International Journal of Environmental Chemistry
2021; 5(2): 38-44
http://www.sciencepublishinggroup.com/j/ijec
doi: 10.11648/j.ijec.20210502.14
ISSN: 2640-1452 (Print); ISSN: 2640-1460 (Online)

Effects of Partial Saturation on Nitrogen Removal and Bacterial Community in Vertical-flow Constructed Wetlands

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To cite this article:

Khadija Kraiem, Hamadi Kallali, Rim Werheni Ammeri, Bessadok Salma, Naceur Jedidi. Effects of Partial Saturation on Nitrogen Removal and Bacterial Community in Vertical-flow Constructed Wetlands. *International Journal of Environmental Chemistry*. Vol. 5, No. 2, 2021, pp. 38-44. doi: 10.11648/j.ijec.20210502.14

Received: July 2, 2021; **Accepted**: July 27, 2021; **Published**: October 19, 2021

Abstract: The laboratory-scale pilot of constructed wetlands has been in operation for six months; (1) an unsaturated vertical flow constructed wetland (UVF-CW), this system was used to represent the classic vertical constructed wetlands, (2) a saturated vertical flow constructed wetland (SVF-CW), to evaluate the effects of the saturated condition on nitrogen removal and composition of the microbial community. The results showed that the saturation condition positiveley influenced the removal efficiencies of the nitrogen, the aeverage removal rate of the total kjeldahl nitrogen increased from 56% in unsaturated vertical flow constructed wetland (UVF-CW) to 63% in saturated vertical flow constructed wetland (SVF-CW). In addition, the microbial communities also was affected by the saturation condition, the relative abundances of nitrifying bacterium in UVF-CW are 13.8% (*Nitrosomonas*), 7.2% (*Nitrosospira*), 18.1% (*Nitrospira*) and 15.3% (*Nitrobacter*). In contrast, in SVF-CW, *Nitrosomonas*, *Nitrosospira*, *Nitrospira* and *Nitrobacter* only accounted for 6.8%, 5.6%, 7.4% and 10.6% respectively. However, the saturation condition seemed to increase denitrifying bacterium more than three times, in unsaturated vertical flow constructed wetland, only *Pseudomonas* (6.5%) and *Paracoccus* (4.85%) were detected, but in saturated vertical flow constructed wetland (SVF-CW), the abundance of *Pseudomonas* (13.08%) and *Paracoccus* (9.74%) were increased, and three other groups of denitrifying bacteria were also detected as *Zoogloea* (3.32%), *Thauera* (5.41%) and *Thiobacillus* (3%), due to the low availability of oxygen, it seems to be beneficial to denitrifying bacteria.

Keywords: Constructed Wetlands, Rural Wastewater, Saturated Bed, Nitrogen Transforming Bacteria

1. Introduction

Excess nitrogen in aquatic ecosystems can cause many problems such as eutrophication, which have negatively effects on biodiversity, climate and human health [1]. Therefore, the remove of nitrogen from waste water it is necessary. Traditional activated sludge technology has been used for nitrogen removal in multiple wastewater treatment plants (WWTPs) due to its high efficiency [2]. However, these conventional technologies require high construction cost and consume more energy [3].

In recent years, constructed wetland (CW) is an ecological technology. Due to its various advantages over traditional wastewater treatment technologies, it has been rapidly developed in wastewater treatment in scattered areas in industrialized counties and low-income countries [4, 5].

These include low construction and operating cost, easier maintenance and good integration into the landscape and promotion of biodiversity [6, 7]. However, the removal of nitrogen in constructed wetlands (CWs) exhibits large fluctuations and is often unsatisfactory [8]. The total nitrogen removal in vertical flow or in horizontal flow constructed wetlands is usually not completely removal, but instead converts it to various nitrogen compounds [9].

In fact, the vertical flow constructed wetlands is an unsaturated systems, which often fed with several pulses intermittently throughout the day, resulting in a high oxygen transmission capacity, that is beneficial to the nitrifying bacteria in the bed [10]. However, horizontal flow constructed wetlands are mainly operated under anoxic/anaerobic conditions, which makes it a suitable environment for denitrification process [11].

Generally, the nitrogen removal limitation in constructed wetlands can be explained by the competition for oxygen by autotrophic and heterotrophic microorganisms heterotrophic [12], and the limit of organic carbon available for the process of denitrification [13]. Vertical flow constructed wetlands have more attention than horizontal flow constructed wetlands due to its less demand for land. [14]. On the other hand, traditional vertical flow constructed wetlands cannot remove total nitrogen (TN) satisfactorily due to the lack of appropriate hypoxic conditions for denitrification [9]. In order to completely remove nitrogen, various types of enhanced vertical flow constructed wetlands have been studied, such as artificial aeration, tidal flow and integrated vertical flow constructed wetlands [15, 16].

There are other operational conditions to enhance the removal efficiency of total nitrogen in vertical flow constructed wetlands, such as recirculation of effluent to improve nitrification efficiency [17] and stepwise feed to enhance carbon source supply to promote denitrification [18].

However, these modifications have increased operating costs and maintenance complexity. The latter strategy uses a partially saturated vertical flow constructed wetland configuration to create anoxic/anaerobic conditions at the bottom of the bed and aerobic conditions at the top of the bed to promote adequate condition for simultaneous nitrification and denitrification [19, 20]. This model is more efficient in total nitrogen removal than traditional unsaturated vertical flow constructed wetlands with sequential nitrification and denitrification [21-23].

In general, all efforts aimed at maximizing the nitrogen removal in constructed wetlands are directly related to the activities of enhancing microbial communities, which are responsible for the conversion of various nutrients in the filter media and rhizosphere biomass [24]. Many studies have evaluated the dynamics of bacterial communities in constructed wetlands. Foladori et al. [25] showed that the number of viable bacteria in the surface layer is 3.7 times that of the deep layer. Adrados et al. [26] characterized the prokaryotic microbial communities of vertical flow constructed wetlands, horizontal constructed wetlands and biological filter sand, and reported higher bacterial activity than archaea in all research systems. Other studies have shown that the diversity of bacterial communities in constructed wetlands may affect the quality of the final effluent [27]. Button et al. [28] pointed out that the metabolic functions of microorganisms determined in different types of constructed wetlands are related to the design of each system and the spatial location in the bed, especially the level of pretreatment.

There is no rigorous research on the knowledge of nitrogen-transforming bacteria in vertical constructed wetlands. Therefore, it is clearly necessary to further understand the kinetics of nitrogen conversion bacteria in unsaturated vertical flow constructed wetlands, especially in partially saturated vertical flow constructed wetlands, in order to increase the total nitrogen removal in a single vertical flow constructed wetland. The purpose of this study is to: (1) compare the conversion and removal efficiency of nitrogen in saturated and unsaturated vertical flow constructed wetlands; (2) identify nitrifying and denitrifying bacteria covered with gravel in unsaturated and saturated zones.

2. Materiel and Methods

2.1. VFCWs Systems and Operation Design

Two laboratory-scale vertical flow constructed wetlands (VFCW) were evaluated in duplicate with the same size and substrate. They operate in parallel, where unit 1 is composed of a partial vertical flow wetland (SVF-CW) (50%), and unit 2 is composed of a traditional unsaturated vertical flow wetland (UVF-CW). A schematic diagram of the laboratory scale of VFCWs is shown in Figure 1. The two laboratory-scale constructed wetlands are made of polyvinyl chloride tube, each having a diameter of 41 cm, a total height of 100 cm, and a media bed height of 80 cm. These units are equipped with a 10 cm coarse gravel (25 to 40 mm) drainage layer at the bottom and a 70 cm main filter layer composed of 2-4 mm gravel. The downwardly applied saturation zone is maintained by the siphon structure at the outlet. Young reed species are collected from local valleys and planted in all vertical flow wetlands.

In order to the growth of plants and biofilms in the medium, the wetland has accumulated water for up to 2 months (50% of rural wastewater and 50% of tap water). After the adaptation period, these systems intermittently fed with rural wastewater at a flow rate of 20 L/d (five batches per day). The operation cycle is divided into a feeding period (3 days) and a rest period (4 days). Table 1 shows the chemical characteristics of influent wastewater.

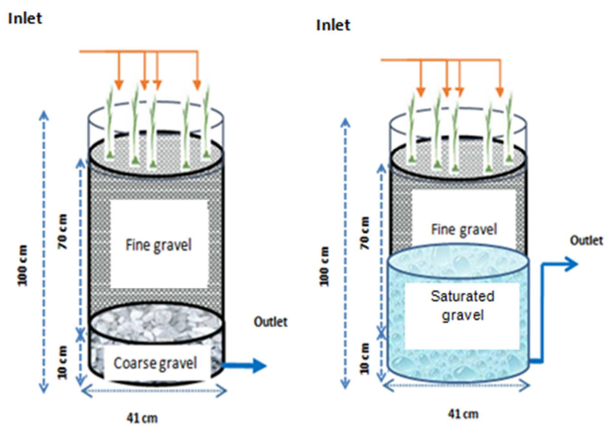

Figure 1. *The schematic diagram for experimental constructed wetlands (CW).*

Table 1. *Characteristics of influent wastewater.*

	DCO (mg/L)	pH	NH_4^+ (mg/L)	NO_3^- (mg/L)	NO_2^- (mg/L)	TNK (mg/L)
Range	1040 -1180	6,.98-8	151-204	1.9-5.4	0-0.1	173-230
Mean	1124	7.45	188	3.4	0.02	202

2.2. Pollutants Sampling and Analyses

During the experiment, water samples were collected twice a week at the beginning and end of each feeding sequence, twice a week. The sample corresponds to a batch of feed. All water samples were filtered through 0.45μm cellulose acetate membrane before analysis. The method to determine the water parameters is as follows: According to the Kjeldahl method [29] Analyze the total Kjeldahl nitrogen (TKN). The analysis of ammonium (NH_4^+) and nitrate (NO_3^-) was performed according to standard methods [20]. The pH value is measured by a pH meter (PHS-3C, China).

2.3. Biomass Sampling and Bacteria Detection

For bacterial analysis of nitrifying and denitrifying communities, 20 grams of bed media were collected in all pilot plants at two different depths (-20) cm and (- 60) cm, and 100 milliliters of deionized water was added to it. The samples are mixed and sonicated in ultrasound for 5 minutes to loosen the biofilm from the medium**.** Then, the sample was centrifuged at 1500 g for 5 minutes. All biomass samples were prepared according to the standard method of fluorescence in situ hybridization (FISH) analysis [31]. To fix the samples, 1ml of each sample (mixed water and biofilm suspension) was centrifuged at 16,000 g for 5 minutes, and then, by adding paraformaldehyde solution (4%) and phosphate buffered saline (PBS) 3:1, According to [32], the recovered particles were fixed at 4°C for 3 hours. Then, the fixed sample was washed twice with 1ml PBS, and finally suspended in a solution of PBS and absolute ethanol and stored at -20°C.

For the quantification of total bacterial cells, considering the Eubacterial domain, a volume of 10 µl of each sample was fixed on a PTFE-printed microscope slide and covered with 1µg.mL^{-1} of 4', 6-diamidino-2-benzene Based indole (DAPI) solution. Table 2 shows the specific probes for nitrifying and denitrifying bacterial communities used in this study. All probes are labeled with the fluorescent pigment Cy3 at the 5'position. The quantification of specific bacteria was performed by directly counting 20 random fields in each well using an epifluorescence microscope (OlympusBX41, Tokyo, Japan). In order to estimate the abundance of cells hybridized with the probe EUB mixture, the cells stained with DAPI were considered to represent 100% of all microorganisms identified by digital images. For the remaining probes, 20 regions were randomly selected, and the cells stained with the probe EUB mixture were considered to be 100% of all bacteria identified by the digital image. The "Microbial Ecology Digital Image Analysis (DAIME)" software was used to determine the relative abundance of nitrifying and denitrifying bacteria from the total DAPI staining [33].

Table 2. *The different probes used in this study.*

Probe	Probesequence;(5’→3’)	Targetedorganisms
EUB338mix	GC(T/A)GCC(T/A)CCCGTAGG(A/T)GT	Equimolar mixture of EUB338I EUB338II And Bacteriadomain, Planctomycetales and Verrucomicrobiales
PAE997	TCTGGAAAGTTCTCAGCA	Pseudomonas
AER66	CTACTTTCCCGCTGCCGC	Aeromonas
TBD121	CTCGGTACGTTCCGACGC	Thiobacillus denitrificans
NEU653	CCCCTCTGCTGCACTCTA	Nitrosomonas
NIT3	CCTGTGCTCCATGCTCCG	*Nitrobacter* spp.
Nsv443	CCGTGACCGTTTCGTTCCG	*Nitrosospira*
PAR124	GGATTAACCCACTGTCACC	Genus Paracoccus

2.4. Statistical Analysis

The average concentration and bacterial abundance were compared by the measured value ± standard error. Use the statistical program STATISTICA software (http://www.statsoft.com) for statistical analysis. The average value was compared by Fisher'shsd test (<0.05).

3. Results and Discussion

3.1. Temperature and pH

During the experiment period, no significant correlation was observed between effluent temperature and saturated zone depth. The average effluent temperature was 23.5 and 25.2°C for SFV-CW and UFV-CW respectively. The temperature range in two VFCWs was suitable for nitrogen removal through nitrification and denitrification process [34-36]**.** The effluent pH was not significantly different in two VFCWs ($p>0.05$). The pH value of VFCWs is 6.98–7.12, which is slightly lower than the pH value of the influent 7.31 (Table 3). The pH range of 6.87–7.31 was favorable for microbial nitrification and denitrification [37].

Table 3. *Influent and effluent of pH and temperature (means±standard deviation).*

Parameter	Influent	Effluent SVF-CW	Effluent UVF-CW
Temperature (C°)	21.74±2	23.5±1.1	25.2±2.3
pH	7.31±0.9	7.02±1.8	6.87±1.2

3.2. Nitrogen Removal

There are significant differences in the removal effect of NH4+-N between the two vertical flow constructed wetlands ($p<0.05$). The NH_4^+-N removal efficiency of UVF-CW and SVF-CW 76.85% and 63.45% respectively (Table 4). The removal of NH4+-N largely depends on the depth of the saturation zone. The removal efficiency of ammonium increases as the depth of the saturation zone decreases. Several studies have shown similar finding [9, 38]. However, These results are inconsistent with those obtained by Pelissari et al. [15] who pointed out that there is no significant difference in ammonium removal efficiency (unsaturated vertical flow and saturated vertical flow approximately 69% ($p=0.928$)).

The unsaturated vertical flow (UVF-CW) had higher ammonium removal efficiencies than partially saturated vertical flow (SVF-CW). This result can be attributed to the higher diffusion of oxygen to the partially unsaturated layer, which is beneficial to the nitrifier development [39].

In contrary, increasing saturation level seemed to have a positive effect on Total Kjeldahl Nitrogen (TKN) removal efficiency. In fact, vertical flow constructed wetlands with saturated layer (SVF-CW) had higher NTK removal than unsaturated bed (UVF-CW) (Table 4). This higher removal efficiency resulted from the development of denitrification process in saturated layer [9, 40].

With regard to NO_2^-–N, this form of nitrogen showed that an increase at both configurations of the systems with respect to the influent, NO_2^-N concentration in the effluents remained above the values in the influent. Due to the fact, this oxidized form of nitrogen is only an intermediate in different nitrogen reaction transformations; it is understandable to find such very low concentrations in this study.

There are significant differences in the removal effect of NO_3^--N between the two vertical flow constructed wetlands ($p<0.05$). Table 4, shows that the removal efficiency of SVF-CW on NO_3^-N is significantly higher than that of UVF-CW ($P<0.05$), indicating that denitrification capacity in the vertical flow constructed wetlands was promoted significantly with the saturated layer. This can be explained by the fact that the saturation layer is produced the required hypoxic condition and enhanced denitrification activity. This finding is consistent with the research conducted in the subsurface vertical flow constructed wetlands [15]. In addition, although autotrophic nitrification-heterotrophic denitrification is the main process of SVF-CW nitrogen removal, other mechanisms probably took place in this system. Other possible mechanisms are ANAMMOX, which could NO_3^-N produce [22, 39]. The increased of nitrate concentration in UVF-CW confirms a lightly higher capacity of UVF-CW for nitrification due to supplementary oxygenation through the unsaturated layer.

Table 4. *Nitrogen concentration at Influent and effluent and removal efficiency of the partially saturated vertical constructed wetland (SVF-CW) and unsaturated vertical constructed wetland (UVF-CW).*

Parameter	Influent ($mg.L^{-1}$)	Effluent ($mg.L^{-1}$)		Removal efficiency (%)	
		UVF-CW	SVF-CW	UVF-CW	SVF-CW
TNK	202±51.3	71±31.3	54±20.3	56±1.7	63±2.71
NH_4^+-N	178±42.1	41.2±1.6	65.05±0.2	41.2±1.6	65.05±0.2
NO_3^--N	3.6±0.8	17.56±2.3	4.26±1.41	-	-
NO_2^--N	0.02±0.1	0.86±1.5	0.1±1.3	-	-

3.3. Influence of Partially Saturated on Nitrifying and Denitrifying Bacterial Communities

In constructed wetlands, the diversity and abundance of microbial communities depends on environmental factors, wastewater types, media types, operating conditions and plant species [14]. Aerobic conditions (inside the media) often support autotrophic nitrification in vertical flow constructed wetlands [17, 41]. In order to better understand the remove of nitrogen obtained in two units with different saturation levels (UVF-CW and SVF-CW), (FISH) analysis were used in this study. Figure 2 shows that the detection of nitrifying bacteria by FISH technology, which shows that higher relative abundance of nitrifying bacteria and higher ratio of nitrite oxidizing bacteria (NOB) to Ammonium oxidizing bacteria (AOB) followed by higher ammonium removal performance were obtained in UVF-CW compared to SVF-CW.

The relative abundances of nitrifying bacteria were significantly different among the two different vertical flow constructed wetlands ($p<0.05$). The relative abundances in UVF-CWwere 13.8% (Nitrosomonas), 7.2% (Nitrosospira), 18.1% (Nitrospira) and15.3% (Nitrobacter). On the contrary, Nitrosomonas, Nitrosospira, Nitrospira and Nitrobacter merely accounted for 6.8%, 5.6%, 7.4% and 10.6% of the total bacteria in SVF respectively (Figure 3). These abundances are higher than that obtained by Guan et al. [42], Wang et al. [43] and Pelissari et al. [9]

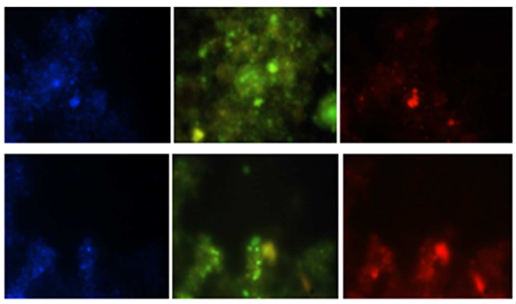

Figure 2. *FISH identification of nitrifying bacteria, DAPI (all DNA (blue)), middle, EUB338 mix probe (all bacteria (fluos, green)) right Nsv443 probe (Nitrosospira (Cy3, red)).*

The presence of saturated media, resulting in the reduction of the aerobic zone (entering the media) [44], which could have played a major role in reducing nitrifying bacteria. This result confirmed the nitrification performance obtained in section 3.4. These results also indicated that *Nitrosomonas* in the two vertical flow constructed wetlands ((UVF-CW and SVF-CW) was more abundant than *Nitrosospira*. This outcome can be explained by the high influent concentration of ammonium (Table 4), forming a community dominated by *Nitrosomonas*, with low substrate affinity but a high maximum activity than *Nitrosospira* [43].

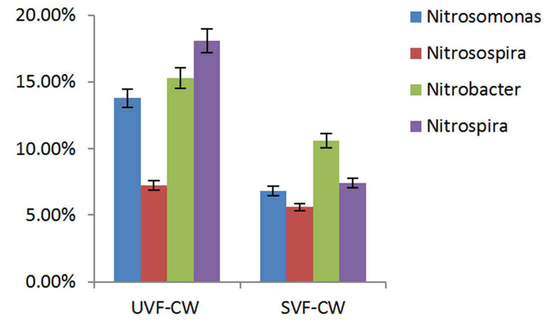

Figure 3. *Relative abundance of nitrifying bacteria in UVF-CW and SVF-CW.*

In contrary to nitrifying bacteria which deceased with increasing saturation level, the denitrifying bacteria increased with increasing of saturation depth. The relative abundances and variety of denitrifying microorganisms were significantly different among the two different vertical flow constructed wetlands ($p<0.05$), Zhang et al. [45] also observed similar findings. Furthermore, under saturated vertical constructed wetland (SVF-CW) denitrifying bacteria increased more than 3 times in comparison to unsaturated vertical constructed wetland (UVF-CW). Foladori et al. [25] showed that due to the different environmental condition of the bed mesdia along the vertical section, the depth has a great influence on the distribution of bacteria. In fact, in (SVF-CW), only *Pseudomonas* (3%), and *Paracoccus* (2%) were detected in this study (Figure 4). These two bacteria are related to the classical denitrification pathway mainly carried out by *Pseudomonas spp*. [46] and with aerobic denitrification and heterotrophic nitrification by *Paracoccus denitrifying* bacteria [47].

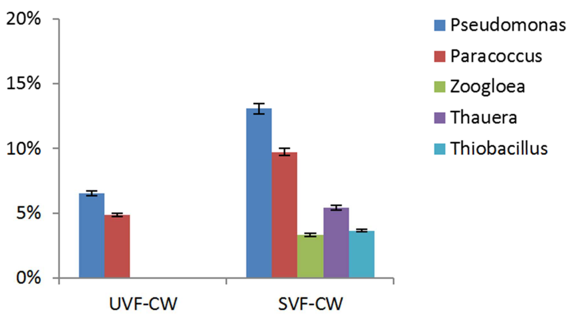

Figure 4. *Relative abundance denitrifying bacteria in UVF-CW and SVF-CW.*

However, in (SVF-CW), *Pseudomonas* (7%) and *Paracoccus* (5%) were increased in abundance but three other groups were also detected as *Zoogloea* (1.8%), *Thauera* (2%) and *Thiobacillus* (3%), owing to low availability of oxygen seems to favor denitrifying bacteria (Figure 4). These results indicated that, besides the classical pathways of denitrification, other pathways of denitrification also participated in nitrogen transformation in saturated zone. In fact, the detection of *Thiobacillus*, which can use inorganic compounds as source of carbon and compounds such as nitrates or nitrites as electron donors was associated to autotrophic denitrification [9].

4. Conclusion

Partially saturated (SVF-CW) configurations were developed to optimize the nitrogen removal in the same filter with low operation costs and low required area. This research highlighted that the application of saturation zone at the bottom can ensure nitrogen removal performance better than classic vertical flow constructed wetlands. FISH analysis in this study indicated that the diversity and the abundance of nitrifying and denitrifying bacteria were affected by the depth of saturated layer:

1) The average abundance of nitrifying bacteria in (UVF-CW) is higher than that in (SVF-CW). These results also showed that *Nitrosomonas* in the two systems was more abundant than *Nitrosospira*, this might be related to the high influent ammonium concentration, which leads, to form a community dominated by *Nitrosomonas*, and its substrate affinity lower, but the activity is higher than *Nitrosospira.*
2) A saturated zone influence was observed on relative abundances and diversity of denitrifying bacteria. In UVF-CW only *Pseudomonas* and *Paracoccus* were detected. However, in (SVF-CW), these bacteria were increased in abundance but three other groups were also detected as *Zoogloea*, *Thauera* and *Thiobacillus*.
3) In addition to the classic denitrification and nitrification, the occurrence of species as *Paracoccus, Thiobacillus denitrificans* and *Thiobacillus thioparus* have also been discovered, which are related to heterotrophic nitrification and aerobic and autotrophic denitrification.

This research is only carried out on a the laboratory scale, so, it is worthwhile to further study the removal effect and application of pilot scale and full scale to obtain more conclusive results. Other studies can focus on the effects of different factors that can improve the performance of nitrogen remove, such as plant species, substrate types. Our group is conducting further research on this topic, such as using a combination of fillers of natural materials (e.g. olive seeds, olive pomace, compost, clay, biochar) to improve the efficiency of nitrogen remove in the constructed wetland wetlands.

Acknowledgements

We wish to thank the Tunisian Ministry of Higher Education and Scientific Research for financial support, and Effluent Treatment and Valorization Laboratory for operational support. We would also like to thank the editors and reviewers for their important comments, which enhanced the previous version of this manuscript.

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