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Bacillus subtilis and *Pseudomona putida* in the removal of organic contaminants in wastewater from food industries

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ABSCTRACT

The purpose of the research was to evaluate the removal of organic contaminants in industrial wastewater from shrimp packing plants by means of a microbial consortium of *Pseudomonas putida* and *Bacillus subtilis*, the study was experimental, with three factors under investigation: application dose, application frequency, and contact time, the initial characterization of the wastewater was carried out according to the Standard Methods for the Examination of Water and Wastewater, the physicochemical parameters showed high concentrations (571 Pt-Co; 51 NTU; 653 mg/L BOD; 1305 mg/L COD; 12.5 mg/L PO_4; and 0.481 mg/L NO_2). The application dose was established based on flow and volume criteria. Bioaugmentation was carried out at three time intervals (24, 48, and 72 hours), with a total duration of 15 days, during which the indicators were monitored every five days. Additionally, the toxicity of the treated water was evaluated through the germination of Lactuca sativa seeds. A reduction in turbidity was observed, reaching 89.2% in T27, as well as in BOD_5 (87%) and COD (85.2%). In terms of nutrients, phosphate removal reached 39.5% and nitrites 28.3%. The analysis of variance confirmed that T27 showed a significant difference among the manipulated factors. The toxicity test revealed that after 15 days, germination levels ranged between 36% and 45%.

Keywords: microbial consortium, bioaugmentation, application dose, contact time, toxicity.

INTRODUCTION

Population growth and human development pose challenges to ecosystems, giving rise to problems such as the deterioration of water quality and metal pollution caused by industries dedicated to food production and processing (Guanoquiza and Antúnez, 2019). 70% of the world's freshwater consumption occurs within the food industry, which is one of the largest consumers of this resource (Bravo et al., 2021), since the amount of water needed for production is related to the type of product to be manufactured, the characteristics of the process, the size of the industry and the methods used in cleaning, such as shrimp packers (Vaibhav et al. al., 2022).

In Ecuador, these industries generate between 5 and 7 m^3 of wastewater for each ton of shrimp processed, as a result of activities such as washing, gutting, peeling, and sorting the product (Bailón and Sablón, 2022), which is notable given that the country is listed as one of the largest exporters worldwide with an annual production that exceeds one million tons (Martínez et al., 2022). This wastewater has pollutants such as organic matter, suspended solids, fats, oils, nitrogen, and phosphorus (Hannan et al., 2022), which, without adequate treatment, can affect receiving water bodies given that only 60% of shrimp packing plants in the country have wastewater treatment plants (Muyulema, 2018).

To address this problem, the use of microbial consortia composed of *Pseudomonas*, *Bacillus*, *Nitrosomonas* and *Nitrobacter* has been proposed as a treatment alternative (Bravo et al., 2020), thanks to their ability to adapt and efficiency to degrade different organic pollutants, optimizing the removal of organic matter, nitrogen and phosphorus, through biotransformation processes and bioaccumulation (Valverde, 2020). In addition, advances in synthetic biology have allowed the development of microorganisms that have greater resistance and biodegradation capacity (Rodríguez and Villegas, 2024).

Biological treatment allows the removal of a high range of contaminants and nutrients, which allows improving water quality, this growing market has boosted the production of microbial consortia (Bravo et al., 2020), reaching a decrease of 86% in phosphorus concentrations, 38–44% ammonium and 90–96% organic matter, among the most commonly used microorganisms for the removal of organic contaminants are bacteria such as Pseudomonas and Bacillus (Bazán and Chiclla, 2023).

This research arose due to the low efficiency of conventional treatment processes in the removal of organic contaminants, so the incorporation of different doses of a microbial consortium in wastewater samples obtained from a shrimp packing plant was evaluated, standing out as a practical and efficient technology in the removal of organic contaminants. contributing to the optimization of conventional treatment conditions. It is hypothesized that the application of microbial consortia in industrial wastewater from shrimp packers presents a significant removal of organic contaminants.

MATERIALS AND METHODS

The present research was carried out in a shrimp packing plant located in the province of Manabí, Sucre canton, kilometer 8 Bahía-Chone, with coordinates 566218 m S and 9927679 m E as indicated in Figure 1.

Type of research

An experimental study will be carried out, which according to Ramos (2021), this approach will allow the validation of the hypotheses raised through experimentation, which is distinguished by the manipulation of the variables, this scope will allow observing the effect of treatments (microbial consortia) under controlled conditions.

Application of the microbial consortium

The microbial consortia to be employed in the process of removing organic matter are composed of *Pseudomona putida*, whose metabolic versatility and genetic plasticity allow it to survive in a wide range of environments, many members of this family can degrade toxic compounds or efficiently produce high-value compounds and, therefore, are of interest both for bioremediation (Puchałka et al., 2008). On the other hand, *Bacillus subtilis* has been shown

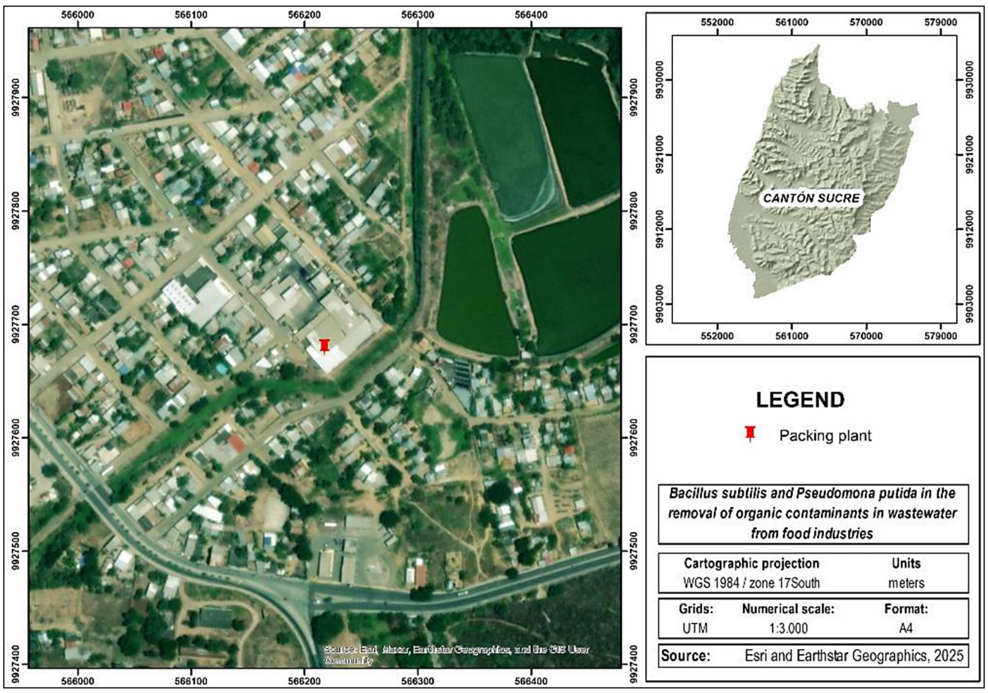

Figure 1. Packing house location map

to significantly decrease COD and BOD_5 values (Pant et al., 2023).

Determination of the optimal consortium dose

To establish the optimal dose of microbial consortium, parameters such as flow rate, initial application dose and maintenance dose based on the volume of water to be treated were considered, in order to ensure the ideal amount for the microorganisms and in turn the maintenance dose that acts as a biofilm where the microorganisms adhere to the surface forming communities and therefore the efficiency of the treatment (Ramesh, 2020), the following application information was taken into consideration for the calculation (Table 1).

Experimental design

In this research, a Completely Randomized Design (BAC) was used to evaluate the effect of 3 factors under study (Mayorga et al., 2022) (A: consortium dose, B: time in contact, C: frequency of application or bioaugmentation), with 27 treatments and 3 replications, as evidenced in Table 2.

Experiment management

The experimentation was carried out on a laboratory scale, in which a volume of 300 ml of wastewater from the shrimp packer was used, to which doses from the consortium were applied and the frequencies of application and contact time were considered, which are the factors under study in the research.

Taking the water sample

The methodology used was adapted to the characteristics of the place, the samples (20 L) were collected in the tributary, in a duly sterilized polyethylene container and were immediately transferred for the installation of the experiment, the sampling standard INEN 2176:2013 was applied for the collection of water samples (Instituto Nacional Ecuatoriano de Normalización [INEN], 2013).

Physicochemical characterization of industrial wastewater

To know the removal of contaminants, the initial and final characterization of industrial wastewater was carried out, the analysis of the parameters to be evaluated (Salgado et al., 2022), were carried out in the environmental chemistry laboratories of the ESPAM-MFL and the laboratory of the shrimp packing plant (Table 3), these analyses were worked according to the Standard methods for the examination of water and wastewater (American Public Health Association, 2020).

Batch system development

An anaerobic digester was developed in a closed system, where wastewater samples were treated in 400 ml amber bottles, this design allowed to evaluate the efficacy of the treatment in a specific period, for this the following materials and operating conditions were used, 300 ml amber bottles with cotton lids, in which the pH parameters (6.5–8) were monitored. Temperature (25 °C) and dissolved oxygen (Tejada et al., 2021), oxygen was not supplied since *Bacillus* is a genus of bacteria that can develop in facultative aerobic or

Table 1. Inoculation characteristics for digesters

Treatment with microbial consortia for digesters		
Flow	First application	Maintenance
30000 Litres	1.5 Litres	0.5 Litres

Table 2. Study factors and levels

Tukey's test	DCA		Levels		
	Treatments	Repetitions	Consortium dosage	Contact time	Application frequency
95%	27	3	A_0 = 15 µl	B_0 = 5 days	C_0 = 24 hours
			A_1 = 30 µl	B_1 = 10 days	C_1 = 48 hours
			A_2 = 45 µl	B_2 = 15 days	C_2 = 72 hours

Table 3. Parameters to be evaluated

Parameters	Units	Method
Chemical oxygen demand	mg/L	5220 D
Biological oxygen demand	mg/L	5210 B
Nitrites	mg/L	4500-NO_2^- B
Phosphates	mg/L	4500-P B.5
Turbidity	NTU	2130 B
Color	PCU	2120 B

anaerobic conditions or form resistant endospores that give it the ability to survive in adverse conditions (Villarreal et al., 2018), while *Pseudomona* it is considered an opportunistic pathogen, since this microorganism is highly versatile and has the ability to tolerate low oxygen conditions (Ochoa et al., 2013).

Removal efficiency

To quantify the removal efficiency of organic pollutants, the following formula proposed by Sperling et al. (2020) was applied to the treatments and their respective replications.

$$\%r = \frac{C_i - C_f}{C_i} \times 100 \tag{1}$$

where: $\%r$ – % removal, C_i – concentration initial, C_f – concentration final.

Establishment and management of the toxicity experiment

To evaluate the toxicity of the wastewater, the germination and growth of 20 lettuce seeds (*Lactuca sativa*) were calculated, this toxicological bioassay is an acute evaluation method (120 hours) designed to analyze the phytotoxic effects of compounds in the germination of seedlings, the parameter evaluated is the inhibition of germination, a stage sensitive to the action of toxic substances (Romero et al., 2021), for this purpose, 100 mm diameter Petri dishes with cotton were used, 25 ml of water corresponding to the treatments and 3 respective repetitions were added to each box, they were covered from exposure to light for 5 days at a controlled temperature of 22 ± 2 °C, they were watered according to the needs of the seedlings with 3 ml every 2 days considering evaporation, untreated wastewater was used as a target for this bioassay (Reynolds et al., 2024).

Relative germination rate (GR) assessment

After the time established for the exhibition, the number of germinated seeds was counted, to measure the germination rate of the seeds, taking into consideration the percentage of seeds that germinated in each of the pots with the equation (Hameed & Khorshid, 2021).

$$GR(\%) = \frac{Nº\ stw}{Nº\ ps} \times 100 \tag{2}$$

where: Nºstw – Nº germinated seeds from the treated water, Nºps – Nº planted seeds.

RESULTS AND DISCUSSION

Optimal dose set

The application of the list of doses proposed in Table 1 determined that for 300 ml of wastewater, 15 microliters and a booster dose of 3.7 µl are necessary, considering three application doses and a defined booster dose. The application of supplementary microorganisms or bioaugmentation allows to improve the degradation of pollutants in dynamic systems, since it contributes to biological stabilization under different environmental conditions, such as competition for nutrients, inadequate pH or unfavorable temperatures (Nzila et al., 2016), the use of bioaugmentation in the treatment of industrial wastewater has been highlighted. emphasizing the efficient decomposition of organic pollutants. The application of bacterial consortia between *Bacillus* and *Pseudomona* has complementary capabilities, since *Pseudomona* is efficient for the degradation of complex organic compounds, while *Bacillus* acts on heavy metals and also provides some stability to biological systems (Mora, 2016).

Initial water characterization

The physicochemical parameters evaluated to the wastewater of the shrimp packing plant are detailed in Table 4, the results were contrasted with the limits of the TULSMA regulations for discharge to the public sewer, it was evidenced that the color, BOD and COD of the water exceed the normative values, while the turbidity, phosphates and nitrites are within the permitted limit. A color greater than 400 Pt-Co is an indicator of the presence of various

Table 4. Initial characterization of industrial wastewater

Parameter	Value	Regulations	Units
Color	571	500	Pt-Co
Turbidity	51	100	NTU
BDO_5	653	250	mg/L
COD	1305	500	mg/L
Phosphates	12.5	15	mg/L
Nitrites	0.481	3	mg/L

chemical, polar and non-biodegradable compounds, among these are substances used in industries such as detergents, caustic agents, oils, latex and acids, these compounds are incompletely eliminated during treatment (Zaharia et al., 2024). This type of wastewater is loaded by suspended particles, usually oils and greases, the turbidity varies according to the processes involved in the industry, such as washing or handling organic products.

High concentrations of BOD_5 indicate a high biodegradable organic load, typical of food industries, this phenomenon is conditioned by the amount of waste generated, the temperature of the water, the mixture between contaminants and the presence of nutrients. COD concentrations in wastewater are indicators of pollutants such as ammonia, nitrites, and organic carbon, from biological waste generated in the processing of the product, and from other chemical treatments applied (Raj, 2009).

Phosphate concentrations above 10 mg/L are related to a high phosphorus content, which comes from processes that require the use of detergents and chemicals, as well as in discarded organic matter, proteins, and phospholipids (Kanu and Achi, 2011). Nitrite concentrations above 0.2 mg/L is an indication of an incomplete nitrification process or accumulation of intermediates, which may be due to a high ammonia load in the system (Suchat and Pattarawan, 2010).

Control parameters for the performance of microorganisms

Once the Batch system is installed, the behavior of the control parameters established in the technical data sheet of the microorganisms is presented, in the proposed treatments, during the 15 days that the trial lasted (Figure 2). It is denoted that the pH maintained an interval of 7–8 in the seven days and with an upward behavior in the following days, this parameter affects the enzymatic activity and solubility of many compounds, a pH outside an optimal range can inhibit biodegradation (Yáñez, 2018), dissolved oxygen was reduced from 2.5 to 0.2 mg/L, which facilitates the transformation of compounds that are resistant to degradation under aerobic conditions, such as halogens or ether bonds. Anaerobic bacteria play a crucial role in this process through reactions such as reducing deshalogenations (Ghattas et al., 2017), the temperature maintained a trend of 25 °C, a factor that affects both the structure of the bacterial community as well as its performance during treatment, since high temperatures influence feed consumption rates, that are not considered significant in terms of biokinetics for the process (LaPara et al., 2000; Ostos et al., 2019).

Removal of organic contaminants

Removal of contaminants in the different treatments (T1-T27) was achieved, turbidity in

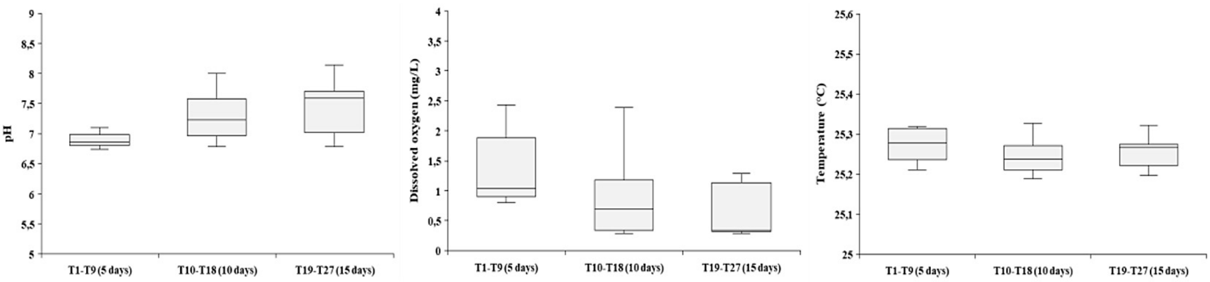

Figure 2. Parameters controlled during experimentation

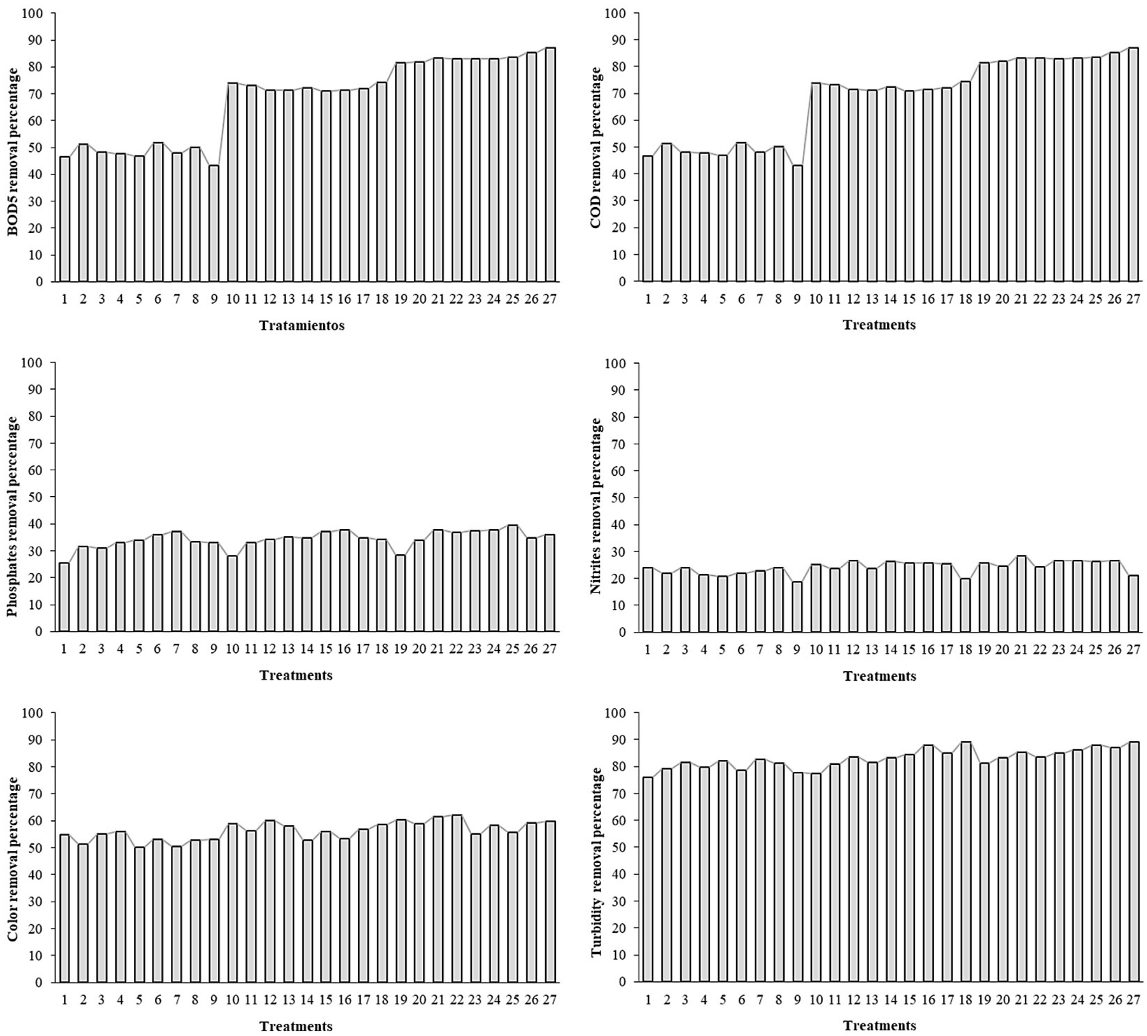

Figure 3. Percentage of removal of organic contaminants

89.2%, BOD 87% and COD 85.2%, evidencing an efficiency in the reduction of organic matter. Regarding nutrients, phosphate (39.5%) and nitrites (28.3%) present maximum removals in the final treatments (T19-T27), which indicates a progressive decrease in these contaminants (Figure 3).

The use of microalgae and native bacteria allowed a decrease in chemical oxygen demand of 36% in 24 hours and 71% in four days, likewise, in a period of seven days, BOD5, ammonium and phosphate were reduced by 80%, 64% and 95%, respectively (Jacome et al., 2021). The performance of a rotary contact biological reactor (RBC) in the treatment of effluent from a shrimp processing plant was evaluated, the results showed a high efficiency in the removal of organic matter, reaching COD removal percentages of 74.5%, 71.9%, 92.3%, 73.3% and 44.6% (Behling et al., 2008). In the tests obtained from the bioremediation of a shrimp farming effluent, it was obtained that treatment 2 was the one that showed the highest efficiency in the removal of organic compounds (99.67%) in 14 days (Navarrete et al., 2022), while Villota (2016) showed that certain species of *Bacillus* and *Pseudomonas* They have the ability to produce biopolymers with flocculant characteristics, which contribute to a reduction in water turbidity of 60.5% and 67% respectively.

Statistical analysis

The pollutant removal data were subjected to Kolmogorov-Smirnov normality tests, the results show that the data comply with a normal distribution ($p > 0.05$). In addition, Levene's test of homogeneity of variances indicated that there are no significant differences between the variances of the groups for all the parameters evaluated ($p > 0.05$), which confirms that the assumption of homogeneity of variances is fulfilled, thus allowing the use of parametric tests.

The table presents the effects of factors A, B and C, as well as their interactions, on the removal of organic contaminants, BOD, COD, phosphates, nitrites, color and turbidity. The results were evaluated according to the significance values (p), allowing the identification of statistically significant ($p < 0.05$) and non-significant interactions in the removal of each parameter. In a general context, the interactions between factors A, B and C showed p values less than 0.05 in several cases, indicating that the combinations of these factors had a statistically significant impact on the removal of certain contaminants. However, some specific interactions yielded high p values (≥ 0.05), suggesting that these combinations did not contribute significantly to the removal of specific contaminants (Table 5).

Factor A showed statistical significance in the removal of phosphates, color and turbidity, but did not have a significant effect on BOD, COD and nitrite parameters, factor B showed significance in the removal of all parameters except nitrites, factor C was significant in the removal of all parameters except for nitrites and turbidity, where the p-value was > 0.05, the A*B interaction showed significance in the BOD and COD parameters, while in the other parameters it was not significant, the A*C interaction was significant in all parameters, except in nitrites, the B*C interaction showed significance in BOD5 and COD, and finally the A*B*C interaction was significant only in BOD and COD.

Tukey's tests determined the treatments that differ from each other, identifying that treatment 27 ($A_2B_2C_2$) is the one that showed the highest mean for BOD_5 with 87.03%, regarding phosphates treatments 27 ($A_2B_2C_2$) and 25 ($A_2B_2C_0$) presented similar means (36% and 39.47%), in nitrites a significant removal was evidenced from treatments 21 ($A_0B_2C_2$) to 26 ($A_2B_2C_1$). with removal percentages from 26% to 28%, in the color parameter treatments 21 ($A_0B_2C_2$) and 22 ($A_1B_2C_0$) showed a removal of 59% and 62%, for turbidity removals of 85% and 89% were achieved from T21 ($A_0B_2C_2$) to T 27 ($A_2B_2C_2$) (Figure 4).

Three different concentrations of a microbial consortium for wastewater treatment were evaluated. Compared to the initial characterization (247.2 mg/L BOD_5), treatment 1 (3×10^8 CFU/mL) reduced the concentration to 199.1 mg/L, treatment 2 (9×10^8 CFU/mL) to 142.9 mg/L, and treatment 3 (1.8×10^9 CFU/mL) achieved the greatest reduction, reaching 132.1 mg/L. The latter proved to be the most effective of the three. The results showed significant differences ($p < 0.05$), with treatment 3 standing out as the most effective in BOD5 removal (Centeno et al., 2019). In this study, T1 and T2 reflected a progressive decrease in the concentration of organic matter that was measured as COD, the mean concentrations in removal were 924.1 to 709.2 mg/L for the control, from 858.0 to 556.3 mg/L for treatment 1 and from 884.4 to 313.1 mg/L for treatment 2. Tukey's test showed significant differences at 70 days of treatment (Díaz et al., 2017).

Nitrite concentrations were found in a range of 0.013-0.118 mg/L for the control, 0.011–0.044 mg/L for T1 and 0.013-0.069 mg/L for T2, evidencing significant differences compared to the control, according to Tukey's test, on the other hand, the average concentrations of orthophosphate during the trials were 6.93 ± 9.45 mg/L in the control, 4.05 ± 2.50 mg/L in T1 and 3.51 ± 2.92 mg/L in T2, showing a time-dependent behavior similar to that of total phosphorus, Tukey's test confirmed significant differences between treatments and control ($p < 0.05$) (Díaz et al., 2017).

Germination rate

The toxicity of the treatments was evaluated in which the wastewater used as a target presented

Table 5. Analysis of variance for the parameters evaluated

Origin	BOD_5	COD	Phosphates	Nitrites	Color	Turbidity
A	.137	.137	.000	.600	.044	.000
B	.000	.000	.000	.053	.000	.000
C	.004	.004	.033	.849	.018	.061
A * B	.000	.000	.820	.968	.862	.144
A * C	.017	.017	.000	.120	.001	.032
B * C	.002	.002	.933	.997	.951	.237
A * B * C	.000	.000	.894	1.000	1.000	.876

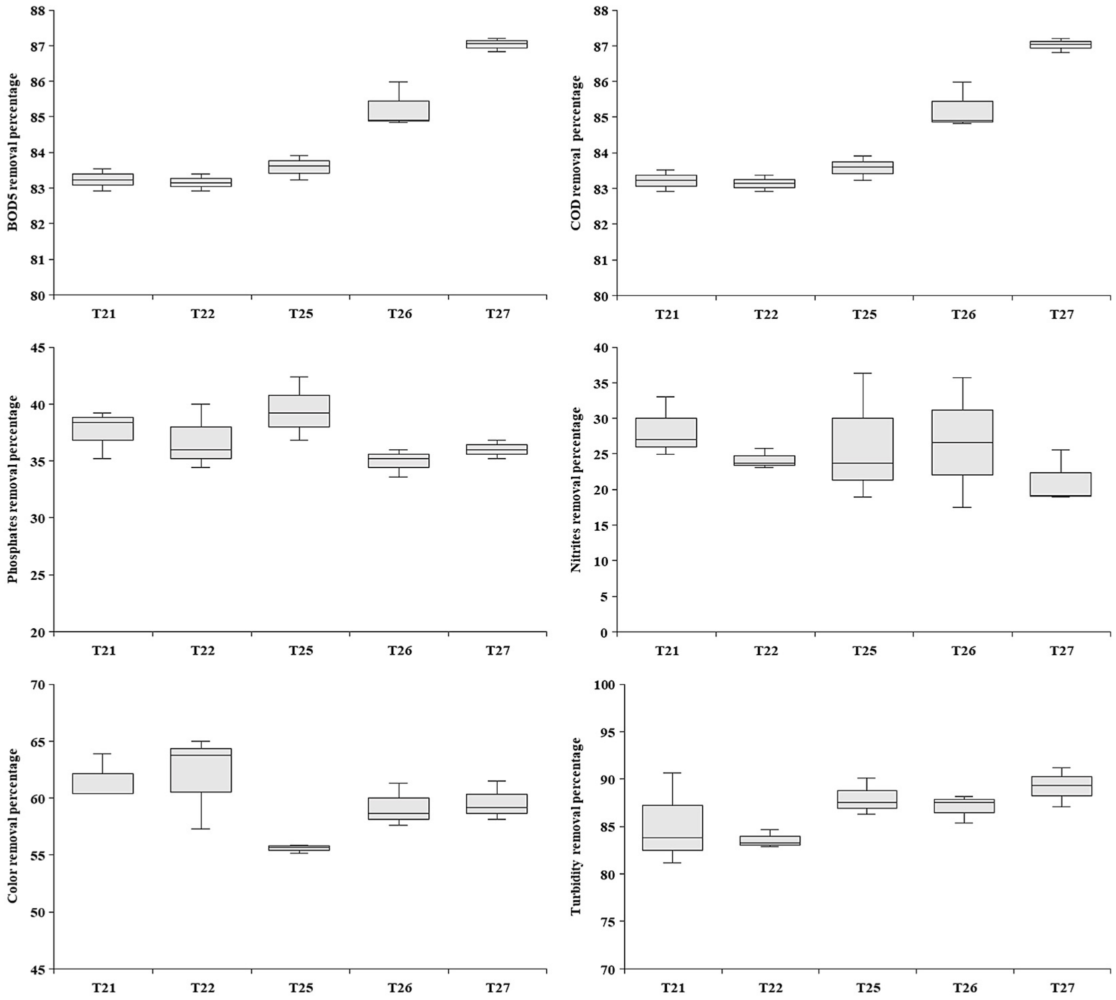

Figure 4. Average of the treatments with the highest removal of pollutants

0% germination, however the treatments applied managed to promote seed germination, showing a trend of increase in germination percentages over time: after 5 days, the treatments achieved between 17% and 22% germination; at 10 days, germination increased to a range of 21% to 29%; and, finally, after 15 days, germination levels between 36% and 45% were reached, these results suggest a progressive reduction in the toxicity of treated wastewater (Figure 5). A study carried out by Rodríguez et al. (2014) in which the toxicity of the water of the Chalma River with L. *sativa* seeds was evaluated, obtained an average germination of more than 70%, with the

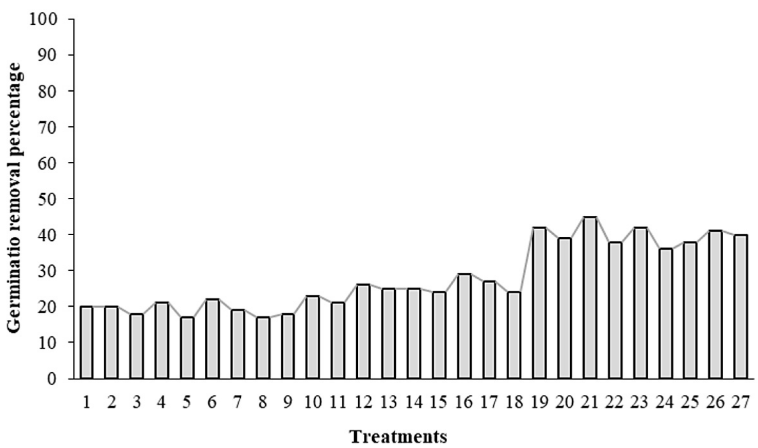

Figure 5. Percentage of seed germination

exception of point 4 where the lowest data was recorded (47.95%) where there was eutrophication of the water body, in the same way in the tests carried out in the effluent of the WWTP of the municipality of Chía by (Solano, 2007), an inhibition in germination was observed in 50% of the seeds, demonstrating that although *L. sativa* is not a representative species of aquatic ecosystems, the results of this toxicological test provide information on the impact of pollutants on plant communities located on the margins of water bodies (Sobrero and Ronco, 2022).

CONCLUSIONS

The highest removal percentages were reached at the end of the experiment (15 days), highlighting the Biochemical Oxygen Demand (87.03%) and the Chemical Oxygen Demand (87.02%), the turbidity and color parameters showed significant removals of 72% and 50%, respectively, from the fifth day of treatment, on the other hand, nitrites and phosphates presented removals of less than 40% throughout the experimental period.

The statistical analysis showed a significance ($p < 0.05$) in treatment 27 (consortium dose: 60 µl; bioaugmentation: 72 hours; contact time: 15 days) for the parameters BOD_5, COD, phosphates and turbidity, positioning it as the most efficient treatment. Finally, the evaluation of the germination rate reflected a decrease in the toxicity of the treated water, reaching an average germination of 40% after 15 days, which confirms the absence of toxic compounds generated by the biological treatment.

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