Removal of organic matter during adaptation of *Nannochloropsis oculata* in livestock waste

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Abstract: Livestock effluents and sludges contain high levels of organic matter, consisting primarily of carbon, hydrogen, oxygen, nitrogen, phosphorus and sulfur (generally in the form of carbohydrates, lipids and proteins). Such waste represents an important resource that can be used to produce some bioproducts. Therefore, different species of microalgae have been studied and used at laboratory scale to carry out processes such as wastewater treatment or aerobic bioconversion, which are presented as sustainable and viable alternatives for the treatment and recovery of organic waste (OW). *Nannochloropsis oculata* can remove contaminants present in organic waste and wastewater by incorporating them into their metabolism through photosynthesis, efficiently producing polyunsaturated fatty acids, sterols, proteins, terpenoids, pigments, among others. The aim of the present study was to evaluate the removal of organic matter during the adaptation of *N. oculata* in poultry wastewater (PWW) and pig manure (PM), obtained from technical and semi-technical plants, respectively, located in the high mountain zone of the state of Veracruz, Mexico. The experiment was carried out in 250 mL discontinuous photobioreactors with a working volume of 200 mL, where 3 inoculum-substrate ratios were studied for each organic residue: 10, 15 and 20% inoculum in PWW and 30, 50 and 70% inoculum in PM. In addition, the conditions of temperature (20 ± 2 °C), illumination (2000 lx), photoperiod of 12/12 (light/dark) and continuous aeration were controlled. The results showed that *N. oculata* was able to reduce the amount of organic matter present in the OW, reaching up to 37.91 and 37.18% reduction of soluble COD in PWW (10%) and PM (70%), respectively.

Keywords: *Nannochloropsis oculata*; poultry wastewater; pig manure.

Introduction

It is estimated that approximately 38 billion metric tons of organic waste (OW) is generated worldwide each year. Such an amount of OW has a significant impact on the emission of large amounts of methane, a result of the anaerobic fermentation process. Therefore, its safe management and disposal has become a global priority (Mashur *et al*., 2021; Xie *et al*., 2023). Currently, some OW, such as agricultural waste, industrial waste, municipal organic waste, sewage sludge, and livestock waste, are being used as potential sources for bioenergy production (Torky *et al*., 2023) and high-value bioproducts (Dhanya *et al*., 2020; Ng *et al*., 2020). These bioproducts can be materials, chemicals, secondary metabolites, and other resources derived from renewable biological matter (Bourlieu *et al*., 2020).

Additionally, the increase in poultry production has led to an increase in the amount of waste produced, including litter, feathers, eggshells, carcasses, blood and wastewater (McGauran *et al*., 2021). The latter represents a serious environmental problem, since it usually contains large amounts of organic matter, which requires appropriate treatment (Vladić *et al.,* 2023). Wastewater treatment aims to reduce contaminant levels in order to minimize the environmental impact of wastewater return flows. Even though, another viable alternative is the reuse of contaminants to generate biomolecules of interest (Jones *et al*., 2021).

In this sense, the development of technologies aimed at protecting the environment, reusing waste, and generating low-cost clean energy has become essential. Microalgae cultures play an important role in the treatment of OW by efficiently recycling contaminants from liquid media and incorporating them into their metabolism to produce biomass (Hernández-Pérez & Labbé, 2014). These biomasses are considered a sustainable resource suitable to produce lipids, polyunsaturated fatty acids, sterols, carbohydrates, proteins, polysaccharides, terpenoids, pigments, among others (Li *et al*., 2023; Liang *et al*., 2019). Each species of microalgae is capable of producing different levels of these intracellular compounds depending on the chemical composition of the culture medium (Manegazzo & Fonseca, 2019).

Nannochloropsis is a genus of unicellular microalgae that was first described taxonomically in 1981. It is widespread in the marine environment. However, it is also commonly found in freshwater due to its important role in the biogeochemical cycling of carbon and other minerals (Tran *et al*., 2022). Among the six identified species of the genus is *N. oculata*, which was originally isolated on the coast of Scotland and has been widely used as a food source in aquaculture, providing a source of omega-3 fatty acids (Ashour *et al*., 2019; Kagan & Makulta, 2015).

The objective of this study was to evaluate the removal of organic matter during the growth of *N. oculata* in Poultry wastewater (PWW) and pig manure (PM), which are typically rich sources of carbon and nitrogen, mainly in the form of carbohydrates, lipids, and proteins (Ng *et al*., 2020). This process was evaluated as a scientific and technological strategy for the valorization of OW. The cells of *N. oculata* are characterized by their resistance to the different environments in which they develop. In addition, it has a high biomass productivity and high lipid content, making it suitable and economical to produce biodiesel, polyunsaturated fatty acids, pigments, etc (Askari *et al*., 2022).

Materials and Methods

Collection, conditioning and characterization of the waste

For the growth experiments of *N. oculata* and with the purpose of comparing the development of said microalgae in media with different concentrations of organic matter, PWW and PM were used. Another important factor was the availability of pig and poultry farms in the state of Veracruz, Mexico. The PWW was collected at the San Antonio Poultry Processing Plant, located in the municipality of Fortin, Veracruz. Sampling was performed using the NMX-AA-3-1980 technique. On the other hand, the PM was obtained in a semi-technical farm located in the municipality of Rafael Delgado, Veracruz. Sampling was carried out using the technique described in Appendix II of NOM-004-SEMARNAT-2002 for residual sludge. Once obtained, the PM was conditioned with a 1:4 dilution to simulate the sludge that results from washing rearing areas. Both samples were kept refrigerated at 4 °C until use.

The characterization of the residuals consisted of the determination of pH by potentiometric method. Total solids (TS) and volatile total solids (VTS) by method 2540 G Standard Methods. Chemical oxygen demand (COD) by the method of standard NMX-AA-030/1-SCFI-2012, as well as soluble COD. total nitrogen by the Kjeldhal method. Proteins from the percentage of total nitrogen by the factor according to the origin of the samples (6.25). Determination of carbohydrates by the Antrone sulfur method. Lipids by the Soxhlet extraction method.

Propagation of Nannochloropsis oculata in synthetic medium and growth in OW.

The species *N. oculata* was purchased from the company Algae Bank. The propagation and growth of the species was carried out in batch photobioreactors with a working volume of 40 mL in the f/2 Guillard culture medium with 1:1 dilution as recommended in the literature (Guillard & Rhyter, 1962). Propagation was carried out under the following controlled conditions: 20 ± 2 °C, white light (2000 lx), pH 6.50 ± 0.20, photoperiod 12/12 (light/dark), continuous aeration (2 L/min) and feeding every 7 days (Chua & Schenk, 2017; Martínez-Macías *et al*., 2018).

The Neubauer chamber was used to determine cell density. Due to the high cell density, a 1:7 dilution was required to count *N. oculata* cells in the central quadrant and Equation 1 was used to determine the number of cells per milliliter.

$$N \cdot f_{ccc} \cdot f_d = \frac{cells}{mL} \quad \textbf{(1)}$$

Where N is the number of cells counted in the central quadrant, $\boldsymbol{f_{ccc}}$ corresponds to the conversion factor of the counting chamber (10^4) and $\boldsymbol{f_d}$ is the dilution factor used.

Subsequently, 250 mL photobioreactors with a working volume of 200 mL were used for the adaptation of the microalgae in OW. Due to the low concentration of organic matter in the PWW, low inoculum concentrations (10, 15 and 20%) were evaluated. These were similar to those used in previous research with the same substrate but different microalgae. High inoculum concentrations (30, 50, and 70%) were used. Such concentrations allowed the growth of *N. oculata* to be evaluated in media with high amounts of organic matter.

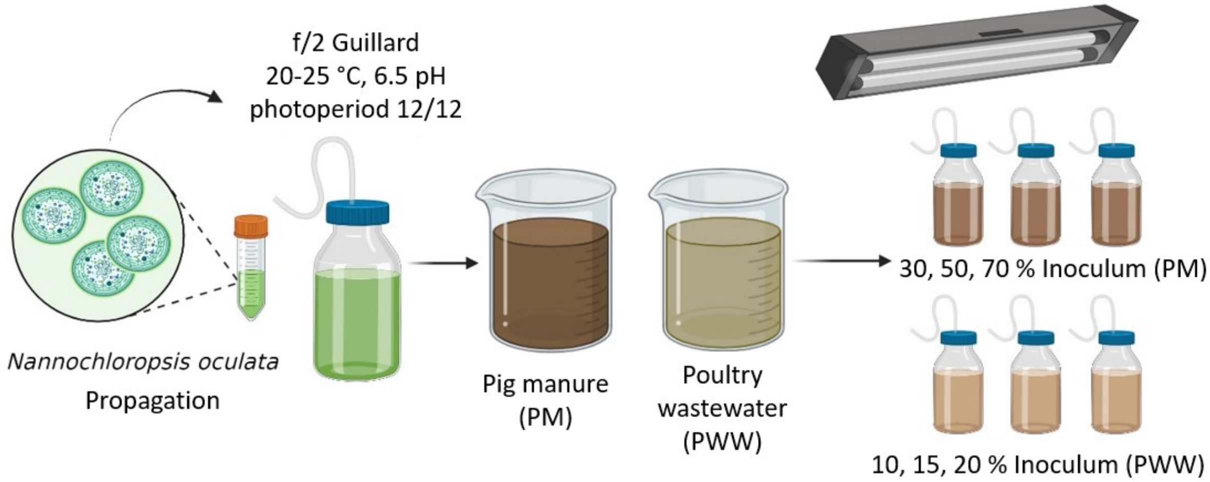


Figure 1. Propagation sequence of the *N. oculata* species in PM and PWW.

The photobioreactors with different concentrations of both wastes were maintained at constant temperature (20 ± 2 °C), white light (2000 lx), 12/12 photoperiod and continuous aeration (2 L/min). All experiments were monitored for 15 days and samples were taken every 24 hours to assess pH and cell density. In addition, initial and final COD were determined to evaluate the removal of organic matter in OW during the 15 days.

Results and Discussion

The results of the physicochemical characterization of the substrates are presented in Table 1. The pH of the PWW can be considered neutral (pH 7.21), which is due to the diet that the birds generally receive: cereals, maize, sorghum, etc. Although the composition varies according to different production practices in different geographical locations (McGauran *et al*., 2021). The pH value obtained in this study is within the range reported by Njoya *et al*. (2019), who mention that for PWW, this parameter reaches values between 6 and 8. In addition, the pH value was close to that suitable for the growth of *N. oculata* (6.50 ± 0.20). Because the sampling was done in the morning, when organic matter concentrations in the effluent are lower, the Total COD (1.52 g/L) was below the average for poultry effluent (4.21 g/L) (Njoya *et al*., 2019). However, both total and soluble COD (1.14 g/L) were ideal for studying the growth of *N. oculata* in substrates with average levels of organic contaminants.

Table 1. Physicochemical characterization of PWW and PM.

Parameter	PWW	PM
pH	7.21 ± 0.15	7.30 ± 0.18
TS (%)	0.66 ± 0.24	4.88 ± 0.95
VTS (%)	87.99 ± 2.08	78.92 ± 2.24
COD (g/L)	1.52 ± 0.23	15.22 ± 1.09
Soluble COD (g/L)	1.14 ± 0.18	4.31 ± 0.36
Electrical conductivity (µS/cm)	830 ± 20	--
Total nitrogen (%)	0.052 ± 0.01	0.35 ± 0.08
Proteins (%)	0.33 ± 0.06	2.18 ± 0.50
Lípids (g/L)	0.41 ± 0.05	0.015 ± 0.00
Carbohydrates (g/L)	0.81 ± 0.12	8.15 ± 0.98

The electrical conductivity represents the salts dissolved in water that decompose into positively and negatively charged ions. Due to the small amount of inorganic matter in the sample, the value obtained (830 µS/cm) was lower than the average reported in the literature (1579 µS/cm) (Yaakob *et al*., 2018). The TS content was 0.66%, which was slightly lower than that reported by Pérez (2023), who obtained 0.78% TS in poultry effluent sampled in the same processing plant. The slight difference can be attributed to the different period, time and sampling point.

On the other hand, the pH of the PM (7.30) was similar to that of the PWW, both values slightly higher than the pH of the f/2 Guillard medium. The TS value was 4.88%, similar to that reported by Estrada García *et al*. (2023), who evaluated the anaerobic bioconversion of pork waste with *Lactobacillus acidophilus* and after subjecting the sample to a 1:4 dilution, they obtained 5.63% TS. Despite the dilution made to the PM, there is a notable difference with the TS value in PWW. Which is caused by the physicochemical characteristics of the media.

In terms of macromolecules, there are notable differences in the values of carbohydrates 8.15 g/L, lipids 0.015 g/L and proteins 2.18% for PM and 0.81 g/L, 0.41 g/L and 0.33% in PWW, respectively. These macromolecules can give an idea of the availability of nutrients for cell growth. They are mainly composed of nitrogen, carbon and phosphorus, which are the essential nutrients to guarantee the minimum growth conditions (Su *et al*., 2022). Carbohydrates in the PM were 10 times higher than in the PWW, which is one of the reasons why *N. oculata* growth differed between substrates. Excess carbon can inhibit cell growth (Menegazzo & Fonseca, 2019). Lipids were higher in the PWW because it is characterized by high concentrations of biodegradable organic matter, colloidal and suspended matter such as fats and cellulose (Stiborova *et al*., 2020).

Nitrogen plays an important role in the metabolism of microalgae, mainly by participating in the formation of proteins. When nitrogen is available in the culture, the concentration of proteins, carotenoids and chlorophyll increases. When this nutrient is limited, it causes an increase in the lipid content of the microalgae (Menegazzo & Fonseca, 2019). Although, like carbohydrates and proteins, total nitrogen in the PM (0.35%) was higher than in the PWW (0.052%), they maintained a similar relationship with respect to the concentration of total COD and TS determined in each sample.

After 1 month of *N. oculata* propagation in f/2 Guillard medium, a cell density of 4.34 $x10^7$ cells/mL was obtained. The f/2 Guillard medium is usually advantageous for the development of *N. oculata* in batch reactors, where they can obtain cell densities between 3.86 $x10^7$ to 3.52 $x10^8$ cells/mL (Martínez-Macías *et al*., 2018).

Figure 2 shows the behavior of *N. oculata* in OW. The PWW presented a higher cell density in the reactors with a lower amount of inoculum (10%), whereas the PM the higher cell density occurred in the reactors with a higher amount of inoculum (70%). Such behavior is attributed to the difference in nutrients between the wastes, since their availability plays an important role in the growth and composition of microalgae, especially nitrogen, which is crucial for the growth of microorganisms and forms a major part of vital macromolecules such as amino acids and proteins (Su *et al*., 2022).

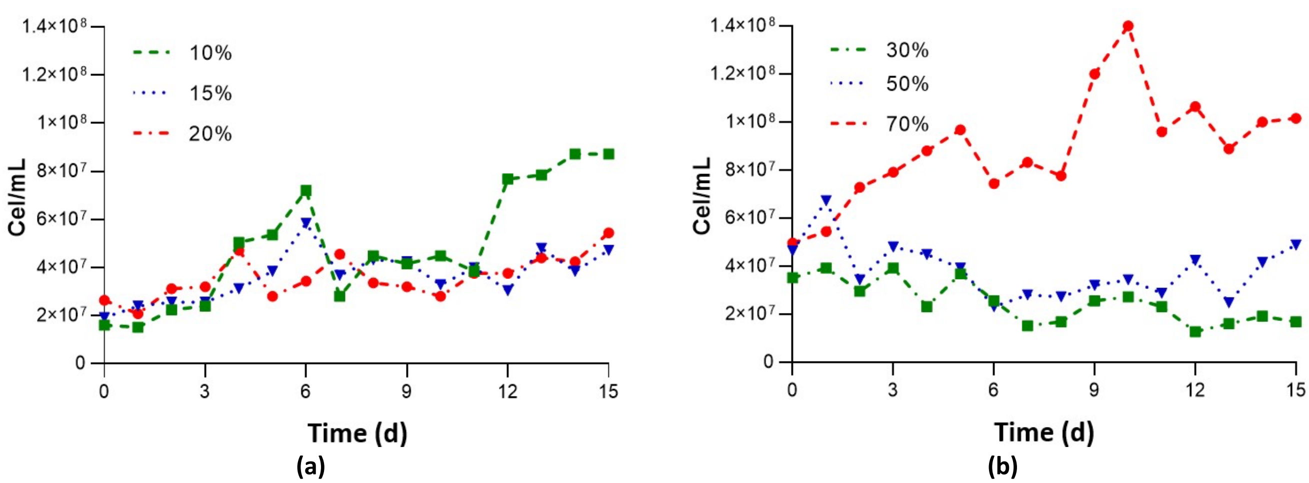
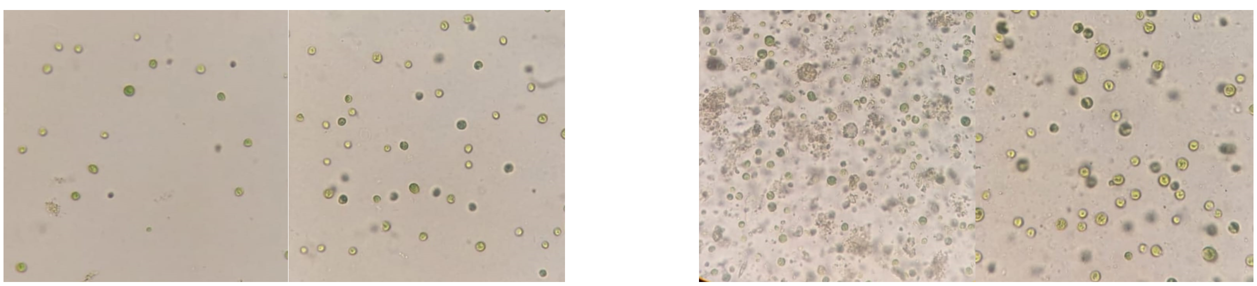


Figure 2. Cell growth kinetics of *N. oculata* in: (a) PWW and (b) PM.

PWW, which is generally produced from the periodic rinsing of poultry processing plants to limit the accumulation of manure and other wastes generated in the different processes (Thoré *et al*., 2021), proved to be a suitable medium for the development of *N. oculata*, since this species was able to survive in the 3 conditions analyzed. As mentioned above, the highest cell density was obtained with 10% inoculum, with an increase from 1.2 x10^7 cells/mL on day 0, to 8.72 x10^7 cells/mL on days 14 and 15. However, with 15%, the greatest growth occurred on day 6 (from 1.92 x10^7 to 5.84 x10^7 cells/mL) and with 20%, the greatest growth occurred on day 15 (from 2.64 x10^7 to 5.44 x10^7 cells/mL). These results are due to the fact that wastewater has a complex physicochemical composition and the nutrients that microalgae need for growth may be limited or excessive depending on the inoculum-substrate relationship (Gutiérrez-Casiano *et al*., 2022).

In PM, a decrease was observed in 2 of the 3 concentrations analyzed. High COD concentrations directly affect turbidity, making it difficult for algae to receive the light intensity necessary for their growth (Gutiérrez-Casiano *et al*., 2022). Another important factor is the presence of microorganisms that can inhibit the growth of the species. In the photobioreactors with 30 and 50% inoculum, there was a decrease mainly due to the inability of the cells to transform large amounts of organic matter. On the other hand, in the photobioreactors with 70% inoculum, a significant growth of microalgae was observed, from 4.96 x10^7 on day 0 to 1.4 x10^8 cells/ml on day 10. The concentrations obtained were higher than those reported by Pachacama *et al*. (2016), who obtained *Chlorella sp*. (1.7 x10^7 cells/ml) and *Synechocystis sp*. (1.04 x10^7 cells/ml) in PM. They evaluated 3 inoculum concentrations (40, 60 and 80%), the maximum concentrations were obtained with an inoculum of 60% for both species. The tests showed that *N. oculata*, like other microalgae species such as *Chlorella vulgaris* and *Isochrysis galbana*, can tolerate extreme conditions such as salinity, temperature, pollutants, and nutrient deficiencies (Ammar *et al*., 2018).

Figure 3 shows the comparison of *N. oculata* cells observed under the light microscope at 40x objective. In a) the PWW with 10% inoculum is observed. Cells and a change in concentration between day 0 and day 15 are clearly visible. In b) the PWW with 70% inoculum is observed. In contrast to the PWW, the cells could not be clearly observed on day 0 due to the high concentration of matter in the sample. On day 15, although the cell concentration is not higher than in PWW, microalgae of larger size and a decrease in the amount of matter present can be clearly seen.



(a) **(b)**

Figure 3. *N. oculata* cells observed under the microscope with the 40x objective at the beginning and end of the growth curves: (a) PWW with 10% and (b) PM with 70%.

The pH of the growth medium has a direct effect on the development and metabolism of microalgae. It is known that neutral pH is favorable for the growth and metabolism of microalgae (Jacob *et al*., 2021). Figure 4 shows the variation of pH values recorded during the growth of *N. oculata*, in both OW the pH had a neutral tendency, presenting values slightly higher than those suggested for the species (6.50-7). This indicates a behavior in which a higher cell density is associated with an increase in pH. Such behavior has been demonstrated in other microalgal species such as *Chlamydomonas reinhardtii*, *Dunaliella parva* and *Anabaena variabilis*, where the removal of carbon from the substrate for its use in photosynthesis and O_2 generation leads to an increase in extracellular pH (Salbitani *et al*., 2021).

Finally, the removal of organic matter was evaluated by the decrease in soluble COD determined on days 0 and 15. In the PWW, experiments with 15 and 20% inoculum showed a decrease of 5.21 and 1.49%, respectively. While with 10% a reduction of 37.91% of the said parameter was obtained. Although the capacity of *N. oculata* to remove contaminants present in the PWW was demonstrated, the percentages were lower than those reported by Aksu *et al*., 2021, who eliminated 76% of COD in a substrate of the same nature pretreated by flotation dissolved air and cultivation of *Chlorella Vulgaris* (12%) for 15 days. Therefore, it is recommended to pre-treat the substrate to increase the removal efficiency of organic pollutants.

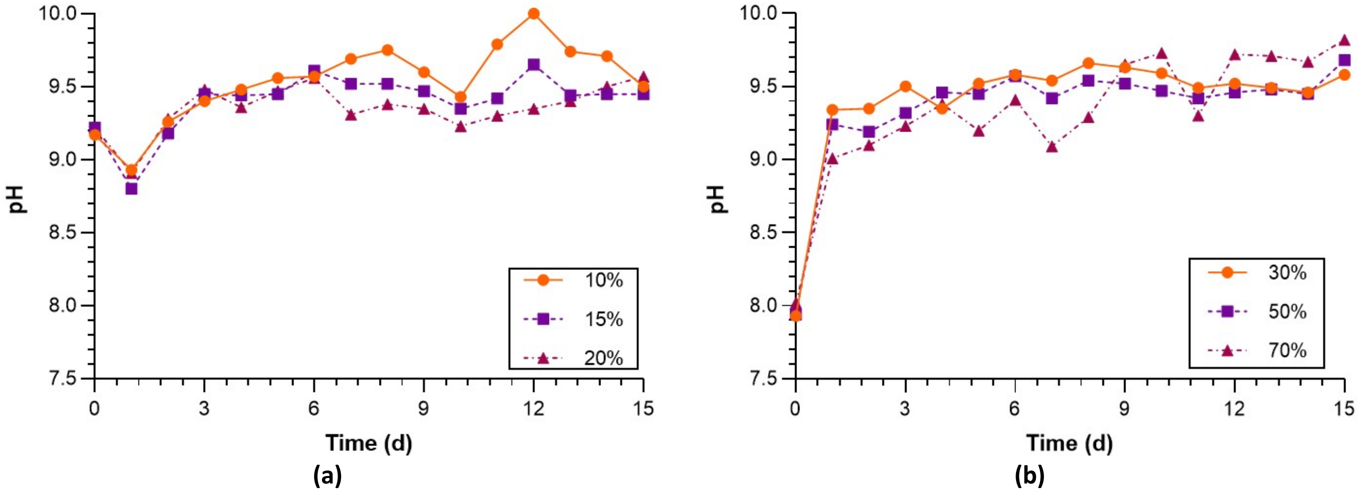


Figure 4. pH values determined during the growth of *N. oculata* in: (a) PWW and (b) PM.

While in PM a decrease of 4.23 and 14.15% was obtained with 30 and 50% inoculum, respectively. The greatest reduction occurred with the highest concentration of microalgae (70%) and was 37.18% of soluble COD. Figure 5 shows the initial (day 0) and final (day 15) COD values graphically. The removal of soluble COD is related to cell growth, since in the experiments with greater cell growth, the removal of said parameter was greater.

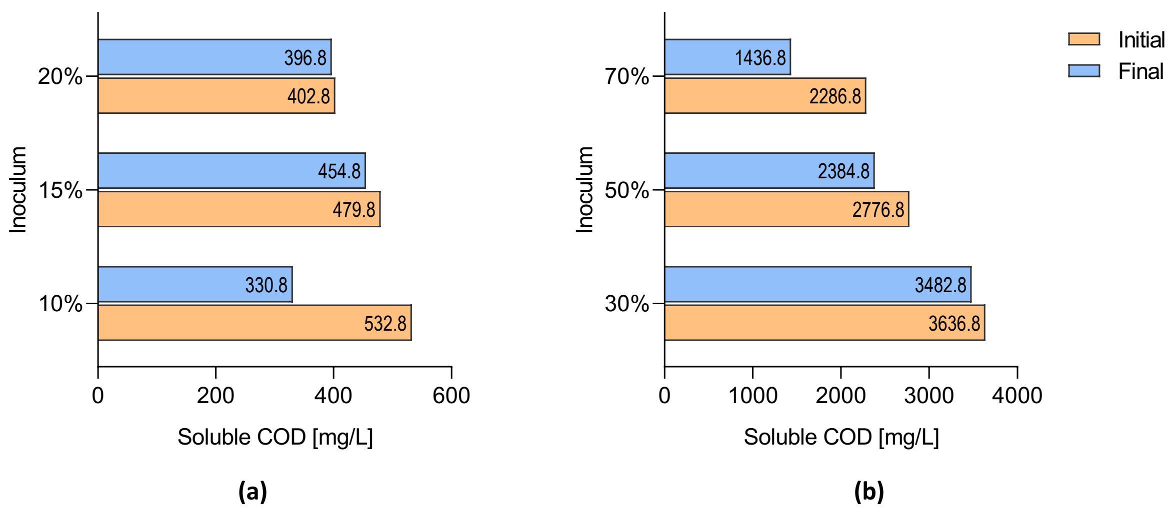


Figure 5. COD decrease during the adaptation process of *N. oculata* at the end of 15 days in: (a) PWW and (b) PM.

Conclusions

The PWW proved to be a suitable medium for the adaptation and growth of *N. oculata*, especially when low inoculum concentrations (10%) were used. The cell densities of microalgae in PWW were similar and even higher than those obtained with f/2 Guillard synthetic medium. Likewise, the organic matter content was reduced in the 3 concentrations studied, reaching up to 37.91% reduction in soluble COD with 10% inoculum. For PM, the microalgae were able to adapt in the photobioreactors with a high inoculum percentage (70%), reaching an elimination of 37.18% of the soluble COD. However, due to the high concentration of organic matter, the microalgae could not grow in substrate concentrations higher than 30%. Due to the high amount of inoculum required, the PM was not viable for the growth of *N. oculata*. Despite the high COD concentrations evaluated in this study, it was demonstrated that it is possible to use some livestock waste as a culture medium for the microalgae *N. oculata*. During the 15 days of adaptation, the microalgae was able to significantly eliminate the organic pollutants present in the waste. Thus, the potential of this microalgae to be used in the treatment of properly conditioned wastewater and sludge was demonstrated. Finally, it is recommended to extend the study time to achieve higher growth and removal efficiencies.

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