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Evaluation of Pre-Chlorinated Wastewater Effluent for Microalgal Cultivation and Biodiesel Production

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Abstract: Microalgae are promising feedstock to produce biodiesel and other value added products. However, the water footprint for producing microalgal biodiesel is enormous and would put a strain on the water resources of water stressed countries like South Africa if freshwater is used without recycling. This study evaluates the utilization of pre-chlorinated wastewater as a cheap growth media for microalgal biomass propagation with the aim of producing biodiesel whilst simultaneously remediating the wastewater. Wastewater was collected from two wastewater treatment plants (WWTPs) in Durban, inoculated with *Neochloris aquatica* and *Asterarcys quadricellulare* and the growth kinetics monitored for a period of 8 days. The physicochemical parameters; including chemical oxygen demand (COD), total nitrogen (TN), and total phosphorus (TP) were determined before microalgal cultivation and after harvesting. Total lipids were quantified gravimetrically after extraction by hexane/isopropanol (3:2 v/v). Biodiesel was produced by transesterification and characterised by gas chromatography. The total carbohydrate was extracted by acid hydrolysis and quantified by spectrophotometric method based on aldehyde functional group derivatization. *Asterarcys quadricellulare* utilized the wastewater for growth and reduced the COD of the wastewater effluent from the Umbilo WWTP by 12.4%. Total nitrogen (TN) and phosphorus (TP) were reduced by 48% and 50% respectively by *Asterarcys quadricellulare* cultivated in sterile wastewater while, *Neochloris* reduced the TP by 37% and TN by 29%. Although the highest biomass yield (460 mg dry weight) was obtained for *Asterarcys*, the highest amount of lipid (14.85 $\pm$ 1.63 mg L^{-1}) and carbohydrate (14.84 $\pm$ 0.1 mg L^{-1}) content were recorded in *Neochloris aquatica*. The dominant fatty acids in the microalgae were palmitic acid (C16:0), stearic acid (C18:0) and oleic acid (C18:1). The biodiesel produced was determined to be of good quality with high oxidation stability and low viscosity, and conformed to the American society for testing and materials (ASTM) guidelines.

Keywords: wastewater; wastewater treatment plant; microalgae; biodiesel; effluent

1. Introduction

Microalgae are photoautotrophic organisms utilizing sunlight and carbon dioxide to generate energy which is stored as lipids. These lipids, usually stored as triglycerides, can be converted to biodiesel, thus the increasing interest in the propagation and cultivation of these organisms [1]. Furthermore, increasing greenhouse gas emissions, concern towards global warming and climate

change has spurred the need for the development of environmentally friendly forms of energy including biodiesel. Biodiesel can be derived from oleaginous feedstock such as oil rich crops but this however, creates a concern of diversion of scarce arable land resources towards energy production instead of food. Microalgae are versatile organisms that require less land space for cultivation and can accumulate a high amount of lipids amongst other advantages. However, it has a large water footprint that is unsustainable for a semi-arid and water stressed country like South Africa, with low average rainfall of 465 mm, which is below the global average of 860 mm [2]. Demand for this important scarce resource is expected to increase due to rapid industrial development, increasing human population, per capita consumption increase, and the resulting impact of human activities on the environment [3]. High water demand and consumption leads to increases in the volume of wastewater generated. Wastewater effluent from wastewater treatment plants (WWTPs) have been identified as a potential solution to the large water requirement for the cultivation of microalgae for biodiesel production.

Wastewater effluent characteristics is dynamic and dependent on several factors including wastewater type, treatment applied, location of treatment plant, climate, population, etc. Agricultural wastewater such as dairy, pig slurry, etc. are known to contain a very high amount of nutrient, while the level of nutrient content varies within municipal wastewater treatment plants [4]. Pre-chlorinated wastewater defined as wastewater in the various stages of the treatment process prior to chlorination contain large amounts of nutrients including Ammonia (NH_4^+), Nitrates (NO_3^-), and phosphates (PO_4^{3-}) which if not properly treated result in eutrophication of natural water bodies [5]. Recovery of value added product from wastes and wastewater is driving a paradigm shift of our perception of wastes leading to the development of technologies for sustainable waste and wastewater processing as well as resource recovery. For example, oleuropein, a biophenol of pharmaceutical value is recovered from the agricultural waste olive leaves [6], highly porous carbon adsorbents useful in dye removals has been recovered from waste rice straw [7], reverse osmosis has been used to generate fresh water for reuse from steel plant wastewater with high salt content [8], and adsorption technology has been used to remove organic impurities in wastewater generated from membrane industries [9]. Microbial fuel cell technology though in its infancy has been touted as a sustainable, less energy intensive and environmentally friendly method of treating wastewater and electricity generation [10], while ammonia and phosphates are recovered from nutrient rich wastewater via struvite precipitation for use as fertilizers [11]. Microalgae can also utilize nutrients in wastewater (phosphates and ammonia) in addition to sunlight and CO_2 for their growth and bioenergy production while simultaneously treating the wastewater hence, it was first proposed and developed in the 1950s as a cheap method of treating wastewater [12]. Since then, other researchers have reported the use of different types of wastewater and microalgae species for the simultaneous remediation and biomass propagation of microalgae for the biodiesel production [13–16]. Mahaptra et al. [17] reported the use of mixotrophic algal consortia for the bioremediation of municipal wastewater and simultaneous lipid accumulation in municipal wastewater in India with nutrient removal reaching as high as 90% and lipid accumulation reaching 28.5% of dry algal biomass.

South Africa possesses an attractive climate for microalgal cultivation and huge untapped microalgal biodiversity which can be applied as feedstock for biodiesel production and other biotechnological application. Utilizing wastewater for microalgal cultivation can reduce the amount of freshwater required for large scale biofuel production while, nutrient in wastewater can reduce cost of biodiesel production with the added benefit of generating wastewater low in nutrient and chemical oxygen demand (COD) fit for discharge into receiving waterbodies. However, there is very little information regarding the suitability of wastewater effluent in South Africa for microalgae cultivation. Hence, this study investigated the potential use of treated pre-chlorinated wastewater effluent from two major WWTPs in Durban, KwaZulu-Natal for growth and biomass propagation of two microalgae isolates, *Asterarcys quadricellulare* and *Neochloris aquatica* for biodiesel production. The phycoremediation potential of the isolates during growth in the wastewater effluent was also established.

2. Materials and Methods

2.1. Sample Collection and Processing

Wastewater samples were collected from the Northern wastewater (29°48′45.62″ S; 30°59′45.62″ E) and Umbilo wastewater treatment plants (29°50′41.431″ S; 30°53′29.122″ E) in Durban, KwaZulu-Natal province of South Africa. The treatment plants are amongst the major wastewater treatment plants in the province and utilize the activated sludge treatment system to treat wastewater. Water samples were collected from the secondary clarifier tank of each wastewater treatment plant in a clean 10 L plastic container previously washed and thoroughly rinsed twice with the sample water before collection. The wastewater samples (5 L) were filtered through Whatmann 1 filter paper to remove solid particles and reduce turbidity before autoclaving at 121 °C for 15 min to sterilize it.

2.2. Determination of Physico-Chemical Parameters of Wastewater Samples

The physico-chemical parameters of the wastewater samples were determined prior to inoculation and after 8 days of inoculation with the microalgae using standard methods. The pH was determined using Hanna Edge pH meter (Hannah Instruments, Woonsocket, RI, USA), Chemical oxygen demand (COD) was determined via the $K_2Cr_2O_7$ assay using Spectroqaunt COD cell test kit (Merck, Darmstadt, Germany) following the manufacturer's instructions, Total nitrogen and phosphates were determined using Spectroquant total Nitrogen and Phosphate cell test kit respectively (Merck, Darmstadt, Germany). Electrical conductivity (EC), salinity, resistivity and total dissolved solids (TDS) were determined using the CDC 401 probe and HQ40d multimeter (HACH, Loveland, CO, USA). Physicochemical parameters were determined in duplicates and results expressed as a mean of the obtained data.

2.3. Microalgal Strain Cultivation and Growth Kinetics

Monocultures of two microalgae strains *Asterarcys quadricellulare* and *Neochloris aquatica*. previously isolated from the Toongati river and maturation pond of the Northern wastewater treatment plant, respectively between May and July 2015, were maintained on commercial BG11 medium (Sigma Aldrich, Darmstadt, Germany) supplemented with trace metals prepared as previously descried by Mutanda et al. [18]. The algal strains were chosen for their high growth rate and biomass accumulation when grown in commercial BG11 medium (Sigma Aldrich). The cells were standardized to an optical density of 1 at a wavelength of 680 nm, centrifuged at 8000 rpm (Beckman Coulter Avanti J-30i, Brea, CA, USA) and washed three times with sterile distilled water and once with sterile wastewater sample to remove every trace of phosphates or nitrogen carried over from the commercial media to the experimental setup. Thereafter, the cells suspended in sterile autoclaved wastewater sample (10%) was inoculated into 800 mL (total volume) of the filtered autoclaved wastewater and unsterilized wastewater samples. The flasks were sealed with cotton wool and placed under cool white light illumination (54.36 μmol/m^2 s^{-1}) with shaking at 180 rpm for 8 days at 30 °C, under ambient air diffusion with a 12:12 h light: dark cycles.

Samples were taken daily over the 8 days incubation period from the purified culture for growth determination at an optical density of 680 nm (OD_{680}) using a Cary 60 UV-Vis Spectrophotometer (Agilent Technologies, Santa Clara, CA, USA) as the algal density indictor. The growth rate per day (GR, per day) was calculated using the equation $GR = (\ln OD_t - \ln OD_0)/t$. where OD_0 is the initial optical density, OD_t is the optical density measured on day t. Every four days, 5 mL of algal cells were filtered through a membrane filter with 0.45 μm pore size (MilliporeSigma, Burlington, MA, USA), washed twice with sterile deionized water and dried in an oven at 40 °C overnight. Prior to filtration, the filter was weighed in a glass dish and reweighed after drying. The experiment was done in triplicate and results expressed as a mean of the data obtained.

2.4. Dry Weight Determination, Lipid Extraction, and Quantitation

Dry weight was determined after 7 days by filtering 10 mL of algal broth through a pre-weighed Whatmann 1 filter paper with a pore size of 11 µm and placed on a Petri dish. The Petri dish and filter paper were dried in an oven at 60 °C for 12 h after which it was allowed to cool to room temperature. The net mass of the microalgal cells was determined by subtracting the weight of microalgae and filter paper from the pre-weighed filter paper. Lipid content was quantified using the gravimetric method previously described by Halim et al. [19]. Lyophilized biomass (50 mg) was finely ground in a mortar and pestle and suspended in 4 mL hexane: isopropanol (3:2 v/v) and vortexed for 5 min. The mixture was sonicated at an interval of 5 min for 15 min at 50% power and 60 pulse to break the cells. The samples were then sealed to prevent evaporation of hexane and agitated at 180 rpm at ambient conditions overnight. The next day the cells were vortexed for 5 min and 1.5 mL of sterile 1% NaCl solution was added to the mixture to induce biphasic phase separation using a separating funnel. The mixture was left to stand until phase separation was observed. After settling, a top dark-green hexane layer containing most of the extracted lipids was collected in a pre-weighed Eppendorf tube and the tubes were heated to dryness in the oven (60 °C) overnight until constant weight to enable gravimetric quantification of the lipid extract. All experiments were carried out in triplicate and results expressed as means of replicate data.

2.5. Biodiesel Production and Characterization

Biodiesel production was carried out using methods previously described by Ramanna et al. [20]. Briefly, dried lipid samples (20 mg) gravimetrically extracted was dissolved in 1 mL of hexane and reacted with methanol containing 5% sulphuric acid as a catalyst (30:1 v/v). The reaction proceeded at 60 °C for 4 h in an incubator with shaking at 200 rpm. Thereafter, the reaction setup was transferred to a separating funnel and washed with a mixture of distilled water and hexane (1:1 v/v) to induce biphasic separation. The organic layer was collected and analysed using gas chromatography-mass spectrometry (GC-MS) (Shimadzu Corp., Kyoto, Japan). The oven temperature was programmed to start at 60 °C and kept at hold for 2 min, then initially increased to 160 °C at a ramp rate of 10 °C min^{-1} and then to 240 °C at a ramp rate of 7 °C min^{-1} and again kept at hold for 1 min. The injector and detector temperature was 250 °C and nitrogen was used as a carrier gas. Identification of lipids was done by comparing the mass spectra of the resolved components using electronic library search routines [21]. Biodiesel properties were estimated using the web version of the BiodieselAnalyzer© version 2.2 [22].

2.6. Evaluation of Carbohydrate Accumulation Potential of Microalgal Isolates

Total carbohydrate extraction was carried out by two-step sulfuric acid hydrolysis followed by spectrophotometric quantification of soluble carbohydrate based on aldehyde functional group derivatization as previously described by Van Wychen and Laurens [23]. Briefly, 25 mg of freeze dried algal biomass was hydrolysed in a 250 µL of 72% (v/v) sulfuric acid at 30 °C for 60 min. Thereafter, 7 mL of sterile Millipore water was added to achieve a concentration of 4% (w/v). The setup was autoclaved at 121 °C for 60 min and cooled to room temperature. The hydrolysate was then filtered through a 0.2 µm nylon filter and the filtrate stored at 4 °C for further analysis.

Spectrophotometric analysis of monomeric sugars was carried out using 500 µL of diluted filtrate (1:50) to which 500 µL each of MBTH and 0.5 N NaOH was added. The setup was carefully vortexed and immediately placed in a preheated dry block at 80 °C for 15 ± 1 min. Thereafter, 1 mL of Ferric solution (made up of 0.5% ammonium sulphate dodecahydrate and 0.5% of sulfamic acid (w/v) in 0.25 M HCl) was added to the mixture. The setup was carefully vortexed and allowed to cool to room temperature after which 2.5 mL of Millipore water was added and vortexed. The optical density was measured at 620 nm using the Cary 60 UV-1800 spectrophotometer (Agilent Technologies, Santa Clara, CA, USA). The concentration of monosaccharide was extrapolated from a standard curve made with

0.25 mg/mL glucose after correcting for dilution. The total carbohydrate content was calculated by multiplying the obtained concentration with the total volume of extract (7.25 mL). The analysis was done in triplicate and the result expressed as a mean of the obtained data.

2.7. Statistical Analysis

In order to arrive at a validated conclusion, the growth rate and physicochemical parameters were subjected to Pearson's correlation to ascertain significant correlation between the obtained data. The statistical analysis was performed using SPSS software package version 25 (IBM Corporation, New York, NY, USA).

3. Results

3.1. Physico-Chemical Profiles of the Treated Wastewater Effluent

The physico-chemical profiles of the treated pre-chlorinated wastewater used in this study are summarised in Table 1. The initial pH, total nitrogen (TN), and total phosphorus (TP), and COD of the wastewater sample from the Northern wastewater treatment works (NWWTW) were found to be 7.66, 0.4 mg L^{-1}, 17.77 mg L^{-1}, and 23.33 mg L^{-1}, respectively, while at the Umbilo wastewater treatment plant (UWWTP), initial values of 7.37 mg L^{-1}, 3.40 mg L^{-1}, 15.03 mg L^{-1}, and 45.67 mg L^{-1}, respectively, were recorded. The N/P ratio of the wastewater samples were 44.42 (NWWTW) and 4.42 (UWWTP). The highest TP and TN reduction at the NWWTW was achieved by *Asterarcys quadricellulare* inoculated in sterilized wastewater and unsterilized wastewater. While at the UWWTP, the highest TP and TN reduction was achieved by *Asterarcys quadricellulare* inoculated in sterilized wastewater and unsterilized wastewater. The salinity of the wastewater from the NWWTW increased after treatment from an initial value of 0.38 up to 0.43 in the unsterilized wastewater inoculated with *Chlorella* spp., while at the UWWTP, salinity was relatively stable and unchanged (Table 1). COD was reduced by up to 12.4% in the wastewater from the UWWTP after 8 days, however an increased COD of up to 30.7% was recorded in the wastewater from the NWWTW. A significant positive correlation was observed between COD and TP ($p < 0.01$; r = 0.937), whereas an inverse correlation between COD and salinity ($p < 0.01$; r = −0.911).

Table 1. Physico-chemical profiles of treated pre-chlorinated wastewater effluent before and after cultivation with microalgae.

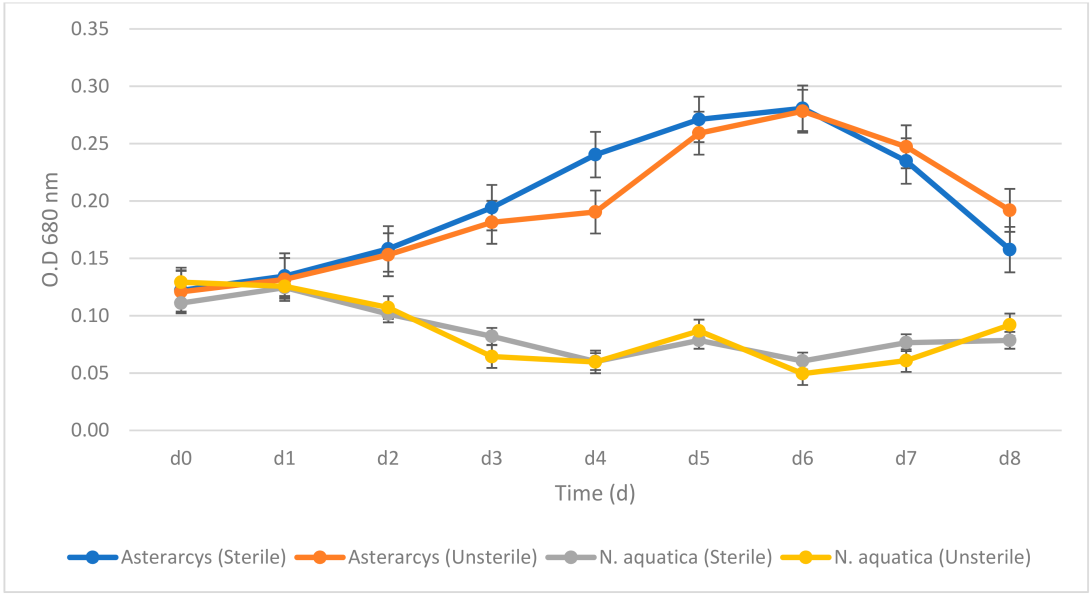
Treatment Plant	Organism ID	pH	Conductivity µS cm^{-1}	Salinity %	TDS mg L^{-1}	Resistivity Ω cm	TP (mg L^{-1})	TN (mg L^{-1})	COD (mg L^{-1})
NWWTW	Before cultivation	7.66	775	0.38	ND	1290	0.40	17.77	23.33
	Asterarcys [a]	8.43	804	0.39	394	1242	0.25	9.20	30.00
	N. aquatica [b]	7.27	867	0.43	425	1151	0.30	12.50	30.50
	Asterarcys [a]	7.62	879	0.42	422	1162	0.20	10.35	28.50
	N. aquatica [b]	7.46	871	0.43	429	1132	0.25	13.55	24.50
UWWTP	Before cultivation	7.37	549	0.26	ND	1822	3.40	15.03	45.67
	Asterarcys [a]	8.33	527	0.25	255	1899	2.30	8.30	40.00
	Asterarcys [b]	7.71	537	0.26	260	1864	2.75	9.20	41.00
	N. aquatica [a]	7.25	547	0.26	265	1826	2.40	12.10	44.00
	N. aquatica [b]	7.45	555	0.27	269	1757	3.10	12.75	41.00

Notes: [a] Sterilized wastewater; [b] unsterilized wastewater.

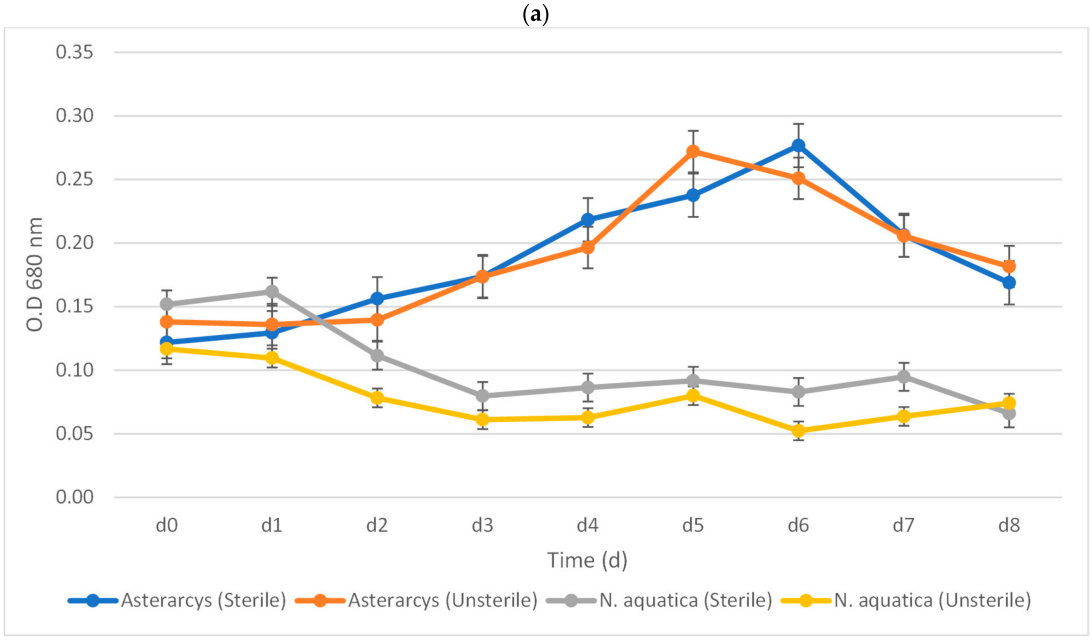
3.2. Growth Kinetics, Biomass Yield, and Total Carbohydrate Content of the Microalgae

Growth profiles of *Neochloris aquatica* and *Asterarcys quadricellulare* in the different wastewater samples are presented in Figure 1a,b. The results suggest *Neochloris aquatica* did not grow in the wastewater (sterilized and unsterilized) from both wastewater treatment plants for biomass

propagation. *Asterarcys quadricellulare* was able to utilise the wastewater achieving a growth rate up to 0.18 g L^{-1} day^{-1} in sterilized wastewater from the NWWTW and 0.17 day^{-1} in the unsterilized wastewater from UWWTP. *Asterarcys quadricellulare* accumulated high biomass ranging from 250 to 460 mg L^{-1} compared to 130 to 180 mg L^{-1} in *Neochloris aquatica*. The highest biomass productivity of up to 65.71 mg day^{-1} was recorded for *Asterarcys quadricellulare*, values which are 2.56-fold higher than those recorded for *Neochloris aquatica* (Table 2). The highest total carbohydrate content (57.9% dry wt.) was recorded in *Neochloris aquatica* cultivated in sterilized wastewater and the lowest (24.29% dry wt.) in unsterilized wastewater.



(**a**)



(**b**)

Figure 1. Growth rate of microalgae cultivated in treated wastewater effluent from the Northern wastewater treatment works (NWWTW) (**a**) and Umbilo wastewater treatment plant (UWWTP) (**b**).

Table 2. Growth kinetics and metabolite accumulation in microalgae cultivated in sterilized and raw treated wastewater effluent.

Treatment Plant	Organism ID	Lipid Dry Wt. (mg L^{-1})	Lipid % Dry Wt.	Total Carbohydrate Content (mg)	Total Carbohydrate % Dry Wt.	Growth Rate (L^{-1} day^{-1})	Biomass Dry Wt. (mg L^{-1})	Biomass Productivity (mg day^{-1})
NWWTW	*Asterarcys* [b]	9.95 ± 0.50	19.90	11.05 ± 0.02	44.20	0.14	250 ± 1.20	35.71
	N. aquatica [a]	10.4 ± 0.71	20.80	14.48 ± 0.10	57.90	0.00	180 ± 0.70	25.71
	Asterarcys [a]	10.95 ± 1.49	21.90	12.61 ± 0.61	50.44	0.18	460 ± 5.28	65.71
	N. aquatica [b]	8.75 ± 0.92	17.50	6.07 ± 0.45	24.29	0.00	163 ± 0.56	23.33
UWWTP	*Asterarcys* [a]	8.25 ± 1.63	16.50	10.77 ± 1.55	43.09	0.15	400 ± 2.77	57.14
	Asterarcys [b]	10.15 ± 1.20	20.30	11.42 ± 0.10	45.68	0.17	433 ± 0.61	61.91
	N. aquatica [a]	14.85 ± 1.63	29.70	9.83 ± 0.96	39.31	0.00	130 ± 0.36	18.57
	N. aquatica [b]	11.35 ± 2.48	22.70	12.82 ± 0.51	51.29	0.00	180 ± 0.26	25.71

Notes: [a] Sterilized wastewater; [b] unsterilized wastewater.

Despite having a low biomass productivity, *Neochloris aquatica* accumulated higher amounts of lipids than *Asterarcys quadricellulare*. *Neochloris aquatica* cultivated in sterilized wastewater from the UWWTP accumulated up to 14.85 ± 1.36 mg (29.7% dry wt.) and 11.35 ± 2.48 mg (22.7% dry wt.) lipids in sterilized and unsterilized wastewater from UWWTP, respectively. In contrast, *Asterarcys quadricellulare* accumulated 10.15 mg (20.3% dry wt.) in the unsterilized wastewater and 8.25 mg (16.5% dry wt.) in the sterilized wastewater. In the wastewater from the NWWTW, *Neochloris aquatica* accumulated comparable amounts of lipids (up to 20.8% dry wt.) to *Asterarcys quadricellulare* (21.9% dry wt.) despite having a lower biomass dry weight and productivity. Significant positive correlation ($p < 0.01$) was recorded between growth rate and dry weight while the dry weight inversely correlated with TN ($p < 0.01$, $r = -0.82$).

Total carbohydrate accumulated by microalgae are presented in Table 2. The total carbohydrate content was highest in *Neochloris aquatica* cultivated in sterilized wastewater from the NWWTW (14.48 ± 0.1 mg) accounting for 57% of its dry weight while the lowest (6.07 ± 0.45 mg) was recorded in *Neochloris aquatica* cultivated in unsterilized wastewater from the NWWTW. Total carbohydrate accumulated by *Asterarcys quadricellulare* ranged from 10.77 ± 1.55 mg (UWWTP) to 12.61 ± 0.61 mg (NWWTW).

3.3. Fatty Acid Methyl Esters (FAME) Profile

Fatty acid profile of the extracted lipid is presented in Figure 2. The fatty acid profile was generally the same irrespective of the wastewater source or sterility. Palmitic acid (C16:0), stearic acid (C18:0) and oleic acid (C18:1) constituted the dominant fatty acids in the microalgae. Minor fatty acids recorded include myristic acid (C14:0), arachidonic acid (C14:0), and pentadecanoic acid (C15:0). The biodiesel property determined in-silico is presented in Table 3. Saturated fatty acids constituted the bulk of the types of fatty acid present ranging from 69.5% to 77.2%. Monounsaturated fatty acids (MUFAs) were the second most dominant type of fatty acid recorded ranging from 22.89% to 34.82%. Polyunsaturated fatty acids were present in small quantity ranging from 0% to 4.75%. Oxidative stability (OS) is dependent on the type fatty acid present in the oil varied and ranged from 36 h to infinity. Viscosity was low with a minimum of 3.96 mm^2/s and a maximum value of 4.13 mm^2/s. Cetane number was high ranging from 64.9 to 66.5. The American society for testing and materials (ASTM) guideline recommended an OS limit of 3 min, viscosity of 1.9 to 6 and a minimum cetane number of 47 [24].

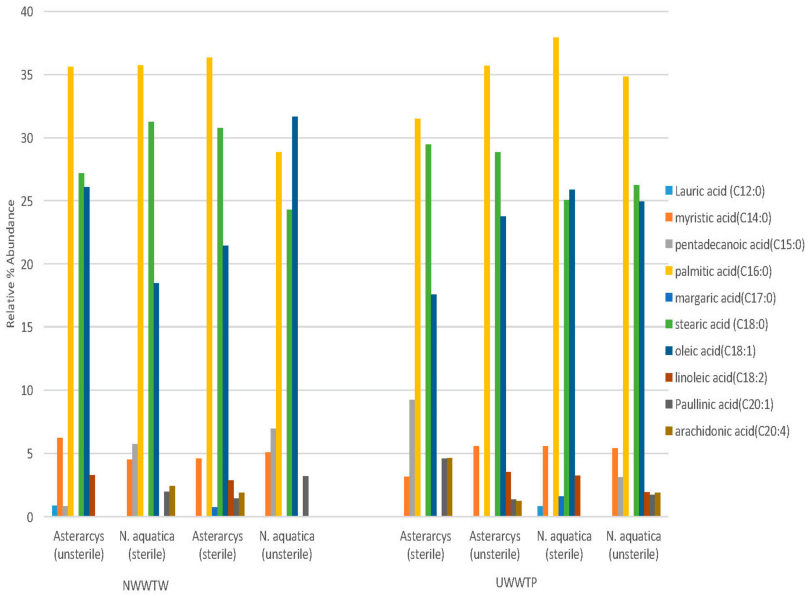


Figure 2. Fatty acid methyl-esters (FAMEs) composition of indigenous microalgae grown in sterilized and raw wastewater effluent.

Table 3. Biodiesel characteristics of microalgae grown in sterilized and raw treated wastewater effluent.

Properties	*Asterarcys* [b]	*N. aquatica* [a]	*Asterarcys* [a]	*N. aquatica* [b]	*Asterarcys* [a]	*Asterarcys* [b]	*N. aquatica* [a]	*N. aquatica* [b]
SFA	70.70	77.16	72.37	65.18	73.26	70.13	70.92	69.50
MUFA	26.05	20.41	22.89	34.82	22.13	25.12	25.86	26.67
PUFA	3.25	2.44	4.75	0.00	4.60	4.74	3.23	3.83
DU	32.55	25.29	32.39	34.82	31.33	34.60	32.32	34.33
SV	209.00	208.31	206.88	207.82	209.98	207.26	209.02	207.89
IV	29.31	26.34	31.95	31.05	34.88	32.93	29.11	33.65
CN	65.82	66.58	65.49	65.58	64.45	65.23	65.86	64.98
LCSF	17.15	19.19	19.00	15.03	15.65	18.00	16.31	16.58
CFPP	37.39	43.80	43.21	30.76	32.69	40.09	34.78	35.62
CP	13.73	13.80	14.12	10.18	−0.15	13.78	14.94	13.32
PP	8.09	8.16	8.50	4.23	−6.98	8.14	9.40	7.64
APE	32.55	25.77	32.82	31.65	31.35	34.45	32.32	34.48
BAPE	3.25	4.88	6.64	0.00	9.20	5.96	3.23	5.71
OS	38.88	Infinity	43.83	Infinity	Infinity	36.09	39.10	63.07
HHV	39.43	39.46	39.49	39.46	39.37	39.47	39.43	39.45
υ	4.06	4.11	4.13	4.10	3.96	4.10	4.06	4.07
ρ	0.87	0.87	0.87	0.87	0.87	0.87	0.87	0.87

Notes: SFA: Saturated Fatty Acid (%); MUFA: Mono Unsaturated Fatty Acid (%); PUFA: Poly Unsaturated Fatty Acid (%); DU: Degree of Unsaturation; SV: Saponification Value (mg/g); IV: Iodine Value; CN: Cetane number; LCSF: Long Chain Saturated Factor; CFPP: Cold Filter Plugging Point (°C); CP: Cloud Point (°C); PP: Pour Point (°C); APE: Allylic Position Equivalent; BAPE: Bis-Allylic Position Equivalent; OS: Oxidation Stability (h); HHV: Higher Heating Value; υ: Kinematic Viscosity (mm^2/s); ρ: Density (g/cm^3); [a] Sterilized wastewater; [b] unsterilized wastewater.

4. Discussion

Compared to available fossil fuel, biofuels from microalgae is more water intensive [25] with an estimated 3726 kg of water, 0.33 kg of nitrogen, and 0.71 kg of phosphate required for the production of 1 kg of microalgae based biodiesel, if freshwater is used without recycling [26,27], and may be unsustainable for a water stressed country like South Africa. Thus, wastewater presents a solution to this dilemma as it contains nitrogen in the form of nitrates and nitrites, phosphates as well as trace metals necessary for algal growth. Treated wastewater effluent is dynamic in its composition of these nutrients and compounds depending on the location of treatment plant, season, and type of treatment applied.

In this study, treated municipal wastewater effluent was accessed for microalgal biomass propagation and subsequent biodiesel production. The treated effluent from the Northern wastewater treatment works (NWWTW) contained low amounts of nitrogen and phosphorus compared to the samples from the Umbilo wastewater treatment plant (UWWTP) while, initial pH values of 7.66 (NWWTW) and 7.37 (UWWTP) as well as low salinity suggests its suitability for the cultivation of freshwater microalgae. COD was reduced by up to 12.4% in the wastewater from the UWWTP after 8 days, however an increased COD of up to 30.7% was recorded in the wastewater from the NWWTW suggesting the excretion of organic compounds such as glycosylic acid into the medium during photosynthesis by the microalgae [28]. Total nitrogen (TN) and phosphorus (TP) were reduced by 48% and 50%, respectively, by *Asterarcys quadricellulare* cultivated in sterile wastewater from NWWTW while, *Neochloris* reduced the TP by 37% and TN by 29%. At the UWWTP, TP and TN were reduced by 32% and 44%, respectively, by *Asterarcys quadricellulare* cultivated in sterile wastewater while 29% and 19% were recorded in *Neochloris aquatica*. The difference in phycoremediation of wastewater by microalgae grown under the same conditions suggests that selecting the right microalgal specie is an important factor to consider when remediating wastewater with microalgae. This is also in agreement with observations by Kim and Kim [29] in their study of phycoremediation potential of tertiary livestock stock wastewater and bioresource recovery.

Sterilization of wastewater did not seem to have any significant effect on the amount of biomass produced or the growth rate of the microalgae, thus raw treated effluent can be used for microalgal cultivation without any pre-treatment steps. However, a significant inverse correlation ($p < 0.01$) exists between the biomass produced and total nitrogen concentration in the water. Several authors have reported the need for a balanced N:P ratio when using wastewater as the growth medium for microalgae for increased growth and nutrient removal [26,30]. In this study, the total nitrogen concentration far exceeded the total phosphorus concentration at the NWWTW with its optimal N:P of 44.2 exceeding the optimal range of 6.8 to 10 [28]. The inability of the microalgae *Chlorella* spp. to utilize treated wastewater for growth was previously reported by Mutanda et al. [18] and it was attributed to the presence of predators such as rotifers and protozoans in the wastewater. However, the inhibited growth observed in *Neochloris aquatica* in both sterilized and unsterilized wastewater coupled with growth of *Asterarcys quadricellulare* in both unsterilized and sterilized wastewater suggests that the ability of microalgae to utilise wastewater for growth may also be specie dependent amongst other factors. Another factor that affects the growth of microalgae in municipal wastewater is the optimal N:P ratio, said to be between 6.8 and 10 [28]. While the UWWTP value of 4.2 is close to the optimum value of 6.8, the N:P of the NWWTW far exceeds the optimum value suggesting a high phosphorus limitation in the wastewater. The growth rate and biomass productivity of microalgae can be improved by supplementing the wastewater with cheap source of nitrogen and phosphorus such as food waste [31] and centrate [32]. The biomass can further be enhanced by the addition of CO_2 from flue gas [33,34]. Though *Asterarcys quadricellulare* had the highest biomass and growth rate when cultivated in wastewater compared to *Neochloris aquatica*, the highest lipid accumulation was recorded in *Neochloris aquatica* (14.85 ± 1.63 mg). This also indicates that a high growth rate or biomass productivity does not necessarily translate to high lipid accumulation and that lipid accumulation potential may vary from specie to specie. This may be a necessary factor to consider when cultivating microalgae in wastewater for large-scale biodiesel production.

Saturated fatty acid; palmitic acid (C16:0), stearic acid (C18:0), and the monounsaturated fatty acid oleic acid (C18:1) were the dominant fatty acids present in the microalgal lipid. Linoleic acid (C18:2) and arachidonic acid (C20:4) are important (omega 6) polyunsaturated fatty acids (PUFAs) that make up the minor components of the microalgal lipid. Polyunsaturated fatty acids are nutritionally important fatty acids essential for infant development with an estimated industrial worth of 11 billion dollars [35]. Therefore, the microalgae are good candidates for optimization of these fatty acids for commercial production. However, the long chain PUFAs; docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), two major essential fatty acids used in baby formula production were not detected in the lipid profile. The biodiesel was deemed to be of high quality as it contained a mix of saturated, monounsaturated, and polyunsaturated fatty acids recommended in the ASTM standard D6751-07 [24]. Lipids containing high amounts of oleic acid have been reported to have a good balance of fuel in terms of its ignition quality, combustion heat, cold filter plug point, oxidative stability, and viscosity, all of which are determined by the fatty acid composition of the biodiesel [36]. Insilco characterization of the biodiesel revealed a quality biodiesel with good oxidation stability ranging from 36 h to infinity well above 3 min limit set by the ASTM standard. Biodiesel viscosity recorded ranged from 3.96 to 4.13 mm^2/s, well within the specified limits of 1.6 and 6 mm^2/s prescribed by the ASTM biodiesel standard for second generation biodiesel [24].

Carbohydrates in microalgae consists mainly of starch found as storage molecule in chloroplasts and cellulose/polysaccharides found as structural components within the cell walls. The potential of microalgae as a promising feedstock for bioethanol production has been reported by several researchers, while the utilization of wastewater for growth by these organisms can drastically reduce the cost of bioethanol production making it competitive with starch rich crop-based feedstock such as sugarcane currently used in bioethanol production [37]. *Chlorella* spp. grown in sterilized wastewater from the NWWTW accumulated the highest amount of total carbohydrate (14.48 ± 0.10 mg) accounting for up to 57.9% of its dry weight and would be excellent candidate as a feedstock for bioethanol production.

5. Conclusions

Pre-chlorinated treated wastewater effluent in Durban was shown to be a feasible growth media for cultivation of microalgae for biofuel production as it possesses adequate physicochemical characteristics for the cultivation of freshwater microalgae. *Asterarcys quadricellulare* utilized the wastewater for growth and reduced the COD of the wastewater effluent from the Umbilo WWTP by 12.4%. Total nitrogen (TN) and phosphorus (TP) were reduced by 48% and 50%, respectively, by *Asterarcys quadricellulare* cultivated in sterile wastewater while, *Neochloris* reduced the TP by 37% and TN by 29%. Biomass yield obtained was as high as 460 mg L^{-1} (dry wt.) while lipid and carbohydrate reached as high as 14.85 ± 1.63 mg L^{-1} and 14.84 ± 0.1 mg L^{-1}, respectively. The dominant fatty acids in the microalgae were palmitic acid (C16:0), stearic acid (C18:0), and oleic acid (C18:1). Though microalgae utilized the wastewater for growth, unbalanced N:P ratios, incomplete nutrient removal, and low biomass yields are some of the challenges that need to be overcome to increase biomass propagation. The growth rate and biomass productivity may be enhanced by supplementation of the wastewater with phosphates to balance the N:P ratio and addition of carbon dioxide [34]. Bacterial contamination and predation were less of an issue in this study as sterilization of the wastewater did not result in any significant increase in the growth rate of the microalgae. Thus, the microalgae can be cultivated without pre-treatment of the wastewater—saving costs. The microalgae also accumulated good amounts of lipids which are desirable for biodiesel production, while the high carbohydrate content also make them good candidates for bioethanol production. Some studies have reported an increase in carbohydrate accumulation after addition of CO_2 [31]. Thus, future research could incorporate this to achieve high carbohydrate content if bioethanol production is the goal.

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