



Population dynamics in mixed cultures of *Neochloris oleoabundans* and native microalgae from water of a polluted river and isolation of a diatom consortium for the production of lipid rich biomass

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The production of biodiesel utilizing microalgae has driven innovation worldwide, especially trying to overcome the current economic and technological limitations of the whole process. Within these efforts, the use of wastewater to cultivate oleaginous microalgae or the use of dual-purpose microalgae–bacteria-based systems that treat wastewater and produce oleaginous microalgae have become an attractive alternative. The aim of this work was to evaluate the population dynamics which occurred in mixed cultures of *Neochloris oleoabundans* with other native microalgae, in mixtures of a synthetic medium (BBM) and water of an urban polluted river. The effect of temperature, nutrient availability and the microscopic monitoring of the population dynamics in such mixed cultures were carried out. Furthermore, the isolation of the predominant consortium of diatoms and the evaluation of its kinetics of growth and its capacity for removal of pollutants was also performed. Results indicated that such green microalgae only predominated in mixtures containing 80% or 60% of the synthetic medium. In mixtures containing a volume of the polluted river higher than 40%, other microalgae predominated, especially diatoms of various genera. The diatom consortium isolated from a 100% of the river's water sampled in spring (April), was formed mainly by a population of *Nitzschia frustulum* and in less extent of *Navicula* sp. It showed a significantly higher specific growth rate when cultivated in water from the river, compared to cultures in synthetic modified diatom medium (MDM) and at 32°C, compared to cultures incubated at 25°C. The consortium was able to remove 95.45% and 95.78% of ammonia nitrogen, 60% and 62.5% of nitrates at 32°C and 25°C, respectively, after 2 days. It also removed 95% of phosphates at 32°C and 67% at 25°C after 4 days from the polluted river. Diatoms also showed significant accumulation of lipids after 10 days of cultivation when stained with Sudan III. In conclusion, such diatom consortium showed a large potential for being used in a dual-purpose system that could treat the water from polluted streams and that could produce lipid rich biomass.

Introduction

Despite the fact that several reports have highlighted the viability of producing biodiesel from microalgae [1–3], some other recent studies utilizing the Life Cycle Analysis (LCA) approach, have indicated the need for decreasing the water footprint and also

diminishing the use of fertilizers to increase the sustainability of the whole process and to decrease the energy input [4–6].

Thus, as a response to the several economic constraints in the entire process of production of biodiesel from microalgae, the use of wastewater for cultivation of the microalgae as a nutrient source has been highlighted by various researchers [7,8]. Furthermore, dual-purpose systems which treat the wastewater and allow the recovery of microalgae rich in lipids and the recovery of other high

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added value products within a biorefinery, have been recently reviewed in depth [9]. However, there are no reports indicating the use of urban polluted rivers for the cultivation of oleaginous microalgae, as a source of water and nutrients and the dynamics of the microalgae population after being inoculated with a specific strain. It is worth to note that the problem of urban polluted rivers is still very acute in some developing countries in which the sanitation infrastructure is still not covering all the cities' needs. In the case of the State of Veracruz in Mexico, a recent study evaluated the quality of the water of the Sordo's watershed in the surroundings of the City of Xalapa. Based on the Quality Index, it was concluded that the tributaries of the Sordo River were highly polluted and that this river became also highly polluted after receiving the stream of the Carneros River [10]. However, the quantification of specific pollutants indicated that organic matter including nutrients such as N and P were the major pollutants. No heavy metals or phenols were detected.

On the other hand, the search for highly valuable strains for production of biodiesel from microalgae, has become one of the priorities in this field [11]. Among the various already reported microalgae with a high potential for biodiesel production [1,12], *Neochloris oleoabundans* is a green microalgae with a high lipid productivity [13]. On the other hand, diatoms of the genus *Nitzschia* have also been reported with potential for the production of biodiesel [14]. The effect of temperature on the growth of *N. oleoabundans* has been investigated under certain specific conditions. It was found that *N. oleoabundans* grew faster at 26°C than at 30°C, irrespective of supplementation with CO₂, when cultures were incubated in Bristol medium at a light intensity of 150 μmol m⁻² s⁻¹ [13]. In other microalgae, it has been reported that temperature has a strong influence on the lipid content. In the case of *Nannochloropsis oculata* an increase in temperature from 20 to 25°C practically doubled the lipid content (from 7.90 to 14.92%). By contrast, in the case of *Chlorella vulgaris* an increase from 25 to 30°C resulted in a decrease of the lipid content from 14.71 to 5.90% [15]. Thus, it is probable that temperature might have an important influence on the growth and lipid content in the case of *N. oleoabundans* cultivated in mixed cultures using water from a polluted river.

The objective of the present work, was to evaluate the population dynamics which occurred in mixed cultures of *N. oleoabundans* (UTEX #1185, USA) with other native microalgae, in mixtures of a synthetic medium (BBM) and water of an urban polluted river, the Sordo River, located in the state of Veracruz, Mexico. More specifically, the objective was to assess the effect of the temperature, nutrient availability and population dynamics in such mixed cultures including the isolation of the predominant consortium of diatoms and the evaluation of its kinetics of growth and its capacity for removal of pollutants.

Materials and methods

Maintenance of the *N. oleoabundans* (UTEX #1185, USA) strain
BBM Synthetic medium (Bold's Basal Medium) prepared according to Barsanti and Gualtieri [16] and added of 1.5% of agar, was used to maintain the strain of *N. oleoabundans* in tubes exposed to 136 μmol m⁻² s⁻¹ (16-hour light/8-hour dark photoperiod) and a constant temperature of 32°C.

Physicochemical characterization of the Sordo River water

Water from the Sordo River was collected in autumn (October and November), during the beginning of the dry season for the cultivation of mixed cultures and in spring (April) for the cultivation of the diatom consortium and it was subjected to various analysis. The COD and BOD₅, were measured according to Standard Methods [17]. Total Phenolic Compounds were determined using the 4-Aminoantipyrine Method (HACH® kit). Total Coliforms, with Coliscan® kit; pH, temperature, total dissolved solids and salinity, were measured with a Hanna® HI9828 multiparameter probe.

Cultivation conditions of mixed cultures

Mixtures of BBM medium with water from the Sordo River were prepared according to Table 1. Two controls were incorporated: sterilized BBM medium and non-sterilized BBM medium, both at an initial pH of 6.6. Cultures were conducted in a fed-batch regimen in Erlenmeyer Flasks (250 mL total volume), containing 100 mL of culture medium and incubated at a light intensity of 66 μmol m⁻² s⁻¹ and at two different temperatures, 25°C and 32°C. Aeration from aquarium pumps (without addition of CO₂) was introduced into the flasks to provide agitation.

Isolation of a native diatom consortium from the water of the Sordo River

Water from the Sordo River collected in April 2012 was used for the isolation of a diatom consortium. 10 mL of this water was inoculated into 90 mL of modified Diatom Medium (MDM), modified from the medium described by Barsanti and Gualtieri [16]. The modification consisted in that it did not contain the EDTA FeNa, nor the biotin and the cyanocobalamin which are included in the original medium. The mixture was prepared in a 250 mL flask and was incubated at 32° and 25°C and at an irradiance of 60 ± 1 μmol m⁻² s⁻¹ with a photoperiod of 16/8-hour light/darkness. After 8 days of cultivation, Petri dishes containing the same modified MDM medium added of 15 g L⁻¹ of agar were inoculated. After 8 days, brownish colonies containing diatoms were transferred again into liquid medium until growth was apparent. Every 6 days, fresh medium (90 mL) was provided to an inoculum of 10 mL.

Growth evaluation

The determination of the Dry Weight (D.W.) was carried out by filtering 10 mL of each sample through filters (Whatman®

TABLE 1

Various treatments involving mixtures of synthetic medium (BBM) and water from the Sordo River

Treatment name	Description
T1	100% Non-sterile BBM
T2	80% BBM + 20% SRW
T3	60% BBM + 40% SRW
T4	40% BBM + 60% SRW
T5	20% BBM + 80% SRW
T6	100% SRW
T7	100% Sterile BBM

BBM: Bold's Basal Medium; SRW: Sordo River's Water.

GF/C 1.2 μm). Membranes were incubated at 105°C till constant weight. Samples were removed from each flask every three days for quantifying D.W. The absorbance of the culture medium was measured at 685 nm according to Wahal and Viamajala [18].

In the case of the growth evaluation of diatoms cultures, counting of the cell number with a Hemacytometer was performed using a Microscope (Leica® DM750PH). Cultures were established in glass reactors (415 mL total volume), containing 300 mL of two types of culture medium: Modified Diatom Medium (MDM) and water from the Sordo river collected in April 2012. Glass reactors were incubated at a light intensity of $60 \pm 1 \mu\text{mol m}^{-2} \text{s}^{-1}$ (with a photoperiod of 16-hour light/8-hour darkness), at two different temperatures, 25°C and 32°C. Air from aquarium pumps was introduced into the glass reactors to provide agitation.

Microscopic monitoring of the mixed cultures

Samples from the cultures were withdrawn every 3 days for observation in a Microscope (Leica® DM750PH) and pictures were taken with an integrated photographic camera. Taxonomic identification of microorganisms in each treatment was performed, using the photographic material.

Analytical techniques

COD and BOD₅ were determined according to the Standard Methods [17]. Nitrates, ammonia nitrogen, phosphates and silicates were quantified using standard spectrophotometric procedures with HACH® kits.

Removal percentages were calculated with the formula:

$$\%R = \left[\frac{(C_i - C_f)}{C_i} \right] \times 100,$$

where C_i and C_f are the initial and the final concentrations, respectively.

Uptake rate of nitrogen consumption was calculated with the formula:

$$N_{\text{cons}} = \frac{(C_i - C_f)}{\Delta T},$$

where C_i and C_f are the initial and the final concentrations of the nitrogen compound (nitrate or ammonia nitrogen), and ΔT is the time differential.

Detection of lipids presence in various microorganisms with Sudan III

Cells from each treatment were stained with Sudan III, according to O'Brien and McCully [19]. A 70% ethanol solution was prepared mixing 70 mL of denatured ethanol (containing 5% methanol, Tecsiquim®) with 30 mL of deionized water. 0.5 g of Sudan III were added to this solution and put immediately in a warm water bath (60–70°C). After three hours, the solution was cooled and then filtered with Whatman® filter paper (No. 4). To stain the cells, 1 mL of samples from each treatment were centrifuged in micro tubes at 12,000 rpm (9660 g) for 2 min. The supernatant was disposed and 150 μL of distilled water were added to the tubes. After suspension of the pellet, 150 μL of the Sudan III ethanolic solution were added and tubes were maintained in a refrigerator (4°C) for 24 hours, before observation in the microscope was carried out.

TABLE 2

Physicochemical characterization of the Sordo River's water used in the cultivation of mixed cultures (Batch 1 collected in November 2011) and in the cultivation of the isolated diatom consortium (Batch 2 collected in April 2012)

Parameter	Batch 1	Batch 2
Chemical Oxygen Demand	188.00 mg L ⁻¹	85.0 mg L ⁻¹
Biochemical Oxygen Demand	169.30 mg L ⁻¹	79.0 mg L ⁻¹
Dissolved Oxygen	4.50 mg L ⁻¹	3.31 mg L ⁻¹
Total Kjeldahl Nitrogen	27.05 mg L ⁻¹	n/d
Ammonia Nitrogen	n/d	4.75 mg L ⁻¹
Nitrates	5.53 mg L ⁻¹	0.40 mg L ⁻¹
Silicates	n/d	50.4 mg L ⁻¹
Total Phosphorous	0.39 mg L ⁻¹	5.31 mg L ⁻¹
Total Phenolic Compounds	0.030 mg L ⁻¹	n/d
Total Coliforms	114,000 cfu 100 mL ⁻¹	n/d
Total Dissolved Solids	193.8 mg L ⁻¹	111 mg L ⁻¹
Temperature	16.5°C	18°C
Salinity	0.25 ppt	0.20 ppt
pH	6.2	6.7

Statistical analysis

The data obtained from the growth evaluation (D.W.) for all treatments was compared using one-way ANOVA. Significance level (α) for all tests was 0.05. The tests were performed with SigmapStat® Software (v.3.0., SPSS Inc.).

Results

Physicochemical characterization of the Sordo River's water

The Chemical Oxygen Demand (COD) and Biological Oxygen Demand (BOD) concentration indicated that there was a significant content of organic matter in the Sordo River's water (Table 2). The N/P ratio was 14.18 and 0.075 taking into account the nitrate concentration over the total phosphorus concentration, for the November and April samples, respectively. This ratio and the rest of parameters, except salinity and pH, are very different for both types of samples, indicating that the water from the river is strongly affected by the season.

Effect of the availability of nutrients on the growth of mixed cultures of *N. oleoabundans* and other native microalgae

Various treatments were evaluated, according to Table 1. The growth profile was evaluated using two different growth monitoring parameters, absorbance of the culture and determination of dry weight (D.W) (data not shown). It is worth to note that the growth profile was different depending on the monitoring method and also on the temperature of incubation. Because it was observed that the cells showed the tendency to aggregate, dry weight was preferred as the method which could provide a better indication of growth. Besides, the color of the cultures changed from green to brown towards the end of the experimental period, especially in treatments with a high proportion of the rivers' water. Thus, it was preferred to carry out the statistical analysis of results using only data from the dry weight curves (Table 3).

TABLE 3

Effect of nutrient availability on the cell density of mixed cultures of *N. oleoabundans* and other native microalgae measured as dry weight (g L^{-1}), in different treatments at two temperatures and various days of incubation

Treatment	32°C			25°C		
	Day 3	Day 6	Day 9	Day 3	Day 6	Day 9
T1	0.620 ± 0.028a	0.735 ± 0.026a	1.010 ± 0.064a	0.365 ± 0.078a	0.440 ± 0.092a	0.850 ± 0.098a
T2	0.940 ± 0.031b	0.880 ± 0.037a	0.665 ± 0.085b	0.445 ± 0.078a	0.360 ± 0.093b	0.185 ± 0.049b
T3	0.735 ± 0.021a	0.605 ± 0.016b	0.275 ± 0.071c	0.450 ± 0.028a	0.285 ± 0.049b	0.200 ± 0.042b
T4	0.690 ± 0.014a	0.485 ± 0.092c	0.310 ± 0.028c	0.460 ± 0.028a	0.335 ± 0.092b	0.165 ± 0.035b
T5	0.665 ± 0.078a	0.360 ± 0.028c	0.480 ± 0.031c	0.465 ± 0.078a	0.325 ± 0.078b	0.125 ± 0.007b
T6	0.585 ± 0.078a	0.165 ± 0.035c	0.355 ± 0.021c	0.440 ± 0.071a	0.210 ± 0.042b	0.155 ± 0.007b
T7	0.920 ± 0.001b	1.020 ± 0.001a	1.530 ± 0.001a	0.525 ± 0.007a	0.630 ± 0.004c	0.965 ± 0.021a

Different letters in each column, indicate significant differences between treatments at any given day; $\alpha = 0.05$.

Treatments T2 and T7 incubated at 32°C, followed a similar trend until day 6 and there were no significant differences ($p > 0.05$) between these two treatments (Table 3). Afterwards, only T7 kept growing, whilst T2 showed a decrease in dry weight. In fact, T7, being sterile BBM medium, reached a maximum cell density of 1.53 g L^{-1} after 9 days of cultivation. In the case of T2, the D.W. reached a maximum of 0.88 g L^{-1} after 6 days and there were no significant differences between T2 and the two control treatments at this time ($p > 0.05$). By contrast, in treatments T3, T4 and T5 containing less percentage of the BBM medium, the dry weight of

the mixed cultures increased only during the first three days, although at a lower rate and afterwards, it decreased indicating deficiency of nutrients. It is interesting to note that T1, which contained only non-sterile BBM medium, followed a similar trend than T3, T4 and T5, during the first 3 days. Later on, it kept growing reaching a maximum D.W. of 1.01 g L^{-1} after 9 days. The maximum specific growth rate (μ_1) calculated for T2 during the first 3 days of cultivation (0.776 d^{-1}), did not show any significant difference ($p > 0.05$), compared to that observed for T7 (0.662 d^{-1}) under the same period. In the case of T6, containing

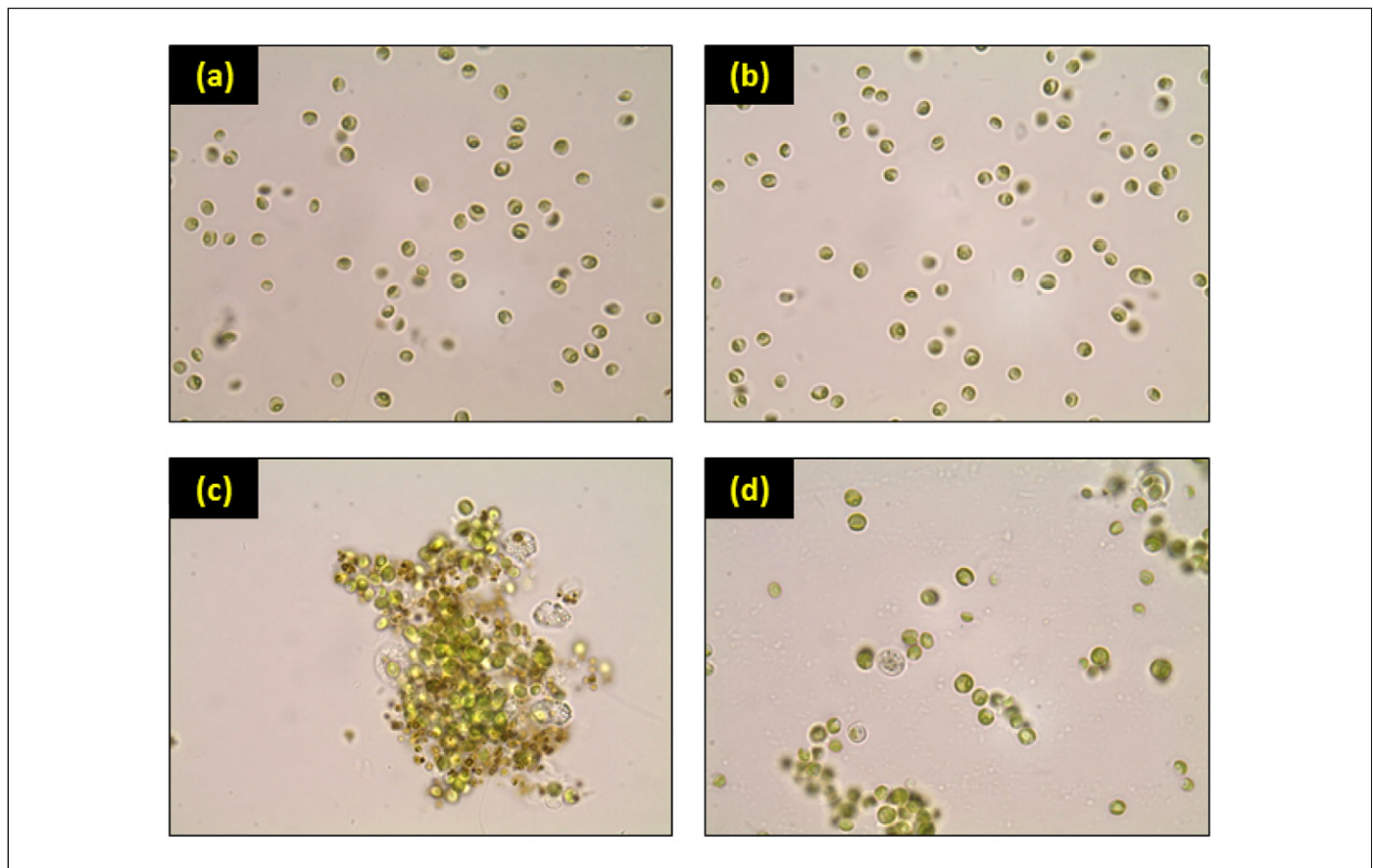


FIGURE 1

N. oleoabundans cultures observed at 100× after 10 days of cultivation: (a) and (b) in sterile and non-sterile BBM, respectively, at 25°C. (c) and (d) in T2 at 25°C and 32°C, respectively.

100% of the SRW, the maximum specific growth rate (μ_1) during the first 3 days was 0.499 d^{-1} and it was significantly different from the one calculated for T2 and T7. Furthermore, growth in this treatment showed a severe loss later on, indicating a high nutrients' deficiency.

In the case of the cultures incubated at 25°C , T7 and T1 followed a similar trend and there were no significant differences ($p > 0.05$) between the maximum cell density reached after 9 days of these two control treatments (Table 3). It is interesting to note that there were no significant differences in cell density reached after 3 days, among all treatments. This means that the cell density promoted by the BBM medium in T1 and T7 after 3 days was similar to the one encountered in all treatments, indicating that nutrients were available in a similar fashion in all treatments, until day 3 of cultivation. However, significant differences between the control treatments T1 and T7 and the rest of the treatments became apparent after 6 days of cultivation and were very clearly established after 9 days of cultivation.

Regarding the effect of temperature on the cell density of mixed cultures after 9 days of cultivation at two different temperatures (Table 3), it was clear that in all treatments, except in treatment T6, the cell density of the mixed cultures grown at the higher temperature tested, were significantly higher compared to the lower temperature tested.

Population dynamics in mixed cultures inoculated with *N. oleoabundans*

In control cultures containing 100% of non-sterile BBM medium (T1) and 100% of sterile BBM medium (T7), it was observed that *N. oleoabundans* was predominant after 10 days of cultivation at both incubation temperatures (Fig. 1a,b). In T2, a fair amount of *N. oleoabundans* was observed after 10 days of incubation (Fig. 1c,d). As expected, in other treatments, the presence of these green microalgae started to decrease as the proportion of the river's water increased. In T3 at 25°C , an important presence of diatoms (Bacillareacea) started to be apparent. *Nitzschia frustulum* (Kützing) Grunow in Cleve et Grunow 1880, *Amphora* sp. and *Navicula* sp. were observed together with *N. oleoabundans* (Fig. 2a). *Scenedesmus obliquus* (Turpin) Kützing 1833, was observed together with *Nitzschia frustulum* at 32°C in T3 (Fig. 2b), although the presence of *N. oleoabundans* was less apparent compared to cultures incubated at 25°C . In T4, after 10 days of cultivation at 25°C , the presence of non-pigmented round cells together with *N. oleoabundans* cells, was observed (Fig. 2c). Such cells were identified as a Chytridiomycete (Div. Chytridiomycota), which is an imperfect fungi, very common in polluted waters with high content of organic matter and frequent parasites of microalgae. These round cells are the sporangium and the small structures inside correspond to zoospores. In the case of T4, after 10 days of cultivation at

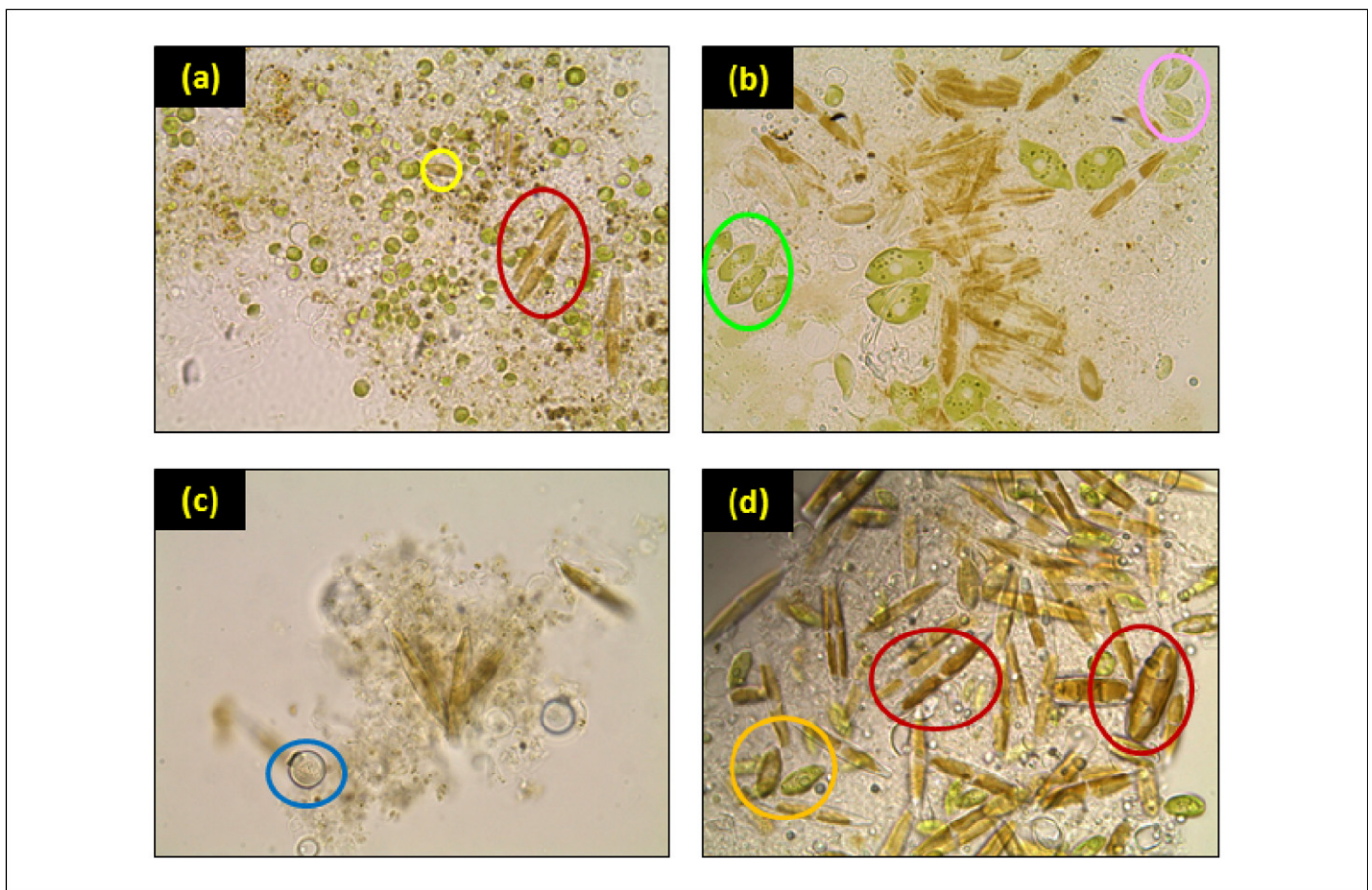
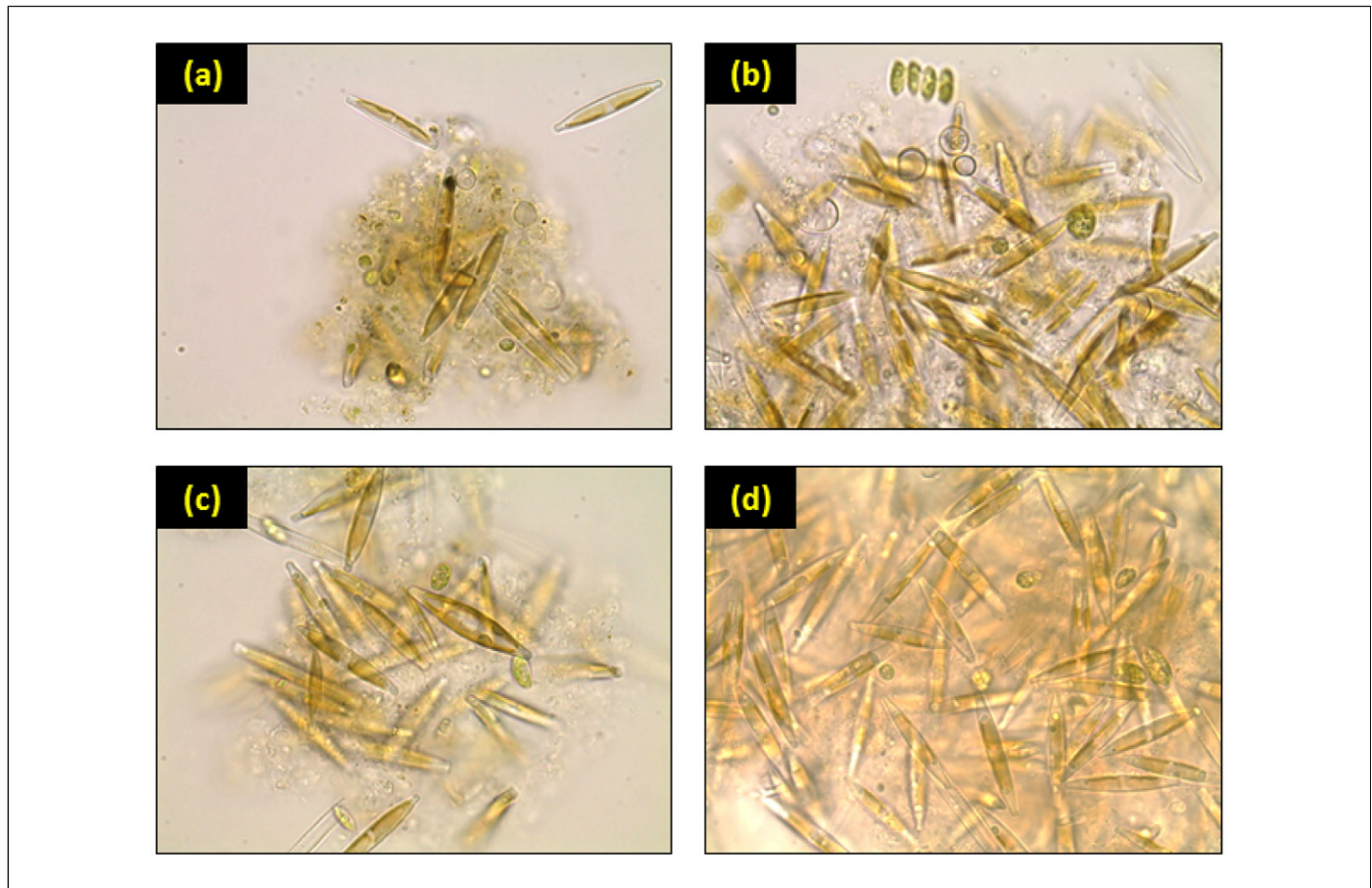


FIGURE 2

Presence of diatoms (Bacillareacea) and other microalgae in various treatments after 10 days of cultivation: **(a)** T3 at 25°C : *Nitzschia frustulum* and *Amphora* together with *N. oleoabundans*. **(b)** T3 at 32°C : *Sc. obliquus* and *Nitzschia frustulum*. **(c)** T4 at 25°C : *N. oleoabundans* cell debris, a Chytridiomycete (non-pigmented round cells) and diatoms. **(d)** T4 at 32°C : *Sc. obliquus* and *N. oleoabundans* together with a large population of *Nitzschia frustulum* can be observed. A Naviculaceae is also present.

**FIGURE 3**

Presence of diatoms (Bacillareacea) and other microalgae in various treatments after 10 days of cultivation: **(a)** and **(b)** T5 at 25°C and 32°C, respectively: *Nitzschia frustulum* was predominant. **(c)** and **(d)** T6 at 25°C and 32°C, respectively: *Nitzschia frustulum* was predominant.

32°C (Fig. 2d), *Nitzschia frustulum* was observed to become predominant, although cells of *Scenedesmus* were also observed. Another diatom of the family Naviculaceae can also be observed, although in a minor proportion compared to *Nitzschia frustulum*.

In the case of T5, which contained only 20% of BBM medium a very abundant population of *Nitzschia frustulum* was observed after 10 days of cultivation at both temperatures (Fig. 3a,b). Finally, in T6, containing 100% of the Sordo river's water, the predominance of *Nitzschia frustulum* was also observed after 10 days of cultivation at 25 and 32°C (Fig. 3c,d).

Kinetics of growth and removal of nutrients of a diatom consortium

Because the predominant population in mixed cultures containing more than 60% of water from the Sordo River, was a mixture of diatoms, isolation of a diatom consortium was carried out. Assessment of its kinetics of growth (Fig. 4), showed that the specific growth rate during the first four days of such consortium was significantly higher in the cultures incubated only with water from the Sordo River (0.0641 and 0.0596 d⁻¹ for 25°C and 32°C, respectively) compared to those cultures prepared using the synthetic medium for diatoms (0.020 and 0.03452 d⁻¹ for 25°C and 32°C, respectively). There was a decline of growth from day 4 to day 6 in all treatments, followed by a new increase from day 6 to day 8 in cultures using the water from the polluted river, although at a

lower specific growth rate (0.0324 d⁻¹ and 0.0479 d⁻¹ for 25°C and 32°C, respectively) compared to the first period of growth.

The diatom consortium was able to remove very effectively the ammonia nitrogen from the Sordo River water, after 2 days (Fig. 5): 95.45% and 95.78% at 32°C and 25°C, respectively. Because the MDM contains very little ammonia nitrogen, the removal was negligible in this medium. The removal of nitrates from the Sordo River's water was 60% and 62.5% at 32°C and 25°C, respectively. Nitrates were also removed from the Diatoms Medium after 2 days: 78.46% at 32°C and 92.30% at 25°C. After 8 days, nitrates were not detected in the culture media.

It is interesting to note that the uptake rate of ammonia nitrogen in the water from the polluted river was 4.348 mg L⁻¹ d⁻¹ in cultures incubated at 32°C and 4.791 mg L⁻¹ d⁻¹ in those incubated at 25°C. By contrast, the uptake rate of nitrate in this type of water was 0.2665 and 0.1250 mg L⁻¹ d⁻¹ at 32°C and 25°C, respectively during the first two days. The uptake rate of nitrate in the MDM was 1.4165 and 1.525 mg L⁻¹ d⁻¹ in cultures incubated at 32°C and 25°C, respectively.

Regarding the removal of phosphates (Fig. 4), the diatom consortium was able to remove 95% at 32°C and 67% at 25°C from the Sordo River's water after 4 days. In the same period of time, the removal of phosphates from the MDM was 40.75% and 18.8% at 32°C and 25°C, respectively. After 10 days, phosphates were not detected in any of the two culture media.

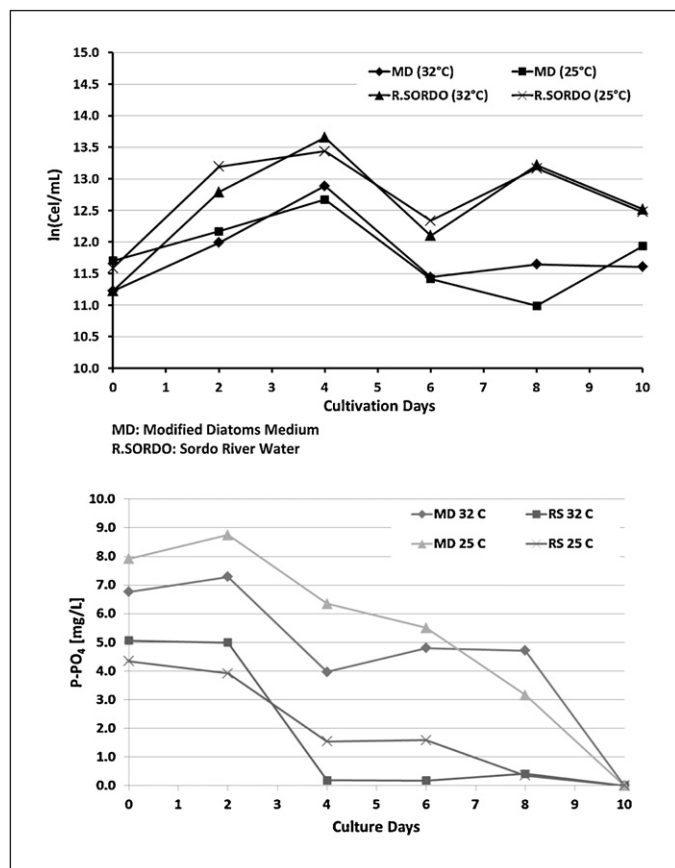


FIGURE 4

(a) Growth curve of the diatom consortium in two culture media (modified diatom medium DM and Sordo River's water) and two temperatures (25°C and 32°C) and (b) phosphate removal.

Presence of lipids in cells of various microalgae

Cells from centrifuged samples from each treatment were subjected to a staining technique with Sudan III as described in the Material and Methods section. The presence of lipids by this technique was only detected in some diatoms (*Nitzschia frustulum*, *N. palea* and a non-identified Naviculaceae) in T4, T5 and T6 after 15 days of incubation at both temperatures tested (Fig. 6). In the rest of the treatments, there was no evidence of the accumulation of lipids at significant levels, neither in *N. oleoabundans*, nor in other microalgae.

Discussion

The characterization of the Sordo River's water collected in November 2011 and in April 2012, indicated that it contained a large amount of organic matter, but most of it was biodegradable, because the value of COD was not very different from the value of the BOD. Besides, the water collected in November 2011 contained a large amount of total Nitrogen which compares with the amount encountered in municipal water in the category classified as 'medium strength' according to Tchobanoglous *et al.* [20]. The nitrate and phosphate content was strongly affected by the time of sampling. However, in the case of the water sampled in spring (April) and used for the cultivation of the diatoms consortium, it resulted in a better medium for the cultivation of such consortium compared to the synthetic medium (DM). Such advantages might

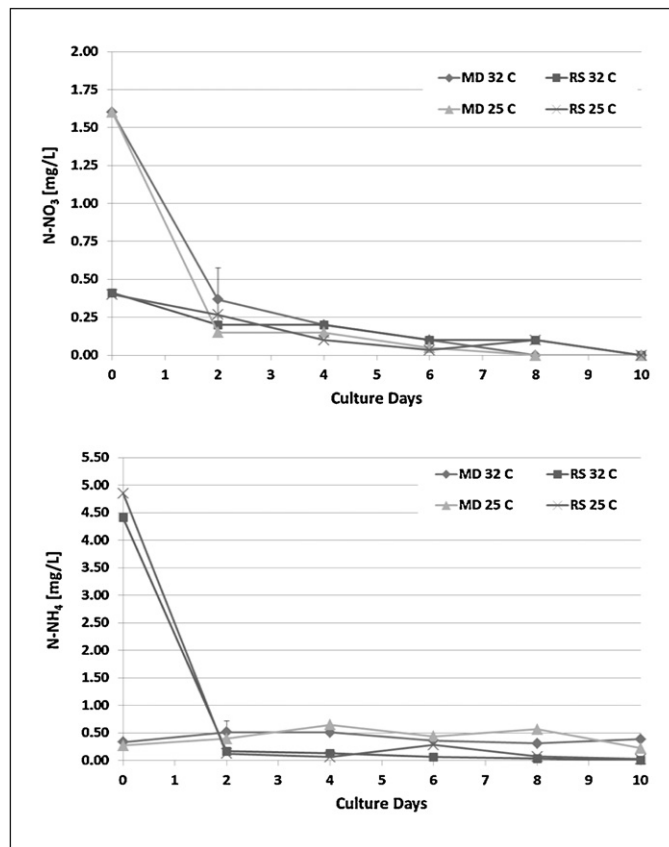
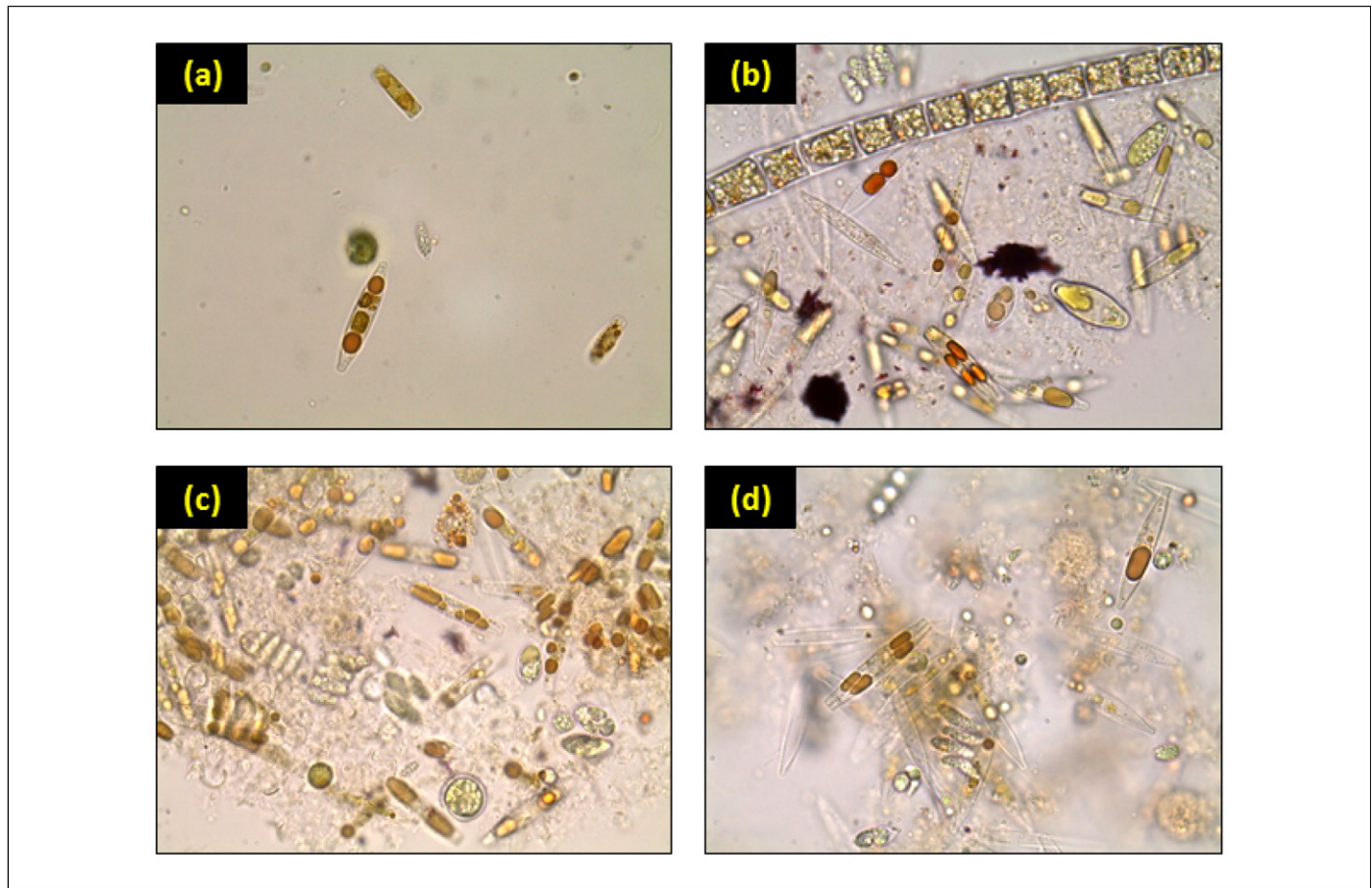


FIGURE 5

Nitrogen concentration changes during the cultivation of the diatom consortium: (a) nitrates and (b) ammonia nitrogen.

be the result of a higher content of nutrients derived from the dissolved organic matter as discussed below (Fig. 7).

Regarding the observed population dynamics in mixed cultures, the presence of *N. oleoabundans* in mixtures containing 80 and 60% water of the Sordo river, could be the result of the utilization not only of the inorganic nutrients dissolved in the river's water and encountered in the BBM culture medium (20 and 40%, respectively), but also of the presence of organic carbon sources in this type of water. The BOD of the river's water was 169.30 mg L⁻¹ in November and 79.0 mg L⁻¹ in April, indicating a high content of organic matter. Mixotrophic growth has been reported in several microalgae including cyanobacteria (*Chlorella vulgaris*, *C. pyrenoidosa*, *Arthrospira platensis* [21], *Cochlodinium polykrikoides*, *Ochromonas tuberculata* and *Cryptomonas* sp., *Chlamydomonas acidophila*, *Nostoc flagelliforme* [22] and *Chlorella minutissima* [23]. In the case of *Chlorella minutissima*, it was found that a medium containing 50% of sterilized municipal wastewater supported 146% more growth than the standard BG 11 medium used as a control medium [23]. The authors explained such higher yield on the basis of the capacity of *C. minutissima* of growing mixotrophically. In fact, mixotrophic growth could explain the very high growth rate observed in this work for T2 (0.44 d⁻¹) during the first 3 days at 32°C since the specific growth rate of *N. oleoabundans* in the control synthetic medium T7 was 0.36 d⁻¹. The latter value is very similar to the specific growth rate of

**FIGURE 6**

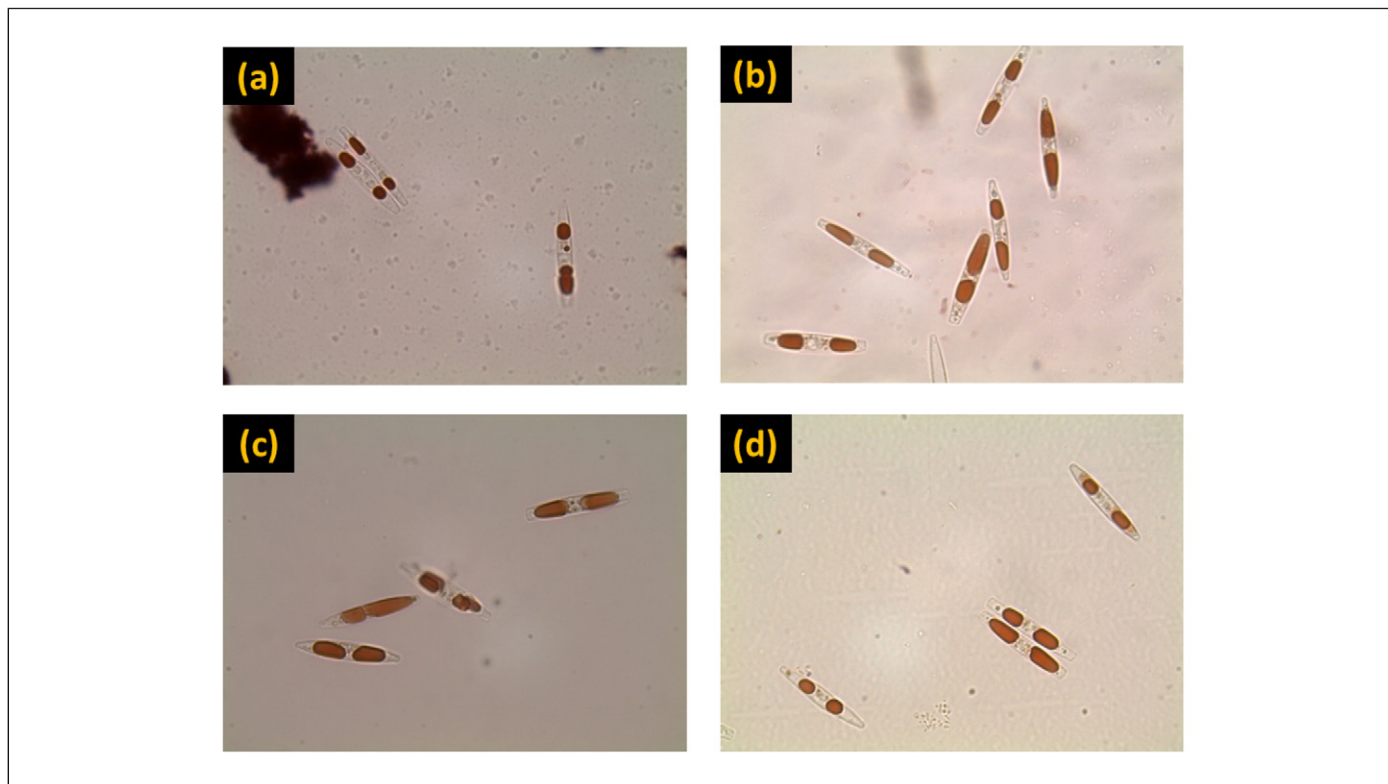
Diatoms in mixed cultures stained with Sudan III in various treatments: **(a)** T4, after 15 days of cultivation, 25°C; **(b)** T6, after 15 days of cultivation, at 25°C; **(c)** T5, after 15 days of cultivation, 32°C; **(d)** T6, after 15 days of cultivation, at 32°C.

0.37 d^{-1} that has been reported for *N. oleoabundans* in an open trough system using BG11 medium [24].

The effect of temperature on the growth of the mixed cultures reported in this work is interesting. The results indicated that in all treatments, except in treatment T6, the cell density of the mixed cultures grown at the higher temperature tested, was significantly higher compared to the lower temperature tested. Thus, it seems probable that during the seasons with temperature around 30–32°C, the mixed cultures could be more productive.

Concerning the native diatoms mainly of the genus *Nitzschia* and *Navicula* which were found to predominate in mixtures with a large proportion of water from the river or in cultures using only the river's water, it is important to mention that diatoms (Bacillariophyceae) are commonly found in rivers and streams. In the case of the present study, diatoms were observed in very small quantities at the beginning of the cultivation period. However, they started to predominate after 8 or 10 days of cultivation, especially in the treatment with 100% of water from the Sordo River. It has been well established that diatoms are more adapted to polluted environments in comparison to other microalgae. In fact, since early work performed by Kobayashi and Mayama [25], it was reported that diatoms were always present in samples from highly polluted rivers (DBO

from 22 to 79 mg L^{-1}) and that the genus *Nitzschia* and *Navicula* were most dominant in all samples. Later on, it was established that diatoms are very useful as environmental indicators in rivers and streams because of three basic reasons: (a) their importance as part of the web chain in the ecosystem, (b) their direct and rapid response to many environmental factors and (c) the facility of their use [26]. Furthermore, a Trophic Diatom Index (TDI) was developed by Kelly and Whitton [27], which became a useful Index for monitoring the trophic status of rivers based on diatom composition. These authors assigned two important values to different diatom taxa: the taxon sensitivity (s) and the indicator value (v). In the case of the diatoms observed in the present study and especially after 10 days in the treatment with 100% of the rivers' water, which were identified to belong to the genus *Nitzschia* or *Navicula*, they have received a taxon sensitivity value in the range of 4–5 according to Kelly and Whitton [27], indicating that they are highly pollution-tolerant and especially that they are in waters containing 0.1 or more than 0.3 mg L^{-1} of Phosphorus (P). In fact, the water from the Sordo River utilized for the cultivation of the mixed cultures, contained 0.39 mg L^{-1} of P. In a recent report of the study of diatom communities as indicators of the ecological status in Mediterranean temporary streams [28], it was found that *Navicula veneta*, *Nitzschia inconspicua*, *Nitzschia*

**FIGURE 7**

Diatom consortium stained with Sudan III in different media and incubation temperatures, after 8 days: **(a)** Modified Diatom Medium, 25°C; **(b)** Modified Diatom Medium, 32°C; **(c)** Sordo River's Water, 25°C; **(d)** Sordo River's Water, 32°C.

frustulum and *Planothidium frequentissimum*, appeared in moderate, poor and bad water streams status classes, which were characterized by high level of organic/nutrient pollution. Thus, it is not surprising that in the present work, *Nitzschia frustulum* was found to predominate in the mixture with 100% of the Sordo River's water.

Regarding the growth kinetics of the diatom consortium cultivated in two different media and at two different temperatures, it is worth noting that the specific growth rate was significantly higher in cultures incubated only with water from the polluted river, compared to the cultures incubated in modified diatom medium (MDM), regardless of the incubation temperature. These results suggest the occurrence of mixotrophic growth, taking advantage of the dissolved organic matter present in the river. It has been shown that the toxigenic diatom *Pseudo-nitzschia australis* is able to utilize both inorganic and organic forms of nitrogen, such as nitrate, ammonium, urea and glutamine [29]. Mixotrophic growth has been shown to occur in other diatoms such as *Phaeodactylum tricornutum* [30]. Furthermore, heterotrophic growth has resulted in very high cell densities of *Nitzschia laevis* [31]. The light intensity used in this work to cultivate the mixed cultures and the diatom consortium ($66 \mu\text{mol m}^{-2} \text{s}^{-1}$), does not seem to limit growth, because a very high cell density of *Nitzschia pusilla* ($1.37 \pm 0.08 \text{ g L}^{-1}$) has been reported, when cultivated in BBM and exposed to a light intensity of $40 \mu\text{mol m}^{-2} \text{s}^{-1}$ [14].

The diatom consortium was very effective at nutrient removal, showing a significantly higher removal percentage of ammonia nitrogen compared to nitrate removal percentage after two days of growth, regardless of the temperature. The uptake rate of

ammonia nitrogen was 16-fold higher than the uptake rate of nitrate at 32°C and 40-fold higher than the nitrate's uptake rate at 25°C. These results are different from other reported for other diatoms, such as *Pseudo-nitzschia australis* that showed preference for nitrate over ammonia nitrogen and other nitrogen sources [29]. Although there are no reports available related to nitrogen uptake in *Nitzschia* sp., it has been reported that phytoplankton in coastal lagoons was well adapted to the seasonal variations in resources, In winter and spring, ammonium was clearly preferred to nitrate as a nitrogen source, but nitrate was an important nitrogen source in summer because of high nitrification rates [32]. In the case of the diatom consortium tested in this work, it seems that the preference for ammonia nitrogen uptake over nitrate uptake might provide a competitive advantage among other microalgae present in a polluted stream. However, further work is required to establish preference of the diatom consortium for various nitrogen sources under various cultivation conditions.

Sudan III stain is a lysochrome (fat-soluble dye), used to stain lipids, lipoproteins, and triglycerides in various biological materials. The detection of lipid accumulation by staining with Sudan III in various microalgae present in each treatment provided interesting results. *N. oleoabundans* cells in samples from T1 and T7, did not show any red intracellular structures, indicating that there was not significant lipid accumulation in these cultures after 15 days. These results could be explained on the basis that the staining technique with Sudan III has a low sensitivity when the cultures contain low percentages of lipids. Gouveia *et al.* [13] reported a constant level of approximately 20% of lipid content during the

whole growth curve in cultures of *N. oleoabundans* under conditions of nitrate sufficient medium, incubation at 26°C and without addition of CO₂. Thus, because the cultures of *N. oleoabundans* used as control cultures in the present work, were under nitrogen sufficient conditions, at least during the first days of cultivation before the nitrogen was totally consumed, no high levels of accumulation of lipids are expected. On the other hand, the observations of accumulation of lipids in diatoms cells, mainly *Nitzschia frustulum*, *N. palea* and a non-identified Naviculaceae) in T4, T5 and T6 after 15 days of incubation at both temperatures tested, indicated the convenience of isolating a diatom consortium. In fact, the results showing that such consortium had a significantly higher specific growth rate in water from the Sordo River compared to the diatom synthetic medium, that they remove efficiently nutrients and that they contain a lot of lipids after 10 days as indicated by the Sudan staining, are encouraging and indicate that these native organisms may have potential for biodiesel production at a low cost. It has been reported that *Nitzschia pusilla* had a lipid content of 48 ± 3.1 as percentage of the cell dry weight, when cultivated in BBM and exposed to a light intensity of $40 \mu\text{mol m}^{-2} \text{s}^{-1}$ [14]. Other reports have indicated that diatoms isolated from freshwater and marine environments, accumulate enough lipids of adequate quality to be considered as potential strains for biodiesel production [33–35]. Work is in progress to assess the lipid productivity of the diatom consortium isolated from the Sordo River using quantitative gravimetric and chromatographic techniques.

Conclusions

Monitoring of the population dynamics in mixed cultures after inoculation of *N. oleoabundans* in different mixtures of BBM and water from the Sordo River, indicated that such green microalgae only predominated in mixtures containing 80 or 60% of the synthetic medium. The effect of temperature on the growth of the mixed cultures was evident and the best performance was observed at 32°C in comparison with the growth observed at 25°C. In mixtures containing a volume of the polluted river higher than 40%, other microalgae predominated, especially diatoms of various genera. The diatom consortium isolated from a 100% of the river's water sampled in spring (April), was formed mainly by a population of *Nitzschia frustulum* and in less extent of *Navicula* sp. Such consortium showed a large potential for being used in a dual-purpose system that could treat the water from the river and that could produce lipid rich biomass. Further work is required to establish larger culture volumes for producing enough biomass for being analyzed by chromatographic methods.

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