MAKERERE  UNIVERSITY

PERFORMANCE OF ROTIFER (*Brachionus calyciflorus* PALLAS) FED ON *Chlorella* CULTURED ON LOCALLY AVAILABLE NUTRIENT MATERIALS

BY

ARINAITWE ANDREW VICTOR IZAARA (BFA) (Mak)

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DECLARATION

I Arinaitwe Andrew Victor Izaara, hereby declare that this work is entirely original and has never been submitted to any University or Institution for any academic award. All sources of information quoted from previous work, have been fully acknowledged. All photos are by Arinaitwe Andrew Victor Izaara.

Signed.....

Date.....16/10/2012.....

Arinaitwe Andrew Victor Izaara (Candidate)

This dissertation has been submitted for examination with the approval of the supervisors below:

Signed

.....Date.....19/10/2012.....

Dr.Gladys Bwanika (BSc., MSc., PhD)

Signed.....Date.....19/10/2012.....

Dr.Lucas Ndawula (BSc., MSc., PhD)

DEDICATION

This work is dedicated to my wife Rebecca and our two daughters; Lisa and Lynnette, who were a continuous motivation towards the completion of this study.

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ACRONYMS

BBM -	Bold's Basal Medium
DAP -	Diammonium Phosphate
DHA -	Di ecosapentaenoic Acid
EDTA -	Ethylenediaminetetraaceticacid
EPA -	Eicosapentaenoic Acid
ESD -	Equivalent Spherical Diameter
FAO -	Food and Agriculture Organization
HPLC -	High-performance liquid chromatography
NWSC -	National Water and Sewerage Corporation
SAG -	*Sammlung von Algenkulturen Gottingen*
UGX -	Uganda Shillings
KWh -	Kilo Watt Hour
MUFAs -	Monounsaturated fatty acids
PUFAs -	Polyunsaturated fatty acids
SFAs -	Saturated fatty acids

ABSTRACT

Locally available nutrient materials (Cowdung, Soybean extracts and Diammonium Phosphate (DAP)/UREA inorganic fertilizer) were used to prepare culture media for growing *Chlorella* spp. as a food base for rotifer culture and compared to the commonly constituted Bold's Basal Medium (BBM) as a control. The production rate of *Chlorella* spp., the effect of intrinsic ammonia and pH on culture performance, duration of culture cycle as well as the differences in resultant fatty acid composition of the algae were tested. The production rate of *B. calyciflorus* fed on this *Chlorella* grown under the four media and the cost effectiveness of using each of the media were investigated. Experiments on algae culture were set in 25L rectangular glass tanks, with 24hour illumination and aeration. Daily reading of ammonia, pH and counts of algae cells/ml were carried out. On the other hand, 10L plastic jerry cans, supplied with 24hour mild bubbling were found suitable for rotifer culture. Daily changes in rotifer density (rotifers/ml) and number of eggs carried per rotifer were recorded. Soybean extract attained the highest density of *Chlorella* per ml, followed by Cowdung and DAP/UREA, while BBM showed least performance. There was a notable positive effect of pH on growth of *Chlorella*. On the other hand, ammonia did not have much impact even at relatively high concentrations (236.095 mg l^{-1}in DAP/UREA). *Chlorella* was found rich in various fatty acids but with a predominance of polyunsaturated fatty acids (>40percent of Total Fatty Acids) in all media.

Monounsaturated fatty acids (MUFAs) were recorded low (<5percent TFA) for Cowdung, BBM and Soybean. DAP/UREA was superior for MUFAs and HUFAs. Comparably, DAP/UREA was found to have about half the quantity of saturated fatty acids (SFA) found in the other three media. The highest number per ml of B. *calyciflorus* was found in the culture grown on Cowdung. This culture also sustained a much healthier culture in terms of production of eggs per rotifer and was also the most cost effective. Further research on how these recorded performances translate into healthy larval fish is required.

CHAPTER ONE

1.0 Introduction

1.1 Background

World production of fish originally depended on wild fish capture, but recent trends indicate stagnation in production in-spite of increased effort, while annual world per capita fish consumption keeps growing by the decade, to about 17.1 kg (FAO, 2010).

Table 1: Showing per capita consumption of fish from the 1960s to 2008

YEAR	Per capita fish consumption (Kg)
1960	9.9
1970	11.5
1980	12.6
1990	14.4
2007	17.0
2008	17.1

(*FAO, 2010*)

Nevertheless, the deficit created by stagnating capture fisheries production, is more than compensated for by production in aquaculture (FAO, 2008). Increasing demand for fish has been strongly associated with up-surging human population (Delgado *et al.*, 2003) coupled with changes in income (Asche and Bjorndal, 1999). Increasing preference for fish in human diet is a contributing factor to its high demand (Delgado *et al.*, 2003). Despite, the notable progress in aquaculture it remains to be exploited to its full potential as well as lack of

sufficient technical personnel, scarcity of quality seed and appropriate feed and lack of comprehensive information for culture of several potential culture species.

Successful domestication of any fish species requires total control of all phases of its life cycle, which necessitates a comprehensive understanding of the fish's reproduction in captivity, subsequent larval nursing and rearing, and grow-out to desired market size. The ontogenic stages in a fish's life cycle demand different food requirements both in presentation and nutrition. Food quality and quantity available to fish are therefore, key determinants of its growth rate and performance. In cultured fish species, the aim is to achieve fast growth and attain maximum size in the shortest time possible for profitable commercial yields.

Fish larvae are generally poorly developed on hatching, and as a result they have to undergo important anatomical, functional and morphological changes during the larval period. This larval stage can be greatly disrupted if nutrition is inadequate and unsuitable. For most cultured species of fish, egg size and larval mouth size are strongly positively correlated. The larval mouth size, in turn determines the particle size that larvae can captured and ingested on commencement of its exogenous feeding.

Zooplanktons play a central role in aquatic ecosystems as larval fish food and as such a channel of energy flow between primary producers and secondary consumers (Banse, 1995). The natural diet of most cultured fish and shellfish species is comprised of a wide diversity of phytoplankton species (diatoms,

flagellates, and micro-algae) and zooplankton organisms (copepods, cladocerans, decapods, larvae, rotifers and ciliates) that grow liberally in the aquatic environment (Lavens and Sorgeloos, 1996). Given that, zooplankton provide food for larval fish, containing vital amino acids, vitamins and minerals, they have been adopted in commercial and ornamental aquaculture as nutritional sources for fish larvae (Lubzens *et al.*, 2001). Nevertheless, larval nutrition still presents a major bottleneck in captive farming of many fish species mainly because of the requirement to present an adequate supply of suitable live starter feed to delicate fish larvae nursed in hatcheries. This necessitates timely controlled mass production of suitable zooplankton for any cultured fish species. This eases heavy dependence on wild harvesting zooplanktons which presents several challenges including diminishing natural reserves, seasonal variability and risks of disease contamination.

While there is recorded success in culturing zooplankton and crustacean as live food for many marine fish species, modest progress has so far been registered in use of freshwater zooplankton for freshwater fish. Thus far, the marine Brine shrimp (*Artemia* spp.) is widely used in fish farming of several freshwater species. On the other hand, attempts to use *Brachionus calyciflorus,* a freshwater rotifer, have recorded some success. As a follow up on this success, the proposed study attempts to investigate the possibility of improving the performance and cost effectiveness of culturing *B. calyciflorus* through using culture media of locally available and cheap ingredients.

1.2 Statement of the problem

Availability of suitable starter feed for fish larvae remains a major constraint in fish hatcheries. In Uganda, the imported Brine shrimp (*Artemia*) is utilized as a starter feed for the hatchery-produced African catfish larvae. Besides being expensive, it is not a suitable feed for other fish species with culture potential that present a much smaller post-hatch larva than the African catfish. A case in consideration is the valuable Nile perch whose domestication attempts are in progress. Rotifers that are relatively smaller, and are now used elsewhere, have not been commercially utilized in Uganda due to lack of adequate protocol for their culture in a local setting. Secondly, innovations to promote utilization of rotifers grown on readily available and possibly cheaper nutrient base are lacking. The commonly used algal culture medium (Bold's Basal Medium) for growing rotifer food constitutes a range of inorganic reagents which are neither handy for a local farmer nor cheap. For this reason, an investigation into the possibility of utilizing locally available nutrient media as alternative culture media for growing suitable food for *B. calyciflorus* rotifers was carried out.

1.3 Objectives

1.3.1 Overall objective

To investigate whether, locally available nutrient materials (Cowdung, Soybean extract and DAP/UREA fertilizer) make culture media for growing *Chlorella* spp. as a food base for rotifer culture.

1.3.2 Specific objectives

I. To determine the production rate of *Chlorella* spp. grown on three (3)

different culture media

II. To establish the fatty acid profiles present in *Chlorella* cultured in the three (3) different culture media

III. To compare the production rate of *B. calyciflorus* fed on *Chlorella* spp. grown under the three (3) culture media

IV. To establish the cost effectiveness of growing *B. calyciflorus* fed on *Chlorella* spp. grown on the three culture media.

1.4 Hypotheses

i. There is no difference in *Chlorella* spp. production rate grown under the selected culture media

ii. There is no difference in the fatty acid profiles present in *Chlorella* cultured using the three nutrient media

iii. There is no difference in the production rate of *B. calyciflorus* culture fed on *Chlorella* spp. grown under the three (3) culture media

iv. There is no difference in cost of production of rotifers using the three treatment nutrient media

1.5 Justification

This study adds to existing knowledge on the use of algae (*Chlorella*) and rotifer (*B. calyciflorus*) as larval fish food in the culture. Emphasis was directed at estimation of *Chlorella* biomass (cells/ml), grown under selected different nutrient media and the potential production of *B. calyciflorus* as the possible

effect of different nutrient media appears to be an important and yet remains relatively unknown.

Production of nutritionally adequate rotifers as starter live food for small-sized larvae is a bench mark for successful seed production of fish species with small post-hatch larvae especially Nile perch. Use of locally available materials to produce rotifers of high nutritional value, and culture sustainability, is desirable for growth of aquaculture since it provides a cost effective and sustainable source of nutrition for fish larvae.

CHAPTER TWO

2.0 Literature review

2.1 Feeding in fish larvae

A clear understanding of the interrelationship between fish larvae size (mouth gape) and food particle size is required for the development of appropriate live feed for aquaculture. The mouth size of first-feeding larvae usually mechanically restricts the size of the food particles which can be ingested. In general, mouth size is correlated with body size, which in turn is influenced by egg diameter and the period of endogenous feeding i.e., yolk sac consumption period (Lavens and Sorgeloos, 1996). Knutsen and Tilseth (1985) showed that in Atlantic cod, the ability of larvae to capture *Artemia* nauplii was positively correlated to mouth gape. Consequently, Atlantic cod larvae from large eggs also survived the shift to exogenous feeding better than those from small eggs. Liem *et al.*, (1999) noted that mouth gape of Marble goby (*Oxyeleotris marmoratus*) larvae limited their ability to utilize food type, particle size, and nutritional composition of the food offered to larvae on hatching underlining its importance to their survival and development.

2.2 Use of rotifers as starter live feeds for fish larvae

Lubzens *et al.*, (1989) noted that rotifers are valuable live food for the culture of larvae of many fish species because of their very small size, their: ability to stay suspended in the water column, high reproductive rate, ability to function normally at high density cultures in addition to their relatively slow swimming motion that makes them useful prey for active larvae. Rotifers of the genus *Brachionus* are widely used as live food for both marine (Lubzens, *et al.*, 1989; Sarma, 1991) and freshwater (Arimoro, 2006) fish larvae. Since rotifers can be

encapsulated with other nutritional ingredients, they can be used as a living food capsule and vehicle which transports adequate supplies of macro-micronutrients, vitamins, digestive enzymes or even antibiotics to the fish larvae (Lubzens, 1987).

The Phylum Rotifera includes four classes; Monogononta, Digonontae, Seisonidaeand Bdelloidae. The lifecycles of Bdeloid and Monogonont rotifers are better studied and the two classes have predominantly freshwater organisms inhabiting unstable environments such as fresh-water lakes and streams that are subject to temporal catastrophic changes (Ricci, 2001). Monogonont rotifers including *B. calyciflorus* have a simple morphology, with a body of three parts: a head, trunk, and foot. Locomotion is facilitated by a circular band of cilia surrounding the head called "corona". A stiff outer shell called lorica covers the body giving it a distinctive shape (Lavens and Sorgeloos, 1996). In *B. calyciflorus* the "lorica" sometimes has characteristic (4) anterior and posterior spines that function as predator defence. Rotifers of the genus *Brachionus* have a cosmopolitan distribution, short generation times, and are easy to culture. *Brachionus* species are regarded as generalist suspension feeders which are capable of collecting and processing numerous small cells simultaneously (Gilbert and Bogdan, 1984).

Reproduction in rotifers is both sexual and asexual, but their primary mode of reproduction is the asexual form known as parthenogenesis (Gilbert, 1974; Ricci, 2001). So long as the environmental conditions are favourable, the female rotifer can produce up to 7 genetically identical eggs simultaneously, which may hatch

in about 12 hrs into 'daughter' cells (Arimoro, 2006). The daughter cells themselves begin to reproduce approximately 18 hours post hatching.

2.3 Feeding in rotifers

2.3.1 Feeding behaviour of rotifers

Rotifers actively graze the water column feeding on particles approximately 1 – 10μm in size (Delbos and Schwarz, 2009). Herbivorous rotifers in nature depend on algae to meet their nutritional demands, and their performance can be limited by availability (quantity and quality) of algae. Similarly, prey size is a key factor in feeding success of rotifers to an extent that the particle size ingested can differ among species of the same genus (Rothhaupt, 1990). Differences observed in *B. calyciflorus* and *B. angularis* indicate that *B. angularis* prefers smaller particles approximately 5μm equivalent spherical diameter (ESD) while *B. calyciflorus* can accommodate larger particles of approximately 10μm ESD.

For mass production of *Brachionus* rotifers, various food types, both natural diets such as algae; (Sarma, 1991) and prepared diets e.g., microencapsulated pellets; (Gatesoupe and Robin, 1981), have been used. Various types of microalgae are continuously being cultured for feeding planktonic rotifers, mainly because production of algae is relatively cheap, both under field and laboratory conditions, (Lucia-Pavon et al. 2001). Two genera of green algae that are widely used include; *Chlorella* and *Scenedesmus* (Dumont *et al.* 1995; Nandini and Sarma 2000; Flores-Burgos *et al.* 2003). A few others, such as; *Chlamydomonas* (La Rocca et al. 1994), *Selenastrum* (Barry *et al.* 1995), and *Ankistrodesmus* (Kilham *et al.* 1998) have also been used. Pen˜ a-Aguado, *et al.*,

(2005) studied population dynamics of *B. calyciflorus* and *Brachionus rubens* fed on algae and yeast, and reported that yeast was inferior to algae as a source of nutrition for both species of rotifers. Moreover, *Brachionus plicatilis* rotifers fed on microalgae showed better viability, larger size and low ciliate contamination compared to those fed on yeast (Nhu, 2004). Trojsova *et al.*, (2008) revealed that the source of algal nutrients influences the growth rate and health of rotifer populations. These observations point to importance of algal nutrition source as a factor that ultimately affects rotifer performance. An investigation on the performance of *B. calyciflorus* fed on *Chlorella*. with varied local nutrient sources under this study therefore, was a measure to assess if some locally available nutrients conferred better nutritional benefits to *Chlorella* sp. than the commonly used media like BBM and DAP-UREA.

2.4 Culture conditions for *Brachionus* rotifers

In culture, physical-chemical conditions have been found to regulate growth of *B. calyciflorus* populations (Lucía-Pavón *et al.*, 2001). Several culture conditions indicating both the range and optimum levels of physical chemical conditions are documented. When *Brachionus calyciflorus* was cultured in reconstituted hard water medium (EPA) at a temperature of 25°C, dissolved oxygen level of > 4mgl^{-1}, Ammonia of <1mgl^{-1}and pH 7.5,and fed on *Chlorella* at a density of 4.5x10^{6} cells ml^{-1}, mean peak population density of 1227±83 individuals.ml^{-1},was achieved (Lucía-Pavón, Sarma, and Nandini, 2001). Hirayama and Kusano (1972), McVey and Moore (1983), Lim and Wong (1997), have all reported an optimum temperature of 25 °C for culture of *B. calyciflorus* rotifers, while Mostary *et al.*, (2007), reported an optimum culture temperature for *B. angularis* of 20°C lowest and 24.5°C highest. Effect of temperature on reproductive

activity of *B. plicatilis* shows that the higher the temperature, the shorter the maturation period (Moretti, 1999).Hoff and Snell (1989) reported optimum pH for *B. calyciflorus* to be 6.0 – 9.0, with upper and lower limits at 9.5 and 4.5 respectively. Ludwig (1993), on the other hand indicates that rotifers can tolerate a wide range of temperatures and pH; 15 - 31°C, and 6-8 at 25°C, respectively. Lavens and Sorgeloos (1996) reported best results at pH 6-8 at 25°C for *B. calyciflorus*. Furthermore, Mitchell and Jobber (1986) reported that a pH of 9.5 and 10.5 gave the highest and lowest capacity for population increase in *B. calyciflorus* cultures and that culturing at a pH of 10 for a short period daily was able to control a predatory species, *Asplanchna*. Besides pH and temperature, ammonia is another critical variable that needs proper monitoring in rotifer cultures. Ammonia exists as NH_3 and NH_4^+ in solution and the ratio of either is influenced by the temperature and pH of the water. High levels of un-ionized ammonia are toxic to rotifers, but concentrations below 1mg/l are reported to be safe (Lavens and Sorgeloos, 1996).

CHAPTER THREE

4.0 Materials and Methods

4.1 Study area

Brachionus calyciflorus rotifers were collected from the concrete aquaria tanks at the Botanical Gardens of the Department of Biological Sciences, at Makerere University. The Sewerage treatment lagoons of the National Water and Sewerage Cooperation – Entebbe (NWSC) were also investigated as a potential source of rotifers, but were found to be susceptible to rapid population changes and inconsistencies in species composition, so it was never considered. Microalgae, *Chlorella* used in these experiments were purified from samples obtained from the NWSC sewerage lagoons –Entebbe.

The experiments were carried out at the Department of Biological Sciences, MUK, Kampala, during 2010 and 2012, details of which are as follows. A mini wet laboratory was set up in the Department of Biological Sciences, Makerere University, where two experimental designs were set up and conducted to address the study objectives. The first experimental set up, examined the suitability of utilizing locally available nutrient materials as culture media for growth of *Chlorella,* and most suitable media identified. The second experiment examined the performance of *B. calyciflorus* fed on *Chlorella* grown on the three different nutrient media was investigated.

4.2 Isolation of *Chlorella*

4.2.1 Experimental set up for purifying *Chlorella* spp.

Water samples containing microalgae were collected from NWSC Sewerage treatment lagoons– Entebbe from which *Chlorella* was isolated. Isolation of *Chlorella* was achieved by a combination of serial dilution and targeted cell

collection by capillary glass pipette, followed by multiple sub-cultures using Bold's Basal Medium (BBM) as modified by SAG, (2007) . Two (2) litres of green water from the NWSC sewage treatment lagoons were filtered with 50μm plankton net and transferred to the laboratory. Then, 10ml of this filtered green water were introduced into a 500ml Erlenmeyer's flask filled up to the 200ml mark and mixed up with cultivation media (BBM) at room temperature (25°C) and the culture kept under a 40W fluorescent tube 24 hours a day for a week.

4.2.1.1 Serial dilution and purification of Chlorella

After one (1) week, using sterile 10 ml pipettes, 9 ml of media (BBM) were dispensed into each of ten test tubes labeled "10-1" to "10-10" to indicate level of dilution (dilution factor). By adding 1 ml of algae sample from the flask above to the first test tube (10-1) and mixing gently, a 10:1 dilution factor was achieved. From the 10:1 dilution, 1 ml was obtained and added to the next test tube ("10-2") and mixed gently to obtain a 10:2 dilution factor. This procedure was repeated for the rest tubes ("10-3" to "10-10"). All the test-tubes were incubated under at room temperature and 40W fluorescent tube (equivalent to 1000 lux) 24 hours a day. The cultures were examined under a microscope after two (2) weeks by withdrawing a small sample using a sterile pipette from each dilution tube. The higher dilution tubes "10-8", "10-9" and to "10-10" had relatively richer and more uniform *Chlorella* stock. Of these three, "10-8" showed better algal densities, but had a mixture of *Scenedesmus* and was selected for inoculating larger Erlenmeyer's flasks in a scale up.

4.2.1.2 Ultraviolet Absorbance Spectrophotometer Ammonia Analysis

Hypochlorite was added to the sample, which reacted with free ammonia in the sample to form monochloramine. An ultraviolet light absorbance spectrophotometer (UV light wavelength intensity meter) was used as an ultraviolet light source and was used to illuminate the sample. Some of the UV light was absorbed by the monochloramine concentration of the sample and the remaining UV light passes through. The UV spectrophotometer measured the UV light that passed through the sample. The ammonia analyser measured the difference in the transmitted UV light versus the amount of UV light generated by the spectrophotometer. This difference in UV light is proportional to the amount of free ammonia in the sample. (Yamazaki, et al., 1992)

4.3 Suitability of utilizing locally available nutrient materials as culture media for growth of *Chlorella*

4.3.1 Preparation of nutrient material using locally available materials for Culture of *Chlorella*

4.3.1.1 Preparation of Soybean extract nutrient medium

Preparation of Soybean-extract medium followed procedure for preparation of leguminous seed cake (Pulse bran: *Vigna mungu*) used by Mostary (2007). Crushed Soybean (700g) was mixed in 20 L tap water in a plastic bucket, and the mixture left to settle for one week. Urea (11g) was added to the bucket, and left to ferment for four weeks. The fermented mixture was filtered through thin cloth to remove and discard all solid materials left due to partial decomposition of Soybean. The supernatant was left to settle for a week, after which it was siphoned to another bucket. Lime (2g/ L), was added to medium to make it clear

followed by Sulphuric acid (H_2SO_4), to correct the pH back to 7 since it might have become acidic from the Sulphuric acid. The resulting medium was made to rest for one week after which it was siphoned to another bucket, autoclaved for 15 minutes at 121°C to obtain a ready to use sterile culture medium.

4.3.1.2 Preparation of cow dung nutrient extract

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 15minutes at 121°C to obtain a ready to use sterile culture medium.

4.3.1.3 Inorganic fertilizer medium

Inorganic fertilizers; Di – Ammonium phosphate (15 g/litre) and Urea (15 g/litre) were dissolved in 1 litre of de-chlorinated tap water. All un-dissolved solids left after stirring for 15 minutes were strained off using a 100 micron zooplankton net to obtain a ready to use medium.

4.3.2 Culture of algae (*Chlorella*) in different nutrient media

Purified *Chlorella* (500ml) obtained from the scaled up cultures was used to inoculate 25 L rectangular glass tanks., filled with water up to the 24.5 L mark to top up. The tanks were then supplied with appropriate (50ml) of each sterile nutrient and continuous aeration supplied through air-lines with perforated air stones, to keep the *Chlorella* cells in constant movement and circulation. This was to 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). The nutrient media were introduced in tanks randomly, with each test nutrient being used in 3 tanks.

Water quality parameters; ammonia and pH were measured twice daily (once in the morning and once in the afternoon at 8 hours interval) for the entire period of the experiment. Ammonia (NH_3) concentration (mg/l) and pH values were determined using Ultraviolet Absorbance Spectrophotometer Analysis. *Chlorella* density was determined once a day for each tank. Counts of cells/ml were taken using a magnification of x200 on an inverted microscope (WILOVERT®) and a *Sedgwick*-Rafter Cell counting chamber. The number of cells per millilitre was calculated according to the formula

$$\text{Number of cellc } *mL^{-1} = \frac{C \times 1000\ mm^3}{A \times D \times F}$$

Where;

- C was the number of cells counted,
- A was the area of field in mm^2,
- D - the depth of a field (*Sedgewick*-Rafter chamber depth) in mm,
- F - the number of fields counted.

4.4 *Chlorella* spp. Fatty acid analysis

4.4.1 Sample collection for fatty acids

Chlorella spp. samples from the four (4) cultures were obtained by taking two (2) 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 transferred to the Department of Chemistry, Makerere University

for fatty acid profiling. The samples were separately homogenized and frozen at −86°Cfor 12 h before further analysis. In each case, the analyses were carried out in triplicate.

4.4.2 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. During the heating process, fatty acids react with the methanol (esterification) and are converted to the corresponding fatty acid methyl esters.

4.4.3 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.

4.4.4 Control medium

The control culture medium was Bold's Basal Medium which is constituted by; Potassium Di-hydrogen Phosphate, Calcium chloride, Magnesium II sulphate, Sodium nitrate, Di-Potassium Hydrogen Phosphate, sodium EDTA, Potassium hydroxide, Iron II sulphate, and Trace Metals.

4.5 Culture of *B. calyciflorus*

4.5.1 Source of *B. calyciflorus*

Seed rotifers were collected from "green" pond (eutrophic) water, (Department of Botany – Botanical Gardens concrete tanks) by selective netting with 100μ and 50μ zooplankton nets. To achieve a culture of only rotifers, 'Basudine' an organophosphoric acid ester was applied at the rate of $1.5mgl^{-1}$ followingAgbone*t al.*, 2002, to kill off copepods, cladocerans, and mosquito larvae but not rotifers, thereby allowing a multi-species rotifer population to flourish. In this experiment, Diazinon PESTANAL® was the organophosphoric acid ester used to control copepods and other predators since its composition is exactly the same as that of 'Basudine'. In order to control ammonia levels in the rotifer culture, NovAqua Plus®™ was applied at the rate of $0.18ml^{-1}$, and refreshed every day.

4.5.2 Identification of *B. calyciflorus*

Identification of rotifers relied on morphological characteristics of the lorica, corona, and mastax (trophi) of dead animals based and behavioural characteristics of live animals. *Brachionus calyciflorus* is a slow swimming rotifer with 4 distinct anterior and posterior spines on the lorica, a feature that is known to be exclusive to the species. Reference to zooplankton identification keys was done to differentiate and isolate *B. calyciflorus* from other rotifers.

4.5.3 Isolation and inoculation

Isolation of *B. calyciflorus* was achieved by using sucker pipettes (Huawei® PIPETTE H100) of 10-30 micron (μ) aperture, coupled with a HUND WETZLER® binocular inverted microscope to pick out individuals with at least one egg sac, placing them in 25ml EPA solution in a 100ml Erlenmeyer's flask, where *Chlorella* was introduced. Wild zooplankton were collected by filtration from either of two sites depending on availability of *B. calyciflorus* using a 50 micron zooplankton net. On filtration, a concentration of zooplankton was shifted to 1liter conical flasks, filled with filtered pond water. Diazinon PESTANAL® was applied at a rate of 1.5mg/l to kill crustacean (Copepods, cladocerans, aquatic insects, mosquito larvae). Inoculum water containing the rotifers was completely renewed with 7.5mg/l Furazolidone (disinfectant). This aimed at achieving a population of pure rotifers. Disinfection consisted of killing free – swimming rotifers but not eggs using a cocktail of antibiotics (Erythromycin 10mg/l, Chlolamphenicol 10mg/l, Sodium oxolinate 10mg/l, penicillin 100mg/l, streptomycin 20mg/l). The eggs were separated from the dead rotifers using a 50micron net and incubated for hatching. The offspring were then used for starting a stock culture.

4.5.4 Population growth and Enumeration of *B. calyciflorus*

Experiments were carried out in 10L plastic jerry cans in 3 replicates per treatment. Well-fed adult *B. calyciflorus* without eggs were transferred into 8Lof the treatment food (*Chlorella* sp. cultured using the different media) suspensions. *Brachionus calyciflorus* were counted at x20 magnification and transferred into fresh algal suspensions (initial algal densities, x 10^4 cells/ml). Once a day, a 1ml sample was taken from which the number of individuals/ml were counted and recorded ten (10) times for each treatment. As well, the number of rotifers with eggs and number of egg sacks carried per rotifer were counted and recorded. A similar experimental set-up running concurrently with algae (*Chlorella* sp.) cultured on BBM served as a control. The experiments run for 7 days by which time productive trends were evident. All experiments were carried out at room temperature 25°C with a 12:12h light: dark cycle. To minimize sedimentation, the experimental tanks were gently bubbled with air from a compressor.

4.5.5 Data Analysis

Multiple regressions was used to ascertain the effect of ammonia and pH on the biomass growth of *Chlorella.* Non-parametric Kruskal-Wallis was used to compare the growth of biomass in the different media. Analysis of variance (ANOVA) was used to compare; means of. *calyciflorus* (individuals/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. While nonlinear regression analysis was used to estimate the effect of nutrient media on the mean number of eggs carried on individual rotifers.

4.6 Cost effectiveness of culture setting

The cost of producing rotifers using Cowdung, Soybean extract and inorganic fertilizer (DAP/UREA) were estimated by calculating the total of the component materials of each medium (cost in Uganda Shillings (UGX))and comparing them to the cost of constituting a commercial nutrient medium, BBM, so as to estimate the most cost effective method. The cost of producing a unit (1L) of nutrient and the mean densities of *Chlorella* spp. and *B. calyciflorus* attained per litre were estimated.

CHAPTER FOUR

4.0 RESULTS

Isolation of *Chlorella* and *B. calyciflorus* from the natural environment is complex and time consuming. However, the procedure described in this study was sufficient to isolate *Chlorella* and *B. calyciflorus* up to 95% purity. Results were obtained from experiments conducted to determine the performance of *Chlorella* spp. on three (3) nutrient media prepared from locally available materials (DAP/UREA, Cowdung extract and Soybean extract), compared with one control (Bold's Basal Medium – BBM), and the subsequent performance of the rotifer *Brachionus calyciflorus*, fed on algae grown using the different nutrient media.

4.1 Production rate of *Chlorella* spp. grown on three (3) different culture media

4.1.1 Production trend 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*. outgrows most unicellular algae apart from blue-green algae, which is a good enough demonstration of possible pure *Chlorella* spp. culture with time (number of days).

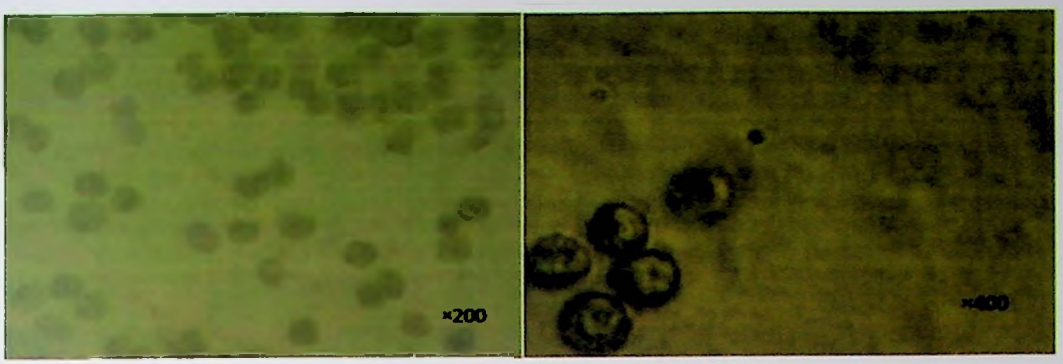


Plate 1: *Chlorella* spp. at x 200 and x 400 as observed under a compound microscope

The growth of *Chlorella* under different culture conditions/media are presented in Fig. 1. The highest density of *Chlorella* (21.53 ± 7.1 × 10^4 cells/ml) was supported by Soybean media.

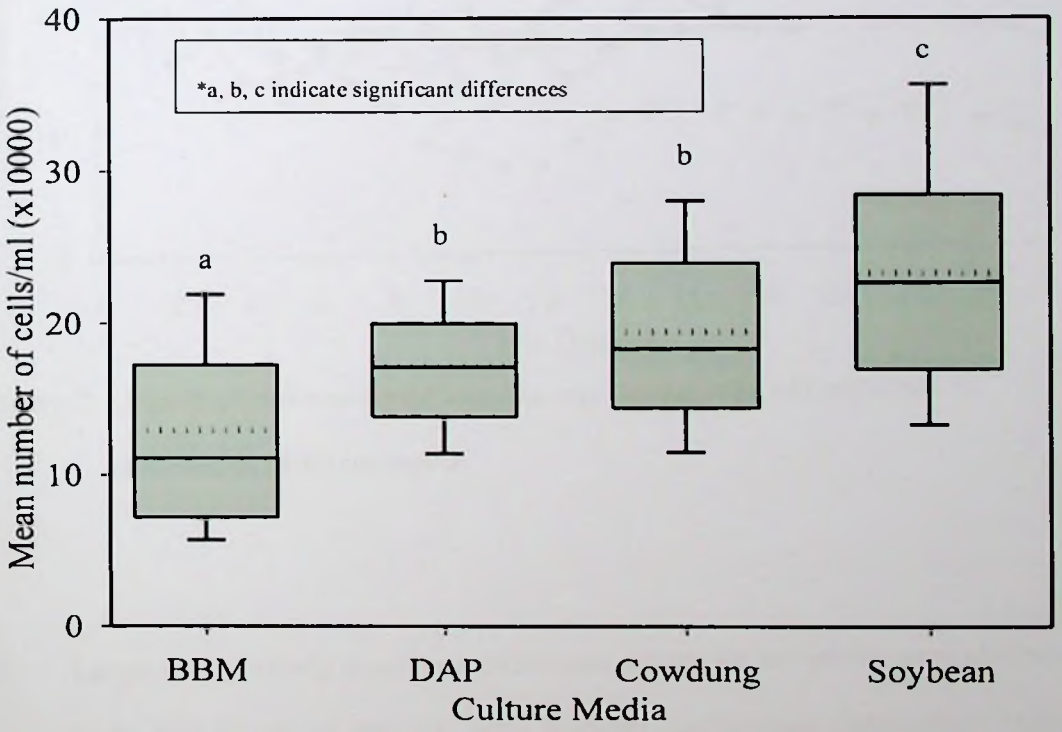


Figure 1: Performance of *Chlorella* spp. on different media

*a, b, c indicate significant differences

To test differences in growth of *Chlorella* in the different media, Kruskal-Wallis non parametric test was performed on non-transformed data. The test revealed

that there was a statistically significant difference in the growth of *Chlorella* in the different nutrient media, (H (3) = 99.024, p=0.000) with mean rank of 198.09 for DAP/UREA, 102.46 for BBM, 257.08 for Soybean and 212.36 for Cowdung media.

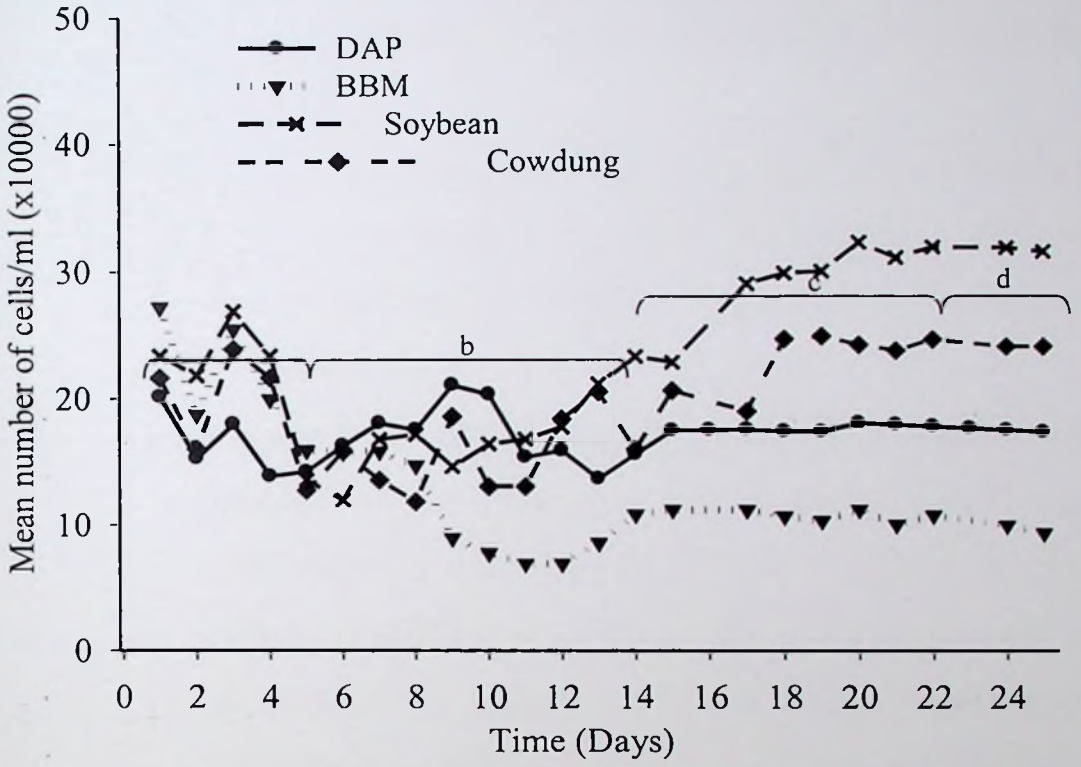


Figure 2: Growth performance of *Chlorella* spp. (mean cells/ml) with time as observed in different media.

Larger and relatively consistent differences among all the media were observed from day 17 up to day 24, with Soybean maintaining consistently higher densities, followed by Cowdung, DAP/UREA and BBM in that order. Soybean and Cowdung were significantly higher than BBM while there were no significant differences between DAP/UREA and BBM between day 17 and day

Growth of *Chlorella* spp. was observed to go through four phases of cell production (Figure 2) including:

a) Section (a) an initial lag phase characterized by a notable decrease in cells per volume from the day of inoculation up to the 5th day, for all the media.

b) Section (b) is characterized by a slow stepwise increase in cell production up to the 14th day. However, BBM appears to reach its maximum in this phase, where it also has the highest production (21 × 10^4 cells ml^{-1}).

c) Section (c) represents the most robust growth of *Chlorella* spp. for a period of 7-8 days. In this phase, Soybean performs better than the rest attaining a maximum of 30.5×10^4 cells ml^{-1}.*Chlorella* spp. appears to continue production (increase numbers cells ml^{-1}) in Soybean medium although the rate of multiplication is notably slowed down, unlike in Cowdung, DAP/UREA and BBM, where it shows a slight but consistent decline.

d) Section (d) is marked by a clear slow-down but stable rate of production of *Chlorella* for all the media with a slight decrease in production for Cowdung, DAP/UREA and BBM, between the 21st and 24th day following inoculation.

4.1.2 Effect of pH and ammonia on the growth of *Chlorella* spp.

The null hypothesis (Ho: $\beta_1 = \beta_2 = 0$, H_a: at least $\beta_1 \neq 0$) was tested at the significance level $\alpha = 0.05$. Transformed data of *Chlorella* biomass

(LogBiomass) was tested against transformed data for ammonia (logAmm) and pH (LogpH). Considering that ANOVA ($F = 12.580$, $p < 0.011$) reveals statistically significant effect of ammonia and pH, on the growth of *Chlorella* biomass, a conclusion to reject the null hypothesis was then arrived at, since p-value $< 0.011 \leq 0.05$, at $\alpha = 0.05$ level of significance. There exists strong enough evidence that at least one of either ammonia or pH is a useful predictor of biomass (cells/ml). Change in *Chlorella* biomass (cells/ml) was predicted, and it was found that ammonia ($\beta = 0.728$ $p = 0.021$), pH ($\beta = 1.094$ $p = 0.004$) were significant predictors ($p < 0.05$) of *Chlorella* biomass. The overall model fit was $R^2 = 0.834$ which implies that about 83.4% of variation in biomass is adequately explained by pH and ammonia by the equation: $y = -20887 + 0.112_{x1} + 9.621_{x2}$.

Table 2: Showing means of *Chlorella* biomass (cells/ml), ammonia concentration (mg/l) and pH

Culture Unit	Mean Biomass	Mean ammonia mg/l	Mean pH
DAPTank1	165245.83	190.21	6.48
DAPTank2	167416.67	180.24	6.63
BBMtank1	101512.50	4.22	6.60
BBMTank2	131125.00	5.84	6.64
SoyTank1	207787.50	7.43	7.00
SoyTank2	215125.00	5.88	6.98
CowdungTank1	177862.50	6.25	6.75
CowdungTank2	181375.00	6.60	6.87

4.2 Growth of *Brachionus calyciflorus* fed on *Chlorella* spp. grown on different culture media

The effect of algal nutrient media, on the growth performance of *B. calyciflorus* was tested using one way Analysis of variance (ANOVA). Figure.3 and Table 5 below show the growth of *B. calyciflorus* fed on algae from different nutrient media.

There were significant differences in abundance of *B. calyciflorus* on *Chlorella* spp. under different media (ANOVA, $F = 21.09$, $P < 0.05$). Growth performance of *B. calyciflorus* fed on *Chlorella* grown on BBM was significantly lower (92.38±83.5 individuals/ml) than the population densities recorded on the other media: 224.00±141.0 individuals/ml on Soybean, 208.50±124.7 on Cowdung and 194.38±106.7 on DAP/UREA (ANOVA, $P< 0.05$).

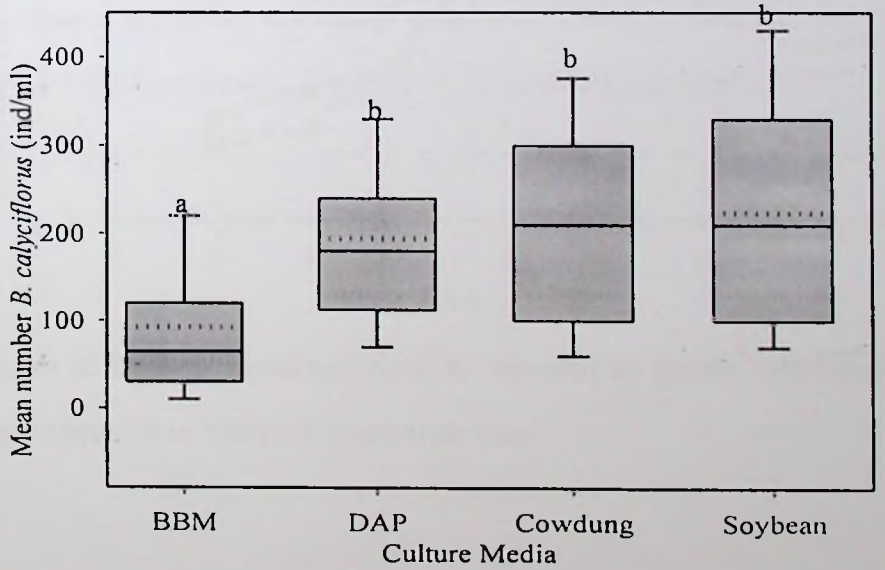


Figure 3: Performance of *B. calyciflorus* on different media

When *B. calyciflorus* was fed on *Chlorella* spp. grown using the test nutrient media, differences were observed in the growth patterns and number of

individual of *B. calyciflorus* (ind.ml^{-1}) (Figure. 4). Generally, three distinct phases of growth, denoted A, B and C in Figure. 4, were observed for each nutrient medium.

A – The first two days are characterized by a slight increment in number of *B. calyciflorus* (ind.ml^{-1})

B – This phase runs from day two today five, and is characterized by rapid increment in number of *B. calyciflorus* (ind.ml-1) except for BBM which drops off on day three.

C – The third and final phase of growth is characterized by declining numbers.

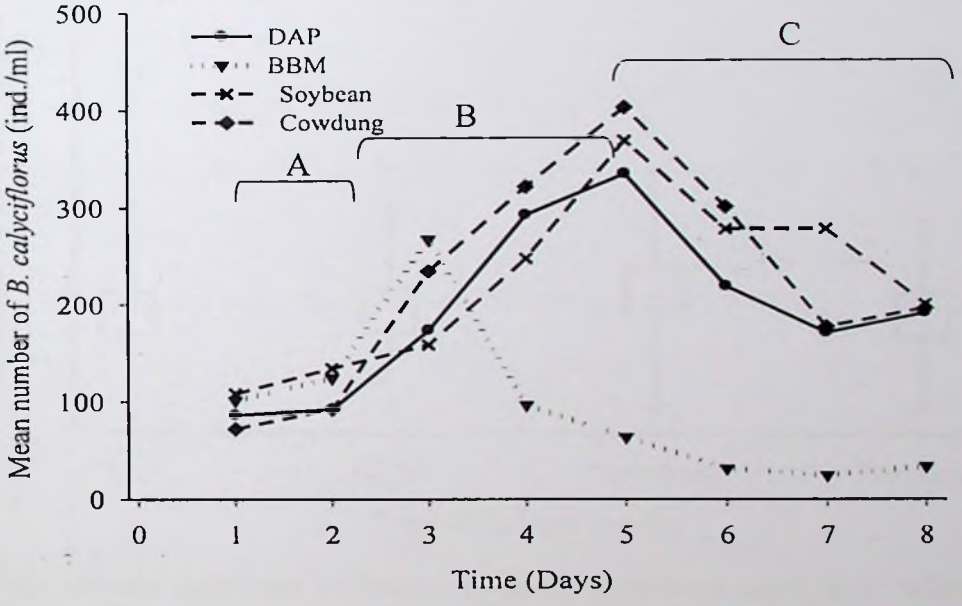
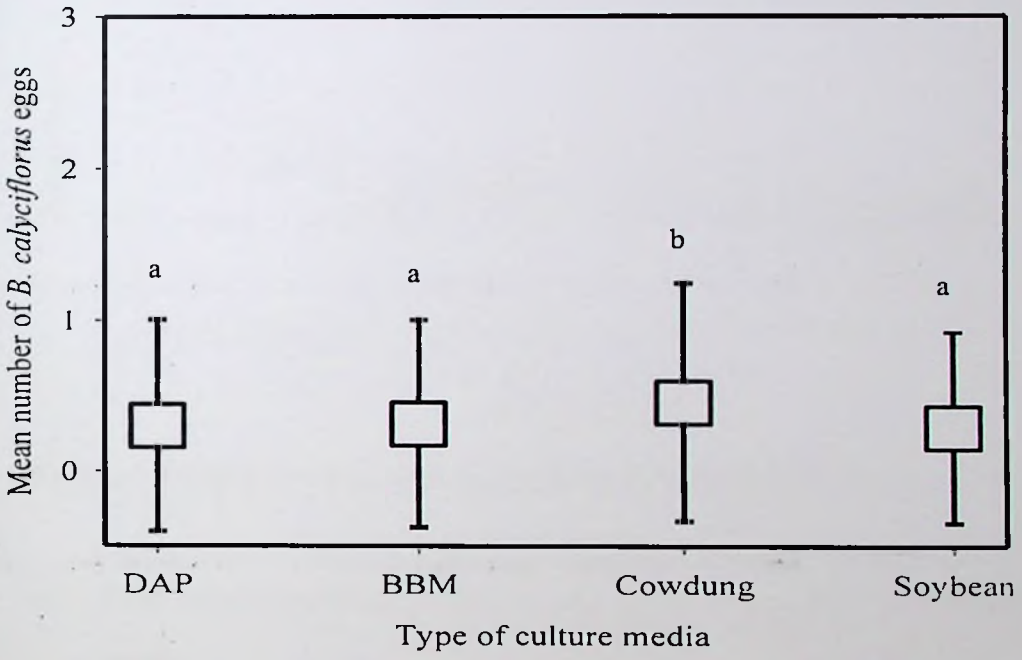


Figure 4: Growth performance of B. calyciflorus (mean rotifers/ml) with time, observed in different media with time

4.3 Changes in rotifer eggs carried on individual rotifers in respect to algal nutrient media and time

Changes in mean number of eggs carried on individual rotifers were investigated in all the media and number of eggs/rotifer in Cowdung (0.46±0.8) was significantly higher (ANOVA, $F = 19.15$, $P < 0.05$) than all media, while no significant difference was observed between Soybean (0.29±0.6), BBM (0.32±0.7) and DAP/UREA (0.30±0.7) (Figure 5).



*a,b indicate significant differences in rotifer growth on algae from different nutrients

Figure 5: Mean number of *B. calyciflorus* eggs carried on individual rotifers in the different media

The mean abundance of eggs on individual rotifers generally decreased gradually with time for all media (Figure 6). Nonlinear regression analysis revealed that with time, the number of days have a significant effect on the number of eggs on

rotifers in DAP/UREA (r^2 = 0.054, P < 0.05), and Cowdung, (r^2 =0.014, P < 0.05), while having no significant effect on BBM (r^2 =0.005, P = 0.07) and Soybean (r^2 =0.002, P = 0.07).

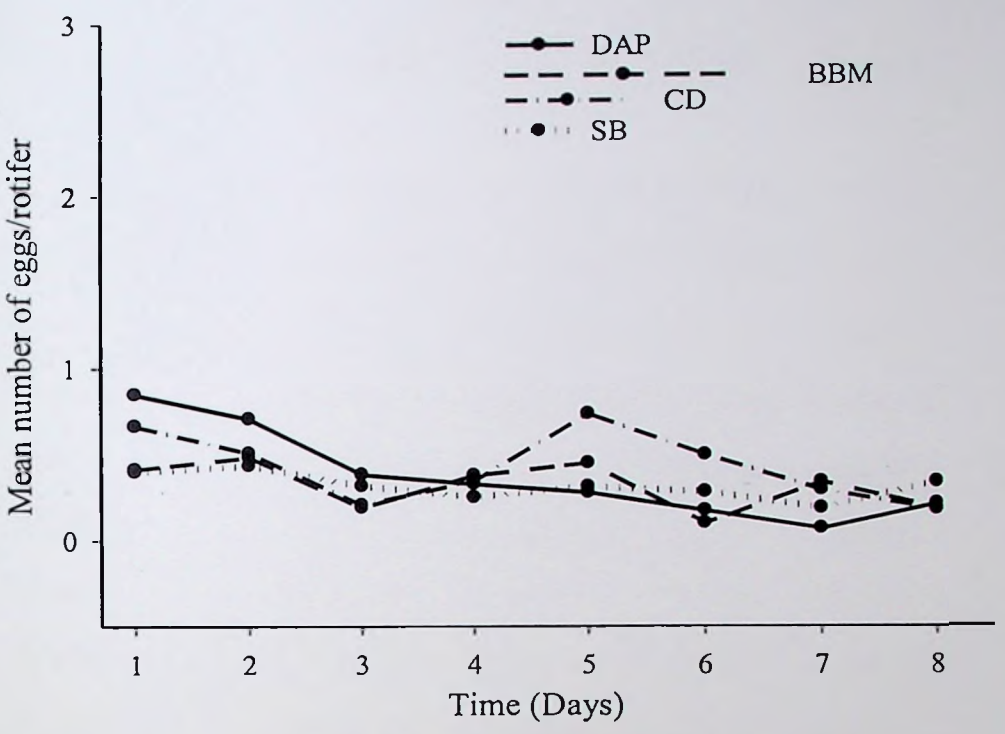


Figure 6: Change in mean abundance of eggs on rotifers with time

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

Table 5 shows variations of fatty acids in the *Chlorella* samples taken from cultures using the different media. PUFAs and MUFAs were significantly higher in DAP/UREA than BBM, Cowdung and Soybean although there was 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 7).

Table 3: Fatty acid composition (expressed as percent of total fatty acids) in Chlorella spp. grown on different nutrient media

Fatty acids Ester	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±0.083	0.75±0.14	0.00±0	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**

4.4.1 Polyunsaturated fatty acids (PUFAs)

Polyunsaturated fatty acids (PUFAs) were the most dominant fatty acids in all the *Chlorella* samples analysed (Table 5). 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).

4.4.2 Monounsaturated fatty acids (MUFAs)

Monounsaturated fatty acids (MUFAs) were generally low in all media (less than 10%) except in DAP/UREA (Figure 8). The most abundant MUFAs were 17:1n9 (12.41±0.32) and8:1n9 (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 7)

4.4.3 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

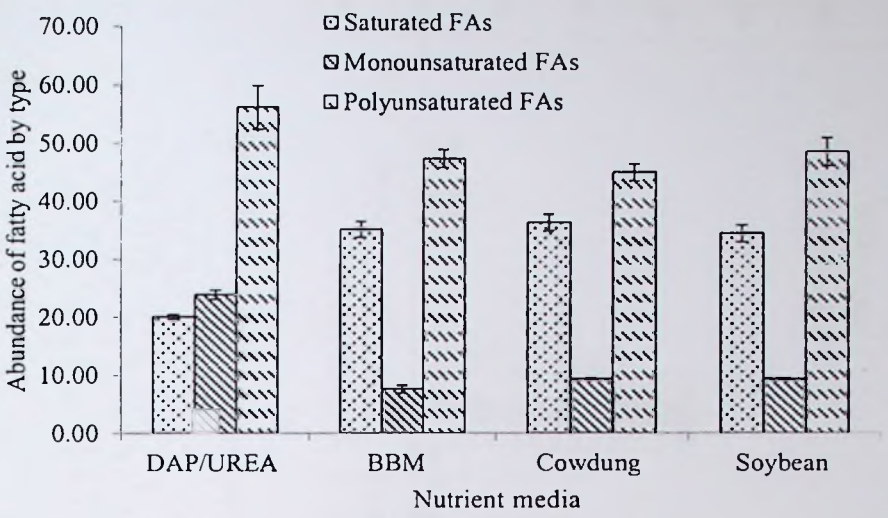


Figure7: Percentage Fatty acid saturation in relation to algal nutrient media

In general, *Chlorella* spp. is rich in unsaturated fatty acids (Figure 8). In these samples, for all nutrient media, unsaturated fatty acids accounted for more than 50% of the total fatty acids. Whereas a few fatty acids could not be identified, all the major fatty acids (by percentage abundance Table 2) present were accounted for.

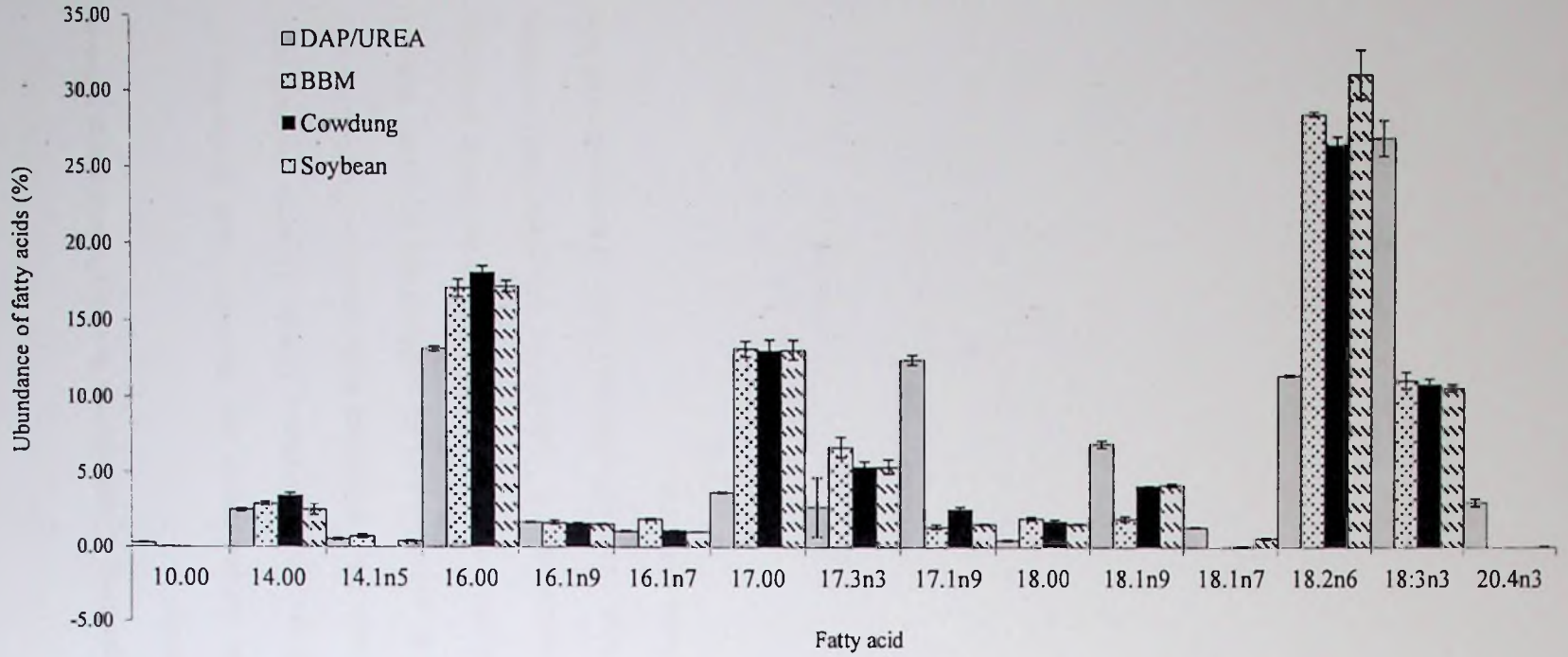


Figure 8: Abundance (mean per cent) of Fatty acids in *Chlorella* spp. grown on different nutrient media

4.5 Cost effectiveness of growing *B. calyciflorus* fed on *Chlorella* spp. grown on different on culture media

The cost of preparing the various media and the subsequent production of *Chlorella* spp. (Table 4) and *B. calyciflorus* (Table 5) were worked out. Cowdung media cost the least, while producing high densities of both *Chlorella* (1,295,333cells/Ush) and *B. calyciflorus* (1,493rotifers/Ush) for every Uganda Shilling spent. The cost of BBM was the highest and it had the lowest minimum returns: *Chlorella* (4,041cells/Ush), *B. calyciflorus* (3rotifers/Ush).Cowdung, DAP/UREA have a very high return cells/shilling (Table 3) and rotifers/shilling (table 4) compared to Soybean and BBM.

Table 4: Cost of producing 1L of nutrient media and subsequent densities of *Chlorella* spp. attained

Media	Minimum Cost/Litre of media (UGX)	Mean *Chlorella* spp. density (±) SD (Individuals/L) ($\times 10^7$) attained	Day Max. *Chlorella* attained	Number of *Chlorella* spp. cells/USh spent on 1L of nutrient
DAP/ UREA	2000	17.12±4.26	9	856000
BBM	320500	12.95±6.77	2	4041
Soybean	66000	23.28±7.962	20	35273
Cowdung	1500	19.43±19.43	18	1295333

Table 5: Cost of producing 1L of nutrient media and subsequent densities of *B. calyciflorus* attained

Media	Minimum Cost/Litre of media (UGX)	Mean *B. calyciflorus* density (±) SD (Cells/L)($\times 10^4$) attained	Day max. *B. calyciflorus* attained	Number of *B. calyciflorus* ind./USh spent on 1L of nutrient
DAP/ UREA	2000	19.44±10.67	5	972
BBM	320500	9.24±8.35	3	3
Soybean	66000	20.85±12.47	5	32
Cowdung	1500	22.40±14.41	5	1493

CHAPTER FIVE

5.0 DISCUSSION

5.1 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. Hecky and Kilham, (1988) and Cloern, (1999), reported that phytoplankton growth is limited by nutrients and other factors such as light and temperature. In this experiment, whereas light and temperature were maintained constant (1000Lux; 25°C), 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 the observations of Dharil *et* al., (1998) who reported continuous positive growth from day one till a maximum growth (day 12; 11.8 × 10^6cell.ml^{-1}, on poultry manure) was attained after which the densities declined.

Soybean extract promoted the highest density in this experiment (39.3 × 10^4cell.ml^{-1}) on by day 23 of period of culture. This value was lower than that which was obtained by Mostary *et* al., (2007) using Pulse bran (*Vigna mungu*) nutrient extract (4.49 × 10^6cell.ml^{-1}). The variation in *Chlorella* densities that were attained by the different media in this experiment were probably a direct function of nutrient availability (especially N, P, and K) in each specific medium. Based on Tiaganides, 1978 in Dharil *et* al., 1998 nutrient limitation of specific nutrients N, P and K strongly control growth of algae in culture limiting nutrients . More specifically, Ashraf *et* al., (2011) reported phosphorus (P) as a major

limiting factor in the growth of *Chlorella*. while Li, *et* al., (2008) showed nitrogen (N) sources below a certain critical value of nitrates to be limiting to cell growth of the green alga *Neochlorisoleo abundans.* From these experiments therefore, it can be concluded 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 progressive concentration increase of *Chlorella* in the soybean medium could be associated with the slower rate of depletion of nutrients. In this study, it was not possible to quantify the rate of nutrient release with time, and the possible subsequent impact on the growth of *Chlorella.* But, Ayuso *et* al., (1996), found that organic matter releases nutrients slowly over a longer period of time, as opposed to rapid release and depletion of nutrient in inorganic fertilizers such as in DAP/UREA and BBM media. The fact that, *Chlorella* performed better in the test nutrient media (soybean and Cowdung) than in the standard inorganic medium (BBM) is desirable for practical application on large scale localized culture of algae for advancement of aquaculture.

5.2 Ammonia and pH changes along the *Chlorella* culture cycle

5.2.1 Changes in pH

There was a significant effect of pH on the growth of *Chlorella* cells only in all media. . Hansen (2002) indicates that slight pH increment in algal cultures is as a result of uptake of inorganic carbon by phytoplankton during photosynthesis. However, Azov and Goldman (1981) concluded 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.

5.2.2 Changes in ammonia

The effect of ammonia on growth of *Chlorella* was found 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(Azov and Goldman, 1981) and has potential to inhibit photosynthesis at high pH levels (Abeliovich and Azov, 1976; Azov and Goldman, 1981). Tam and Wong (1996) reported that 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}). In this study maximum ammonia (DAP/UREA; 236.095 mg l^{-1}) was well within limits and yet it had a notably significant impact on the growth rate of the *Chlorella* 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 (Azov and Goldman, 1981).

5.3 Performance of *B. calyciflorus* in the four nutrient media

It is evident that *B. calyciflorus* responds differently when fed on *Chlorella* spp. grown using different nutrient media as demonstrated by the growth rates, (ind.ml^{-1}). In general, these results agree with earlier studies by Dahril, *et* al., (1998) on the same species which revealed variation in growth rates of *B. calyciflorus* when fed *Chlorella* spp. grown on different animal manures. An investigation on the effect of food quality on the life history of *B. calyciflorus* (Jensen and Verschoor, 2004) reported a reduction in somatic growth and reproduction in *B. calyciflorus* fed on Nitrogen limited (N-Limited) and Phosphorus limited (P-Limited) algae, although rotifer lifespan was not affected. In this study, the biochemical analyses of the test nutrient media were not carried out, but the notable variation in *B. calyciflorus* production rate between BBM

(control) and the test media (DAP/UREA, Cowdung and soybean) strongly suggests that DAP/UREA, Cowdung and soybean media may contain superior quantities of N and P, compared to those in BBM whose effect on the algae is reflected in the growth of rotifers.

5.4 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 (MacCarthy and Patterson, 1974). 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 by Mutlu *et* al., (2011) demonstrated that total fatty acids were highest at high N (35.6% for 100% N) media. Furthermore, Li, *et* al, (2008) in his study on the effects of nitrogen sources on lipid accumulation in *Neochloris oleoabundans* concluded that high lipid accumulation in algal cells were easier obtainable with N-rich nutrient media.

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) (Khozin-Goldberg et *al.* 2002) and subsequent synthesis of docosahexaenoic (DHA) (Pereira et *al.*, 2004). 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* (Namulawa *et al.*, 2010), suggesting that they might have associated functions with egg development.

5.5 Cost effectiveness of nutrient media

Cost-effectiveness analysis involved comparing the cost of preparing a single unit (L) of a particular nutrient medium in monetary units (Uganda shillings) with its efficacy measured in algal density (cells.mL^{-1}) and rotifer density (ind.mL^{-1}) attained using the specific nutrient medium. BBM nutrient medium was most expensive to constitute on a small scale since the minimum quantity packaged units make it difficult to purchase smaller quantities. As a result, the price of constituting 1L remains high and translates into very low return on investment. BBM is therefore more suitable for very large scale operations, where it enjoys better economies of scale compared to the rest. Cowdung, DAP/UREA and Soybean media however, are easily available in the communities and provide a suitable alternative to BBM. In addition, Cowdung and Soybean media can sustain cultures for a much longer time as compared to the inorganic ones.

CHAPTER SIX

6.1 Conclusions

1. Different nutrient media facilitate different growth rates of *Chlorella*. 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. However, in order to stimulate rapid growth in the initial stages of the culture, it may yield faster growths to blend Cowdung or Soybean medium with an inorganic fertilizer like DAP/UREA.

2. A healthy balance between level of ammonia and changes in pH in algal culture using Cowdung and Soybean facilitates healthy *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. Noteworthy however, is the fact that *Chlorella* under high ammonia should never be introduced directly to the Rotifer culture as this would cause a sudden crush of the culture. In this study, it was found appropriate to concentrate clean algae by removing all the culture water before feeding the rotifers.

3. Differences in fatty acid composition of algae exist depending on the type of nutrient used. *Chlorella* has high levels of unsaturated Fatty acids (in this case >60% of Total Fatty acids) which makes it a very desirable food for rotifers, and subsequently, fish that require Highly Unsaturated Fatty Acids (HUFAs) in their early stages of development.

4. *Chlorella* spp. grown on Soybean Cowdung and DAP/UREA facilitate higher rotifers densities in culture than BBM with Cowdung maintaining high production rates evidenced by the higher mean number of eggs carried per rotifer, which are both indicators of culture health, fecundity and sustainability of culture.

5. Cowdung nutrient media was the most cost effective of all the four media, tested, registering the highest densities of both *Chlorella* spp. and *B. calyciflorus* per unit cost (1 shilling).

6.2 Recommendations

- The fact that the test media in this study yielded better results than the standard BBM, is an indication that the latter can be replaced by these nutrient media for improved live larval food production at a cheaper cost. Besides, the suggested nutrient media are locally available, easily obtainable and handy to a local farmer. Nevertheless, several detailed investigations are needed to strengthen this recommendation, including these that follow.
- Analyses of the specific elements of the nutrient media used in this study were not possible within the limits of this study. It is my recommendation that further investigations be carried out to determine the specific nutrients present in the various nutrient media.

- There is need to investigate the effect of combining DAP/UREA with 1) Cowdung, and 2) Soybean media on the growth performance of *Chlorella*, and *B. calyciflorus*, and the resultant fatty acid concentrations in *Chlorella* spp. and *B. calyciflorus*.

- Further studies need to be conducted to ascertain what specific types and quantities of fatty acids transferred to rotifers from the DAP/UREA, BBM, Cowdung and Soybean cultures.

- Rotifers (*B. calyciflorus*) fed *Chlorella* spp. grown on these nutrients need to be tested on larval fish so as to ascertain whether the effect on algae is carried over and reflected in the life cycle and development of larvae.

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Appendix 1:Soybean nutrient extract: Improvised bioreactor for controlled Soybean fermentation, with gas expulsion hose-pipe fitting:

Appendix 2: Cost of constituting various nutrient media

Cowdung	**Amount purchased g /ml**	**Cost price**
Cowdung	1000	1500

DAP/UREA	**Amount purchased g / ml**	**Cost price**
DAP	1000	1200
UREA	1000	1200

Soybean	**Amount purchased g / ml**	**Cost price**
Soybean	1000	3600
UREA	1000	1200
Sulphuric acid	100	20000
Plastic bucket	1	22000
Hose pipe vent	3	9000
Polythene Bag (HD)	3	6000
Rubber tape	3	3000
Lime	1000	1200

BBM item	**Amount purchased g / ml**	**Cost price**
Potassium di-hydrogen phosphate (KH_2PO_4)	250	25000
Calcium chloride ($CaCl_2.2H_2O$)	250	20000
Magnesium Sulphate ($MgSO_4.7H_2O$)	250	20000

Sodium Nitrate ($NaNO_3$)	250	15000
Di-Potassium hydrogen Phosphate (K_2HPO_4)	250	25000
Sodium chloride (NaCl)	250	500
Sodium EDTA (Na_2EDTA)	250	50000
Potassium hydroxide (KOH)	250	15000
Concentrated Sulphuric Acid (H_2SO_4)	100	20000
*Trace Metal Solution:		
Boric Acid (H_3BO_3)	250	25000
Manganese II chloride ($MnCl_2.4H_2O$)	250	20000
Zinc II Sulphate ($ZnSO_4.7H_2O$)	250	20000
Sodium Molybidnate ($Na\ MoO_4.5H_2O$)	250	20000
Copper II Sulphate $CuSO_4.5H_2O$	250	20000
Cobalt Nitrate $Co(NO3)_2.6H_2O$	250	25000