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BIOTECHNOLOGY

Chlorella sp. inoculum doses affect ethinylestradiol removal in a wastewater treatment plant in the Peruvian Andes

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ABSTRACT. The microalgae *Chlorella* sp. have demonstrated efficiency in the removal of pharmaceutical contaminants. However, there is no agreement on the inoculum dose since it depends on the contaminant concentration and other very specific parameters in each case. This study aims to evaluate the effect of *Chlorella* sp. inoculum doses on ethinylestradiol (EE) removal from wastewater treatment plant effluent in Celendín district of the province of Celendín, Cajamarca region, Peru. Four doses of inoculum (0, 100, 200, and 300 mL) were tested at a $2{,}1 \times 10^6$ cell·mL^{-1} microalgae cell concentration and 4 mg·L^{-1} of ethinylestradiol in photobioreactors. The final concentration of ethinylestradiol was determined after 20 days through High Performance Liquid Chromatography (HPLC). It was evidenced that a dose of 300 mL·L^{-1} of *Chlorella* sp. could remove 96,49% of ethinylestradiol from wastewater, unlike the other tested concentrations, which were below 80,92% removal. It was concluded that at a higher dose of *Chlorella* sp. inoculum, a higher ethinylestradiol removal percentage was observed in a wastewater sample from a treatment plant in the Peruvian Andes.

Keywords: Microalgae; effluent bioremediation; synthetic estrogen.

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Introduction

The Peruvian Andes constitute an ecosystem of significance at geographic, cultural, economic, and environmental levels. In addition to its high diversity in species of flora, fauna, climates, and ecosystems, they are an important source of fresh water for the country. Its highlands capture humidity from the air and feed rivers and lagoons, which supply local communities, mountains, and agricultural areas. Thus, the conservation of the Peruvian Andes is essential for the region's development.

Wastewater treatment plants are designed to purify and reuse wastewater generated by human activities. These wastewater treatment plants are essential to protect the environment and improve the quality of life of the local population. However, in many highland Peruvian communities, the absence of adequate sewage systems results in inadequate wastewater disposal. This fact not only contaminates water sources but also poses a risk to public health. There are 202 wastewater treatment plants in Peru, of which 171 are operational. Among them, 104 are located on the Peruvian coast, while only 67 are distributed in mountainous and jungle regions. (SUNASS, 2022).

Wastewater is the effluent resulting from various human activities and can contain a wide range of contaminants. Population growth, followed by the rapid advance of industrialization, has caused serious environmental degradation, within emerging contaminants found in waste and surface waters (Kasonga et al., 2020).

Pharmaceutical compounds are considered emerging contaminants in wastewater (Rzymski et al., 2017). These compounds, originating from hospitals, domestic medical treatments, agricultural runoff water, and landfill leachates, are discharged into aquatic environments, posing risks to aquatic life and human health. (Hena et al., 2020; Khan et al., 2020). Pharmaceutical compounds have various detrimental effects on the environment and aquatic fauna, including the transmission of antibiotic-resistance genes, damage to microbial communities, alterations in life cycles, reduced fertility, and changes in sexual characteristics due to hormones. (Barrantes et al., 2022). These harmful effects are manifested even at low doses, while its stable structure resists disintegration and consequently induces greater persistence in the environment; therefore, it remains biologically active.

Among this group of drugs; contraceptives, which were designed as a family planning system, are seen as an important factor to decrease unsustainable population growth and the negative effects it brings to the economy, the environment, and efforts to achieve development (Meneses, 2018).

Ethinylestradiol (EE) is a structural analog of the natural estrogen, 17 β-estradiol (E2). It is the most common active ingredient in contraceptives and is also used in estrogen replacement therapies (Durcik et al., 2023). It has been reported that ethinylestradiol (EE) excreted by humans is not effectively removed in conventional wastewater treatment plants. As a result, it easily enters the aquatic environment and subsequently affects the organisms inhabiting it. (García et al., 2020). These compounds result in disruptions to soil microbial communities, inhibit growth, and reduce microbial activity, thereby affecting denitrification rates. (Stephen et al., 2022). These estrogens, once they deposit in the environment, can endanger the reproduction and development of aquatic fauna, even at very low concentrations (nanograms per liter) (Hom et al., 2021) and below the detection limit of certain instruments (Li et al., 2020). The current wastewater treatment methods are often inefficient and inadequate for pharmaceutical compound removal, posing a serious environmental concern. This issue is further aggravated by the absence of effective regulations. Thus, steroid hormones and their structural analogs are a critical environmental problem. Studies have observed that in conventional wastewater treatment plants, only 18-32% of pharmaceutical waste is degraded by secondary treatment, with disposal increasing by 30-65% with tertiary treatment (Khan et al., 2020).

Estrone estrogens 17β-estradiol and 17α-ethinylestradiol are not completely removed or degraded by conventional treatment plants. These estrogens are often persistent and released daily into water bodies, which can later serve as drinking water sources (Spindola et al., 2018). The most frequently identified drugs in wastewater are sexual hormones (estradiol, ester, ethinylestradiol, 17β-estradiol) and anti-epileptic drugs (Grdulska & Kowalik, 2020). In this regard, Kumar et al. (2021) point out that the presence of estrogens in the environment are due to discharges and/or excretions coming from natural and anthropogenic sources. These estrogens vary from men (1.6, 3.9 and 1.5 μg) to women (3.5, 3.8 and 4.8 μg) and in pregnant women, showing a daily excretion of estriol up to 6.0 μg.

Microalgae are renowned for their capacity to absorb and accumulate various compounds from the environment, including pollutants.In addition, some species can metabolize and degrade organic compounds (Chan et al., 2022). The effectiveness of bioremediation depends on several factors, including environmental conditions, initial pollutant concentration, nutrient availability, and the adaptation of microorganisms to specific conditions. *Chlorella* sp. has shown efficacy in removing ethinylestradiol and other steroids almost completely under favorable seasonal conditions, although these conditions greatly influence the biomass concentrations and microbial diversity present in the cultures (Parladé et al., 2018; Huang et al., 2019; Bano et al., 2021).

Contamination of water, soil, sediments, and other environmental media by estrogens is an emerging health problem. Therefore, the present study aims to evaluate the effect of *Chlorella* sp. inoculum doses on the removal of EE from wastewater treatment plant effluent in Celendin district of the province of Celendín, Cajamarca region, Peru.

Materials and methods

Wastewater effluent sampling

20 L of wastewater final effluent was collected from the Celendin wastewater treatment plant (9242243.70 N, 815313.60 E). This volume of water was transferred without preservation to the water laboratory of the Autonomous National University of Chota, where it was vacuum filtered to eliminate organic and inorganic impurities, presence of cellular debris that could interfere with EE removal. EE concentration in the effluent sample was 0.0034 mg L^{-1}. Then, these samples were enriched with this compound with 4 mg of EE for experimental purposes.

Chlorella sp. inoculum: isolation and scaling

1 L of *Chlorella* sp. UTEX 2714 strain culture was acquired from the National University of Santa, Chimbote, Peru. The 1 L culture was separated into 5 equal amounts of 200 mL and gauged to 500 mL with drinking water in Erlenmeyer flasks. F/2 Guillard's Algae Food - Part A and F/2 Guillard's Algae Food - Part B were added as

culture medium at a rate of 2.1000 mL^{-1} of each nutritional supplement. The cultures were maintained at an average temperature of 21.40 °C, with white fluorescent light of 36 Watts and intensity of 2000 lux, pH 7.03, and agitation through air injection, by aeration pumps. The supplemented culture reached a density of $2.1x10^6$ $cell{\cdot}mL^{-1}$ of *Chlorella* sp. after 15 days (It was verified by counting in a Neubauer chamber).

Chlorella sp. inoculum doses determination in photobioreactors

1 L test tubes were used as photobioreactors. 1 L of wastewater effluent, previously vacuum filtered through 0.22 µm filters, was used. Three *Chlorella* sp. culture doses were used: 100, 200, and 300 mL, with a population density of $2.1x10^6$ $cell{\cdot}mL^{-1}$; to which 4 mg of EE (previously diluted in 1 mL of methanol) were added, according to Tawfik et al. (2022) (Table 1). The photobioreactors were equipped with a continuous aeration system to facilitate oxygen ingress, which also promoted wastewater homogenization and prevented microalgae precipitation. The photoperiod consisted of 12:12 h and the system was exposed to artificial light (daylight) with an intensity of 2000 Lux, at room temperature (25 ± 2°C). After 5 days, samples were collected to determine EE concentration. In addition, pH, dissolved oxygen, and water temperature were measured, parameters evaluated directly in each photobioreactor with a multiparameter, which were validated at the Regional Water Laboratory of the Regional Government of Cajamarca through the SM 4500-O C method. Azide Modification for oxygen concentration and the SM: 4500-H+B method for pH.

Physicochemical parameters included dissolved oxygen which ranged between 16.2-17.0 $mg{\cdot}L^{-1}$ and temperature between 22-24.56 °C. The experimental treatments have pH values ranging from 7.0 to 7.2, which are considered optimal for EE removal (Huang et al., 2019).

Table 1. *Chlorella* sp. inoculum doses tested in photobioreactors.

N°	Treatment	Description	Ethinylestradiol (mg L^{-1})
1	T_1	100 mL L^{-1} of *Chlorella* sp. fresh biomass in wastewater	4
2	T_2	200 mL L^{-1} of *Chlorella* sp. fresh biomass in wastewater	4
3	T_3	300 mL L^{-1} of *Chlorella* sp. fresh biomass in wastewater	4

Microalgae and ethinylestradiol concentration for the experiment.

Sample preparation for Ethinylestradiol quantification

15 mL sample from each photobioreactor was deposited into conical tubes which were centrifuged for 15 min at 1500 rpm. 4 mL of the supernatant was extracted with a syringe and transferred to a new conical tube for centrifugation. (previously filtered, using a sterile simplex syringe filter of 0.54 µm). Then, 1 mL of methanol (previously filtered, according to the previous description) was added; it was slowly stirred until a homogeneous mixture was obtained. The samples were placed in a cooler with ice packs, to maintain the temperature below 4°C and to be sent to the laboratory for high-performance liquid chromatography (HPLC) analysis (Alcántara, 2021).

Ethinylestradiol analysis by high-performance liquid chromatography

Sample analysis was carried out at the Biochemistry Laboratory of Cayetano Heredia Peruvian University. Recovered EE was quantified through HPLC using the technique described by Daniel & De Lima (2014). 20 µL of the eluate (sample) and standard solutions were administered through an automatic injector in the HPLC-DAD system model 1260 (Agilent, USA). Isocratic separation was performed on a Zorbax SB 5 µm, 150 x 4.6 mm column. The mobile phase consisted of a mixture of Acetonitrile: Water: phosphoric acid (50: 50: 0.05, v/v). The column oven was maintained at 40 °C and the flow rate was 1.0 mL min^{-1}. EE detection was performed at 280 nm. In addition, linearity, stability, precision, limit of quantification, and detection analyses were performed.

Ethinylestradiol removal efficiency

EE removal percentage was estimated as follows:

The average concentrations of EE were calculated for each treatment of the photobioreactor system using HPLC. These values were then utilized to determine the percentage of EE removal for each treatment and control.

$$ER\ \% = \frac{Ci\ ethinylestradiol - Cf\ ethinylestradiol}{Ci\ ethinylestradiol} * 100$$

ER%: ethinylestradiol removal percentage efficiency; Ci ethinylestradiol: ethinylestradiol initial concentration (4 mg·L^{-1} of the administered dose); Cf ethinylestradiol: ethinylestradiol final concentration at different treatments.

Results and discussion

Ethinylestradiol removal efficiency average values obtained for the three treatments were T1: 70.65%, T2: 80.92%, and T3: 96.49%. In addition, significant differences between treatments were found using the Kruskal-Wallis test (p-value: 0.02732), proving that higher removal efficiency of EE from enriched wastewater is obtained by using a dose of 300 mL·L^{-1} of *Chlorella* sp. (Figure 1).

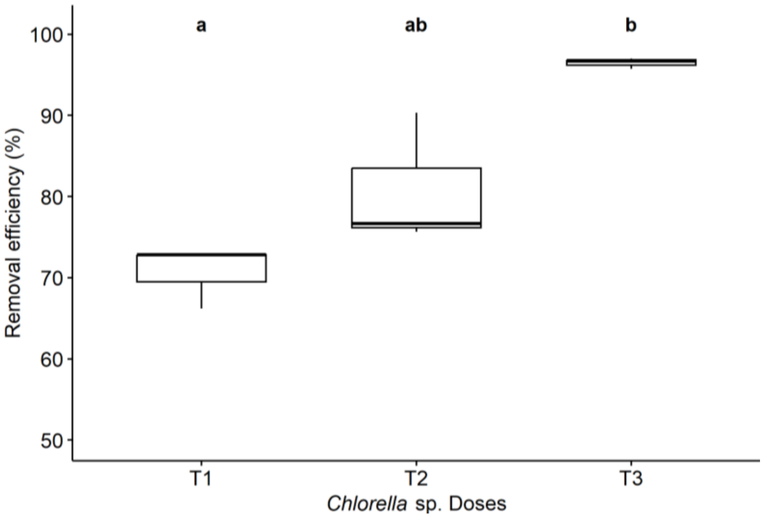

Figure 1. EE removal efficiency values from wastewater enriched with 4 mg·L^{-1} of EE.

Chlorella sp., a microalgae known for its ability to remove contaminants from water, has been investigated as a possible solution for EE removal. EE removal efficiency values found are in a similar range as reported by Ruksrithong & Phattarapattamawong (2019) who removed between 52-99% of synthetic estrogen at concentrations of 100 mg L^{-1} dry weight of microalgae and similarly Huang et al. (2019) between 65-92%. Moreover, they exceeded the removal reported by Xuelian and Kumud (2019) who obtained removal efficiency values between 30-57%. Wang et al. (2019) refer EE degradation efficiency through biotransformation processes with values above 65% applying *Chlorella*. In their study, Xuelian & Kumud (2019) reported similar findings using *Chlorella vulgaris*, achieving EE removal efficiency values ranging between 65% and 91%.

It is important to address how different doses of *Chlorella* sp can affect EE removal. Molinuevo et al. (2019) stated that higher doses of microalgae might enhance the ability to absorb and degrade EE due to the increased absorption surface area provided by a larger number of microalgal cells, which translates to higher metabolic capacity and cell density. This fact allows adaptation to the environment and enzyme production. However, practical and economic limitations may arise with the use of high doses of microalgae. At these levels, cells can experience oxidation and inhibition of their cellular processes (Durcik et al., 2023). Oxidative stress, changes in cellular composition, toxin accumulation, and growth inhibition are some of the impacts caused by the lack of monitoring and periodic analysis of treatment systems (Henn et al., 2020).

Other research such as those developed by Huang et al. (2019), Parladé et al. (2018), Jun et al. (2017), Ruksrithong and Phattarapattamawong (2019), and Hena et al. (2020) have shown that *Chlorella* sp. can metabolize and adsorb organic compounds, including EE. Its removal efficiency may depend on aeration, temperature, pH, lighting periods, and nutrient availability, which allow microalgae to adapt (Chan et al., 2022). In addition to *Chlorella* sp., the concentration doses, environmental conditions, and the presence of other contaminants in wastewater—such as organic compounds, heavy metals, nutrients, and suspended solids—affect EE removal efficiency. While high removal efficiency can be achieved under controlled conditions, this efficiency decreases in field conditions due to variability and concentration. In this regard, Spindola et al. (2018), Garcia et al. (2020), De Jesus (2020), and Rodriguez et al. (2022) mention that in conventional treatment systems, EE removal is inefficient. Therefore, it is vital to use the characteristics of actual wastewater samples instead of an ideal solution to ensure the adaptability and tolerance of microalgae. In this way, the elimination of predatory microorganisms from microalgae can be verified, thus promoting the efficiency of the biological process.

It should be considered possible side effects or environmental impacts associated with the use of *Chlorella* sp. in large quantities which can generate an algal bloom due to the availability of nutrients in the wastewater (Plöhn et al., 2021), in a long-term period or after scaling up. Should also be taken into account the possibility that the microalgae can be recovered and reused as demonstrated by Pablo et al. (2021), Molinuevo (2019), and Khan et al. (2023) in the generation of biofuels, biofertilizers, wastewater treatment, production of chemical inputs, and bioactive compounds, carbon sequestration, and environmental monitoring. Aspects related to the industrial-scale feasibility and practical implementation of this strategy for EE removal in wastewater treatment plants can also be addressed. The implementation of microalgae for ethinylestradiol (EE) removal in wastewater treatment plants requires a comprehensive approach. From strain selection to culture system design and optimization, the aim is to maximize EE uptake. Constant monitoring and analysis are crucial for ensuring long-term efficacy. Biomass harvesting and utilization in sustainable alternatives are essential, and ongoing assessment of EE concentration ensures compliance with environmental regulations. This strategy emphasizes the importance of continuous research and sustainability in industrial-scale environmental solutions (Varela et al., 2023).

Population growth in the presence of ethinylestradiol at different doses of *Chlorella* sp.

Population density of *Chlorella* sp. in the photobioreactors after 21 days was 3.1x10^{6} cell⊠mL^{-1} for the concentration of 100 mL⊠L^{-1}, 4.0x10^{6} cell⊠mL^{-1} for 200 mL⊠L^{-1} and 4.9x10^{6} cell⊠mL^{-1} for 300 mL⊠L^{-1} as shown in (Figure 2).

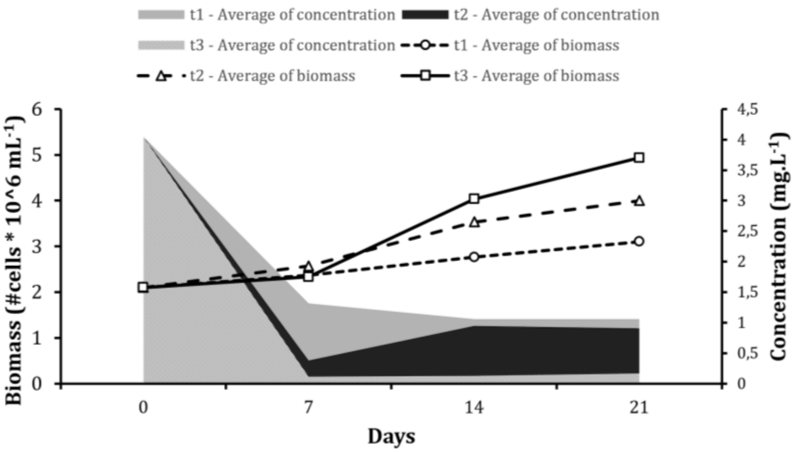

Figure 2. *Chlorella* sp. growth curves at different population densities (T1: 100 mL·L^{-1}; T2: 200 mL·L^{-1}; T3: 300 mL·L^{-1}) in the presence of 4 mg·L^{-1} of EE, regarding the Gompertz model.

Considering the effects of EE on *Chlorella* sp. growth it is worth mentioning that at low inoculum doses, minimal impact of EE on *Chlorella* sp. growth may be observed. This microalgae can adapt to the presence of EE or show initial resistance. At intermediate doses, a more pronounced response was observed, as exposure to moderate concentrations of EE influenced the growth rate of *Chlorella* sp. In this regard, Yingxiao et al. (2023) reported that the estrogenic activity of EE generates hormonal action and photosynthetic promotion in aquatic organisms that influence their growth rate. Cellular metabolism modulation is considered a process that favors and promotes the growth conditions of this microalgae when exposed to estrogenic compounds (Hardegen et al., 2023).

Considering *Chlorella* sp. response to increasing inoculum doses, it is suggested that at low doses the adaptability and reproduction phase may be prolonged (Khalaji et al., 2023). This could influence the EE removal efficiency process. At intermediate doses, a balance between *Chlorella* sp. concentration and the presence of EE is likely to be reached, which could result in a higher removal efficiency of the pharmaceutical compound. At high inoculum doses, competition between Chlorella sp. cells may increase, potentially leading to limitations in available resources and negatively affecting population growth. The sustainability of using higher inoculum doses should be discussed in terms of long-term viability and associated costs. EE removal efficiency could be a determining factor in the choice of inoculum dose; however, it is also essential to consider other factors such as the energy required to maintain optimal growth conditions.

On the other hand, there may be genetic or metabolic adaptations in *Chlorella* sp. in response to the presence of EE, which could influence its ability to grow and remove the compound. The main removal process of EE is through biodegradation by enzymatic action of this microalgae. Its lipid bilayer and the presence of exudates and mucilage, initially generate an adherence to its wall, passing through a process of absorption within it, to finally convert and/or biotransform the contaminant (Hena et al., 2020).

This removal level may be due to biodegradation processes, which are facilitated by the optimal conditions for the growth and development of microalgae. In this regard, Plöhn et al. (2021) suggest that adsorption processes appear to precede the biodegradation of some pollutants. Additionally, the biodegradation of estrogen by microalgae occurs either directly through metabolites generated by heterotrophic microorganisms or through the action of extracellular enzymes. Ruksrithong & Phattarapattamawong (2019), claim that these estrogen removal processes may be due to biodegradation mechanisms or adsorption processes to a lesser degree. Wang et al. (2019) emphasize estrogen degradation rather than mere uptake or adsorption by microalgae, highlighting the importance of these processes in recommending the use of microalgae for advanced wastewater treatment to remove both nutrients and estrogens.

In terms of growth dynamics, the Gompertz model is a commonly used equation to describe the growth of biological populations. (Figure 2) illustrates that the exponential phase is most pronounced when a dose of microalgae secures the initial population base, which then decreases after a conditioning phase. This could be due to the consumption of nutrients present in the wastewater matrix. Hejna et al. (2022) consider that the exponential onset due to adaptability allows the cellular base to experience population growth. On the other hand, Kasonga et al. (2020) state that growth slowdown is compromised by nutrient depletion due to nutrient consumption. In addition to making a correlation in relation to the removal of EE. The greater the population growth of microalgae, the greater the removal percentage.

Chlorella microalgae population growth modeled by Gompertz exhibits an initial exponential phase, followed by a gradual deceleration. As shown in (Table 2), it is observed that the key parameters of growth evolution (μ - λ) influence the magnitude and speed of response to EE stimulus, determining the carrying capacity and growth stability (Bano et al., 2021). Li et al. (2020) point out that these parameters can be optimized to efficiently understand and exploit the biotechnological potential of *Chlorella* sp. in biomass production applications.

Table 2. Population growth parameters according to the Gompertz model.

Treatment (mL·L^{-1})	C	μ	λ
T1: 100	1.64e+06	5.66e+04	0.52
T2: 200	2.28e+06	1.42e+05	2.86
T3: 300	3.22e+06	2.70e+05	6.20

C logarithmic difference between final and initial population, μ growth rate, λ time of highest growth rate in days.

Conclusion

It was found that the use of *Chlorella* sp. at 300 mL·L^{-1} can represent an effective bioremediation treatment for ethinylestradiol with up to 96.49% removal efficiency so that its detrimental effects on aqueous ecosystems and living beings are avoided.

The doses of *Chlorella* sp. affect the elimination of EE. High doses of inoculum improve absorption and degradation due to greater cell density and metabolic capacity. However, EE can cause cellular oxidation and oxidative stress, inhibiting the growth of microalgae.

The efficiency of contaminant removal by microalgae, such as *Chlorella* sp., depends on factors such as aeration, temperature, pH, lighting, and nutrients, which favor their adaptability and growth.

The main elimination process of EE is through biodegradation by enzymatic action. *Chlorella* sp., its lipid bilayer, and the presence of exudates and mucilage, initially generate adhesion to its wall, going through an absorption process within it, to finally convert and /or biotransform the EE.

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