# Performance of three pilot-scale hybrid constructed wetlands for total coliforms and *Escherichia coli* removal from primary effluent – a 2-year study in a subtropical climate

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## ABSTRACT

Three pilot-scale two-stage hybrid constructed wetlands were evaluated in order to compare their efficiency for total coliforms (TCol) and *Escherichia coli* removal and to analyze their performances in two 1-year periods of experimentation. System I consisted of a horizontal flow (HF) constructed wetland (CW) followed by a stabilization pond. System II was also configured with a HF CW as a first stage which was then followed by a vertical flow (VF) CW as a second stage. System III was configured with a VF CW followed by a HF CW. In the first year of evaluation, the HF–VF system was the most effective for TCol removal ($p < 0.05$) and achieved a reduction of 2.2 log units. With regard to *E. coli* removal, the HF–VF and VF–HF systems were the most effective ($p < 0.05$) with average reductions of 3.2 and 3.8 log units, respectively. In the second year, the most effective were those with a VF component for both TCol and *E. coli* which underwent average reductions of 2.34–2.44 and 3.44–3.74 log units, respectively. The reduction achieved in *E. coli* densities, in both years, satisfy the World Health Organization guidelines that require a 3–4 log unit pathogen reduction in wastewater treatment systems.

**Key words** | disinfection, Latin America, pathogens, subtropical climate

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## INTRODUCTION

The incidence of waterborne diseases associated with pathogenic organisms in partially treated or untreated domestic wastewater discharged to the environment is widespread in areas of developing countries with poor sanitation. In Mexico, more than a quarter of the 112 million population lives in rural communities in which the number of municipal wastewater treatment plants has been increasing slowly (Zurita *et al.* 2012). In these areas, the direct or indirect use of untreated wastewater for crop irrigation is still a common practice. It is well-known that the use of untreated municipal wastewater in an agricultural setting poses risks to human health mainly due to the potential presence of excreta-related pathogens (viruses, bacteria, protozoan and multicellular parasites).

Constructed wetlands (CWs) are one of the most widely used ecological wastewater treatment systems in the world due to their proved capacity for the removal of practically any pollutant from a variety of wastewaters. However, their use is still low in Latin America, specifically in Mexico (Zurita *et al.* 2012). Constructed wetlands are a low-cost option known to act as excellent biofilters for the reduction of bacteria of anthropogenic origin (Ávila *et al.* 2013a; García *et al.* 2013a, b). They are capable of reaching nearly 100% removal of parasitic eggs (Ávila *et al.* 2013a) due to longer retention times in comparison to more expensive and energy-intensive conventional technologies (Sharafi *et al.* 2012). These systems can be used in centralized systems or *in situ* to generate reclaimed water which can be safely reused in agriculture (Vymazal 2005; Cirelli *et al.* 2012). The removal of pathogens takes place through several mechanisms, such as the exposure to biocides excreted by

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macrophyte roots, adsorption to the media and organic matter, natural die-off and predation by nematodes, protozoa and rotifers (Kadlec & Wallace 2008). Despite the high efficiency of CWs for pathogen removal, one-stage systems are usually not sufficient to achieve the desired levels of indicator organisms to ensure the absence of pathogens (Marecos do Monte & Albuquerque 2010; García *et al.* 2013a, b). Therefore, many authors argue that only through the use of hybrid constructed wetlands, it is possible to reach such a quality of reclaimed water to fulfill the World Health Organization (WHO) guidelines for restricted and unrestricted irrigation (Kim *et al.* 2006; Barros *et al.* 2008; Reynoso *et al.* 2008). According to the most recent WHO guidelines, a 3–4 log unit pathogen reduction should be achieved by wastewater treatment in order to protect the health of those working in wastewater-irrigated fields as well as to protect the health of those consuming wastewater-irrigated food crops. For the last case, the achievement in wastewater treatment should be accompanied by post-treatment health-protection control measures providing together an additional reduction of 2–4 log unit pathogen, so that the target of a global reduction of 6–7 log unit might be possible (WHO 2006; Mara & Bos 2010).

Although the potential of two- or three-stage hybrid constructed wetlands has been evaluated for pathogen removal, most of the studies have been carried out in cold climate regions of central and northern Europe (Ávila *et al.* 2013a) and only a few cases have been reported in tropical and subtropical areas of Latin America (García *et al.* 2013a, b). In addition, many of these studies have evaluated the efficiency of CWs for pathogen reduction as an advanced treatment stage after a conventional wastewater treatment system, rather than as secondary treatment (Leto *et al.* 2013; Rühmland & Barjenbruch 2013). In Latin America there is a great need to protect human health by the removal of pathogens from wastewater with low-cost systems. In consequence, the study of hybrid constructed wetlands is required to find out the necessary combinations to be implemented in rural and medium-size communities for wastewater treatment. Therefore, in this study, three pilot-scale two-stage hybrid systems were evaluated in order to compare their efficiency for total coliforms (TCol) and *Escherichia coli* removal from primary effluent and to analyze their performances in two 1-year periods of experimentation.

## METHODS

### Description of the wetland systems

The entire description of the systems can be found in Zurita & White (2014). Briefly, three two-stage hybrid constructed wetlands (HCWs) were evaluated in duplicate. System I consisted of a horizontal flow CW followed by a stabilization pond (HF–SP). A water level of 35 cm was maintained in HF wetlands. The CWs were continuously fed with a theoretical hydraulic retention time of 3 days. The effluent from the CWs flowed by gravity to the stabilization ponds. System II was also configured with a horizontal flow CW as a first stage, which was then followed by a vertical flow CW as a second stage (HF–VF). The horizontal flow CW operated in the same way as in system I, but the effluent was collected in a tank and pumped intermittently every 2 h onto the substrate of the vertical flow CW. System III was configured with a vertical flow CW followed by a horizontal flow CW (VF–HF). The vertical flow CW was intermittently fed by a pump programed to discharge 2.8 L every 2 h onto the surface, specifically over the plant without a distribution system. The effluent flowed by gravity to the next stage. A total flow rate of ~200 L/d of wastewater was treated and distributed equally among the three HCWs. The design hydraulic loading rate for the HF–CW, VF–CW and SP were 6.9, 14.5 and 6.8 cm/d. The horizontal flow CWs were each planted with six (25–30 cm height) individual *Zantedeschia aethiopica* plants and the vertical flow CWs were planted with one individual adult plant of *Strelitzia reginae*. After 8 months of experimentation (from September to April 2010), the *Z. aethiopica* plants were replaced with *Canna indica* due to the fact that the former plants desiccated during the dry season, characterized by low air humidity and high ambient temperatures. Ground tezontle rock was used as the media in all the CWs, with a fine granulometry ($d_{10}$ of 0.645 mm, $d_{60}$ of 2.3 mm, uniformity coefficient of 3.6) and porosity of 54%. The systems were evaluated during 2 years. The altitude in this area is

1,530 m, the average precipitation is 810 mm/year and the average temperature is 21 °C.

## Water quality parameters

The systems were fed with primary effluent from the beginning but allowed to stabilize for 4 months and then monitored weekly for the following 8 months during the first year and every 2 weeks in the second year. TCol and *E. coli* were quantified by the Colilert method (IDEXX Laboratories, Inc., Westbrook, Maine, USA). Colilert simultaneously detects total coliforms and *E. coli* in water. It is based on IDEXX's patented defined substrate technology. The coliforms metabolize Colilert's nutrient-indicator, *O*-nitrophenyl-β-D-galactopyranoside, due to their ability to produce the β-galactosidase enzyme; this turns the samples to a yellow color. *Escherichia coli*, in turn, metabolize Colilert's nutrient-indicator, 4-methylumbelliferyl-β-D-glucuronide, because of their ability to produce β-glucoronidase enzyme, which allows the samples also to fluoresce under a 365 nm UV light. Colilert was run in a multiple format by using Quanty-Tray/2000. Most probable numbers (MPNs) tables provided by IDEXX Laboratories, Inc. were used to find the MPNs after incubation at 35 °C for 24 h. In addition, measurements of chemical oxygen demand (COD), total nitrogen (TN), total phosphorus (TP), total suspended solids (TSS), dissolved oxygen, pH and conductivity were taken at the influent and effluent of each system in order to better characterize the wastewater and the systems. The water quality parameters were determined as described in *Standard Methods for the Examination of Water and Wastewater* (APHA, AWWA & WEF 2005). A potentiometer (Thermo Scientific 3 Star) and a dissolved oxygen meter (Orion 3 Star Thermo Electron) were used to measure pH and conductivity, and dissolved oxygen, respectively. Samples were analyzed immediately after they were taken in the Quality Environmental Laboratory at the university.

## Data analysis

A randomized block design was used to analyze the data in this study. Multifactor analysis of variance (ANOVA) was carried out using the Statgraphics Centurion XVI software package to check differences among treatments by using both mean percentage removal and monthly mean percentage removal in the treatments. A significance level of $p = 0.05$ was used for all statistical tests. When a significant difference was observed between treatments in the ANOVA procedure, multiple comparisons were made using the least significant difference test for differences between means.

# RESULTS AND DISCUSSION

## Performance of the systems during the first year

The performance of the three hybrid systems with regard to additional pollutants and control parameters during the first period of evaluation is shown in Table 1. The wastewater

**Table 1** | Performance summary for the three hybrid constructed wetlands with respect to COD, TN, TP, TSS and control parameters during the first period of evaluation

| | | System I: HF–SP | | System II: HF–VF | | System III: VF–HF | |
|---|---|---|---|---|---|---|---|
| | Influent | Stage 1 | Stage 2 | Stage 1 | Stage 2 | Stage 1 | Stage 2 |
| COD (mg/L) | 273.5 ± 145.5 | 96.9 ± 49.9 | 277.8 ± 126.7 | 95.4 ± 48.5 | 56.9 ± 21.5 | 66.7 ± 25.1 | 55.8 ± 23.3 |
| Total N (mg/L) | 139.5 ± 73.4 | 108.3 ± 70.4 | 60.4 ± 26.4 | 108.2 ± 57.5 | 111.6 ± 59.8 | 134.5 ± 56.5 | 102.9 ± 57.5 |
| Total P (mg/L) | 12.4 ± 5.8 | 11.8 ± 5.7 | 11.4 ± 6.5 | 12.1 ± 5.8 | 12.2 ± 5.6 | 11.3 ± 5.6 | 12.4 ± 6.2 |
| TSS (mg/L) | 61.8 ± 38.0 | 12.3 ± 6.7 | 138.3 ± 87.4 | 8.3 ± 5.6 | 4.6 ± 2.6 | 10.6 ± 10.1 | 4.5 ± 2.9 |
| DO (mg/L) | 1.5 ± 0.97 | 5.5 ± 1.4 | 8.8 ± 4.0 | 4.7 ± 1.6 | 4.3 ± 1.3 | 6.9 ± 1.2 | 5.2 ± 1.4 |
| pH | 8.2 ± 0.25 | 8.0 ± 0.34 | 8.1 ± 0.36 | 8.2 ± 0.19 | 6.4 ± 0.55 | 6.7 ± 0.44 | 6.8 ± 0.44 |
| Conductivity (μS/cm) | 1797 ± 810 | 1774 ± 865 | 1387 ± 659 | 1693 ± 847 | 1369 ± 657 | 1381 ± 627 | 1457 ± 897 |

Average ± SD, $n \sim 33–35$. DO, dissolved oxygen.

had a high content of N, mainly in the form of ammonium (Zurita & White 2014).

### System efficiency for total coliform removal

The concentration of TCol in the influent is in the range of a weak untreated domestic wastewater (Table 2) (Metcalf & Eddy 2003).

In the first stage of the three HCWs, the VF wetlands (VF–HF system) registered a significantly higher efficiency for TCol removal ($p < 0.05$) in comparison to the HF beds in the HF–SP and HF–VF systems (Table 2 and Figure 1(a)–1(c)). The lower average reduction in HF beds (1.08 and 1.1 log units) was probably due to the reintroduction from birds observed directly on the higher exposed area (0.48 $m^2$) in this type of wetland owing to the poor coverage of plants. The obtained results are close to the low limit of the range of 1.2–2.2 reported by Morató *et al.* (2014) in HF CWs in a Mediterranean climate. Apparently, the performance of HF CWs was not affected by the replacement of the macrophytes planted initially (Figure 1(a) and 1(b)). With respect to the second stage, an increase in TCol concentrations was observed in the SPs (Table 2 and Figure 1(a)) of the HF–SP system. The well-known capacity of TCol to reproduce in surface water when stimulated by high nutrient availability (Tyagi *et al.* 2006) and the reintroduction from birds observed in the ponds were probably responsible for these results. Similar findings have been reported by other authors in free surface water wetlands (Ávila *et al.* 2013b). In contrast, in the other two systems, TCol was significantly reduced ($p < 0.05$) with respect to the first stage. In this way, the superiority of CWs to ponds for TCol removal is clear, which is common according to Kadlec & Wallace (2008) when the inlet concentration is in the range of $10^4$–$10^6$ CFU per 100 mL. On the other hand, these findings reaffirm that TCol is not the best indicator organism to evaluate the efficiency of natural treatment systems for pathogen removal due to the fact that it is ubiquitous in water and soils (Kadlec & Wallace 2008) and capable of growing even in non-polluted waters (Tyagi *et al.* 2006). As a result of the performance of the two stages, during the first period of evaluation the HF–VF system was the most effective for TCol removal ($p < 0.05$) (Table 2 and Figure 1(b)).

### Systems efficiency for *E. coli* removal

The reduction of *E. coli* in the first stage of the three systems was high and similar ($p > 0.05$) (Table 2 and

**Table 2** | Performance summary for the three hybrid constructed wetlands with respect to total coliforms and *E. coli* during the first year

| | **Concentrations** | | | |
|---|---|---|---|---|
| | **TCol × $10^4$ (MPN/100 mL)** | ***E. coli* × $10^4$ (MPN/100 mL)** | **Removal (%)** | |
| Influent | 250 ± 99 | *160* ± 68 | | |
| System I (HF–SP) | | | | |
| Stage 1 (HF) | 20 ± 6.3 | 3.1 ± 0.96 | 92 *(1.1)* | 98.06 (*1.71*) |
| Stage 2 (SP) | 38 ± 14 | 0.42 ± 0.14 | (*−0.28*)[a] | 86.42 (*0.87*) |
| | | | **84.8 (*0.82*)** | **99.74 (*2.58*)** |
| System II (HF–VF) | | | | |
| Stage 1 (HF) | 21 ± 6.6 | 3.1 ± 1.3 | 91.6 (*1.08*) | 98.06 (*1.71*) |
| Stage 2 (VF) | 1.6 ± 0.57 | 0.10 ± 0.03 | 92.38 (*1.12*) | 96.58 (*1.49*) |
| | | | **99.36 (*2.20*)** | **99.93 (*3.20*)** |
| System III (VF–HF) | | | | |
| Stage 1 (VF) | 11 ± 4.1 | 3.8 ± 0.62 | 95.6 (*1.36*) | 97.63 (*1.62*) |
| Stage 2 (HF) | 7.1 ± 2.5 | 0.021 ± 0.006 | 30.00 (*0.19*) | 99.44 (*2.18*) |
| | | | **96.92 (*1.55*)** | **99.99 (*3.80*)** |

[a] Negative removal (an increase in the output concentration).

Average (×$10^4$) ± standard error of the mean. Reduction in logarithmic units is in parentheses. Entire system removal percentages as well as global reduction in logarithmic units are in bold font.

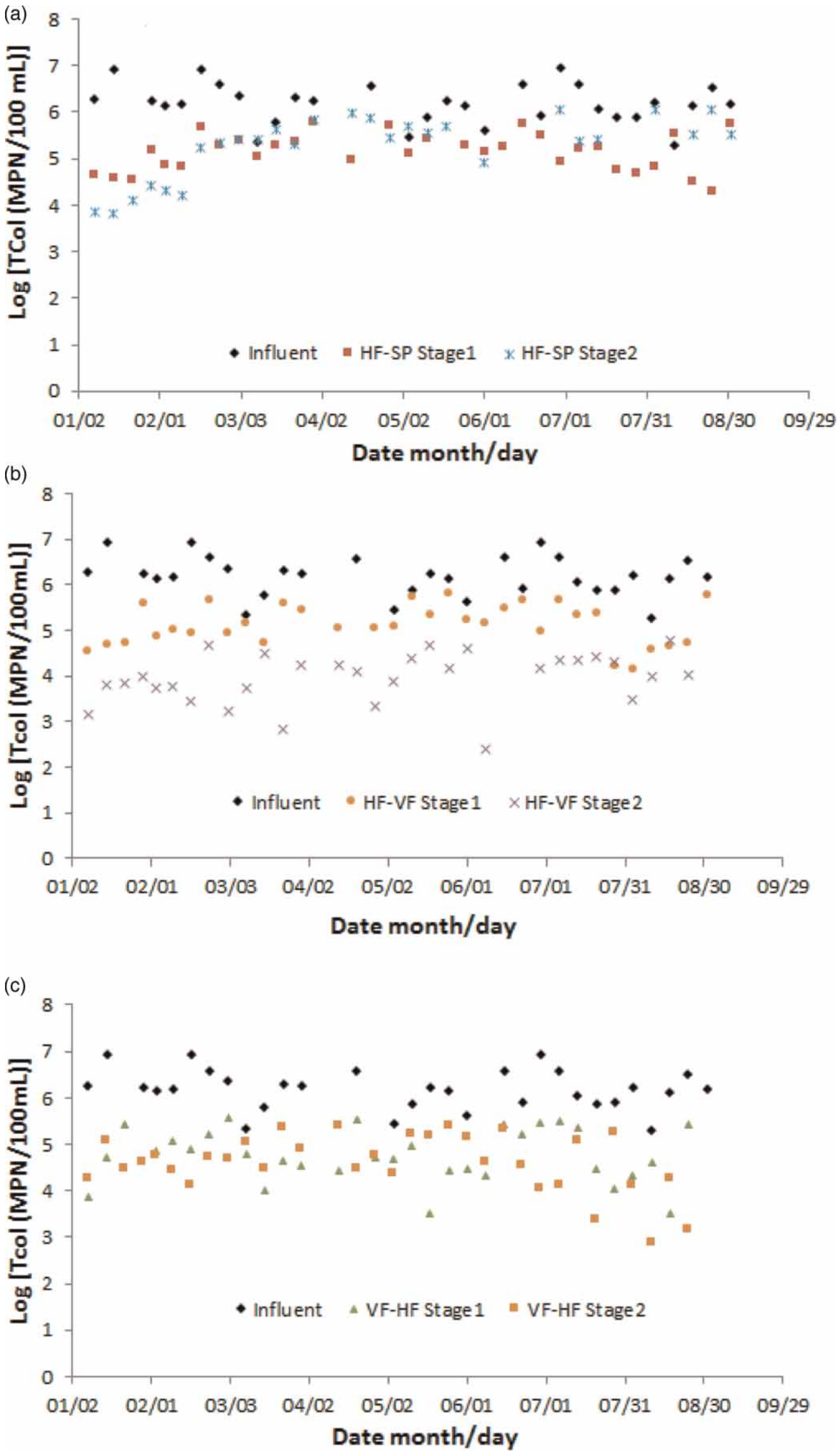

**Figure 1** | Removal of total coliforms in the three HCWs: (a) HF-SP system, (b) HF-VF system, (c) VF-HF system.

Figure 2(a)–2(c)). Probably, the appropriate selection of design factors such as the fine granulometry of the substrate and shallow HF beds contributed to these results. Moreover, the efficiencies of the two types of CWs fall in the range of 1.4–2.3 log units, obtained by Morató *et al.* (2014) in HF CWs designed with the purpose of evaluating pathogen removal. These authors concluded that some design factors are crucial to determine the efficiency of HF beds for pathogen removal, such as those mentioned above. In the second stage, in all cases an additional reduction of *E. coli* was observed (Figure 2(a)–2(c)), but the highest reduction was reached in the HF wetlands of the VF–HF system ($p < 0.05$); probably due to the direct discharge of the oxygenated effluent from VF units which allowed aerobic conditions in some extent in these CWs (as will be discussed later), along with the presence of macrophytes.

When comparing the global results during the first year of monitoring, it was found that the HF–VF and VF–HF systems were the most effective for *E. coli* removal ($p < 0.05$) in comparison to the HF–SP system, reaching similar reductions between 3 and 4 log units (Table 2). These results satisfy the aforementioned 2006 WHO guidelines that require a 3–4 log unit pathogen reduction by a wastewater treatment system, as a first step, to protect the health of those working in wastewater-irrigated fields and those consuming wastewater-irrigated food crops (Mara and Bos 2010). Moreover, if considering the previous settling stage of the wastewater, the pathogen log reduction during the whole wastewater treatment system would probably have been a little higher. In addition, these results fulfill the recommended required effluent quality of 1,000 MPN/100 mL of *E. coli* for irrigation of eaten-uncooked root crops (WHO 2006) and for reclaimed water reuse for agricultural irrigation purposes established in Mexico's current national guideline (SEMARNAT 1996). Similar to these findings, García *et al.* (2013a, b) reported a removal of 3.8 log units of *E. coli* in VF-HF wetland systems in a tropical area of Latin America.

## Performance of the systems during the second year

The concentration of additional pollutants in both the influent and the three systems regarding the second year were similar to the first period of evaluation (Table 3).

### System efficiency for total coliform removal

Throughout the second year, both HF–VF and VF–HF systems were equally effective and superior to HF–SP systems ($p < 0.05$) for TCol removal (Table 4). The monthly efficiency of the systems is reported in Figure 3(a)–3(c). No significant difference was found when comparing the efficiencies in the two defined seasons in this region, namely dry (October–May) and rainy season (June–September) ($p > 0.05$).

The global removal of the VF–HF system increased in comparison to the first year ($p < 0.05$) because of the better performance of the HF beds in the second stage. Also, the performance of HF wetlands (as first stage) in both HF–SP and HF–VF systems improved ($p < 0.05$) and was superior to the VF wetlands in the VF–HF system ($p < 0.05$) (Table 2 and Figure 2(b)) contrary to the first year. The performance of the VF wetlands remained analogous during the two periods of evaluation ($p > 0.05$). These results suggest that the HF beds achieved a higher maturity after 1 year in operation which made them more efficient for TCol removal. The presence of well-developed *C. indica* which reached a more than 2 m height might have strongly contributed to the pathogen removal. Significant higher TCol reductions ($p < 0.05$) were found when comparing the efficiency with small plants (May–August 2010) and well-established plants (September 2010 to August 2011) in all the HF CWs (mean log unit reduction of $0.62 \pm 0.15$ (SE) and $1.53 \pm 0.11$ (SE) with small and tall plants, respectively). An average reduction in the range of 1.21–1.72 log units was achieved in the HF beds in the three systems, values superior to the annual average of 0.93 log units in HF wetlands reported by Vymazal & Krôpfelová (2008). With regard to the HF–SP system, similar to the first year, an increase in TCol concentration was registered in every sample campaign (Figure 3(a)).

### Reduction of *E. coli* in the three HCWs

The average efficiency of the systems per month is shown in Figure 4(a)–4(c). Analogous to the first period of experimentation, the HF–VF and VF–HF systems were the most effective for *E. coli* removal (Table 4 and Figure 4)

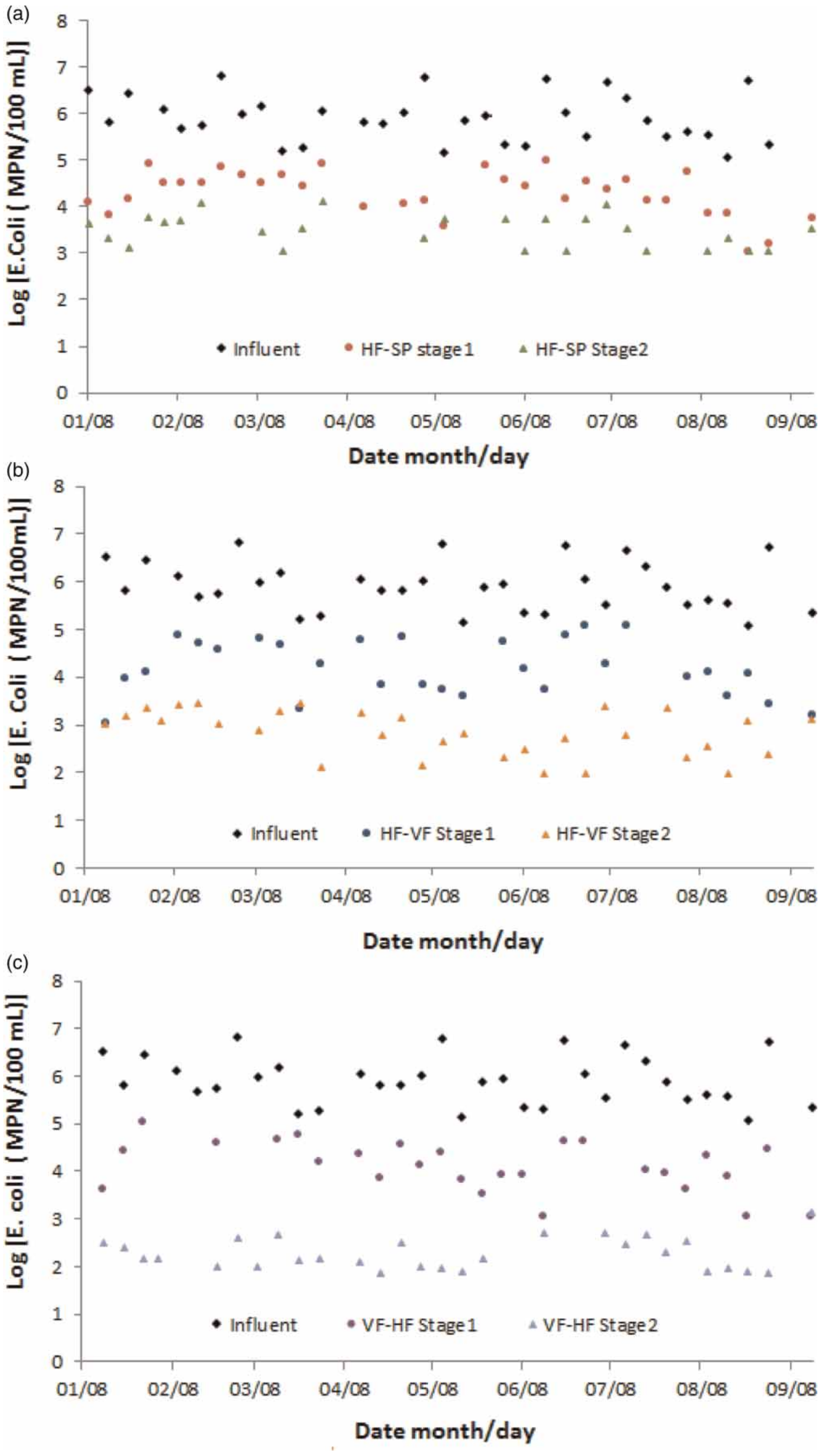

**Figure 2** | Removal of *E. coli* in the three HCWs: (a) HF-SP system, (b) HF-VF system, (c) VF-HF system.

($p < 0.05$). The reductions of 3–4 log units ($p > 0.05$) in *E. coli* densities were similar to those in the first year ($p > 0.05$). With regard to the individual performance of the CWs, the HF wetlands placed in the HF–SP and HF–VF systems were equally effective and better than the VF wetlands installed in the VF–HF system ($p < 0.05$). The efficiency of the HF wetlands (as first stage) increased significantly ($p < 0.05$) during the second year; contrary to the VF wetlands whose efficiencies remained the same ($p < 0.05$). According to many authors, plants play an important role in improving pathogen removal in constructed wetlands through several physicochemical mechanisms (Stottmeister *et al.* 2003). As mentioned above, during the second year, the HF beds reached a higher maturity noticeable by a vigorous growth of *C. indica* plants which probably allowed the higher efficiencies for *E. coli* removal. Similar to TCol removal, when the efficiencies with small and well-established plants were compared, significantly better results with the latter ones were obtained ($p < 0.05$) (mean log unit reduction of 1.52 ± 0.20 (SE) vs. 2.31 ± 0.15 (SE) and mean log unit reduction of 1.60 ± 0.22 (SE) vs. 2.21 ± 0.15(SE) for system I and system II, respectively). Leto *et al.* (2013) reported in a Mediterranean climate that the capacity of HF CWs improved significantly under densely vegetated conditions. Also, Azaizeh *et al.* (2013) found that *E. coli* lowered in 1.7 log units in a high-density planted system while decreased only in 0.7 log units in a low-density planted system. Moreover, this particular species has a higher evapotranspiration rate (ET) in comparison to other species like *Z. aethiopica* and *Typha latifolia* (data not shown). In general, high ET improves global treatment performances in constructed wetlands (Chazarenc *et al.* 2010). In HF wetlands, high ET might have slowed down the flow increasing the hydraulic retention time (HRT), exposing *E. coli* to the hostile environment predominant in CWs during a longer time and causing inactivation.

In the second stage of the three systems, in all cases, an additional reduction of *E. coli* was observed, but the highest reduction was reached in the HF wetlands of the VF–HF system similar to the first year of experimentation ($p < 0.05$). Some measurements taken inside these beds at the final section indicated average positive values of oxidation-reduction potential (ORP) (130 mV) and relatively high average concentrations of OD (2.8 mg/L), because of

**Table 3** | Performance summary for the three hybrid constructed wetlands with respect to COD, TN, TP, TSS and control parameters during the second year

| | | System I: HF–SP | | System II: HF–VF | | System III: VF–HF | |
|---|---|---|---|---|---|---|---|
| | **Influent** | **Stage 1** | **Stage 2** | **Stage 1** | **Stage 2** | **Stage 1** | **Stage 2** |
| COD (mg/L) | 235.2 ± 166.8 | 60.2 ± 39.4 | 257.6 ± 143.3 | 43.3 ± 15.8 | 35.0 ± 17.0 | 48.9 ± 41.1 | 54.5 ± 36.9 |
| Total N (mg/L) | 99.8 ± 44.6 | 69.4 ± 35.5 | 42.4 ± 24.2 | 69.7 ± 35.2 | 58.6 ± 23.1 | 76.9 ± 32.2 | 53.4 ± 41.0 |
| Total P (mg/L) | 8.1 + 3.4 | 5.2 + 2.5 | 6.7 + 3.0 | 5.4 + 2.5 | 5.5 + 2.0 | 6.31 + 3.0 | 0.93 + 1.1 |
| TSS (mg/L) | 65.6 ± 50.4 | 4.8 ± 3.2 | 99.6 ± 61.3 | 4.2 ± 3.1 | 4.1 ± 3.1 | 5.0 ± 3.8 | 3.8 ± 3.5 |
| DO (mg/L) | 3.0 ± 2.4 | 6.7 ± 1.3 | 8.0 ± 2.1 | 5.5 ± 1.1 | 6.2 ± 1.0 | 7.6 ± 1.0 | 5.8 ± 1.7 |
| pH | 8.2 ± 0.30 | 7.6 ± 0.22 | 8.0 ± 0.38 | 7.7 ± 0.22 | 6.8 ± 0.0.70 | 6.8 ± 0.83 | 6.0 ± 0.92 |
| Conductivity (µS/cm) | 1418 ± 492 | 1397 ± 511 | 1116 ± 375 | 1319 ± 523 | 1036 ± 404 | 1045 ± 366 | 1151 ± 578 |

Average ± SD, $n \sim 23$–26.

**Table 4** | Performance summary for the three hybrid constructed wetlands with respect to total coliforms and *E. coli*, during the second year

| | **Concentrations** | | | |
|---|---|---|---|---|
| | **TCol × $10^4$ (MPN/100 mL)** | ***E. coli* × $10^4$ (MPN/100 mL)** | **Removal (%)** | |
| Influent | 420 ± 8.8 | 220 ± 84 | | |
| System I (HF–SP) | | | | |
| Stage 1 (HF) | 11 ± 4.8 | 1.3 ± 0.7 | 97.38 *(1.58)* | 99.41 *(2.23* |
| Stage 2 (SP) | 81 ± 34 | 0.43 ± 0.24 | *(−0.87)*[a] | *66.57 (0.48)* |
| | | | **80.71 (*0.71*)** | **99.80 (*2.71*)** |
| System II (HF–VF) | | | | |
| Stage 1 (HF) | 8 ± 4.3 | 1.9 ± 1.1 | 98.10 *(1.72)* | 99.14 *(2.06)* |
| Stage 2 (VF) | 1.5 ± 0.88 | 0.10 ± 0.08 | 81.25 (*0.72*) | 94.58 (*1.28*) |
| | | | **99.64 *(2.44)*** | **99.95 *(3.44)*** |
| System III (VF–HF) | | | | |
| Stage 1 (VF) | 31 ± 18 | 7.6 ± 4.4 | 92.62 (*1.13*) | 96.55 (*1.46*) |
| Stage 2 (HF) | 1.9 ± 1.2 | 0.04 ± 0.02 | 93.87 (*1.21*) | 99.51 (*2.31*) |
| | | | **99.55 (*2.34*)** | **99.98 (*3.74*)** |

[a] Negative removal (an increase in the output concentration).

Average (×$10^4$) ± standard error of the mean. Reduction in logarithmic units is in parentheses. Entire system removal percentages as well as global reduction in logarithmic units are in bold font.

the direct discharge of the oxygenated VF wetland effluent on the HF beds. It is well-documented that aerobic conditions are hostile to indicator organisms.

The impact of the season (dry and rainy) in the global effectiveness of the systems for *E. coli* removal was not significant ($p > 0.05$) despite the change in the environmental conditions, such as temperature and the presence of rain. This behavior demonstrates the robustness of the hybrid systems in subtropical–tropical climates for the removal of pathogens, in contrast to what was argued by García *et al.* (2013a, b).

For agriculture irrigation it is important to reduce pathogen levels before wastewaters are used for crop irrigation (WHO 2006; Jimenez *et al.* 2010); the higher the reduction, the better the system. The results during the two periods of evaluation definitively demonstrated that the HF–SP system is the least effective one for pathogen removal and this is owing to the presence of SP instead of a VF CW. Other authors have demonstrated that SP are less effective than CWs for pathogen removal (García *et al.* 2008) and its land area requirement can be up to three times the area per person equivalent to what would be required for HF CW to treat the same amount of wastewater (Mburu *et al.* 2013). However, the HF–SP system is the simplest, lowest-cost and lowest-maintenance option and could be preferred in developing countries where skilled labor and economic resources are scarce. The VF CW is the type of constructed wetlands with the least land area requirement but its O&M cost increases even with respect to HF CWs, mainly because of the need for intermittent feeding by pumping which implies electricity consumption; furthermore, it requires skilled labor for design, construction and monitoring (Morel & Diener 2006). This means that for poor rural areas of Latin America where there is more availability of land, the HF–SP could probably be the most recommended system. Furthermore, the log unit reduction reached in the effluent of the HF–SP system could be combined with other locally feasible health-protection measures (type of irrigation and/or type of crops, etc.) in order to achieve the health-based target in practice that is defined by the WHO (2006).

## CONCLUSIONS

This study, performed in a subtropical climate, confirms the results reported by other authors with regard to the capacity

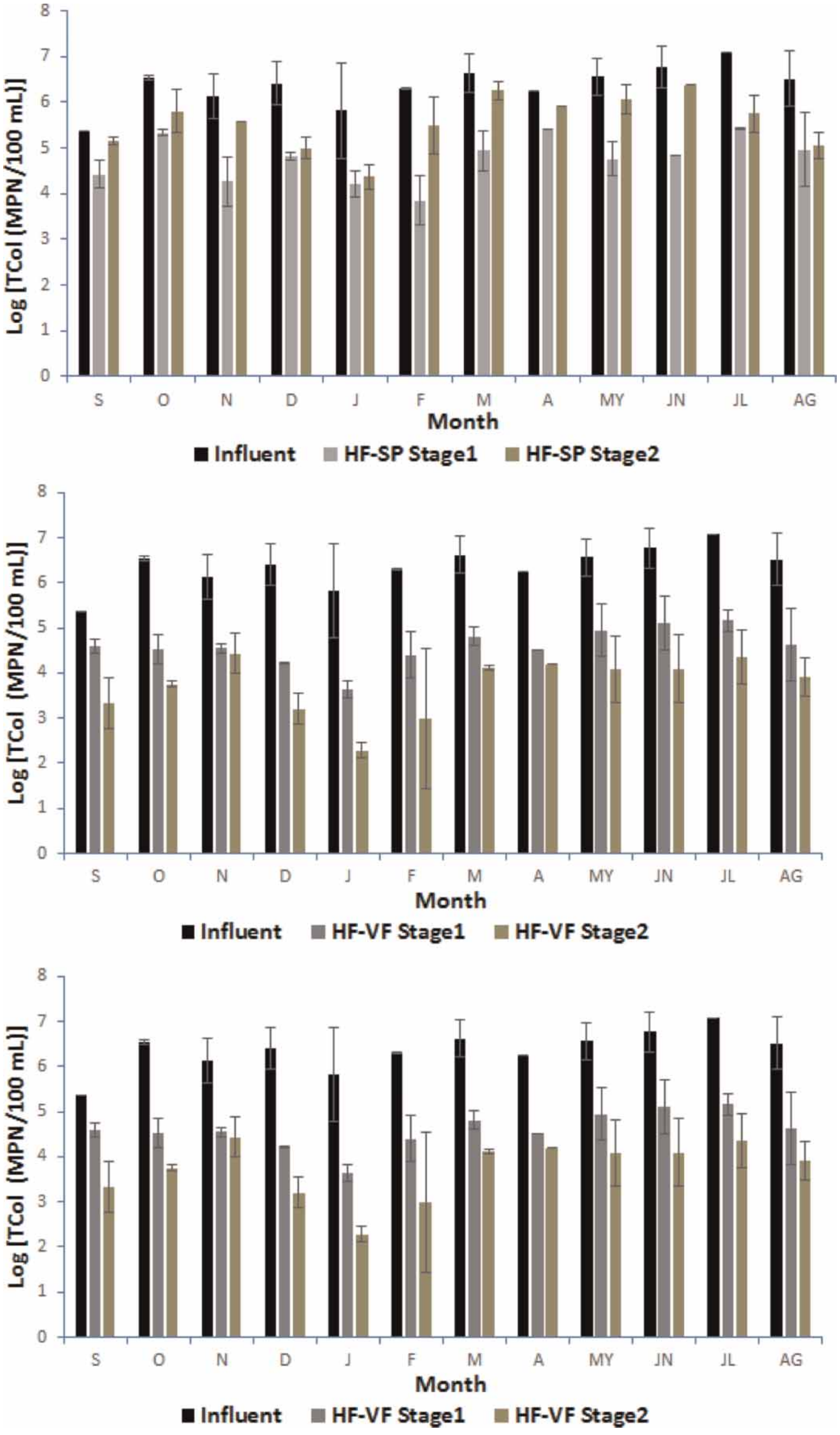

**Figure 3** | Removal of TCol in the three HCWs ($n \sim 2$–3; mean ± SD): (a) HF-SP system, (b) HF-VF system, (c) VF-HF system during the second year. S, September; O, October; N, November; D, December; J, January; F, February; M, March; A, April; MY, May; JN, June; JL, July; AG, August.

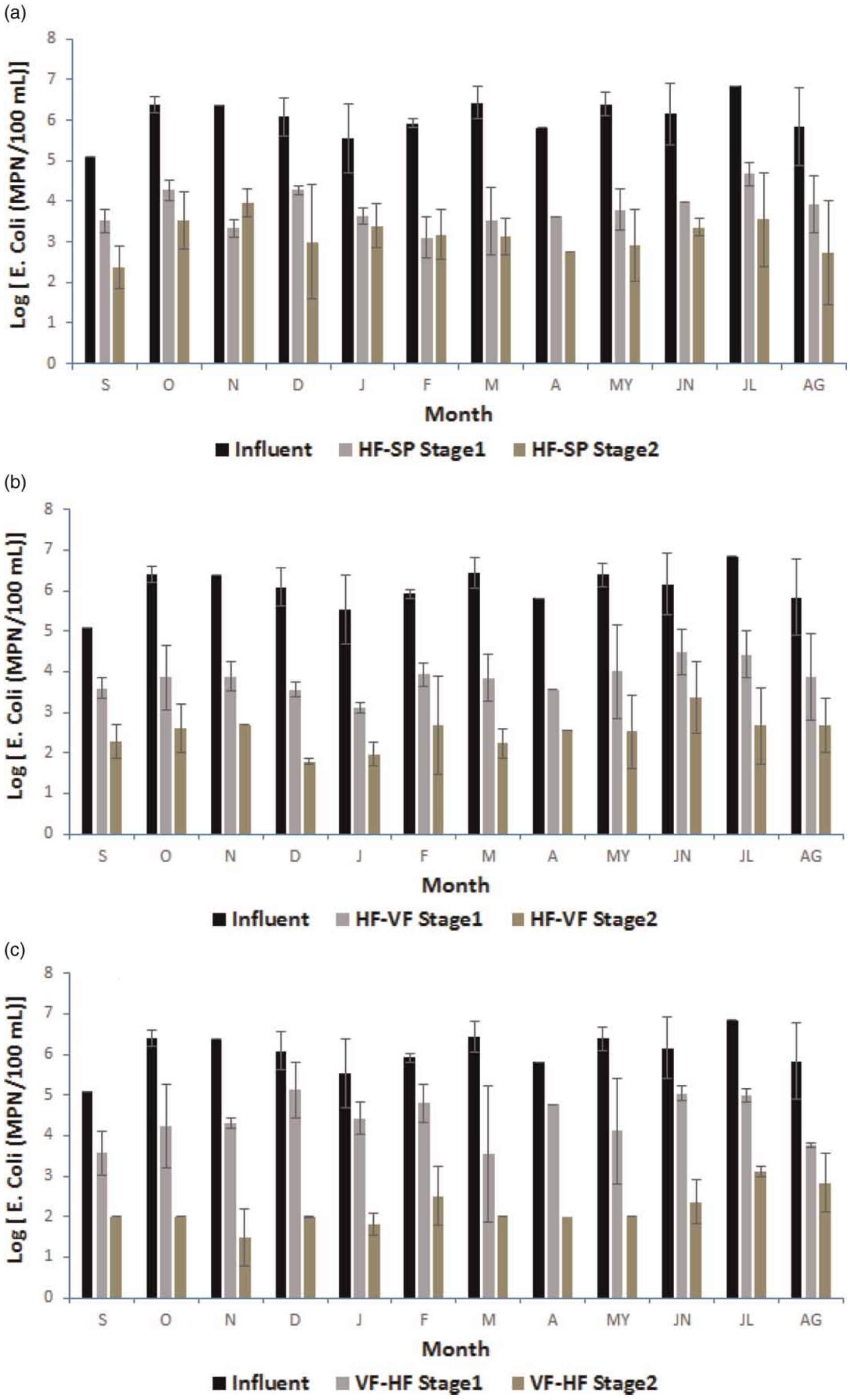

**Figure 4** | Removal of *E. coli* in the three HCWs ($n \sim 2$–3; mean ± SD): (a) HF-SP system, (b) HF-VF system, (c) VF-HF system throughout the second year. S, September; O, October; N, November; D, December; J, January; F, February; M, March; A, April; MY, May; JN, June; JL, July; AG, August.

of hybrid constructed wetlands for pathogen removal. In addition, it was found that *E. coli* is a more reliable indicator organism than TCol, which exhibited an evident capacity to reproduce in the evaluated systems. The three hybrid constructed wetlands were highly effective during the two periods of evaluation. However, only those that included a VF component were capable of lowering the *E. coli* density in 3.20–3.83 log units, fulfilling the WHO guidelines for wastewater treatment systems, as a first step, to protect human health. Furthermore, the final effluent concentration in these two systems complies with the <1,000 MPN/100 mL Mexican standard for treated wastewater reuse in agriculture. Although the difference was not significant, the VF–HF system tended toward a better performance for *E. coli* removal when compared to the HF–VF system during the two periods of evaluation. The efficiency of the HF beds improved during the second year of evaluation because of the maturity reached by the systems, noticeable through the presence of well-developed macrophytes. This was possible due to the fact that in tropical and subtropical climates, the growing season ordinarily lasts all year in contrast to cold temperate climates where macrophytes exhibit a growing–senescence cycle. Despite this improvement in individual treatments, the global efficiency of both HF–VF and VF–HF systems for *E. coli* removal did not significantly improve in the second year.

With these results, we have demonstrated that in tropical and subtropical climates, it is possible to remove harmful pathogenic organisms by using at least two-stage hybrid constructed wetlands with a pre-treatment stage. These types of low-cost technologies capable of producing disinfected reclaimed wastewater without the use of expensive disinfectants are needed in poor areas of Latin America where the reuse of raw wastewater in agriculture is endangering human health.

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