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Removal of Pathogenic Bacteria in a Horizontally Fed Subsurface Constructed Wetland Hybrid System

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Abstract: The management of effluents and their treatment is a fundamental issue in water management, the removal of different types of contaminants is another relevant issue for public health and the environment. Bacteria are one of the main types of contaminants in untreated water discharged to receiving bodies. The objective of this research was to evaluate the removal efficiency of pathogenic bacteria in a horizontal feeding subsurface artificial wetland that treats wastewater originated from the Boca de Río Technological Institute, Veracruz, Mexico. A hybrid system composed of seven cells with three types of substrates and ornamental type vegetation was designed; the indicators evaluated were the concentration of total and fecal coliforms and the efficiency of bacterial removal in the stages of the system. The artificial wetland system demonstrated a significant reduction ($p < 0.05$) between the different cells of the system. The values of pathogenic bacteria removal obtained in the wetland were higher than 99% in the cells of the system and times. In conclusion, it was identified that the interaction of the components of this system and its operation under the climatic seasons of the site influenced the removal efficiency of pathogenic bacteria, allowing optimal removal efficiency.

Keywords: residual water; effluents; constructed wetland; pathogenic bacteria; substrates

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1. Introduction

Water is a vital element for all forms of life on the planet, and the drinking water is a limited resource. However, the accelerated growth of the population and its inadequate management generates a greater demand for this resource to cover population and industrial needs worldwide [1,2].

The increase in the required volumes of the water resource destined for the different anthropogenic activities and its inadequate management has contributed to the increase in the generation of wastewater, which contain contaminants of various origins such as pathogenic bacteria, that are responsible for more than 90% of poisonings and waterborne diseases when this wastewater, are discharged into receiving bodies without any prior treatment [3,4].

The treatment of wastewater in Mexico represents a national problem, a percentage less than 40% of wastewater receives some type of treatment [5]. Wastewater is generally discharged in receiver bodies in an adequate manner, lacking any type of previous treatment or these are inefficient in the total removal of pollutants [3]. Therefore, there is a need to develop innovative technologies capable of being adapted to different geographical, cultural, social and political conditions of the country; in addition, the necessary tools and assistance should be incorporated to involve municipalities so that they can perform recovery and reuse of treated wastewater works [6].

The problem of the development of wastewater treatments is that they are self-sufficient, efficient, economically viable and in addition, provide solutions that manage to imitate natural processes, leading to the development of technologies such as constructed wetlands [2,7,8]. These are defined as systems in which the development of a culture of rooted macrophytes occurs on a waterproof gravel bed where through the action of these are possible a series of complex physical, chemical and biological interactions through which the water fluent residual is progressively and slowly treated [9]. Constructed wetlands have proven to be a viable alternative for the treatment of wastewater, since they can simulate the processes that occur in natural wetlands [2,10–12].

The diversity of currently existing constructed wetlands is wide from systems such as surface flow [9,13]; including those with subsurface horizontal flow [14–16]; vertical and the hybrid type, in which anaerobic microorganisms and emergent plants are used [17–19]. The variations have contributed to the fact that this technology allows reaching optimal percentages of removal of various contaminants and pathogenic microorganisms through the interaction of its components as the microorganisms, vegetation and substrates [2,9,20].

Hybrid wetland systems are considered as one of the most common and efficient systems in the designs of artificial wetlands, because they have reduced area requirements for the system, an increase in the efficiency of the treatment and removal of pollutants is generated; as well as, avoid obstructions in the system [13,17,19]. The implementation of variations in hybrid-type wetland systems allows the removal of pathogens such as total coliforms and *Escherichia coli*, as a calcareous substrate as the tezontle and ornamental type vegetation to treat wastewater produced in an academic institution [18]. Other substrate variations have included the used a calcareous substrate of marine shells as a support material for the treatment of municipal wastewater, which had a previous management of aerobic-anaerobic type [21].

The type of substrate and vegetation used in different types of wetlands has generated variations in water quality; in a horizontal subsurface flow wetland system with river gravel and volcanic stone substrates, and the *Phragmites australis* and *Typha latifolia* macrophytes, an improvement was obtained in the organoleptic characteristics of the residual water, pH and the removal percentages of BOD5 and COD greater than 90% [10]. In contrast, Solís-Silván et al. [2] used gravel as a support medium for a constructed wetland with surface flow with the macrophytes *T. domingensis* and *Eichhornia crassipes*, and one of a subsurface type with *Paspalum paniculatum* and *Cyperus articulatus*; they reported that the highest efficiency of pollutant removal from wastewater was obtained in the free-flowing wetland using *T. domingensis*.

The presence of different types of pathogenic microorganisms is considered as one of the main indicators of microbiological contamination in wastewater [22,23]. Although there are different species of these microorganisms, total and fecal coliforms are used as the main cause of bacterial contamination in the treatment of wastewater [11,12,24].

Therefore, it is considered that the indicators that provide the most appropriate information on the microbiological quality of the water are the bacteria removal index and the type of pathogens present in the water [15,18,22,25]. However, there are other factors that must be considered together given their influence on the removal of different contaminants; these include the climatic conditions of the site, the characteristics of the water to be treated and the design of the system [3,12,13]. The objective of this work was to evaluate the efficiency in the removal of pathogenic bacteria when using different substrates and ornamental type vegetation in a subsurface artificial wetland system with horizontal feeding, designed for the treatment of wastewater generated at the Technological Institute of Boca from the river.

2. Materials and Methods

2.1. Study Area

The artificial wetland system is located within the facilities of the Technological Institute of Boca del Río (ITBOCA) in the city of Boca del Río, Veracruz México. The study area has a direct influence with the Jamapa river basin located in the central region of the State of Veracruz, at coordinates 18°45′ and 19°13′ N, and 95°56′ y 97°16′ O; it originates in the Pico de Orizaba and finally discharges into the Gulf of Mexico [26–28]. The lower portion of the basin is located immediately to the discharge zone of this artificial system. The basin area receives the contribution of important effluents, but is also strongly impacted by municipal discharges from the cities of Boca del Río-Veracruz and Medellín [29–32].

2.2. Artificial Wetland Design

The artificial wetland system of subsurface flow with horizontal type feeding (HAFSSH), was designed in a surface area of 139 m^2, with a slope of 1.5%, feeding of 31.5 m^3/d and the hydraulic retention time (TRH) was 2–3 days. The HAFSSH system is made up of seven cells, most of which contain 0.6 m in height of various substrates and ornamental type vegetation (Table 1). The variations in the components in each cell have the objective of treating the wastewater generated from the activities in the institution where said system was installed.

Table 1. Distribution of the components in the HAFSSH cells.

	Sites	Substrates	Substrate Height	Ornamental Vegetation
STAGE 1	C_0	Tributary (initial feed)		
	C_1	Stony Calcareous	0.40 m 0.20 m	*Cannaindica*
	C_2	Stony Calcareous type 1 Calcareous type 2	0.30 m 0.10 m 0.20 m	*Alpinia purpurata*
	C_3	Stony Inert Stony	0.15 m 0.30 m 0.15 m	*Xanthosoma robustum, Heliconia psittacorum*
STAGE 2	C_4	Stony	0.60 m	*Cyperus papyrus, Equisetum arvense*
	C_5	Stony	0.40 m	*Pistia stratiotes*
	C_6	Stony	0.60 m	*Iris germanica, Spathiphyllum wallisii, Pennisetum purpureum, Crossandra, Ruellia brittoniana*
	C_7	Stony	0.60 m	*Cyperus papyrus, Ruellia brittoniana, Pennisetum purpureum, Amaranthus*

The implementation of the HAFSSH system were considered two stages, the first covered the initial feeding (C_0) to the discharge of the effluent from cell 3 (C_3). The distribution of the variations in the arrangement of the types of substrates and ornamental plants had the objective of demonstrating the efficiency of bacterial removal; furthermore, the base of the system has as a principle the architecture of the wetland built for the treatment of wastewater implemented by Amaya [33]. The first three cells had a combination of alternative substrates of stone, calcareous and inert types; furthermore, different types of ornamental vegetation were interspaced in these. the second stage corresponded to the tributary of C_4 to the effluent of C_7; the greatest diversity of ornamental vegetation was placed in these. while, in the last stage, the cells contain only a stone substrate as a means of support for vegetation and treated wastewater was obtained as a product.

The HAFSSH was fed through a 2500 L capacity tank; it had a location adjacent to the system that allowed the pumping and storage of wastewater originating from the activities of the institute, such as: coffee shops, laboratories, offices, rainwater and sanitary. The mobilization of the wastewater, in the system was due to the gravity of the slope; the isometric design with the system specifications was carried out according to the specifications of Amaya [33].

The effluent obtained at the end of the system was stored in a tank with a capacity of 1100 L to be pumped to another tank with a capacity of 10,000 L. Meanwhile, the final discharge of the treated water generated in this system used the lower Jamapa river basin as a receiving body in the transition zone of the river and the marine zone.

2.3. Collection and Analysis of Water Samples

The collection of samples was carried out according to the climatic seasons in the municipality of Boca de Río, differentiating the rainy, northern winds and dry seasons. The collection period was from August 2019 to May 2020. Meanwhile, the sample collection sites had as their main criterion the distribution of the substrates in the different cells of the wetland, derived from this, the flow inputs and outputs were chosen, selecting a total of eight sampling sites.

The collection, transport and storage of the samples were carried out in accordance with the specifications of the standard methods for the analysis of water and wastewater from APHA-AWWA-WPCF [34] and the Mexican norm NMX-AA-042-SCFI-2015 [35]. 100 mL of sample were collected in sterile bags, these were transported in a cooler with a temperature below 4 °C to the Institute's Aquatic Resources and Research Laboratory (LIRA), following the specifications of the NMX-AA-102-SCFI-2006 [36] and NMX-AA-042-SCFI-2015 [35].

The analyzes of the samples were carried out immediately upon receipt at the LIRA laboratory, where quantitative analyzes were carried out to determine the concentration of pathogenic bacteria measured as total coliforms (UFC/100 mL) and fecal coliforms (NMP/100 mL); in accordance with what is described by the official Mexican standards NMX-AA-102-SCFI-2006 [36] and the Standard Methods for the Analysis of Water and Wastewater [34].

The detection and enumeration of coliform organisms, thermotolerant coliform organisms and *Escherichia coli*, was carried out by filtration through a cellulosic membrane, a subsequent culture in a differential lactose medium and calculation from numbers of colonies. The filtrate considered a volume of 10 mL through a 0.45 µm cellulose membrane; This membrane was then placed in m-Endo Les Agar and subsequently incubated for 24 h at 35–37 °C [NMX-AA-102-SCFI-2006 [36]. The number of colonies with a golden appearance that grew in the culture medium was counted, expressed as colony-forming units per 100 mL (UFC/100 mL) in Equations (1) and (2), where:

$$\frac{\text{Coliform colonies (total)}}{100\ \text{mL}} = \frac{\text{Coliform colonies counted} \times \text{Reference volume}}{\text{mL of filtered sample}} \quad (1)$$

$$\frac{\text{UFC}}{100} = \frac{\text{Coliform colonies counted} \times 100}{\text{mL of filtered sample}} \quad (2)$$

The determination of the concentration of fecal coliforms was carried out using as a reference the NMX-AA-042-SCFI-2015 [35]; by culturing in a liquid medium contained in multiple tubes and calculating their most probable number in wastewater and treated wastewater samples. Dilutions were made to carry out the inoculation of 1 mL in a series of test tubes with sodium lauryl sulfate broth medium, these tubes were incubated for 24 and 48 h at 35 ± 0.5 °C. After the culture period, the tubes that presented turbidity and gas production were reseeded in a more selective confirmatory medium (EC broth), and incubated for 24 h at 44.5 ± 0.2 °C. At the end of the 24-h period, the sowing was carried out in EC broth, the tubes were examined and those that showed turbidity and gas production were recorded as positive with the presence of thermotolerant microorganisms and *E. coli*.

The calculation of the "most probable number (MPN)" of coliform organisms, thermotolerant coliform organisms and *E. coli* were expressed as contained in 100 mL of the sample from the number of positive tubes in the confirmatory results, using the following Equation (3).

$$\text{MPN}/100\ \text{mL} = \frac{\text{Number of positive tubes} \times 100}{\sqrt{\text{mL of sample from negative tubes} \times \text{mL of sample in all tubes}}} \quad (3)$$

Total coliforms were determined in all component sites of the system; while fecal coliforms were only determined in four sites, which were: the initial feeding (C_0), cell 3 effluent (C_3) effluent from cell 7 (C_7) and treated wastewater.

2.4. Removal Efficiency Calculation

The evaluation of the removal of pathogenic bacteria was based on the equation proposed by García et al. [37]; This considers as a base the values belonging to the concentration of total and fecal coliforms corresponding. Where: ERP = Pathogen removal efficiency in the system in%; C_e = Bacterial concentration in the effluent; C_0 = Bacterial concentration in the influent (4).

$$\text{ERP} = \frac{C_0 - C_e}{C_0} \times 100 \quad (4)$$

2.5. Statistical Análisis

A statistical analysis was performed with TIBCO Statistica 14.0.0.15 software (TIBCO Software Inc., Palo Alto, CA, USA). The data were transformed to the natural logarithm of the bacterial concentration. Comparisons of the wetland components were carried out, which included factors such as the system sites, the seasons of the year and their effect on the total and fecal coliform concentrations in the components of each cell and stage of the process. The data were analyzed using the levene test to verify the normality adjustment and the homogeneity of variance of the groups from the value obtained from p. The data showed to have a non-normal distribution ($p > 0.05$) with a 95% confidence. Therefore, an analysis of variance for non-parametric data was performed using the Kruskal-Wallis test [38,39]; to determine significant differences existing between the bacterial concentration of CT and CF, between cells of the wetland system and sampling times.

3. Results

3.1. Concentration of Total Bacteria by Site and Time of Year

The average concentration of total coliforms in each of the sites of the system presented the minimum concentrations in the effluent of sites C_3 and C_7 located in each stage. The highest concentration occurred in the initial feeding (C_0); Table 2. Likewise, significant statistical differences ($p < 0.05$) were detected between the sites of the system in relation to the average concentration of total coliforms, particularly these were detectable in the sites C_0 and C_1 that corresponded to stage 1 with respect to site C_7 in stage 2 of the system (Figures 1 and 2).

Table 2. Average concentration (Mean ± SD) of total coliforms (UFC/100 mL) in the cells of the HAFSSH system.

STAGE 1 [UFC/100 mL]			
C_0	**C_1**	**C_2**	**C_3**
876,667 ± 1,369,535 ^{a}	520,833 ± 836,352 ^{a}	29,167 ± 51,031 ^{a,b}	5833 ± 12,007 ^{a,b}
STAGE 2 [UFC/100 mL]			
C_4	**C_5**	**C_6**	**C_7**
17,500 ± 26,786 ^{a,b}	25,833 ± 34,988 ^{a,b}	70,000 ± 119,038 ^{a,b}	833 ± 2041 ^{b}

Different letters express statistically significant differences ($p < 0.05$), with a confidence level of 95%.

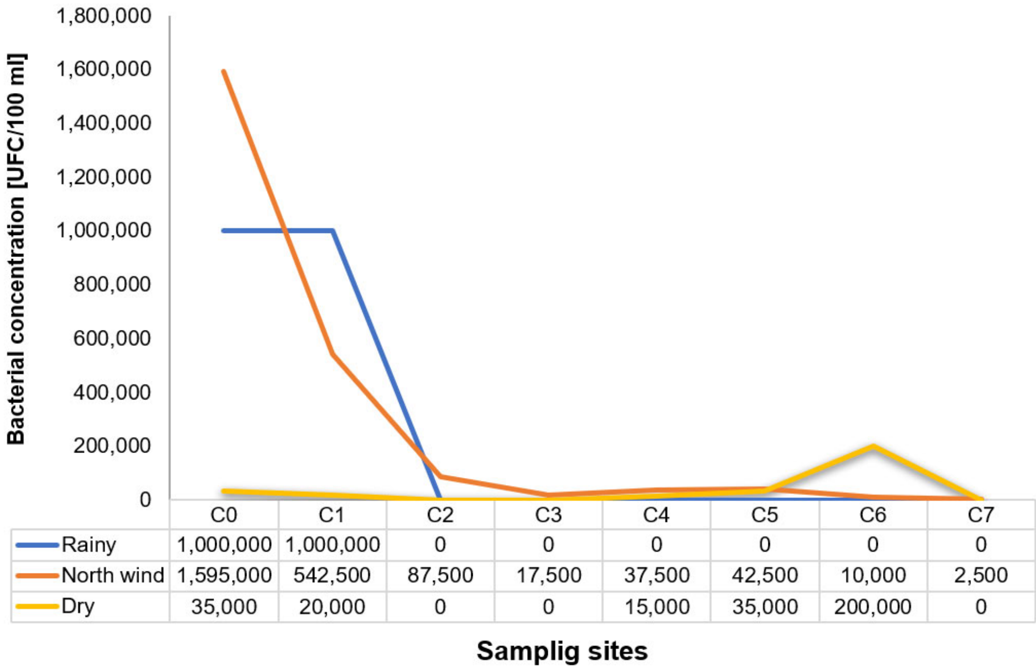

Figure 1. Concentration of total coliforms in cell water and times of the year in the HAFSSH system.

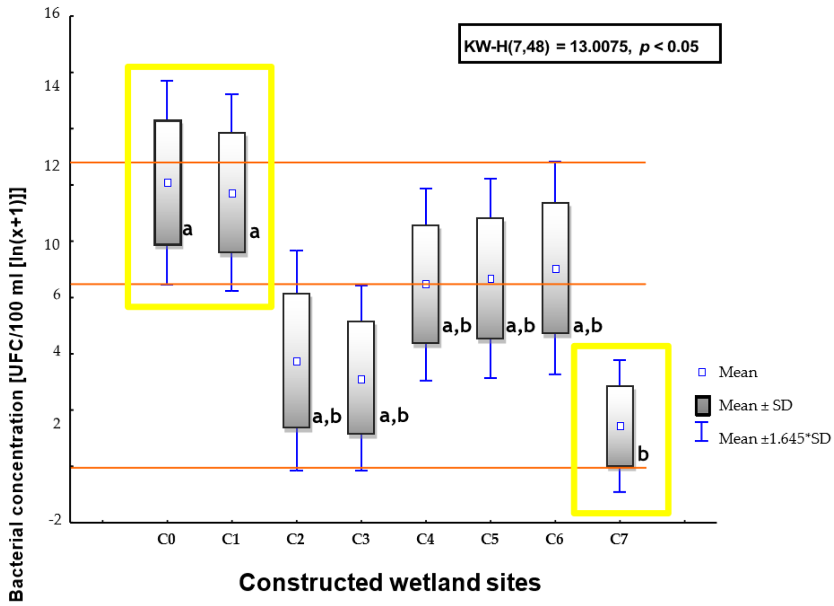

Figure 2. Bacterial concentration with HAFSSH sites. Values with different letters express data with statistically significant differences, with a confidence level of 95%.

The maximum concentration of total coliforms was recorded during the north-wind season at site C_0 with a value of 1,595,000 UFC/100 mL (Table 3). In contrast, the concentration of these pathogens had a value of 0 UFC/100 mL during the rainy seasons in different cells of the wetland system. During the dry season, this trend was only detected in sites C_2, C_3 and C_7 (Table 3). The average value of total coliforms in the entire system during the study had a value of 193,333 ± 609,415 UFC/100 mL. It was detected that there were statistically significant differences ($p < 0.05$) between the seasons of the year and the average concentration of total coliforms (Figure 3).

Table 3. Concentration (Mean ± SD) of total coliforms (UFC/100 mL) in the different HAFSSH sites and season.

	Sites	Rainy Season	Coliforms [UFC/100 mL] North-Wind Season	Dry Season
Stage 1	C_0	1,000,000 ± 1,414,214	1,595,000 ± 2,213,244	35,000 ± 21,213
	C_1	1,000,000 ± 1,414,214	542,500 ± 731,856	20,000 ± 0
	C_2	0 ± 0	87,500 ± 53,033	0 ± 0
	C_3	0 ± 0	17,500 ± 17,678	0 ± 0
Stage 2	C_4	0 ± 0	37,500 ± 45,962	15,000 ± 7071
	C_5	0 ± 0	42,500 ± 53,033	35,000 ± 35,355
	C_6	0 ± 0	10,000 ± 7071	200,000 ± 141,421
	C_7	0 ± 0	2500 ± 3536	0 ± 0

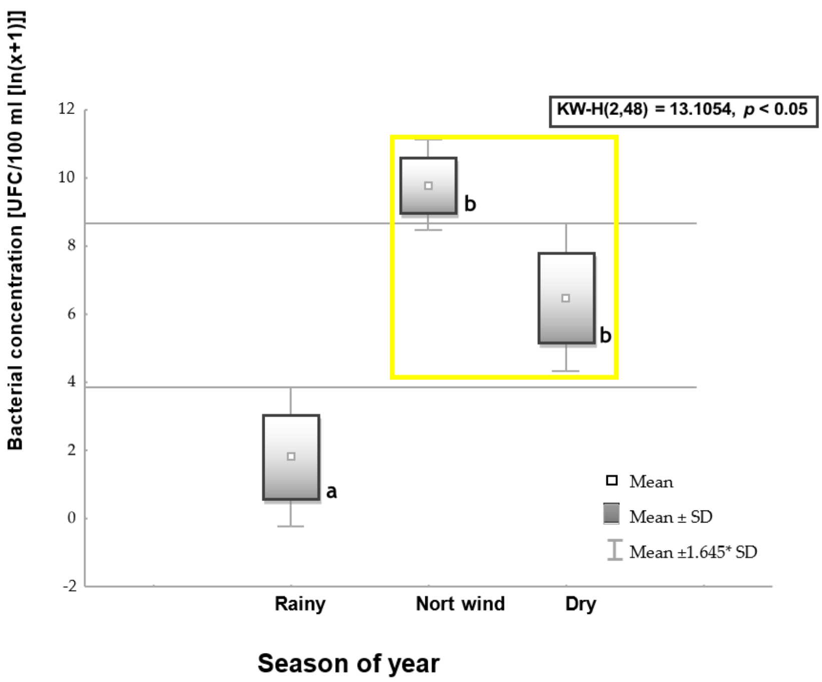

Figure 3. Concentration of total coliforms at different seasons in the wetland system. Values with different letters express data with statistically significant differences, with a confidence level of 95%.

The average bacterial concentration in the north-wind season of 291,875 UFC/100 mL, with maximum and minimum values of 1,595,000 and 2500 UFC/100 mL at C_0 and C_6 sites, respectively. The maximum and minimum concentrations obtained in the rainy season were 1,000,000 and 0 UFC/100 mL, respectively, with a total average of 250,000 CFU/100 mL for this season. Finally, in the dry season, the maximum and minimum values were 200,000 CFU/100 mL at site C_6 and 0 CFU/100 mL at C_2, C_3 and C_7; meanwhile, the total average at this time was 38,125 CFU/100 mL (Table 3). This season was the one that presented the greatest variations in bacterial concentrations in the different components of the system.

3.2. Concentration of Fecal Bacteria by Site and Season

The average concentration of fecal coliforms had a maximum value of 1,118,050 ± 1,924,138 at site C_0, followed by C_3 with 1881 ± 2172 (Table 4); meanwhile, the minimum concentration was 724 ± 649 (MPN/100 mL). There were significant statistical differences ($p < 0.05$) in relation to the concentration of fecal coliforms in the three sites analyzed in the system (Figure 4). However, no significant statistical differences ($p > 0.05$) were detected in the concentration of fecal coliforms during the seasons (Table 5).

Table 4. Concentration (Mean ± SD) of fecal coliforms at sites of the HAFSSH.

C_0	C_3	C_7
[MPN/100 mL]		
1,118,050 ± 1,924,138 [a]	1881 ± 2172 [b]	724 ± 649 [b]

Different letters express statistically significant differences ($p < 0.05$), with a confidence level of 95%.

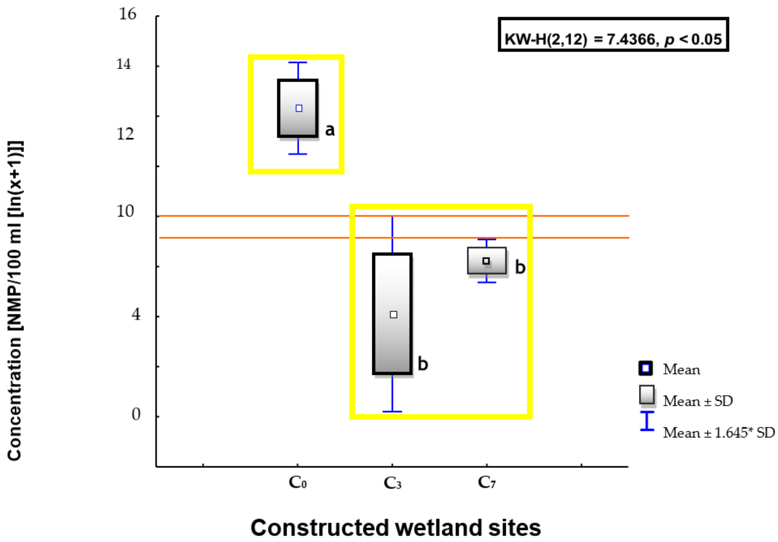

Figure 4. Average concentration of fecal coliforms at the sites of the HAFSSH system. Values with different letters express data with statistically significant differences, with a confidence level of 95%.

Table 5. Concentration (Mean ± SD) of fecal coliforms ([NMP/100 mL) at the sites and times in the HAFSSH system.

		Fecal Coliforms [MPN/100 mL]		
Stage	**Site**	**Rainy Season**	**North-Wind Season**	**Dry Season**
E1	C_0	15,000 ± 0	2,196,170 ± 2,541,153	39,931 ± 9971
	C_3	0 ± 0	3762 ± 0	0 ± 0
E2	C_7	0 ± 0	1137 ± 717	312 ± 261

The maximum concentration of fecal coliforms detected in the system was 2,196,170 NMP/100 mL at site C_0 during the north-wind season, while the minimum values were obtained in the dry season with 312 ± 261 and 0 MPN/100 mL at sites C_7 and C_3, respectively (Table 5). The total average concentration of fecal coliforms during the north-wind season was 733,690 MPN/100 mL and a concentration of 2,196,170 and a minumum of 1137 MPN/100 mL at sites C_0 and C_7, respectively (Table 4). While, in the dry season the total average concentration was 13,414, the maximum value in that season was obtained at site C_0 with 39,931 MPN/100 mL.

3.3. Removal Efficiency (ER) of Pathogenic Bacteria of the HAFSSH System

The ratio of the effluent from cell 3 and effluent from cell 7 was considered for the evaluation of the removal efficiency of pathogenic bacteria between Stages 1 and 2 of the system. The removal efficiency of total coliforms during the rainy and dry seasons reached percentages of 100% in both steps (Table 6). Meanwhile, during the north-wind season, the maximum removal efficiency was obtained at site C_3 with 98.90%.

Table 6. Removal efficiency (%) of total and fecal coliforms in the HAFSSH system.

Seasons	Rainy Season		North-Wind Season		Dry Season	
	CT	CF	CT	CF	CT	CF
ER C_3 (STAGE 1)	100%	100%	98.90%	99.95%	100%	99.22%
ER total (STAGE 2)	100%	100%	94.84%	99.95%	100%	99.22%

Abbreviations: CT = total coliforms; CF = Coliformes fecales; ER: Removal efficiency.

Regarding the percentage of removal with the indicator of fecal coliforms (FC), these were determined only in two seasons (Table 6). The percentages obtained from these bacteria were the same at site C_3 and in the effluent of the HAFSSH system during the north-wind season, a value of 99.95% was reported; likewise, in the dry season a value of 99.22% was obtained in both stages.

4. Discussion

4.1. Concentration of Pathogenic Bacteria (Total and Fecal Coliforms) in the System

The concentrations of total and fecal coliforms presented similar dynamics in the cells of the HAFSSH system; nevertheless, the main differences can be attributed to various factors that included environmental conditions and system components, among the main of. Furthermore, the configuration of the main parameters that directly influence the elimination rates of microorganisms in constructed wetlands include the composition of the treated wastewater and various operational parameters that include from the dimensions of the system, the hid regime raulico, the starting entry level, microbial biofilm, retention time, seasonal fluctuations and plant species in the system [40,41].

The bacterial concentration dynamics is influenced by various environmental factors that affect microbial growth, such as temperature, pH, nutrient availability, dissolved oxygen, among others [39,42,43]. Herrera-Melián et al. [16] indicated that the elimination of some bacteria such as *E. coli* occurs faster under aerobic conditions; and in the case of vertical sand filters, a reduction of oxygen transfer occurs in the vertical flow and an effect is generated on nitrification as it is a very sensitive process to oxygenation.

The significant decrease in the concentration of coliforms throughout the system used in this investigation may be the result of the complex dynamics among all the conditions and components of the system. In addition, the weather conditions which influence parameters such as light periods and increased winds during times of north winds and rain. The concentration of fecal coliforms is negatively affected by solar radiation in shallow ponds and positively influenced by the tributary in deep ponds; in addition, the concentration of these bacteria is negatively affected by the ph in almost all of the system analyzed [44]. The high evapotranspiration at the installation site of the wetland contributed to generate water stress in the vegetation that is part of the system and influences the capacity of reduction and elimination of pollutants from the treated water [45]. Likewise, the sensitivity of pathogenic microorganisms to ultraviolet radiation has been highlighted, so that when entering the system part of them die and another fraction of these enter the system being found in the residual water in solid fraction or suspended [43,46,47]. In addition, variations of parameters such as temperature is an important factor in the elimination of pathogens in a constructed wetland [48].

The C_4 and C_5 sites were the ones that stood out for an increase in the concentration of total coliforms, being maximum in the C_5 site, which could be associated with the conditions of this site that influenced the growth/reproduction of these microorganisms. The vast majority of pathogenic bacteria tend to die because the HAFSSH conditions are not ideal for their growth or survival, since they are also often preyed on by protozoa, nematodes and rotifers [46,47,49]. Meawhile, another part of pathogenic bacteria are removed through sedimentation mechanisms, filtration through the substrates and adsorption by the bacterial biofilm formed on the substrate of the system. Nevertheless, the dynamics of bacterial concentration is influenced by environmental factors, as written above.

The maturation of the system may have an influence on the bacteria concentration in the artificial wetland system; Calheiros et al. [40] analyzed the bacterial communities during a period of three consecutive years, reported that the structure and composition of these bacterials are influenced mainly by the year, more than by the time of year or the wetland zone.

The total coliform bacteria are not the most appropriate indicator organism to assess the degree of disinfection in systems that include natural treatment processes such as constructed wetlands [19]. Indicated that these microorganisms have the capacity to reproduce when the conditions are adequate; which confirms the importance of *E. coli* is a more recommended indicator organism to evaluate the efficiency of removal of pathogenic microorganisms [2,18,19]; therefore, the characteristics of these micro-organisms should be considered to maintain or increase their populations in constructed wetlands. Calheiros et al. [40] reported that although the efficiency of total coliform removal in wastewater did not vary significantly throughout the times; the concentrations of *E. coli*, significant differences were found between warm and cold times of the year.

The types of materials implemented in constructed wetlands may also have an effect on their efficiency and the environmental impact of their construction. Herrera-Melián et al. [16] indicated the clogging problems with the use of sand in some stages of a constructed wetland may generate differences in the removal according to the height between the different components of the system. Likewise, Zurita Martínez et al. [18] indicated that the wetland sites in which total coliforms occurred, presented adequate conditions for these micro-organisms to grow and reproduce; therefore, the design of the wetland system can be influenced to avoid those favorable conditions for pathogenic microorganisms.

The presence of floating vegetation provides different conditions in system, these include limiting the diffusion of oxygen and blocking the passage of solar radiation [30]. An increase in coliforms can be associated with these sites, as they are areas with greater outdoor exposure and are more susceptible to harboring pathogenic bacteria of environmental origin due to dispersion effects. Mosso et al. [49] indicated that the atmosphere is a medium for the dispersion of many types of microorganisms, that come from other environments, which can explain the differences obtained in this investigation during the north-wind season and that the maximum concentrations have been recorded then in relation to the other seasons.

Macrophytes have been suggested to possess several properties that make them an important component of constructed wetlands; these range from the physical type such as the stabilization of the surface of constructed wetlands and the prevention of clogging of the matrix [2,19]. In addition, ornamental-type vegetation, being successfully adapted to humid environments, provides oxygen-rich conditions thanks to its roots, this leads to a greater growth of various types of microorganisms such as protozoa, nematodes and rotifers capable of preying on pathogenic bacteria. It has been indicated that macrophytes provide adequate conditions for physical filtration in the wetland system by having a greater surface area for microbial growth; In addition, they contribute to the transfer of oxygen to the rhizosphere in a variable way [11,19,40].

4.2. HAFSSH Efficiency in the Removal of Pathogenic Bacteria

The removal efficiency results obtained in the present investigation had a similar behavior to those reported by in other investigations. The removal of these microorganisms depends on the removal mechanisms that the components of the system promote [41]. Calheiros [40] indicated that there are different bacterial communities associated with the type of substrate used in the aritificial wetland. The design of the system influences its removal capacity, since he reported that the removal of total and fecal coliforms is higher in the horizontal flow subsurface feeding wetlands in relation to those of vertical feed; in the first they usually reach removals of 88.1 to 92.6% and while in the others values of 65.1 and 85.6% were achieved [19].

Zurita-Martinez et al. [18], reported percentages of removal of microorganisms higher than 90% in a system of hybrid artificial wetlands with vertical and horizontal feeding made up of ornamental type vegetation and tezontle as a substrate medium. In contrast, Quintero-García et al. [11] reported lower removal values in a subsurface flow artificial wetland coupled with a fixed bed with *Chlorella* sp. microalgae and *Heliconia psittacorum* macrophyte, achieving a reduction of total and fecal coliforms of 87% and 88%, respectively. Also, Shukla et al. [50] reported in a horizontal underground flow constructed wetland with macrophytes *T. latifolia* and *Commelina benghalensis*, registered that the maximum elimination percentage obtained in the implemented system for total, fecal, and e. coli coliforms obtained values of 64, 61, and 52%, respectively. Although, these values may be considered minimum and indicated that the use of the wetland improved the quality of treated domestic wastewater.

The removal values obtained in this investigation had values of 99 and 100%, indicating that the combination of substrates and use of macrophytes contributes a higher percentage of elimination of pathogenic bacteria. Herrera-Melián et al. [16] reported a similar removal for *E. coli* and total coliforms in hybrid wetlands with horizontal groundflow with multiple stages, with a value of 99,998%. In contrast, Torres-Guerra et al. [51] reported that the effectiveness of the wetland system is 80% removal of microbiological parameters; however, they indicated that they identified variations in the removal efficiency of bacteria associated with the macrophyte species used, since *Phragmites australis* was 30% more efficient in the removal of total coliforms and thermotolerant coliforms compared to the *Cyperus papyrus* species.

The variations in the efficiency of removal of contaminants from wastewater associated with the macrophyte species, the types of wetland system, the design of the system, the sustrates used. Therefore, the reduction of the number of bacteria is a complex process. it is established by the interaction of physical, chemical and biological factors [19]. Plants have an active role in the bacterial pathogen purification mechanism in the wetland, since the number of total coliforms and *E. coli* associated with the roots of plant presented a higher concentration than in the substrate in the entry zone of the system [40].

The type of design has proven to be an important indicator of the variations reported in the efficiency of bacteria removal in different wetland systems. In the free-flow artificial wetland with the species *T. domingensis* presented the highest pollutant removal efficiency; that the subsurface flow wetland with the species *Paspalum australis* also presented high removal efficiencies, followed by the free flow type with *Eichhornia crassipes*. In contrast, they highlighted that the subsurface flow wetland in which *Cyperus articulatus* was used presented the lowest removal of the pollutants analyzed [2].

The pathogenic bacteria removal values indicated that the interaction of the components of the first three cells contributed to reach an optimal removal efficiency in the effluent in Stage 1 in relation to the concentration of pathogenic bacteria. The previous values were higher than those reported by Galindo et al. [21], since they obtained efficiencies in the removal of total and fecal coliforms of 97.24 and 94.63%, with the implementation of a biofilter with a calcareous substrate of sea shells as support material. In contrast, Torres-Guerra et al. [51] indicated that the efficiency of their wetland prototype was not completely effective in the removal of pathogenic bacteria and that another removal method was required, as a secondary treatment so that the wetlands fulfill the function of a tertiary treatment that allows obtaining removal of parameters within required limits.

4.3. Regulations on the Discharge of Effluent Water in Mexico

The quality of the effluents and their final discharge site are crucial issues to preserve the health of ecosystems. Coinciding with the above, Castañeda-Chávez and Lango-Reynoso [31] and Castañeda-Chávez et al. [52] indicated that there is a diversity of chemical and microbiological compounds that the Jamapa river basin receives; Likewise, they reported that these discharges come from different sources of contamination such as industry, waste, sewage, agricultural and urban runoff, and the accumulation of sediments.

Therefore, the reduction of pathogenic bacteria with the HAFSSH system represents a decrease in the contribution of microbiological contaminants, since, as indicated, the Jamapa River is the discharge site for many of the activities carried out. throughout this basin. According to the NOM -001-SEMARNAT-1996 [53], the maximum allowable limit for wastewater discharges to national waters and assets, as well as discharges to the soil (use in agricultural irrigation) has a value of 1000 and 2000 as the most probable number (MPN) of fecal coliforms per 100 mL. Therefore, the concentrations of fecal coliforms obtained in the effluent (C7) of the HAFSSH, and based on the reference values of the official Mexican standard, it is concluded that the data obtained are within the reference values of the norm (Table 7). The concentrations of fecal coliforms obtained in the effluent (C7) of the HAFSSH, and based on the reference values of the official Mexican standard, it is concluded that the data obtained are within the reference values of the norm (Table 7).

Table 7. Permissible limits of the concentration of fecal coliforms in the effluent with the NOM -001-SEMARNAT-1996.

Reference Value NOM-001-SEMARNAT-1996	Rainy	Season North-Wind	Dry
1000 a 2000 MPN/100 mL complies: Yes/No	0 MPN/100 mL Yes	1137 MPN/100 mL Yes	311.5 MPN/100 mL Yes

In accordance with the update of NOM-001-SEMARNAT-2021 [54], in which it establishes the permissible limits of contaminants in wastewater discharges in receiving bodies owned by the Nation; contamination by pathogens would be determined by the concentration of *Escherichia coli* in units of MPN/100 mL. Therefore, reference values of 1000, 1200, and 1400 MPN/100 mL were established as maximum permissible limits for the monthly average, daily average, and instantaneous value of *E. coli* concentration. According to the above, the MPN/100 mL concentrations obtained comply with the values established by NOM-001-SEMARNAT-2021 [54], because the results correspond to instantaneous values.

The efficiency in the removal of pathogenic bacteria during the monitoring period was greater than 95% in the different sites of the system. In accordance with the above, compliance with the microbiological quality of the water discharge from the wetland is carried out, since according to NOM-001-SEMARNAT-1996 [53] and NOM-001-SEMARNAT-2021 [54], the residual water treated in the HAFSSH system can be discharged in the vicinity of the Jamapa River without causing damage to aquatic life and public health. In contrast, Torres-Guerra et al. [51] indicated that although their wetland systems reduced parameters such as pathogenic bacteria by 80 to 89%, their results did not achieve compliance with the permissible limits established by the ECA for fecal coliforms with 1000 MPN/100 mL, for their use as irrigation water.

Constructed wetlands, although it has been shown that they have a positive effect on the removal of pollutants such as microbiological those; the source of the system components must be considered. The extraction and transport of gravel and sand have an environmental impact that can be reduced through the use of residual materials such as agroforestry waste [16]. In the case of this investigation, we helped to mitigate the effect of plastic pollution by carrying out the use of pet material from plastic bottles as an inert substrate; in addition, to contribute to provide a treatment to the waters discharged in receiver bodies such as the Jamapa river.

5. Conclusions

The components of the HAFSSH system demonstrated a marked influence on the efficiency of microorganisms removal. The results obtained in the removal of pathogenic bacteria when treating the wastewater in the system were the main indicator of the removal capacity.

By treating wastewater in the HAFSSH system, it contributes to reducing the contribution of pathogenic bacteria to the lower basin of the Jamapa River, and the environmental pressure generated by the discharge of untreated wastewater to the final stretch of this basin is reduced that discharges directly into the central area of the Gulf of Mexico.

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