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Evaluation of microalgae's (*Chlorella* sp. and *Synechocystis* sp.) pollutant removal property: Pig effluent as a live stock discharge

Leonidas Pachacama[1,2*], Juan Ortiz Tirado[1], Jessica Duchicela[2], Bangeppagari Manjunatha[1], Rajesh R. Kundapur[3], Bugude Rajeswari[4]

[1]Department of Life Sciences, Laboratory of Water Resources and Aquaculture, Universidad de las Fuerzas Armadas-ESPE, P.O.BOX: 171-5-231B, Sangolquí, Ecuador, South America. [2]Carrer of Engineering Biotechnology, Universidad de las Fuerzas Armadas–ESPE, Sangolquí, Ecuador. [3]Department of Zoology, University of Pune, Pune, India. [4]Department of Botany, Yogi Vemana University, Kadapa, India.

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ABSTRACT

The ability of microalgae to remove nitrogen and phosphorus from wastewater has been used in recent years as an alternative treatment for discharges from livestock slurry, which generate a negative environmental impact on vulnerable ecosystems. With this background and the feasibility of using microalgae, we have evaluated the effect of *Chlorella* sp. and *Synechocystis* sp., in removing contaminants from the pig manure collected from El Prado ESPE. Slurry samples were collected, filtered and autoclaved, and the supernatant was further diluted to three different concentrations of 40%, 60% and 80%. The microalgal growth and pollutants removal property was evaluated up to 15 days in batch culture. The cell density was determined by counting in a Neubauer hemocytometer, and the pollutants removal was analyzed by standard colorimetric methods. The microalgae *Chlorella* sp. showed a maximum cell growth of $1.70 \pm 0.09 \times 10^7$ cells/mL at 60% effluent concentration on day 6. While *Synechocystis* sp. showed a maximum growth of $1.04 \pm 0.05 \times 10^7$ cells/mL, at 60% concentration on day 9. On the other hand, there exists a competition when microalgae used as a consortium. The cell growth of *Chlorella* sp. was higher at all concentrations compared to *Synechocystis* sp.. Overall, efficiency of pollutant removal were between 40% and 90%, which demonstrate the feasibility of using microalgae in tertiary swine wastewater treatment.

INTRODUCTION

The pollution resulting from wastewater of animal origin which contains high loads of nitrogen and phosphorus has created serious threats to the aquatic environment, the main problems are eutrophication of waters, air pollution by volatilization of ammonia and land degradation (Godos *et al.*, 2010). In Ecuador, the pork sector presented a dynamic growth in recent years and equally, posing an environmental threat because of their improper disposal. The ability of microalgae to remove nitrogen and phosphorus from wastewater has allowed the use of microalgae cultures as tertiary treatment, presenting great advantages over physical and chemical conventional systems, because they do not generate secondary pollutants and presents an efficient recycling of nutrients (De la Node and De Pauw 1988). Furthermore, the use of microalgae in the wastewater treatment reduces costs by not adding chemicals, and at the same time recovering nutrients as biomass that could be used as fertilizer, animal nutritional supplement, and bio fuels (Kim and Park 2007). Microalgae from the genera *Chlorella, Scenedesmus, Botryococcus, Spirulina* and *Phormidium* microalgae have been used in the treatment of industrial and animal wastewater, due to its particular tolerance to the conditions of the effluents and high removal efficiency (Pittman *et al.*, 2011). Furthermore, the use of microalgae for nutrient removal from wastewater in swine has been previously established (Zhu *et al.*, 2013). Wastewater from the pig production units at El Prado farm contains a high concentration of inorganic nutrients, proving to be a suitable medium for the growth of microalgae. The concentration of nitrogen and phosphorus in wastewater has a direct influence on the growth kinetics and is closely related to efficient nutrient removal (Goldberg and Cohen 2006).

** Corresponding Author*
Pachacama Leonidas, Department of Life Sciences, Laboratory of Water Resources and Aquaculture, Universidad de las Fuerzas Armadas–ESPE, P.O. BOX: 171-5-231B, Sangolqui, Ecuador, South America.
Email: lepachacama1@espe.edu.ec

This study evaluated the optimal conditions for the growth of microalgae, the efficiency of NH_4^+ and PO_4^+ removal, the reduction of Chemical Oxygen Demand (COD) and Biodegradable Oxygen Demand (BOD), using pig effluent as a wastewater.

MATERIALS AND METHODS

Microalgae strain and pre-culture conditions

The microalgae *Chlorella* sp. and *Synechocystis* sp. were provided by the Department of Biotechnology at Universidad de las Fuerzas Armadas, Ecuador and preserved in leaf Nitrofoska and complete BG11 medium. The Nitrofoska leaf medium composed of the following ingredients (g/L): N (300), P (100), K (100), Mg (6), S (40), Mn (2), Fe (2), Cu (1), Zn (0.6), B (1.5) and Mo (0.1). Furthermore, the BG11 complete medium consisted of (g/L): $NaNO_3$ (1.5), $MgSO_4.7H_2O$ (7), $CaCl_2.2H_2O$ (3.6), $K_2HPO_4.3H_2O$ (4), EDTA (0.1), Na_2CO_3 (2), H_3BO_3 (2.86), $MnCl_2.4H_2O$ (1.81), $ZnSO_4.7H_2O$ (0.22), $NaMoO_4.5H_2O$ (0.39), $CuSO_4.5H_2O$ (0.8), $Co(NO_3)_2.6H_2O$ (0.05), $FeCl_3$ (0.22), additionally added (35) NaCl. The growth of microalgae was performed in two plastic bottles of 5 liters capacity each one, maintained at a temperature of 20 ± 2°C, irradiation (78.8 μmol quanta m^{-2} s^{-1}) and with the continuous aeration (Jonte *et al.*, 2003).

Culture of *Chlorella* sp. and *Synechocystis* sp. in swine wastewater

The pig effluent was collected from the pig rearing units at El Prado farm - IASA I –Universidad de las Fuerzas Armadas - ESPE, Ecuador. The pre-treatment of wastewater was filtered in a plastic strainer N° 8 to remove large solid and insoluble particles. After filtration, the sample was autoclaved for 20 min at 121°C, and stored at 4°C for 2 days for sedimentation of any visible solid particles (Zhu *et al.*, 2013). The supernatant was used in the growth assays and nutrient removal. The characteristics of raw and autoclaved wastewater are summarized in Table 1.

Table 1: Characteristics of raw and autoclaved swine wastewater used in the experiment.

Parameter	Original Concentration	Autoclaved Concentration
NH_4^+ (mg/L)	203 ± 3.08	189 ± 1.47
Conductivity (uS)	960.25 ± 22.78	905.25 ± 9.72
DBO_5(mg/L)	2875 ± 47.87	2580 ± 52.28
DQO (mg/L)	3525 ± 43.3	3333.3 ± 54.43
PO_4^+ (mg/L)	99.75 ± 2.02	94 ± 2.12
pH	6.55 ± 0.06	7.55 ± 0.16
TDS (mg/L)	525 ± 24.77	467.5 ± 11.98
Temperature (°C)	20.88 ± 0.24	20.65 ± 0.12

The autoclaved supernatant was diluted with distilled water at concentrations of 40%, 60%, and 80% slurry. A volume of 850 ml of swine wastewater of above mentioned dilutions were placed in different 1.5 L bottles. A volume of 150 mL of micro algal culture with a cell density of $5x10^7$ cells/mL determined in a Neubauer hemocytometer was added to each bottle. Nine treatments were specified in the study. *Chlorella* sp. and *Synechocystis* sp. individually and in consortium with effluent dilutions of 40%, 60% and 80% was evaluated.

Analytical procedures

Sampling and nutrients analysis

For the analysis of nutrient removal a volume of 10 ml was collected at the beginning and end of the batch culture. The samples were centrifuged at 1000 rpm for 20 min, the resultant supernatant was further diluted and analyzed for NH_4+ and PO_4+ content using the spectrophotometer (SpectroFlex WTW 6600). COD was determined by the oxidation of potassium dichromate ($K_2Cr_2O_7$) method and Oxitop WTW was used for BOD_5 measurement. The removal rate was obtained using the following equation:

$$\boldsymbol{Removalrate}\ \% = [(C_0 - C_i)/C_0]x100\ \% \text{ (Zhu } \textit{et al.}\text{, 2013)}$$

Where C_o and C_i are the mean values of the concentration of nutrients at the initial time (t_o) and time (t_i), respectively.

Determination of microalgae growth

Cell number was determined by cell count with Neubauer hemocytometer under the light microscope. Cell density was determined using the following equation:

$$\boldsymbol{DC_{inoculum}} = Nx10^4xFD \text{ (Bermúdez } \textit{et al.}\text{, 2004)}$$

Where N is the average number of cells in relation to quadrants used in Neubauer hemocytometer, 10^4 is the conversion factor of 0.1 μL to 1 mL and FD is the dilution factor.

The specific growth rate (μ) in an exponential phase was measured using the following equation:

$$\mu(\boldsymbol{day^{-1}}) = \ln\left(\frac{N_2}{N_1}\right)/(t_2 - t_1) \text{ (Zhu } \textit{et al.}\text{, 2013)}$$

Where N1 and N2 are defined as cell density (cells mL^{-1}) at time t_1 and t_2, respectively. All data were expressed as a mean and standard error.

RESULTS

Microalgae growth

The growth characteristics of *Chlorella* sp., *Synechocystis* sp. and consortium (*Chlorella* sp. - *Synechocystis* sp.) under the three concentrations of slurry were evaluated up to 15 days, as shown in Fig. 1. The curves illustrate all phases of characteristic growth of microalgae batch culture.

In case of *Chlorella* sp., the lag phase was not apparent in all concentrations. In the exponential phase a significantly increased cell density (P <0.0001) was observed on day six. Meanwhile, the specific growth rate μ in 40%, 60% and 80% slurry concentrations was found to be 0.13, 0.16 and 0.17 $days^{-1}$, respectively. In the stationary phase, fluctuations between 6 and 12 days were observed. The maximum cell density (1.70 ± 0.09 $x10^7$ cells/mL) was achieved at 60% slurry concentration.

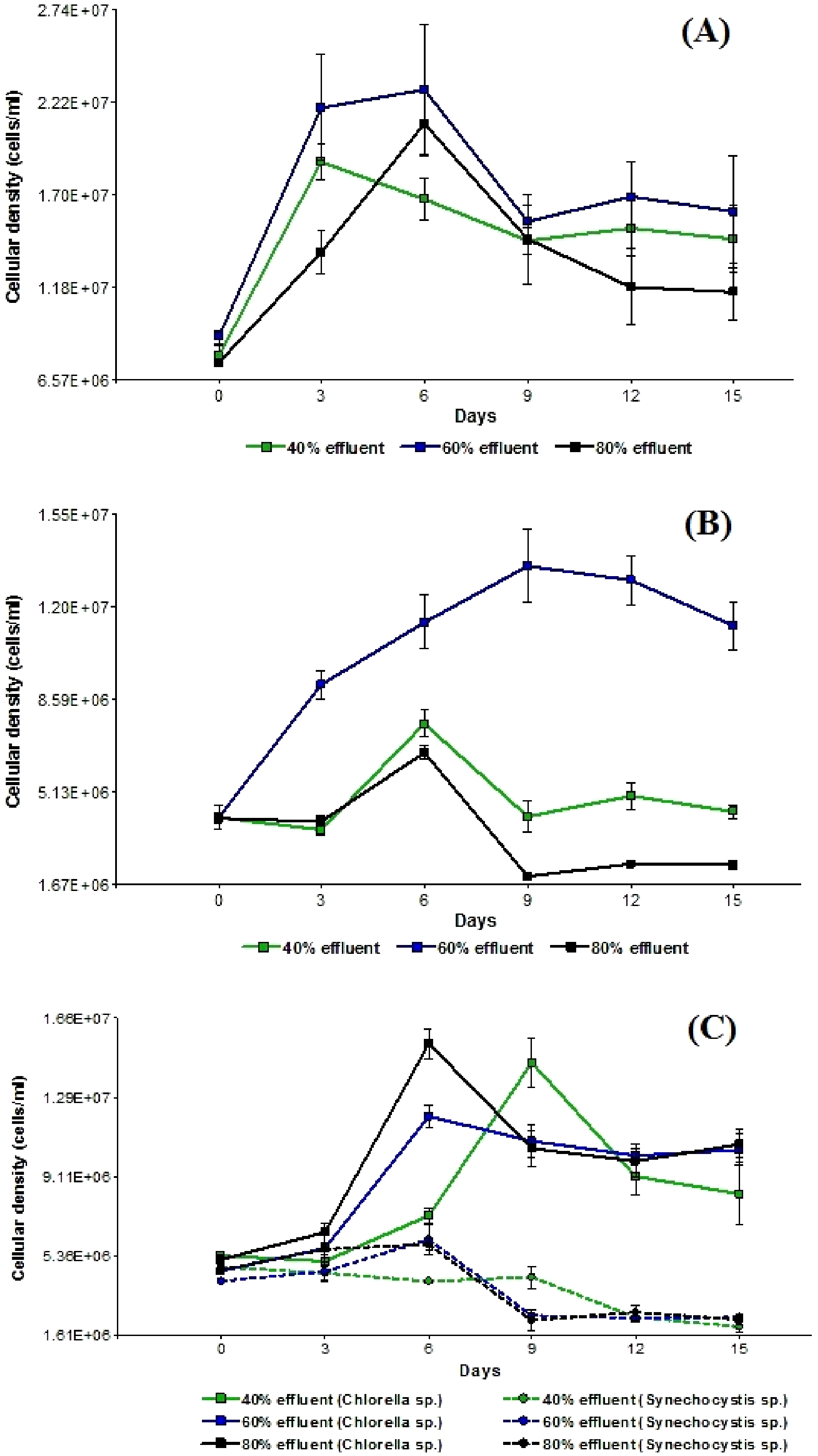

Fig. 1: Growth curves for three concentrations of pig manure within 15 days (mean ± S.E.). Slurry concentration was 40%, 60% and 80%. Concentrations were specified as a treatment in all cases of this article. (A) Growth profile of *Chlorella* sp. (B) Growth Profile of *Synechocystis* sp. (C) Growth profile of microalgal consortium (*Chlorella* sp. - *Synechocystis* sp.). Statistically different values (p<0.0001). Error bars correspond to standard deviation of triplicate cultures (n=27).

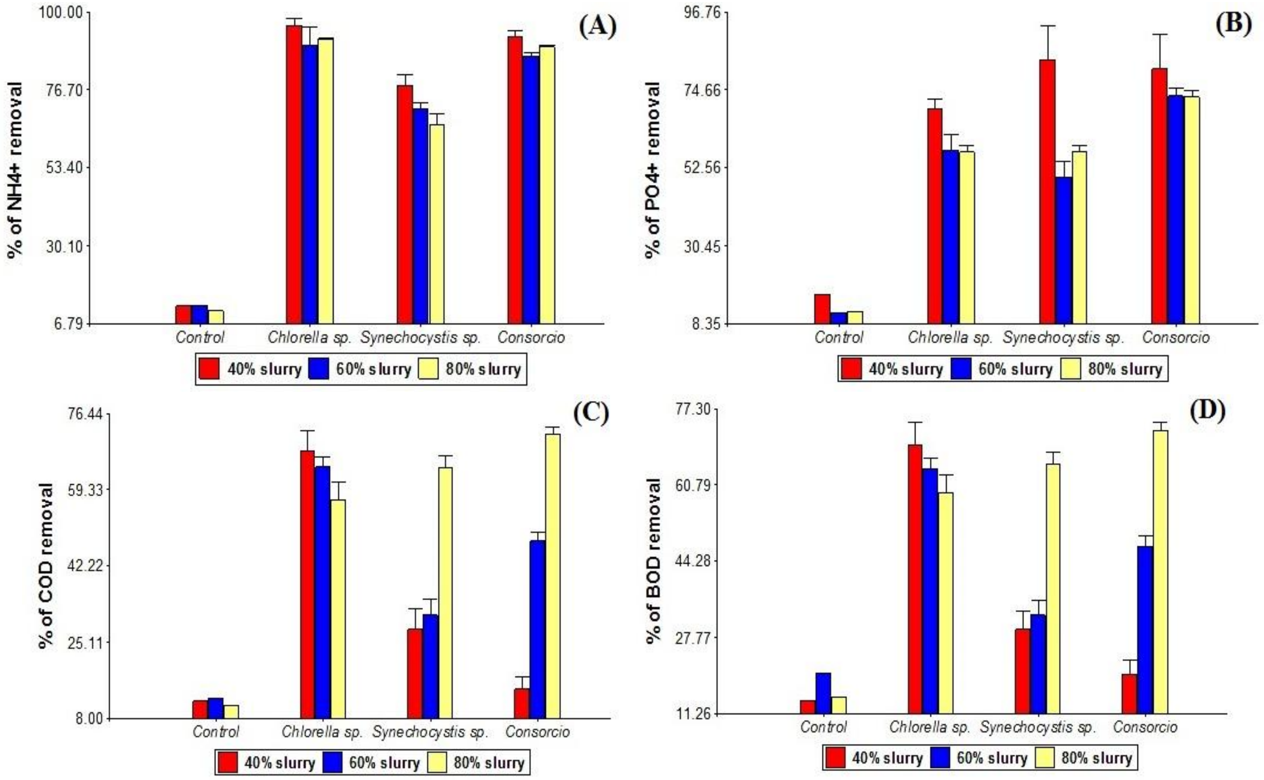

Fig. 2: Nutrient removal by microalgae (*Chlorella* sp., *Synechocystis* sp. and microalgal consortium) in three concentrations of pig manure within 15 days (Mean ± S.E.). Slurry concentration was 40%, 60% and 80% diluted with distilled water. (A) Percentage of NH_4^+ removal. (B) Percentage of PO_4^+ removal. (C) percentage of COD removal. (D) percentage of BOD removal. Statistically different values ($p<0.0001$). Error bars correspond to standard deviation of triplicate cultures (n=30).

In case of *Synechocystis* sp., a lag phase in 40% and 80% slurry concentrations within the first three days was observed, while the exponential phase showed early at begin of batch culture in of 60% concentration. The stationary phase was shown between the days 9 and 15. In 40% and 80%, there was adecline in cell density between day 6 and 9 before reaching the stationary phase. The specific growth rate in exponential phase in all concentrations was 0.10, 0.17 and 0.08 days^{-1}, respectively. The maximum cell density reached was 1.04 ± 0.05 x10^7 cells/mL at 60% slurry concentration. In the microalgal consortium, a wide latency phase was observed in *Synechocystis* sp. for all concentrations, in contrast to *Chlorella* sp. was observed within the day 3 of batch culture. On the other hand, in *Chlorella* sp. with compare to other concentration there was a delay in reaching an exponential phase at 40% concentration was observed. In case of *Synechocystis* sp. exponential phase was not evident, a decline in growth occurred between days 6 and 9, followed by a stationary phase until the end of the batch culture. The specific growth rate showed by *Chlorella* sp. at all concentrations were 0.10, 0.16 and 0.18 days-1, respectively. While for *Synechocystis* sp. it was 0.01, 0.06 and 0.03 day -1, respectively. As per as achiveing maximum cell density was concerned, for both species it was observed at 60% and 80% concentration, for *Chlorella* sp. it was 8.93 ± 0.55 x10^6 cells/mL and for *Synechocystis* sp. it was 9.66 ± 0.69 x106 cells/mL, respectively.

Nutrient Removal

Removal property of ammonium (NH_4^+), phosphate (PO_4^+), and variations observed in COD and BOD, values in different concentrations of swine wastewater upto 15 days in batch culture are shown in Fig. 2. Ammonium decreased dramatically in the presence of *Chlorella* sp. and the microalgal consortium, whereas *Synechocystis* sp. showed a lower efficiency relative to other treatments, with respect to control, the removal percentage was between 10% and 12%. The *Chlorella* sp. showed a highest removal efficiency (95.92%) at 40% slurry concentration. Concomitantly, in all treatments, the percent efficiency was in the range between 66% to 95%.

With respect to the phosphate concentration, within 15 days of culture, *Synechocystis* sp. showed a greater removal (75.41%) than the other treatments. While *Chlorella* sp. showed a removal percentage 69.45% and the microalgal consortium showed 80.56%, at 40% slurry concentration. Furthermore, at concentrations of 60% and 80% removal rates were between 50% and 73.08%.

COD was drastically decreased in all treatments. The *Chlorella* sp. showed anaverage removal porcentage of 64.27%. On the other hand, *Synechocystis* sp. and the microalgal consortium showed removal percentages of 64.44% and 71.85% respectively at 80% slurry concentration. In addition, the *Synechocystis* sp. did not show much difference in removal percentage at 60% and 80 % slurry concentration which was 28% to 31.25%. In the microalgal consortium a slight removal (14.67%) was noted at 40%. In relation to the control group, the percentages were lower between 11% and 12% for all concentrations of wastewater.

Furthermore, as BOD is directly related to the COD, so the percentage reductions were similar in both parameters. The highest percentages of reduction in BOD were observed at 80% concentration by *Synechocystis* sp. it was 65.39% and for the microalgal consortium it was 72.62%. With respect to *Chlorella* sp. there was no significant difference in the reduction of BOD between different slurry concentrations. At 40% slurry concentration, 69.34% percentage of removal was observed.

DISCUSSION

Microalgal Growth

Microalgae like other microorganisms, has four stages of growth: latency, exponential, stationary and decline (Li *et al.,* 2011). In the treatment with *Chlorella* sp. no stationary phase was observed, due to the fact that the inoculum was at the exponential phase (Li *et al.,* 2011). Similarly, *Chlorella* sp. can be adapted to different concentrations of wastewater with a lower latency phase at day 1 (Ryu *et al.,* 2014). Subsequently, there was a decline in growth before reaching the stationary phase, which may be due to low light availability resulting from high cell density (Li *et al.,* 2011).

As per as the *Synechocystis* sp., is concerned, there was a latency period observed in first three days at 60% and 80% slurry concentration which is in support of previous studies (Ding *et al.,* 2015). Subsequently, a difference in an exponential phase was observed, followed by a decay before reaching the stationary phase. The large decay was observed microalgal culture could be related to the depletion of certain nutrients, primarily nitrogen and carbon (Li *et al.,* 2011).

In microalgae consortium (*Chlorella* sp.- *Synechocystis* sp.), a lag phase was observed in the first 3 days for both species. Subsequently, *Chlorella* sp. showed evident exponential phase, whereas *Synechocystis* sp. cellular growth started to decline. This might be due to green algae (*Chlorella* sp.) which has a high demand for nitrogen and phosphorus in relation to other species; plus nitrogen absorption is favored when the phosphorus concentration is relatively high (Prescott 1968).

On the other hand, the competition between cyanobacteria (*Synechocystis* sp.) and microalgae (*Chlorella* sp.), limits availability of nutrients, and considering the advantage of *Chlorella* sp. takes advantage of this to assimilate nutrients (Prescott 1968), which results decline in the growth of *Synechocystis* sp. Finally, both species enter stationary phase, around nineth day, presenting *Synechocystis* sp. with lower cell density.

Nutrient Removal

High removal percentages of NH_4^+ and PO_4^+ were achieved in all treatments. Previous studies have reported that algae can assimilate NH_4-N, nitrate and organic nitrogen simple forms such as urea, acetic acid, and amino acids, from wastewater (Su *et al.,* 2011). Moreover, absorbed nitrogen is used by microalgae for the synthesis of proteins, nucleic acids, and phospholipids (Zimmo *et al.,* 2003). The removal of NH_4^+ from wastewater by microalgae could be affected by precipitation of NH_4^+ and separation of NH_3. Separation of NH_3 usually occurs in an alkaline medium, high temperature and the presence of abundant urea in wastewater (Matusiak *et al.,* 1976). In previous studies, high rates of removal in municipal wastewater were determined, where the main form of soluble nitrogen was ammonia (Woertz *et al.,* 2009).

The nitrogen removal is dominated by the absorption of nutrients by microalgae and bacteria during growth, on the other hand, the volatilization of ammonia at high pH (Min *et al.,* 2011), could influence the percentages of removal of certain treatments. With regard to the control treatments without microalgae inoculums, the low percentage of NH_4^+ removal could be due to abiotic processes such as chemical precipitation and gasification of ammonia at high pH (Ji *et al.,* 2013).

In relation to phosphorus removal from pig wastewater, it was mainly used and consumed by *Synechocystis* sp. and the microalgal consortium. One of the factors related to the disposition of phosphorus in the medium is the pH. The increase in pH in cultures could contribute to the precipitation of phosphorus and increased phosphate absorption by microalgae (Ruiz Marin*et al.,* 2010), which could be directly related to the given percentages of removal in this study. The increase in pH above 8 in the solution inoculated with microalgae could cause coagulation and adsorption of inorganic phosphate (Li *et al.,* 2011). Furthermore, it is known that the microalgal biomass contains only 0.5-3.3% phosphorus (Richmond 2004), therefore, a high percentage of phosphorus may have been removed by sedimentation. The energy generated by the oxidation of phosphorous under aerobic conditions is used by microalgae for cell growth and metabolism (Lananan *et al.,* 2014). Also, ammonium consumption is directly related to the concentration of phosphorus, which acts as a constraint on growth; the ideal range of N:P (nitrogen-phosphorus) for microalgal growth is 6:3 (Ramos Tercero *et al.,* 2014). Similarly, the phosphate may play a significant role in the synthesis of valuable products such as astaxanthin and PUFAs (Chen and Chen 2006). On the other hand, COD and BOD values were decreasedwhen inoculated with *Chlorella* sp. (90%). While treatments with *Synechocystis* sp. and microalgal consortium, it was 60% and 70%, respectively. The COD is related to carbon levels in the effluent, thus reducing this parameter could be attributed to carbon is a necessary macronutrient for microalgal growth. Furthermore, since this organic carbon and light in the medium is considered a mixotrophic growth, in which the CO_2 and organic carbon are simultaneously assimilated (Min *et al.,* 2011).

It has previously been reported that the removal of volatile organic compounds (VOCs) resulted in approximately 20% of COD removal of swine wastewater (Zhang and Jahng 2010), therefore, removal percentages in the control group could be directly related to the separation of VOCs. With respect to COD removal percentages of *Chlorella* sp., it has found that the *Chlorella* metabolic pathway can be altered supplying organic substances (organic acids, glucose, etc.) allow it to adapt to heterotrophic growth rather autotrophic (Eny 1951). Furthermore, the COD removal correlates with growth of *Chlorella* sp., the highest cell density, could be related to a heterotrophic growth which is faster metabolic pathway (Burrell *et al.*, 1984).

With regard to treatment with the microalgal consortium, *Synechocystis* sp. improved the efficiency of nutrient removal by *Chlorella* sp., because *Synechocystis* sp. is capable of bioconverting contaminants in stable forms of ammonia and phosphorus (Lananan *et al.*, 2014), which could subsequently assimilated by *Chlorella* sp., where in the cell density of *Chlorella* sp. it was significantly greater than the density of *Synechocystis* sp. In this study, the swine wastewater was autoclaved before inoculation with microalgae. However, the air supply compressor not sterilized (membrane filter, etc.), so the culture could contain bacteria introduced by air (Zhu *et al.*, 2013), which could contribute to the degradation of pollutants (Chen *et al.*, 2012). The microalgae can improve bacterial activity by releasing certain extracellular compounds like glycolic acid (Wang *et al.*, 2009), while bacterial growth can improve microalgal metabolism, reducing oxygen concentrations in the environment and releasing growth promoting factors (Gonzalez and Basan 2000), or degrading large compounds into assimilable forms for microalgae (Zhu *et al.*, 2013). Furthermore, bacterial growth can be harmful to the microalgal cultivation because it increases the pH, alters temperature or release inhibitory metabolites (Gonzalez and Basan 2000). Also could form a bacterial layer on the walls of the container, affecting the microalgal photosynthesis by interfering the penetration of light (Zhang *et al.*, 2012). Therefore, the interaction between microalgae and bacteria is difficult to predict, since is directly related to environmental conditions.

CONCLUSION

Porcine wastewater with three different concentration levels were efficiently treated with *Chlorella* sp., *Synechocystis* sp. and microalgal consortium in batch culture upto 15 days. *Chlorella* sp. and microalgal consortium largely assimilated the NH_4^+ with percentages of 92.69% and 89.62%, respectively. Meanwhile, the PO_4^+ was assimilated in greater proportion by *Synechocystis* sp. with 75.41%. Regarding COD, *Chlorella* sp. presented a removal efficiency of 64.27%, being the highest in relation to other treatments. The specific growth rate of microalgae *Chlorella* sp., *Synechocystis* sp. and the microalgal consortium inoculated in wastewater at different concentrations was in ranges from 0.13 to 0.17 (day $^{-1}$), from 0.10 to 0.13 (day $^{-1}$) and from 0.04 to 0.18 (day $^{-1}$), respectively. The *Chlorella* sp. showed a maximum cell growth of 1.70 ± 0.09 x10^7 cells/mL at 60% effluent concentration on day 6. While *Synechocystis* sp. has a maximum growth of 1.04 ± 0.05 x10^7 cells/mL, at 60% concentration on day 9. During interaction of microalgae as a consortium cell growth of *Chlorella* sp. was higher at all concentrations compared to *Synechocystis* sp.

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CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest regarding the publication of this paper.

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