

Algae bioremediation of swine and domestic wastewater promotes a reduction of coliforms and antibiotic-resistant bacteria

Itzel Y. López-Pacheco^a, Georgia María González-Meza^{a,b}, Reyna Berenice González-González^{a,b}, Roberto Parra-Saldívar^c, Elda M. Melchor-Martínez^{d*}.

^a Tecnológico de Monterrey, School of Engineering and Sciences, Monterrey, 64849, Mexico.

^b Tecnológico de Monterrey, Institute of Advanced Materials for Sustainable Manufacturing, Monterrey 64849, Mexico.

^c Cranfield University, Magan Centre of Applied Mycology, Cranfield, MK43 0AL, UK

^d Universidad Autonoma de Nuevo Leon, Facultad de Medicina, Monterrey 64460, Mexico

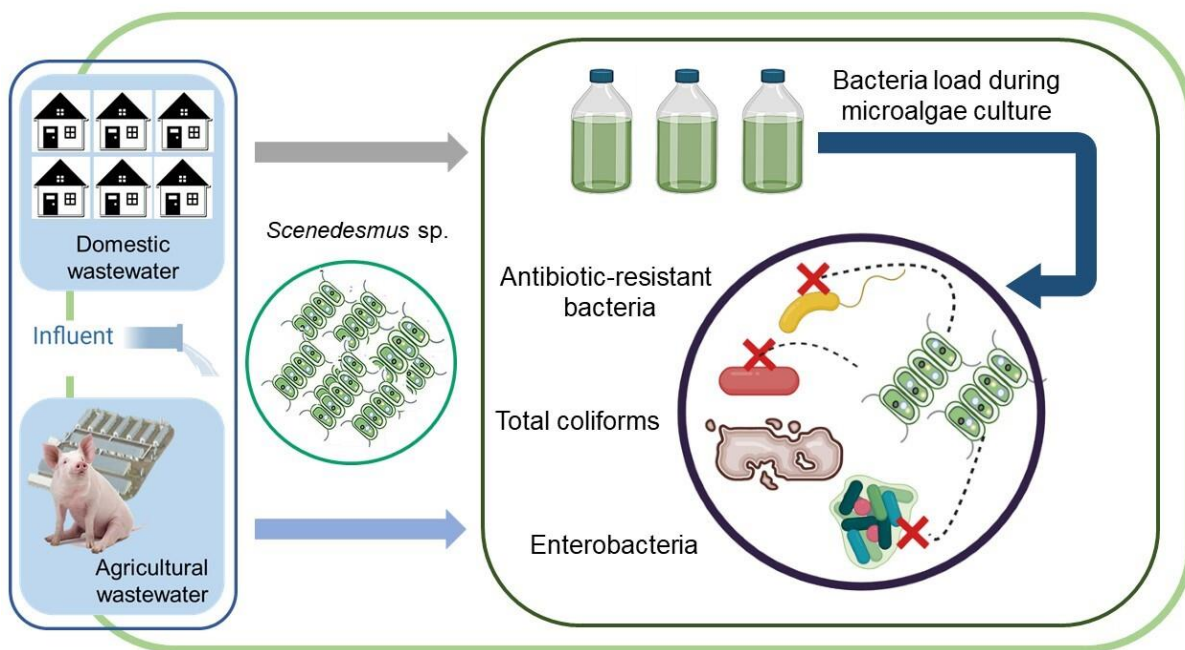
*Corresponding author e-mail: elda.melchor@tec.mx (E.M. Melchor-Martínez).

Abstract

The microbiological load that wastewater may contain is an important factor to consider in wastewater treatment to avoid water bodies contamination and has taken on great relevance due to the possible presence of antibiotic-resistant bacteria. This study investigates the feasibility of bacteria control by phycoremediation treatment using *Scenedesmus* sp. in two types of wastewater (domestic and swine wastewater). It was determined the cell growth of microalgae culture, and the reduction of total coliforms and enterobacteria load throughout ten days of experiment. In addition, the removal of antibiotic-resistant bacteria was performed using five different antibiotics commonly used in clinical diagnosis: Ampicillin, Tetracycline, Ciprofloxacin, Sulfamethoxazole, and Ceftriaxone. The results shown a significant decrease in total coliforms and enterobacteria in the phycoremediation process, it was removed up to 98% of total coliforms [from $(8.7 \pm 2.31) \times 10^4$ to $(1.6 \pm 0.17) \times 10^3$ CFU mL⁻¹] in swine wastewater and 99% in domestic wastewater [(3.6

$\pm 0.31) \times 10^5$ to $(2 \pm 0.05) \times 10^3$ CFU mL⁻¹]. Significant reduction in the case of sulfamethoxazole-resistant bacteria by microalgae in swine wastewater from $[(1.47 \pm 0.05) \times 10^5$ to $(5.3 \pm 0.57) \times 10^3]$ and domestic wastewater $[(4.9 \pm 0.15) \times 10^4$ to $(2.9 \pm 0.36) \times 10^3]$. These findings demonstrate the versatility and effectiveness of the phycoremediation system since the general microbial control to most specific of antibiotic-resistant bacteria in wastewater, demonstrating its great potential to reduce the risk of public health issues in urban and rural areas.

Graphical abstract



Keywords: Total coliforms, Enterobacteria, Antibiotic resistance, Microalgae, *Scenedesmus sp.*

1. Introduction

Population growth increases urbanization and the demand for goods and services, such as the use of water and food, increasing environmental pollution in water bodies. It is estimated that the volume of wastewater produced globally will reach 471 billion m³ in 2030 and 574 billion m³ in 2050 (Qadir et al., 2020). Since there is and is expected to be a water shortage in various cities in the future to the point that

it is estimated that by 2050, half of the global population will face water scarcity (He et al., 2021). Therefore, wastewater treatment is of great importance to achieve good recirculation of the resource and reduce this problem.

It has even been estimated that worldwide the use of antibiotics has increased by up to 40% in a range of 20 years (2000-2018) (Browne et al., 2021). Particularly, the COVID-19 pandemic caused an increase in indiscriminate drug use, which increased the concentration of antibiotics such as ciprofloxacin, azithromycin, and amoxicillin in domestic wastewater (Harrington et al., 2022). These antibiotics can inhibit the bioactivity of activated sludge, hindering the efficiency of traditional wastewater treatment (Chen et al., 2021). Domestic and livestock wastewater, containing higher antibiotic levels, act as a medium to facilitate horizontal gene transfer in aquatic environments, posing a public health risk. This gene transferring increases the abundance of resistance genes, which promotes the spread of resistant bacteria (Sun et al., 2023).

On the other hand, fecal indicator microorganisms, such as *Enterobacteriaceae* and *E. coli*, which commonly persist in wastewater, are exposed to these antibiotic residues, and can increase resistance genes presence (Nguyen et al., 2021). In consequence, wastewater is being a source of antibiotic-resistance genes and antibiotic-resistant bacteria, which increases the risk of waterborne diseases and reduces therapeutic efficacy for humans and animals against bacterial infections. The WHO classifies antibiotic-resistant bacteria as one of the three most significant threats to public health (Serwecińska, 2020).

Pig farming in the production sector is responsible of the highest generation of wastewater (Doreau et al., 2012). Swine wastewater harbors bacteria such as fecal coliforms, *E. coli*, and antibiotic-resistant bacteria due to the use of antibiotics in intensive farming systems (Savin et al., 2020). A study conducted in two sites near the discharge of swine wastewater after primary treatment revealed the presence of fecal Coliforms (140,000 CFU/100 mL⁻¹) and *E. coli* (5,400 CFU/100 mL⁻¹) in surface water (Heaney et al., 2015). Due to this situation, both in the discharge of swine

wastewater and in domestic water, searching for technologies that can reduce the presence and translocation of bacteria in the environment must be encouraged, especially to avoid a public health outbreak.

There are effective techniques for removing bacteria from wastewater including antibiotic resistant bacteria, such as ultrasound and ozone, these are expensive and require specific conditions. However, more efficient, cost-effective, and adaptable wastewater treatment technologies are needed to treat this global concern situation (Foroughi et al., 2022). The bioremediation of domestic and swine wastewater based on microalgae is an alternative to these technologies (Hu et al., 2020). Microalgae are photosynthetic organisms able of CO₂ capture with great potential for bioremediation without producing secondary pollution, generating biomass for valorizing high-value compounds (López-Pacheco et al., 2021). Unlike conventional treatments, which are expensive and energy-intensive, microalgae bioremediation is a circular and sustainable alternative, the cost of performing it, range between \$0.18 and \$1.69 per m³ of treated water (Chaudry, 2021). In addition, it allows to valorize the generated biomass for elaborating biotechnological products, such as antimicrobial metabolites for example, long-chain fatty acids and pigments, which can assist in microbiological control in wastewater (Ansa et al., 2012). The biocontrol could be due to the microalgae outer layer adsorbs particles, including bacteria, and after biomass separation techniques these bacteria can be removed from the treated water(Inuwa et al., 2023).Likewise, some parameters (light, pH, temperature) of the microalgae culture could reduce the survival of some kind of bacteria(Liu et al., 2020).

Therefore, this study evaluates the feasibility of phycoremediation with *Scenedesmus* sp. for the elimination of bacteria load associated to total coliforms and enterobacteria in two types of wastewater: domestic and swine wastewater. The reduction of antibiotic-resistant bacteria was monitored in domestic and swine wastewater on a laboratory scale, and five types of antibiotics (Ampicillin, Tetracycline, Ciprofloxacin, Sulfamethoxazole and Ceftriaxone) were tested. Finally,

a general overview of the possible implementation of the phycoremediation system in urban and rural communities was designed.

2. Materials and methods

2.1 Reagents and equipment

The reagents used for culture medium preparation, carbendazim and antibiotics were purchased from Sigma-Aldrich (Estado de Mexico, Mexico). The pH was measured using a Thermo Scientific™ Orion™ 3-Star Benchtop pH Meter. Petrifilm plates for total coliforms and Enterobacteriaceae were purchased from 3M™ Petrifilm™ (Michigan, USA).

2.2 Inoculum culture conditions

The strain *Scenedesmus* sp. was purchased from UTEX (Austin, TX, USA). BG11 culture medium was used to perform experiments. The stock culture was maintained at 21 ± 1 °C with continuous light at $100 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ and with $1.5 \text{ L m}^{-2} \text{min}^{-1}$ of aeration (OPTIMA 4.5-Watt pump with an air filter of $0.20 \mu\text{m}$). The stock culture was maintained until reaching $20 \times 10^6 \text{ cell mL}^{-1}$ according to previous conditions reported (López-pacheco et al., 2021).

2.3 Swine and domestic wastewater as a culture media for *Scenedesmus* sp.

Swine wastewater after primary treatment (separation of solids) was obtained from the swine farm “Ana Margarita”, located in Allende, Nuevo Leon, Mexico (25.279731°N , $100.019186^\circ \text{W}$). The domestic wastewater was obtained after the primary treatment (Solids separation) from a local domestic WWTP (Dulces Nombres WWTP) located in Nuevo León, México (25.737784°N , $100.069270^\circ \text{W}$). The average temperature at the time of sampling was 35°C . Each wastewater sample was stored at 4°C until its use.

A volume of 100 mL of *Scenedesmus* sp. (Initial concentration: $6 \times 10^6 \text{ cell mL}^{-1}$) was used as seed culture to inoculate a 1000 mL glass bottle. The experimental setup was developed by a general factorial design (**Table 1**). In the case of the control condition for cell growth, it was used BG11 as a culture medium. The sparging and

temperature of both cultures was maintained at the same conditions as in Section 2.2., in this case it not was monitoring the temperature and the dissolved oxygen in the cultures. All treatments were conducted in triplicate. The biomass concentration of *Scenedesmus* sp. cultures was evaluated every 24 hours. Each sample of 5 mL of culture was measured to monitor the cell growth by cell count in a Neubauer chamber. The pH was measured every five days.

Table 1. Experimental setup of swine and domestic wastewater as a culture media for *Scenedesmus* sp.

Swine wastewater				
Treatment	Run	Blocks	Bi-distilled water	Wastewater
Control	1	2	50%	50%
SW4	2	2	50%	50%
SW3	3	2	65%	35%
SW2	4	2	75%	25%
SW1	5	2	90%	10%
Control	6	3	50%	50%
SW4	7	3	50%	50%
SW3	8	3	65%	35%
SW2	9	3	75%	25%
SW1	10	3	90%	10%
Control	11	1	50%	50%
SW4	12	1	50%	50%
SW3	13	1	65%	35%
SW2	14	1	75%	25%
SW1	15	1	90%	10%
Domestic wastewater				
Control	1	2	20%	80%
D4	2	2	20%	80%
D3	3	2	40%	60%
D2	4	2	60%	40%
D1	5	2	80%	20%
Control	6	3	20%	80%
D4	7	3	20%	80%
D3	8	3	40%	60%
D2	9	3	60%	40%
D1	10	3	80%	20%
Control	11	1	20%	80%
D4	12	1	20%	80%

D3	13	1	40%	60%
D2	14	1	60%	40%
D1	15	1	80%	20%

SW: Swine wastewater (SW1 -SW4). D: Domestic wastewater (D1-D4).

2.4 Total coliforms and enterobacteria determination in swine and domestic wastewater under phycoremediation treatment

The cultures were sampled by taking 10 mL of the culture on days 0, 2.5, 5, 7.5, and 10. Samples were stored at 4°C until further analysis. For total coliform analysis, it was used 3M™ Petrifilm™ Coliform Count Plate; 1 mL of each homogenized sample was placed on the plates and incubated for 24 hours at 35° C. For enterobacteria analysis, it was used 3M™ Petrifilm™ Enterobacteriaceae Count Plates; 1 mL of each homogenized sample was placed on the plates and incubated for 24 hours at 35° C.

2.5 Antibiotic resistant bacteria determination in swine and domestic wastewater under phycoremediation treatment

The cultures were sampled by taking 10 mL of the culture on days 0, 5, and 10. Samples were stored at 4 °C until further analysis. For antibiotic resistant bacteria determination in wastewater during the phycoremediation process, it was used the heterotrophic plate count method. Five types of antibiotics were used, as follow conditions: Ampicillin (32 mg L⁻¹), Tetracycline (16 mg L⁻¹), Ciprofloxacin (4 mg L⁻¹), Sulfamethoxazole (50.4 mg L⁻¹) and Ceftriaxone (8 mg L⁻¹). Each antibiotic was used to prepare 1 liter of solution of the culture medium. The selection of antibiotics was determined in order to consider different types of antibiotics [Ampicillin referred to penicillin class; Tetracycline to tetracycline class; Ciprofloxacin to quinolone class; Sulfamethoxazole to Sulfonamides class; and Ceftriaxone to cephalosporin class] (Cheng et al., 2020). Bacteriological agar (BD Bioxon, Estado de Mexico, Mexico) was used as a culture medium for this experiment. In order to prevent fungal growth 400 µg L⁻¹ of carbendazim was added to the culture. Carbendazim was previous reported to prevent fungal growth, and it have not affected the count of antibiotic-resistant colonies (Mahan et al., 2005). All samples were diluted by serial ten-fold

dilutions and spread on the plates with each antibiotic. Plates were incubated for 48 h at 37° C before antibiotic resistant bacteria determination.

2.6 Data analysis

All data were analyzed by Minitab 21 (Minitab Inc.). The correlation of variables such as microalgae growth, lipids production and pH with total coliforms and Enterobacteria presence was processed by a Pearson correlation analysis ($\alpha=0.05$).

3. Results and Discussion

3.1 Swine and domestic wastewater as a culture media for *Scenedesmus* sp.

Scenedesmus sp. had consistent cell growth in both samples of wastewater (**Figure 1**). In the control condition, it was obtained 2×10^6 cell mL⁻¹ (0.17 g L⁻¹) and 4×10^6 cell mL⁻¹ (0.29 g L⁻¹) on the 5th and 10th culture day, respectively. These results are similar to those reported by (Rodas-Zuluaga et al., 2021), in which *Scenedesmus* sp. reached approximately 0.3 g L⁻¹ on the 10th culture day using BG11 as growth medium. In swine wastewater (**Figure 1a**), a greater result in SW1 treatment (10% swine wastewater) was obtained, even in this treatment, a higher biomass concentration than the control condition was observed (7×10^6 cell mL⁻¹ at 10th culture day). This behavior could be explained because of swine wastewater has a large amount of nutrients, and water was not as cloudy due to dilution. In studies on *Chlorella vulgaris* JSC-6, it grown in 10-fold diluted swine wastewater obtained greater cell growth compared to 20-fold diluted wastewater (Wang et al., 2015), which are comparable to those obtained in this study. On the other hand, secondary swine wastewater treatments showed slight growth, similar than the case of SW2 [25% swine wastewater].

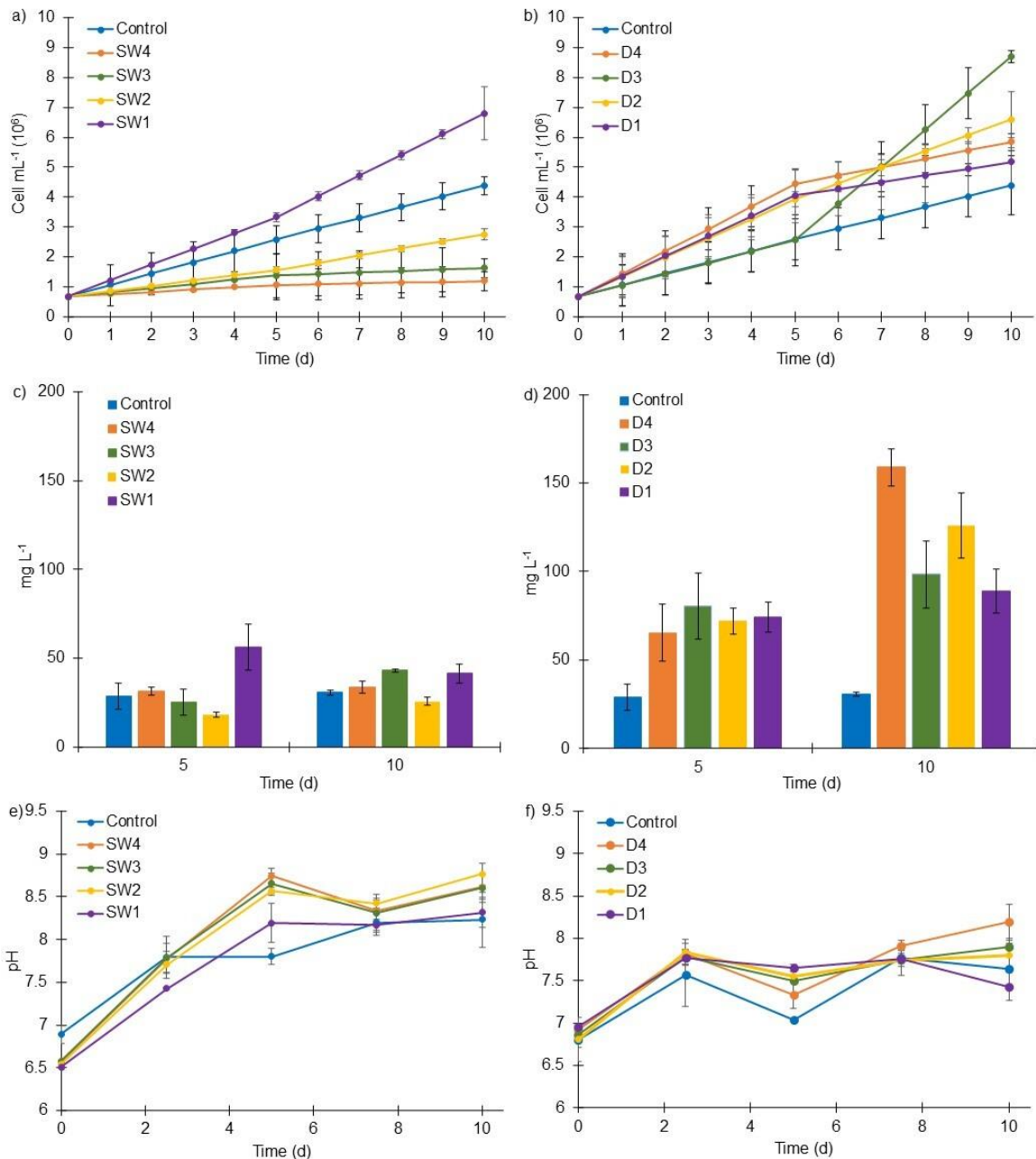


Figure 1. Measurements of microalgae growth in wastewater (Cell mL^{-1}), lipids production ($mg L^{-1}$) and pH. Cell growth of *Scenedesmus* sp. grown in **a)** swine wastewater and **b)** domestic wastewater. The samples for cell growth were taken every day. Lipids production of *Scenedesmus* sp. grown in **c)** swine wastewater and **d)** domestic wastewater. The samples for lipids production were taken every five days of the experiment. pH determination of *Scenedesmus* sp. culture grown in **e)** swine wastewater and **f)** domestic wastewater. The samples were taken every 2.5 days of the experiment. All points were sampled in triplicate.

Domestic wastewater had shown relevant results with a higher proportion of wastewater compared with swine wastewater experiments, this may be because of the contaminant load present in swine wastewater and its turbidity. The analysis of D3 treatment (60% domestic wastewater) released a higher biomass concentration in the whole experiment (9×10^6 cell mL⁻¹ at 10th culture day [0.58 g L⁻¹]) (**Figure 1b**). Similar to these results, *Scenedesmus acutus* reached 0.6 g L⁻¹ on the 10th culture day after grown in pre-treated municipal wastewater (Sacristán de Alva et al., 2013). Comparing the results of D2 treatment (40% domestic wastewater) showed consistent cell growth, and 7×10^6 cell mL⁻¹ were measured at the 10th culture day. Finally, it was noted using 80% of domestic wastewater (D4) resulted in increased microalgae growth, reaching 6×10^6 cell mL⁻¹ by the 10th culture day, this is because the amount of nutrients is greater than in the other treatments, and in this case the turbidity was not conducted to cell death. Interestingly, there has been reported an excellent cell growth of *Scenedesmus* sp. SD07 in municipal wastewater; the best condition was observed using 75% domestic wastewater (Silambarasan et al., 2023). Considering their results and those from the present study, a range between 60-80% of wastewater could be a suitable formulation of medium for microalgae growth.

The data analyzed regarding the lipids production in the cultures using swine wastewater (**Figure 1c**), have shown higher lipids production in SW1 treatment (10% swine wastewater), where it was obtained 56 mg L⁻¹ of lipids on the 5th culture day. There is a correlation between the cell growth in swine wastewater treatments and the lipids production that can be explained this behavior. Meanwhile the treatments using domestic wastewater, revealed a significant enhanced lipids production in all experiments compared with the control. The composition of the culture medium once again could promote the cell growth correlated with the lipids metabolism in the algae. The experiment D4, was monitored releasing 65 mg L⁻¹ and 158 mg L⁻¹ on the 5th and 10th culture day, respectively (**Figure 1d**).

The pH was monitored through the culture kinetics. In swine wastewater, specific SW1 and SW2 experiments, were noted an increase of pH from 6.50 to 8.31 and 6.54 to 8.76, respectively (**Figure 1e**). Not relevant modification was observed in

domestic water treatments. The most notable change was on D4 experiment, in which pH was varying from 6.91 to 8.19 (**Figure 1f**). Previous reports determined that photosynthetic activity could increase pH, even using a buffer in the culture medium than can mitigate significant changes (Xie et al., 2020). Secondly explanation about the pH increments is related to nitrogen removal from the medium that was mentioned in other studies and was supported in present observations in the control conditions (Scherholz& Curtis, 2013).

3.2 Total coliforms and enterobacteria determination in swine and domestic wastewater under phycoremediation treatment

Total coliforms are some bacteria strains considered as indicators of microbial quality of water (USEPA, 2023). These organisms are commonly found in the feces of humans and animals (Patel et al., 2014). Generally, coliform bacteria mainly but not restrictive belong to four genera of the Enterobacteriaceae (Halkman and Halkman, 2014).

The monitored total coliform concentration (CFU mL⁻¹) showed a notable decreasing in values from $(8.7 \pm 2.31) \times 10^4$ to $(1.6 \pm 0.17) \times 10^3$ in SW1 treatment (**Figure 2a**). Foremost in SW3 experiment (35% swine wastewater) and secondly in SW2 experiment (25% swine wastewater) a 2-log reduction was observed in both. In experiments using domestic wastewater (D4 treatment), the same fact was observed as a reduction in total coliform concentration (CFU mL⁻¹) from $(3.6 \pm 0.31) \times 10^5$ to $(2 \pm 0.05) \times 10^3$ (**Figure 2b**). The total coliform growth was reduced in order of 2 log in experiments using domestic wastewater.

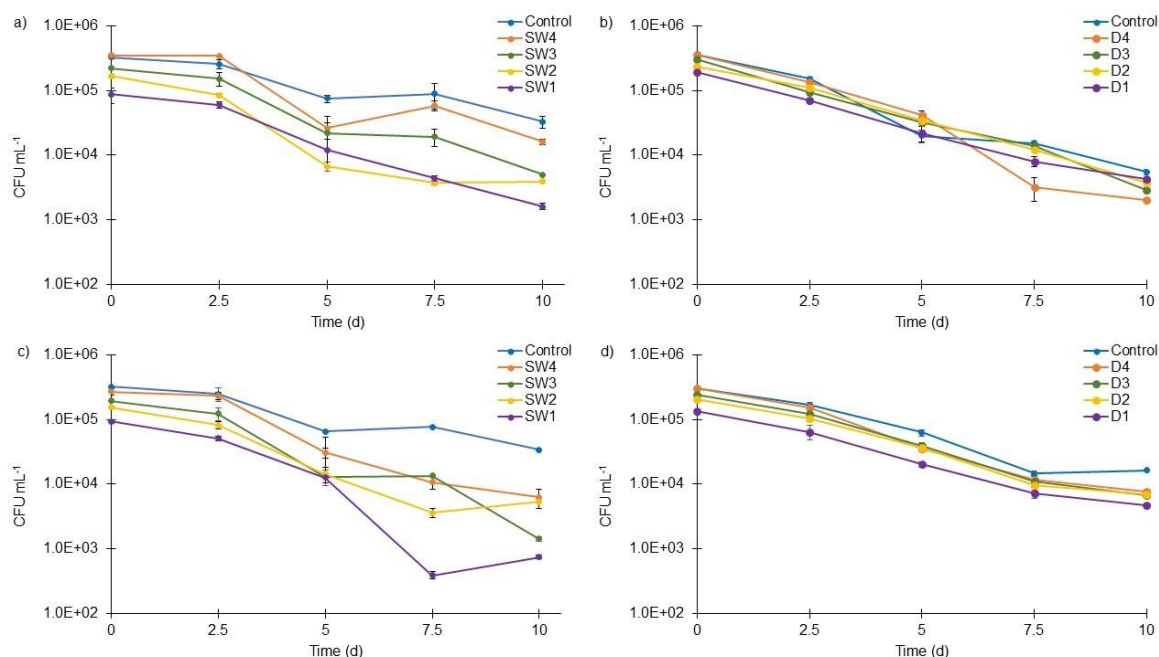


Figure 2. Total coliforms and enterobacteria (CFU mL⁻¹) determination in wastewater under phycoremediation treatment. **a)** Total coliforms determination in swine wastewater. **b)** Total coliforms determination in domestic wastewater. **c)** Enterobacteria determination in swine wastewater. **d)** Enterobacteria determination in domestic wastewater. All points were sampled in triplicate. The samples were taken every 2.5 days of the experiment.

These results are in agreement with similar studies, for example, Slompo et al. (2020) reported a 68% reduction of the total coliform load (Log reduction: 0.76) of swine manure and sanitary wastewater mixture by *Chlorella sorokiniana* (Slompo et al., 2020). Normally, other types of treatments have also seen a reduction in the coliform load of at least 50% (Bhat and Danek, 2012), this percentage is a point of comparison with the results obtained in the phycoremediation experiments of this work.

The most relevant result was observed in D4 experiment, and it can be attributed to the exposure of microalgae to higher stress due to the initial bacterial concentration and contaminant load. A second explanation for this behavior may be due to the relationship between microalgae cell growth and bacteria control. We observed a high correlation between the microalgae cell growth and the decrease in total coliforms presence in swine wastewater treatments [SW1 ($r = -0.908$, $p = 0.000$),

SW2 ($r = -0.858$, $p = 0.000$), SW3 ($r = -0.974$, $p = 0.000$), and SW4 ($r = -0.895$, $p = 0.000$)]. Also, in domestic wastewater treatments a high correlation between both variables was found in all treatments [D1 ($r = -0.977$, $p = 0.000$), D2 ($r = -0.944$, $p = 0.000$), D3 ($r = -0.724$, $p = 0.002$), and D4 ($r = -0.978$, $p = 0.000$)]. These results lead us to consider that there is indeed an inverse correlation relationship in these two variables, especially when observing the same behavior in both wastewaters at different concentrations. In this sense, this study determines that the growth of microalgae could have an effect on reducing the presence of total coliforms in the culture, which is also confirmed by observing a greater reduction in total coliforms presence in the SW4 and D4 treatment compared to the control condition.

Regarding the impact of microalgae metabolism in bacteria control, lipids production could have a significant impact (Michelon et al., 2021). Fatty acids belong to the group of primary metabolites identified in algae and have shown recognized antibacterial properties being effective against many gram-positive and negative bacteria. The effectiveness of these metabolites could be dependent on the degree of unsaturation and fatty acid chain length (Michelon et al., 2021). Saturated fatty acids lack or have little antimicrobial activity due to they are not water soluble contrary to unsaturated lipids such as DHA, linoleic acid and oleic acid which remain their lipophilic composition and can be absorbed through the cell layers (Alsenani et al., 2020). There are several statements to explain the antibacterial action of fatty acids. For example, they are able to disrupt the cell membranes and released the genetic material to the extracellular compartment. In addition, they are inhibitors of the cell wall biosynthesis and can alter the peptide glycan synthesis in gram-positive bacteria (Casillas-Vargas et al., 2021)

So, in this study, it was shown that there is a high correlation between the microalgae lipids production and the decrease of total coliforms load in some swine wastewater treatments [SW1 ($r = -0.856$, $p = 0.003$), SW3 ($r = -0.700$, $p = 0.036$), and SW4 ($r = -0.992$, $p = 0.000$)]. Also, in domestic wastewater treatments a high correlation between both variables was observed in all treatments [D1 ($r = -0.992$, $p = 0.000$), D2 ($r = -0.914$, $p = 0.001$), D3 ($r = -0.990$, $p = 0.000$), and D4 ($r = -0.809$,

p= 0.008)]. These results lead us to consider that there is an inverse correlation relationship in these two variables, especially when observing the same behavior in both wastewaters. In this sense, this study determines that the lipids production of microalgae could have an impact on reducing the presence of total coliforms in the culture.

Variables such as light and aeration could be influenced the removal of total coliforms in wastewater because there was noticed a reduction in the control condition (only wastewater) that was placed in the experiment. The monitoring load in swine wastewater was from $(3.5 \pm 0.2) \times 10^5$ to $(3.3 \pm 0.67) \times 10^4$ meanwhile, in domestic wastewater was from $(3.6 \pm 0.2) \times 10^5$ to $(5.5 \pm 0.5) \times 10^3$. Previous reports found that aeration (dissolved oxygen) and constant light inhibit the growth of pathogenic bacteria, both factors could be related to generate a photo-oxidation process causing damage to bacteria cells by affecting the cytoplasmic membrane (Pompei et al., 2023). However, the most relevant effect was demonstrated by the microalgae growth enhancing the removal of the bacteria. This behavior could be clearly shown in SW4 and D4 experiments, compared with the control that was running with the same wastewater concentration (**Figure 2**).

Enterobacteriaceae has gained clinical relevance as a gram-negative bacteria family, including some strains of *Escherichia coli* such as *E. coli* O 157:H7, *Yersinia* spp., and *Citrobacter freundii* (Pérez Guerrero et al., 2014). Antibiotic-resistant strains belong to *Enterobacteriaceae* can be commonly found in a large amount of waste from meat production and processing areas (e.g. pork meat production) (Homeier-Bachmann et al., 2021).

In the experiments using swine wastewater, a higher decrease in the Enterobacteria concentration (CFU mL⁻¹) was observed. The most relevant values were obtained from SW1 treatment $(9.3 \pm 0.66) \times 10^4$ to $(7.4 \pm 0.5) \times 10^2$ and SW3 and SW2 treatments as well, they showed at least 2 log reduction(**Figure 2c**). In the other side, domestic wastewater experiments showed to be competitive with at least 1 log reduction. For example, in D3 experiment (60% of domestic wastewater) the

reduction was from $(2.4 \pm 0.07) \times 10^5$ to $(6.7 \pm 0.01) \times 10^3$ and in the D4 experiment the values ranged from $(3.1 \pm 0.06) \times 10^5$ to $(7.8 \pm 0.38) \times 10^3$ (**Figure 2d**).

There are a few studies in which the phycoremediation and inhibition of bacteria growth have been reported. Pompei et al. (2023) found that the phycoremediation of rural wastewater removed *E. coli* (Pompei et al., 2023). A similar study carried out by (Ruas et al., 2018), reported that *Chlorella* sp. reduced 99% of *Enterococci* (Log reduction: 2.6), and 98% of *E. coli* (Log reduction: 2.2) in domestic wastewater at 25 days of cultures. However, there are needed future studies to validate the hypothesis in which bacterial removal can be enhanced if the culture were maintained extending the time of cultivation, as it was observed in this study (Michelon and Viancelli, 2023).

Therefore, the evidence of high correlation between microalgae cell growth and the decrease in enterobacteria load was substantial. In the SW1-SW4 experiments high correlation was observed [SW1 ($r = -0.907$, $p = 0.000$), SW2 ($r = -0.877$, $p = 0.000$), SW3 ($r = -0.977$, $p = 0.000$), and SW4 ($r = -0.941$, $p = 0.000$)]. Same case was elucidated in D1-D4 experiments [D1 ($r = -0.992$, $p = 0.000$), D2 ($r = -0.952$, $p = 0.000$), D3 ($r = -0.801$, $p = 0.000$), and D4 ($r = -0.986$, $p = 0.000$)]. These correlations were notable also in the measurements of total coliforms. Additionally, the correlation between the bacterial load and the lipids production by the microalgae denoted that the presence of high concentration of these hydrophobic compounds had an effect in the inhibition of the bacterial growth. A higher correlation on swine and domestic wastewater treatments was observed[SW1 ($r = -0.853$, $p = 0.003$), SW3 ($r = -0.688$, $p = 0.041$), SW4 ($r = -0.998$, $p = 0.000$), D1 ($r = -0.995$, $p = 0.000$), D2 ($r = -0.922$, $p = 0.000$), D3 ($r = -0.995$, $p = 0.000$), and D4 ($r = -0.800$, $p = 0.010$)].

Previously, it was shown that higher pH values could influence the viability of bacteria (Liu et al., 2020). Therefore, to validate this correlation, the pH values were monitored in the culture medium during all experiments. Finally, as it was expected a higher correlation between the treatments and total coliforms load was observed[SW1 ($r = -0.979$, $p = 0.000$), SW2 ($r = -0.989$, $p = 0.000$), SW3 ($r = -0.954$, $p = 0.000$), SW4 ($r = -0.874$, $p = 0.000$), D1 ($r = -0.784$, $p = 0.001$), D2 ($r = -0.841$, $p = 0.000$), D3

($r = -0.909$, $p = 0.000$), and D4 ($r = -0.796$, $p = 0.000$)]. Similarly, it was analyzed in the experiments based on enterobacteria control [SW1 ($r = -0.991$, $p = 0.000$), SW2 ($r = -0.986$, $p = 0.000$), SW3 ($r = -0.964$, $p = 0.000$), SW4 ($r = -0.896$, $p = 0.000$), D1 ($r = -0.721$, $p = 0.002$), D2 ($r = -0.826$, $p = 0.000$), D3 ($r = -0.840$, $p = 0.000$), and D4 ($r = -0.747$, $p = 0.001$)]. Based on the correlation results, it is clear that microalgae growth, lipid concentration and pH have a synergistic effect on the bacterial control as a multifactorial process. The interaction between these variables make sense, because high values of pH induced stress in the microalgae culture leading to high lipid productivity and at the end the accumulated fatty acids produce the substantial bacterial load reduction.

3.3 Antibiotic resistant bacteria determination in swine and domestic wastewater under phycoremediation treatment

The resistance of bacteria against antibiotics occurs because of the ability of the bacteria to defend against the compounds designed to control them. This problem has become of great relevance throughout the world in recent years (CDC, 2022). Discovery of new drugs and the development of strategies to reduce the tolerance of these organisms to antibiotics has become a global priority (Metz and Shlaes, 2014). The effectiveness of wastewater treatment in reducing the exposure of the environment and organisms and human beings to these bacterial strains is declared an urgent call for action by all countries.

Ampicillin is known as a β -lactam antibiotic which is commonly used to control infections caused by gram-positive bacteria (Castle, 2007). In the present study, ampicillin-resistant bacteria showed critical values in domestic wastewater [$(3.5 \pm 0.85) \times 10^4$] than in swine wastewater [$(2.97 \pm 0.46) \times 10^4$] (**Figure S1**). Phycoremediation has been implemented to reduce ampicillin-resistant bacteria in wastewater. After the microalgae treatment, swine wastewater showed a diminishing ampicillin-resistant bacteria concentration (CFU mL⁻¹). For example in SW4, it was from $(2.27 \pm 0.8) \times 10^4$ to $(4.63 \pm 0.51) \times 10^3$ (**Figure S1a**). Additionally, in domestic

wastewater experiments, the same fact was observed, decreasing values in ampicillin-resistant bacteria concentration (CFU mL⁻¹) from $(3.53 \pm 0.85) \times 10^4$ to $(3.1 \pm 0.36) \times 10^3$ in D4 treatment (**Figure S1b**), and the D1-D3 treatments, it reached at least 1 log reduction. Our results were compared with literature, and similar effects were noted in a study reported by Cheng et. al, they detected a reduction of ampicillin-resistant bacteria in algal bioremediation systems of municipal wastewater (4 log reduction) using *Galdieria sulphuraria* (Cheng et al., 2020).

Tetracycline is an antibiotic used to treat gram-positive and gram-negative bacteria infections such as *Listeria monocytogenes* (Scholar, 2007a). In this study, higher values of tetracycline-resistant bacteria were found in swine wastewater [$(1.3 \pm 0.15) \times 10^5$] compared with domestic wastewater samples [$(5 \pm 1.6) \times 10^3$] (**Figure S1**). It could be related to the fact that tetracycline is the most widely employed antibiotic in pig industry and tetracycline-resistance genes are commonly observed in pig microbiome (Lekagul et al., 2019).

In swine wastewater experiments, a reduction on tetracycline-resistant bacteria concentration (CFU mL⁻¹) was observed. The values ranged from $(1.4 \pm 0.1) \times 10^5$ to $(4.3 \pm 0.57) \times 10^3$ in SW4 treatment (**Figure S1c**). Similarly, the treatments SW1-SW3, showed considerable reduction on the concentration, the values monitored were 5.8×10^4 , 9.7×10^4 , and 9.8×10^4 , respectively. Similar behavior was observed in domestic wastewater experiments. Treatment D2, showed the highest removal from $(5.3 \pm 0.57) \times 10^3$ to $(6.3 \pm 2.08) \times 10^2$ (**Figure S1d**). Treatments D1 and D4, were impacted and reduced up to 3.9×10^3 and 2.5×10^3 , respectively. Previously, it was found that microalgae growth in wastewater could reduce tetracycline resistance genes (e.g., *tetO*, *tetW*, and *tetX*) presence in municipal wastewater after the 4th culture day, this finding supported the notable reductions on the tetracycline-resistant bacteria concentration here established (Inuwa et al., 2022).

Ciprofloxacin is used to treat diseases in humans (e.g., typhoid fever, urinary tract and chronic bacterial prostatitis) and animals such as pigs (Jilany Khan et al., 2015). Ciprofloxacin-resistant bacteria was found in a higher concentration in swine wastewater [$(9.6 \pm 0.57) \times 10^4$] compared with domestic wastewater [$(4.0 \pm 1.0) \times$

10³] (**Figure S2**). More than 80% of pig farms use ciprofloxacin to treat bacterial infections, and ciprofloxacin resistance bacteria occurrence has increased by up to 5% from 2015 to 2017 in the European Union (O'Neill et al., 2020).

The experiments performed using swine wastewater (SW4), showed a higher decrease in ciprofloxacin-resistant bacteria load (CFU mL⁻¹) from $(9.67 \pm 0.57) \times 10^4$ to $(1.07 \pm 0.11) \times 10^3$ (**Figure S2a**). Also, other treatments (SW1, SW2, and SW3) showed a reduction in ciprofloxacin-resistant bacteria load. In domestic wastewater experiments, also it can be shown a reduction in ciprofloxacin-resistant bacteria concentration (CFU mL⁻¹) from $(3.33 \pm 0.57) \times 10^3$ to $(4.0 \pm 1.0) \times 10^2$ in D2 treatment (**Figure S2b**). In the other treatments such as D1, it reached up to 1 log reduction in the 10 days of cultivation.

Ceftriaxone is one of the most used antibiotics because it has a wide range of action; thus, it can control different bacteria while having a low toxicity risk. This antibiotic is used in different bacterial infections such as abdominal and skin infections, pneumonia, among others (Ayele et al., 2018). In this study, Ceftriaxone-resistant bacteria was found in a higher concentration in swine wastewater [$(3.4 \pm 0.11) \times 10^4$] than in domestic wastewater [$(1.0 \pm 0.11) \times 10^4$] (**Figure S2**). Swine wastewater has a greater number of bacteria resistant to different antibiotics, having bacteria multidrug-resistant to three or more antibiotics (Yang et al., 2021).

The studies of swine wastewater showed a higher decrease in ceftriaxone-resistant bacteria load (CFU mL⁻¹) from $(2.63 \pm 0.15) \times 10^4$ to $(2.3 \pm 0.28) \times 10^3$ in SW2 treatment (**Figure S2e**). SW1, SW3, and SW4 were analyzed and same behavior was observed regarding the removal of ceftriaxone-resistant bacteria of 8.1×10^3 , 1.2×10^4 , and 9.6×10^3 , respectively. Domestic wastewater experiments, particularly D4 treatment showed the higher ceftriaxone-resistant bacteria concentration removal [from $(1.0 \pm 0.1) \times 10^4$ to $(2.8 \pm 0.26) \times 10^3$] (**Figure S2f**). and in the other treatments such as D2, reached at least 1 log reduction from $(5.67 \pm 0.5) \times 10^3$ to $(4.67 \pm 0.57) \times 10^2$ at 10 days of cultivation.

Sulfamethoxazole is a derivate of sulfonamide commonly employed in urinary tract, and some systematic infections (Scholar, 2007b). Similar to other antibiotics, when sulfamethoxazole is administrated, it was not completely metabolized by organisms, then it ends up in waste, mainly in wastewater (Samrot et al., 2023). The monitoring of wastewater used in the present study showed significant load of sulfamethoxazole-resistant bacteria in swine wastewater $(1.48 \pm 0.02) \times 10^5$ compared load with domestic wastewater $(5.23 \pm 0.25) \times 10^4$ (**Figure 3**).

At the end of the phycoremediation treatment using swine wastewater, the load of sulfamethoxazole-resistant bacteria (CFU mL⁻¹) diminished from $(1.47 \pm 0.05) \times 10^5$ to $(5.3 \pm 0.57) \times 10^3$ in SW4 treatment (**Figure 3a**). A diminished value of sulfamethoxazole-resistant bacteria load was demonstrated in domestic wastewater experiments as well. D3 treatment reported the highest removal from $(4.9 \pm 0.15) \times 10^4$ to $(2.9 \pm 0.36) \times 10^3$ (**Figure 3b**). Similarly, D1, D2, and D4, experiments were able to reduce up to 4.2×10^4 , 4.3×10^4 and, 4.6×10^4 , respectively. This study made possible to compare the action of the microalgae in the removal of resistant bacteria.

Considering the results in section 3.3 in which resistant bacteria to ampicillin, tetracycline ciprofloxacin, ceftriaxone, and sulfamethoxazole found in domestic and swine wastewater, the most relevant finding was that bacteria resistant to sulfamethoxazole was particularly sensible to the microalgae treatments. It has been reported that phycoremediation of municipal wastewater could enhance the reduction of sulfonamides resistance genes (e.g., *sul1*, *sul2*) after the 16th culture day and other studies demonstrated a slight reduction in *sul2* presence after the 4th culture (Inuwa et al., 2022). Moreover, other investigations on phycoremediation validated the lower values of CFU mL⁻¹ of ampicillin, sulfamethoxazole, ciprofloxacin, and tetracycline resistant bacteria in at least 1 log in *Galdieria sulphuraria* culture (Cheng et al., 2020) and collected from various sources of wastewater (domestic and primary production) (Suchandan et al., 2024).

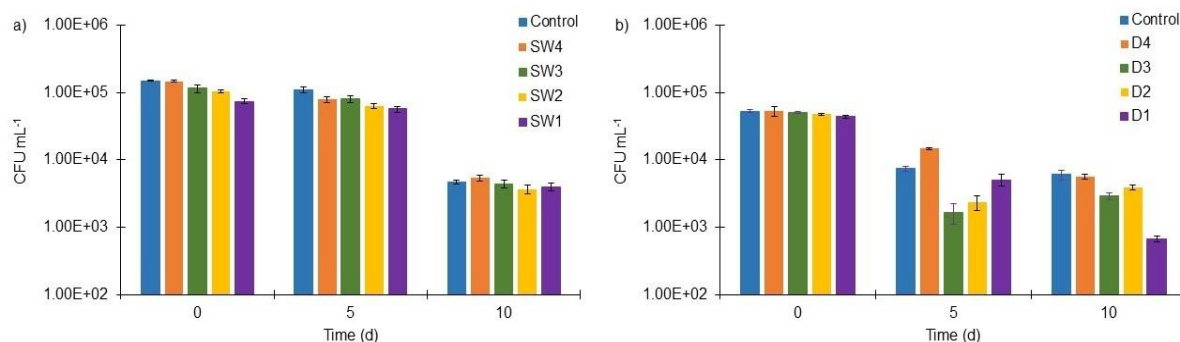


Figure 3. Sulfamethoxazole resistant bacteria (CFU mL⁻¹) determination in swine and domestic wastewater under phycoremediation treatment. **a)** Sulfamethoxazole-resistant bacteria determination in swine wastewater. **b)** Sulfamethoxazole-resistant bacteria determination in domestic wastewater. All points were sampled in triplicate. The samples were taken every five days of the experiment.

Section 3.2 discussed how physical changes like pH values can influence the bacteria removal, this can also occur with the antibiotic-resistant bacteria. Previous reports demonstrated that high pH values and the parameters of the aeration could be enhanced the photosynthetic activity of microalgae (Pompei et al., 2023). Other investigations in microalgae culture, expressed relevance to high pH and high dissolved oxygen (20 mg O₂ L⁻¹) values regarding with the viability of bacteria in at least the half-life of bacteria (Liu et al., 2020). Other factors reported in literature that can impact in the bacteria removal are temperature, in that way, warm temperatures (>20 °C) could increase pathogenic bacteria reduction (Liu et al., 2020) and photooxidation could enhance bacteria removal, due to the damage on cells and DNA (P. Kumar et al., 2022).

Therefore, giving support to the fact of the microalgae metabolism influences the inhibition of bacteria through the action of compounds or metabolites such fatty acids as was mentioned above. Ansa et al (2012) found that Chlorophyll *a* and its high concentration in algae (>13 mg L⁻¹) could enhance bacteria removal (Ansa et al., 2012). Other authors have already determined that microalgae grown in swine wastewater can produce a series of compounds in different concentrations (e.g. fatty acids, vitamins) that they do not produce regularly. Methyl linoleate, pinolenic acid, stearidonic acid, and linoleic acid altered the bacteria survival (Michelon et al., 2021). Section 3.2 showed the correlation that exists between cell growth, lipids production

and the reduction of total coliforms and enterobacteria, antibiotic resistant bacteria could be influenced by the same factors. Finally, there is a huge volume of literature in which extracellular microalgae compounds such as exopolysaccharides could enhance bacterial removal (Androutsopoulou and Makridis, 2023). Although exopolysaccharides were not analyzed in this study, their production could also be related to the reduction of antibiotic-resistant bacteria.

The results on the reduction of antibiotic-resistant bacteria support that microalgae treatment can influence the microbiological control, and this could represent a solid alternative for wastewater treatment plants, reducing chlorine use at the final stages of treatment. Furthermore, an ideal utilization and driving with the drawbacks of microalgae treatment systems in rural and urban areas can help to reduce the spread of these antibiotic-resistant bacteria, which are a global public health problem.

3.4 Future perspectives on phycoremediation as a tool to ensure the health and safe of urban and rural communities

Phycoremediation treatment could be implemented in urban and rural communities to control bacteria presence in wastewater. In urban areas, this could be incorporated into the actual wastewater treatments, after secondary treatment preferably. In the case of rural areas, this technology could be implemented both to treat domestic wastewater and wastewater from productive processes as it was shown in this study. Mostly, in these areas there is no type of liquid waste treatment installed, therefore, this technology can be an interesting option for these types of communities and reduce the risk of infection and translocation of pathogenic bacteria. Bacterial control by microalgae can uphold the reduction of chlorine use in wastewater treatment. This can be beneficial because some compounds in wastewater can become toxic through this practice (organochlorine compounds) (Parveen et al., 2022).

Some large-scale wastewater treatment projects have been established previously. In Spain, it was implemented a pilot plant to treat 11.7 m³ of agricultural wastewater. In that system, it was removed at least 90% of the phosphorus and nitrogen of wastewater (Rueda et al., 2020). In urban areas also some pilot plants were established, for example, a 1,600 L thin-layer reactor was established to treat urban wastewater. In this case, it was removed 90% of nitrogen and 80% of COD (Sánchez Zurano et al., 2020). These pilot projects lead us to consider the feasibility of using the phycoremediation process for large-scale wastewater treatment. Regarding public regulations, there are general regulations on the microbiological aspect such as the NOM-063-ECOL-1994 in Mexico (Secretaria de Gobernacion, 2025); however, most only establish the total coliform count that must be present at the system outlet. However, given the severity of the problem, new regulations must be implemented, although antibiotic-resistant bacteria are a topic that is only just beginning to be studied. Although establishing new regulations can be complicated for all sectors involved, the problem of antibiotic-resistant bacteria and its importance to public health requires the necessary effort.

It is important to note that the application of microalgae in wastewater treatment can reduce the contaminant load in wastewater. For example, microalgae culture could remove at least 29% of BOD and COD, and 83% of nitrates load in livestock wastewater (Blanco-Vieites et al., 2024). Phycoremediation can also remove emerging contaminants including antibiotics (Eheneden et al., 2023). For example, *Chlorella pyrenoidosa* could remove approximately 90% of tetracycline (initial concentration: 10 mg L⁻¹) (Tang et al., 2022). In this way, this technology not only reduces the contaminating load of antibiotic-resistant bacteria but also reduces the concentration of antibiotics present in the wastewater, therefore, this type of treatment can be very beneficial for the environment and public health.

In addition to wastewater treatment, the use of this technology has another important advantage for the environment and Sustainable Development Goals; carbon capture. It was determined before that per kilogram of microalgae biomass generated, 1.8 kg of carbon is captured (Adamczyk et al., 2016). In this way, the

implementation of this technology could help achieve carbon neutrality in rural communities and mainly in urban ones (López-Pacheco et al., 2021).

The microalgal biomass generated by this bioremediation process could be used to generate value products such as biofertilizers, biofuels, pigments, and bioplastics, among others (Arora et al., 2021). However, it is necessary to evaluate the possible bioaccumulation of certain toxic compounds that may have remained in the biomass because of wastewater treatment and the best processing route must be explored to avoid the translocation of these compounds. An overview of the phycoremediation process is shown in **Figure S3**.

The phycoremediation process could be very useful both in rural and urban areas, as well as in primary productive processes. In this way, it was estimated the possible production of biomass that could be generated considering that the wastewater from different areas will be treated through the phycoremediation process (**Figure S4**). It was estimated that in Mexico City Metropolitan Area (21 million of persons), it could be produced at least 167131 tons of microalgae biomass and 25069 tons of lipids at year. Although these results may not seem so profitable economically, the production of other valuable by-products that increase the income from the use of technology can be sought, in addition to contemplating other elements such as carbon capture, which under this scenario would be able to capture around 300836 tons of CO₂.

In the case of rural areas, it was estimated that using the wastewater of a population of 2500 people, at least 20 tons of biomass (3 tons of lipids) could be produced at year and 34 tons of CO₂ could be captured by the system. Phycoremediation of wastewater could be implemented also in livestock production systems like swine meat production. It was estimated that if pork production systems in Mexico could establish bioremediation of wastewater by microalgae systems at least 3.4 million tons of biomass (0.5 million tons of lipids) could be produced at year and 6.1 million tons of CO₂ could be captured in this process.

In this sense, it can be concluded that the installation of a microalgae wastewater treatment system can have great benefits such as the production of microalgae biomass as was discussed in this study, especially when comparing it with other systems, where it could not be obtained any economic benefit for maintenance and installation. By this way, in phycoremediation processes, obtaining products of low, medium or high value could be possible. Without counting the benefit to the environmental and public health value, as is the case of the reduction of antibiotic-resistant bacteria. However, to learn more about the viability and feasibility of establishing these systems in rural and urban areas, a techno-economic and life cycle assessments study must be carried out.

4. Conclusions

Phycoremediation treatment was proposed as an integral tool to reduce the microbiological load in domestic and swine wastewater. In the present study, the algae treatment reduced the total coliforms and Enterobacteria load in both wastewater sources. In swine wastewater, it was removed up to 98% of total coliforms $[(3.5) \times 10^5 \text{ CFU mL}^{-1}]$ and 99% of enterobacteria load $[(9.3) \times 10^4 \text{ CFU mL}^{-1}]$ in SW1; these removal rates were observed in the treatment with the highest biomass production. Similar results were observed in domestic wastewater, in which it was removed up to 99% of total coliforms $[(3.5) \times 10^5 \text{ CFU mL}^{-1}]$ and 97% of Enterobacteria $[(3.0) \times 10^5 \text{ CFU mL}^{-1}]$ in D4 experiment. Furthermore, removal of at least 1 log could be observed in the presence of antibiotic-resistant bacteria in all treatments with both samples of wastewater. The notable case was Sulfamethoxazole-resistant bacteria in domestic and swine wastewater, it was reduced $(4.6) \times 10^4 \text{ cell mL}^{-1}$ in D3 and $(1.4) \times 10^5 \text{ cell mL}^{-1}$ in SW4, respectively. There are several factors that could influence bacterial control such as pH, aeration, and algae metabolites production. The advantages of using microalgae as principal or secondary wastewater treatment, are focused on carbon capture, biomass production leading to the by-products generation and removal of contaminants, including the reduction of the chlorination. Therefore, algae treatment can revolute

wastewater management in rural and urban areas mitigating the environmental impacts and reducing the public health risk.

Credit authorship contributions statement

Itzel Y. Lopez-Pacheco: Conceptualization, methodology, formal analysis, investigation, data curation, investigation, writing—original draft preparation, writing—review and editing and visualization. **Georgia Maria Gonzalez-Meza:** methodology, data curation, and writing—review and editing. **Reyna Berenice Gonzalez-Gonzalez:** data curation, writing—review and editing. **Roberto Parra-Saldívar:** writing—review and editing, visualization, supervision, project administration, funding acquisition. **Elda M. Melchor-Martínez:** data curation, writing—review and editing, supervision, project administration, funding acquisition.

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Conflicts of Interest

The authors declare no conflict of interest.

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López-Pacheco, Itzel Y.; González-Meza, Georgia María; González-González, Reyna Berenice; Parra-Saldívar, Roberto; Melchor-Martínez, Elda M.

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