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Effect of type of nutrient media on the biomass and fatty acid profiles of microalgae (*Chlorella* spp.)

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Abstract
Locally available nutrient materials (Cow dung, Soybean extracts and Diammonium Phosphate (DAP)/UREA) were used to prepare culture media for growing *Chlorella* spp. and compared to the commonly constituted Bold's Basal Medium (BBM) as a control. Algae was cultured in 25L rectangular glass tanks with 24 hour illumination and aeration. Ammonia, pH and counts of algae cells/ml were recorded daily for 24 days. *Chlorella* spp. production rate, effect of intrinsic ammonia and pH on culture performance, duration of culture cycle and the di fferences in resultant fatty acid composition of the algae were tested. Results indicated a significant effect of culture media on the growth performance of *Chlorella* spp. ($F = 3.42$, $P< 0.05$), although the interaction between time (day) and nutrient media was also significant ($F = 17.27$, $P< 0.05$). Bold's Basal Media had significantly lower mean abundance of *Chlorella* spp. per millilitre than all the other media ($F = 20.65$, $13.57\pm7.1\times10^4$, $P< 0.05$). However, Soybean media supported significantly higher densities of *Chlorella* spp. than other media ($F = 20.65$, 20.65, $21.53\pm7.4\times10^4$, $P< 0.05$). The abundance of *Chlorella* in DAP/UREA and Cow dung media did not differ significantly ($F = 20.65$, $17.01\times10^4\pm4.3$ and $18.43\pm6.0\times10^4$, $P = 0.46$ respectively). There was a notable positive effect of pH on growth of *Chlorella* whereas ammonia did not have much impact even at relatively high concentrations (236.095 mg l^{-1} in DAP/UREA). Of the fatty acids in *Chlorella*, polyunsaturated fatty acids (>40% of Total Fatty Acids) were predominant in all media. Monounsaturated fatty acids (MUFAs) were recorded low (<5% TFA) for Cow dung, BBM and Soybean. DAP/UREA was superior for MUFAs and HUFAs. The results from this study demonstrated the feasibility of cultivating *Chlorella* spp. using locally prepared nutrients.

Keywords: Nutrient media, *Chlorella* spp, Biomass and fatty acid profiles

1. Introduction
The feeding of fish larvae is a major challenge in fish hatcheries and this is indeed hindering the full commercialization of most domesticated fish species and subsequent success in hatchery based seed production for grow out and bait customers [1]. It is an established fact that the success of any hatchery operation will depend mainly on the availability of the basic food. Phytoplankton can provide food for early stage crustaceans and zooplanktons to feed fish larvae. Phytoplankton therefore constitute a base for early fish nutrition, [2] supplying vital fatty acids, vitamins, minerals, energy and have been widely adopted in hatcheries [3]. *Chlorella* spp, given the correct quantity of nutrients, light, pH and temperature [4], attains incredible rates of multiplication [5], and can withstand a wide range of temperatures 5-42 ^{0}C (optimum 20-30 ^{0}C) [6], while growth is inhibited at pH 11 (optimum 6-8). Nitrogen and phosphorus are the two main nutrients that influence phytoplankton growth [7]. *Chlorella* spp. is rich in linoleic and linolenic fatty acids [8], which improves the nutritional quality of the zooplankton foraging on it. Also, *Chlorella* spp. has appreciably higher crude protein (50% of dry weight) than that of best plant sources used as animal fodder. Laboratory based culturing of micro algae using inorganic media is costly to many fish farmers due to insufficient availability of the different ingredients of the culture media. This study was therefore undertaken to exploit the prospects of culturing *Chorella* spp. using locally available nutrient materials and ultimately augment the production of locally available zooplanktons (rotifers, cladocerans, copepods) as alternative starter fish larval feeds to the imported Brine Shrimp in Uganda.

2. Materials and Methods

2.1 Study area

Seed microalgae (*Chlorella* spp) was isolated from sewerage treatment lagoons at the National Water and Sewerage Corporation-Entebbe and isolated in a wet lab at Department of Zoology, Entomology and Fisheries sciences of Makerere University between August 2015 and February 2016.

2.2 Experimental set up for purifying Chlorella spp.

The purification and isolation of *Chlorella* was followed the methods developed by Lee and Tamaru [09]. Serial dilution and multiple sub-cultures were obtained using Bold's Basal Medium (BBM) and as modified by SAG [10]. All serial dilution cultures were incubated at room temperature with continuous aeration and exposure to 40W fluorescent light (1000lux) for one week after which they were examined microscopically.

2.3 Preparation of nutrient media (Soybean nutrient extract, Cow dung nutrient extract, Inorganic fertilizer medium and Bold's Basal Medium)

Preparation of Soybean-extract medium followed procedure for preparation of leguminous seed cake [11], by substituting Pulse bran (*Vigna mungu*) with full grain soya bean. The resulting nutrient solution was autoclaved for 15 minutes at 121ºC to obtain a ready to use sterile culture medium. Farm yard manure (cow dung 200g/l) was boiled for 1hour in a litre of water and strained to remove excess solids on cooling. Two litres of water (tap water) were added to dilute and the mixture and left to stand for two days. The mixture was autoclaved for 15 minutes at 121ºC to obtain a ready to use sterile culture medium. Inorganic fertilizers; Di – Ammonium phosphate (15 g/litre) and Urea (15 g/litre) dissolved in 1 litre of de-chlorinated tap water was used as inorganic fertilizer medium. All un-dissolved solids left after stirring for 15 minutes were strained off using a 100µ zooplankton net to obtain a ready to use medium. Bold's Basal Medium was used as control and was constituted as modified by SAG [10]

2.4 Culture of algae

Chlorella spp. was cultured in 25 L rectangular glass tanks, supplied with continuous aeration through perforated air stones, to keep the *Chlorella* spp. cells in constant motion, prevent settling and facilitate maximum exposure to light. The cultures were also supplied with 24 hour constant lighting using a single 40W (daylight) fluorescent tube (equivalent 1000Lux). Water quality parameters; ammonia and pH were measured daily for the entire period of the experiment by photo-spectrometry. Counts of cells/ml were taken using a magnification of x200 on an inverted microscope (WILOVERT®) and a *Sedgwick*-Rafter Cell counting chamber.

2.5 Chlorella spp. Fatty acid analysis

2.5.1 Sample collection for fatty acids

Chlorella spp. samples from the four cultures were obtained by taking two litres of the 'green' water and centrifuging at 4000rpm, to obtain a concentrate of algae (*Chlorella* spp.) in 1 ml clean dry test-tubes. The samples were immediately separately homogenized and frozen at −86 °C for 12hr before further analysis. In each case, the analyses were carried out in triplicate.

2.5.2 Esterification of the fatty acids

Dry hydrogen chloride gas was bubbled through anhydrous methanol (HPLC grade) in a flask immersed in an ice-bath and its concentration periodically monitored by the increase in mass of the methanol. The ensuing hydrogen chloride gas (7.2g) dried by passing it through conc. H_2SO_4 was bubbled it into methanol (100ml) to make methanol/2MHCl solution. Samples of the *Chlorella* spp. weighing approximately 30 to 50 mg were transferred to thick walled glass tubes to which 1cm^3 of the acidified anhydrous methanol was added. Nitrogen gas was then flushed through the tubes which were then securely sealed with Teflon-lined screw caps and left for a period of 2 h in an oven set at 90 °C by a thermostat.

2.5.3 Extraction of the fatty acid methyl esters (FAME)

The resulting fatty acid methyl esters (FAME) were extracted from the mixture by solvent extraction using a water-hexane solvent system. To make methyl esters less soluble in methanol phase, about half of methanol was evaporated under a stream of nitrogen gas and 0.5cm^3 water was added followed by 1cm^3 of hexane the tube was shaken for 3 minutes**.** After**,** the mixture was centrifuged at 1500rpm for 3 minutes. The FAMEs were obtained from the upper hexane phase of the partition by siphoning. A second extraction was performed after addition of hexane (1cm^3) to the residual mixture and repeating the same procedure as described above. The extracts were then cooled and stored under refrigeration until Gas Chromatography analysis.

2.6 Data analysis

Analysis of variance (ANOVA) was used to compare; means of population densities of *Chlorella* (cells/ml) and the mean percentage fatty acid types present in *Chlorella*, while analysis of covariance (ANCOVA) was used to verify the effect of time (day) on the growth performance of *Chlorella*. General linear model regression was used to test the effect of pH and ammonia on the growth performance of *Chlorella* among the media.

3. Results

3.1 Production of isolated *Chlorella* spp. under the culture media

This study achieved 95% growth of isolated *Chlorella* spp. (Plate.1) with a limited mixture of *Scenedesmus* spp. (5%). It was observed that *Chlorella* out-grows most unicellular algae apart from blue-green algae, which is a good enough demonstration of possible pure *Chlorella* spp. culture with time.

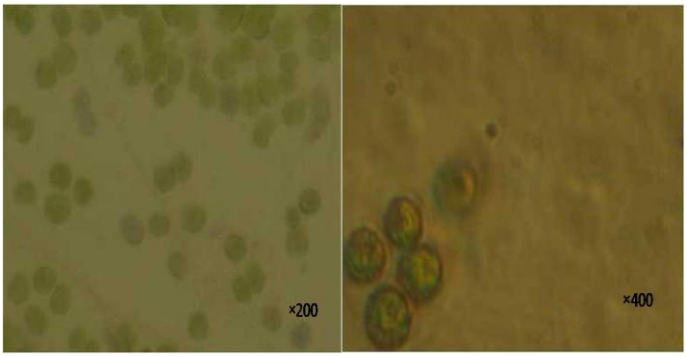

Plate 1: Chlorella spp. at x 200 and x 400 as observed under a compound microscope

The type of culture media showed a significant effect on the

growth of *Chlorella* spp. (ANOVA, F = 3.42, $P< 0.05$), although the interaction between time (day) and nutrient media was also significant (ANCOVA, F = 17.27, $P< 0.05$). The performance of *Chlorella* spp. on different media is depicted in Figure. 1 and BBM had significantly lower mean abundance of *Chlorella* spp. than all the other media (ANOVA, F = 20.65, $13.57\pm7.1\times10^4$, $P< 0.05$). In contrast, Soybean media supported significantly higher densities of *Chlorella* spp. than other media (ANOVA, F = 20.65, 20.65, $21.53\pm7.4\times10^4$, $P< 0.05$). The abundance of *Chlorella* on DAP/UREA and Cowdung media did not differ significantly (ANOVA, F = 20.65, $17.01\times10^4\pm4.3$ and $18.43\pm6.0\times10^4$, P = 0.46).

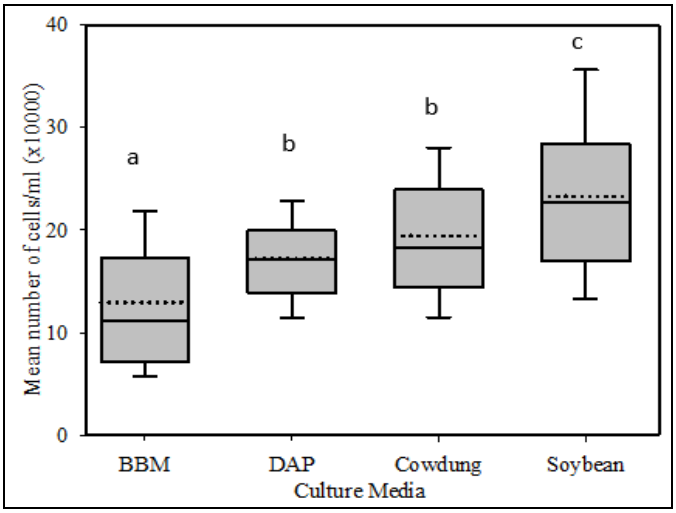

Fig 1: Performance of *Chlorella* spp. on different media

Growth trends revealed no significant differences among media for the first 12 days after which the trends and differences in density become clearly separated (Fig. 2). BBM remains significantly lower than DAP/UREA, Cowdung and Soybean Period DAP/UREA performs above all other media, although differences between DAP/UREA, Cowdung. In the end, the superior media was soybean followed by cowdung and DAP/UREA respectively.

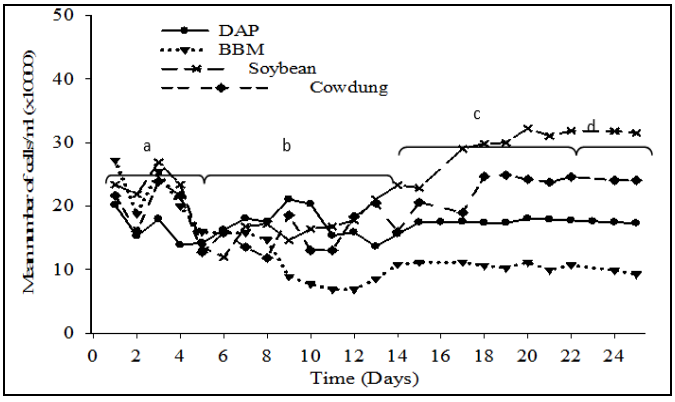

Fig 2: Growth performance of *Chlorella* spp. (mean cells/ml) with time as observed in different media.

3.2 Effect of pH and ammonia on the growth of Chlorella spp

General linear model (GLM) regression analysis was used to explore the effect of pH and ammonia on the growth and multiplication of *Chlorella* spp. on individual culture media. There was a significant effect of pH on growth and multiplication of *Chlorella* spp. only in BBM but not in any of the other media (Table 1).

Table 1: Linear regression analysis exploring the effect of pH and ammonia on the growth performance of *Chlorella* spp. on different media

Media	pH (p-value)	Ammonia (p-value)
DAP	0.065(0.60)	0.006(0.96)
BBM	0.375(0.001)*	0.103(0.35)
Soybean	-0.082(0.48)	-0.20(0.08)
Cowdung	0.089(0.45)	-0.003(0.98)

*significant at $p< 0.05$

Fatty acid composition of Chlorella spp. grown using different nutrient media

The analysis of fatty acids in the *Chlorella* spp. samples indicated variation in fatty acid composition among media (Table 2). PUFAs and MUFAs were significantly higher in DAP/UREA than BBM, Cowdung and Soybean although there were no significant difference in PUFAs between BBM, Cowdung and Soybean. On the other hand, SFAs in DAP/UREA were significantly lower than in BBM, Cowdung and Soybean media which showed no significant differences among themselves (Figure 3).

Table 2: Fatty acid composition (expressed as percent of total fatty acids) in *Chlorella* spp. grown on different nutrient media

Fatty acids	DAP/UREA	BBM	Cowdung	Soybean
	n=3	n=3	n=3	n=3
10.00	0.28±0.01	0.02±0.03	0.00±0.00	0.00±0.00
14.00	2.52±0.12	2.94±0.12	3.46±0.19	2.55±0.38
16.00	13.15±0.15	17.10±0.57	18.10±0.41	17.20±0.37
17.00	3.68±0.04	13.15±0.51	13.00±0.75	13.08±0.63
18.00	0.47±0.07	1.93±0.12	1.74±0.11	1.56±0.04
Total SFAs	**20.10±0.39**	**35.14±1.35**	**36.30±1.46**	**34.39±1.42**
14.1n5	0.55±0.08	0.75±0.14		0.43±0.05
16.1n9	1.68±0.05	1.66±0.12	1.61±0.07	1.55±0.05
16.1n7	1.08±0.04	1.90±0.07	1.12±0.04	1.05±0.00
17.1n9	12.41±0.32	1.38±0.16	2.54±0.19	1.56±0.05
18.1n9	6.87±0.24	1.90±0.18	4.07±0.03	4.16±0.11
18.1n7	1.34±0.06	0.00±0	0.04±0.07	0.0±0.066
Total MUFAs	**23.92±0.80**	**7.60.±0.66**	**9.39±0.41**	**9.34±0.32**
17.3n3	2.69±1.98	6.69±0.69	5.35±0.38	5.39±0.46
17.3n9	12.41±0.32	1.38±0.16	2.54±0.19	1.56±0.05
18.2n6	11.37±0.06	28.24±0.15	26.23±0.57	30.84±1.60
18:3n3	26.68±1.15	11.03±0.54	10.77±0.37	10.57±0.21
20.4n3	3.00±0.25	0.00±0	0.00±0	0.03±0.06
Total PUFAs	**56.14±3.76**	**47.34±1.54**	**44.90±1.5**	**48.39±2.39**

3.3.1 Polyunsaturated fatty acids (PUFAs)

Polyunsaturated fatty acids (PUFAs) were the most dominant fatty acids in all the *Chlorella* samples analysed (Table 2). The most abundant of PUFAs, in all four treatments, was Linoleic acid (18:2n6). *Chlorella* (Soybean) had the highest percentage of Linoleic acid (30.34±1.60), followed by BBM (26.26±0.15), Cowdung (26.23±0.57) and DAP/UREA (11.37±0.06). Another PUFA present in large quantities is Linolenic acid (18:3n3), which is most abundant in DAP/UREA (26.68±1.15), and present in the other media in approximately equal but much lower amounts than in DAP/UREA:BBM, (11.03±0.54), Cowdung, (10.77±0.37) and Soybean, 10.57±0.21).

3.3.2 Monounsaturated fatty acids (MUFAs)

Monounsaturated fatty acids (MUFAs) were generally low in all media (less than 10%) except in DAP/UREA (Table 2). The most abundant MUFAs were 17:1n9 (12.41±0.32) and 8:1n9 (Elaidic acid) (6.87±0.24), both in DAP/UREA,

although other MUFAs (14:1n5, 16:1n7 and 18:1n7) were identified, albeit, in very small quantities (<5%) (Figure 3)

3.3.3 Saturated fatty acids (SFAs)
Saturated fatty acids were found in *Chlorella* grown on all four media. SFAs were dominated by 16:00 (palmitic acid), with Cowdung (18.10±0.41), Soybean (17.20±0.37) and BBM (17.10±0.57) registering the higher concentrations than can be found in DAP/UREA (13.15±0.15). The other SFA present in relatively high amounts was 17:00 (daturic acid) in similar order as palmitic acid; with Cowdung (13.00±0.75), BBM (13.15±0.51) and Soybean (13.08±0.63) had similar quantities while DAP/UREA (3.68±0.04) is comparably very low.

4. Discussion
4.1 Performance of *Chlorella* spp. grown on different culture media
It is evident that culture medium had a positive effect on growth performance of *Chlorella* spp. Inorganic nutrients are known to be major stimulants for growth of phytoplankton in aquatic systems. Previous reports agree that that phytoplankton growth is limited by nutrients [12, 13] as well as light and temperature. In this experiment, whereas light and temperature were maintained constant (1000Lux; 25ºC), variation in growth performance of algae can be explained by the interaction between the different nutrient media provided and the duration of the culture. In the first four days, the growth of the algae appears to decline perhaps as the algae establish in the different media. This observation is however, different from previous observations in which continuous positive growth was noted from day one [14] till a maximum growth (day 12; 11.8×10^6cell.ml^{-1}, on poultry manure) was attained after which the densities declined.

The highest density obtained in this experiment (by day 23; 39.3×10^4cell.ml^{-1}) on soybean-extract medium was lower than that which was obtained by [12] using Pulse bran (*Vigna mungu*) nutrient extract (4.49×10^6cell.ml^{-1}). The variation in *Chlorella* spp. densities that were attained by the different media in this experiment were probably a direct function of nutrient levels (especially N, P, and K) in each specific medium which were the key limiting nutrients [15]. More specifically, phosphorus (P) was reported as a major limiting factor in the growth of *Chlorella* [16]. While nitrogen (N) sources whose value of nitrates is below certain levels were shown to be limiting to cell growth of the green alga *Neochlorisoleo abundans* [17]. From these experiments therefore, it can be inferred that Soybean extract has higher levels of, either, N, P or both followed by Cowdung and DAP/UREA (which were similar) and can, consequently, support higher densities of *Chlorella* spp. in culture than BBM.

The continued increase in density of *Chlorella* in the soybean medium is indicative of the slower rate of depletion of nutrients, and similar performance is exhibited by Cowdung. It's been stated that organic matter releases nutrients slowly over a longer period of time [18], as opposed to rapid release and depletion of nutrient in inorganic fertilizers as seen in DAP/UREA and BBM media. The fact that, *Chlorella* spp. performed better in the test nutrient media than in the standard inorganic medium (BBM) is desirable for practical application on large scale localized culture of algae for advancement of aquaculture.

4.2 Ammonia and pH changes along the *Chlorella* culture cycle
There was a positive relationship between pH and growth of *Chlorella* cells only in BBM. Slight pH increment in algal cultures is as a result of uptake of inorganic carbon by phytoplankton during photosynthesis [19]. Moreover, it's been shown that pH played no role in determining the magnitude of inhibition of photo-assimilation of carbon, apart from establishing the degree of dissociation of nontoxic NH_4^+ to toxic NH_3[20]. The effect of ammonia on growth of *Chlorella* was found not significant, in this study. In its ionized (NH_4^+) form, ammonia is not toxic to algae, however, when in unionized form (NH_3) it is known to be toxic [21] and has potential to inhibit photosynthesis at high pH levels [22]. Ammonia does not have effect on *Chlorella vulgaris* unless the concentration of ammonia is too low (10 mg N l^{-1}) or very high (750 and 1000 mg N l^{-1}) [23]. In this study, maximum ammonia (DAP/UREA; 236.095 mg l^{-1}) was well within limits and therefore had no significant impact on the growth rate of the *Chlorella* spp. in all the media, perhaps due to the combination of low algal biomass and strong pH buffering commonly exhibited by freshwater environments unlike in algal waste water treatment systems [22].

4.3 Fatty acids
Chlorella spp. cultured using BBM was just as rich in 18:3 PUFAs + MUFAs as Cowdung and Soybean which could be as a result of its chemical constitution, being deliberately enriched with cations Mg^{2+}which is known to influence total cellular fatty acids in *Chlorella* spp. and K^+ found to be good for production of 18-C unsaturated fatty acids especially 18:3 [22]. This also points to the possibility that Soybean and Cowdung are not lacking in K^+ and Mg^{2+}. High PUFA+HUFA were recorded in *Chlorella* spp. grown on Cowdung and Soybean extracts. This can be attributed to higher high Nitrogen content in the two nutrient media. Recent work demonstrated that total fatty acids were highest at high N (35.6% for 100% N) media [23]. Furthermore, a study on the effects of nitrogen sources on lipid accumulation in *Neochlorisoleo abundans* concluded that high lipid accumulation in algal cells were easier obtainable with N-rich nutrient media [23].

The presence of α-Linolenic acid (ALA) (18.3n3) and Linoleic (LA)(18.2n6) in varying quantities for *Chlorella* spp. grown with Soybean and DAP/UREA suggests that there is potential for the two (organic and inorganic) nutrient media, when used in combination, to complement each other and produce an algae rich in both fatty acids (LA and ALA), which are vital in the biosynthesis of eicosapentaenoic (EPA) [24] and subsequent synthesis of docosahexaenoic (DHA) [25]. Both ALA and LA fatty acids are critical in the early growth and development of fish. Monounsaturated fatty acids (17: 1n9 and 18:1n9) and saturated fatty acid (17:00) were found to be plentiful in fat and eggs of *Lates niloticus* [26], suggesting that they might have associated functions with egg development.

5. Conclusion
In this study, Soybean and Cowdung nutrient extracts support higher densities of *Chlorella* spp. than DAP/UREA and BBM for longer periods, which make them more stable media for continuous culture of algae. In order to stimulate rapid growth in the initial stages of the culture a blend of Cowdung or Soybean medium with an inorganic fertilizer like DAP/UREA may be required. A healthy balance between level of

ammonia and changes in pH in algal culture using Cowdung and Soybean facilitates strong *Chlorella* culture, and although DAP/UREA has higher levels of ammonia than the rest of these media, it does not cause major limitation to the growth and multiplication of *Chlorella* spp. Different nutrient media facilitate varying fatty acid composition of algae. *Chlorella* has high levels of unsaturated Fatty acids (in this case >60% of Total Fatty acids) which makes it a very desirable food for zooplankton, and subsequently, fish that require Highly Unsaturated Fatty Acids (HUFAs) in their early stages of development.

5.1 Recommendation
Test media in this study yielded better results than the standard BBM suggests that BBM can successfully be replaced with these nutrient media for improved live larval food production at a cheaper cost. There is a need however, to investigate the effect of combining DAP/UREA with either Cowdung or Soybean media on the growth performance of *Chlorella* and the resultant fatty acid concentrations in *Chlorella* spp.

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