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

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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 ± 0.09 x10⁷ cells/mL at 60% effluent concentration on day 6. While *Synechocystis* sp. showed a maximum growth of 1.04 ± 0.05 x10⁷ 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 present 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).

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This study evaluated the optimal conditions for the growth of microalgae, the efficiency of NH₄⁺ and PO₄³⁻ 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 Nitrofoksa and complete BG11 medium. The Nitrofoksa 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): NaNO₃ (1.5), MgSO₄.7H₂O (7), CaCl₂.2H₂O (3.6), K₂HPO₄.3H₂O (4), EDTA (0.1), Na₂CO₃ (2), H₂BO₃ (2.86), MnCl₂.4H₂O (1.81), ZnSO₄.7H₂O (0.22), NaMoO₄.3H₂O (0.39), CuSO₄.5H₂O (0.8), Co(NO₃)₂.6H₂O (0.05), FeCl₃ (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 – 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.

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 NH₄⁺ and PO₄³⁻ content using the spectrophotometer (SpectroFlex WTW 6600). COD was determined by the oxidation of potassium dichromate (K₂Cr₂O₇) method and Oxitop WTW was used for BOD₅ measurement. The removal rate was obtained using the following equation:

\[
\text{Removal rate} = \frac{[C_0 - C_i]}{C_0} \times 100\% \quad (Zhu \ et \ al., \ 2013)
\]

Where C₀ and Cᵢ are the mean values of the concentration of nutrients at the initial time (tᵢ) and time (tₑ), 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:

\[
DC_{inoculum} = N \times 10^6 \times FD \quad (Bermúdez \ et \ al., \ 2004)
\]

Where N is the average number of cells in relation to quadrants used in Neubauer hemocytometer, 10⁶ 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:

\[
\mu (day^{−1}) = \ln \left( \frac{N_2}{N_1} \right) / (t_2 − t_1) \quad (Zhu \ et \ al., \ 2013)
\]

Where N₁ and N₂ are defined as cell density (cells mL⁻¹) at time t₁ and t₂, 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 microalgal 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 x10⁷ cells/mL) was achieved at 60% slurty 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).
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 a decline 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 $1.04 \pm 0.05 \times 10^7$ 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. was observed within the day 3 of batch culture. 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 achieving maximum cell density was concerned, for both species it was observed at 60% and 80% concentration, for *Chlorella* sp. it was $8.93 \pm 0.55 \times 10^6$ cells/mL and for *Synechocystis* sp. it was $9.66 \pm 0.69 \times 10^6$ cells/mL, respectively.

**Nutrient Removal**

Removal property of ammonium ($\text{NH}_4^+$), phosphate ($\text{PO}_4^{3-}$), 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 an average removal percentage 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 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 ninth day, presenting *Synechocystis* sp. with lower cell density.

**Nutrient Removal**

High removal percentages of NH$_4^+$ and PO$_4^{3-}$ 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 (Zimno et al., 2003). The removal of NH$_4^+$ from wastewater by microalgae could be affected by precipitation of NH$_3^+$ 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 (Li 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 decreased when 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 up to 15 days. *Chlorella* sp. and microalgal consortium largely assimilated the NH₄⁺ with percentages of 92.69% and 89.62%, respectively. Meanwhile, the PO₄³⁻ 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 x10⁷ cells/mL at 60% effluent concentration on day 6. While *Synechocystis* sp. has a maximum growth of 1.04 ± 0.05 x10⁷ 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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