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Urban Secondary Sewage: an Alternative Medium for the Culture of *Tetraselmis chuii* (Prasinophyceae) and *Dunaliella viridis* (Chlorophyceae)

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ABSTRACT

The effect of different concentrations (10, 20, 30 and 40%) of urban secondary sewage on the growth of Tetraselmis chuii (Prasinophyceae) and Dunaliella viridis (Chlorophyceae) was examined to verify the possibility of its use as an alternative culture medium for these species. Stocks and 700mL duplicate batch cultures were maintained under controlled laboratory conditions. 30% and 40% additions were the most efficient for the growth of both species. Statistical analysis of the adjusted growth (cell.mL^{-1}) and biomass (μg chlorophyll-a.mL^{-1}) curves performed using the Chi-square test ($p<0.05$) demonstrated significant differences between the nutritive medium f/2 and the 40% addition used for these two species. The results suggested that some of the concentrations tested here yielded satisfactory cell densities and growth rates when compared with other culture media (macroalgae, bovine and chicken manure extracts). Thus it could be used by semi intensive aquaculture systems.

Key words: Microalgae, culture, urban secondary sewage, alternative medium

INTRODUCTION

The culture of planktonic marine algae in Brazil, begun at the Oceanographic Institute of São Paulo during the late 60´s, where the first attempts to isolate microalgae were developed. Many works related to culture methodologies, physiologic and ecophysiologic characteristics of various species and their use as bioreactors have been studied (Aidar-Aragão and Vieira, 1986). In the Northeast region of Brazil, this activity has expanded, although in a discrete way, concomitantly with aquaculture, being directly related to the production of live food as a source of nutrients for larval stages of different organisms used in local aquaculture. In the last decade, the aquaculture activities expanded around the world and one of the major challenges facing algologists is reducing production costs while maintaining reliability. Approximately 90% of the 14.5 million metric tons of aquaculture-produced animals in 1993 were reared using phytoplankton as a feed source during one or more stages (Duerr et al., 1998). Favored genera of microalgae for larval feeds include *Chaetoceros* Ehremberg, *Thalassiosira* Cleve, *Tetraselmis* Stein, *Isochrysis* Parke, and *Nannochloropsis* Hibberd (Duerr et al., 1998).

Algal production for feeds is divided into intensive monoculture for larval stages of bivalves, shrimp, and certain fish species, and extensive culture for

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grow out of bivalves, carp, and shrimp. These organisms are fed directly and/or indirectly to the cultured larval organism. Indirect feeds consist of providing the algae through artemia, rotifers, and daphnia, which are, in turn, fed to the target larval organisms. At a calculated cost of 50/kg dry algae equivalent, there is significant pressure to replace algae with prepared diets. The largest algal production (100,000 liters/day) facilities support bivalve larval and nursery cultures (Duerr et al., 1998). The Prasinophyte *Tetraselmis*, for example, is an important live feed organism for shrimp/prawn hatchery operations because of its high nutritional value and easy of culture. It can replace *Brachionus plicatilis* and *Artemia* nauplii as live diet during the protozoeal and mysis feeding stage **(**Ronquillo-Jesse et al., 1997).

In the microalgae laboratory of the Department of Oceanography of the Federal University of Pernambuco, many works on the use and efficiency of alternative culture media have been carried out, contributing to the reduction of costs generated by the use of natural or artificial media frequently used in this region. Alternative media such as sugar cane mineralized syrup (Koening et al., 1988), and chicken manure (Koening et al., 1990) were successfully bioassayed.

Previous studies carried out using secondary sewage, in the culture of mixed marine microalgae species (Dustan and Menzel, 1971; Dustan and Tenore, 1972; Goldman and Stanley, 1974), as well as unialgal cultures (Yoneshigue-Braga et al., 1977; Costa et al., 1999), served as subsidies to the development of the experiments with the Prasinophyceae *Tetraselmis chuii* Butcher and the Chlorophyceae *Dunaliella viridis,* which had the objective of testing the efficiency of urban secondary sewage as a nutrient source for the culture of these species.

MATERIAL AND METHODS

Urban secondary sewage collection and characterization

The urban secondary sewage used in the experiments was collected from a State secondary urban treatment plant (Companhia Pernambucana de Saneamento-COMPESA) where treatments were resumed to solids extraction and reduction of the biological oxygen demand (BOD) to the levels admitted by Brazilian's legislation.

In the laboratory, the samples of sewage were filtered through a 25mm, 3μm glass microfibre filters (GF/C) mounted on a Millipore filtration unit and sterilized by autoclaving. The chemical composition of the raw sewage was studied with respect to the major dissolved nutrient salts necessary for algae growth like nitrite-N, nitrate-N and phosphate-P.

Cell cultures

No axenic stocks of *Dunaliella viridis* and *Tetraselmis chuii* were obtained from the Enterprise of Agricultural Research of Rio Grande do Norte S/A (ENPARN-"Rio Grande do Norte"-Brazil). All stocks were grown in 60mL flasks in batch cultures containing f/2 medium (Guillard, 1975) in seawater at 30 ‰ salinity, and maintained in a controlled environment room at 22 ± 1°C, under a continuous light regime by day-light fluorescent tubes (light intensity of 100μmol. $m^{-2}.s^{-1}$). Stocks were kept in exponential growth phase by transferring then to new culture medium every 2 weeks. Experiments with different urban secondary sewage concentrations were carried out after acclimation of the stocks to respective experimental conditions during a period of 15 days.

Growth experiments

Growth of *Dunaliella viridis* and *Tetraselmis chuii* was tested in 21-22 days experiments at concentrations of 10, 20, 30 and 40% of urban secondary sewage (f/2 medium and filtered seawater as controls) under the same conditions of salinity, light intensity and temperature described before for the acclimation of the stocks. For each treatment duplicate 700mL batch cultures kept without forced aeration were grown in Erlenmeyer flasks. Subsamples of 5mL were collected daily, from which aliquots of 0.0018mL were counted to determine cell densities. For this, the Neubauer chamber was used, counting the number of cells with the help of the binocular optic microscope Bausch and Lomb, and expressing them in average values of $cell.mL^{-1}$.

Specific rates of increase (daily average growth rates) were calculated during the exponential phase of each experimental unit according to Guillard (1973). Total chlorophyll-*a* concentrations were determined through subsample filtrations of 2.5mL to 10.0mL, of each experimental unit, at 4 days intervals. After the

filtration, the method of Creitz and Richards (1955) was followed, readings with a Micronal spectrophotometer, in wavelengths of 630nm, 645nm, 665nm and 750nm. The calculations of the chlorophyll-*a* concentrations were performed according to the formula of Parsons and Strickland (1963), with the results being expressed in $\mu g.mL^{-1}$. Nutrient dissolved concentrations were analyzed at the beginning, the seventh day and at the end of the experiments, according to methods described by Strickland and Parsons (1972), and Grasshoff et al. (1983). Values of pH were mesured during analysis.

All the results were expressed in average values. Adjustment curves (by CAJUS computer program) for the number of $cell.mL^{-1}$ and μg chlorophyll-$a.mL^{-1}$ (biomass) in relation to the time of culture of the two tested species were calculated according to the formula:

$$\text{Nº of cell. (or } \mu g \text{ chlorophyll-}a.mL^{-1}) = \beta_0\, t\, e^{-\beta_1 t} + \varepsilon_I$$

where β_0 and β_1 are parameters of the model at time of culture t (time in days) and ε_I is the error associated to each observation. To compare the microalgae growth models, the Chi-square test was used to the trust level of 5% ($p<0.05$) (see Costa et al., 1999).

Parcels and replicates employed during the experiments were abbreviated using the first letter of the specific epithet followed by the number that represents the tested addition (D0, D10, D20, D30, D40 and T0, T10, T20, T30,T40 represents *Dunalliella viridis* and *Tetraselmis chuii* with sewater and sewage additions of 10, 20, 30, 40%, respectively).

RESULTS

The highest cellular densities ($cell.mL^{-1}x10^3$) for *Dunaliella viridis* and *Tetraselmis chuii* experiments were observed in the control enriched with the medium f/2 (Guillard, 1975). *Dunaliella viridis* presented a maximum population density of 1,536.25 x 10^3 $cell.mL^{-1}$ on the seventh day (experimental unit D40). *Tetraselmis chuii*, with the same addition, presented 1,316.25 x 10^3 $cell.mL^{-1}$ on the eighteenth day of the experiment (Fig. 1a). The highest daily growth rates obtained for these two species were 2.11 $divisions.d^{-1}$ (30% addition) and 2.28 $divisions.d^{-1}$ (40% addition), respectively. When compared statistically to the significance level of 5% ($p<0.05$), significant differences were found between all the tested additions (Table 1). The highest biomass (Chlorophyll-*a*) registered for *Dunaliella viridis* and *Tetraselmis chuii* were 1.44$\mu g.mL^{-1}$ and 1.11$\mu g.mL^{-1}$, both obtained with the medium f/2. This control unit was significantly different ($p<0.05$) from all the tested additions. The 40% addition provided chlorophyll-*a* concentrations of 0.56$\mu g.mL^{-1}$ and 0.25$\mu g.mL^{-1}$ for *Dunaliella viridis* and *Tetraselmis chuii*, respectively (Fig. 1b).

For *Dunaliella viridis*, significant differences were not observed ($p<0.05$) between seawater and 10%, nor for 20% and 30% additions. For *Tetraselmis chuii,* significant differences were not registered ($p<0.05$) between the following treatments: 10 and 20, 10 and 40, 20 and 30, 20 and 40, and 30 and 40% additions (Table 1).

The most elevated concentrations of nitrite-N, nitrate-N and phosphate-P for both species were registered on the first day, with respective values of 0.593$\mu moles.L^{-1}$ (20% addition), 17.368$\mu moles.L^{-1}$ (40% addition) and 34.843$\mu moles.L^{-1}$ (40% addition). The nitrite-N minimum concentration for *Dunaliella viridis* (0.017$\mu moles.L^{-1}$, in the experimental unit D40), was registered on the seventh day of culture. For nitrate-N and phosphate-P the minimum concentrations were respectively 2.887$\mu moles.L^{-1}$ and 0.738$\mu moles.L^{-1}$ on the same day referred above, but at 30% addition. *Tetraselmis chuii* experiments presented smallest nitrite values on the 22nd day, in the treatment T40 (0.001$\mu moles.L^{-1}$), while for nitrate and phosphate these were 1.878$\mu moles.L^{-1}$ and 0.308$\mu moles.L^{-1}$ registered at 40% addition, on the seventh day (Fig. 2 a, b and c).

In the *Dunaliella viridis* experiments, the highest reduction in nitrite concentration (96.5%), was observed in a 22 days period at 40% addition. Nitrate and phosphate concentrations were reduced 80.2% (40% addition) and 97.5% (30% addition), respectively, in a seven-day period. In the different treatments employed for *Tetraselmis chuii*, highest reductions of 99.9% (T40-22d period), 86.40% (T30-7d period), and 99.1% (T40-7d period) were observed for nitrite, nitrate and phosphate concentrations.

Table 1 - Non-linear values of the parameters of the mathematical model used to carry out the statistical analysis ($p<0.05$) of the cell density and biomass data obtained for the species *Tetraselmis chuii* and *Dunaliella viridis* .

Specie	Treatments (%)	Mathematical model $n°=\beta_0 .t^{e.\beta 1.t}$				Computed statistic	
		β_0		β_1			
		Cell density	Biomass	Cell density	Biomass	Cell density	Biomass
T. chuii	0+	43.252	0.002	0.008	0.007	a	a
	f2•	253.769	0.106	0.003	0.003	b	b
	10	599.670	0.002	0.008	0.007	c	c
	20	133.683	0.039	0.083	0.008	d	cd*
	30	156.948	0.061	0.007	0.100	e	de*
	40	158.227	0.038	0.005	0.007	f	cdef*
D. viridis	0+	209.881	0.019	0.311	0.078	a	a
	f2	1,063.220	0.417	0.147	0.130	b	b
	10	238.908	0.005	0.151	0.117	c	ac*
	20	367.649	0.009	0.176	0.129	d	d
	30	445.445	0.140	0.174	0.144	e	de*
	40	555.686	0.189	0.154	0.133	f	f

+ Seawater

• f_2 culture medium (Guillard, 1975).

*No significant differences between tested treatments.

DISCUSSION

The concentrations of nitrite, nitrate and phosphate obtained with additions of urban secondary sewage were in most cases higher than values obtained with alternative media employed by other authors, such as the organic extracts of *Macrocystis pyrifera* (L.) C. Agard macroalgae, bovine and chicken manure aerobically biodigested for the culture of *Pavlova lutheri* (Droop); chicken manure extracts for the culture of *Tetraselmis chuii* and *Dunaliella tertiolecta* Butcher, and different additions of mineralized and non-mineralized sugar cane syrup solution for the culture of *Tetraselmis chuii* (Paniágua-Michel et al., 1987; Koening et al., 1988; Koening et al., 1990). Some alternative culture media have been used in the culture of different microscopic algae used in aquaculture systems. Experiments carried out using *Scenedesmus falcatus* Chod. and *S. quadricauda* (Turp.) de Breb. (Chlorophyta) for mobilizing nutrients of pig manure showed that algal production has often been substantial (up to 315 g $dm.m^{-2}.week^{-1}$) (Dabbadie, 1994). Arrendondo-Figueroa et al. (1998) testing liquid sheep and cow manures as alternative culture media for the growth of three species of *Chlorella* Beyerinck (*C. vulgaris, C. pyrenoidosa* and *C. minutissima*-Chlorophyta) concluded that the three algal species grew in both liquid manures media as well as in the control medium (bold-basal medium). These authors also reported that these organic compounds presented the same efficiency of the control considering the cell density, the chlorophyll concentration and the nutritional value of the culture with respect to the content of lipids, carbohydrates and proteins. They suggested that liquid sheep and cow manures could be used as a low-cost alternative growth media for the production of these microalgal species for use in aquaculture.

With urban sewage, population densities ($cell.mL^{-1}$) registered at the experimental unit T40 (1,316.25 x 10^3 $cell.mL^{-1}$), were higher than those obtained with agricultural fertilizers, fish extracts and bovine manure, urban refuse extracts, and chicken manure extracts for the culture of *Tetraselmis chuii* (Yamashita and Magalhães, 1984; Oliveira and Koening 1984; Triani et al., 1986; Koening et al., 1990). Under the same conditions and additions employed for *Tetraselmis chuii*, *Dunaliella viridis* attained a highest density of 1,536.25 x 10^3 $cell.mL^{-1}$ (D30). This cellular concentration was higher than the previously reported values for different additions of raw sewage from a municipal plant for the culture of *Dunaliella tertiolecta* (Yoneshigue-Braga et al.,1977).

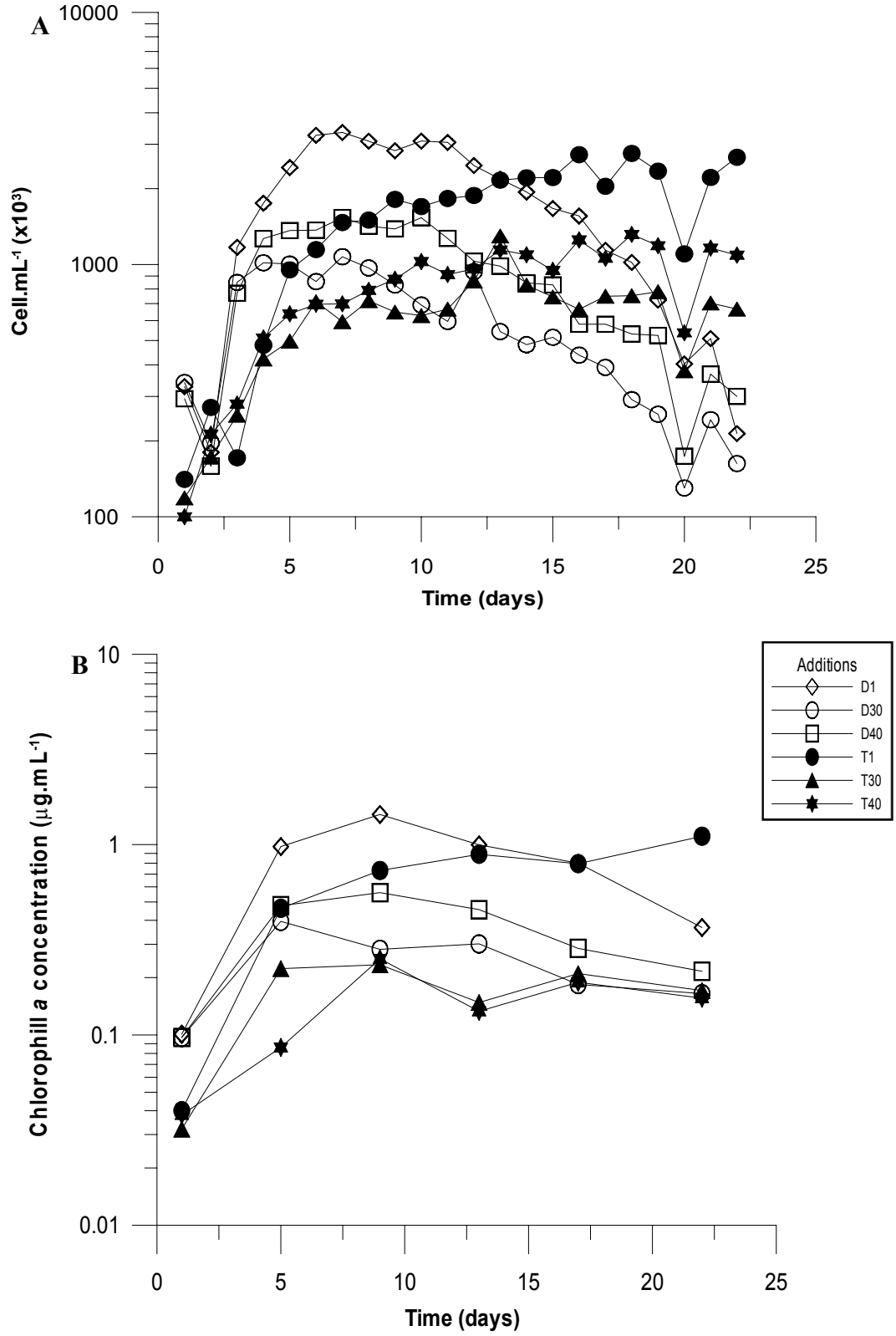
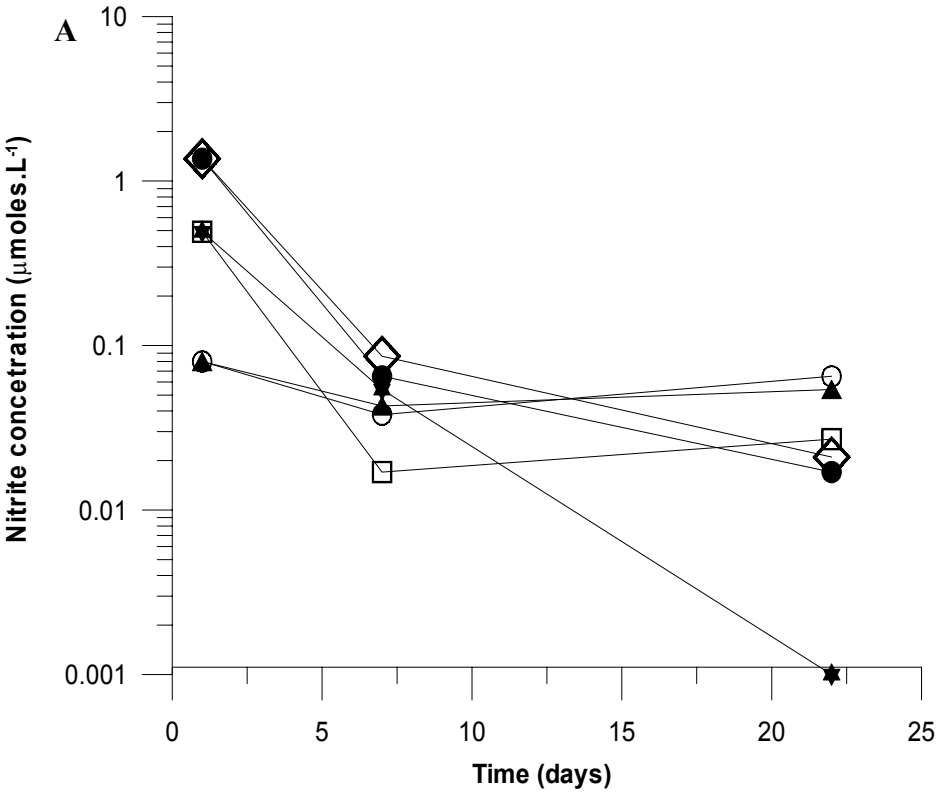
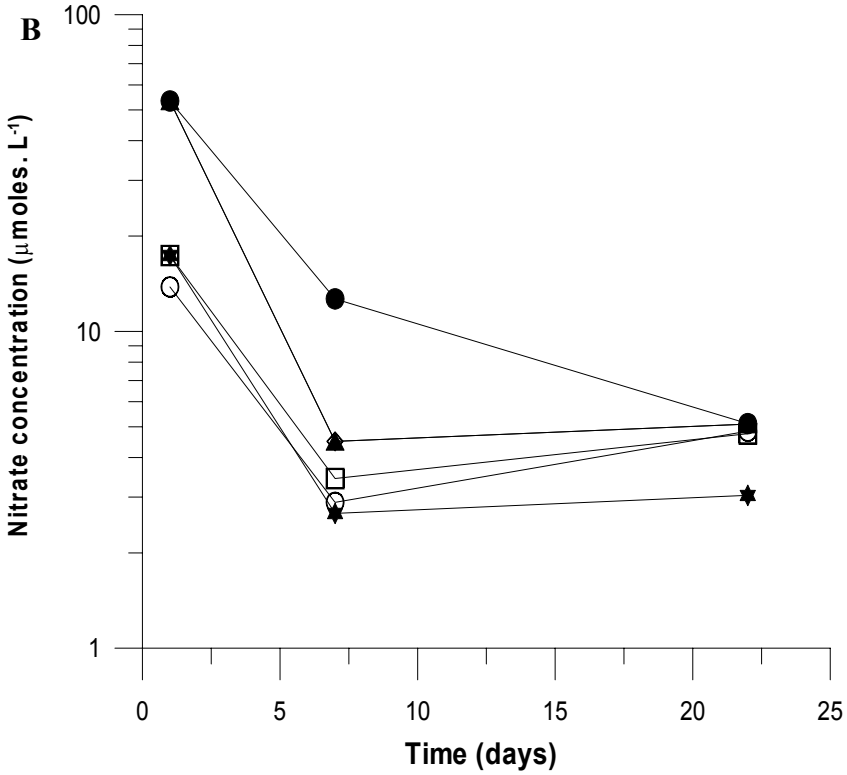


Figure 1 - Daily population density (cells.mL^{-1} x 10^3 - A) and chlorophyll *a* concentration (µg.mL^{-1} - B) in some of the experimented additions (f/2 control media, 30% and 40% additions) used for the species *Dunaliella viridis* (D1, D30, D40) and *Tetraselmis chuii* (T1, T30, T40) represented in logarithm scale.


A
10
1
0.1
0.01
0.001
Nitrite concetration (μmoles.L-1)
0
5
10
15
20
25
Time (days)


B
100
10
1
Nitrate concentration (μmoles. L-1)
0
5
10
15
20
25
Time (days)

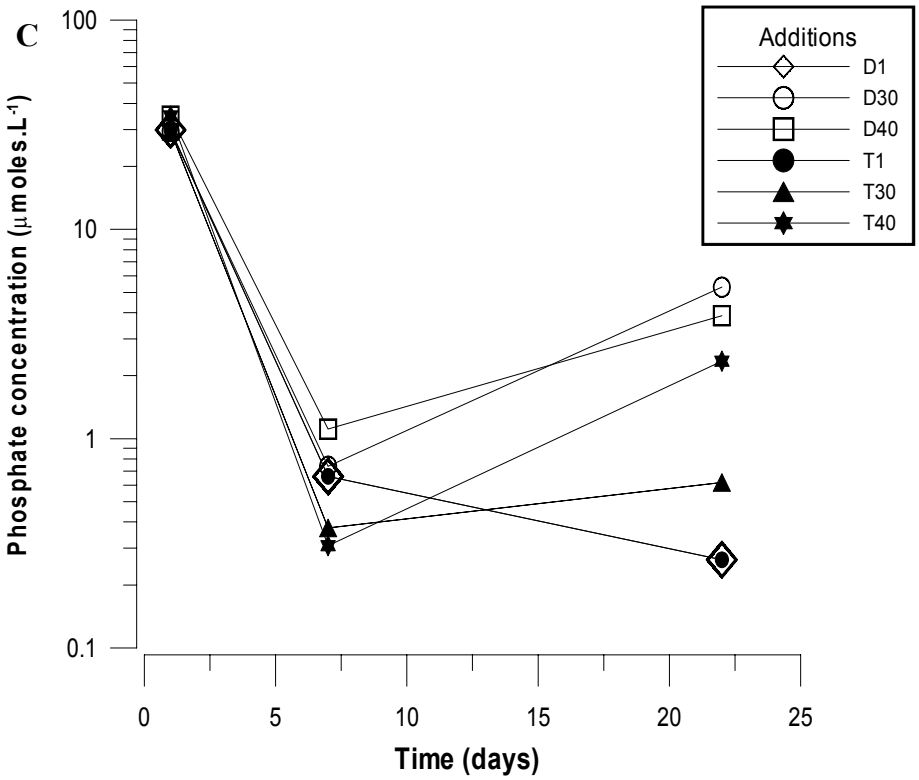


Figure 2 - Nitrite-N (A), nitrate-N (B) and phosphate-P (C) concentrations (represented in logarithm scale) of the experimental units (f/2 media control, 30% and 40% additions) employed during the bioassays carried out with *Dunaliella viridis* (D1, D30, D40) and *Tetraselmis chuii* (T1, T30, T40).

For 30 and 40% additions the values obtained were compatible with those observed with other alternative culture media employed in Brazil and other countries for seeding fish ponds (semi intensive aquaculture systems) and for large scale microalgae production (Yamashita and Magalhães, 1984; Oliveira and Koening 1984; Triani et al., 1986; Koening et al., 1990).

Chlorophyll-*a* maximum concentrations registered for these species were 0.56μg.mL^{-1} and 0.25μg.mL^{-1} for D40 and T40, respectively. Although these values were higher than those reported by Koening et al. (1988), other authors obtained either similar values, or as in most of cases, higher values than those observed for the secondary sewage (Koening et al., 1990). Moreover, these results were lower than those reported by Costa et al. (1999), when using the same additions of secondary sewage for the culture of *Thalassiosira* sp. and *Chaetoceros gracilis* Schütt (Chrysophyceae).

These results are compatible with the other alternative media tested by some researchers and supported the data obtained by Goldman et al. (1974) and Goldman and Stanley (1974), about the possible utilization of sewage/seawater mixtures with mixed and monoalgal populations designed for algae production, that could be used by semi intensive aquaculture systems of some countries.

Results of Khalil and Hussein (1997) demonstrated that total aerobic bacterial count was very low (9.3 X 10^2 g^{-1}) in the edible muscles of fish grown in secondary-treated effluent and complied with the WHO guidelines (less than 10^5 g^{-1}), and the accumulation levels of heavy metals were within the acceptable limits when compared to the international legal standards for hazardous elements in fish and fishery products. Van-Der-Heever and Frey (1996 a and b) studying aspects of certain metals (Chromium and mercury; iron and manganese) in tissues of the African sharptooth catfish, *Clarias gariepinus*, kept in treated sewage effluent on human health aspects, found that Chromium concentrations could exceed international limit values and could be a health hazard to consumers only if fish was

consumed in excess. With respect to the others metals (mercury, iron and manganese) they reported that concentrations approached the maximum permissible levels in the liver and kidneys of catfish suggesting that these organs were not recommended for human consumption. These results showed that algae grown with urban secondary sewage would give satisfactory results if applied as food for herbivore fish without being hazardous elements in fish and fishery products.

Khalil and Hussein (1997) reported that primary and secondary treated waste effluents were successfully used to grow the Nile tilapia *Oreochromis niloticus* (L.) in field experiments. These authors observed that the growth rate of fish reared in treated wastewater was significantly higher than that of fish reared in the natural habitat. They also reported that chemical and bacterial analyses indicated that there was no evidence of any public health hazard associated with treated wastewater reuse in aquaculture. However, they pointed out that the risks, if any, to the fish growers, processors and consumers should be evaluated, especially that related to viral pathogens.

The fact that experimented species were able to reduce dissolved nutrient salt concentrations to levels 90% lower than those obtained at the beginning of the bioassays, would constitute an important alternative to the tertiary treatment of sewage in treatment plants and could be used in the treatment of stabilization lagoons. Similar results were found in experiments using *Scenedesmus falcatus* Chod. and *S. quadricauda* (Turp.) de Breb. (Chlorophyta) for mobilizing nutrients of pig manure showing that algal removal of ammonia sometimes reached 100% (Dabbadie, 1994). Although our results showed that treated secondary sewage could promote algal growth at the same extent observed for other alternative culture media, it would be necessary to conduct some microbiological, chemical and field growth tests with some algae species before its application in semiintensive aquaculture systems.

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RESUMO

Foi estudado o efeito das diferentes concentrações de esgoto urbano secundário (10%, 20%, 30% e 40%) sobre o crescimento de *Tetraselmis chuii* (Prasinophyceae) e *Dunaliellla viridis* (Chlorophyceae) para verificar a possibilidade do seu uso como meio de cultura altenativo para estas espécies. Culturas stocks duplicatas de 700mL foram mantidas sob condições de laboratório controladas. As adições de 30% e 40% foram as mais eficientes para o crescimento de ambas as espécies. Análises estatísticas para o ajuste do crescimento (cel.mL^{-1}) e curvas de biomassa (µg clorofila-a.mL^{-1}) em relação ao tempo de cultura desenvolvidas usando Chi-square test ($p<0.05$), demonstraram diferenças significativas entre o meio nutritivo f/2 e a adição de 40% usada para estas duas espécies. Os resultados sugerem que algumas das concentrações testadas, rendem densidades celulares e taxas de crescimento satisfatórias quando comparados com outros meios de cultura (macroalga, estratos de esterco bovino e de galinha) sendo um meio altenativo que pode ser usado para sistemas de aquicultura semi intensivos devido aos baixos custos e fácil aquisição.

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