELLA MINUTISIMA*

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ABSTRACT
Four microalgae - *Nannochloropsis oculata, Chlamydomonas moewussi, Tetraselmis suecica, Chlorella minutissima*- were grown in suspension culture in pure sea water and pure sea water with an enrichment media over a 10 day period. Our results indicate that pure sea water is an adequate medium for the growth of microalgae over a 10 day period in a non- continuous suspension medium for three of the microalgae used. The generation times for the microalgae grown with the enhancement media are *Nannochloropsis oculata* 35.2 h, *Chlamydomonas Moewussi* 16.8 h, *Tetraselmis suecica* 16.3 h, and *Chlorella minutissima* 36.4 h. The generation times for the microalgae grown in pure sea water (without the enhancement media) are *Nannochloropsis oculata* 32.4 h, *Chlamydomonas moewussi* 25.6 h, *Tetraselmis suecica* 15.2 h, and *Chlorella minutissima* 32.3 h. The microalgae *Chlamydomonas moewussi*- a soil water isolate grew significantly faster with the pure sea water plus the enrichment media with a generation time of 16.8 h, as compared to 25.6 h without the enrichment media. The other three microalgae (sea water isolates) grew faster in pure sea water than in sea water plus the enhancement media. *Chlamydomonas moewussi* completely die off by day 8 in both suspensions. The generation times and growth curves for the microalgae were calculated from cell counts. A growth equation was used to mathematically calculate the generation times.

Keywords: *Microalgae, Suspension Culture, Pure seawater, Enrichment Media, Generation Time, Cell Counts, Growth Equation*

INTRODUCTION
Algae are organisms that grow in watery environments and use light and carbon dioxide to create biomass. There are two classes of algae: macroalgae and microalgae (Thomas, 2002). Macroalgae- measured in inches- are large multicellular algae which are normally seen in ponds. These large multicellular algae are called seaweeds (Thomas, 2002). Microalgae are measured in micrometers and are tiny unicellular algae that normally grow in suspension with the aquatic environment (Thomas, 2002).
Natural seawater is a complex medium containing more than 50 known elements in addition to large but variable amounts of organic compounds (Harrison and Berges, 2004). Chemicals added to seawater contain trace elements and the often improve algal growth (Harrison and Berges, 2004). For microalgal culture the direct use of natural sea water is seldom acceptable, and may be the preferred base if large quantities are required, if a good source is readily available, or if open ocean species are being cultured in the laboratory (Harrison and Berges, 2004). For near shore microalgae, the salinity may vary seasonally and large phytoplankton blooms may alter the organic compounds in sea water and various additions must be made to the sea water to enhance algal growth (Harrison and Berges, 2004).
The macronutrients considered important for the growth of microalgae are nitrogen, phosphorus, and Silicon (Parsons *et al.*, 1984; Brzezinski, 1985). The ambient ratio of these macronutrients in natural sea water is often similar to the ratio required by microalgae (Harrison and Berges, 2004). Three vitamins- Vitamin B 12, (cyanocobalamin), Thiamin, and Biotin are normally added to enrichment media but few microalgae require all three (Provasoli and Carlucci, 1974). HEPES (N-[2-Hydroxyethyl] piperazine-N'-

[2-ethanesulphonic] acid was used extensively in fresh water media (McFadden and Melkonian, 1986). However, when HEPES was used in comparison with other buffers MOPS, TRIS, Glycine, and TAPS ([2-hydroxy-1, bis (hydroxymethyl) ethyl] amino-1-Propanesulfonic acid, it was concluded that TRIS and TAPS provided the maximum growth with minimal pH change in marine dinoflagellates (Loeblich, 1975).The most common metal chelator is EDTA which has been known to inhibit the growth of some marine species (Muggli and Harrison, 1996).

Growing microalgae remains an art as well as a science, and most researchers tend to settle for the media that work for the species they are growing rather than engage in comparisons to determine which media are best (Harrison and Berges, 2004). Media preferences are not normally supported by comprehensive qualitative comparisons (McLachlan, 1973). Any media comparison should be based on the use of the media by the researcher (Harrison and Berges, 2004). The three broad categories of general use for growth media are culture maintenance, biomass yield and growth rate experiments (Harrison and Berges, 2004). Growth rates are straightforward to measure using cell counts (Brock, 1974). The growth of single celled organisms in suspension has also been monitored using turbidity and light scattering measurements (Held, 2011). Another criterion for media evaluation is whether the original morphology of the cells is maintained (Harrison *et al.,* 1980).

Microalgae are recognized as potentially good sources for biofuel production because of their high oil content and rapid biomass production (Christi, 2007). Algal biomass contains three main components, namely, carbohydrates, protein, and lipids/natural oil (Spoloare *et al.*, 2006). The bulk of lipid/ natural oil made by microalgae is in the triglycerol form making it a potential source for biodiesel production (Christi, 2007). During the early stages/phase of growth, green algae produce large amounts of polar lipids and polyunsaturated C-16 and C-18 fatty acids (Piorreck *et al.*, 1984). Near the stationary phase of growth the green algae produce neutral lipids in the C-16 and C-18 saturated fatty acids forms (Christi, 2007).

In this study we compared the growth of four microalgae in pure sea water and pure sea water plus an enrichment media over a 10 day period using cell counts and a growth equation.

MATERIALS AND METHODS

Growth of Microalgae in Suspension Culture

(1) Growth of Microalgae in Pure Sea water (With- out Enrichment Media)

The sea water was collected 5 miles off the coast of Boca Raton, Florida, USA. The sea water was sterilized using a 0.22 um membrane filtration from Corning (Cat. 431098) and stored in 1 liter sterile containers.

12 test tubes containing 200 ml of sea water were inoculated with microalgae. The algal cultures were obtained from Utex.

For *Nannochloropsis oculata* 2 ml of culture solution (Utex 2164) was added to 3 of the test tubes containing 200 ml sterile pure sea water and labeled.

For *Chlamydomonas Moewussi* 2 ml of culture solution (Utex 1054) was added to 3 of the test tubes containing 200 ml of sterile pure sea water and labeled.

For *Tetraselmis suecica* 2 ml of culture solution (Utex LB 2286) was added to 3 of the test tubes containing 200 ml of sterile pure sea water and labeled.

For *Chlorella minutissima* 2 ml of culture solution (Utex LB2341) was added to 200 ml of sterile pure sea water and labeled.

Nannochloropsis oculata, *Tetraselmis suecica*, and *Chlorella minutissima* are sea water isolates, whereas *Chlamydomonas moewussi* was isolated from soil water.

All algae were grown at room temperature 30^{o} C with a continuous light source and a pH 7.2. All tubes containing microalgae were agitated twice daily.

The experiments were replicated three times.
2. Growth of Microalgae in Pure sea water plus Enrichment Media
The microalgae suspension cultures were set up as for pure sea water above. However, to each test tube 5 ml of Utex Enrichment Media was added and adjusted to pH 7.2.
The experiments were replicated three times.
National Science Foundation-The University of Texas at Austin- Utex Enrichment Media for Sea water Medium.

Component	Amount	Final Concentration
1. NaNO3 (Fisher BP 360-500)	4.7g/2L	27.65 mM
2. Na2Glycerophate-5H2O (Sigma G650)	0.7g/2L	1.6 mM
3. ESFe Solution	325ml/2L	
4. P-11-Metal Soln.	325ml/2L	
5. HEPES buffer (Sigma H-3375)	6.5g/2L	14mM
6. Vitamin B12	3ml/2L	
7. Biotin Vitamin Soln.	3ml/2L	
8. Thiamin Vitamin Soln.	3ml/2L	

Counting Cells for Growth Analysis
Every 48 h, after agitation, one drop of each microalgae suspension was counted using a Wolfe binocular microscope fitted with a digital microscope eye piece (MA 88) and attached to a Lenovo Lap top computer.The samples were loaded on a Neubauer improved Haemocytometer, a digital image was captured, the cells counted using a tally counter and recorded. The magnification used was X 100.

RESULTS AND DISCUSSION

Tables 1 and 2 show the cell counts of the four microalgae grown in suspension culture. Three microalgae *Nannochloropsis oculata, Tetraselmis suecica and Chlorella minutissima* showed exponential growth over the 10 day period when grown in pure sea water without the enrichment media (Table 1; Figure 1).The same three microalgae also showed exponential growth over the 10 day period when grown in pure sea water plus the enrichment media (Table 2; Figure 2). However, the soil water isolate, *Chlamydomonas moewussi* showed exponential growth in both suspension culture (Table 1; Table 2; Figure 1, Figure 2) until day 6. At day 8 the cells of *Chlamydomonas moewussi* disappeared from the culture suspensions. This might be due to poor acclimation, nutrient depletion or toxicity.

Table 1: The Cell Counts of Microalgae Grown in Pure Seawater without Enrichment Media (Average of 3 Experimental Replicates)

MICROALGAE	TIME				
	Day 2 (X 1000)	Day 4 (X 1000)	Day 6 (X 1000)	Day 8 (X 1000)	Day 10 (X 1000)
Nannochloropsis Oculata	5.98	15.4	212.5	400.0	637.5
Chlamydomonas Moewussi	1.1	8.0	60.0	0	0
Tetraselmis Suecica	7.0	54.0	145.0	187.5	357.5
Chlorella Minutissima	4.0	10.0	18.3	24.5	54.0

Table 2: The Cell Counts of Microalgae grown in Pure Seawater with Enrichment Media (Average of 3 Experimental Replicates).

MICROALGAE	TIME				
	Day 2 (X 1000)	Day 4 (X 1000)	Day 6 (X 1000)	Day 8 (X1000)	Day 10 (X1000)
Nannochloropsis Oculata	71.0	199.0	225.0	301.0	585.2
Chlamydomonas moewussi	4.5	16.5	22.5	0	0
Tetraselmis Suecica	7.5	66.5	69.3	79.2	85.0
Chlorella Minutissima	5.1	14.2	28.5	50.0	66.5

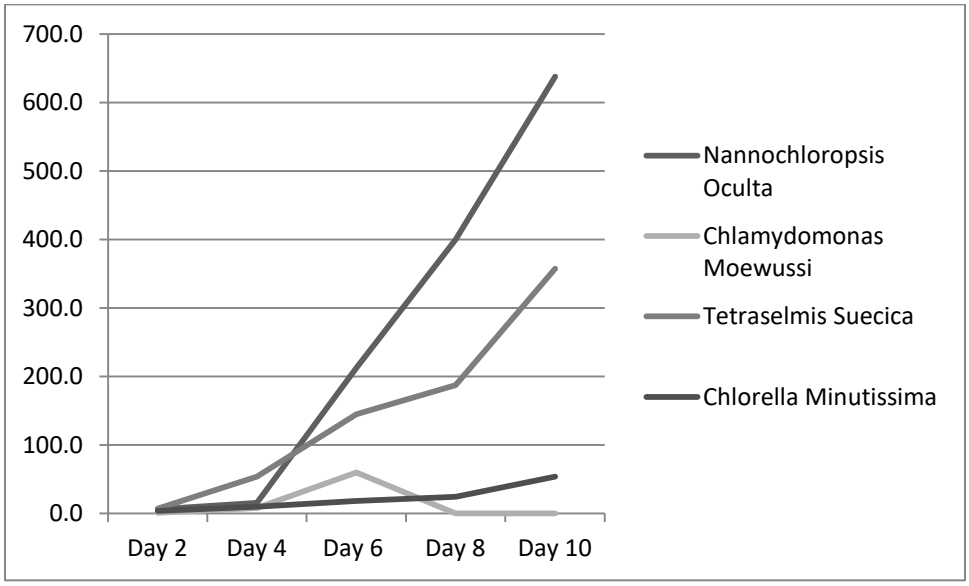
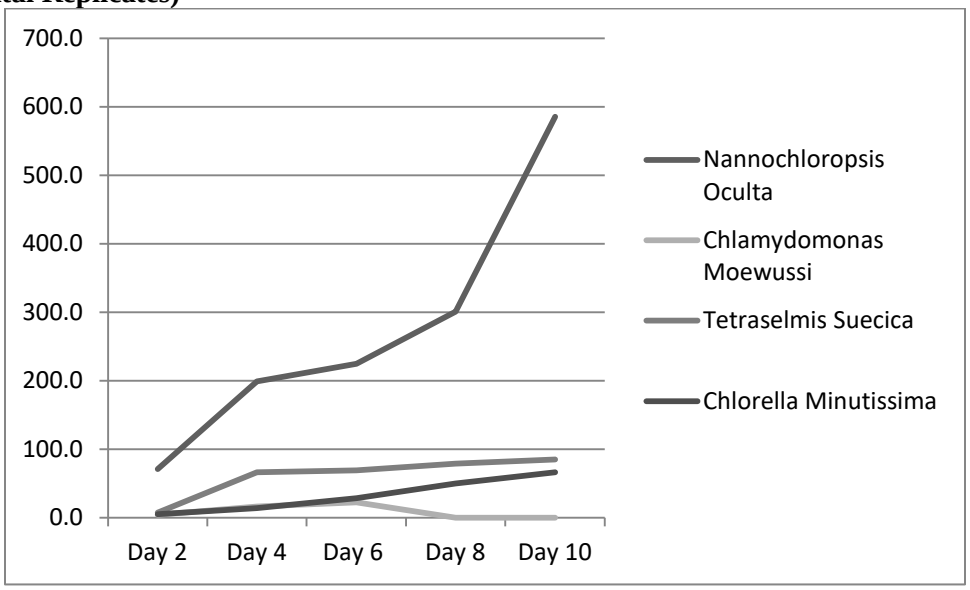


Figure 1: The Growth of Microalgae in Pure Sea Water without Enrichment Media (Average of 3 Experimental Replicates).

From the growth analysis (10; equation 1, Tables 3 and 4) it is evident that three microalgae *Nannochloropsis oculata*, *Tetraselmis suecica*, and *Chlorella minutissima* grew faster in pure sea water than pure sea water with the enrichment media. The generation times for the three microalgae in pure sea water are, *Nannochloropsis oculata* 32.4 h, *Tetraselmis suecica* 15.2h, and *Chlorella minutissima* 32.3h, whereas the generation times the same three microalgae in pure sea water plus the enrichment media are 35.2h, 16.3h and 36.4h respectively. The soil water isolate (*Chlamydomonas moewussi*) grew faster with pure sea water plus the enrichment media than pure sea water alone with a generation time of 16.8h and

25.6h respectively. This might be due to the presence of HEPES which might enhance the growth of fresh water species (McFadden and Melkonian, 1986).

Figure 2: The growth of Microalgae in Pure Sea water with Enrichment Media (Average of 3 Experimental Replicates)



The Generation times and Growth constant were calculated using the following equation (Brock, 1974).

K= log Xt-log Xo/0.301t

Where Xo=Number of Cells at Initial time

Xt=Number of Cells at a later time

t= Length of time from Xo to Xt

K=Growth Rate Constant, expressed as the number of doublings per unit time.

1/K=Generation time, the time required for the population to double.

Table 3: The Growth Analysis of Microalgae Grown in Pure Sea water with Enrichment Media (t= 48h)

MICROALGAE	GROWTH CALCULATIONS			
	Xo (X1000)	Xt (X1000)	K	1/K
Nannochloropsis *Oculata*	5.98	15.4	0.0284	35.2h
Chlamydomonas Moewussi	1.1	8.0	0.0596	16.8h
Tetraselmis Suecica	7.0	54.0	0.0614	16.3h
Chlorella Minutissima	4.0	10.0	0.0275	36

Table 4: The Growth Analysis of Microalgae in Pure Sea water without Enrichment Media (t=48h).

MICROALGAE	GROWTH CALCULATIONS			
	Xo (X1000)	Xt (X 1000)	K	1/K
Nannochloropsis Oculata	71.0	199.0	0.0309	32.4h
Chlamydomonas Moewussi	4.5	16.5	0.0391	25.6h
Tetraselmis Suecica	7.5	66.5	0.0656	15.2h
Chlorella Minutissima	5.0	14.0	0.0310	32.3h

Our results indicated that pure sea water is a better medium for the growth of three microalgae, *Nannochloropsis oculata*, *Tetraselmis suecica* and *Chlorella minutissima* than pure sea water with the enrichment media. We can conclude that pure sea water contain an adequate amount of all nutrients required to grow *Nannochloropsis oculata*, *Tetraselmis suecica*, and *Chlorella minutissima* (the sea water isolates) over a 10 day period since these organisms grew faster in pure sea water than pure sea water plus the enrichment media over the same period. The results also suggest that the soil water isolate (*Chlamydomonas moewussi*) can be grown in pure sea water and pure sea water plus the enrichment media over a 6 day period. The growth analysis of microalgae can be used to determine the exponential/near stationary phase that is important for the production of lipids and hence biodiesel for commercial use.

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