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Influence of chlorothalonil on the removal of organic matter in horizontal subsurface flow constructed wetlands

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This study investigates the effects of chlorothalonil (CLT) on chemical oxygen demand (COD) and dissolved organic carbon (DOC) in pilot-scale horizontal subsurface flow constructed wetlands (HSSFCW) planted with *Phragmites australis*. Physicochemical parameters of influent and effluent water samples, microbial population counting methods and statistical analysis were used to evaluate the influence of CLT on organic matter removal efficiency. The experiments were conducted on four planted replicate wetlands (HSSFCW-Pa) and one unplanted control wetland (HSSFCW-NPa). The wetlands exhibited high average organic matter removal efficiencies (HSSFCW-Pa: 80.6% DOC, 98.0% COD; HSSFCW-NPa: 93.2% DOC, 98.4% COD). The addition of CLT did not influence organic removal parameters. In all cases CLT concentrations in the effluent occurred in concentrations lower than the detection limit of the analytical method. Microbial population counts from HSSFCW-Pa showed significant correlations among different microbial groups and with different physicochemical variables. The apparent independence of organic matter removal and CLT inputs, along with the CLT depletion observed in effluent samples demonstrated that HSSFCW are a viable technology for the treatment of agricultural effluents contaminated with organo-chloride pesticides like CLT.

Keywords: *Phragmites australis*, chlorothalonil, constructed wetlands, chemical oxygen demand, dissolved organic carbon, biofilm.

Introduction

The use of pesticides in agricultural production has allowed food production to support an ever-increasing global population. Extensive use of pesticides however has polluted water, land and placed organisms that consume agricultural products, including humans, at risk.[1,2] Numerous studies have linked pesticides to the occurrence of different types of cancer in humans.[3,4] Exposure to pesticides, even at low doses, has been shown to alter the function of the immune system, affecting lymphocyte and autoantibody levels, and causing hypersensitivity.[5,6] Acute contact of skin or mucus with certain pesticides can cause inflammation, itching and dermatitis.[7] Inhalation of pesticides can cause irritation of the respiratory tract, coughing, dysphonia and other allergic reactions.[8] One of the more commonly used pesticides in the agricultural sector is chlorothalonil (CLT; 4,5,6,-tetrachloro-1,3-benzodicarbonitrile). This agro-chemical represents 15% (by weight) of all fungicides used in the world annually. In the United States, its annual consumption is estimated to be between four and six million kilograms. CLT is most commonly applied in the U.S. to peanut crops (34%), tomato crops (12%) and golf courses (10%).[9] The annual consumption of CLT exceeds 8.7 million kg in Colombia, where it is commonly used as a fungicide on tomato, potato and banana crops.[10]

CLT occurs as a stable crystalline solid at room temperature. It maintains its stability in the presence of ultraviolet light in both the solid state and in aqueous media, including acidic aqueous solutions. In alkaline solutions,

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CLT is moderately stable but slowly hydrolyzes above pH 9.0.[11] Its solubility in water is relatively low (0.6 mg L^{-1} at 25°C), leading to moderate mobility in sandy soils (log $K_{oc} = 3.14$).[12] The CLT half-life in soil ranges from 1.5 to 3 months. In aerobic or anaerobic aqueous environments, CLT half-life may be as little as 8 and 5 days, respectively.[13]

Horizontal subsurface flow constructed wetlands (HSSFCW) have recently emerged as a viable alternative technology for treating agricultural wastewater polluted with pesticides.[14,15] HSSFCW depuration systems operate primarily through chemical reactions within microbial biofilms that develop in the granular material and macrophyte roots structures of the soil matrix.[16,17] The toxicity of pesticides such as CLT has been documented for a large number of microorganisms.[18,19] The primary influence that pesticides exert on biological processes in wetland treatment systems is to reduce efficiency of organic matter removal by microbial populations. Although pesticides and organic matter are well-known components of agricultural effluents, their interactions have not been widely studied.[20,21] The effect of CLT on the removal of organic matter is therefore unknown. This research quantified the removal of organic matter in the presence of CLT using pilot-scale HSSFCW. The study aimed to establish the influence of CLT on organic matter removal efficiency, and further ascertain the utility of these treatment systems for the depuration of agricultural effluents.

Materials and methods

Experimental procedures

This study used five fiberglass HSSFCW chambers (1.0 m long × 0.6 m wide × 0.6 m high) installed at the research building of the University of Antioquia, Colombia (6° 15′37.58″ N, 75° 34′5.08″ W, 1532 m). The HSSFCW contained gravel beds packed in plastic mesh and were monitored with piezometers located at influent and effluent (0.05 m diameter). Each HSSFCW included a 0.3 m thick gravel layer infiltrated with water to a level of 0.2 m. Gravel clasts were 3.2–6.4 mm in diameter, with a specific gravity of 2.5. Four HSSFCW were planted with *Phragmites australis* (6 plants m^{-2}; HSSFCW-Pa), and one unit served as an unplanted control (HSSFCW-NPa). The HSSFCW were equilibrated using a constant flow (4.6 mL min^{-1}) of domestic wastewater for one month. Following the equilibration period, synthetic sewage[22] containing CLT was introduced to each HSSFCW. Influent injections included three different pesticide concentrations (85 μg L^{-1}, 240 μg L^{-1} and 385 μg L^{-1}) accompanied by three different concentrations of glucose (20 μg L^{-1}, 100 μg L^{-1} and 150 μg L^{-1}), applied to each HSSFCW. The influents were introduced continuously starting with the lowest concentration of CLT. CLT concentrations in the influent were selected so as not to exceed the maximum solubility of the pesticide in water (25°C), and concentrations of glucose were based on the range observed from polluted river systems in Colombia.

Sampling and analysis

Sampling was carried out simultaneously at the inlet and outlet apertures of each HSSFCW. The HSSFCW-NPa was fully equilibrated by the end of the experiment, with 20 mg L^{-1} glucose measured in influent and effluent. Prior to sampling, each HSSFCW was infiltrated with 100 and 150 mg L^{-1} glucose. Samples were collected 5, 10 and 15 days after each treatment for four months. Dissolved oxygen (DO), pH, redox potential (ORP), water temperature and electrical conductivity were measured *in situ* according to standard methods.[23]

Dissolved organic carbon (DOC) was quantified by wet combustion (phosphoric acid 5% v/v and sodium persulfate) and voltammetry with a non-dispersive infrared detector using a model 1010 OI analytical carbon analyzer. Blank samples were filtered prior to analysis (0.45 μm) and chemical oxygen demand (COD) was determined by flame photometry using a Linus Nanocolor 500D photometer.

CLT was quantified using an Agilent Technologies model 6890 gas chromatograph (GC) with an auto-injector and microcapture electron detector. The GC used a HP-5 column (30 m × 0.322 mm × 0.25 mμ; methylpolysiloxane stationary phase [2.8 mL min^{-1}]), and N_2 carrier gas (60 mL min^{-1}). The temperature program ran from 80°C to 180°C at 30°C min^{-1} and then from 180°C to 205°C at 3°C min^{-1}. The temperatures of the injector and detector were 290°C and 300°C, respectively. For each analysis, a 1 mL sample volume was injected in splitless mode. Calibration curves were prepared using analytical grade CLT (98% purity; Chem Service).

To quantify bacterial communities, gravel from the packed beds was carefully removed. Biofilms attached to clast surfaces were collected following the procedures described by Morató.[24] The biofilms were removed by sonicating the clasts for 3 minutes in 100 mL of sterile NaCl (0.9%). The rinse solution was collected in sterile glass containers and sealed. Biofilm material was homogenized by mechanical or manual stirring. Rinse solutions were subjected to a fourfold serial dilution (10^{-1} to 10^{-4}). Duplicated dilutions were plated by inoculating (1 mL) several types of media, selected according to the type of microbial communities under investigation. Communities analysed included (i) total heterotrophic bacteria, (ii) *Pseudomonas spp*. and iii) anaerobic bacteria. Heterotrophic bacteria were grown on Plate Count Agar (Merck®), and *Pseudomonas-Aeromonas* on Selective Agar GSP (Merck®). Anaerobic species were grown on Brewer Anaerobic Agar (Merck®). Bacterial culture estimates were performed according to standard methods.[23]

Statistical analysis

Statistical analysis of the results was carried out using SPSS (version 16) and R software packages. Preliminary analysis established some basic statistical assumptions concerning the dataset. The Kolmogorov-Sminorv test was used to verify normal distributions among dependent variables. Mixed models were applied to compare the effects of different experimental conditions on microbial population data. The association between the physicochemical and microbial population variables was evaluated with the Spearman correlation test. The Kruskal-Wallis test was used to identify significant differences in the removal efficiencies of DOC and COD associated with different concentrations of CLT and wetland types. Except for the Spearman correlation test ($p \leq 0.05$ and $p \leq 0.01$), all statistical parameters were calculated at a significance level of $p \leq 0.05$.

Results

Physicochemical parameters

Basic statistics for the physicochemical parameters of the HSSFCW experiments are presented in Table 1. The parameters showing the greatest degree of variability were ORP (VC = 0.6842 to -2.877) and DO (VC = 1.210 to 1.4415). Average temperature ranged from 19.7°C to 28.4°C and showed relatively little variation within that range (σ = 1.7°C to 2.5°C). Average DO in influent samples (3.2 mg L^{-1} to 3.3 mg L^{-1}) and its associated variation (σ = 1.3 mg L^{-1} to 1.4 mg L^{-1}) showed a marked decrease in effluent samples from all HSSFCW (46.88% to 54.55% for DO average values and 71.4% to 76.9% for standard deviations). Statistical parameters associated with pH measurements exhibited the same behavior. Average influent conductivity ranged from 1312.7 μS cm^{-1} to 1322.7 μS cm^{-1}. Conductivity decreased in effluent samples from all HSSFCW by 7.3% to 21.0% relative to that measured in influent samples, but effluent samples showed greater variation in conductivity (σ = 174.2% to 262.9% of respective influent values). Average influent ORP ranged from −112.7 mV to −78 mV whereas effluent ORP ranged from −49 mV to 228 mV.

Organic matter removal

DOC removal. DOC removal efficiency in HSSFCW-NPa varied from 86.9% to 97.9% with an average median removal efficiency of 93.2% (Figs. 1a, 1b and 1c). Minimum median removal (87.6%) was observed for the 240 μg L^{-1} CLT and 100 mg L^{-1} glucose application (Fig. 1b). The greatest difference between average and median removal efficiencies was associated with the 240 μg L^{-1} CLT and 150 mg L^{-1} glucose application (0.3%; Fig. 1b). The 85 μg L^{-1} CLT and 150 mg L^{-1} glucose application gave the maximum standard deviation in removal efficiency (σ = 1.8%; Fig. 1a).

DOC removal efficiency in HSSFCW-Pa varied from 30.7% to 94.2% with an average median removal efficiency of 80.6% (Figs. 1d, 1e and 1f). The 240 μg L^{-1} CLT and

Table 1. Physicochemical parameters from influent and effluent samples of pilot-scale horizontal subsurface flow constructed wetlands with *Phragmites australis* (HSSFCW-Pa) and unplanted (HSSFCW-NPa).

Wetland type	*Sampling site*	*Parameter*	*Units*	*N*	*Median*	*Mean*	*SD*[a]	*VC*[b]	*Minimum*	*Maximum*
HCFHSS-NPa	Influent	T[c]	°C	18	24.90	24.30	2.50	0.103	18.30	27.70
		DO[d]	mg L^{-1}	18	3.40	3.20	1.30	0.406	0.80	5.30
		pH	Units	9	5.16	5.21	0.75	0.144	3.68	6.39
		Cond[e]	μS cm^{-1}	18	1287.50	1312.70	177.20	0.135	1008	1674
		Redox	mV	18	−124	−112.17	64.20	0.572	−227	−20
	Effluent	T	°C	18	25.70	25.10	2	0.080	20.10	28.30
		DO	mg L^{-1}	18	1.60	1.70	0.30	0.176	1.30	2.30
		pH	Units	18	3.56	3.68	0.83	0.226	2.32	6.65
		Cond	μS cm^{-1}	18	911.50	1037	308.60	0.298	697	1646
		Redox	mV	18	267	228	156	0.684	−277	383
HCFHSS-Pa	Influent	T	°C	108	25	24.30	2.30	0.095	18.50	28.40
		DO	mg L^{-1}	100	3.60	3.30	1.40	0.424	0.40	6.90
		pH	Units	36	5.72	5.64	0.3628	0.064	4.58	6.30
		Cond	μS cm^{-1}	108	1291.00	1322.7	175.00	0.132	1006	1730
		Redox	mV	103	−115	−78	1140	1.461	−192	318
	Effluent	T	°C	107	24	23.90	1.70	0.071	19.70	28.10
		DO	mg L^{-1}	95	1.40	1.50	0.40	0.267	0.80	2.85
		pH	Units	105	5.72	5.46	0.96	0.176	3.50	8.78
		Cond	μS cm^{-1}	106	1154	1226.70	468.90	0.382	418	2350
		Redox	mV	103	−99	−49	141	−2.877	−219	371

[a]Standard deviation, [b]Variation coefficient, [c]Temperature, [d]Dissolved oxygen, [e]Electrical conductivity.

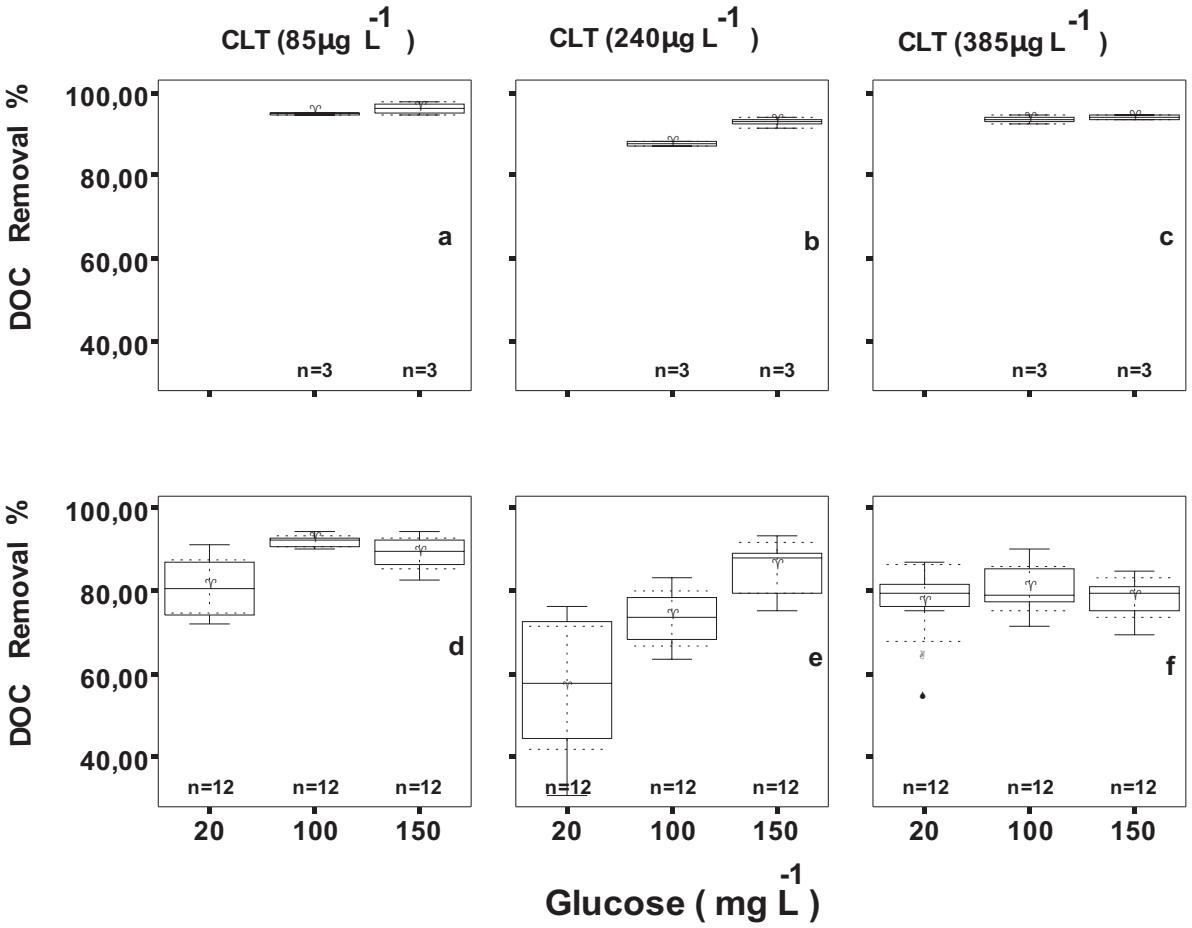

Fig. 1. Percentage removal of dissolved organic carbon (DOC) in pilot-scale HSSFCW without emergent macrophytes (a, b and c) and planted with *Phragmites australis* (d, e and f), given different input concentrations of chlorothalonil (CLT) and glucose. The black circles show average values and the dotted lines indicate standard deviations. The solid lines within the boxes represent median values. The boxes represent interquartile ranges that include 50% of the values. The solid parallel lines outside the box represent minimum and maximum values. The star and circle represent outliers and extreme values, respectively.

20 mg L^{-1} glucose application gave the minimum median removal efficiency (57.5%; Fig. 1e). The greatest difference between average and median removal efficiencies (6.34%) was observed for the 85 μg L^{-1} CLT and 100 mg L^{-1} glucose application (Fig. 1d). The 240 μg L^{-1} CLT and 20 mg L^{-1} glucose application gave the maximum standard deviation ($\sigma = 14.8\%$; Fig. 1e).

COD removal. COD removal efficiency in HSSFCW-NPa varied from 67.2% to 100% with an average median removal efficiency of 98.4% (Figs. 2a, 2b and 2c). The minimum median removal (97.5%) was observed for the 240 μg L^{-1} CLT and 100 mg L^{-1} glucose application (Fig. 2c). The 85 μg L^{-1} CLT and 100 mg L^{-1} glucose application gave the greatest difference between average and median removal efficiencies (10.9%) and the maximum standard deviation (18.9%; Fig. 2c).

COD removal efficiency in HSSFCW-Pa varied from 48.8% to 100% with an average median removal efficiency of 98.0% (Figs. 2d, 2e and 2f). Minimal median removal (85.3%) was associated with the 85 μg L^{-1} CLT and 20 mg L^{-1} glucose application (Fig. 2d). The greatest difference between average and median removal efficiencies (15.7%) occurred with the 240 μg L^{-1} CLT and 20 mg L^{-1} glucose application (Fig. 2e). The 385 μg L^{-1} CLT and 20 mg L^{-1} glucose application gave the maximum standard deviation ($\sigma = 23.2\%$; Fig. 2f).

Biofilm bacterial populations

Total heterotrophic bacteria count. The average number of total heterotrophic bacteria counted from influent samples of HSSFCW-NPa ranged from 4.6 to 6.9 (4.0×10^4 CFUmL^{-1} to 8.4×10^6 CFUmL^{-1}), whereas effluent samples contained an average heterotrophic bacteria count of 4.7 to 5.4 (4.5×10^4 CFU mL^{-1} to 2.3×10^5 CFUmL^{-1}; Fig. 3a). The 85 μg L^{-1} CLT and 150 mg L^{-1} glucose application gave the maximum variability in heterotrophic

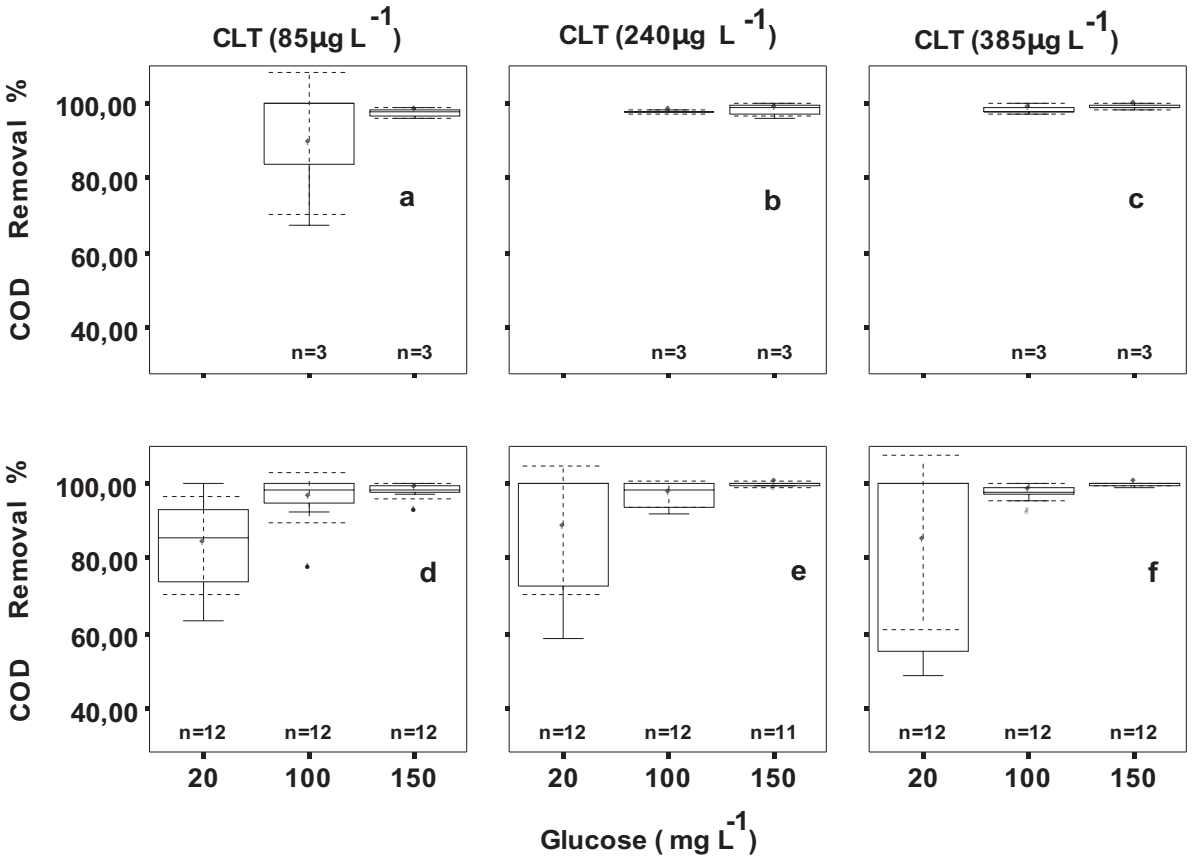

Fig. 2. Percentage removal of chemical oxygen demand (COD) in pilot-scale HSSFCW without emergent macrophytes (a, b and c) and planted with *Phragmites australis* (d, e and f), given different input concentrations of chlorothalonil (CLT) and glucose. The black circles show average values and the dotted lines indicate standard deviations. The lines within the box represent median values. The boxes represent interquartile ranges that include 50% of the values. The solid parallel lines outside the box represent the minimum and maximum values. The star and circle represent outliers and extreme values, respectively.

bacteria count ($\sigma = 0.67 \times 10^2$ CFU mL^{-1}) observed in influent samples (Fig. 3a). The 385 μg L^{-1} CLT and 100 mg L^{-1} glucose gave the maximum variability ($\sigma = 0.08 \times 10^2$ CFU mL^{-1}) observed in effluent samples (Fig. 3a).

Average number of total heterotrophic bacteria counted from influent samples of HSSFCW-Pa ranged from 6.3 to 7.3 (1.8×10^6 CFUmL^{-1} to 2.1×10^7 CFUmL^{-1}). Counts from effluent samples were lower, ranging from 5.1 to 6.5 (1.1×10^5 CFUmL^{-1} to 3.3×10^6 CFUmL^{-1}; Fig. 3a). The 240 μg L^{-1} of CLT and 150 mg L^{-1} glucose application gave the maximum variability in counts observed for both influent samples ($\sigma = 0.05 \times 10^2$ CFU mL^{-1}) and effluent samples ($\sigma = 0.08 \times 10^2$ CFU mL^{-1}; Fig. 3a).

Pseudomonas spp. count. The average number of *Pseudomonas spp.* counted from HSSFCW-NPa ranged from 3.3 to 6.0 (2.10×10^3 CFU mL^{-1} to1.03×10^7 CFU mL^{-1}) in influent samples, and from 2.7 to 4.2 (4.8×10^2 CFU mL^{-1} to 1.5×10^4 CFU mL^{-1}) in effluent samples (Fig. 3b). The 85 μg L^{-1} CLT and 150 mg L^{-1} glucose application gave the maximum variability for both the influent ($\sigma = 0.4 \times 10^2$ CFU mL^{-1}) and effluent samples ($\sigma = 1.7 \times 10^2$ CFU mL^{-1}; Fig. 3b).

The average number of *Pseudomonas spp.* counted from HSSFCW-Pa ranged from 4.2 to 6.7 (8.4×10^4 CFU mL^{-1} to 7.3×10^6 CFU mL^{-1}) for influent samples, and from 3.2 to 5.3 (1.3×10^3 CFU mL^{-1} to 2.0×10^5 CFU mL^{-1}) for effluent samples (Fig. 3b). The 85 μg L^{-1} CLT and 150 mg L^{-1} glucose application gave the maximum variability observed among influent samples ($\sigma = 0.06 \times 10^2$ CFU mL^{-1}). The 240 μg L^{-1} CLT and 150 mg L^{-1}) glucose application gave the maximum variability observed among effluent samples ($\sigma = 0.15 \times 10^2$ CFU mL^{-1}; Fig. 3b).

Anaerobic bacteria count. The average number of anaerobic bacteria counted from HSSFCW-NPa ranged from 5.9 to 6.6 (7.0×10^4 CFU mL^{-1} to 4.0x 10^6 CFU mL^{-1}) for influent samples, and from 2.2 to 4.3 (2.2×10^2 CFU mL^{-1} to 2.1×10^4 CFU mL^{-1}) for effluent samples (Fig. 3c). The 85 μg L^{-1} CLT and 150 mg L^{-1} glucose application gave the maximum variability for both effluent samples

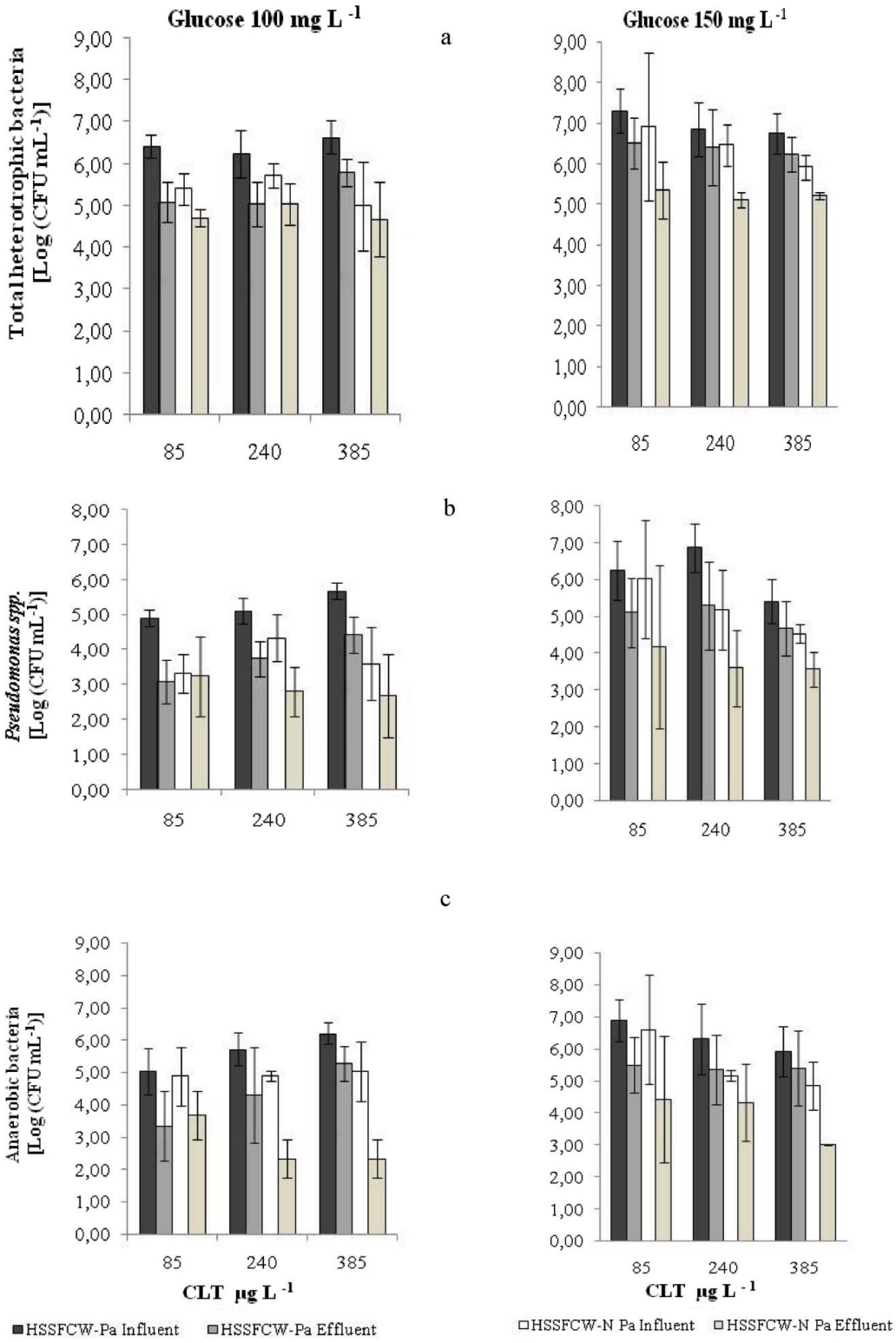

Fig. 3. Microbial population counts from biofilms in pilot-scale HSSFCW without emergent macrophytes (HSSFCW-NPa) and planted with *Phragmites australis* (HSSFCW-Pa), given different input concentrations of chlorothalonil (CLT) and glucose: a) Total heterotrophic bacteria, b) Anaerobic bacteria, c) *Pseudomonas spp.* (color figure available online).

($\sigma = 1.0 \times 10^2$ CFU mL^{-1}) and influent samples ($\sigma = 0.5 \times 10^2$ CFU mL^{-1}; Fig. 3c).

The average number of anaerobic bacteria counted from the HSSFCW-Pa ranged from 5.0 to 6.9 (1.1×10^5 to 7.5×10^6 CFUmL^{-1}) in influent samples, and 3.3 to 5.5 (2.2×10^3 to 3.1×10^5 CFUmL^{-1}) in effluent samples (Fig. 3c). The 240 μg L^{-1} CLT and 100 mg L^{-1} glucose application gave the maximum variability observed among effluent samples ($\sigma = 0.3 \times 10^2$ CFU mL^{-1}). The 240 μg L^{-1} CLT and 150 mg L^{-1} glucose application gave the maximum variability observed among influent samples ($\sigma = 0.13 \times 10^2$ CFU mL^{-1}; Fig. 3c).

Discussion

Except in the case where glucose was added at 150 mg L^{-1} in HSSFCW-NPa, the addition of CLT exerted a relatively strong negative effect ($P \leq 0.05$) on DOC removal efficiency. From a technical and practical standpoint, the reduction in DOC removal efficiencies by CLT was not so great that it would preclude the use of HSSFCW as a treatment for agricultural wastewater. These experiments achieved average median removal efficiencies (80.6% to 93.2%) that match or exceed those reported for other biological treatment systems.[25]

The decrease in DOC removal efficiency associated with higher CLT inputs (given varying concentrations of glucose) was an expected result. The average removal efficiency in each type of HSSFCW was higher for the 85 μg L^{-1} CLT application than for the 385μg L^{-1} CLT application, and lowest for the 240 μg L^{-1} CLT application (Fig. 1). The lower removal efficiency associated with the 240 μg L^{-1} CLT application may be due to the adaptation of microbial populations participating in organic matter degradation in response to a threshold concentration of the pesticide. Significant differences ($p \leq 0.05$) in DOC removal between the two types of HSSFCW were observed for the entire range of CLT and glucose applications (except for the 240 μg L^{-1} CLT and 150 mg L^{-1} glucose application). HSSFCW-NPa exhibited greater removal efficiencies (average 93.2%) than HSSFCW-Pa (average 80.6%). This suggests that the emergent macrophyte *P. australis* inhibited dissolved organic matter removal. These results are consistent with those reported in other studies.[26,27,28] The apparent reduction in dissolved organic matter removal could actually be due to the addition of carbon exuded by macrophyte root structures into effluent fluids.[29,30] The environmental science community has not reached consensus concerning this mechanism. Studies have not identified consistent trends in organic matter removal from wetlands that include macrophytes versus those that do not.[17,31,32] On the other hand, some studies have noted that organic matter removal is significantly higher in wetlands containing macrophytes.[27]

CLT had no effect ($p \leq 0.05$) on the removal efficiency of COD (except in the case where glucose was added at 150 mg L^{-1} in HSSFCW-Pa). The independence of COD and CLT variables contrasted trends observed for the DOC variable, suggesting that different processes perform organic carbon removal. DOC removal is primarily a function of microbial processes, which are strongly affected by CLT. Physical processes such as filtration facilitate the removal of particulate organic material and are not directly affected by the presence of CLT. These may contribute to the removal of COD, also exerting effects on microbial processes that eliminate dissolved organic material.

The average removal efficiency of COD for both types of HSSFCW increased with increasing concentrations of CLT, independent of glucose concentrations (i.e. $COD_{385\ \mu g/L\ CLT} > COD_{240\ \mu g/L\ CLT} > COD_{85\ \mu g/L\ CLT}$; Fig. 2). These results were difficult to interpret.

Studies addressing the influence of macrophytes in wetlands have attributed observed organic matter removal to physical effects. These include plant root structures that provide attachment surfaces for microbial communities[33,34] and special sites within their rhizospheres that facilitate adsorption, filtration and other microbiological processes. These processes would allow roots to produce the necessary oxygen required for the oxidation of organic matter.[27] The present study found no differences ($P \leq 0.05$) in the removal efficiency of COD between the planted and unplanted HSSFCW. The average median removal efficiency was basically equal in both types of HSSFCW (HSSFCW-NPa: 98.4%; HSSFCW-Pa: 98.0%). DOM removal exceeded that observed for the COD, and was comparable to that observed for other biological treatment systems.[25] This suggests that the emergent macrophyte *P. australis* does not produce the physical effects necessary for facilitating the removal of all organic fractions.

In all cases, CLT concentrations in the effluent were below the detection limit of the analytical method. Retention of the pesticide by the HSSFCW was most likely due to adsorption[35,36] and biodegradation.[18,36]

The two types of HSSFCW exhibited significant differences in their total heterotrophic bacteria, anaerobic bacteria and *Pseudomonas spp.* ($P \leq 0.05$). The differences indicate that *P. australis* strongly influences the composition of microbial communities within subsurface biofilms. These findings are consistent with those reported in other studies,[37,38] that have shown that plants exert a strong effect on the aforementioned communities through rhizosphere interactions.

Variables associated with HSSFCW-NPa observations showed few correlations (Table 2). Further research with more advanced analytical tools is necessary to determine causal factors operating in HSSFCW-NPa systems.

The negative correlation between the bacterial populations and DO concentrations for the HSSFCW-Pa (Table 2) indicates that microbial biofilms develop in spite of low DO concentrations. These findings support the assumption that

Table 2. Spearman correlation matrix for physico-chemical and microbiological variables measured in pilot horizontal subsurface flow constructed wetlands with *P. australis* (HSSFCW-Pa) and unplanted (HSSFCW-NPa). Values in parentheses indicate the number of samples.

Variable	*pH*	*T°C*	*Electrical conductivity*	*Dissolved oxygen*	*Redox potential*	*% COD removal*	*% DOC removal*	*Total heterotrophic bacteria*	*Pseudomonas spp.*	*Anaerobic bacteria*
HCFHSS-NPa										
pH	1									
T°C	0.094 (18)	1								
Electrical Conductivity	−0.107 (18)	−0.051 (18)	1							
Dissolved Oxygen	−0.201 (18)	−0.306 (18)	0.130 (18)	1						
Redox Potential	−0.307 (18)	0.105 (18)	0.038 (18)	−0.197 (18)	1					
% COD removal	0.179 (18)	−0.263 (18)	−0.105 (18)	0.028 (18)	−0.190 (18)	1				
% DOC removal	−0.010 (18)	0.062 (18)	0.447 (18)	−0.240 (18)	0.032 (18)	−0.106 (18)	1			
Total heterotrophic bacteria	0.145 (17)	0.025 (17)	−0.088 (17)	0.187 (17)	−0.515 (17)[1]	−0.228 (17)	−0.017 (17)	1		
Pseudomonas spp.	−0.297 (17)	0.239 (17)	0.095 (17)	0.006 (17)	−0.263 (17)	−0.109 (17)	0.283 (17)	0.721 (17)[2]	1	
Anaerobic bacteria	0.294 (17)	−0.085 (17)	0.332 (17)	−0.022 (17)	−0.347 (17)	0.183 (17)	0.359 (17)	0.213 (17)	0.257 (17)	1
HCFHSS-Pa										
pH	1									
T °C	0.236 (105)[1]	1								
Electrical Conductivity	0.310 (104)[2]	0.080 (106)	1							
Dissolved Oxygen	0.049 (93)	0.004 (95)	−0.002 (94)	1						
Redox Potential	−0.483 (101)[2]	−0.150 (103)	−0.271 (102)[2]	0.259 (91)[2]	1					
% COD removal	0.117 (105)	−0.060 (107)	0.433 (106)[2]	−0.150 (95)	−0.193 (103)	1				
% DOC removal	0.238 (105)[1]	0.070 (107)	0.425 (106)[2]	−0.084 (95)	−0.084 (103)	−0.003 (107)	1			
Total heterotrophic bacteria	0.373 (101)[2]	0.221 (103)[1]	0.495 (102)[2]	−0.364 (91)[2]	−0.565 (99)[2]	0.122 (103)	0.299 (103)[2]	1		
Pseudomonas ssp.	0.269 (101)[2]	0.227 (103)[1]	0.303 (102)[2]	−0.284 (91)[2]	−0.567 (99)[2]	0.038 (103)	0.101 (103)	0.864 (104)[2]	1	
Anaerobic bacteria	−0.116 (65)	0.027 (67)	−0.127 (67)	−0.352 (67)[2]	−0.376 (67)[2]	−0.092 (67)	−0.089 (67)	0.663 (68)[2]	0.728 (68)[2]	1

[1]significant correlation at 0.05
[2]significant correlation at 0.01

microbial biofilm communities are spatially organized in response to environmental conditions, especially DO and ORP variations that occur at different depths within HSSFCW.

Positive correlation between bacterial populations counted and the percentage of DOC removed from the HSSFCW-Pa (Table 2) indicates high growth rates and rapid consumption of soluble organic compounds similar to that observed in other studies of these treatment systems.[39]

Temperature observations (Table 1) fell within the range reported for the efficient removal of organic matter by biological processes.[40] The positive correlation between temperature and the total heterotrophic bacteria and *Pseudomonas spp.* counts in HSSFCW-Pa (Table 2) indicates that this mesophilic range favored microbial growth.

The observed pH (Table 1) values also facilitated the development of microbial biofilms. In HSSFCW-Pa, pH values fell within the range recommended by the U.S. Environmental Protection Agency (5.0–9.0).[41] The presence of *P. australis* in HSSFCW-Pa coincided with higher pH values observed in the effluent. This effect was likely due to the transfer of oxygen through macrophyte roots and removal of carbon dioxide from the water column, which together would increase pH levels.[42]

The positive correlation between the pH variable and total heterotrophic bacteria and *Pseudomonas spp*. counts in HSSFCW-Pa (Table 2) suggests that higher pH conditions enhance growth dynamics, metabolic processes and nutrient assimilation.

The electrical conductivity of the HSSFCW effluent exceeded that recorded for influent samples (Table 1). Higher conductivity may result from the release of salts by macrophytes and the presence of products derived from the mineralization of organic matter.[43] The positive correlations between conductivity and total heterotrophic bacteria and *Pseudomonas spp.* counts in HSSFCW-Pa (Table 2) are consistent with these mechanisms. Metabolic activity favors the decomposition of organic matter into basic ionic components. The presence of these ions manifests as higher conductivity of fluids downstream from sites of microbial activity.

DO concentrations in HSSFCW effluent samples were lower than those observed in influent samples (Table 1). Similar trends have been reported in other studies,[44,45] which suggested that organic matter degradation by wetland microbial communities creates a high oxygen demand. Comparison of HSSFCW behavior with that of natural soil can help further explain the observations of lower DO. Saturation or flooding in natural soils often causes an oxygen deficit leading to anaerobic and anoxic conditions.[34,43,46,47] Wetlands exhibit similar behavior but have higher DO inputs given their larger flow rates.[44] The negative correlations between the DO variable and total heterotrophic bacteria, anaerobes and *Pseudomonas spp.* counts for the HSSFCW-Pa (Table 2) may reflect a decrease in DO as a result of catabolic microbial processes.

Significant positive correlations were observed between microbial population counts and different physicochemical variables for HSSFCW-Pa (Table 2).

The ORP of influent and effluent samples from HSSFCW showed negative and positive values, respectively (Table 1). Statistical analysis also revealed negative correlations between ORP and counts for total heterotrophic bacteria, *Pseudomonas spp*. and anaerobic bacteria in HSSFCW-Pa (Table 2). The high degree of variability in the ORP variable as well as its contrasting behavior for influent and effluent sample points (relative to that of the microbial counts; Fig. 3) suggests that the negative correlation coefficients observed between ORP and microbial counts may not be significant.

The higher ORP levels in effluent samples indicate that carbon mineralization and associated oxygen demand was lower in the absence of *P. australis*, which would provide source material through the exudation of organic material by its roots. This interpretation is supported by the negative ORP values observed in influent and effluent samples from HSSFCW-Pa (Table 1). In contrast to observation from HSSFCW-NPa, these negative ORP values indicate active reduction processes operating within the planted systems.[48,49] Planted systems also showed a consistent negative correlation between redox potential and microbial population counts. Average concentrations of DO were consistent with this interpretation (Table 1).

Conclusions

The emergent macrophyte *P. australis* does not improve the removal of organic material from HSSFCW. The presence of *P. australis* decreased the removal efficiency of dissolved organic carbon (DOC) but did not affect the removal of total organic matter. Experiments showed that HSSFCW can reduce CLT concentrations to levels below the detection limit of highly sensitive analytical equipment (GC with electron mircocapture detector) given CLT input concentrations of up to 385 μg L^{-1}. From a technical and practical standpoint, CLT does not influence the removal of organic matter in HSSFCW systems. Removal efficiencies in the presence of CLT are comparable or superior to those achieved by other biological treatment systems. HSSFCW can therefore serve as viable alternatives for the treatment of agricultural effluents polluted simultaneously with organic matter and CLT. Larger scale studies are necessary to assess the long-term influence of CLT on the removal efficiency of organic matter. This study also identified significant statistical relationships between: 1) physicochemical variables, 2) biofilm microbial population and 3) removal efficiencies. Better understanding of these relationships can enhance the design of future wetland treatment systems.

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