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A N I N T E R N A T I O N A L J O U R N A L

Cultivation of *Nannochloropsis* sp. in Brackish Groundwater Supplemented With Municipal Wastewater as a Nutrient Source

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ABSTRACT

The aim of this work was to study growth potential of the green microalgae Nannochloropsis *sp. using brackish groundwater from a well in the semi-arid northeast region of Brazil as culture medium. The medium was supplemented with (%) 19.4, 22.0, 44.0 and 50.0% of municipal wastewater after UASB treatment as a low-cost nutrient source. The results showed that the culture tested was capable of growing in the brackish groundwater even at salinity levels as low as 2 ppt. Furthermore it was shown that municipal wastewater could be used as a sole nutrient source for* Nannochloropsis *sp.*

Key words: Microalgae, brackish groundwater, municipal wastewater, *Nannochloropsis sp.*

INTRODUCTION

The use of microalgae is very diverse and includes from environmental indicators to industrial use. Lipids obtained from microalgae have received growing attention during the last decade for the production of biofuels. Many species of microalgae can be induced to accumulate substantial amounts of lipids, leading to a high oil yield (Sheehan et al. 1998; Cheirsilp and Torpee 2012). The average lipid content varies between 20 and 50% (Demírbas 2009; Kanda et al. 2012; Liam and Philip, 2012), but under certain conditions some species can reach 90% of dry weight (Illman et al. 2000; Chiu et al. 2009). Microalgae feed on inorganic nutrients and several species are capable of removing nitrogen and phosphorus from the effluents (Chisti 2007; Pérez-Martínez et al. 2010; Pittman et al. 2011). They are also capable of converting the nutrients to biomass at much higher rates than the conventional cultures of oleaginous plants. They do not occupy agricultural land for the cultivation, require only water and atmospheric CO_2 associated with sunlight for growth (Mata et al. 2010; Huan et al. 2010).
The mass production of microalgae faces several challenges to become economically feasible, since the costs associated with the cultivation are still higher than the value of the final product, i.e. biodiesel (Schenk et al. 2008). One of the strategies to resolve this problem is the use of a low cost culture medium. Some species of microalgae are capable of growing in the water of low quality (saline or brackish), including municipal, industrial and agricultural wastewater, as well as removing nitrogen and phosphorus from these effluents (Pérez-Martínez et al. 2010; Pittman et al. 2011).

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The arid and semiarid regions of the world occupy around 40% of the earth's surface and due to increasing anthropogenic pressures, destruction of native vegetation, soil degradation, accelerated processes of desertification and global climate change, the proportions of these areas have increased substantially in recent decades (Barbosa 2006). The Brazilian northeastern semi-arid region represents approximately 18.2% of the nations territory and 70.6% of the Northeast region. Large areas of this region exhibit favorable conditions for microalgae cultivation, because the temperature presents little variation, insolation is high and large quantities of groundwater are saline, or brackish, and therefore unfit for human consumption and irrigation, making these areas unsuitable for agriculture. At the same time, it is a semi-arid region with a relatively high population density compared to other semi-arid regions in the world with over 20 million people, representing more than 10% of Brazil's population. In this context, the semi-arid region of the Brazilian Northeast becomes an interesting region for the cultivation of microalgae on a large scale, offering space, sunlight, as well as low cost cultivation medium (brackish, or saline groundwater) and nutrient sources (municipal wastewater).

This study aimed to evaluate the growth potential of the green microalgae *Nannochloropsis sp*. in culture medium using brackish groundwater from the Brazilian semi-arid region and municipal wastewater as a nutrient source.

MATERIALS AND METHODS

Microalgae, medium composition and preparation of culture media

The species selected for cultivation was a strain of *Nannochloropsis sp.* obtained from the governmental aquiculture project Bahia Pesca and maintained in the algae collection of the Laboratory for Bioenergy and Catalysis (LABEC), located at the Polytechnical School, Federal University of Bahia (EP UFBA). The microalgal species was chosen due to its high content of triglycerides, as reported in literature (Chisti 2007; Moazami et al. 2012; Talukder et al. 2012) and based on previous studies evaluating the growth of several species present in the algae collection in culture medium with saline water indicating the best results for *Nannochloropsis* sp. The wastewater used as a nutrient source was collected at the treatment station in Praia do Forte (12 ° 39'S, 38 ° 5'W), Bahia, 80 km north of Salvador. The wastewater was removed after the anaerobic treatment in an UASB reactor and analyzed at the Laboratory for Residues and Effluents (LABRE), also situated at EP UFBA. The brackish water used for the preparation of culture media was collected from a well in the region of Ipirá (12 º 09'S 39 º 44'W), Bahia, Brazil and analyzed at the Laboratory of the Department of Environmental Engineering (LABDEA), EP UFBA, following the procedures described in the Standard Methods for the Examination of Water and Wastewater (Apha 2005).

Medium composition

Nutrient addition to the culture media was based on the modified Conway Medium (Walne, 1979) consisting of the nutrients ($mg.L^{-1}$): Na_2EDTA, 45; $NaNO_3$, 100; H_3BO_3, 33.6; Na_2HPO_4, 20; $MnCl_2.4H_2O$, 0.36 ; $FeCl_3.6H_2O$, 1.3, 1mL L^{-1} of trace metal solution ($ZnCl_2$, 2,1 $g.L^{-1}$; $CoCl_2.6H_2O$, 2 $g.L^{-1}$; $(NH_4)6Mo_7O_{24}.4H_2O$, 0,9 $g.L^{-1}$; $CuSO_4.5H_2O$, 2 $g.L^{-1}$) and vitamin solution 1 $mL.L^{-1}$ (Tiamina, 100 $mg.L^{-1}$; Cianocobalamina, 2 $mg.L^{-1}$; Biotina, 1 $mg.L^{-1}$). Growth media with Conway nutrients were prepared using either diluted seawater (80% seawater, 20% distilled water) (C- controls), or brackish groundwater (SG). Growth media receiving municipal wastewater as a nutrient source (SGMW) were prepared with brackish groundwater and varying volumes of wastewater (expressed in % of the total volume of the culture medium): V_1 =19%, V_2 = 22%, V_3 = 44% and V_4 = 50%. Sterilization by autoclaving during 15 min was performed only for the controls in the first experiment. All other culture media were inoculated without sterilization.

Experiments

In a first step, the overall ability of *Nannochloropsis sp*. to grow in brackish groundwater and to use the municipal wastewater as a nutrient source was evaluated. Culture media C, SG and SGMW were prepared in duplicates. Then evaluated the growth of *Nannochloropsis* sp in SG (controls) and SGMW medium with four different wastewater concentrations: V_1 =19%, V_2 = 22%, V_3 = 44% and V_4=50%.The cultivation tests were performed by adding 10 mL of inoculum to 150 mL of culture medium in aerated Erlenmeyer flasks of 250 mL, which were maintained at 22 ºC and a light intensity of approximately 60 μmol photons m^{-2} s^{-1}. The monitoring of the cultures was performed by cell counting in a Neubauer counting chamber (Boeco Germany) using an optical microscope (Axiostar plus, Carl Zeiss) during 14 to 16 days. When the cultures reached the stationary phase, biomass production was evaluated by the determination of dry weight gravimetrically as follows: fiberglass filter paper (45 µm, Macherey-Nagel) was dried until weight remained constant. Then 30 mL of each culture was filtered and filters were placed to dry at 70°C for 24h. Filters were weighed and the dry biomass was determined by the difference between the initial and final filter weight.

RESULTS AND DISCUSSION

Groundwater and wastewater analysis

Table 1 shows some characteristics of the brackish groundwater compared to seawater, used for the preparation of culture media for *Nannochloropsis* sp.

Table 1 - Chemical composition of groundwater from the well in Ipirá, Bahia, Brazil and sea water from Ondina Beach, Salvador, Bahia, Brazil sampled December 7th 2010.

Parameter (mg L^{-1})	Brackish groundwater (well)	Sea water	Methods
Chloride	2170	21072	SM 4500-Cl B 21ªEd
Salinity *	3920	38068	-
Phosphate	0.11	<0.01	SM 4500-CP D 21ªEd
Nitrate	8.73	0.03	SM 4500-NO3 E 21ªEd
Total Nitrogen	8.30	0.80	HACH 10071 ou 10072 (LR e HR)
Suspended solids	<10	16.60	SM 2540D 21ªEd

* calculated by the JPOTS formula (UNESCO, 1966): S(‰) = 1,80655 Cl(‰)

The wastewater used in the experiments was removed after the anaerobic treatment in an UASB reactor at the treatment station in Praia do Forte, Bahia, Brazil and was analyzed. The results are shown in Table 2.

Table 2 - Characterization of the municipal wastewater after UASB reactor at the treatment station Praia do Forte, Bahia, Brazil.

Parameter	Concentration
Ammonia	39,20 mg NH_3-N L^{-1}
TKN*	54,88 mg TKN L^{-1}
soluble P	4,97 mg $(PO_4)^{3-}$-P L^{-1}
pH	7,18
Alkalinity	345 mg $CaCO_3$ L^{-1}

*TKN = Total Kjeldahl Nitrogen

Cell growth monitoring

The first test was carried out in order to evaluate whether the selected *Nannochloropsis* sp. could grow in the medium with brackish water and would be able to utilize nutrients from municipal wastewater. Figure 1 shows that the growth curves were similar in all the three culture media. Cultures in Conway medium prepared with brackish groundwater (SG) and those in brackish groundwater with addition of V=19% municipal wastewater (SGMW) did not differ considerably from the control cultures. Therefore, it could be concluded that it was possible to use the brackish groundwater with municipal wastewater as a nutrient source as culture medium of for the strain of *Nannochloropsis* sp. No contamination by other microorganisms was observed in the SGMW cultures, which was in accordance with Macalta (2011) who considered marine microalgae as more suitable for large scale production, because the salinity of the medium avoided contamination.

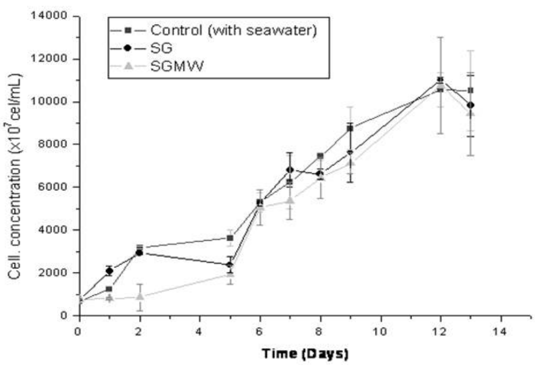

Figure 1 - Growth curve of *Nannochloropsis* sp. (control - cultivation in modified Conway medium prepared with diluted sea water; SG - cultivation in modified Conway prepared with brackish groundwater; SGMW – cultivation in brackish groundwater with addition of wastewater as a nutrient source).

The amount of dry biomass obtained from each culture medium at the end of the experiment is shown in Table 3. Final biomass concentrations obtained in three culture media were quite similar, not showing a considerable difference between them Comparing with the results obtained by Gris (2011), who obtained a maximum biomass concentration of 0.2182 $g.L^{-1}$ (under culture conditions of 45 $mg.L^{-1}$ of $NaNO_3$, 68 μmol photons. $m^{-2}.s^{-1}$ and T = 24°C), the biomass concentration obtained in this study was higher, with 0.9 $g.L^{-1}$ (using wastewater as a nutrient source, light intensity of 60 μmol photons. $m^{-2}.s^{-1}$ and T = 22°C). These results confirmed that the selected species was able to grow in the culture medium with wastewater.

In the following experiments, the wastewater addition was increased randomLy in order to evaluate if the biomass concentration could be enhanced for higher nutrient availability. Figure 2 shows the cell concentrations ($x10^7$ $cel.mL^{-1}$) obtained for each variation of wastewater volume added. It could be observed that the cell density of the control (Conway medium with bracksih groundwater) was higher than the cell concentrations in the experiments with wastewater addition.

Table 3 - Quantification of cell concentration and dry biomass in the first experiment (C – Conway medium with sea water; SG – Conway medium with saline groundwater; SGMW – saline groundwater with 19% wastewater addition).

Experiment	Cell concentration ($x10^7 cel.mL^{-1}$)	Dry biomass ($g.L^{-1}$)
C	10,5	1,86
SG	9,8	1,1
SGMW	9,4	0,9

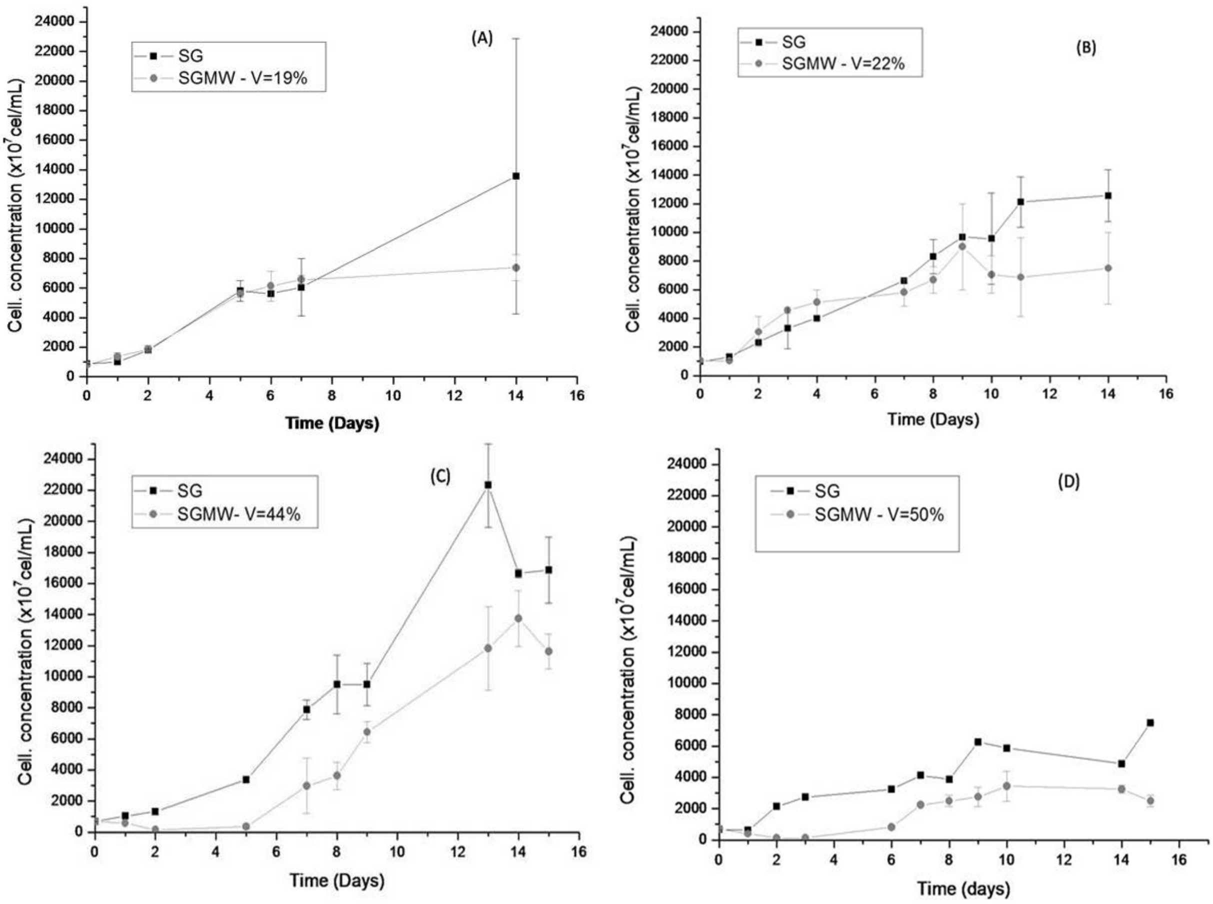

Figure 2 - Growth curves showing the evolution of cell density (10^7 $cel.L^{-1}$) of *Nannochloropsis* sp. in controls (SG – Conway medium with brackish groundwater) and with four different volumes of wastewater added (SGMW): (A) 19.4%, (B) 22%, (C) 44%, (D) 50%.

It could also be observed that increasing wastewater addition from 19 to 22% did not increase cell concentration considerably (Table 4), which might have been due to the relatively small increase of 3%. But with the addition of 44%, cell growth was almost two times as high as compared to the addition of 19 and 22% the wastewater. At a volume of 50%, a reduction in cellular growth was observed both in the control and wastewater cultures. As the reduction also occurred in controls, it could not be attributed to excess of wastewater addition. Comparing the results of dry biomass achieved with each volume of wastewater added to the values of the respective controls (Table 4), it was seen that the addition of 19.4% and 22% of wastewater did not lead to a reduction of biomass production, while the higher additions (40 and 50%) led to 20-30% reduction in comparison to the controls. Reduction at higher volumes of wastewater could be attributed to some kind of inhibitory substance present in the wastewater, or to the decrease in the salinity due to the higher volume of the wastewater added.

While highest cell concentrations were obtained for a wastewater volume of 44%, biomass concentration did not vary much between the volumes of 19nto 44%, being highest at 44%. This result confirmed that the biomass production was not directly related to cell number, since this also depended on the cell size. Cell growth and biomass concentration were comparable in the first (Table 3) and in the second experiment (Table 4). Compared to cell density values published for *Nannochloropsis* in the literature, reaching up to 52.9 x10^7cel.mL^{-1} in an outdoor experiment (588L coiled tube reactor, 23-31ºC, 530 µmol photons m^{-2} s^{-1}) (Briassoulis et al. 2010), the values determined in this study (batch culture, 22ºC, 60 µmol photons m^{-2} s^{-1}, aeration) were relatively low. The main factor responsible for this difference might be the different light intensities, which direct influenced the photosynthesis, and therefore the growth. Higher temperatures may accelerate the organism's metabolism, enhancing growth as well. Another possible factor for low cell density is the pH, which determines the availability of CO_2 and HCO^{3-} for algal photosynthesis. As pH was not controlled, pH values varied between 9.0 and 10.0 after the first culture days, which ensured that at least 68% of the dissolved inorganic carbon (DIC) was present in a form that could be utilized by the algae (Knud-Hansen 2006).

Table 4 - Quantification of cell concentration and dry biomass in the second experiment (Control (SG) – Conway medium in brackish groundwater; SGMW – Brackish groundwater with addition of different volumes of municipal wastewater: 19.4%, 22%, 44% and 50%).

	CONTROL (SG)		**SGMW**	
Wastewater volume (%)	**Cell concentration (x10^7cel.mL^{-1})**	**Dry biomass (g.L^{-1})**	**Cell concentration (x10^7cel.mL^{-1})**	**Dry biomass (g.L^{-1})**
19.4	13.5	0.84	7.4	0.85
22	12.5	0.95	7.5	0.97
44	16.6	1.20	13.8	0.96
50	7.5	0.53	2.5	0.39

Another phenomenon observed during the experiments with higher volumes of wastewater was that the test cultures had a yellowish color compared to the controls during all days of culture, indicating that the cells of *Nannochloropsis* sp. changed to a stress condition. This stress could be due to the absence of a nutrient, or the decreasing salinity as a consequence of wastewater addition to the brackish groundwater. Pigment production by *N. gaditana* as a response to nutrient deficiency (P, N, S), or salinity (15 to 100 ppt) were investigated by Froján et al. (2007) and Lubián (2000), respectively. Nutrient limitation, especially S limitation, increased the production of carotenoids, while no clear effect of salinity on the pigment contents was found. The influence of salinity on biomass and lipid production by *Nannochloropsis* sp. has been investigated, concentrating on the influence of hypersalinity stress on the growth performance of these microalgae (Renaud and Parry 1994; Pal et al. 2011; Gu et al. 2012). Salinity ranges investigated ranged from 10 to 55‰, with no significant differences in the growth rate and biomass production found in the range of 10 to 35‰, decreasing with salinity above this level. No references could be found reporting growth behavior of *Nannochloropsis* at salinity levels lower to 10‰. The results obtained in the present work showed that the strain of *Nannochloropsis* sp. tested was able to grow at a salinity as low as 2‰ (50% wastewater addition).

The brackish groundwater used in the present study originated from a well in the semiarid northeast region of Brazil. This region has a high potential for microalgae biomass production due to low variation in temperature range, high solar radiation, presence of high amounts of brackish and saline ground water inappropriate for consumption or irrigation, as well as the presence of large areas unsuitable for agricultural use (Pereira et al. 2011). Furthermore, the Brazilian semiarid region, unlike many other semiarid regions of the world, is densely populated, with over 20 million inhabitants, representing more than 10% of the total population of the country (Cirilo et al. 2008), which implies a corresponding water consumption and wastewater production. The cultivation of microalgae species that tolerate brackish groundwater as a growth medium and are able to use domestic wastewater as a nutrient source, such as the strain of *Nannochloropsis* tested in the present work, could present a sustainable economic alternative for the local population as well as contributing to wastewater treatment and deposition in this region.

CONCLUSION

The possibilities of the economical use of microalgal biomass are well known. Species of the genus *Nannochloropsis* have been evaluated worldwide for biomass production, as they are able to produce high amounts of lipids, which can be used for biodiesel production. Besides this, these organisms can produce pigments as carotenoids under certain conditions. The strain of *Nannochloropsis* sp. tested was able to grow in brackish groundwater from a well in semi-arid arid northeastern region of Brazil with domestic wastewater as the only source of nutrients. The species was able to grow in low salinities, as groundwater had a salinity ten times lower than that of seawater. Both of these resources, brackish groundwater and domestic wastewater are widely available in the Brazilian Semi-Arid Northeast, a region with a low economic development. The cultivation of microalgal species such as the strain of *Nannochloropsis* tested has the potential to turn itself into a sustainable economic alternative for this region and may contribute to wastewater treatment and deposition.

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