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Available online: 16 May 2011

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This work used subsurface flow constructed wetlands, planted with *Phragmites australis*, using 2 water depths and 2 sizes of granular material, in order to find the optimal conditions for the removal of chlorpyrifos and dissolved organic carbon (DOC) from synthetic wastewater. In addition, some bacterial groups were identified which formed the biofilm present in subsurface flow constructed wetlands used in the removal of chlorpyrifos. In samples taken from influents and effluents of the wetlands, chlorpyrifos was quantified by gas chromatography (GC μ-ECD), DOC by an organic carbon analyser and bacterial groups using conventional microbiology, according to Standard Methods. The highest values of chlorpyrifos (97.9%) and DOC (80.1%) removal were found with granular material having diameters within 3.18–6.35 mm and according to water column depth (0.4 m) were 97.8% and 79.7%, respectively. The bacterial groups quantified in the biofilm were total heterotrophic, revivable heterotrophic, total coliforms, facultative sporulated, Pseudomonads, denitrifying bacteria and sulphate-reducing bacteria. Some bacteria showed little development, probably due to the pesticide and/or the anaerobic conditions of the systems (negative redox potential and dissolved oxygen (DO) concentrations approaching zero). It was proven that subsurface flow constructed wetlands, in adequate conditions, are able to eliminate organic matter and chlorpyrifos.

Keywords: subsurface flow constructed wetlands; chlorpyrifos; dissolved organic carbon; *Phragmites australis*

1. Introduction

In Colombia, agricultural activity has been carried out which permanently uses a lot of pesticides that can contaminate soils, rivers, lakes and reservoirs [1,2]. It is very common in rural areas to find domestic wastewater contaminated with pesticides such as chlorpyrifos. According to the Colombian Agricultural Institute (ICA) [3], in the year 2004 in this country was consumed 2000 t and 3000 m^3 of organophosphates, of which 34.3% corresponded to chlorpyrifos. The United States Environmental Protection Agency (US-EPA) [4] estimated that, in the year 2001, the overall consumption of pesticides

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worldwide was 5.1 billion pounds of active principles of which 24.0% corresponded to insecticides.

Chlorpyrifos (O,O-diethyl-O-[3,5,6-thrichloro-2-pyridinyl phosphorothioate) is an organophosphorus pesticide [5,6] extensively produced worldwide [4], broadly used to control insects and arthropods in agriculture [7,8] and at a domestic level [9,10]. The excessive use of this substance increases risks for human health, animals and the environment [11–14]. This kind of organophosphate also has toxic effects in amphibians and other organisms [2,15]. Chronic human exposure to this agrochemical and to water contaminated with this substance [16,17] can produce long-term mutagenic and neuro-logical effects [18], visual disturbances [19,20] as well as affect different stages of the female reproductive cycle and respiratory and cardiovascular systems [21–23]. The main physicochemical properties of this agrochemical are presented in Table 1.

Subsurface flow constructed wetlands (SSFCW) have shown to be viable alternatives in domestic and agricultural wastewater treatment [24–26] for their efficiency in removing organic matter, nitrogen and phosphorus [27–29]. These purification systems are optimal when they use water depths lower than 0.6 m, where water level is maintained from 0.1 to 0.5 m under the gravel layer [30] because, with these conditions the roots and rhizomes of the macrophytes show better development and have positive effects in water purifying processes [31], avoiding insect proliferation in tropical areas.

Table 1. Relevant physicochemical properties of chlorpyrifos [34].

Property	Unit	Value
Melting point	°C	41.0 and 43.5
Vapour pressure at 25°C	mm Hg	1.87×10^{-5}
Density	$g\,(cm^3)^{-1}$	1.39
Solubility in water at 23°C	$mg\,L^{-1}$	2.00
Solubility in organic solvents		Acetone, ethanol, ethyl acetate, hexane, acetonitrile
Partition coefficient octanol/water Log Kow		4.70–5.11
Volatilisation in water $t_{1/2}$	d	3.5–20
Persistence in water $t_{1/2}$	d	0.2–0.3, 0.5–4.0
Photolysis $t_{1/2}$	d	21–28
Soil sorption coefficient Koc		6070, 8498
Adsorption coefficient Kd	$mg\,g^{-1}$	13.4–1.862 depending on soil type
Exothermic decomposition	°C	>30
Boiling point	°C	160
Half life in water	d	20 to 30
Half life in soil	d	10 to 120
Molecular structure		
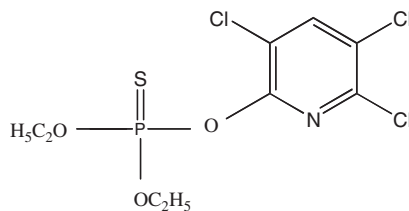
Chlorpyrifos

Microorganisms from the biofilm formed on the gravel of SSFCW, are vital for the degradation of organic matter and organic contaminants, allowing the purification of wastewater [32]. A fraction of the degraded organic matter is incorporated into the microorganisms for their cellular growth. For this reason, organic contaminants are an important carbon and nutrient source for microbial activity in SSFCW [33]. Investigations regarding wastewater treatment in wetlands are abundant, but very few investigations have been carried out regarding the treatment of wastewater contaminated with pesticides [34] and even fewer concerning the impact of toxic contaminants upon some microbial populations associated to these systems.

The objective of this study was to test two water layer depths and two granular material sizes which allowed high removals of chlorpyrifos and dissolved organic matter from synthetic wastewater in SSFCW planted with *Phragmites australis* to be accomplished. Furthermore, it permitted the identity of some bacterial groups that formed the biofilm associated to these systems and those which were involved in the pesticide removal.

2. Experimental

2.1 *Location and assembly of the SSFCW*

The investigation was carried out with 4 pilot wetlands (WA, WB, WC, WD) (Figure 1), built in fibreglass, 1.0 m long × 0.6 m wide × 0.6 m deep and planted with *Phragmites australis* (12 plants per m^2). The wetlands were fed with synthetic wastewater [35]

PAI: Piezometer A influent. **PBI:** Piezometer B influent. PCI: Piezometer C influent, PDI: Piezometer D influent
PAE: Piezometer A effluent. **PBE:** Piezometer B effluent. PCE: Piezometer C effluent, PDE: Piezometer D effluent
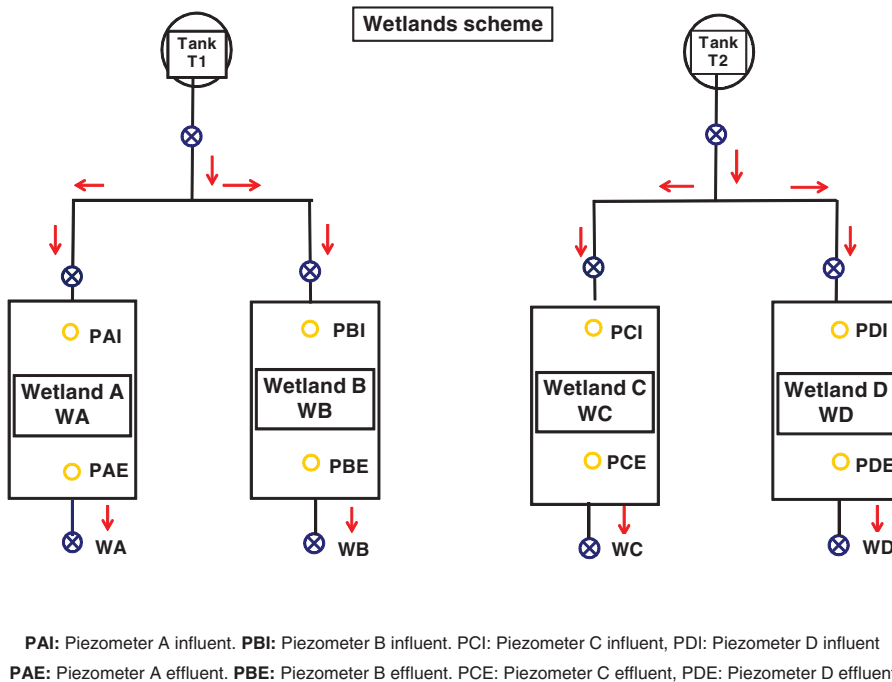
Figure 1. Subsurface flow constructed wetland (SSFCW) assembly.

Table 2. Configuration of the conditions of the subsurface flow constructed wetlands (SSFCW).

Type of constructed wetland	Water depth (m)	Gravel bed height (m)	Gravel diameter (mm)	Area (m^2)	Porosity %	Flow (cm^3/min)
WA	0.2	0.3	12.70–25.40	0.6	53	6.3
WB	0.2	0.3	3.18–6.35	0.6	39	4.6
WC	0.4	0.5	12.70–25.40	0.6	53	12.6
WD	0.4	0.5	3.18–6.35	0.6	39	9.3

contaminated with chlorpyrifos and $1.7 \, L \, d^{-1}$ of loading rate, controlling the input and output flow with free pass valves for a hydraulic retention time of seven days [36]. This has been considered in literature as that which is necessary to degrade organic matter and nutrients [37]. The water column depth varied between 0.2 and 0.4 m. The granular material used in the SSFCW was of non calcareous quartz (98.0%), with a high roughness and size of 3.18–6.35 mm and 12.70–25.40 mm. Table 2 shows all conditions for the SSFCW.

2.2 Sampling

2.2.1 Physicochemical sampling

Eight samples were taken from the influent and effluent of each wetland to determine the concentrations of chlorpyrifos and DOC. The influent of each wetland had $22.1 \, mg \, L^{-1}$ of DOC and $474.6 \, \mu g \, L^{-1}$ of chlorpyrifos, concentrations that are similar to natural sources contaminated with chlorpyrifos in Colombia. Furthermore, the chlorpyrifos concentration used in the experiments is lower than its solubility limit in water ($2 \, mg \, L^{-1}$, at $25°C$). Samples were taken on days 1, 7, 11 and 15 after the beginning of each treatment. Dissolved oxygen (DO), pH, chemical oxygen demand (COD) and water temperature were measured *in situ*, following normalised methods [38].

2.2.2 Microbiological sampling

Two PVC piezometers were installed, one next to the input of the SSFCW and another next to the output. Inside each piezometer a plastic mesh basket containing granular material of the same size as the SSFCW was installed. On days 5, 11 and 15, granular material was collected from the plastic mesh basket. The biofilm was extracted from granular material by sonication in sterile saline solution at 0.9%. Successive dilutions were made up to 10^{-4} (1 : 10000) in peptone water at 1.0%. Afterwards, 1.0 mL of sample was plated with culture media specific to each bacterial group. Plates were incubated at $37°C$ for 48 h and the procedure was continued with conventional microbiology, with the exception of revivable heterotrophic bacteria that were incubated at room temperature. The count of $CFU \, mL^{-1}$ was made according to Standard Methods [38] to estimate the number of present microorganisms in the samples. The most probable number method ($MPN \, 100 \, mL^{-1}$) was used to determine the concentration of total coliforms, by means of multiple fermentation tubes in BRILA broth. In order to determine the possible

participation of the bacteria from the biofilm of the granular material of the wetlands during the degradation of the chlorpyrifos, the following experiments were carried out.

Gravel of 3.18 to 6.35 mm was put into two erlenmeyers of 200 mL along with synthetic water (pH 7.0) containing chlorpyrifos, one erlenmeyer having a chlorpyrifos concentration of $1.0\,mg\,L^{-1}$ and the other having a concentration of $2.0\,mg\,L^{-1}$. Previous to this stage the 6 identified bacterial groups in the wetlands were cultivated in the laboratory in tubes and plates. Samples were taken from the culture at five separate times and inoculated on the gravel that would be placed in the erlenmeyers. The concentration of the chlorpyrifos by gas chromatography was determined at the beginning of the experiment and on the seventh day.

2.3 *Procedure of extraction and quantification of the chlorpyrifos*

A volume of 10.0 mL of sample was taken, it was filtered with membranes of cellulose $0.45\,\mu m$ and afterwards an extraction in solid phase using a C18 cartridge of 3.0 mL/ 500 mg Macherey-Nagel® was carried out. The cartridge was conditioned with hexane-ethyl acetate, acetone, methanol and water. The sample was immediately passed through the cartridge which was then dried with a suction apparatus and finally the chlorpyrifos was eluted with hexane-ethyl acetate (50 : 50). The final volume was 10.0 mL, with a percentage of recovery of 96.2%.

For each sample, chlorpyrifos was quantified using a Agilent Technologies 6890 plus® gas chromatograph, with an electron capture micro detector, a Splitless autosampler, a HP-5 column (5% phenylmethylpolysiloxane) and helium as the carrier gas at $1\,mL\,min^{-1}$. Oven temperatures were: Initial $60°C\,min^{-1}$ for 0 min; ramp $1:40°C\,min^{-1}$ from 60°C to 200°C for 0 min; ramp $2:10°C\,min^{-1}$ to 240°C, for 2 min. Temperatures of the injector and the detector were 290°C and 300°C, respectively. Using the Chemstation® software, real-time chromatograms were registered and standard solutions prepared using 99.5% pure chlorpyrifos (Chem Service®).

2.4 *Quantification of DOC*

The DOC was quantified in an IO-Analytical 1010 organic carbon analyser, with humid combustion, a non-dispersive infrared detector and a $10.0\,\mu L$ loop. Each sample was filtered with nylon acrodiscs $(0.45\,\mu m)$. All methods were validated in the laboratory of the Diagnosis and control of contamination (GDCON, UdeA) research group, following criteria established by the AEFI [39].

2.5 *Statistical analysis*

To determine the distribution of the results for each variable, the Kolmogorov-Smirnov test ($p > 0.05$ or $p < 0.05$) was applied. The presence of significant differences was established by a bilateral analysis of variance by Friedman's hierarchy. The Wilcoxon sign test was used to determine differences between specific results [40]. The calculations were made with statistical programs such as 'Statistical Package for the Social Sciences' SPSS® version 16 and Microsoft Office Excel®. The data for determining some populations of bacterial communities was statistically analysed by variance comparison tests and multiple regression analysis, using Statgraphics Plus® version 4.1.

3. Results and discussion

None of the variables measured in this study presented a normal distribution (Kolmogorov-Smirnov test, $p < 0.05$), therefore nonparametric procedures were applied, using the median of each variable for the analysis of results.

3.1 *Behaviour of chlorpyrifos, DOC and nutrients in SSFCW*

In this investigation, the configuration of SSFCW was studied, according to the gravel size and the water layer depth which allowed highest chlorpyrifos and DOC removal. With the finest gravel (3.18–6.35 mm) the highest removal of chlorpyrifos (97.9%) and dissolved organic matter (80.1%) (Table 3) was accomplished, because this size has greater surface area and therefore, greater bacterial population.

With the water depth of 0.4 m, where there was a greater amount of gravel, the removals of chlorpyrifos and the COD were slightly greater than when a depth of 0.2 m was used. However, the difference in the removal of contaminants between the two depths was not significant (*p*-value > 0.05) (Table 3). These depths allowed a better distribution of roots and rhizomes in the granular medium [41] and helped to optimise the removal of organic matter [42].

Regarding the nutrients, high removals were achieved without noticeable statistical differences between the gravel sizes used (Table 3). Likewise, the removals of phosphate and ammonium were higher in wetlands with a water depth of 0.4 m [43,44].

DOC and other nutrients are removed by microorganisms and plants and in the case of chlorpyrifos, chemical and biochemical processes can participate in the extraction. Microorganisms [45–48] can degrade xenobiotic compounds like pesticides, the bacterial activity being influenced by the environmental conditions of the wetlands and the roots of the plants. Hydrolysis is one of the most important chemical processes in the degradation of some pesticides. Biochemical processes and hydrolysis in medium acids [49] form 3.5.6-trichloro-2 pyridinol (TCP), a compound very stable in water [50–52] and whose product was identified in this work. The physical processes also can participate in removal of chlorpyrifos, like adsorption and absorption to the roots and rhizomes of the plants, and adsorption to the surface of the granular material [53,54]. However, in this work it was proven that chlorpyrifos was not absorbed into granular material.

Table 3. Removal percentage of chlorpyrifos, dissolved organic carbon (DOC) and nutrients according to gravel diameter and water depth in SSFCW.

Conditions SSFCW	Removal %			
	DOC	Chlorpyrifos	COD	NH_4^+
Gravel diameter: 3.18–6.35 mm	80.1	97.9	73.7	85.8
Gravel diameter: 12.70–25.40 mm	75.1	96.6	74.9	87.5
Water depth: 0.2 m	75.2	96.3	78.7	85.8
Water depth: 0.4 m	79.7	97.8	70.7	87.5

Notes: Determination for parameters (n = 7).
DOC: dissolved organic carbon.
COD: chemical oxygen demand.

The degradation of organic material produced a diminution of DQO, an assimilation of nutrients by plants and microorganisms as well as a diminution of ammonium and phosphates. In the case of pH diminution, this occurred due to the formation of CO_2 or acetic acid in anaerobic processes that were carried out in the lower part of wetland that normally showed negative redox potential in the piezometer. The DO diminution was due to the consumption of oxygen in aerobic processes of the wetland (Table 4) [43,44,55–58].

3.2 *Effect of chlorpyrifos in bacteria groups identified in wetland*

3.2.1 *Identification of some bacterial groups*

In this study, six bacterial groups were identified in the biofilm associated to the wetlands, and their population ranges are shown in Table 5. In general, these six bacterial groups increased their population after day 11 of each experiment. The total heterotrophic bacteria formed a large part of the biofilm adhered to the gravel and plant rhizosphere in the SSFCW. These bacteria play an important part in the degradation of organic xenobiotic compounds such as chlorpyrifos. According to Bastardo and Rosales [59], total heterotrophic bacteria are the most unstable bacteria regarding changes and

Table 4. Removal of chlorpyrifos, DOC and variation of the concentration of the parameters physicochemical in the SSFCW.

			Effluent							
			WA		WB		WC		WD	
Parameters	Unit	Influent	Value	%	Value	%	Value	%	Value	%
Chlorpyrifos	$\mu g\,L^{-1}$	474.6	25.1	94.7	12.9	97.3	14.6	96.9	6.7	98.6
DOC	$mg\,L^{-1}$	22.3	6.1	72.9	4.4	80.1	5.1	77.0	4.4	80.1
COD	$mg\,L^{-1}$	180.7	36.8	79.6	40.4	77.7	47.5	73.7	58.2	67.8
DO	$mg\,L^{-1}$	4.6	1.8	–	2.5	–	2.2	–	2.8	–
pH	unit	6.6	3.7	–	5.8	–	4.1	–	6.1	–
NH_4^+	$mg\,L^{-1}$	62.3	12.5	79.9	17.1	72.6	16.2	74.0	13.2	78.8
NO_3^-	$mg\,L^{-1}$	0.2	0.0	79.3	0.1	64.3	0.0	93.7	0.0	96.9

Table 5. Maximum and minimum values of bacterial groups identified in the SSFCW.

		Values	
Bacterial groups	Unit	Min	Max
Total heterotrophic	$CFU\,mL^{-1}$	55	$\geq 6.500 \times 10^4$
Revivable heterotrophic	$CFU\,mL^{-1}$	37	$\geq 6.500 \times 10^4$
Total coliforms	MPN	3	$\geq 6.500 \times 10^4$
Facultative sporulated	$CFU\,mL^{-1}$	110	≥ 2.400
Pseudomonads	$CFU\,mL^{-1}$	60	$\geq 6.500 \times 10^4$
Denitrifying	$CFU\,mL^{-1}$	100	$\geq 6.500 \times 10^4$
Sulphate-reducing	Absence-Presence	–	–

Note: 6.5×10^4 correspond to the maximum value when the bacterial population is uncountable.

fluctuations of abiotic factors in a given system, behaviour that was seen in this study. Although the group of revivable heterotrophic bacteria had a lower population (between 37 and 6500×10^4 CFU mL^{-1}) than the total heterotrophic bacteria (between 55 and 6500×10^4 CFU mL^{-1}), it had a similar pattern of behaviour to the latter. These reviled and revivable bacteria also have an important function within the biofilm because of their participation in the biodegradation and/or transformation processes of organic and toxic compounds.

Included in the group of sporulated bacteria are the genera *Bacillus* sp. and *Clostridium* sp. that produce endospores. These give them advantages over other bacterial groups like their resistance to fluctuation of diverse environmental factors and to the action of chemical compounds, such as pesticides [60]. This bacterial group was the one which showed highest resistance to the chlorpyrifos and for that reason, its development in the biofilm was the most stable. From the group of the nitrifying bacteria, the genera *Pseudomonas* sp., *Klebsiella* sp., *Clostridium* sp. and *Bacillus* sp. stood out because of their cooperation in the degradation of the pesticide [57]. This bacterial group showed development in the biofilm which was very similar to that of the total heterotrophic group.

From the Pseudomonales family, the genera Pseudomonas was evaluated, which has proven in earlier studies, to be capable of using several xenobiotic compounds as energy and carbon sources, pesticides included [61]. The initial population of Pseudomonas, after feeding the SSFCW with water containing chlorpyrifos, was very low (between 35 and 215×10^4 CFU mL^{-1}). In general, this bacterial family did not have an optimal development during the biofilm formation, possibly due to the amount of nutrients and toxic substances which could have limited its growth and activity [62]. The reducing bacteria of sulphate that was identified qualitatively (presence-absence) presented little growth and little development, attributable to the aerobic and anoxic conditions of the wetlands. The total coliforms bacterial group was the one that presented greater fluctuations, probably as a result of the competition of nutrients with the other bacteria, especially with the total heterotrophic.

3.2.2 *Distribution of bacterial groups*

According to Figure 2, the bacterial groups identified presented atypical distributions in the influent and effluents of the wetlands, with outliers appearing in the family of the Pseudomonas, probably due to significant fluctuations in population dynamics, mainly caused by the presence of the pesticide. Total heterotrophic bacteria in the influent of the wetlands, with an atypical negative distribution, had the highest median compared to the other groups. Meanwhile the nitrifying and Pseudomonales families in the effluent, with a better distribution of data, presented the lowest median. The facultative sporulated (anaerobic) bacteria in the effluent, with lower dispersion and higher data symmetry, presented the highest median compared to the other bacterial groups. This was because this group resisted important fluctuations in the physicochemical variables of DO, COD and nutrients, therefore consequently its population dynamics were more stable.

3.2.3 *Identification of bacterial groups that participated in the removal of chlorpyrifos in wetlands*

In the experiments with bacteria identified in gravel tanks 98.6% of chlorpyrifos degradation was obtained when 0.2 mg of pesticide was used and 89.4% when 0.4 mg
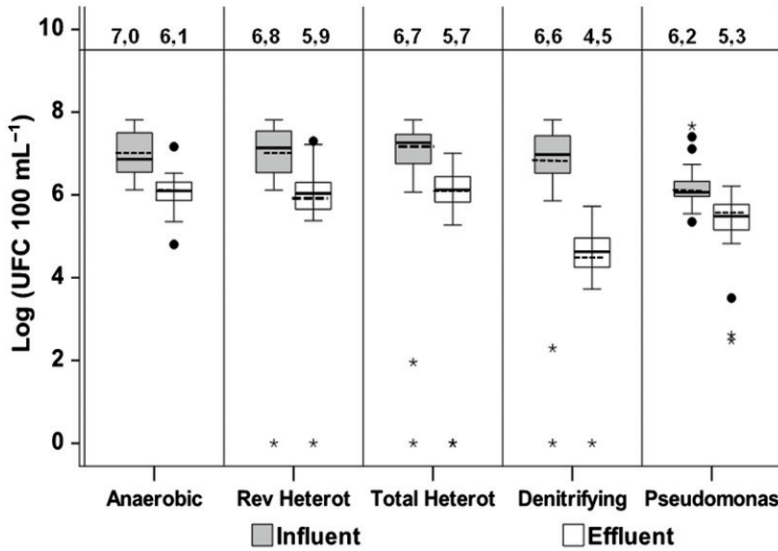
Figure 2. Distribution of bacterial groups associated with chlorpyrifos removal in the piezometers installed in the influent and effluent of the SSFCW.

Table 6. Removal of chlorpyrifos by bacterial groups identified in wetlands.

Chlorpyrifos				
Initial		Final		
Concentration $(mg\,L^{-1})$	Amount (mg)	Concentration $(mg\,L^{-1})$	Amount (mg)	Removal (%)
1.0	0.2	0.0128	0.00256	98.7
2.0	0.4	0.1042	0.02084	94.2

of pesticide was used (Table 6). Mainly the degradation was a biochemical process due to the action of microorganisms present in the biofilm of the granular material; however, which six bacterial families participated in it could not be determined.

The six bacterial groups identified in the wetland experiments were identified in other work with samples contaminated with chlorpyrifos [63]. Lakshmi *et al*. [63] isolated bacteria from soils contaminated with chlorpyrifos and molecularly and morphologically the bacterial species *P. fluorescence*, *Brucella melitensis*, *Bacillus subtilis*, *Bacillus cereus*, *Klebsiella* sp., *Serratia marcescens* and *P. aeruginosa* were identified. This is a typical case of bioremediation, in which wetlands eliminate the pesticide and its main degradation product, both being toxic compounds [64].

4. Conclusions

The SSFCW were efficient in the removal of chlorpyrifos, dissolved organic matter and nutrients. Particularly, the highest removals occurred in the wetlands with the finest

gravel size (3.18–6.35 mm) and the deepest water layer. However, with a more shallow water layer, high removal of the same substances was also observed.

With the working conditions for the wetlands in this investigation, bacterial groups were developed and formed biofilms where the degradation processes of pesticide and DOC were executed in greater proportion. The identified bacterial groups can live in the presence of toxic compounds like chlorpyrifos, and some participate in the degradation of pesticide. However, the fact that some were present in low populations like the total coliforms and Pseudomonas was probably due to the presence of chlorpyrifos in the wetlands.

This investigation demonstrated that subsurface flow wetlands with a granular material size of 3.18–6.35 mm inch and a water depth of 0.4 m are effective in removing toxics like the chlorpyrifos pesticide, which due to its extensive use in agriculture, is a potential threat to human beings, the biota and the environment.

Acknowledgement

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