BRAZILIAN JOURNAL OF PETROLEUM AND GAS
Journal Homepage: www.portalabpg.org.br/bjpg
A publication of the ABPG BRAZILIAN ASSOCIATION OF R&D IN PETROLEUM AND GAS

NANNOCHLOROPSIS OCULATA GROWTH IN PRODUCED WATER: AN ALTERNATIVE FOR MASSIVE MICROALGAE BIOMASS PRODUCTION

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ABSTRACT

Produced water (PW), extracted as a petroleum byproduct, presents high contents of impurities and, therefore, must be treated before being released into the environment. This clean-up process increases oil production costs. Microalgae are photosynthetic microorganisms that can produce biofuels and treat effluents. This work tested the viability of growing marine microalgae species in culture medium with PW. In the first experiment, the marine microalgae *Nannochloropsis oculata* was inoculated in culture media containing different concentrations (0, 50, and 100%) of PW. In the second experiment, the *N. oculata* adapted to grow in PW was re-inoculated into media with different proportions of this effluent (0, 50, and 100%) to evaluate a possible adaptation. *N. oculata* presented significant growth in diluted and pure PW. However, pre-adaptation did not result in higher biomass production. These results indicate that *N. oculata* can grow in this effluent and generate bio-products.

KEYWORDS

produced water; microalgae; *Nannochloropsis oculata;* oil and gas; biotechnology of microalgae

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doi:10.5419/bjpg2014-0011

1. INTRODUCTION

The oil exploitation is one of the most important industrial activities of modern society and petroleum has many other applications in chemical industry besides fuel production. However, this activity generates an important effluent called produced water (PW), that is, the formation water, which is extracted along with petroleum. This geologic water has varying concentrations of impurities such as inorganic salts, aliphatic and aromatic hydrocarbons, phenols, metals, radionuclides, and added elements used in the separation of water and oil in the production line. Moreover, due to the presence of high amounts of NaCl in its composition, produced water is a matter of great concern **(Campos et al. 2002)**. The treatment of this water is, thus, necessary in order to avoid environmental impacts, besides the increase in oil production costs. The characteristics and amounts of PW depend on the geological formation of the reservoirs, the age of the oil well, and the procedure used in the extraction of the oil. A new oil field produces, on average, 5 to 15% of PW, but as the oil wells get depleted the amount of PW increases to 90% of total volume extracted, greatly exceeding the volume of oil produced **(Hansen & Davies, 1994)**.

In offshore areas, the produced water that is not re-injected in the well could be discharged directly into the ocean, which has great capacity of dilution. However, in onshore fields, which account for approximately 23% of the Brazilian oil production, PW must be treated before being discarded in the near coastal region. In this case, the discarded PW must attend the legal demands that regulate effluents characteristics **(CONAMA, 2007)**. Even if re-injected in the wells, PW must be treated to remove compounds such as suspended solids, gases and bacteria that induce corrosion **(Stephenson, 1992)**.

With the advancement of oil and gas exploitation technologies and the growth of environmental concerns, bioremediation techniques have been used to improve the management and treatment of effluents, such as produced water. Many bioremediation methods use microorganisms to remove or convert the contaminants into less toxic components. Organisms like bacteria, fungi, and microalgae have been used in many bioremediation procedures **(Kao & Wang, 2000)**.

Microalgae are unicellular organisms that make photosynthesis due to the presence of chlorophyll *a* and constitute the basis of food webs of most aquatic environments. These microorganisms have important applications in the food and pharmaceutical industries and, more recently, have been pointed out as an alternative for the production of biofuels, especially the biodiesel **(Chisti, 2007)**. The microalgae biomass and its extracts have substances like polyunsaturated fatty acids, proteins and pigments with antioxidant and immunological properties, besides enzymes, antibiotic elements and vitamins. Lipids and sugars produced by the microalgae can also be used in the biofuel production **(Slade & Bauen, 2013)**.

The biodiesel production has received considerable attention in recent years because it is biodegradable, renewable, and nontoxic. This fuel is nowadays produced from animal fat, or oil seed of some superior plants. However, more recently, the microalgae has been considered as a potential feedstock, due to its high photosynthetic efficiency, rapid growth, and high biomass production. Nevertheless, the large-scale production of this organism still presents some financial challenges such as the high costs of resources to produce and recover the produced microalgae biomass. The high cost of nutrients such as nitrogen and phosphorus, used in culture media, is certainly a limiting factor for microalgae mass production, and represents an obstacle to the expansion of this economic activity. Moreover, the use of the abovementioned nutrients in microalgae production could compete directly with agriculture demands, in case production increases considerably to attend the biodiesel demand of countries.

It is well known that produced water has high concentrations of nitrogen elements and phosphate **(Ahmadun et al., 2009)**. Therefore, this effluent could be used for microalgae growth, representing an important alternative for culture media normally employed in large-scale microalgae production, contributing to reduce the cost and the environmental impact caused by this oil industry effluent. Moreover, the growth of microalgae in PW could contribute to clean up this effluent, and to produce bioelements of high commercial value.

Researchers of the Laboratory of Phytoplankton and Marine Microorganisms of the Federal University of Rio Grande (FURG), southern Brazil,

Table 1. *Nannocloropsis oculata* in three (3) treatments with different concentrations of produced water.

Treatment	Microalgae Inoculum (L)	f/2 medium (L)	Produced water (L)	Total volume (L)
0%	0.3	0.7	-	1
50%	0.3	0.35	0.35	1
100%	0.3	-	0.7	1

have isolated thirteen strains of microalgae capable of growing in produced water and removing some pollutants of this effluent **(Mendes et al., 2010)**. Nevertheless, these species did not present high production rates. Therefore, it would be interesting to evaluate other microalgae species already employed in aquaculture that are resistant, present high production rates and produce elements of commercial value.

The Eustigmatophycea *Nannochloropsis oculata*, a species of marine microalgae, shows high production rates, being cultivable even under adverse environmental conditions. This microalgae produces, among other elements, large amounts of polyunsaturated fatty acids like the EPA (C20:5), which is of great interest to the pharmaceutical and food industries **(Borges et al., 2011)**. For these reasons, this microalga was chosen to be tested in culture media with different amounts of produced water.

2. MATERIALS AND METHODS

The studies were conducted with the marine microalgae *Nannocloropsis oculata* (Eustigmatophycea) from the culture collection of the Laboratory of Phytoplankton and Marine Microorganisms of the Federal University of Rio Grande (FURG) (code NANNOCUL-1).

Two experiments were carried out to evaluate the growth conditions of unadapted and pre-adapted *N. oculata* in medium with different concentrations of produced water. In the first experiment, the *N. oculata* was cultured, initially, in f/2 medium **(Guillard, 1975)**, periodically inoculated (3:10) in a new culture medium after reaching the exponential (LOG) growth phase. This process was repeated until the culture media reached a volume of 9L. Then, the 9L culture was divided into three treatments, (1L triplicates), with the following amounts of PW: 0; 50; and, 100%, as described in Table 1.

In the second experiment, the microalgae that had grown in pure produced water of experiment 1 (treatment 100%), was re-inoculated (3:1) only in PW until reaching a volume of 9L. When it reached the LOG phase, after 7 days, the culture was inoculated in culture media containing 0; 50; and 100% PW, following the same steps described before.

Both experiments lasted for 19 days, and the flasks with the cultures were kept in a germination chamber (Fanem model 347-CDG), with irradiance of 100 µ mol photons m^{-2} s^{-1}, 12L:12D photoperiod, and temperature 25°C. Final salinity of all culture media was 25.

The produced water used in the experiments was collected in the Processing Unit of Fluids Treatment of Petrobras in Guamaré, Northeast Brazil, and the compounds of this effluent are described in Table 2.

Carbon dioxide was introduced into the samples through constant air flow.

To evaluate the *Nannochloropsis oculata* cell growth in both experiments, water samples were collected every two days from each treatment and cells were counted in Neubauer chamber, using transmitted light microscope (Nikon), with 100x final magnification.

The microalgae growth rate (µ) was determined during the LOG phase as follows:

$$[\mu = \ln X_t - \ln X_0 . (T - T_0)^{-1}] \quad (1)$$

Where, X_0 and X_t are the cell abundances at the initial final period of the LOG phase.

The cells yield was determined subtracting the highest cell abundance from the minimum value **(Stein, 1984; Schelegel,1986)**:

$$(X = X_{max} - X_0) \quad (1)$$

Statistical differences among the treatments were evaluated through the analysis of variance (ANOVA- one way) followed by Tukey's *ad hoc*

Table 2. Compounds of produced water of the Processing Unit of Fluids Treatment of Petrobras, in Guamaré, Northeast Brazil, and the parameters accepted in the relevant legislation **(CONAMA, 357/Art.34)**.

PARAMETERS	RESULTS (mg/L)	RES.357 (mg/L)
pH (adm)	7.35	5.0 - 9.0
salinity (NaCl)	2	7
oil and greases	23.4	20
arsenic	0.001	0.5
barium	0.3358	5
boron	1.2	5
cadmium	< 0.004	0.2
lead	< 0.01	0.5
cyanide	0.064	0.2
cuprum	< 0.01	1
trivalent chrome	< 0.01	0.5
hevalent chrome	< 0.01	0.5
tin	< 0,01	4
iron	< 0.01	15
fluoride	1.43	10
manganese	< 0.01	1
mercury	< 0.001	0.01
nickel	< 0.008	2
total ammonial nitrogen	1.11	20
silver	< 0.001	0.1
selenium	< 0.0030	0.3
sulfides	0.1	1
zinc	0.013	5

multiple comparisons test ($p \leq 0.05$). The homoscedasticity and normal distribution of the data were previously tested and appropriate data transformation (logarithmic) were conducted, when required **(Zar, 1996)**.

3. RESULTS AND DISCUSSION

In the first experiment, the treatment with 0% of produced water (Control - f/2 medium) presented initial abundance of 535 × 10^4 cel. mL^{-1}, and the highest abundance of 2599 × 10^4 cel. mL^{-1}. In the treatment with 50% produced water, the initial cell abundance was 371 × 10^4 cel. mL^{-1}, and maximum abundance 1510 × 10^4 cel. mL^{-1}. At pure produced water (100%), the *N. oculata* reached the maximum abundance on the 9th day with 617 × 10^4 cel. mL^{-1}, whereas the initial abundance in this treatment was 346 × 10^4 cel. mL^{-1}. There were statistical differences among maximum abundances of all treatments (Fig. 1).

The highest microalgae yield was reached at the 0% treatment, but both growth rates (μ) and yield did not present statistical differences among treatments. The calculated growth rates (μ) varied from 0.06 d^{-1} (100%) to 0.13 d^{-1} (0%), see Table 3.

In the second experiment, the initial cell abundance in the 0% treatment was 357 10^4 cel/mL. This treatment also presented the highest abundance (1459 × 10^4 cel.mL^{-1}), while in the 50%, the initial cell abundance was 174 ×10^4 cel.mL^{-1}. On the treatment of 100%, the initial cell abundance

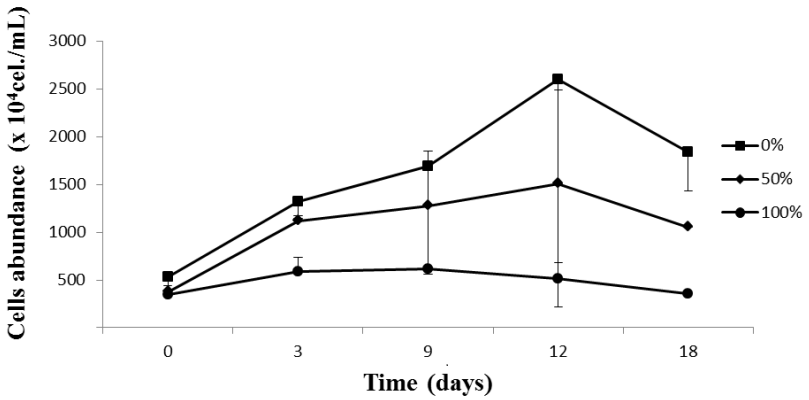

Figure 1. Experiment 1 - Cell abundance (10^4 cel. mL^{-1}) of *Nannochloropsis oculata* inoculated in culture media with different concentrations of produced water (0, 50 and 100%).

Table 3. Growth rate (μ) of experiments 1 (microalgae not adapted to the effluent) and 2 (with adaptation prior to the effluent) and yield ($X = X_{max} - X_0$) experiments 1 and 2.

	GROWTH RATE			CELL YIELD		
	0%	**50%**	**100%**	**0%**	**50%**	**100%**
EXPERIMENT 1	0.13	0.12	0.06	2063	1139	271
EXPERIMENT 2	0.08	0.22	0.09	1102	466	353

was 527× 10^4 cel.mL^{-1}. The maximum cell number in both treatments was 640 and 880 ×10^4 cel.mL^{-1}, respectively (Fig. 2). In this experiment, the highest cell yield also occurred in 0% (1102 ×10^4 cel.mL^{-1}), and the growth rate of this treatment was 0.08 d^{-1}. The growth rate of the 100% treatment (0.22 d^{-1}) was bigger than the Control, but the cell yield in this treatment only reached 353 ×10^4 cel.mL^{-1} (Tab. 3). The cell yield and growth rates did not present statistical differences.

The *N. oculata* without previous adaptation (experiment 1) showed better performance, with higher cell abundance and yield, in the 50 to 100% treatments ($p < 0.05$) (Tab. 3) than in the same treatments in the second experiment. However, growth rates in treatments 50 and 100% of experiment 2 were higher than the Control (0%), indicating a possible adaptive advantage of pre-adapted cells regarding the growth speed, but not of biomass production.

The growth rates (μ) determined in experiments 1 and 2 (Table 3) are smaller than those mentioned by **Spolaore et al. (2006)** (0.86 d^{-1}), but was in the same order of that calculated by Converti et al. (2009), which achieved 0.13 μ d^{-1} using f/2 medium. However, the cell yield reached in both experiments were close to that found by Meinerz (2007) (2882 × 10^4 cel.mL^{-1}), especially in the 50% produced water treatment of experiment 1, justifying the use of this effluent as culture media for the *Nannochloropsis oculata.*

It is very likely that the physiological stress caused by the compounds present in produced water generated the lower results achieved in both experiments, although it must be considered that the 50 and 100% treatments of the second experiment showed higher values of μ, similar to the 0% treatment, but with lower biomass production. Factors such as excess of nutrients, presence of metals and other organic compounds may explain the lower biomass production in treatments where produced water was added. However, according to **Wood et al. (2005)** the physiological adjustments necessary for a strain of microalgae to be

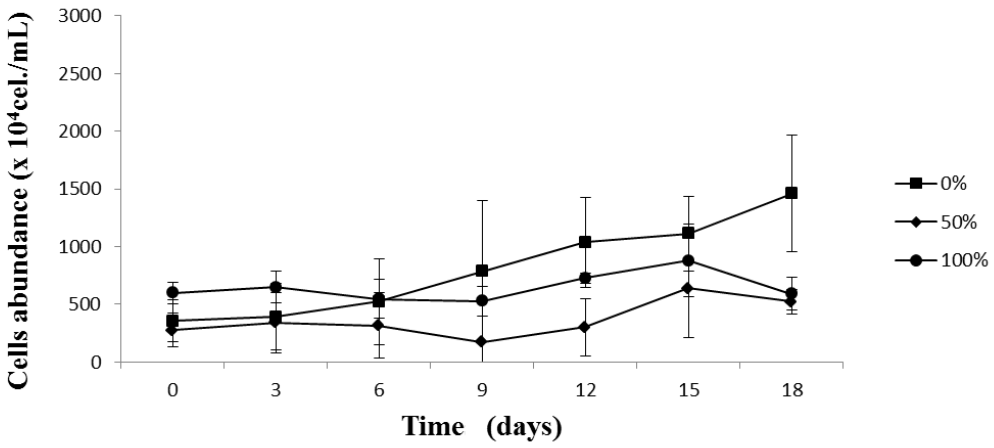

Figure 2. Experiment 2 - Cell abundance (10^4 cel. mL^{-1}) *Nannochloropsis oculata* pre-adapted to grow produced water, inoculated in culture media with different concentrations of produced water (0, 50 and 100%).

considered acclimated are only achieved after successive transfers in the stressful medium, and several generations after the initial inoculums, where efficient adaptation of the microalgae is achieved for the new growing conditions.

Some algae are able to withstand extreme conditions of salinity, temperature, and contaminants, having a competitive advantage over other more sensitive microalgae.

Moreover, many of these algae can produce allelopathic compounds, having a negative effect on other microalgae species and even other organisms, inhibiting their growth and proliferation. The use of this strategy to manage contamination would be an interesting tool, representing a promising way for further microalgae massive production **(Mendes & Vermelho, 2013)**.

Microalgae of the genus *Nannochloropsis* have already been tested in other effluents, with good results. For example, **Bianchini et al. (2006)** achieved higher growth and biomass of microalgae *N. oculata* in medium with effluent of super-intensive production of the white shrimp *Litopenaeus vannamei,* compared to cultures grown in f/2 medium. **Jiang et al. (2011)** also found that the *Nannochloropsis sp* showed better growth rates and biomass production in treatments with 50% of domestic sewage and attributed this result to the greater availability of nutrients and light on cultivation.

The influence of different salinities and concentrations of dissolved nutrients (N and P) in the growth rate of *N. oculata* was studied by **Meinerz (2007)**. The author observed that, despite this microalgae being highly adaptable to environmental variations, its optimal growth is governed by the interaction of temperature and salinity.

Traditional chemical and mechanical treatments used to clean up produced water are complex and expensive **(Tellez et al., 2002)** and depend very much on the specific characteristics of the generated produced water. On the other hand, the use of microalgae for the purification of produced water may represent an important advance in the bioremediation of this effluent, although further studies are necessary to corroborate this hypothesis.

4. CONCLUSIONS

The microalgae *Nannochloropsis oculata* showed great potential for cultivation in produced water generated from oil production. Growth in culture media with 50% of the effluent produced significant biomass in rates similar to those achieved in traditional culture systems. The culture in pure produced water (100%) was also possible, although showing smaller microalgae biomass yields. Further studies should be conducted in order to determine the performance of long pre-adapted microalgae, as well as its lipid production, fatty acid profile and other parameters of biotechnological interest as pigments and carbohydrates. Similarly, the bioremediation capacity of the *Nannochloropsis oculata* should be determined especially for the removal of metals and organic compounds.

ACKNOWLEDGEMENTS

The author Alessandra Arriada had an ANP - PRH27 (Programa de Recursos Humanos 27 - ANP Agência Nacional do Petróleo) Scholarship, and Paulo Cesar Abreu is a research fellow of the Brazilian Council for Science and Technology (CNPq) under the Brazilian Ministry of Science, Technology, and Innovation.

5. REFERENCES

Ahmadun, F.R.; Pendashteh, A.; Abdullah, C.C.; Biak, D.R.; Madaeni, S.S.; Zurina, Z. Review of technologies for oil and gas produced water treatment. **Journal of Hazardous Materials**, v. 170, p. 530–551, 2009.
http://dx.doi.org/10.1016/j.jhazmat.2009.05.044

Bianchini, R.; Ohser, S.; Avila, R.; Braga, M.V.; Cunha, P.; Lamarca, C.P.; Santos, M.E. Produção de biomassa e teores de carbono, hidrogênio, nitrogênio e proteína em microalgas. **Ciência Rural**; v. 36, p. 760-1767, 2006.

Borges, L.V.; Morón-Villarreyes, J.A.; D'Oca, M.G.M.; Abreu, P.C. Effects of flocculants on lipid extraction and fatty acid composition of the microalgae *Nannochloropsis oculata* and *Thalassiosira weissflogii*. **Biomass and Bioenergy**; v. 35, p. 4449–4454, 2011.
http://dx.doi.org/10.1016/j.biombioe.2011.09.003

Campos, J.C.; Borges, R.M.H.; Oliveira Filho, A.M., Nóbrega, R.; Sant'Anna, Jr. G. Oilfield wastewater treatment by combined microfiltration and biological processes. **Water Research**; v36, p. 95-104, 2002. http://dx.doi.org/10.1016/S0043-1354(01)00203-2

Chisti, Y. Biodiesel from microalgae. **Biotechnology Advances**, v. 25, p. 294-306, 2007. http://dx.doi.org/10.1016/j.biotechadv.2007.02.001

CONAMA, Conselho Nacional do Meio Ambiente. Resolução nº 393 de agosto de 2007. Ministério do Meio Ambiente, Brasília.

Converti, A.; Casazza, A.; Ortiz, E.; Perego, P.; Del Borghi, M. Effect of temperature and nitrogen concentration on the growth and lipid content of *Nannochlorpsis oculata* and *Chlorella vulgaris* for biodiesel production. **Chemical Engineering Process**; v. 48, p. 1146-1151, 2009. http://dx.doi.org/10.1016/j.cep.2009.03.006

Guillard, R.R.L. Culture of phytoplankton for feeding marine invertebrates, In:Smith, WL & MH Chanley (Eds.) **Culture of Marine Invertebrate Animals**. Plenum, New York, p. 29-60, 1975. http://dx.doi.org/10.1007/978-1-4615-8714-9_3

Hansen, B.R.; Davies, S.R.H. Review of Potential Technologies for the Removal of Dissolved Components from Produced Water. **Chemical Engineering Research**; v. 72, p. 76-88, 1994.

Jiang, Z.; Shengjun, L.; Xiaolei, F.; Zhiman, Y.; Rongbo, G. Biomass and lipid production of marine microalgae using municipal wastewater and high concentration of CO2. **Applied Energy**; v. 88, p. 3336–3341, 2011. http://dx.doi.org/10.1016/j.apenergy.2011.03.043

Kao, C.M.; Wang, C.C. Control of BTEX migration by intrinsic bioremediation at a gasoline spill site. **Water Research**; v. 34, p. 3413-23, 2000. http://dx.doi.org/10.1016/S0043-1354(00)00070-1

Meinerz, L.I. **Influência da temperatura, salinidade e nutrientes dissolvidos (N e P) no cultivo de microalgas estuarina e costeira**, Dissertação Mestrado em Aquicultura, Furg, Rio Grande, RS, 2007.

Mendes, L.B.; Cunha, P.C.R.; Montes D'Oca, M.; Abreu, P.C.; Primel, E. Method for removing pollutants from produced water. **US Patent** 7955505 B2, 2010.

Mendes, L.B.; Vermelho, A.B. Allelopathy as a potential strategy to improve microalgae cultivation. **Biotechnology for Biofuels** v. 6, p. 152, 2013. http://dx.doi.org/10.1186/1754-6834-6-152

Schelegel, H.G. **General microbiology**, USA. Cambridge University Press, 1986.

Slade, R.; Bauen, A. Micro-algae cultivation for biofuels: cost energy balance, environmental impacts and future prospects. **Biomass and Bioenergy**; v. 53, p. 29-38, 2013. http://dx.doi.org/10.1016/j.biombioe.2012.12.019

Spolaore, P.; Joannis-Cassan, C.; Duran. E.; Isambert, A. Commercial applications of microalgae. **Journal of Bioscience and Bioengineering**; v. 101, p. 87-96, 2006. http://dx.doi.org/10.1263/jbb.101.87

Stein, J.R. Handbook of phycological methods. Culture methods and growth measurements, 2th ed. **Cambridge Univ. Press**. 1984.

Stephenson, M.T. Components of produced water: a compilation of industry studies. **Journal of Petroleum Technology**; v. 44, p. 548–603, 1992. http://dx.doi.org/10.2118/23313-PA

Tellez, G.T.; Nirmalakhandan, N.; Gardea-Torresdey, J.L. Performance evaluation of an activated sludge system for removing petroleum hydrocarbons from oil field produced water, **Advances Environmental. Research**; v. 6, p. 455–470, 2002. http://dx.doi.org/10.1016/S1093-0191(01)00073-9

Wood, A.M.; Everroad.,R.C.; Wingard, L.M. Measuring growth rates in microalgal cultures. In: Andersen, R. A. Algae Culturing Tecniques. United States of America: **Elsevier Inc**., p. 201-209, 2005.

Zar, J.H. **Biostatistical analysis**. Prentice Hall, New Jersey, 1996.