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Tequila Vinasse Treatment in Two Types of Vertical Downflow Treatment Wetlands (with Emergent Vegetation and Ligninolytic Fungi)

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Abstract: The aim of this study was to evaluate and compare the efficiency of two types of vertical downflow wetlands (VDFWs) (with the presence of ligninolytic fungus *Trametes versicolor* and planted with *Iris sibirica*) for the treatment of tequila vinasses (TVs) as a secondary treatment; control systems with only a filter medium were also included. The systems operated with a 7-day run/resting mode of operation. Various water quality parameters were analyzed in both the influent and the effluents, namely total suspended solids (TSS), chemical oxygen demand (COD), biological oxygen demand (BOD_5), total organic carbon (TOC), pH, electrical conductivity (EC), true color and turbidity, total phosphorus (TP), total nitrogen (TN), etc. The two types of VDFWs as well as the control treatment were effective in reducing the different pollutants ($p < 0.05$); however, planted systems showed a tendency toward higher efficiencies. With an influent concentration of 49,000 mg L^{-1} and an organic loading rate of 4942 g COD $m^{-2}d^{-1}$, the COD reduction was around 40% in the planted systems, while in the other two, the reduction was 35%. Furthermore, TSS removals were 36, 20 and 16% in the VDFWs with vegetation, ligninolytic fungus and control systems, respectively. These results suggest that the fungus *Trametes versicolor* did not develop the desirable enzymatic expression for pollutant removal, probably as a result of the absence of aerobic conditions in the systems. Therefore, more research is needed to achieve a better fungal performance in VDFWs.

Keywords: *Trametes versicolor*; *Iris sibirica*; tequila vinasses; treatment wetlands; chemical oxygen demand

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1. Introduction

Tequila, a world-famous alcoholic beverage, is produced mainly in the state of Jalisco in western Mexico. In recent years, the production of this alcoholic beverage has grown impressively, increasing from 309.1 million liters in 2019 to 651 million liters in 2022 [1]. Tequila vinasses, generated during the production of tequila, are liquid residues that are difficult to treat due to the high concentration of different pollutants such as organic matter, total suspended solids and total dissolved solids, and features such as a dark reddish-brown color and an acidic pH, among others [2]. On average, 10–12 L of vinasses is produced per liter of tequila (up to 20 L) and high volumes are disposed of without any treatment or only cooled and neutralized [3,4]. Although large and medium-size factories, in general, provide a treatment for their vinasses, micro- and small factories argue that they do not have the economic resources to invest in treatment plants [4].

In this scenario, it is necessary to look for alternative treatment processes to conventional systems, which are characterized by high construction, operation and maintenance

costs. Treatment wetlands (TWs) could be an option, as they represent a technology considered an economical and environmentally friendly option for wastewater treatment [5]. TWs have been evaluated and used for the treatment of industrial effluents such as effluents from tanneries or the petrochemical industry. Specifically for the treatment of tequila vinasse, there are some studies that demonstrate their feasibility. Montoya et al. [2] used horizontal subsurface horizontal flow wetlands and vertical upflow wetlands to treat tequila vinasse diluted in different percentages with domestic wastewater. With 40% dilution, the authors obtained COD removal percentages of 95.4 and 95.8% and TSS removal of 91.8 and 95.9% with horizontal and vertical wetlands, respectively. On the other hand, Tejeda et al. [6] evaluated, in a microcosm of a wetland, the resistance of four plant species to diluted tequila vinasse. In general, although all the plants showed stress symptoms, they were able to survive even at concentrations of 5000 mg L^{-1} of COD (approximately), and *I. sibirica* was the species that presented the greatest resistance to vinasse.

On the other hand, ligninolytic fungi have also been successfully tested for the treatment of tequila vinasse. In this regard, Garzón-Zúñiga et al. [7] evaluated aerobic biofilters for the treatment of diluted tequila vinasse (with distilled water) inoculated with *Phanerochaete chrysosporium* and *Trametes versicolor*. The biofilters had *Ficus benjamina* wood chips as the filter medium. The highest COD removals were obtained at a 40/60 dilution (vinasse/distilled water), namely 72% with *Phanerochaete chrysosporium*, 73% with *Trametes versicolor* and 66% for the biofilter without fungus. With this dilution, the COD concentration was around 5500 mg L^{-1}. In another study, Retes-Pruneda et al. [8] evaluated the fungi Pleurotus ostreatus 7992 and *Trametes trogii* 8154 in liquid medium for the treatment of diluted vinasses (50 and 75% vinasse/water dilution) and found the highest removal of BOD_5 and COD at 75% dilution with 10 g of *T. trogii* 8154 biomass. According to the authors, the high concentrations of contaminants in the vinasse activated the ligninolytic enzyme system of both fungi. It is important to highlight that in this study, the initial concentrations of BOD_5 and COD were 82,000 and 100,000 mg L^{-1}, respectively.

Due to the ability of both TWs and ligninolytic fungi to treat tequila vinasses, in this study, the aim was to evaluate and compare the efficiency of two types of vertical downflow wetlands for the treatment of raw tequila vinasses: one type planted with *Iris sibirica* and the other type with the ligninolytic fungus *Trametes versicolor* placed as part of the filter medium.

2. Materials and Methods

2.1. Production and Immobilization of Trametes Versicolor

Trametes versicolor was the selected species due to its preliminary evaluation in lab-scale vertical wetlands for the treatment of TVs [9]. The procedure for fungus biomass production was performed according to the methodology described by Pickard et al. [10]. This methodology is recommended for the production of enzymes by fungi. Enzymes allow fungi to remove pollutants from TVs. Briefly, portions of fungi pre-seeded on PDA (potato dextrose agar) (Becton Dickinson de México, Estado de México, México) (1 cm × 1 cm) were mixed, using a Dremel rotor (Bosh Group, México) and a stainless steel container, with approximately 100 mL of cereal buffer medium for 40 s. Subsequently, this mixture was inoculated into 2 L flasks containing 1 L of the same buffer medium (made with fiber cereal at 2% *w*/*v*, at pH 6) and stirred at 120 rpm at room temperature for 12 days to promote the growth of fungi in the form of pellets. The fungal biomass was filtered with a vacuum pump using pieces of blanket cloth as a filter and poured into plastic containers (8.5 cm in diameter) which were placed in a freezer at −20 °C. In this way, 1.1 cm thick pellet fungi were obtained (Figure 1a). On the other hand, some portions of the fungal biomass were inoculated into sterilized dried corncobs and luffa (around 10 in diameter) (Figure 1b,c) and allowed to grow for around 12 days (Figure 1c).



(a) (b) (c)

Figure 1. *Trametes versicolor* (**a**) as frozen pelleted fungi, (**b**) inoculated into sterilized dried corncobs and (**c**) grown on dried luffa.

In order to demonstrate the viability of the fungus both at the beginning of the experiment and at the end of it, inoculation tests were carried out in PDA medium, in addition to observation under a fluorescence microscope of the biomass stained with a solution of fluorescein diacetate (FDA) (Sigma-Aldrich Co., Spruce St.) (3.06×10^{-5} M). All tests were carried out in duplicate and with fungi extracted from different parts of each of the VDFWs. From each system, 8 boxes were processed and 8 samples were observed under a microscope.

2.2. Experimental Setup

The TWs for this study were constructed using PVC pipes 1.20 m high and 0.254 m in diameter, with a circumscribed tube of 0.0762 m in diameter, with the function of aerating the substrate and thus promoting aerobic conditions in the system. Tezontle was used as the filter medium, with an average diameter of between 1 and 4 cm. This material is widely used in Mexico as a filter medium in TWs.

Three systems were implemented in duplicate. In a pair, the ligninolytic fungus inoculated on dried luffa and pelleted fungus were placed on the upper surface of the VDFWs exposed to the atmosphere (along the last 0.3 m of the height of 1.2 m) (VDFWs-LF) (Figure 2). In each wetland, approximately 11 pieces of luffa and a mass of pelleted fungus (381 g wet basis) were added. The objective of using two forms of fungal immobilization, including growth on luffa, was to promote the drainage of the vinasses, preventing their excessive accumulation and thus favoring the survival of the fungi. Another pair of VDFWs were planted with an individual of *Iris sibirica* about 30 cm high (VDFWs with vegetation). Finally, two systems with only a filter medium were included in order to determine the effect caused by the ligninolytic fungus or the plant (control VDFWs).

It is important to highlight that the raw vinasses were subjected to a sedimentation process for around 5 days before being fed to the systems, with the purpose of reducing the high load of suspended solids contained in the raw vinasses. After the settler, the vinasses were homogenized and then fed to the systems (Figure 2). The systems were fed with a flow rate of 5 Ld^{-1} divided into 4 pulses every 6 h by means of peristaltic pumps (MasterFlex, model L/S 07522-20). The systems operated with a 7-day run/resting mode of operation. This way of operating is similar to the French systems, in which one of the advantages is the preservation of aerobic conditions in the substrate when operating with rest periods [5], thus allowing the mineralization of organic matter [11].

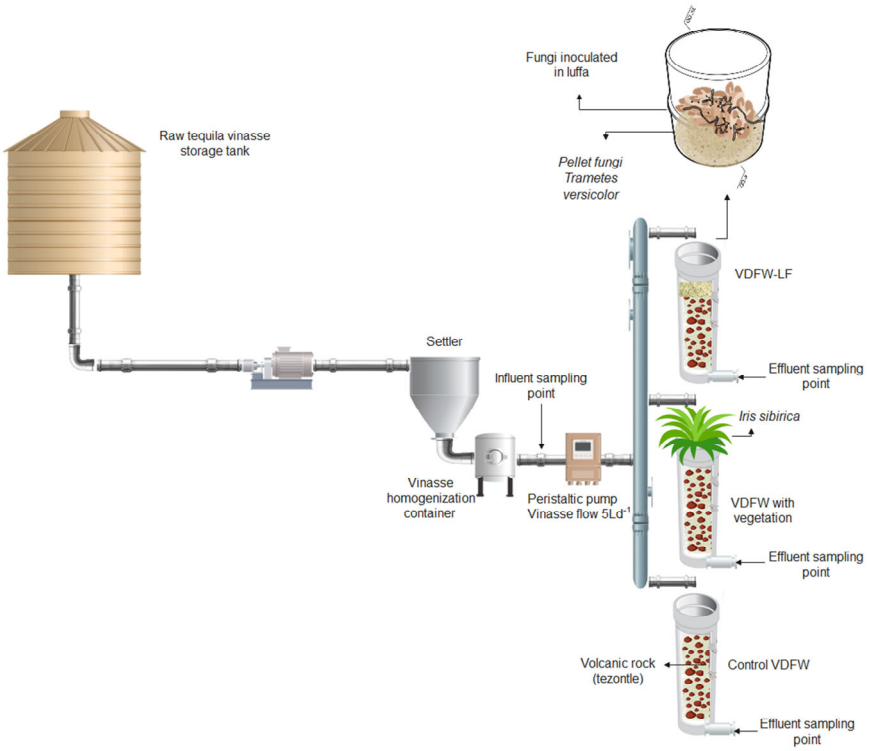


Figure 2. Experimental setup.

2.3. Evaluation of the Systems for the Treatment of TVs

2.3.1. System Monitoring

The systems were monitored for 33 weeks, with different water quality parameters in the influents and effluents of the systems measured on a weekly basis. The parameters measured were total suspended solids (TSS), total dissolved solids (TDS), true and apparent color, turbidity, total nitrogen (TN), phosphorus, total organic carbon (TOC), biological oxygen demand (BOD_5), chemical oxygen demand (COD) and fats and oils, based on standard methods for the analysis of water and wastewater [12]. On the other hand, and with the purpose of monitoring the internal conditions of the systems, the parameters of pH, electrical conductivity (EC) and dissolved oxygen (DO) were measured in situ every 3 days in the influent and effluents. Finally, the effluents were measured daily to verify the effect of evapotranspiration and correct the concentrations of pollutants.

2.3.2. Statistical Analysis of Data

The concentration values of each pollutant in the raw tequila vinasses were compared with the effluent concentrations in each VDFW throughout the experimentation period. Since the results did not present a normal distribution, a randomized complete block design was used and the Kruskal–Wallis test was performed. When significant differences were observed between treatments, a notch analysis was conducted. The software used for data analysis with a significance level of 0.05 ($\alpha = 0.05$) was Statgraphics Centurion XVI.

3. Results and Discussion

3.1. Behavior of Parameters Measured In Situ in the VDFWs

Figure 3a shows that in the three VDFWs, the pH increased ($p < 0.05$) in the effluents from an average value of 6.2 units in the influent, and varied in the effluents in the range of values close to neutrality with no difference between the systems. The fact that the pH varied in the range of neutral values is appropriate since microbial activity and plant development is adequate with such pH values. These changes in pH variation were probably due to two processes. The first is the reduction of sulfates under anoxic conditions through the action of bacteria, thus generating alkalinity (carbonates) and resulting in a pH

increase [13]. This is supported by the fact that the presence of sulfate has been reported in tequila vinasse [14]. The second process is denitrification, during which alkalinity is also produced [15].

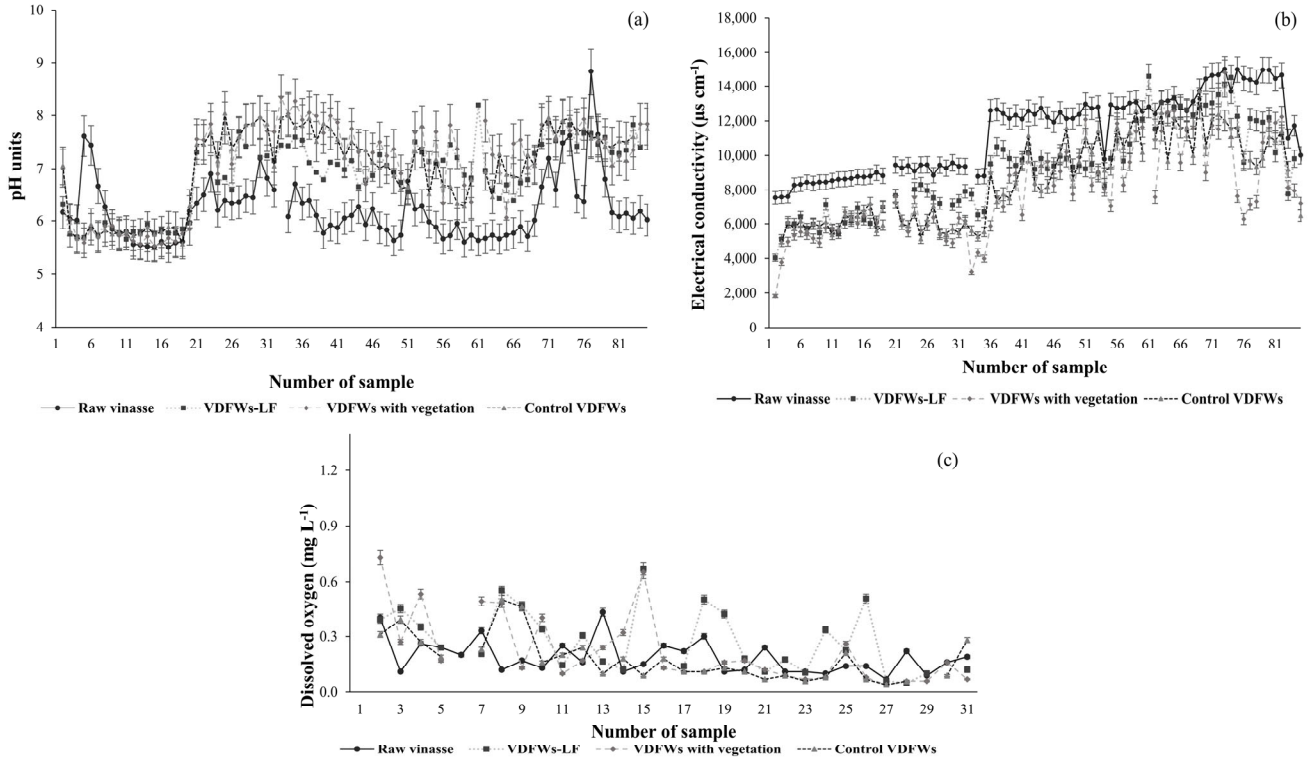


Figure 3. Behavior of pH (**a**), electrical conductivity (**b**) and dissolved oxygen (**c**) of treated tequila vinasse in the influent and effluents of VDFWs throughout the research period.

On the other hand, the EC showed a tendency to decrease in the 3 systems (Figure 3b), and such reduction was significant ($p < 0.05$) but without difference between the treatments. In this case, the average value in the influent was 11,316 µs cm^{-1}, while in the VDFW-LF, VDFW with vegetation and control VDFW, the average values were 9248, 7946 and 8507 µs cm^{-1}, respectively. These results were expected, since contaminants related to conductivity (for example, metal ions) are removed by processes such as absorption or adsorption and precipitation [16,17]. Furthermore, it can be observed that in the VDFWs with plants, the EC values were lower compared to the other two systems, which may be related to the probable effect of mineral uptake by the vegetation [18]. Finally, the values corresponding to dissolved oxygen are shown in Figure 3c. In all three systems, an increasing trend can be observed; however, these increases were not statistically significant ($p > 0.05$).

3.2. Viability Tests on the Trametes vesicolor Fungus Used in Vertical Wetlands

The evaluation of fungal viability with the FDA fluorescence technique is based on the fact that metabolically active cells are capable of hydrolyzing FDA intracellularly, producing fluorescein, a compound that emits fluorescence. On the other hand, PDA agar is a common medium used for growing fungi in the laboratory. It is a basic medium composed of dextrose, potato extract and agar [19]. The viability results of the fungus are presented in Figure 4a,b. The growth of the fungus was observed both at the beginning and at the end of the experiment, as was the greenish coloration characteristic of the FDA fluorescence. Based on this, it can be stated that the fungus remained viable throughout the experimental phase.

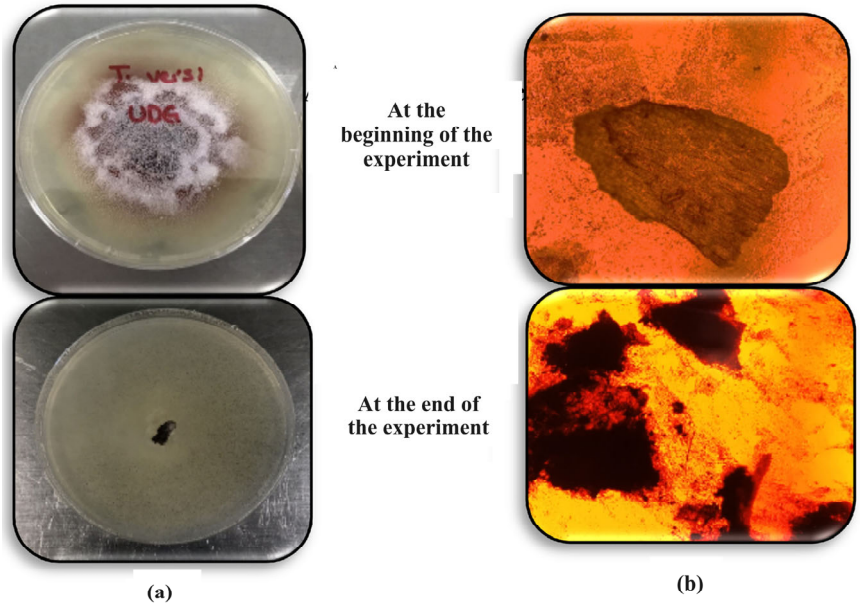


Figure 4. *Trametes versicolor* fungus growth tests in PDA medium (**a**) and fluorescence tests with FDA (**b**).

3.3. Efficiency of the Systems for the Treatment of TVs

Table 1 shows the initial characteristics of the vinasse along with the removal percentages obtained with each of the treatments. This summarizes and facilitates the interpretation of the results that will be discussed in the following sections. It is important to mention that the removal percentages were calculated with the average concentrations of the influent and effluents during the 33 weeks of monitoring.

Table 1. Characteristics of the raw vinasses and removal percentages obtained in the three systems (mean values ±95% confidence interval).

		Removal Percentage		
	Influent Concentrations	**VDFW-LF**	**VDFW with Vegetation**	**Control VDFW**
TSS (mgL^{-1})	781 ± 401	20	36 [a]	16
TDS (mgL^{-1})	28,716 ± 4626	32	39	34
Apparent color (Pt-Co)	15,430 ± 2698	25	36 [a]	24
Turbidity (NTU)	500.36 ± 197.61	32	46 [a]	27
True color (Pt-Co)	6400 ± 1417	17	21	21
COD (mgL^{-1})	49,423 ± 12,933	35	40	35
DBO_5 (mgL^{-1})	22,855 ± 5578	35	43	35
TOC (mgL^{-1})	16,032 ± 9151	43	55	44
Total phosphorus (mgL^{-1})	678 ± 826	11	11	10
Total nitrogen (mgL^{-1})	306.94 ± 59.79	26	36	26
Fats and oils (mgL^{-1})	0.27 ± 0.13	42	46	38

Note: [a] Statistically different from the rest of the treatments.

3.3.1. Total Suspended Solids and Total Dissolved Solids

The statistical analysis revealed for both TSS and TDS that the removal was significant ($p < 0.05$). The main mechanisms for the removal of suspended solids in vertical wetlands are sedimentation and filtration [5]. The removal of TSS (Figure 5a) was higher and statistically different in the VDFW with vegetation, since the removal percentage was 36%, compared to 20 and 16% for the VDFW-LF and control VDFW, respectively (Table 1). These removal efficiencies for TSS are low if they are compared to those reported by Abdelhakeem et al. [20], who obtained removals between 61 and 81% in a vertical subsurface flow constructed wetland used to treat raw sewage. However, the authors applied in their

system a solid loading rate (SLR) between 30 and 34 g TSS $m^{-2}d^{-1}$, which is 2.29 times lower than the load used in this study (78.1 g TSS $m^{-2}d^{-1}$). In addition, these removals are quite minor compared to those obtained by Montoya et al. [2], whose values were 91.8 and 95.9% of TSS for horizontal and vertical treatment wetlands, respectively, but with an SLR of 8.67 and 10.84 g TSS $m^{-2}d^{-1}$. It should be noted that the SLR indicates the applied suspended solids loading per unit surface area of the system. It is an important parameter to determine the capacity of the VDFWs to receive solids, which could eventually block the spaces in the pores of the substrate and thus affect the hydraulics of the system.

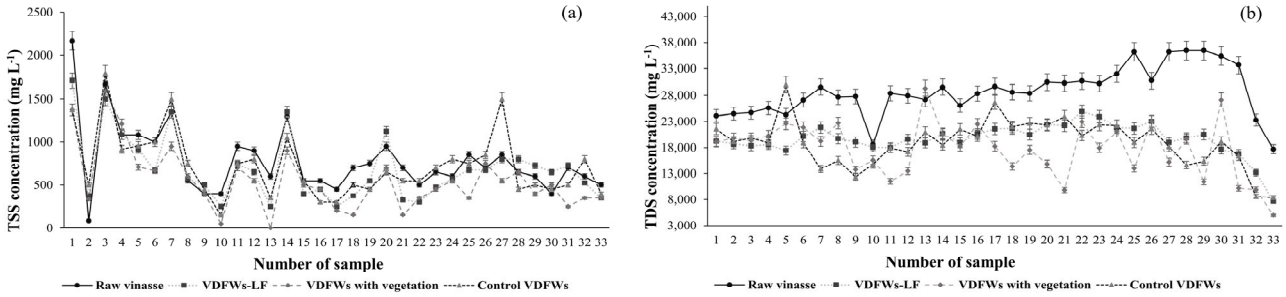


Figure 5. Behavior of TSS (**a**) and TDS (**b**) of treated tequila vinasse in the influent and effluents of VDFWs throughout the research period.

Regarding TDS (Figure 5b), a significant reduction ($p < 0.05$) was also found with the three systems (although there was no significant difference between them), with the VDFW with vegetation being the one that showed a tendency to higher values, at 39% (32 and 34% for the VDFW-LF and the control VDFW, respectively). It is important to emphasize that these removal efficiencies were achieved with an average concentration of 28,716 mg L^{-1} in the influent, a common value in TVs (23,000 to 42,000 mg L^{-1}) [21]. On the other hand, these efficiencies could be considered somewhat low, but it is important to take into consideration the high TDS concentration and also the high applied hydraulic loading rate (HLR) (10 cm d^{-1}). In fact, these removal efficiencies are higher than the 15% obtained by Yadav et al. [22] in vertical wetlands, with an HLR of 15 cm d^{-1} and an average concentration of 0.65 mg L^{-1} of TDS when treating domestic wastewater.

3.3.2. Apparent Color, Turbidity and True Color

Apparent color is due to the color resulting from substances suspended and dissolved in water, while true color is due only to dissolved substances [12,23]. The results for these parameters and turbidity are shown in Figure 6a–c. A clear reduction can be observed with the three types of systems, and the statistical analysis indicated that such reductions were significant ($p < 0.05$). Again, the VDFWs with vegetation presented the highest average removals, with 36 and 46% for apparent color and turbidity (Table 1). This was expected, since vegetated systems were the most efficient for TSS removal.

On the other hand, color may be associated with suspended substances such as phytoplankton or clay, and in the case of dissolved substances, the presence of natural organic substances such as humic matter, especially fulvic acids, can cause a yellow-brown color of the water [24]. In the specific case of TVs, the removal of these substances plays a very important role, since if TVs are dumped into water bodies, this characteristic prevents the penetration of light, which in turn can affect photosynthetic activity [25]. This decreases the concentration of dissolved oxygen and extinguishes aquatic life [26–29]. It is also important to mention that the characteristic brown color of these residues is due to the fact that they contain phenolic compounds and melanoidins [29,30], which are heterogeneous, nitrogen-containing compounds generated by the Maillard reaction [31].

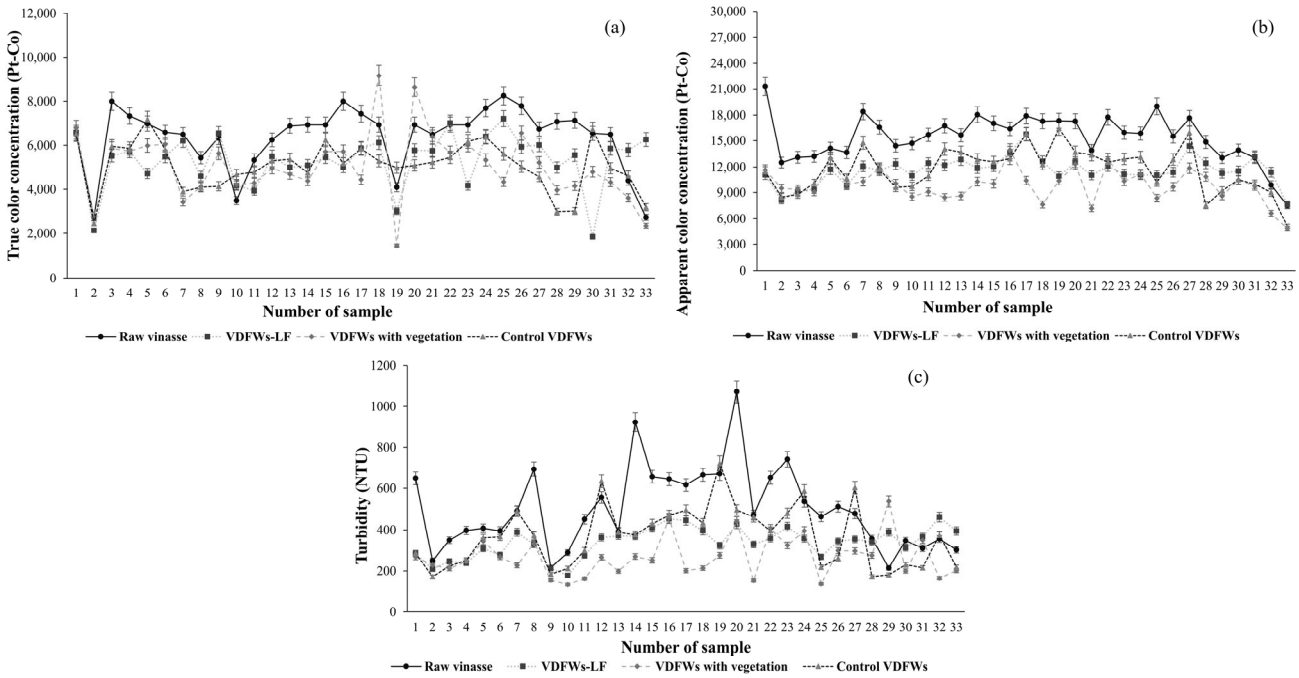


Figure 6. Behavior of true color (**a**), apparent color (**b**) and turbidity (**c**) of treated tequila vinasse in the influent and effluents of VDFWs throughout the research period.

True color removal, due to the recalcitrant nature of the aforementioned compounds [32,33], is generally difficult to achieve. In this study, the average removals of the true color were very similar in the three systems (17% for the VDFW-LF, 21% for the VDFW with vegetation and 21% for the control VDFW). Therefore, it is probable that the mechanisms responsible for true color removal included interaction with the filter medium as well as biodegradation [30,34,35]. In this regard, Montoya et al. [2] reported color removal when treating TVs in two types of constructed wetlands (horizontal subsurface flow wetlands and vertical upflow wetlands) of approximately 86%, which they attributed to a probable biodegration by the bacterial population that grows mainly near the rhizomes of CW plants [36]. With respect to the VDFW-LF, a higher removal was expected, since there are investigations where a reduction in the color of distillery residues has been reported [37,38]. These color reductions have been attributable to processes such as absorption on the mycelium or due to the enzymatic and extracellular activity (of laccase, manganese peroxidase and lipase) that characterizes fungi. Therefore, our results suggest that the fungal enzyme system did not develop properly.

3.3.3. Organic Matter (COD, BOD_5 and TOC)

In general, removing organic matter from TVs is crucial due to its negative impacts on the environment if TVs are discharged without proper treatment. In a water body, the organic load causes the proliferation of microorganisms that deplete the oxygen dissolved in the water, destroy aquatic animals and plants, and make contaminated water bodies more difficult to be used as sources of potable water [39]. On the other hand, its anaerobic decomposition can lead to the generation of bad odors. In soils, the organic matter content could mineralize and alter the nitrogen and carbon cycles, increasing the emission of greenhouse gases such as methane, carbon dioxide and nitrous oxide under conditions of high humidity [40]. In this study, the organic matter removal results (Figure 7) showed a clear reduction in the three systems, and the VDFW with vegetation presented the best performance. These reductions were significant ($p < 0.05$) but without difference between treatments. The average removal percentages obtained were 35%, 40% and 35% for COD (Figure 7a), 35%, 43% and 35% for BOD_5 (Figure 7b) and 43%, 55% and 44% for TOC (Figure 7c), for the VDFW-LF, VDFW with vegetation and control VDFW, respectively

(Table 1). In this way, the influence of macrophytes is evident, since in the two systems without vegetation, the removal percentages for BOD_5 and COD are the same, and that of TOC is very similar. It is well known that plants contribute by enabling the development of microbial populations in an oxidized rhizosphere [41]. Other mechanisms that are also responsible for organic matter removal in TWs are sedimentation and filtration [42]. Regarding the VDFW-LF, the removal percentages being practically equal to those of the control system could indicate the absence of the enzymatic expression that characterizes fungi (as aforementioned), which is responsible for the efficient elimination of the organic load found in different studies [8,43,44].

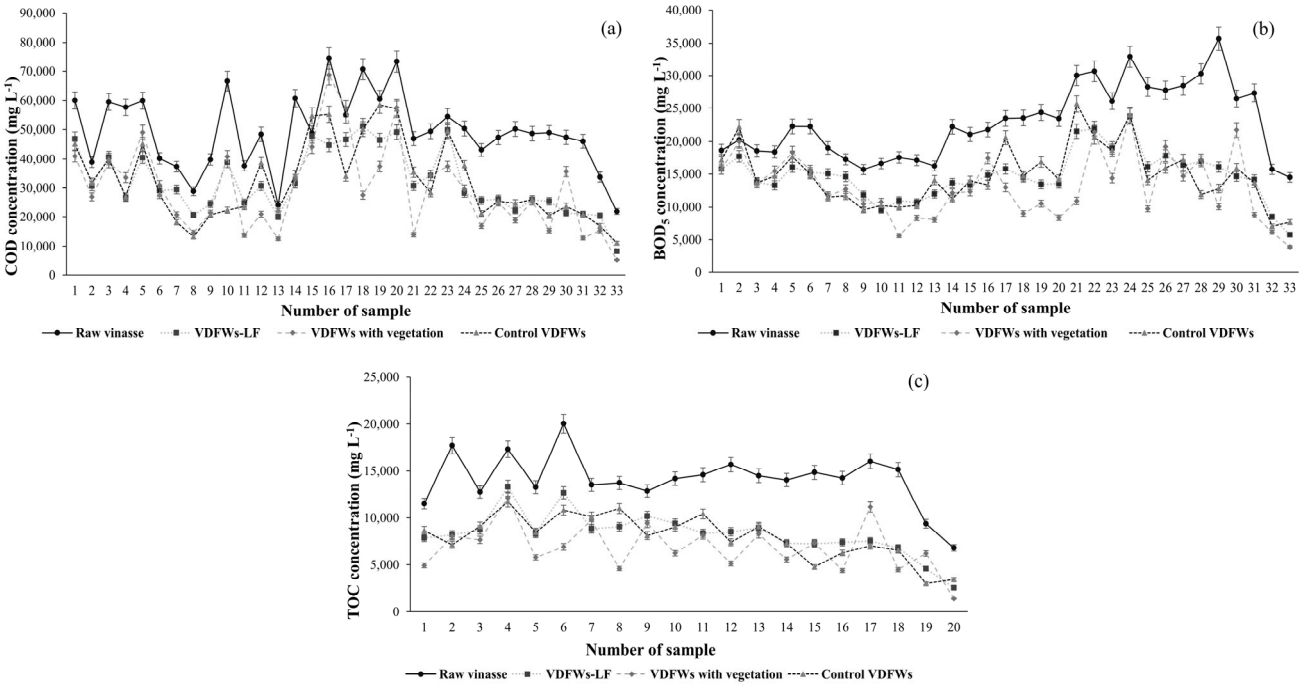


Figure 7. Behavior of COD (**a**), BOD5 (**b**) and TOC (**c**) of treated tequila vinasse in the influent and effluents of VDFWs throughout the research period.

On the other hand, it is necessary to highlight that the organic loading rate (OLR) applied to the systems was very high (4942 g COD $m^{-2}d^{-1}$), since it exceeds approximately 16 times the 300 g COD $m^{-2}d^{-1}$ reported as one of the highest values applied in vertical TWs [5,45]. Therefore, although the obtained results of removal could be considered low, they are actually very relevant when taking into account that the concentrations in the influent were very high. Furthermore, Arévalo-Durazno et al. [46], in a study for the treatment of raw wastewater derived from a combined sewer system with a pilot-scale modified first-stage French vertical flow constructed wetland, obtained a COD mean removal efficiency of 53 ± 18% with an average influent COD of 187 mg L^{-1}, which reflects the importance of the results obtained in this research (average influent concentration of 49,000 mg L^{-1} of COD).

On the other hand, the biodegradable fraction or organic matter is measured as BOD_5 and TOC, while both the biodegradable and non-biodegradable fraction are measured as COD. It is interesting to note that the average ratio of BOD_5/COD in the influent was 0.46, while in the effluents the values were 0.46, 0.44 and 0.46 for the VDFW-LF, VDFW with vegetation and control VDFW, respectively. This indicates that tequila vinasse has high biodegradable organic matter content, since with a BOD_5/COD between 0.4 and 0.6, the presence of biodegradable substances is significant [47]. Additionally, these ratios, without changes in the effluents, suggest that another stage of treatment with TWs could reduce the organic matter content in the TVs. It is important to note that effluent biodegradability based on this relationship varies depending on the particular characteristics of the vinasses;

in this way, biodegradability has been found to be as low as 0.02 (for cachaca vinasse) [48], similar to the TV in this study (0.43) [49] or higher (0.75) [50]. On the other hand, when observing little or no change in the BOD_5/COD ratios of the treatments, it could be assumed that microbial or biological activity was not responsible for the removal of organic matter, but rather some other mechanism such as adsorption was responsible. However, there are studies that have shown that although adsorption in wetlands is an important mechanism in the removal of organic matter, it is not comparable to microbial degradation, which far exceeds the adsorption process [51]. Furthermore, in many studies it has been shown that in planted systems, vegetation promotes microbiological enrichment compared to non-planted systems [41]. This supports what was found in this study, in the sense that microbial processes were carried out, since the removals of organic matter in the systems were greater in the VDFW with vegetation (although without significance).

3.3.4. Total Phosphorus

Phosphorus reduction in the three evaluated systems (Figure 8) was not significant ($p > 0.05$), although a trend was observed toward lower values in the effluents compared to the influent. When calculating the average removal percentages, these were 11, 11 and 10%, for the VDFW-LF, VDFW with vegetation and control VDFW, respectively (Table 1). Phosphorus is an essential macronutrient for agriculture, so the reuse of treated TVs for crop irrigation could be an option [29]. On the contrary, if poorly treated TVs are discharged into water bodies, the presence of phosphorus causes many negative environmental impacts. The most important is eutrophication, which produces algal blooms, the growth of harmful aquatic plants and low availability of oxygen in water bodies, as well as other undesirable consequences [52]. On the other hand, the ability of constructed wetlands to remove phosphorus is an issue that has to be considered due to their poor removal efficiency [53]. The removal potential of this parameter in TWs is mostly dependent on the substrate, which also plays a crucial role in the overall phosphorus retention [54].

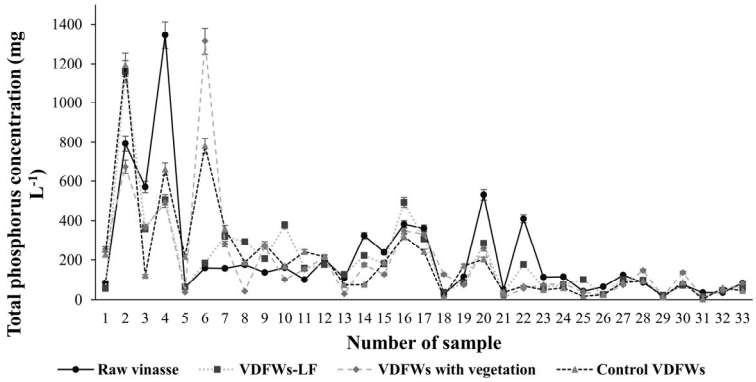


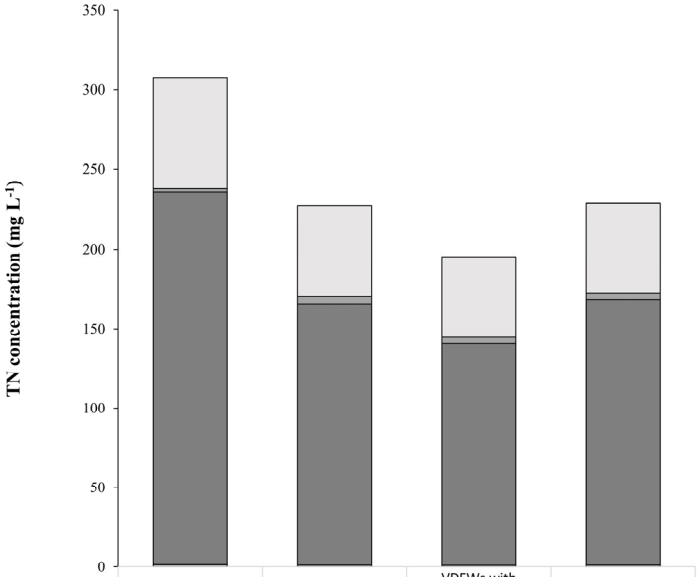
Figure 8. Behavior of total phosphorus of treated tequila vinasse in the influent and effluents of VDFWs throughout the research period.

There are studies that indicate that the substrate is responsible for around 50% of the phosphorus removal compared to components such as plants [54–56]. Considering that phosphorus is removed through sorption and/or precipitation, the Ca, Fe and Al content of a substrate is important in efficient phosphorous removal. In Ca- and Mg-based materials, precipitation is the main process for phosphorous retention, while surface complexation on or in Al- and Fe-based materials is the other way to remove this nutrient [57–59]. In addition, it is important to consider the sensitivity that these materials have to pH, so that for Mg and Ca oxide/hydroxide materials the retention is enhanced at high pH values, while for the Fe and Al oxide-based materials, phosphorus retention is strongest at a low pH [58–62]. Finally, it is worth mentioning that there is a wide variety of different types of materials for phosphorus retention, and they could be classified as natural materials (e.g.,

apatite, bauxite, dolomite, gravel, etc.), industrial byproducts (e.g., coal ash, fly ash, ochre, oil shale, etc.) and manufactured products (e.g., filtralite P, Leca, LWA, etc.) [63].

3.3.5. Total Nitrogen

Figure 9 shows a clear reduction in total nitrogen concentration in the three systems, and such reductions were statistically significant ($p < 0.05$). The removal percentages in the systems were 26, 36 and 26% for the VDFW-LF, VDFW with vegetation and control VDFW, respectively (Table 1). Similar to the other pollutants, the VDFW with vegetation showed the best performance. Regarding each of the chemical species, in all systems the concentrations of nitrite, nitrate and organic nitrogen were reduced, and in the case of ammonium, its concentration always increased.



	Raw vinasse	VDFWs-LF	VDFWs with vegetation	Control VDFWs
Organic N.	69.62	56.81	50.80	56.23
Ammonium N.	2.23	5.28	4.04	4.50
Nitrate	234.09	164.23	139.61	167.09
Nitrite	1.32	1.01	0.94	1.04

Figure 9. Behavior of total nitrogen and the species that make it up in treated tequila vinasse in the influent and effluents of VDFWs throughout the research period.

In treatment wetlands, nitrification–denitrification mediated by microorganisms is considered the most common route for the removal of total nitrogen [64–66]. In vertical wetlands, it has been reported that total nitrogen removal is minimal or non-existent, due to the efficient aeration of the substrate (which prevents conditions from being anoxic or anaerobic) that promotes nitrification, as well as the low concentration of carbon available for denitrification [65,67–69]. The latter is measured by the C/N ratio, since when this is less than 2.5, denitrification is limited [65,70]. In the systems in this study, the probable denitrification that took place could explain the reduction in nitrate. In this process, facultative heterotrophic bacteria use nitrogen oxides as electron acceptors and organic matter as an electron donor [5,66,70]. As mentioned before, this is known to be a very limited process in vertical wetlands due to the prevailing oxygenation conditions; however, due to the minimal concentrations of oxygen in TVs, as well as the high availability of carbon (COD) (C/N = 161), it was possible for this process to take place. With respect to the higher removal in the VDFW with vegetation, this is explained by the fact that the plants use nitrate as a nutrient for their growth [5].

With respect to the removal of organic nitrogen, it could presumably be due to an ammonification phenomenon, which can take place in both aerobic and anaerobic conditions, although it proceeds more rapidly in oxygen-rich layers [5]. During this process, microor-

ganisms break down small organic molecules containing an amino group (such as amino acids, amino sugars, urea and nucleotides) to release $NH4^+$. It is carried out through a wide variety of metabolic routes, whether intracellular or extracellular. Generally, most bacteria and fungi, as well as many phyla of unicellular photosynthetic eukaryotes, can perform such a transformation. Therefore, the level of ammonification is partially controlled by the size and activity of the microbial community. Furthermore, ammonification is also limited by the availability of dissolved organic carbon [71]. Analyzing the behavior of the systems in this study, and considering the greater oxygenation of the substrate provided by the action of the roots of the plants in the VDFW with vegetation, it was to be expected that in the latter the amount of ammonium would be greater. However, this did not happen, since, as can be seen in Figure 9, the VDFW with vegetation presented the lowest concentration of ammonium. This was probably due to the fact that the plants assimilated ammonium; many species use both ammonium and nitrate as nitrogen sources [72,73].

3.3.6. Fats and Oils

Figure 10 shows that there was a reduction in fats and oils in the three systems and it was significant ($p < 0.05$), but without difference between treatments. It can be seen that the concentration of fats and oils was very low. However, other authors have found concentrations up to 100 mg L^{-1} [21]. Furthermore, it is worth mentioning that in the three systems a good reduction was obtained, ranging between 38 and 46% (Table 1), with the VDFW with vegetation once again registering the highest removal. Such removals in the three systems are attributed to a filtration effect in the substrate (tezontle). In the case of planted systems, the better efficiency can be attributed to the presence of hydrocarbon-degrading bacteria in the rhizosphere of the wetland plants [74].

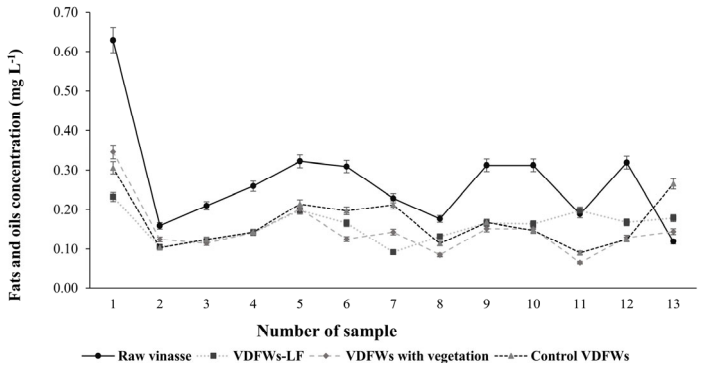


Figure 10. Behavior of fats and oils of treated tequila vinasse in the influent and effluents of VDFWs throughout the research period.

The removal of these types of pollutants from TVs is desirable because of the negative impacts they can cause. When these compounds come into contact with water, they cause an unwanted visual appearance and a strong odor. Fats and oils have low solubility and density, so they form an insoluble film on the water surface, which reduces the level of aeration and sunlight penetration. Furthermore, they have a high toxicity and, therefore, an adverse effect on aquatic biota and humans.

4. Conclusions

The results of this study demonstrate the treatment capacity of VDFWs for tequila vinasses, which are considered high-strength effluents. The TVs were fed with raw vinasses and the applied organic load exceeded by about 16 times what is reported in the literature as a high organic load value for a conventional vertical wetland. With such values, the systems with vegetation were able to reduce the COD concentration by 40%, while the systems with fungi and control treatment were able to reduce it by 35%. Considerably good results were also obtained for other pollutants and parameters such as BOD_5, TSS,

nitrogen, apparent and true color, etc. Therefore, the results obtained open the possibility of incorporating the use of vertical wetlands as part of a treatment train for the treatment of tequila vinasse. Furthermore, VDFWs with fungi are a promising option that requires future studies.

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