Evaluation of nutrients removal (NO₃-N, NH₃-N and PO₄-P) with *Chlorella vulgaris*, *Pseudomonas putida*, *Bacillus cereus* and a consortium of these microorganisms in the treatment of wastewater effluents


**ABSTRACT**

In this research removal of NH₃-N, NO₃-N and PO₄-P nutrients from municipal wastewater was studied, using *Chlorella vulgaris*, *Pseudomonas putida*, *Bacillus cereus* and an artificial consortium of them. The objective is to analyze the performance of these microorganisms and their consortium, which has not been previously studied for nutrient removal in municipal wastewater. A model wastewater was prepared simulating the physicochemical characteristics found at the wastewater plant in Chapala, Mexico. Experiments were carried out without adding an external carbon source. Results indicate that nutrient removal with *Chlorella vulgaris* was the most efficient with a removal of 24.03% of NO₃-N, 80.62% of NH₃-N and 4.30% of PO₄-P. With *Bacillus cereus* the results were 8.40% of NO₃-N, 28.80% of NH₃-N and 3.80% of PO₄-P. The removals with *Pseudomonas putida* were 2.50% of NO₃-N, 41.80% of NH₃-N and 4.30% of PO₄-P. The consortium of *Chlorella vulgaris–Bacillus cereus–Pseudomonas putida* removed 29.40% of NO₃-N, 4.2% of NH₃-N and 8.4% of PO₄-P. The highest biomass production was with *Bacillus cereus* (450 mg/l) followed by *Pseudomonas putida* (444 mg/l), the consortium (205 mg/l) and *Chlorella vulgaris* (88.9 mg/l). This study highlights the utility of these microorganisms for nutrient removal in wastewater treatments.

**Key words** | *Bacillus cereus*, *Chlorella vulgaris*, consortium, nutrient removal, *Pseudomonas putida*, wastewater treatment

**INTRODUCTION**

Environmental issues are becoming profound problems because of the possible risk of pollution of ecosystems, due to the increasing population, urbanization and industrialization (Rawat et al. 2011). The most profound issues include the excessive generation of wastewater and the release of this water into freshwater resources, which are giving serious challenges to the scientific community, in terms of sustainability of our planet and global warming, for the present and the future generations. Therefore, the mixing of untreated wastewater in aquatic bodies is one of the major issues that are changing the stability of ecosystems (Renuka et al. 2014). Further, the presence of excess
nutrients such as nitrogen and phosphorus in untreated wastewater causes a dense growth of aquatic plants and the death of animal life from the lack of oxygen, leading to a total degradation of water bodies (Khan & Ansari 2005).

These practices also cause negative effects, such as an increment of consumption of dissolved oxygen (DO) and the contamination by toxic chemicals. The increase in eutrophication by large amounts of phosphorus and nitrogen results in a degradation of aquatic ecosystems and the deterioration of water quality and a potential hazard to human health. The above is due to the fact that the nitrate in the gastrointestinal tract can be reduced to ions of nitrite (Wang et al. 2010). Traditionally, treatments in aerobic and anaerobic reactors are undertaken but their high costs, large amount of generated sludge and high energy consumption have made these treatments less viable. Therefore, there is a need to develop new technologies, which must be environmentally friendly, effective, inexpensive and with the possibility of being used in industrial applications (Doria et al. 2012). At the end of 2015, Mexico had 2,477 municipal wastewater treatment plants in operation, giving treatment to a volume of 120.9 m³/s; 57% of the total wastewater produced at that time (CONAGUA 2015). Bioremediation using microorganisms such as microalgae and bacteria to treat wastewater has been reported as one option because these microorganisms have high requirement of nitrogen and phosphorus for growth (Mata et al. 2012). The wastewater also provides a convenient medium to supply most of the necessary nutrients for the growth of these microorganisms. *Chlorella vulgaris* can tolerate extreme environmental conditions such as those found in wastewater, showing an efficiency of 80% for the elimination of nutrients in wastewater, such as ammonia nitrogen, nitrate nitrogen and total phosphorus in certain conditions (Pittman et al. 2011).

Bacteria such as *Pseudomonas putida* and *Bacillus cereus* are usually present in wastewater. The first is a Gram-negative microorganism, and it is found in soil, freshwater and saltwater and it can consume all organic substrates such as sugars, amino acids, alcohols, hydrocarbons, humic acids and even some synthetic pesticides (Borde et al. 2002). The second microorganism, is a Gram-positive bacterium, and it is a promising microorganism for the bioremediation of contaminated sites with chromium; in addition, its ability to sporulate under adverse conditions makes it a promising prospect in terms of resistance of the organism to changing conditions which may occur in the bioremediation of contaminated environments (Ramirez-Ramirez & Benitez-Campo 2013).

### Consortium of microalgae–bacteria

In the natural aquatic environment, microalgae always co-exist with bacteria. The interactions between microalgae and bacteria are many (Cole 1982). Combined systems of algae-bacteria can take up nitrogen and phosphate for growth (He et al. 2009, improving the water quality (Gonzalez et al. 1997). The bacteria can break down organic matter and assimilate phosphorus using the O₂ produced by photosynthesis of algae (Munoz & Guieysse 2006). The algae release a variety of organic substances, composed of proteins, lipids and nucleic acids. All these molecules serve as substrates for the bacterial growth (Abed et al. 2007). The growth of algae is promoted for the bacteria (Hernandez et al. 2009). Previous reports mentioned that the interaction of the consortium *Chlorella vulgaris–Bacillus cereus–Pseudomonas putida* increases the growth of the microalgae (Qu et al. 2014). The molecule of oxygen produced in the photosynthesis process by the algae is used as an electron receptor for the bacteria so it can degrade the organic matter. Symbiotic interactions between microalgae and bacteria can reduce the biochemical oxygen demand (BOD) in treated wastewater (Oswald 2005).

This study was conducted to evaluate the potential of *Chlorella vulgaris*, *Pseudomonas putida*, *Bacillus cereus* and a consortium of *Chlorella vulgaris–Pseudomonas putida–Bacillus cereus* for nutrients removal from municipal wastewater, to try to find an alternative method for the improvement of water quality. The aim of this work is to study the capacity of these microorganisms to remove NO₃-N, NH₃-N and PO₄-P in municipal wastewater. Experiments about the removing of these nutrients employing a single bacterium or the microalga or a consortium of the three are presented.

### MATERIALS AND METHODS

#### Wastewater collection and analyses

The wastewater was collected from a treatment plant located at Chapala, Jalisco, Mexico. Samples were collected in clean plastic jars and transported to the laboratory and stored at 4°C for further analysis. Physicochemical parameters such as alkalinity, carbonate, hardness, DO BOD, and concentration of NO₃-N, NH₃-N and PO₄-P were determined using *Standard Methods* (APHA 1998). Once the characterization of wastewater (Table 1) had been carried out, a model wastewater with the same
characteristics was formulated. This model wastewater was used in the experimental part of this work in order to have a better control of the initial physicochemical characteristic of the water.

**Cell cultures**

The organisms used were *Chlorella vulgaris* ATCC (UTEX 29), *Pseudomonas putida* (ATCC 700007) and *Bacillus cereus* (ATCC 9592). *Chlorella vulgaris* was inoculated in a liquid Bold’s Basal Medium and the bacteria were inoculated in tryptone soy broth. Both cell cultures were carried out at 25 °C for 21 days. The inoculum of microalga was carried out under photoperiods of 12/12 hours of light/dark (Markou & Georgakakis 2014).

The inocula were adjusted to 0.5 optical density (OD) at 600 nm for *Chlorella vulgaris, Pseudomonas putida* and *Bacillus cereus*. However, the preparation of the inoculum for the consortium was prepared with a ratio of 3:1 of OD (Guo & Tong 2014). The culture of *Chlorella vulgaris* has an OD of 0.5; *Pseudomonas putida* and *Bacillus cereus* have an OD of 0.16 respectively. The experiments were carried out in 1 l bottles, containing 900 ml of model wastewater and 100 ml of the corresponding inoculum. The bottles were maintained at 25 °C with an agitation of 100 rpm and photoperiods of 12/12 light/dark. It is important to mention that no source of carbon was added to any of the inocula (microalga and bacteria). The systems were analyzed at different times to record their growth.

**Model wastewater**

The model wastewater was prepared using distilled water and adding the following concentration (mg/l) of compounds; 293.32 of CaCO3, 53.2 of NaHCO3, 140.98 of NaCl, 251 of MgCl2.6H2O, 5.54 of KNO3, 20.72 of NH4Cl and 2.66 of KH2PO4. Finally the pH was adjusted to 7.7 and the model wastewater was sterilized to use it in the experiments.

**Percent of removal**

The percent removal (PR) of nutrients was calculated by using the following the equation:

$$PR = \frac{(C_0 - C)}{C_0} \times 100 \hspace{2cm} (1)$$

where $C_0$ (mg) is the initial mass of the corresponding nutrient in the wastewater; $C$ (mg) is the final mass of nutrient left in the model wastewater at the experimental time (Renuka et al. 2015).

**Biomass determination**

The determination of biomass was carried out with 6 ml of sample, which was centrifuged at 14,000 rpm for 15 minutes, and the pellets obtained were dried at 60 °C until a constant weight (Arredondo & Voltolina 2007).

**RESULTS AND DISCUSSION**

**Nutrient removal in model wastewater**

The accumulative removals for each nutrient were calculated using Equation (1). The removal of NO3-N by *Chlorella vulgaris, Pseudomonas putida* and *Bacillus cereus* and the consortium of the microorganisms is shown in Figure 1. The removal of NO3-N with *Chlorella vulgaris* is shown in Figure 1(a); the removal starts approximately after 24 hours. *Chlorella vulgaris* is able to remove 24.0% (initial mass 1.19 mg and final mass 0.904 mg), *Pseudomonas putida* (Figure 1(b)), 2.5% (initial 1.19 mg, final 1.13 mg), and *Bacillus cereus* (Figure 1(c)) 8.4% (initial 1.19 mg, final 1.09 mg), all of them during a period of 168 hours. The consortium (Figure 1(d)) was able to remove 29.4% (initial 1.19 mg, final 0.840 mg) in 240 hours. In Figure 1, it is also shown that the consortium of *Chlorella*
*Chlorella vulgaris*, *Pseudomonas putida* and *Bacillus cereus* had the highest percentage removal of NO$_3$-N, followed by *Chlorella vulgaris*. *Chlorella vulgaris* is the dominant microorganism in the consortium and the bacteria give robustness to the system (Ruiz et al. 2014). *Pseudomonas putida* and *Bacillus cereus* are able to remove the NO$_3$-N; this happens when there is not enough organic matter available in the medium. The metabolism of bacteria decreases, since this medium was not supplemented with organic matter or some source of glucose: therefore bacteria systems make adjustments to maintain energy, which is why the concentration of NO$_3$-N remains almost stable at certain time (Zhao et al. 2009).

The accumulative removal of NH$_3$-N for the four systems is shown in Figure 2. *Chlorella vulgaris* (Figure 2(a)) was able to remove 80.6% (initial mass 38.5 mg, final mass 7.46 mg) in the model wastewater, and *Pseudomonas putida* (Figure 2(b)) and *Bacillus cereus* (Figure 2(c)) were able to remove 41.8% (initial 38.5 mg, final 22.40 mg) and 28.8% (initial 38.5 mg, final 27.38 mg) respectively, all of them during a period of 168 hours. However, the artificial consortium (Figure 2(d)) was able to remove just 2.52% (initial 40.85 mg, final 39.82 mg) in 240 hours. From the results it is shown that *Chlorella vulgaris* is the most efficient microorganism removing NH$_3$-N. The above behavior is due to the fact that the microalgae used primarily NH$_3$-N for their growth and when this source is almost finished they use NO$_3$-N or another source of nitrogen from the environment. That behavior is due to the fact that a set of enzymes of *Chlorella vulgaris* is deactivated when ammonium is
present in the medium (Ruiz et al. 2011). According to different reports the order of use of the nitrogen source for microalgae is generally NH$_3$ > NO$_3$ > NO$_2$ (He et al. 2013). This phenomenon occurs when the nutrient medium has available NH$_3$$^+$; the microorganisms did not use another nitrogen source until the ammonia nitrogen was depleted (Cromar et al. 1996). Dortch et al. (1982) explained that this behavior might be due to the low energy needed for the absorption process of NH$_3$-N in the microalgae.

The accumulative removal of PO$_4$-P for the four system is shown in Figure 3. Chlorella vulgaris (Figure 3(a)) removes 4.3% (initial mass 0.00209 mg, final mass 0.00200 mg), Pseudomonas putida (Figure 3(b)) 4.3% (initial 0.00209 mg, final 0.00200 mg), and Bacillus cereus (Figure 3(c)) 3.8% (initial 0.00209 mg, final 0.00201 mg), all of them during a period of 168 hours. The consortium (Figure 3(d)) is able to remove 8.6% (initial 0.0025 mg, final 0.0021 mg) during 240 hours. This research found that the microorganisms used in the study for removal of PO$_4$-P, are poorly efficient. However, they were able to maintain the concentration of PO$_4$-P below 0.003 mg/l since the bacteria such as Pseudomonas and Bacillus are able to remove phosphates under aerobic conditions, and this nutrient can be accumulated in granular form or as a short chain of polyphosphate, in the periplasmic area of the bacteria (Streichan & Schön 1991; Hupfer et al. 2008). However, there is little information about the interactions involved in this process, because the subject of study has been poorly elucidated (Sinkko et al. 2011). Zhao et al. (2009) found that in treatment with Bacillus cereus when
an external source of carbon was not added to the medium, the bacteria could take the organic matter that might be available in the medium. In the case of the microalgae this cannot remove nitrogen without the presence of phosphate in the wastewater, and vice versa, because both nutrients are essential for its growth (Cai et al. 2013). Chinnasamy et al. (2010) found that microalgae consortia were able to remove 99.8% of NO₃-N and 96.6% of PO₄-P during 72 hours of treatment, using industrial wastewater with high levels of CO₂, with the presence of Chlorella vulgaris in the treatment, because this microalgae has the ability to capture phosphate from the wastewater, since the microalgae can store the excess of phosphorus (Markou & Georgakakis 2011). In the study of Zhao et al. (2014), A microalgae–bacteria consortium was able to remove 95% of PO₄-P. However, this did not happen in our study, because in our study we found that Chlorella vulgaris, Pseudomonas putida and the artificial consortium are capable of removing only 4.3% of PO₄-P in the model wastewater at 168 hours, followed by the consortium (8.4%) in 240 hours. The importance of this research is that the consortium used has not been previously studied for the removal of these nutrients, and it was found that this consortium is appropriate to remove phosphorus (PO₄-P) nutrient.

**Biomass productivity**

Biomass was monitored as dry weight, for Chlorella vulgaris, Pseudomonas putida, Bacillus cereus and the consortium (Figures 1–3). Chlorella vulgaris was able to produce the maximum concentration of biomass, 88.9 mg/l, in 48 hours; Pseudomonas putida 444.4 mg/l, and Bacillus cereus 450 mg/l in 168 hours. The consortium was able to produce a maximum concentration 205 mg/l in 144 hours.
The production of biomass using *Chlorella vulgaris* in the wastewater model remained constant because the microalga only took the necessary nutrients to stay active (Renuka et al. 2015); however, the nutrients contained in the model wastewater do not produce any inhibition. *Bacillus cereus, Pseudomonas putida* and the consortium have a higher biomass production because these microorganisms were able to grow while removing nutrients.

**CONCLUSION**

In this work, the microorganisms and the consortium of them were able to remove the nutrients present in the wastewater model (NO₃-N, NH₃-N and PO₄-P). *Chlorella vulgaris* was the microorganism having the highest removal percentage of NH₃-N, 80.62%, with a biomass production of 88.9 mg/l during 168 hours. It was found that the consortium had the highest removal percentage of PO₄-P, 8.40%, with a biomass production of 205 mg/l in 240 hours. For *Bacillus cereus and Pseudomonas putida*, the NH₃-N removal was 27.6% and 40.97%, and NO₃-N removal was 3.8% and 4.30% for each microorganism with a biomass concentration of 450 mg/l and 444 mg/l, respectively.

**REFERENCES**


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