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BIOREMEDIATION OF WASTEWATER ORIGINATE FROM AQUACULTURE AND BIOMASS PRODUCTION FROM MICROALGAE SPECIES - *NANNOCHLOROPSIS OCULATA* AND *TETRASELMIS CHUII*

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Abstract

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The cultivation of microalgae in wastewater leads to the removing of nutrients and at the same time, produces biomass which can be further exploited as a biofuel. At the same time, *Tetraselmis* sp. and *Nannochloropsis* sp. have a high nutritional value, and for this reason they have been widely used as a food supply in the aquaculture industry for hatchery grown herbivores. The aim of current study was to compare the biomass accumulation of two microalgae species *Nannochloropsis oculata* and *Tetraselmis chuii* cultivated in wastewater originate from recirculation aquaculture system (RAS) and explore their abilities for nitrogen and phosphorus compound removal. A bioreactor consisted of 500 ml Erlenmeyer flasks, containing 250 mL of wastewater from semi closed recirculation aquaculture system. The cultures were maintained at room temperature (25-27ºC) under a fluorescent light with a light:dark photoperiod of 15 h: 9 h, with sterile air containing 2% (v/v) CO_2. Optical density, chlorophyll and carotenoids were measured for the biomass evaluation for a 10 days growth period. In our study *N. oculata* removed 78.4% of total nitrogen and 92% of nitrate. At the end of experiment for *T. chuii* cultivation in wastewater, phosphorus decreased by 79%, which was by 26.7% higher, compared to that of the phosphorus removal rate of *N. oculata*.

Key words: biomass, *Nannochloropsis oculata*, *Tetraselmis chuii*, RAS, wastewater

Introduction

Microalgae are used in different aspects. Microalgae are one of the most promising sources for biodiesel production because of their high photosynthetic efficiency and their faster growth rate as compared to any other energy crop (Minowa et al., 1995). They reproduce quickly and can be harvested every day (Haag, 2007).

The microalgae depend of many factors in the laboratory or in nature, such as temperature, light, salinity and nutritional factors that influence the growth, physiological activities and biochemical composition (Alsull and Omar, 2012). Microalgae are able to assimilate nitrogen from a variety of sources (Paasche and Kristiansen, 1982; Queguiner et al., 1986; Lund, 1987; Dortch, 1990; Cochlan and Harrison, 1991; Page et al., 1999). Ammonia, nitrite, nitrate and many-dissolved organic nitrogen's (urea, free amino acids and peptides) are considered as the main nitrogen sources for microalgae (Abe et al., 2002; Soletto et al., 2005; Converti et al., 2006). Microalgae have been widely used for nutrient removal in wastewater (Hammouda et al., 1995; Craggs et al., 1997; Hoffmann, 1998; Olguin, 2003; Borges et al., 2005). They are considered one of the most efficient, environmentally friendly, relatively low-cost and simple alternative wastewater treatments compared to conventional wastewater treatment techniques (Hii et al., 2011).

Some authors have worked on this topic in the area of aquaculture (Wang, 2003; Lefebvre et al., 1996; Hussenot et al., 1998) nevertheless the number of articles connected with wastewater treatment with algae in aquaculture are very limited.

Some of the algae species (*Nannochloropsis oculata and Tetraselmis chuii*) are interesting and important microorganisms in the field of biotechnology because of their high lipid content, higher proteins, essential fatty acids (Ghezelbash et al., 2008). From the other side *Tetraselmis* sp. and *Nannochloropsis* sp. have a high nutritional value, and for this reason, they have been widely used as a food supply in the aquaculture industry for hatchery-grown herbivores (Alsull and Omar, 2012).

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The use of algal species (*Nannochloropsis* sp. and *Tetraselmis* sp.) in wastewater treatment in aquaculture could offer the combined advantage of removing nutrients and biomass production at the same time providing an interesting opportunity.

The aim of current study was to compare the biomass accumulation of two microalgae species *N. oculata* and *T. chuii* cultivated in wastewater originate from recirculation aquaculture system (RAS) and explore their ability for nitrogen and phosphorus compound removal.

Material and Methods

Microalgae strain

N. oculata (SKU: 100-NOC00-50) and *T. chuii* (SKU: 100-TCH00-50) were delivered from Algae depot – USA (www.algaedepot.com). The trials were conducted in the algal laboratory of the Department of biology and aquaculture (Trakia University, Bulgaria).

Cultivation

The cells in an exponential period were inoculated (10%, v/v) in a liquid medium. Cultivation was initiated in 500 mL Erlenmeyer flask containing 250 mL relevant medium (Figure 1).

The cultures were maintained at room temperature (25-27ºC) on a fluorescent light with a light:dark photoperiod of 15 h: 9 h. Sterile air containing 2% (v/v) CO_2 was aerated into the flask through an air sparger at the bottom of the flasks.

Two experiments were carried out, each of them with a 10 days duration.

Experiment 1

Growth rate of N. oculata and T. chuii cultivated in wastewater originate from aquaculture

The first experiment was conducted in 4 variants:

1. N. oculata cultivated in wastewater originate from semi – closed RAS;

2. N. oculata cultivated in modified f/2 medium;

3. T. chuii cultivated in wastewater originate from semi – closed RAS;

4. T. chuii cultivated in modified f/2 medium

The composition of modified f/2 medium was as follows (per liter): 29.23 g NaCl, 1.105 g KCl, 11.09 g $MgSO_4.7H_2O$, 1.21 g tris-base, 1.83 g $CaCl_2$. $2H_2O$, 0.25 g $NaHCO_3$, and 3.0 mL of trace elemental solution. The trace elemental solution (per liter) included 75 g $NaNO_3$, 5 g $NaH_2PO_4.H_2O$, 4.36 g Na_2 EDTA, 3.16 g $FeCl_3.6H_2O$, 180 mg $MnCl_2.4H_2O$, 10 mg $CoCl_2.6H_2O$, 10 mg $CuSO_4.5H_2O$, 23 mg $ZnSO_4.7H_2O$, 6 mg Na_2MoO_4, 100 mg vitamin B1, 0.5 mg vitamin B12 and 0.5 mg biotin (Chiu et al., 2009).

Experiment 2

Comparison of nutrient removal efficiency in wastewater originate from semi – closed RAS from algal species N. oculata and T. chuii

Two variants of experiment were tested:

1. Nutrient removal efficiency in wastewater originate from semi – closed RAS from *N. oculata;*

2. Nutrient removal efficiency in wastewater originate from semi – closed RAS from *T. chuii;*

Both experiments were conducted in three replications (Figure 1).

The wastewater

The wastewater used in both trials originated from semi – closed recirculation aquaculture system (semi – closed RAS), before it was cleaned by a mechanical and biological filter. The samples of wastewater were filtered through 25 mm, 3 µm glass microfiber filters (GF/C) mounted on a Millipore filtra-

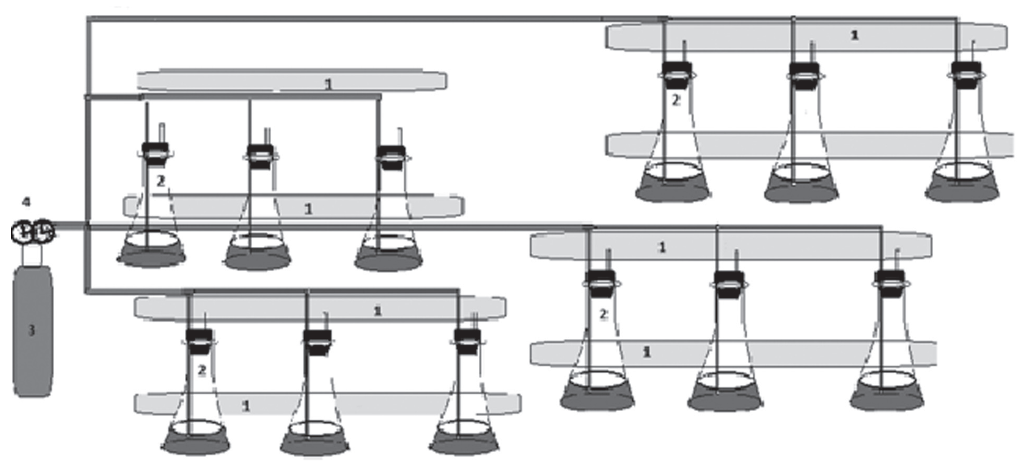

Fig. 1. Bioreactor used in conducted trials

1). Luminiscent lamps; 2). Erlenmeyer flasks; 3). CO_2 bottle; 4). Manometer.

tion unit. In the wastewater from the semi – closed RAS the same quantity of salt like in modified f/2 medium was added.

Determination of optical density, chlorophyll and carotenoid content in samples

The growth of *N. oculata and T. chuii* were measured via spectrophotometry (DR 2800). Optical density for biomass growth factor was determined at wavelength 650 nm. 1 ml of sample was appropriately diluted with deionized water and the absorbance of the sample was read at 650 nm.

The isolation of pigments from algae cells included the following procedures: harvesting 2 ml of microalgae cells by centrifugation at 10000 rpm, two times for 3 min and discarding the supernatant, suspension of cells in 2 ml methanol/water 90:10 v/v and mixing of Vortex for 1 min, heating of the suspension for half an hour in a water bath at 60ºC, cooling of the samples at room temperature, centrifugating the suspension (10000 rpm for 3 min) and discarding the supernatant with dissolved pigments. The absorbance of the pigments extract (665, 652 nm for chlorophyll content (a) and 470, 666nm for carotenoids content) was recorded by using spectrophotometer. The chlorophyll (a) content was computed ($mg.l^{-1}$) according Porra et al. (1989) and carotenoid content was computed ($mg.l^{-1}$) according Lichtenthaler (1987).

Determination of hydrochemical parameters

Samples for hydrochemical analysis were taken in the start of the trial, at 2, 4, 6, 8 and 10 days after the start of the experiment. The samples were centrifuged at 300 rpm for 10 min, for freeing them from algal cells (Lee and Lee, 2002).

The measurement of pH was conducted with a portable combined meter and with a pH probe (Hach Lange).

Other analyzed hydrochemical parameters were measured spectrophotometrically with spectrophotometer DR 2800 (Hach Lange). The methods and range of tests which were used during the experiment are shown in Table 1.

Statistical analysis

The data from the measurements were analyzed by variance analysis (ANOVA) in order to determine the effects and differences of treatment using ANOVA single factor test at $p< 0.05$. The SPSS program was used.

Results

Experiment 1

Growth rate of N. oculata and T. chuii cultivated in wastewater originate from aquaculture

The both algae species *N. oculata* and *T. chuii* showed a good growth potential in the f/2 medium as well as in wastewater from RAS in experiment 1 (Figure 2). The optical density of *T. chuii* was 1.2 and for *N. oculata* it reached a value of 1.1 in the f/2 medium responding in this way to 16.6% and 12.7% higher optical density respectively in the f/2 medium compared with their values when wastewater was used as a growing media. The differences were statistically proven (Table 2).

In the tested condition, the highest amount in chlorophyll content was determinate in *T. chuii* cultivated in f/2 medium (8.86 $mg.l^{-1}$). The chlorophyll content was higher in both tested algae, with 23.5% for *T. chuii* and with 21.2% for *N. oculata* when f/2 media was used like growing media compared with their values received when experimental strain were cultivated in wastewater and differences were statistically proven (Table 2). The chlorophyll content in the *T. chuii* strain cultivated in wastewater from RAS did not differ significantly from the amount of chlorophyll in the *N. oculata* when this alga were cultivated in the f/2 medium (Figure 3).

In our study the quantity of carotenoids in *T. chuii* were higher (2.89 $mg.l^{-1}$) in cultures grown in the f/2 medium, compared with the carotenoids of *N. oculata* (2.6 $mg.l^{-1}$) cultivated

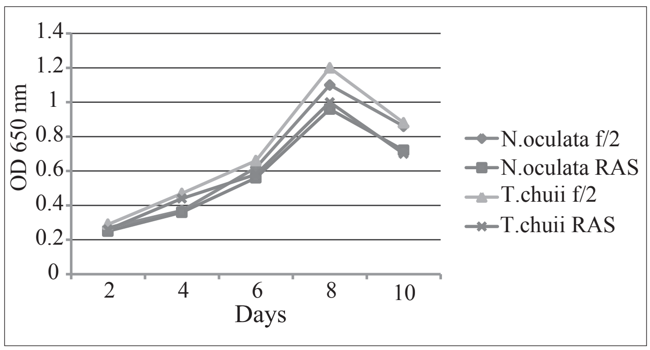

Fig. 2. Optical density of *N. oculata* and *T. chuii* (at 650nm) for 10 days in f/2 medium and wastewater from RAS

Table 1
Methods and range of tests used for monitoring the water quality parameters during the experiment 2

Quality parameters	Determination method	Measuring range ($mg.l^{-1}$)
Nitrite – nitrogen	Diazotization	0.015 – 0.6
Nitrate - nitrogen	2.6 dimethylphenol	5 - 35
Total nitrogen	Koroleff digestion + 2.6 dimethylphenol	5 - 40
Phosporus (ortho + total)	Phosphormolybdenum blue	0.05 – 1.5 mg/l PO_4-P 0.15 – 4.5 mg/l PO_4

in the same medium (Figure 4). For *T. chuii* cultivated in the f/2 medium the quantity of carotenoids was with 19.4% higher compared with its quantity when growing process were accomplished in wastewater originated from RAS, and the differences were statistically proven (Table 2)**.** *N. oculata* strain, cultivated in the f/2 medium showed a carotenoid quantity higher by 13.1%, than that which we received for the same strain when wastewater from RAS was used as a cultivation media, and the difference was statistically proven (Table 2)**.**

Experiment 2

Nutrient removal efficiency in wastewater originate from semi – closed RAS from algal species N. oculata and T. chuii

During our trial, the measured pH varied from 6.5 to 7.5 in both tested algae (Figure 5) and the differences in this parameter were not statistically proven (Table 3).

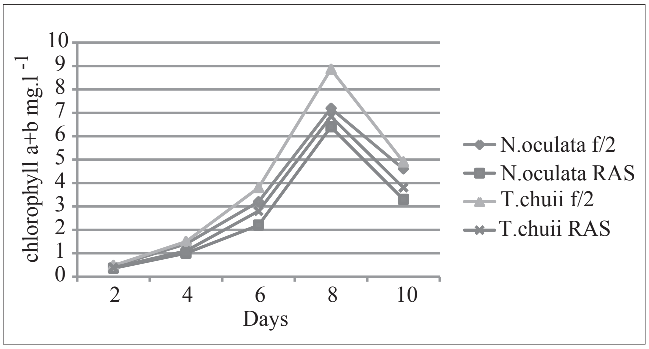

Fig. 3. Chlorophyll (mg.l^{-1}) of *N. oculata* and *T. chuii* for 10 days in f/2 medium and wastewater from RAS

At the beginning the nitrite was 0.96 mg.l^{-1}, and at the end of the experiment their quantity reduced down to 0.15 mg.l^{-1}

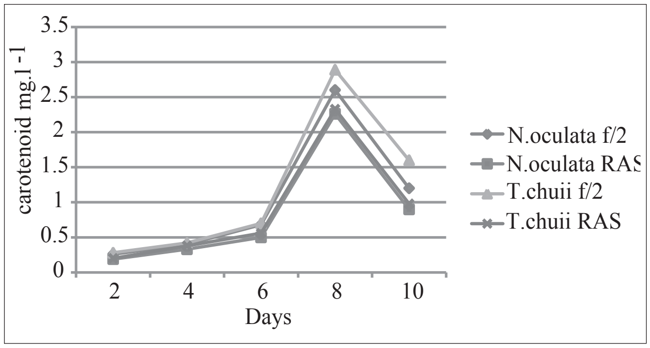

Fig. 4. Carotenoid (mg.l^{-1}) of *N. oculata* and *T. chuii* for 10 days in f/2 medium and wastewater from RAS

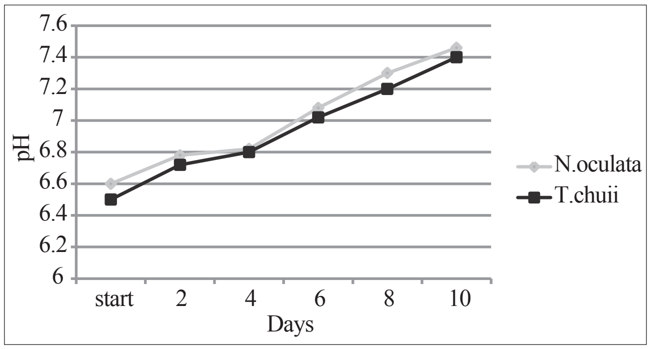

Fig. 5. Changes in the pH during the experiment 2

Table 2

Average values of growth parameters of *N. oculata and T. chuii* grown in f/2 media and wastewater from RAS during the experiment 1

x±Sx Parameters	*N. oculata* f/2	*N. oculata* RAS	*T. chuii* f/2	*T. chuii* RAS
optical density	0.639±0.10	0.582±0.07**	0.694±0.1	0.619±0.06**
Chlorophyll a (mg.l^{-1})	3.368±7.1	2.652±5.6**	3.908±10.7	2.986±6.4*
Carotenoid (mg.l^{-1})	1.028±0.9	0.836±0.7**	1.178±1.1	0.89±0.7*

P≤0.05*, P≤0.01**

Table 3 Average values of hydrochemical parameters during the experiment 2

Parameters	*N. oculata*	*T. chuii*	*p*
	x±Sx	x±Sx	
Nitrate	14.38±1.1	17.09±8.9	*
Nitrite	0.46±0.07	0.53±0.07	***
Total nitrogen	18.17±7.9	22.6±11	ns
Total phosphorus	3.04±0.03	2.34±0.8	ns
pH	7.00±0.1	6.94±0.1	ns

P≥0.05-ns,*P≤0.05, **P≤0.01, ***P≤0.001;

for *N. oculata* and 0.2 $mg.l^{-1}$ for *T. chuii* (Figure 6), which responded approximately 6 - 7 times lower values in nitrite at the end of the experiment compared with its value at the start and the differences were statistically proven at a high level of significance ($P \leq 0.001$) (Table 3)**.**

During the experiment all measured quantities of nitrates were higher in *T. chuii* than those which we found for the *N. oculata* strain (Figure 7), and the differences between the two species were statistically proven ($P \leq 0.05$) (Table 3). In the end of experiment the quantity of nitrate reduced down to 4.2 $mg.l^{-1}$ for *T. chuii* and 2.0 $mg.l^{-1}$ for *N. oculata*, in this way *N. oculata* showed a 7.7% better removal efficiency in the nitrates compared with that accumulated by *T. chuii*.

The same tendency was found for total nitrogen, its quantity was reduced at the end of trial in wastewater used for the cultivation of *N. oculata* by 78.4%, while in *T. chui* – by 69.5%. (Figure 8), but the differences were not statistically significant ($P \geq 0.05$) (Table 3).

The efficiency in total phosphorus removal from wastewater was better in wastewater used for *T .chuii* cultivation when compared with wastewater, which contained *N. oculata* (Figure 9). At the beginning a high level of phosphorus compounds (3.4 $mg.l^{-1}$) were measured. At the end of experiment for *T. chuii,* cultivation in wastewater phosphorus decreased by 64.7%, while for *N. oculata* only by 14.7%.

Discussion

The production of high biomass concentrations is the first step of the cultivation process for microalgae. For biomass growth microalgae depend on a sufficient supply of a carbon source, organic nutrients present in the water and light to carry out photosynthesis.

Our data concerning the optical density of *T. chuii* culture grew in wastewater from semi-closed RAS are similar to these received from Costa et al. (2004) when *T. chuii* was cultivated in urban secondary sewage and they observed population density of 1.3.

Chan (2011) conducted experiment by cultivating the microalgae in wastewater from a fish farm and established that they can promote the growth of *Chlorella* sp. and *Nannochloropsis* sp. and they obtained a 90% growth rate of Nannochloropsis sp. during the experimental period.

Borges et al. (2005) received a two times lower quantity of chlorophyll A (3.08 $mg.l^{-1}$) when they used *Tetraselmis* sp. for treatment of fish farm effluents.

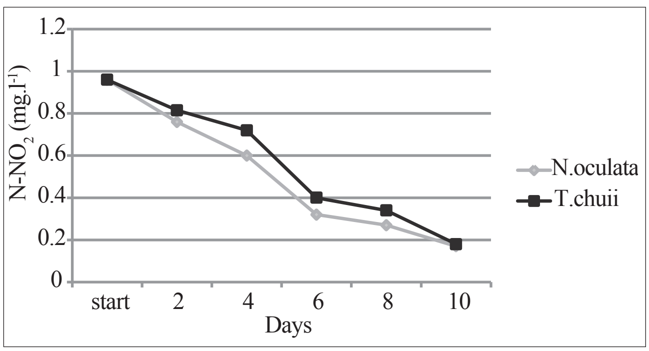

Fig. 6. Changes in the concentrations of nitrite during the experiment 2

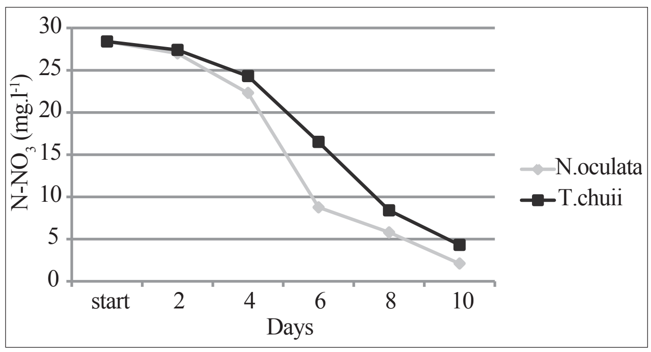

Fig. 7. Changes in the concentrations of nitrate during the experiment 2

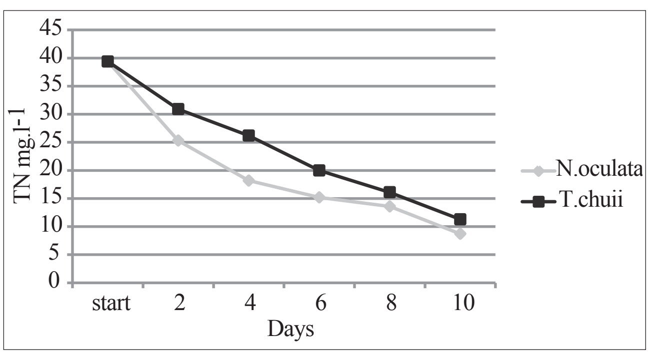

Fig. 8. Changes in the concentrations of total nitrogen during the experiment 2

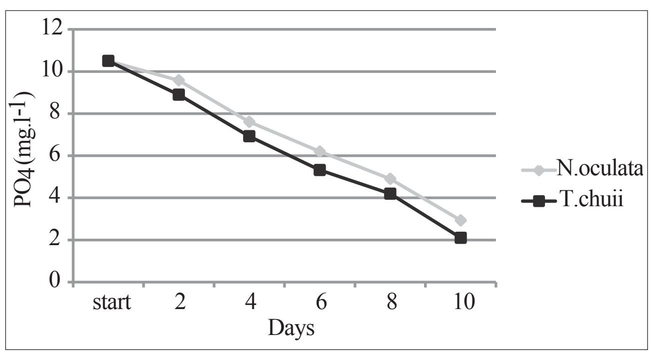

Fig. 9. Changes in the concentrations of phosphorus the experiment 2

In our study the carotenoids and chlorophyll a quantity were higher in wastewater from RAS in comparison with the results of other authors (Gu et al., 2012; Ghezelbash et al. 2008) cultured *N. oculata* and *T. chuii* in f/2 media. The cultivation of these two algae species in aquaculture wastewater in the current study showed a good growth of the strains and the data was comparative to that of their development in the control medium.

Arrendondo-Figueroa et al. (1998) stated that some algal species showed the similar growing potential when they are cultivated in wastewater, as well as when they are cultivated in the control medium. The above authors tested sheep and cow manures as an alternative culture media for the growth of three species of *Chlorella* and concluded that the algal species grew well in manure mediums as well as in the control medium. They also proved that organic compounds presented in the manure medium are assimilated with almost the same efficiency as that of the control-growing medium.

The pH of the wastewater affects many of the bio-chemical processes associated with algal growth and metabolism and the availability and uptake of nutrient ions. pH could not only influence algal growth but also nitrogen removal efficiency in wastewater treatment (Park and Craggs, 2010; Park et al., 2011). Due to the photosynthetic CO_2 assimilation, pH usually increases in algal cultures (Borowitzka, 1997; Chevalier et al., 2000).

Costa et al. (2004) used the urban secondary sewage as a growing media for *T. chuii* cultivation and observed high reductions of nitrite (99.9%) and nitrate (86.40%) for 7 days period.

Lowrey (2011) conducted an experiment with Tetraselmis sp. cultivating in 10%, 25% and 33% dairy wastewater concentration. He established that Tetraselmis sp. took up 49% of the total nitrogen from 10% wastewater, 18% total nitrogen from 25% wastewater, 51% total nitrogen from 33% wastewater.

Dalrymple et al. (2013) used wastewater as a growing media for algae production in photobioreactor and found out that total nitrogen uptake was just below 60%.

Although nitrogen is available to microalgae in various forms, nitrate, ammonium ions, and urea are the dominant forms (Syrett, 1981).

According Hii et al. (2011) conducted experiment with cultivation of *Nannochloropsis* sp. in wastewater and established that at the end of the trial, 33.24 % of nitrates were removed. In our study *N. oculata* removed 78.4% of total nitrogen and 92% of nitrates in wastewater originate from semi – closed RAS. Ammonia and nitrate uptake were retarded when the growth of Nannochloropsis sp. approached the stationary phase. We did not measure the ammonium concentration in current study, because in previous experiment (unpublished data) wastewater from semi - closed RAS showed lower quantity of ammonium and this led as a result to a higher accumulation of alternative nitrogen sources such as nitrite and nitrate. Hii et al. (2011) which stated that after ammonium removal from algae they are capable to remove nitrite and nitrate confirmed our assumptions. Ammonium ions are preferentially taken up, followed by nitrate (Levasseur et al., 1990; Levasseur et al., 1993).

Han et al. (2008) studied integrated cultivation for aquaculture wastewater purification and algal biomass production and established that the average removal rate of total nitrogen, nitrate and nitrite was more than 80% for each; the removal rate of total phosphorus was 94.17%.

Menke et al. (2011) after culture of Nannochloropsis sp. for 10 days grown in 75% wastewater, nitrogen and phosphorus was respectively reduced to 80.4% and 72.8%.

Patel et al. (2012) experimented how microalgae differ in uptake of phosphorus from wastewater. They established that *Nannochloropsis* sp. removed phosphorus poorly and *Tetraselmis* sp. removed 79.4% of total phosphorus.

According Costa et al. (2004) conducting an experiment with *T. chuii* cultivated in urban secondary sewage account that the phosphate concentration decreased by 97% on the 7th day of the trial. In our experiment the phosphorus decreased by 79% in the end of the trial.

The concentrations of nitrite, nitrate and phosphate obtained of wastewater were in most cases higher than values obtained with alternative media. Goldman et al. (1974) and Goldman and Stanley (1974) obtained data for possible utilization of seawater with mixed and monoalgal populations designed for algae production, that could be used by semi-intensive aquaculture systems.

Conclusion

Our results showed that wastewater from aquaculture could promote good algal growth of *N. oculata and T. chuii* to a similar extent as observed for the f/2 culture media. *N. oculata and T. chuii* can be used for nitrogen and phosphorus removal in aquaculture and they could take a part in biological treatment of wastewater originate from such production.

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