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Supplementing wastewater with NPK fertilizer as a cheap source of nutrients in cultivating live food (*Chlorella vulgaris*)

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Abstract

Introduction: The decline in fishery resources from the wild has led to an ever increasing focus on aquaculture in recent years. With increasing aquaculture of animal species, there is an increasing need for suitable microalgae in the production of these animals. However, cultivation of microalgae in expensive pure chemical media is one of the major challenges facing large-scale cultivation of microalgae.

Purpose: The present study investigated the suitability of aquaculture wastewater (AWW) supplemented with NPK (nitrogen:phosphorus:potassium) fertilizer as a cheap source of nutrient to cultivate a microalga *Chlorella vulgaris* (*C. vulgaris*).

Methods: *C. vulgaris* with an initial cell density of 0.8×10^6 cells/mL was batch cultured in AWW supplemented with NPK at 0.1, 0.5, 1.0 g/L and BBM for 20 days under laboratory conditions using 2000 mL Erlenmeyer flasks. The proximate composition, chlorophyll, minerals, and vitamins analysis of *C. vulgaris* biomass were done using standard analytical methods.

Results: The highest values in optical density (4.872 ± 0.025), dry cell weight (2.858 ± 0.015 g/L), specific growth rate (0.2097 ± 0.0038 day^{-1}), and biomass productivity (0.1701 ± 0.0007 g/L/day) were obtained in *C. vulgaris* grown in AWW + 1.0 NPK medium. The total chlorophyll, protein, lipid, and carbohydrate content of the microalgae biomass were in the range of 0.05–0.862%, 44.062–57.089%, 17.064–23.260%, and 15.217–21.896%, respectively. Furthermore, microalgae grown in AWW + 1.0 NPK showed good vitamin and mineral content compared to BBM grown alga.

Conclusion: These findings indicated that the AWW + 0.1 NPK, AWW + 0.5 NPK, and AWW + 1.0 NPK are potential growth media for *C. vulgaris* cultivation and can replace the BBM medium, which is very expensive and less accessible to users.

Keywords: *Chlorella vulgaris*, Microalgae, Aquaculture wastewater, NPK

Introduction

With dwindling wild fishery catches since 2000, there is an ever increasing global effort in increasing aquaculture output (Ahmed et al. 2019), to ensure food security for all and keep up with the increasing per capita fish consumption. Since then, world aquaculture output (in tons) has more than doubled (32.4×10^6 in 2000 to 82.1×10^6 in 2018), with the output anticipated to increase by 60–100% over the next 20–30 years (FAO 2020). During the same period, Africa recorded over 5-fold increase in output from 399.9×10^3 to 2195×10^3 (FAO 2020). With increasing aquaculture of animal species, there is an increasing need for suitable microalgae in the production of these animals. Microalgae play a vital role in supplying energy, essential compounds, such as protein, lipid, carotene,

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vitamins, amino acids, polyunsaturated fatty acids, and essential minerals required for high-quality aquaculture production (Singh et al. 2014). Further on, microalgae in aquaculture are used as live food for rearing larvae and juveniles of many species of commercially important molluscs, crustaceans, fish species, and for zooplankton used in aquaculture food chains. Spolaore et al. (2006) demonstrated that the use of microalgae by fish larvae enhances their reproduction, giving the animal's better immune system and health.

The microalga (*C. vulgaris*) is one such example widely used in aquaculture as a promising source of protein, carbohydrates, essential fatty acids, dietary fibers, and minerals (Ahmad et al. 2020). Apart from aquaculture, *C. vulgaris* has become a good candidate for biofuel production due to its rapid growth rate and high lipid content (Koller et al. 2014). It is also used as a healthy food, and nutrition supplements for animals and human consumption, because of the quality of proteins that they produce (Ramaraj et al. 2015). Besides the high levels of protein (51–58%), carbohydrates (12–17%), lipids (14–22%), and nucleic acids (4–5%), *C. vulgaris* contains appreciable amounts of valuable vitamins and minerals (Kim et al. 2010; Mata et al. 2010). This microalga can also accumulate pigments such as chlorophyll *a* and *b*, β-carotene, and xanthophylls which are used for enhancement of the pigmentation in fish and as a colorant for foods, drugs, and cosmetics (Guedes et al. 2011).

Despite of the several benefits from this microalga, its commercial scale production is still not cost-effective if algae are required in large amount (Di Caprio et al. 2015; Nayak et al. 2016b). Xia and Murphy (2016) reported that in large-scale production, nutrient requirements can be up to 50% of the total cost of biomass production. Its high production costs have triggered efforts to find cheaper and economically feasible approach for its culture. Studies by Nasir et al. (2015), Luo et al. (2016), and Hemalatha and Venkata Mohan (2016) have reported that the microalgae can be grown using aquaculture wastewater instead of the expensive synthetic medium often used as it contains all the necessary nutrients required for algal growth. Nevertheless, the composition of aquaculture wastewater depends on the nature and quantity of feed, the species being reared, stocking density, water replacement frequency, and the type of system in operation. The low or imbalanced nutrient concentration in wastewater may be a major challenge in cultivating microalgae using wastewater which often leads to low biomass yield and poor quality (Cai et al. 2013; Lu et al. 2015b). For example, De Lourdes et al. (2017) cultivated *C. vulgaris* by using real wastewater from a wastewater treatment plant and only recorded a maximum dry weight of 0.267 ± 0.031 g/L. Due to the low or inconsistency of nutrients compositions in aquaculture wastewater, some studies recommended the supplementation of nutrients as an alternative method to overcome the nutrient limitations in wastewater (Cai et al. 2013; Lu et al. 2015a).

According to the work of Ammar (2016) and Mahmood and Mohsin (2017), different agricultural fertilizers were used as a nutrient source as potential alternatives to reduce microalgal production costs. In that context, commercial fertilizers can be used as a nutrient source for cultivation and economically viable production of microalgae. Previous studies have mainly been focused on the culture of microalgae by using aquaculture wastewater (Ramanna et al. 2014; Caporgno et al. 2015; Hawrot-Paw et al. 2020). In the current study, commercial fertilizer NPK (20:20:10) purchased from the local market at a very low price of 1 US $ for 1 kg was added in aquaculture wastewater (AWW) to determine if these additions could enhance the biomass production of *C. vulgaris* under laboratory condition. The present study therefore aimed to develop inexpensive culture medium by using aquaculture wastewater and NPK fertilizer for mass production of *C. vulgaris* whose biomass composition was evaluated for its potential use as a feed ingredient in aquaculture.

Materials and methods

Isolation of microalgae

Chlorella vulgaris was isolated from freshwater fish ponds with pH of 8.5 at Fisheries Education and Training Agency (FETA) in Mwanza, Tanzania, using a standard plating method. Twenty grams of agar was mixed with 1 l of autoclaved Bold's Basal Medium (BBM) in a conical flask and boiled for 20 min. After boiling, the agar solution was poured on sterilized glass petri dishes (100 × 15 mm) and allowed to cool for 2 h. Five milliliters of algal sample was transferred into media plate and spread uniformly across the surface; the plates were then placed at a light intensity of 5000 lux under room temperature (27–30 °C). After 15 days, cell colonies were observed to grow on the surface, and the best individual colonies were picked up by using a sterile syringe needle and transferred to the culture tubes containing liquid BBM and placed at the same conditions. After 14 days, when the color change was observed in the culture tube, a sample of 5 mL was taken and checked under the light microscope to see the isolated algal strain. Identification was carried out using a guide provided by (Shubert and Gärtner 2015).

Preparation of microalgal growth media

Aquaculture wastewater (AWW) was collected from African catfish pond at the University of Dar es Salaam, Department of Aquatic Science and Fisheries at Kunduchi, Dar es Salaam. Immediately after collection, the

AWW was sterilized by autoclaving for 15 min at 121 °C and stored in a refrigerator (4 ± 2 °C) for 2 days for sedimentation of any visible solid particles (Zhu et al. 2013). The supernatant was collected and used as the microalgae culture medium. Water quality parameters such as temperature (T), dissolved oxygen (DO), and pH were analyzed at the time of wastewater collection using a multiparameter equipment (multi-3430 WTW, Germany). Ammonium (NH_4^+) and phosphorus (P) were analyzed using indophenol blue and ascorbic acid method respectively, following the procedures described by (Allen 1989), whereas nitrate (NO_3) was analyzed using cadmium reduction method as described by (Emteryd 1989). The calcium (Ca), sodium (Na), magnesium (Mg), iron (Fe), manganese (Mn), zinc (Zn), and copper (Cu) were determined using Atomic Absorption Spectrophotometer (AA240 Varian, USA).

Experimental setup

The microalgae were cultured using AWW in the laboratory. The inoculum of *C. vulgaris* was pre-cultured in 1000 mL conical flasks using BBM at a light intensity of 5000 lux, temperature (28 ± 1 °C) and constantly mixed (150 rpm). The nutrient composition and costs for 1 l of BBM medium is depicted in Table 1. At the exponential growth phase, the microalgae cells were collected and cultured in five different media. The first and second media were BBM (control) and AWW, respectively. Third, fourth, and fifth media were AWW supplemented with NPK fertilizer at concentrations of 0.1, 0.5, and 1.0 g/L, respectively. All media components were sterilized by autoclaving at 121 °C for 15 min. The microalgae were batch cultured in 1000 mL Erlenmeyer flask containing 800 mL of medium and 200 mL of *C. vulgaris* with the initial cell density of *C. vulgaris* of 0.8 × 10^6 cells/mL in all treatments. All experiments were conducted at a controlled environment of temperature (28 ± 1 °C) maintained using air condition, illumination intensity (5000 ± 10 lux) measured using vertex VXLM-636 light meter, photoperiod (16:8 light:dark cycle) adjusted by electricity timer and continuous aeration was provided by aerators. The pH values in all treatments were measured using a pH meter (Fisher Scientific AB 15 Accumet Basic, Singapore) and maintained at the pH of 9–10 by adding 5 M sodium hydroxide (NaOH) or 3 M hydrochloric acid (HCL). The microalgae were cultured for 20 days and all treatments were carried out in triplicate.

Estimation of microalgae culture media cost

The estimation of cultivation media costs was done by considering only the concentration and the price of each reagent used to make 1 l of the medium. Other expenses like taxation, electricity consumption, and transport cost were not considered. The prices of all reagents and commercial fertilizer used were obtained from https://www.alibaba.com and https://www.sigmaaldrich.com. The AWW was obtained for free.

Determination of microalgae growth

Microalgal growth was monitored by measuring dry cell weight, optical density, and photosynthetic pigments (total chlorophyll and carotenoids). The optical density (as an indicator of cell density) was determined daily

Table 1 Nutrient composition and cost estimation for BBM growth medium

Components	Concentration (mg/L)	Price (US $)/L
Di-potassium hydrogen orthophosphate (K_2HPO_4)	18.75	0.001032
Potassium di-hydrogen orthophosphate (KH_2PO_4)	43.75	0.00072
Magnesium sulfate $MgSO_4.7H_2O$	18.75	0.050565
Sodium nitrate ($NaNO_3$)	62.5	0.003125
Calcium chloride ($CaCl_2.2H_2O$)	6.25	0.00089
Sodium chloride (NaCl)	6.25	0.000405
EDTA tetrasodium salt (EDTA-Na_4)	5	0.00112
Potassium hydroxide (KOH)	3.1	0.000453
Ferrous sulfate ($FeSO_4$.7H2O)	0. 498	0.07569
Boric acid (H_3BO_3)	1.142	0.012738
Zinc sulfate ($ZnSO_4.7H_2O$)	0. 0353	0.000021
Manganese chloride ($MnCl_2.4H_2O$)	0. 0058	0.000004
Cupric sulfate ($CuSO_4.5H_2O$)	0.0063	0.000014
Cobaltous nitrate ($Co(NO_3)_2.6H_2O$)	0.002	0.000002
Sodium molybdate ($Na_2MoO_4.2H_2O$)	0.0048	0.000006
Total costs		0.14679

using a UV–Vis spectrophotometer (UV 6305, Genway, UK) at a wavelength of 688 nm. Microalgae dry weight (biomass concentration, g/L) was determined every 2 days, where a sample of 10 mL was taken from each treatment and filtered using a pre-weighed glass fiber filter (Whatman GF/F) and oven dried at 105 °C for 24 h. The biomass (dry weight) was weighed by electronic analytical balance XPE 105 (Mettler-Toledo, Switzerland). The specific growth rate, SGR (μ, day^{-1}) of algal culture is a measure of the increase in biomass over time and it was calculated according to (Liang et al. 2013)

$$SGR = \ln(W_2 - W_1)/(T_2 - T_1) \quad (1)$$

Where W_2 and W_1 are the biomass concentrations (g/L) at T_2 and T_1 respectively.

The biomass productivity, P_B (g/L/day) was calculated by the method of (Liang et al. 2013)

$$PB = (B_1 - B_0)/(T_1 - T_0) \quad (2)$$

Where B_0 and B_1 are the mean dry biomass concentration at the times T_0 and T_1, respectively.

The determination of total chlorophyll content in *C. vulgaris* cells was done using a spectrophotometric technique, as described by (Quarmby and Allen 1989). The chlorophyll content within the algal samples was extracted by dissolving well grinded 1 g of *C. vulgaris* biomass into 50 mL aqueous acetone (85% v/v) and stored at 4 °C for 24 h. Twenty-five milliliters aliquot of the extract was added to 50 mL of diethyl ether in a separating funnel and mixed well. The ether layer was washed with distilled water until all the chlorophyll passed into the ether layer. The water layer was then decanted, and the ether phase was transferred into a volumetric flask and anhydrous sodium sulfate (Na_2SO_4) was added for drying out the water. The absorbance of ether containing chlorophyll was measured at 660 and 643 nm using the UV–Vis spectrophotometer (Shimadzu) and the total chlorophyll content of the *C. vulgaris* was calculated using Eq. 3.

$$\text{Total chlorophyll } (\%) = \frac{\text{C (mg/L)} \times \text{ ether solution (mL)} \times \text{acetone extraction (mL)}}{10^4 \times \text{acetone aliquot (mL)} \times \text{sample weight (g)}} \quad (3)$$

Where C = chlorophylls in ether solution = 7.12 × OD660 + 16.8 × OD643: whereby OD = optical density

Biochemical composition analysis

Due to sample size requirements, the biomass composition analysis was done only for the growth media which showed good results in growth parameters analyzed. At the end of the experiment, the *C. vulgaris* biomass was harvested by centrifugation at 978.02×*g* for 10 min. The collected biomass was then dried at 100 °C to constant weight for protein, lipid, carbohydrate, minerals, and vitamin analyses.

Microalgae lipid extraction

Total lipids from microalgae biomass were extracted based on the procedure described by (Bligh and Dyer 1959). Accurately weighed 5 g of *C. vulgaris* biomass was mixed with chloroform, methanol and water in the proportion of 1:1:0.8 respectively and homogenized for 2 min under oxygen-free nitrogen (OFN) with cooling. The chloroform and water were then added to give a final solvent ratio of chloroform:methanol:water solvent ratio of 2:2:1.8. The mixture was filtered to remove biomass residues and transferred to a graduated cylinder where the volume of the chloroform layer was recorded. The solvent was separated into two layers (chloroform and aqueous methanol layers) using a separating funnel. An aliquot of the lower chloroform layer was pipetted and weighed using a pre-weighed, clean, and dry evaporation dish. The solvent was then oven dried at 40 °C for 30 min for lipid recovery and the remaining lipids were cooled in a desiccator and weighed. The percentage of lipid in *C. vulgaris* biomass was calculated using the equation below:

$$\text{Total lipids } (\%) = \frac{\text{residue weight (g)} \times \text{volume of chloroform layer (mL)} \times 10^2}{\text{aliquot (mL)} \times \text{sample weight (g)}} \quad (4)$$

Determination of protein contents

The total protein content in the *C. vulgaris* biomass was determined using semi-micro Kjeldahl digestion method (Emteryd 1989; Quarmby and Allen 1989). Here, 4 g of *C. vulgaris* biomass was digested with a strong sulfuric acid in the presence of selenium catalyst to convert nitrogen compounds into ammonium form. The ammonium concentration was then determined by using indophenol-blue colorimetric method and the nitrogen content was calculated using the Eq. (5) below. The percentage crude protein present in algal biomass was calculated by multiplying the nitrogen content by the conventional factor of 6.25.

$$\text{N } (\%) = \frac{{NH_4}^+ - \text{N (mg)} \times \text{solution volume (mL)}}{10^4 \times \text{aliquot (mL)} \times \text{sample weight (g)}} \quad (5)$$

Determination of carbohydrate contents

The total soluble carbohydrate (CHO) was determined in the algal biomass by using Anthrone method as described by (Allen 1989). Five grams of *C. vulgaris* biomass was mixed with 30 mL of distilled water in 100 mL conical flask and boiled at boiling point of water for 2 h.

The sample was allowed to cool at room temperature and filtered through a Whatman filter paper No. 44. An aliquot of a clear sample solution was placed into the test tube and the anthrone reagent was added. The solution was allowed to cool, and its absorbance was measured at 625 nm. The percentage soluble carbohydrate in the *C. vulgaris* biomass was calculated based on the formula below.

$$\text{Soluble carbohydrate }(\%) = \frac{\text{C (mg)} \times \text{extraction volume (mL)}}{10 \times \text{aliquot (mL)} \times \text{sample weight (g)}} \quad (6)$$

Whereby C = mg glucose obtained from the graph

Vitamins composition analysis

Extraction of vitamins from *C. vulgaris* biomass was done by mixing 0.5 g of sample with 100 mL of 95% ethanol in a conical flask. The mixture was vigorously shaken for 15 min to ensure complete extraction. The extract was centrifuged for 10 min and filtered using Whatman No. 1 filter paper. To remove ethanol and obtain clear extracts, the sample was placed in a rotary evaporator (Gmbh & Co.KG, Germany) under reduced pressure at 40 °C. The obtained extract was then kept at 4 °C until analyses. Vitamin A (as beta- carotene) was determined according to the method of (Nagata and Yamashita 1992). One hundred milligrams of dried extract was vigorously stirred with 10 mL of acetone-hexane mixture (4:6) for 1 min and filtered through Whatman No. 4 filter paper. The absorbance of the filtrate was measured at 453, 505, and 663 nm. Content of beta carotene was calculated according to the following equation:

$$\text{Beta – carotene (mg/100 mg)} = 0.216\ A_{663} - 0.304 A_{505} + 0.452\ A_{453} \quad (7)$$

Determination of vitamin B complex present in microalgae extracts was done according to the method of (Rajput et al. 2011). The working standard solution for the standard vitamins and microalgae samples were prepared by dissolving a known weight of the standard vitamin and extracts in a known volume of distilled water into a conical flask. Vitamins B1, B2, B3, B6, B12, and C were determined from riboflavin, nicotinamide, pyridoxine hydrochloride, cyanocabalamin, and ascorbic acid stock solutions, respectively. The absorbance of the working solutions, *C. vulgaris* samples, and blank were read at 430 nm for vitamin B1, 444 nm for vitamin B2, 450 nm for vitamin B3, 650 nm for vitamin B6, 530 nm for vitamin B12, and 450 for vitamin C using a UV-visible spectrophotometer (Jenway 6305).

Mineral composition analysis

To determine the mineral composition of *C. vulgaris* biomass, the biomass was digested using nitric perchloric acid method as described by (Jones 1984). Approximately 0.5 g (dry weight) of *C. vulgaris* biomass from each treatment was weighed into a beaker. To the samples, a mixture of 5 mL of concentrated nitric acid ($HNO_{3)}$ and 1 mL of per-chloric acid ($HClO_4$) in the ratio of 5:1 was added. The solution was heated at 120 C until the disappearance of the brown fumes which indicated the complete digestion of the organic matter. The solution was then cooled and diluted with distilled water up to 100 mL solution. The Ca, Mg, Fe, K, Mn, Na, and Zn concentration of the digested biomass were determined using Atomic Absorption Spectrophotometer (AA240 Varian, USA).

Statistical analysis

The data were presented as mean ± standard error (SE). Statistical analysis was carried out by using R software (Version 3.6.3). Normally and not normally distributed data were tested using one-way analysis of variance (ANOVA) and Kruskal-Wallis respectively. Tukey's (ANOVA) and Dunn (Kruskal-Wallis) post hoc test were used to check the significant difference among the treatment means. A p value of less than 0.05 was considered statistically significant.

Results

Microalgae growth

The physico-chemical composition of the AWW and the growth media cultivation costs are shown in Tables 2 and 3, respectively. The statistical analysis showed significant variation in the optical density (χ^2 = 66.824, df = 4, p = 0.000,

Table 2 The physico-chemical parameters of AWW

Parameter	Unit	Value
pH		7.770 ± 0.033
Temp.	C	27.300 ± 0.409
DO	mg/L	5.000 ± 0.153
NH_4^+	mg/L	0.163 ± 0.002
NO_3^-	mg/L	4.043 ± 0.035
P	mg/L	1.776 ± 0.144
Ca^{2+}	mg/L	164.533 ± 1.618
Zn^{2+}	mg/L	8.333 ± 1.380
Mn^+	mg/L	6.067 ± 0.371
Mg^{2+}	mg/L	130.667 ± 2.324
Cu^{2+}	mg/L	13.200 ± 1.331
Na^+	mg/L	304.667 ± 11.153
K^+	mg/L	85.200 ± 1.792
Fe^{2+}	mg/L	117.667 ± 2.040

Table 3 Microalgae cultivation media estimation cost

Growth medium	Growth medium cost (US$/L)
BBM	0.14679
AWW	0
AWW + 0.1 NPK	0.0001
AWW + 0.5 NPK	0.0005
AWW + 1.0 NPK	0.001

Fig. 1), dry weight (χ^2 = 31.404, df = 4, p = 0.000; Fig. 2), biomass productivity (F = 887.660, df = 4, p = 0.000; Fig. 3), specific growth rate (F = 158.600, df = 4, p = 0.000; Fig. 3) and total chlorophyll content (F = 46.612, df = 4, p = 0.000; Fig. 4) of *C. vulgaris* for different growth media. All the microalgae growth parameters analyzed increased with the increase of NPK concentration in AWW. The optical density and dry weight of *C. vulgaris* cultured in BBM, AWW + 0.1 NPK, AWW + 0.5 NPK, and AWW + 1.0 NPK were significantly higher than that grown in AWW (p = 0.000). The highest values of optical density (4.872 ± 0.025) and dry weight (2.858 ± 0.015 g/L) were recorded in microalgae cultured in AWW + 1.0 NPK, followed by AWW + 0.5 NPK, AWW + 0.1 NPK, BBM, and finally AWW. Additionally, significantly higher amount of biomass productivity (0.170 ± 0.001 g/L/day) and specific growth rate (0.210 ± 0.004 μ, day^{-1}) were found in *C. vulgaris* grown in AWW + 1.0 NPK than those raised in all other media (p = 000). The statistical analysis also showed significantly higher amount of chlorophyll content (0.862 ± 0.087%) in microalgae cultured in AWW + 1.0 NPK than those cultivated in the rest of the media including BBM (p < 0.05).

Biochemical composition

The protein, lipid, and carbohydrate contents of *C. vulgaris* biomass are shown in Fig. 5. The statistical analysis showed that there was a significant effect of the cultivation media on the protein (F = 337.140, df = 3, p = 0.000), lipid (F = 38.674, df = 3, p = 0.000), and carbohydrate (F = 216.830, df = 3, p = 0.000) content of microalgae. Cells grown in AWW + 1.0 NPK medium had significantly higher protein content (57.089 ± 0.186%) than those grown in the other media (p = 0.000). On the other hand, a significantly higher percentages of lipid (23.260 ± 0.484%) and carbohydrate (21.896 ± 0.169%) contents were observed in *C. vulgaris* biomass cultivated in AWW + 0.1 NPK than those grown in the rest of the media (p = 0.000).

Furthermore, the growth media had a significant impact on vitamins A, C, B2, B1, B3, and B6 content of the microalgae biomass tested (p < 0.05; Table 4). *C. vulgaris* cultured in BBM, AWW + 0.5 NPK and AWW + 1.0 NPK had significantly higher vitamin A content than those grown in AWW + 0.1 NPK medium (p < 0.05). Moreover, significantly higher vitamin C, B1, and B2 content of *C. vulgaris* was recorded in AWW + 1.0 NPK when compared with those cultured in other cultivation media (p < 0.05). A significantly higher amount of vitamin B3 content was found in AWW + 0.1 NPK, AWW + 0.5 NPK, and AWW + 1.0 NPK (p < 0.05) than in BBM. The vitamin B6 of microalgae grown in AWW + 1.0 NPK was significantly higher than those cultured in BBM (p = 0.013) but no significant difference was observed with those cultured in AWW + 0.1 NPK and

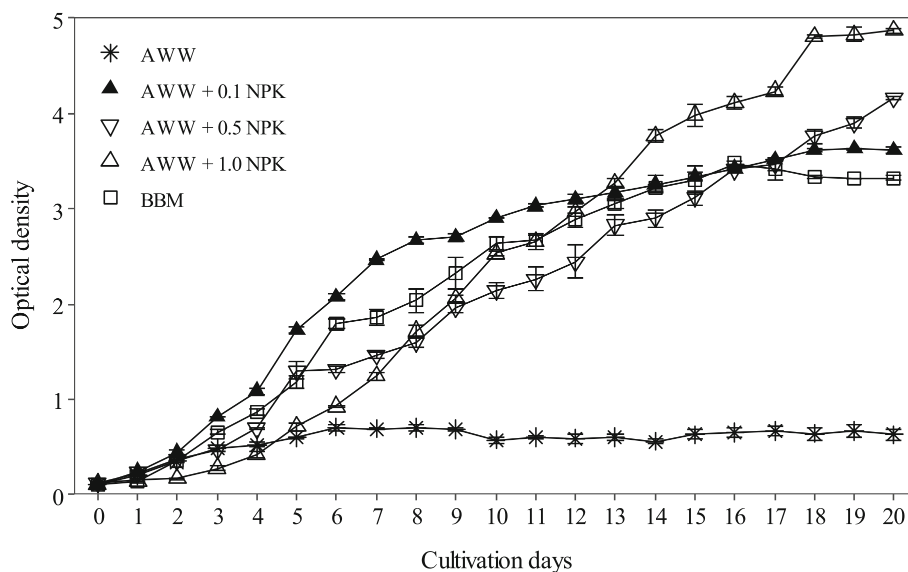

Fig. 1 Optical density of *C. vulgaris* cultured in different cultivation medium

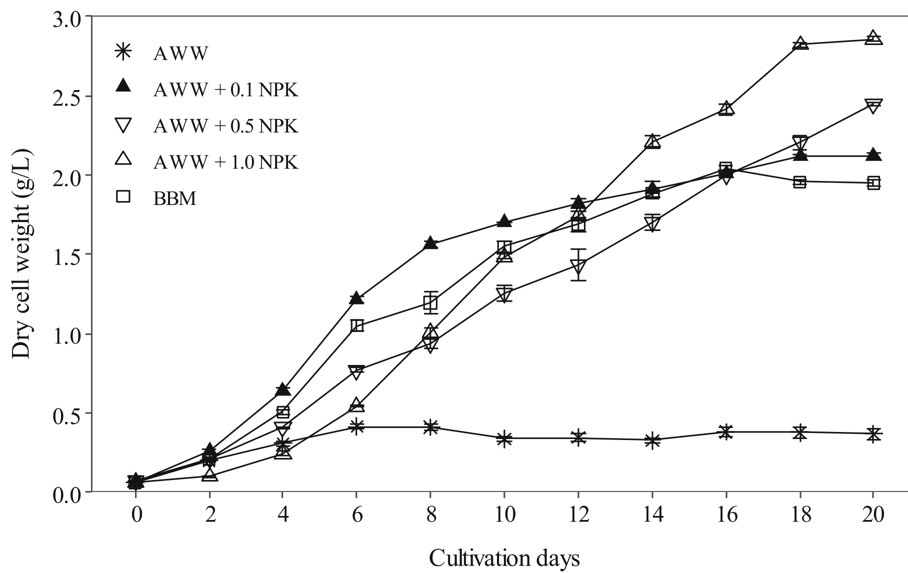

Fig. 2 Dry cell weight of *C. vulgaris* cultured in different cultivation media

AWW + 0.5 NPK ($p > 0.05$). In addition, the cultivation media did not show any significant difference in vitamin B12 content of the microalgae investigated ($p > 0.05$; Table 4).

Mineral components of *C. vulgaris* are given in Table 5. We observed significant variations in the calcium, iron, magnesium, potassium and zinc content of *C. vulgaris* biomass for the different cultivations medium used ($p < 0.05$). Cells grown in BBM medium had a statistically higher amount of calcium, iron and magnesium content than those grown in AWW + 0.1 NPK ($p < 0.05$); however, no significant variation was recorded with the other growth media ($p > 0.05$). Significantly lower amount of sodium content in BBM than in AWW + 0.5 NPK was observed ($p = 0.013$), though no significant variation was found with AWW + 0.1 NPK and AWW + 1.0 NPK ($p > 0.05$). Microalgae raised in AWW + 0.1 NPK had statistically higher potassium content than those cultured in BBM ($p = 0.386$). Also, potassium value for *C. vulgaris* grown in BBM did not show significant difference with those cultivated in AWW + 0.5 NPK and AWW + 1.0 NPK ($p > 0.05$). Zinc content of *C. vulgaris* cultured in BBM was significantly higher than that grown in AWW + 1.0 NPK ($p = 0.013$). But no significant variation was

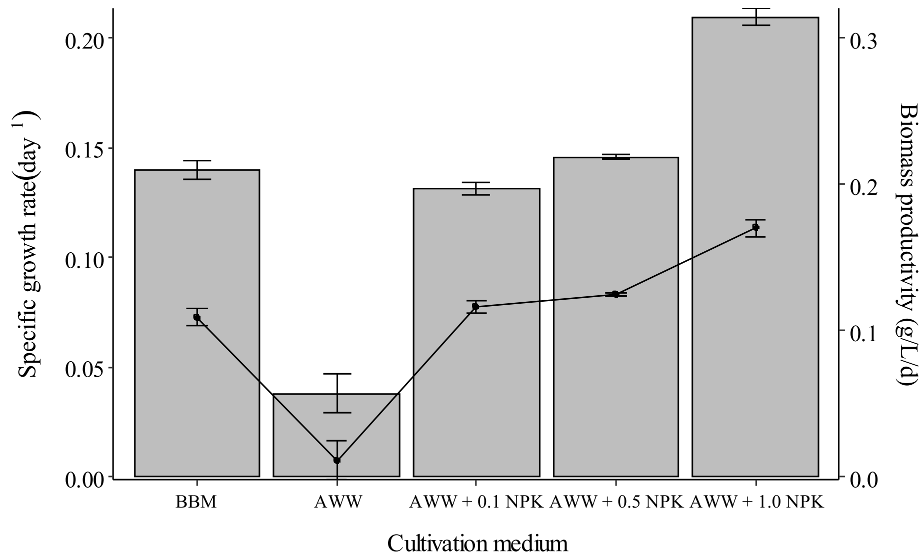

Fig. 3 Specific growth rate (bar graph) and biomass productivity (line graph) of *C. vulgaris* in different cultivation media

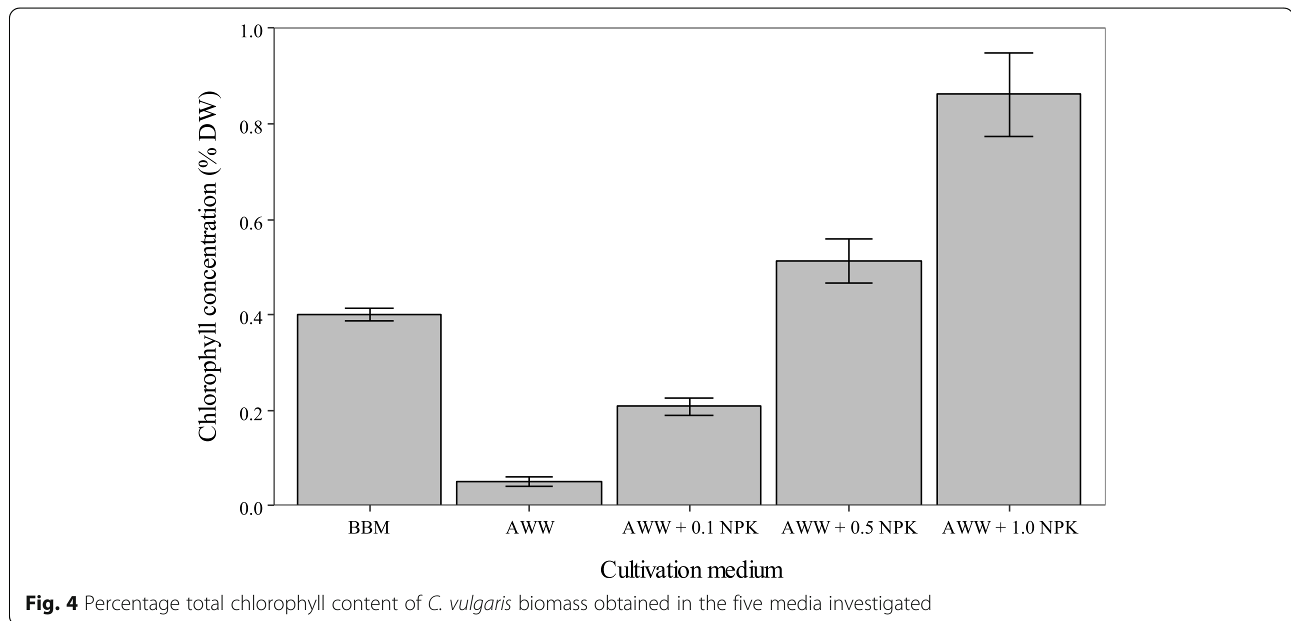

Fig. 4 Percentage total chlorophyll content of *C. vulgaris* biomass obtained in the five media investigated

found with the rest of the media ($p > 0.05$). In addition, the growth media did not show any significant difference in manganese content of *C. vulgaris* biomass ($p = 0.057$).

Discussion

Microalgae growth

Nutrient is among the key factor that greatly affects the microalgae growth rate and their production of various bio-chemicals (George et al. 2014; Mahmood and Mohsin, 2017). However, for large-scale production, it poses an economical challenge to provide excess nutrients (Di Caprio et al. 2015; Nayak et al. 2016a). The present study was done to investigate the effect of AWW supplemented with NPK fertilizer as a low-cost growth medium for production of *C. vulgaris*. The growth parameter analyzed were optical density, biomass content, biomass productivity, total chlorophyll content, and specific growth rate. The specific growth rate is the most important parameter for assessing algal growth. It determines both the time required for achieving the maximum algal biomass concentration during the batch culture and the hydraulic retention time required for high biomass productivity during a continuous culture (Ruiz et al. 2013). In the current study, the highest specific growth rate of 0.209 day^{-1} was recorded in

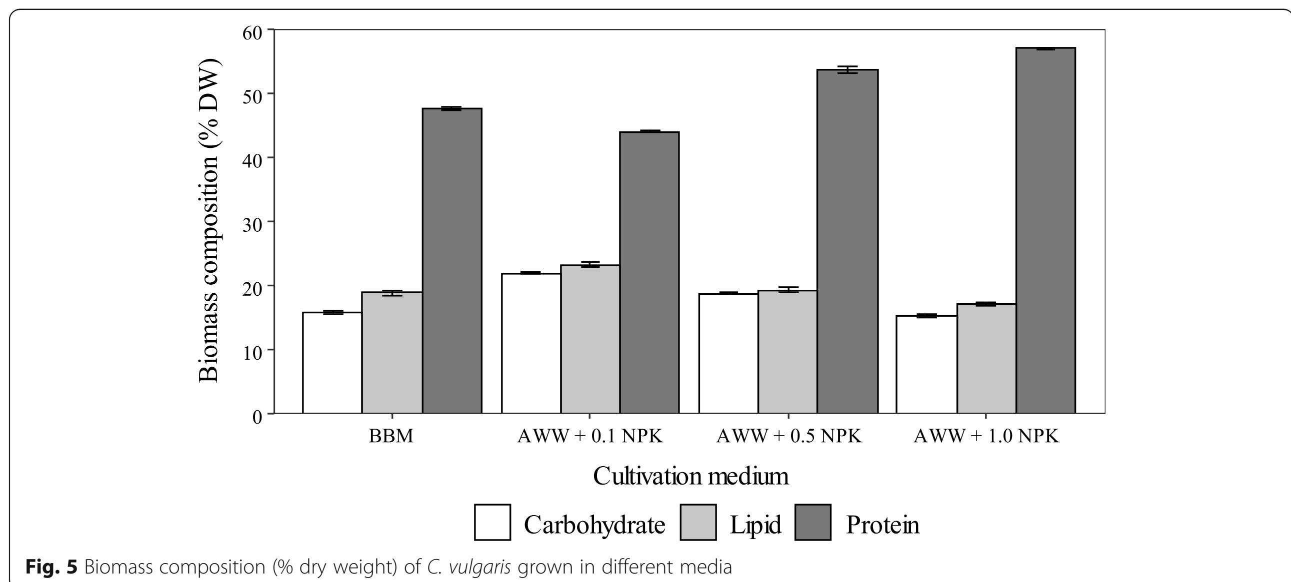

Fig. 5 Biomass composition (% dry weight) of *C. vulgaris* grown in different media

Table 4 Vitamins content of C. vulgaris biomass grown in different cultivation media

Vitamins	Cultivation medium			
	BBM	AWW + 0.1 NPK	AWW + 0.5 NPK	AWW + 1.0 NPK
A (beta-carotene)	84.900 ± 1.375	57.500 ± 3.027	87.200 ± 1.931	89.500 ± 1.752
C (ascorbic acid)	6.533 ± 0.102	7.053 ± 0.072	9.190 ± 0.040	9.513 ± 0.012
B1 (thiamine)	1.830 ± 0.012	1.693 ± 0.018	1.786 ± 0.034	2.063 ± 0.048
B2 (riboflavin)	2.113 ± 0.067	2.053 ± 0.043	3.158 ± 0.040	3.536 ± 0.132
B3 (niacin)	16.673 ± 0.135	20.193 ± 0.043	21.056 ± 0.260	21.470 ± 0.667
B6 (pyridoxine)	0.414 ± 0.007	0.941 ± 0.010	0.984 ± 0.009	1.052 ± 0.006
B12 (cobalamin)	0.001 ± 0.000	0.001 ± 0.000	0.001 ± 0.000	0.002 ± 0.000

microalgae cultured in AWW + 1.0 NPK. This value is within those reported in the literature for *C. vulgaris*. Gao et al. (2014) observed specific growth rates of 0.277 day^{-1} and 0.108 day^{-1} when *C. vulgaris* was grown in membrane and conventional photobioreactor respectively, using treated sewage medium. On the other hand, De Lourdes et al. (2017) reported a growth rate of 0.205 day^{-1} on their study of tolerance and nutrients consumption of *C. vulgaris* growing in mineral medium and real wastewater under laboratory condition.

The chlorophyll content of microalgae is an important parameter as it indicates the physiological status of the culture (Martínez-Roldán et al. 2014). In our study, media with high nutrient concentrations were found to support the accumulation of pigments which indicate that the physiological activity in *C. vulgaris* was affected by nutrient concentrations. The highest total chlorophyll content was observed in microalgae cultured in AWW + 1.0 NPK (0.862 ± 0.090%). These results are in accordance with that of Paes et al. (2016) who reported values ranging from 0.72 ± 0.07% to 1.05 ± 0.06 for *Chlorella* sp under nitrogen starvation culture condition.. Contrary to our results, Ribeiro et al. (2019) obtained optical density of 0.519 ± 0.002 and 0.849 ± 0.003 when cultured *Chlorella sorokiniana* in NPK and BBM media respectively. These values are much less than the present findings (0.629 ± 0.059 – 4.872 ± 0.025), probably due to differences in media composition and concentration used (Richmond 2004).

Furthermore, the maximum biomass productivity of *C. vulgaris* was recorded in AWW + 1.0 NPK (0.17g/L/day). This value is higher than that of Gao et al. (2016) who obtained the biomass productivity of 0.043 g/L/day when cultivated *C. vulgaris* in a membrane photobioreactor, using aquaculture wastewater as a nutrient medium. Nevertheless, Tan and Zhang (2016) in their study on *C. pyrenoidosa* growth in diluted anaerobically digested activated sludge in outdoors obtained highest dry weight of 2.430 g/L against 2.858 g/Lin our study. Furthermore, De Lourdes et al. (2017) recorded a maximum dry weight of 0.267 ± 0.031 g/L in cells of *C. vulgaris* cultured in real wastewater from a wastewater treatment plant. This observation can be largely attributed to nutrients differences resulting from the addition of NPK fertilizer in AWW (Cai et al. 2013; Lu et al. 2015a). The results of this work agree with those reported by Cai et al. (2013) and Lu et al. (2015a), where they emphasized that supplementation of nutrient in wastewater can enhance microalgae growth; however, it depends on the initial value of the nutrient concentrations of the wastewater used and the requirements of the selected microalgal strain. Results presented here agree with those reported by Bhatnagar et al. (2011) where they found that the addition of 1% w/v glucose and 250 mg/L of N as $NaNO_3$ into wastewater yielded the highest biomass

Table 5 The mineral content of C. vulgaris biomass raised in different growth media

Minerals	Cultivation medium			
	BBM	AWW + 0.1 NPK	AWW + 0.5 NPK	AWW + 1.0 NPK
Calcium (Ca)	95.303 ± 0.294	40.1667 ± 0.032	43.390 ± 0.240	85.050 ± 0.932
Iron (Fe)	24.303 ± 0.070	20.700 ± 0.311	23.240 ± 0.040	24.127 ± 0.118
Magnesium (Mg)	165.470 ± 0.973	120.963 ± 0.333	123.240 ± 0.200	133.240 ± 1.151
Sodium (Na)	887. 980 ± 1.000	917.000 ± 2.633	991.487 ± 0.637	922. 843 ± 0.919
Potassium (K)	1113.647 ± 0.557	1320.860 ± 1.560	1315.517 ± 1.740	1318.923 ± 0.559
Zinc (Zn)	2.157 ± 0.015	1.330 ± 0.021	1.310 ± 0.015	1.187 ± 0.015
Manganese (Mn)	1.447 ± 0.052	1.123 ± 0.058	1.140 ± 0.012	1.103 ± 0.012

concentration compared to BG11 addition and without any supplementation. In another study, Ramanna et al. (2014) supplemented wastewater with urea (1.5 g/L) as a cheap N source for the cultivation of *Chlorella sorokiniana* and found 23% increase in biomass production when compared to the control wastewater without any supplementation. Furthermore, Ansari et al. (2017) grew *Chlorella sorokiniana* in aquaculture wastewater with sodium nitrate supplementation and found comparable biomass yields to the synthetic medium. The authors also observed high-nutrient removal and proposed that treated water can be used for aquaculture. The current results indicated that the AWW + 0.1 NPK, AWW + 0.5 NPK, and AWW + 1.0 media are ideal for *C. vulgaris* culture and can be used as substitute for the BBM medium which is expensive and less accessible to poor aquaculture farmers.

Biochemical composition

One of the most noteworthy nutritional characteristics of *Chlorella* is its high protein content which contains all essential amino acids, demonstrating that *Chlorella* could be used as healthy food for humans and feed for fish and other livestock (Becker 2007). It has been reported that the nitrogen concentration in the medium can influence the protein content of microalgae, whereby having a higher amount of nitrogen in the cultivation medium can induce protein synthesis in microalgae (Martínez et al. 2000). Our data show that cells grown in AWW + 1.0 NPK had a higher percentage of protein content (57.400%) in their biomass, and this could be due to its composition, as this medium was rich in nitrogen and had higher algal biomass. Such protein content was higher than that of *C. vulgaris* (50.640%) cultivated in the 100-fold diluted Monosodium glutamate wastewater (MSGW) by (Ji et al. 2014) and lower than the value reported (about 63.500%) by Abreu et al. (2012).

Lipid accumulation by microalgae depends on the species and is affected by medium composition and cultivation conditions (Richmond 2004; Chen et al. 2011). Kim et al. (2010) and Mata et al. (2010) reported the average lipid content of *C. vulgaris* of approximately 14–22% under common conditions. In this study, the total lipid content ranged from 16.400–23.300% and the highest value was detected in the biomass cultivated in AWW + 0.1 NPK medium. These values were much lower than that found in *C. vulgaris* (42%) cultivated using industrial dairy waste as organic carbon source (Abreu et al. 2012). Furthermore, the present finding of lipid content was higher than that of Song et al. (2013) who cultivated *C. vulgaris* in BG11 medium and obtained a value of 20.820 ± 1.020% of dry weight. The difference in lipid contents of microalgae biomass cultured under different growth media in our study may be attributed to the variation in nitrogen contents in the cultivation medium. It is known that under nitrogen deficiency or limitations, the metabolic pathway of carbon assimilation diverts from protein synthesis to lipid or carbohydrate production as carbon and energy storage (Chandra et al. 2014; Kim et al. 2014). Similar results have been reported in the other green algae *Chlorella* sp. and *Chlamydomons* sp. (e.g., Shen et al. 2015).

Carbohydrate is one of the major components in *C. vulgaris* biomass, and its production can vary as a result of changes in growth conditions, such as temperature and light intensity and nutrient media characteristics including the concentration of nitrogen, phosphates, and iron (Illman and Scragg 2000; Liu et al. 2008). The carbohydrate content of algae in the present study ranged from 15 to 22% which is within the range of carbohydrate contents in *Chlorella* sp. reported in the literature (Batista et al. 2013; Beuckels et al. 2015; Lu et al. 2015b). The highest content of carbohydrate (22%) was in the biomass cultivated in AWW + 0.5 NPK medium, which are also characterized by a lower concentration of nitrogen, especially in the form of nitrate. Our results are agreeing with the literature data, which indicate that under limited nutrient conditions, cell division slows down but carbon uptake continues (through photosynthesis) which leads to a higher concentration of carbon-rich metabolites such as carbohydrates or lipids in the biomass of algae (González-Fernández and Ballesteros 2012; Vitova et al. 2015)

Apart from main compounds such as proteins, lipid, and carbohydrates, living organisms require several micro-nutrients for survival. These micronutrients act as either co-enzymes or as active electron/proton carriers in the breakdown process of the macro-nutrients. One such group of important micro-nutrients is vitamins (Koyande et al. 2019). *C. vulgaris* is a very rich source of nearly all the important vitamins such as vitamin A (in the form of beta-carotene), vitamin C, vitamin E, and vitamin B such as thiamine (B1), riboflavin (B2), niacin (B3), pantothenic acid (B5), pyridoxine (B6), folic acid (B9), and cobalamin (B12) (Safi et al. 2014). These vitamins have great potential benefits to animal and human health; since they are used to nourish the body, detoxify, and normalize intestinal function, as well as stimulate the immune system and regenerate cells. Even though vitamins are required in a small amount to maintain good health, lack of them can cause serious diseases in humans (Heudi et al. 2005). The cells grown in AWW + 1.0 NPK had highest content of all vitamins analyzed when compared to other cultivation media. The highest vitamin B1, B3, B6, B12, and C obtained in our study are in accordance with those achieved in past studies for *C. vulgaris* (Maruyama et al. 1997; Panahi et al. 2012; Andrade et al. 2018). The vitamin A content recorded in

this study was much lower when compared with the study by (Panahi et al. 2012) who obtained the vitamin A content of 180 mg/100g in their study investigating the effects of *Chlorella vulgaris* as adjunctive therapy for dyslipidemia. This variation could be a result of different composition and concentrations of media used as it was reported that environmental factors, particularly nutrient status, light, and temperature not only affect photosynthesis and productivity of cell biomass, but also influence the pattern, pathway, and activity of cellular metabolism and thus dynamic cell composition (Richmond 2004)

As illustrated in Table 5, *C. vulgaris* is a good source of important minerals such as Ca, K, Mg, Na, Fe, Zn, and Mn. These minerals are considered to be essential for cellular metabolism in all animals including fish (Paul and Mukhopadhyay 2016). For instance, iron is essential for the production of hemoglobin, myoglobin, cytochromes, and many other enzyme system and its deficiency has been reported to induce anemia in trout and carp (Paul and Mukhopadhyay 2016). Further on, zinc is an essential component of enzymes which participate in many metabolic processes including synthesis of carbohydrate, lipid, and protein synthesis and it is also a cofactor of the superoxide dismutase enzyme, which is involved in protection against oxidative processes (Mann and Truswell 2009). Potassium is associated with intracellular fluid balance and volume, carbohydrate metabolism, protein synthesis, and nerve impulses (Safi et al. 2014). Magnesium is important in maintaining normal and constant nervous activity and muscle contraction; hence, magnesium deficiency in human organism can lead to depression and symptoms of suicidal behavior (Diehl 2002). The mineral components obtained in this study were within the range of reported elemental composition of *Chlorella* biomass (Tokuşoglu and üUnal, 2003; Panahi et al. 2012).

Conclusion

The present study was done to formulate a low-cost culture medium by using aquaculture wastewater and NPK fertilizer for cultivation of *C. vulgaris.* The best growth performance in respect of optical density, dry weight, specific growth rate, biomass productivity, total chlorophyll content, and biochemical composition were observed in *C. vulgaris* grown in AWW + 1.0 NPK medium. Also, the *C. vulgaris* grown in AWW + 0.1 NPK, AWW + 0.5 NPK showed good results in biomass composition when compared with *C. vulgaris* grown in BBM. These results indicated that it is feasible to use AWW supplemented with NPK fertilizer medium in place of the synthetic medium (BBM) to cultivate *C. vulgaris* for feed production in aquaculture. More importantly, these media are inexpensive since the NPK fertilizer is widely available in the local market and the AWW is found at a lower or no cost. Therefore, the use of these media will reduce the mass production costs of highly nutritive *C. vulgaris* and improve the aquaculture sector.

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Involvement of human and animals in the study
N/A

Authors' contributions
Conceptualization, Kulwa Mtaki, Margareth S. Kyewalyanga and Matern S.P. Mtolera; experimental design, Kulwa Mtaki, Margareth S. Kyewalyanga and Matern S.P. Mtolera; data analysis, Kulwa Mtaki; manuscript drafting and writing, Kulwa Mtaki, Margareth S. Kyewalyanga and Matern S.P. Mtolera; funding acquisition, Margareth S. Kyewalyanga and Matern S.P. Mtolera; supervision, Margareth S. Kyewalyanga and Matern S.P. Mtolera. All authors read and approved the final manuscript.

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