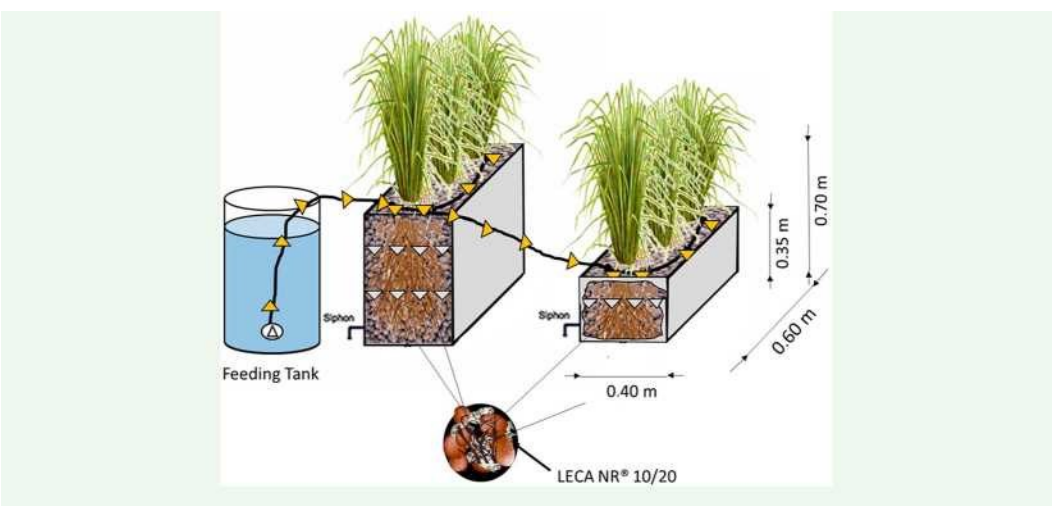
Nitrogen removal in vertical flow constructed wetlands: influence of bed depth and high nitrogen loadings

Adelaide Almeida, Krzysztof Józwiakowski, Alina Kowalczyk-Jusko, Piotr Bugajski, Karolina Kurek, Fátima Carvalho, Anabela Durao, Carlos Ribeiro & Magdalena Gajewska

Adelaide Almeida[a,b], Krzysztof Józwiakowski[c], Alina Kowalczyk-Jusko[c], Piotr Bugajski[d], Karolina Kurek[d], Fátima Carvalho[a,b], Anabela Durao[e], Carlos Ribeiro[a] and Magdalena Gajewska[f]
[a]Department of Technologies and Applied Sciences, Polytechnic Institute of Beja, Beja, Portugal; [b]FibEnTech - Materiais Fibrosos e Tecnologias Ambientais, Covilhã, Portugal; [c]Department of Environmental Engineering and Geodesy, University of Life Sciences in Lublin, Lublin, Poland; [d]Department of Sanitary Engineering and Water Management, University of Agriculture in Kraków, Kraków, Poland; [e]Department of Engineering, Polytechnic Institute of Beja, Beja, Portugal; [f]Department of Water and Wastewater Technology, Gdansk University of Technology, Gdansk, Poland

KEYWORDS
Ammonium nitrogen mass load; ammonium toxicity; bed depth; subsurface vertical flow bed; Vetiveria zizanioides

ABSTRACT
The aim of the study was to evaluate the nitrogen removal and its effects on the plant's growth and leaves morphology. using two subsurface vertical flow (VF bed), with different depths (0.24 m^2 x 0.70 m; 0.24 m^2 x 0.35 m) and nitrogen load increments. The VF bed were planted with Vetiveria zizanioides, filled with light expanded clay aggregates (Leca®NR 10/20) and fed in parallel mode with synthetic wastewater. High ammonium nitrogen concentration ([NH+-N] from 68 ± 3 to 290 ± 8 mg L^{-1}) was used without toxicity symptoms in plants, although the effects of ammonium nitrogen load were stopped the growth of the plants. Significant differences between ammonium nitrogen removed in each VF bed obtained for total nitrogen ($TN_{infl.}$) > 27 ± 0.8 g m^{-2} d^{-1}. The nitrification was contributed to ammonium nitrogen removal because was found higher values of nitrate and nitrite in the effluent. These values were more higher in VF bed 1 than in the VF bed 2, since ammonium nitrogen removal were also more higher in VF bed 1 than in the VF bed 2. Total nitrogen mass balance was carried out and the results show that the nitrification/denitrification process occurred with nitrogen plants uptake. It was observed that the VF bed depth has an influence on all nitrogen removal processes. As higher the depth root system it is seemed to favour the creation of zones with different oxidations conditions that allow the nitrogen compounds to be removed intensively.



1. Introduction
Rapid expansion of urban areas and industrial development are often associated to wastewater generation. The wastewater, such as industrial and agricultural wastewater, urban drainage, sewage and landfill leachate contain high nitrogen compounds that when discharged without enough treatment, can contribute to eutrophication by depleting dissolved oxygen (DO). Nitrogen exists in wastewater in both forms (organic and

inorganic). Organic nitrogen can be present e.g. in amino acids, urea, uric acid, is converted in ammonium ($NH4^+$) by ammonification. High concentrations of $NH4^+$ in water can be toxic to aquatic organisms and at last to human beings, when it is converted to nitrate (NO_3^-) [1-3]. Therefore, it is necessary to treat the wastewater previously to be discharged in the environment. Treatment using wetlands (TW) can be a viable technology to treat various types of wastewater, such as domestic and industrial once it has advantages of low-cost and easy operation in comparison to the conventional technologies [1,4,5]. TW is considered as a complex bio-reactor, where several physical, chemical and biological processes occur providing a stable condition to remove pollutants [1,6,7].

Nitrogen compounds transformation and removal are accomplished by biological processes (e.g. ammonification, nitrification, denitrification, plant uptake, biomass assimilation, dissimilatory nitrate reduction), and physicochemical processes (e.g. ammonia volatilization and adsorption). Recently new nitrogen removal routes were discovered (e.g. Anemoi and Canon processes) [1,7-11]. The nitrogen compounds removal routes are extremely dependent on several factors, namely: pH, temperature, dissolved oxygen (DO), feeding mode, hydraulic load (HL), hydraulic retention times (HRT), plants harvest and bed depth [1,12-14]. Some of those factors are related to each other. So, DO and its diffusion into the TW depend on: the plants species, type of the flow (vertical flow or horizontal flow, intermit- tent or continue mode), surface area geometry, HL and HRT. The wastewater characteristic and pollutants com- position, play also, an important role in nitrogen removal mechanisms [1,13-18].

The HRT is determined by the flow rate, that means that is dependent on HL and therefore on the surface area, depth, substrate porosity, as well as plants root and substrate depth [1,13]. Greater amounts of HL pro- motes a quicker passage of waste water through the substrate, thus reduces the optimum contact time. Nevertheless, greater H_L can contribute to oxygen diffusion inside the matrix, which can increase nitrification. In opposite, lack of oxygen can inhibit the nitrification process, but, predominant anoxic conditions can promote denitrification [8,19]. When HL increases, the wastewater mass load, causes nitrogen mass load applied increases too, contributing to the decreasing in removal efficiencies [15,20,21].

The substrate depth has also a significant effect on the effectiveness of pollutants removal, due to the adsorption of pollutants in substrate can occur as well as the quantity and activity of microorganism sand the growth conditions of plants [22,23]. On the other hand, is a crucial factor in determining which plant types will become established, and it also influences the biochemical reactions responsible for removing contaminants by affecting the redox status and DOlevel in TW[13]. Garcia et al. [24] observed differences in the transformations of pollutants in wet- lands subsurface horizontal flow beds (HF) with different depths (0.27 and 0.5 m). Similarly, Garcia et al. [25] evaluate the effect of depth on the removal of selected contaminants in subsurface horizontal flow beds (HF) for 3 years. The results [25] indicated that bed depth of 0.27 m removes better chemical oxygen demand (COD), biochemical oxygen demand (BOD), ammonia and dissolved reactive phosphorus. In addition, experiments carried out by Aguirre et al. [26] to investigate the effect of bed depth on organic matter removal efficiency in HF concluded that the relative contribution of different metabolic pathways varies with bed depth.

Prochaska and Zouboulis [27], in VF bed planted with Phragmites australis, observed that COD, total nitrogen (TN), nitrate (NO_3-N) and ortho-phosphate (PO^{3-}-P) removal varied significantly with bed depth and observed also that the majority of COD and $PO4^{3-}$-P removals occurred at the lower layer of the VF bed at the standard depth of 0.6 m (i.e. at depths between 0.4 and 0.6 m below the wetland's surface). So according to these researchers, there was no need to increase the bed depth from the standard 0.6-1.0 m. However, certain authors [28] recommend a depth of 1 m to avoid fast water penetration through the wetlands substrate, which would result in low pollutant removal rates. Bed depth higher may provide the possibility of developing compact CWs, which are less intensive in terms of land requirements. It may also provide the possibility to apply this technology for various industries where land availability is a big constraint [29]. The plant used in this study Vetiveria zizanioides has stiff and erect steams which can stand up to high velocities flow, it has massive and complex root system that grows very quickly, which penetrates to deeper layers of soil or substrate. The root depth in some applicationsreaches3-4m in the first year [30]. This study aimed: (i) to evaluate the possibility ofusing a VF bed to nitrogen removal, as well as to study the effect of bed depth, 0.35 and 0.70 m, respectively, on the VF bed treatment performance in respect to nitrogen removal (ii) to analyse the effect of increasing nitrogen load on the nitrogen removal and in V. zizanioides leaves growth and on its morphology.

2. Materials and methods

2.1. Vertical flow constructed wetland system

The experimentalworkwas developed usingtwo subsur- face vertical flow beds (VF bed), with different depth (VF

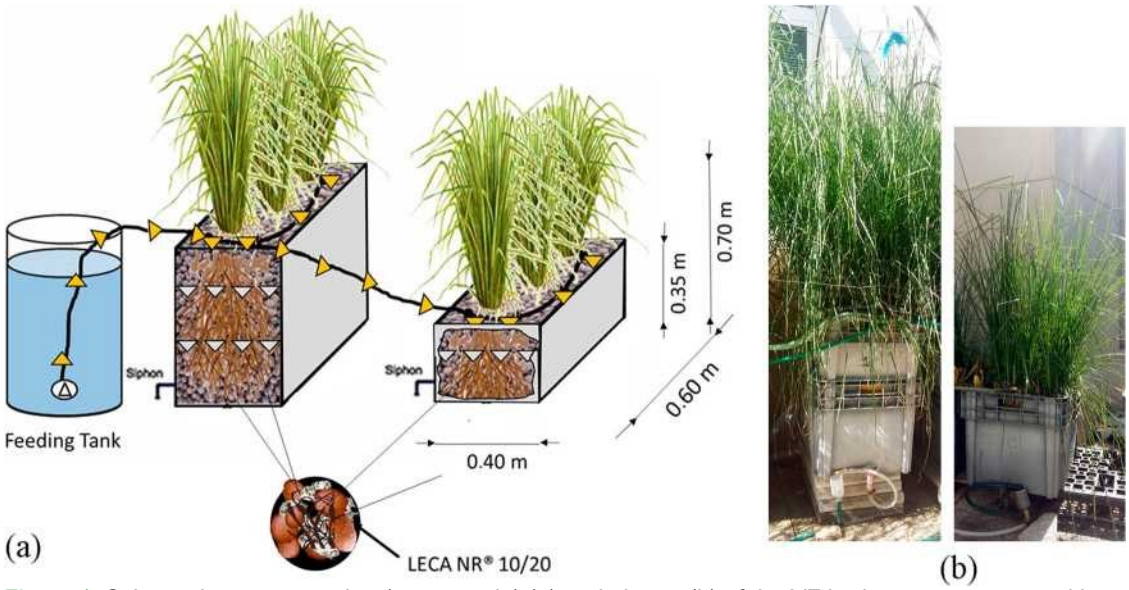


Figure 1. Schematic representation (not at scale) (a) and picture (b) of the VF bed system, composed by two beds with different depth (VF bed 1 (0.24 m^2x 0.70 m) and VF bed 2 (0.24 m^2x 0.35 m)).

Table 1. Trial number, hydraulic load applied (H_L), total nitrogen load (TN), sodium hydrogencarbonate [$NaHCO_3$], ammonium ([NH_4^+–N]), nitrate ([NO_3^-–N]), nitrite ([NO_2^-–N]) and total nitrogen ([TN]) concentrations in influent of VF bed 1 and VF bed 2. Values are means ± standard deviation (SD), ($n \geq 10$).

	Trial number	H_L ($L\ m^{-2}\ d^{-1}$)	TN ($g\ m^{-2}\ d^{-1}$)	[$NaHCO_3$]	[NH_4^+–N]	[NO_3^-–N]	[NO_2^-–N]	[TN]
				($mg\ L^{-1}$)				
7 April–26 April	1	59 ± 2	5 ± 0.4	–	68 ± 3	6 ± 2	6 ± 7	79 ± 7
12 May–03 Jun	2	60 ± 3	9 ± 0.4	–	142 ± 6	8 ± 2	5 ± 4	155 ± 3
15 Jun–14 July	3	172 ± 5	14 ± 0.8		68 ± 3	6 ± 2	6 ± 3	79 ± 3
20 July–15 Aug	4	171 ± 6	27 ± 1.1	150 ± 2	143 ± 9	8 ± 2	5 ± 2	155 ± 4
20 Aug–2 Sept	5	355 ± 9	107 ± 1.3		290 ± 8	9 ± 3	4 ± 1	303 ± 5

bed 1 (0.24 m^2 x 0.70 m) and VF bed 2 (0.24 m^2 x 0.35 m)) (Figure 1), planted with V. zizanioides. A slope of 2% was created at the bottom of the VF bed to allow easier drainage. The VF beds were filled with support matrix of light expanded clay aggregates (Leca®NR 10/20). The beds were fed in parallel and operated in continuous mode, from the same feeding tank (125 L), using two submersible pumps (Eheim-2400, Deizisan, Germany). The influent was distributed through a network sprinklers in the surface of the VF bed.

2.2. Experimental influent and operational condition

The VF beds were fed with artificial wastewater, prepared by mixing in tap water, ammonium sulphate, ammonium chloride, potassium nitrate and sodium nitrite reagents as nitrogen sources, as well as sodium hydrogen carbonate to supply alkalinity (Table 1). All reagents were purchased from Merck KGaA, Darmstadt, Germany. A mineral medium was used and prepared according to Almeida et al. [15], whose reagents were purchased from Merck KGaA, Darmstadt, Germany. This study was carried out in five successive experimental trials (Table 1). Both VF beds were performed under similar experimental conditions. The influence of depth of beds, hydraulic load (HL), total nitrogen load (TN), ammonium ([NH_4^+ -N]), nitrate ([NO_3^- -N]), nitrite ([NO_2^- -N]) and total nitrogen ([TN]) concentration were tested (Table 1).

The trials with the lowest HL were conducted firstly and between each trial, the VF beds were acclimatized to the new loading rate, before the treatment performance was measured. Three different HL were used and three different total and ammonium nitrogen concentrations, which gave origin to five different ammonium, nitrate, nitrite and total nitrogen loads applied ($p < .05$).

2.3. Monitoring of climatic conditions and plant growth

These trials were carried out in a well-established 5-year- old VF beds, with a plant density higher than 120 plants m^{-2}, installed in the campus of the Polytechnic Institute of Beja, Portugal. The climate in Beja is influenced by its distance from the coast. Beja has relatively cool winters compared to coastal Portugal, while summers are long and hot. There is more rainfall in the winter than in the summer. The average temperature is 16.1°C and the average annual rainfall is 581 mm. In August, the hottest month of the year, the average temperature is about 23.8°C, although in the last few years, the maximum daily temperature reached 44°C. The lowest mean temperature of the year has been observed in January, with a value of 9.6°C, in the last 3 years the temperature values were higher in January, there have been observed average

daily values of 20°C. The air and soil temperature were measured daily, at the same time. Soil temperature was measured using two thermometers inserted into each one VF bed and air temperature with another one suspended on the wall near the VF bed system.

2.4. Wastewater sampling collection and analysis

Every weekday, from Monday to Friday, wastewater samples were taken and the flow rate was measured in inlet and outlet of each VF bed, during the investigation period (from 7th April to 20th September). In those samples, the dissolved oxygen (DO), electrical conductivity (EC) and pH were immediately measured [31]. After pre-treatment and reagent addition, ammonium ([NH+ -N]), nitrate ([NO_3 -N]), and nitrite ([NO_2 -N]) concentrations were analysed using the methods as described in Standard Methods of APHA [31].

EC was measured using a Jenway 4510 conductivity Meter. A WTW inoLab pH Level 1 electrode was used for pH determination. Total dissolved solids (TDS) were calculated according to the following expression: TDS (mg L^{-1}) = 640EC [32]. DO was determined by the Winkler Method or iodometric method with azide modification [31].NH_4^+-N was determined after a distillation step using a Distillation Unit Buchi K-350, followed by titrimetric method with sulphuric acid, 0.02 M. The NO_2^- -N was determined through formation of a reddish purple azo dye produced at pH 2.0-2.5 by coup- ling diazotized sulphanilamide with N-(1-naphthyl)-ethy- lenediamine dihydrochloride (NED dihydrochloride), at 543 nm using a Pharmacia Ultrospec 2000 UV/VIS spec- trophotometer. The NO_3^- -N concentrations were analysed using the Cd-reduction technique. So, nitrate is reduced almost quantitatively to NO_2^- -N in the presence of cadmium (Cd). Thus, the NO_2^--N produced was deter- mined according to the same method used to nitrite, mentioned previously.

2.5. Plant growth and tissue analysis

The VF bed has a surface area of 0.24 m^2 covered with V. zizanioides. At the start of the study, the plant leaves were cut to a height around 15 cm. Vetiveria zizanioides plants were visually inspected on a weekly basis for toxicity signals. Twenty plants were randomly selected from each bed, and the height of leaves was monitored during this experiment. The aboveground tissues (leaves) were cut and analysed at the beginning and at the end of the experimental work. Three independent determinations were performed.

The concentration of the essential elements and nutrients, namely Total Kjeldhal Nitrogen (TKN), phosphorus (P), calcium (Ca), magnesium (Mg), sodium (Na), potassium (K) and dried matter were deter- mined. Vetiveria zizanoides leaves were cut into small pieces, well mixed and oven dried to constant weight at 70°C for 48 h to moisture determination, the remaining residue was put in a mufflefurnaceat temperatures of 500-550°C for 4-8 h to get ash [33,34]. The ash was dissolved using 10 mL of hydro- chloric acid 3 M to determine the concentration ofCa, Mg,K,NaandP.

Concentrations of Ca and Mg were determined by atomic absorption spectrophotometry (Varian), K and Na by flame photometry using a Model 410 Corning Flame Photometer. P after digestion liberate orthophosphate, that was determined by ascorbic acid method at 470 nm using a Pharmacia Ultrospec 2000 UV/VIS spectrophotometer. The TKN content of aboveground dry biomass was determined digesting 1 g of dried leaves during 3 h, 385 ± 2°C, using a Kjeldahl digester 2520 Auto Rack by a distillation step in a Distillation Unit Buchi K-350 [35].

2.6. Data treatment and statistical analysis

Results were statistically verified using software 'Statitistica 12.0' (StatSoft, Inc., USA). Differences in wastewater quality between influent and effluent of the VF bed were tested using ANOVA at the significance level of $p<.05$. Post-hoc (a posteriori) Tukey's test was used to determine differences between means of specific variables. All results were presented as means ±standard deviation calculated for $n > 10$.

2.6.1.Removal efficiency

Removal efficiency ofeach parameter (h_m) was obtained by applying the following Equation (1)

$$\eta_m = ((H_{Li}C_i - H_{Le}Ce)/(H_{Li}C_i)) \times 100 \qquad (1)$$

where HLiand HLe are the hydraulic load (L m^{-2} d^{-1}), and Ci and Ce are the concentration (mg L^{-1}), respectively, in the inlet and outlet of the VF bed.

2.6.2.Hydraulic retention times calculations

The hydraulic retention time (HRT) was established from Equation (2) [36]:

$$HRT = V/Q_i = AYP/Q_i \quad (2)$$

where Qi (L d^{-1}) is the influent flow rate, measured every day in the inlet of the bed; A is the surface area of the bed (m^2); V (m^3) is the bed volume; Y (m) is the flow depth; P is the porosity, which expresses the space available for the water to flow through the matrix, roots and other solids in the subsurface constructed wetland system [36]. Rainfall data were not used because the VF beds were covered by a transparent plastic greenhouse to avoid dilution in the wastewater concentration. Evapotranspiration was not calculated because the flow rate in inlet and outlet the VF bed was measured every day.

3. Results and discussion

3.1. Temperature

Air temperature (Tair) was monitored daily and in the same assay a great thermic amplitude was observed: the maximum Tair was 44°C (trial 3) and minimum in trial 1 Tair= 5°C (Figure 2).

The temperature was often referred as important to the wetland performance, namely to organic matter and nitrogen removal, once these processes are considered depending on microbial activity and vegetal biomass present. Thus, plants growth, photosynthetic rate among others are depending on the wastewater and the local temperature, that interfere with the

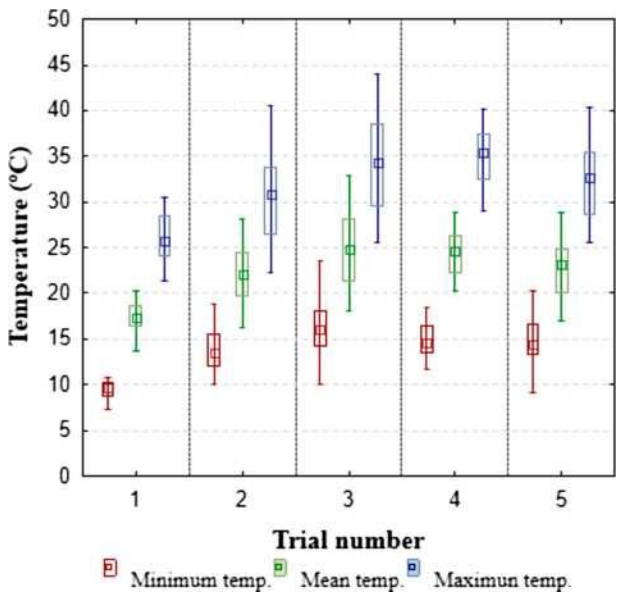


Figure 2. Air temperature observed during the trials. The vertical lines separate the trials.

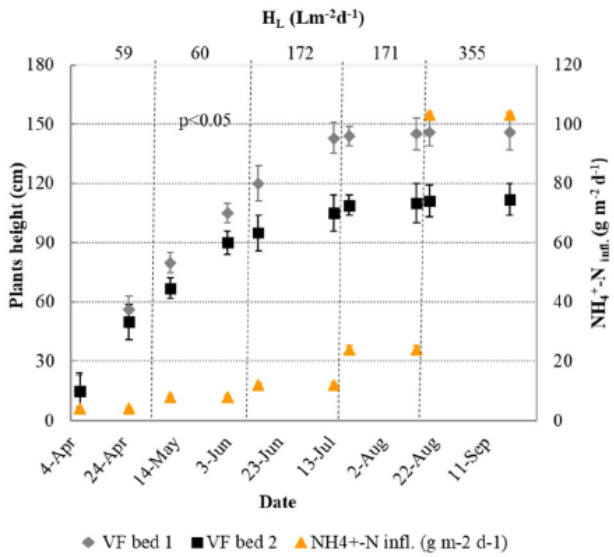


Figure 3. *Vetiveria zizanioides* growth in VF bed 1 and VF bed 2. The vertical lines separate the trials.

nitrogen removal [37]. It was founded by Stefanakis and Tsihrintzis [38] that organic matter (COD and BOD5) and TKN removal, from a synthetic wastewater, in a VF bed feeding in intermittent mode, was favoured by the temperature increase. However, there are some controversies about the temperature influence on the system performance. Prochaska et al. [39] found that a VF bed nitrification occurred when temperatures were close to 7°C, but Cooper et al. [17] and Prochaska et al. [39] stated that the minimum temperatures for nitrates production vary from 4°C to 5°C. It is important to refer that when temperatures are higher, DO has a tendency to decrease, which may contribute to decreasing the organic matter and ammonium nitrogen removal.

In this study, the minimum air temperature reached was about 5°C, taking into account that was referred by Prochaska et al. [39], then temperature did not interfere negatively with the microbial community development. Regarding V. zizanioides, Dudai et al. [40] observed under Mediterranean conditions, that air temperature >15-23°C and long days, influenced plants growth, and consequently nitrogen uptake by plants increased (Figures 3 and 4).

3.2. Growth of wetland plants

During the whole study period, plants grew very well without obvious symptoms of toxicity or nutrients deficiency. The results show a similar pattern growth in both beds.

Plants height rose quickly, after they have been cut at the beginning of the experimental work, reaching a stationary growth state, in the two last trials, since 20 July till the end of the trials. The average height was of 146 ± 9 and 112 ± 8 cm, for VF bed 1 and VF bed 2, respectively, (Figure 3). However, from the beginning of the trial 2, significant differences were observed in plants growth, between the VF bed ($p < .05$).

As said before there are some factors as being able to interfere with plants wetland growth, namely the ammonium nitrogen excesses cause reduction in photo- synthesis and nutrients uptake by plants so, the plants can show various toxicity symptoms such as chlorosis, growth inhibition and finally death. It has been accepted that there are great differences in ammonium tolerance among plant species [41-44]. It was found in literature various ammonium nitrogen concentration referred as toxic for plants, namely from 0.5 to 10 mM (from 7 to 140 mg L^{-1} of NH+ -N) [41-43]. Tarvorasak et al. [45] observed that ammonium concentrations of 10 and 15 mM (from 7 to 210 mg L^{-1} of NH_4^+-N) inhibited plants growth, root length and new sooth production in Pennisetum purpureum x P. americanum. However, Li et al. [46] referred that Typha angustifolia was not significantly affected by ammonium concentration below 28 mM (400 mg L^{-1} of NH_4^+-N).

The EC is often referred as salinity or/and sodium con- centration in wastewater, but it is also referred as able to inhibit the plants growth [30,47,48]. As we can see later the EC and the sodium concentration observed in this study in the VF bed influent was always lower than the values reported as toxic for the plants [30,47], the stop- page of leaves plant growth observed was not due to these parameters.

In this work, five different ammonium nitrogen loads were generated from three increasing concentrations of ammonium nitrogen (varied from 68±3 to 290 ±8 mg L^{-1}) and three HL. Therefore, the plant's growths were analysed in function of the applied loads (ammonium nitrogen). It can be concluded that: during the first three trials the leave plants had grown exponentially. Although, in fourth and fifth trial carried out with loads of 24 ±1.1 and 103 ±1.0 g m^{-2} d^{-1}, respectively, the plants leaves presented the same height. So, this stopped growth can be attributed to their life cycle or the high ammonium nitrogen load applied. However, it seems that the most plausible reason is the applied load because the stoppage of growth occurred at the beginning of the fourth trial. Regarding the bed depth it is also possible to conclude that it had an effect on the growth of the plants, due to, the plants of VF 2 has shown less high.

So, we can conclude that the VF bed depth has an influence on the plants growth, probably due to in the VF bed 1 there was more vertical space, that allowed the V. zizanioides root system, which is finely structured, have grown quickly. Although [30] reported that V. zizanioides roots can grow up to 4 m in the first year. In other hand, Vymazal [6] noted that the bed depth can limit the space for increasing plants root growth. The results shows that ammonium nitrogen load, together with the VF bed depth bed were the most important factors to V. zizanioides leaves growth and consequently to ammonium nitrogen removal. Photo- synthesis activity as well as root activity might result in more oxygen release into the VF bed, contributing to aerobic conditions creation which interferes with ammonium removal.

3.3. Nutrient and mineral elements in plants

The concentration of some mineral elements and nutri- ents (Ca, Mg, Na, K, TKN and P) in the V. zizanioides leaves was analysed, after cutting. The results show that at the start of the trials K and P concentrations, in the VF bed 1 V. zizanioides leaves, were significantly different from those observed in the VF bed 2 ($p < .05$) (Figure 4).

The remaining parameters (TKN, Ca, Mg and Na) did not show significant differences at the beginning of the trials between the VF bed (Figure 4). The concen- tration of Ca and TKN showed a tendency to increase due to the ammonium nitrogen exposition, with signifi- cant differences between the values at the end of the

sampling period and the both VF beds ($p < .05$). The depth of the VF bed probably also contributed to this.

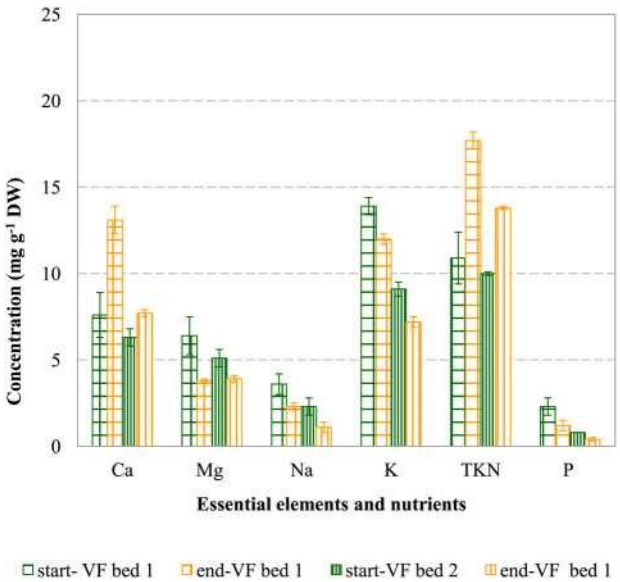


Figure 4. Vetiveria zizanioides leaves essential elements (calcium(Ca), magnesium (Mg), sodium (Na), potassium (K)) and nutrients concentration (Total Kjeldhal Nitrogen (TKN), phosphorus (P)), at the star and at the end of the trials, in the VF bed 1 and VF bed 2.

The concentration of Mg, Na, P decreased in both VF beds, due to the V. zizanioides exposition at the high ammonium nitrogen concentration, being significantly different at the start and at the end of the sampling period, in each VF bed ($p < .05$). Some results obtained in this study were different than those reported by Britto and Kronzucker [41].

According to [41] the plants fed with ammonium nitrogen, decreases all cations concentrations particularly K and the P uptake increase. However, in our study, it was observed that when ammonium nitrogen increased, Ca and TKN uptake were increased too but P uptake decreased. In general, the concentration of all cations and nutrients, at the end of the trials, were smaller in the VF bed 2 than in the VF bed 1. Therefore, in addition to high ammonium nitrogen, VF bed depth also interferes with the composition of the plants leaves.

3.4. Influence of ammonium nitrogen load and VF bed depth in pH, electrical conductivity and dissolved oxygen variation

The results show that pH in the influent was practically constant, with an average value of 7.5 ± 0.2, during whole experimental period (Figure 5(a)). The pH variation in effluent was different in both VF bed. So, pH effluent of the VF bed 1, in the first and second trials (done with TNinfl. of the 5 ± 0.4 and 9 ± 0.7 g m^{-2} d^{-1}, respectively), was significantly lower in effluent than in the influent ($p <.05$) (Figure 5(a)). The minimum pH observed in effluent was 3.9 in the second trial (TNinfl. of 9 ± 0.7 g m^{-2} d^{-1}) because during the nitrification process the effluent alkalinity is consumed, causing a pH decrease [49]. To prevent such a situation, NaHCO3 was added in the feeding reservoir, from the third trial (done with TNinfl.= 14 ± 1.1 g m^{-2} d^{-1}) till the end of the sampling period. Thus, the alkalinity was kept constant, avoiding a substantial pH decrease [7]. In the remaining trials, there were no significant differences between pH in the effluent and influent ($p > .05$) (Figure 5(a)).

The pH decrease in effluent was not observed in the VF bed 2 (Figure 5(a)), remaining practically constant during the experimental period, although the NaHCO3 addition in the feeding tank had only occurred from the third test assay on. In this VF bed the ammonium nitrogen removal also occurred, but in minor extension than in the VF bed 1, as it can be seen later, when the ammonium nitrogen removal will be studied (Figure 6). Thus, once the influent was not buffered in the first two trials (1 and 2), it was only possible to evaluate the effect of VF bed depth in these trials. Therefore, it was found that the pH in the effluent of VF bed 1 decreased to an acid range, most likely because the ammonium nitrogen removal had occurred in larger scale than in the VF bed 2, with H^+ production.

The average EC in the influent varied from 1.6 ± 0.2 to 2.9 ± 0.3 mS cm^{-1} (Figure 5(b)). The raising observed was due to the increase of ammonium nitrogen concentration and the addition of NaHCO3 (Table 1).

There were no significant differences between influent and effluent average EC, in both beds ($p > .05$). It can be observed a slight increase in TDS for the effluent when comparing the influent and the effluent of each trial. Nevertheless, no significant differences were detected ($p > .05$). These results indicate that there was no retention of salts into the VF bed. The EC as already referred is often associated with wastewater salinity. High salt concentrations impose osmotic and ionic stresses in plants, in plants morphology and physiology [50].

Edelstein et al. [51] found that under optimum

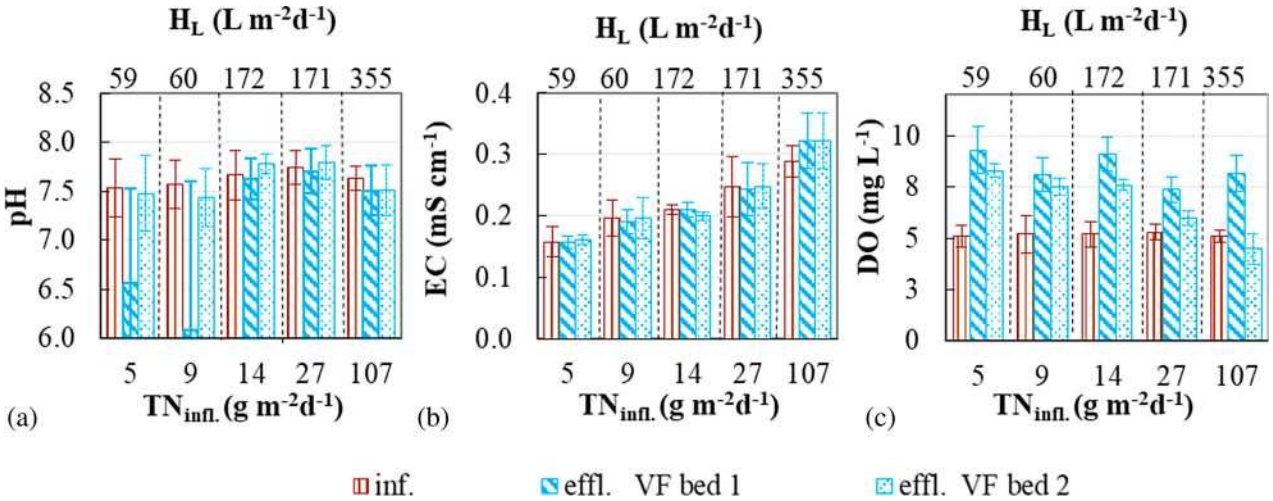


Figure 5. pH (a), EC (b) and DO (c) variation in influent and effluent of the VF bed 1 and VF bed 2 with hydraulic load (HL) and and total nitrogen in influent ($TN_{infl.}$). The vertical lines separate the trials.

fertilizer conditions, the salinity threshold for V. zizanioides, was between 3 and 6 dSm^{-1}. Truong [30] observed that V. zizanioides can survive in saline soil with EC up to 47.5 mS cm^{-1}, its salinity threshold is at EC of the 8 mS cm^{-1} and soil EC values of 10 and 20 mS cm^{-1} reduce yield by 10% and 50%, respectively. As in our study influent EC was smaller than the one reported as susceptible to cause reduction yield in V. zizanioides, then this parameter was not problematic for the development and plants growth, as previously mentioned.

The DO influent was practically constant, with an average of 5.2 ± 1.2 mg L^{-1} and without significant differences between trials ($p > .05$). Anaerobic conditions were never detected, in influent and effluent of both VF bed (Figure 5(c)). Although the operational conditions applied in both beds were identical, DO in VF bed 1 effluent showed a tendency to increase in all trials, when compared with influent ($p < .05$). DO in VF bed 2 effluent also increased during the experimental period, except for the last trial (done with TNinfl. of 107 ± 1.3 g m^{-2} d^{-1}), in which DO in effluent was smaller than in the influent, but without significant differences ($p > .05$). The ammonium nitrogen removal occurred in all trials, probably by nitrification, which is a process that consumes DO. The main pathways of oxygen transfer in the VF bed are atmospheric diffusion and plant-mediated oxygen transfer. So, DO transfer varied with plant species and season, and convective flow of air within the pore space of the matrix [52,53]. VF bed is generally considered to be a highly aerobic system, since wastewater drains vertically through the planted matrix, allowing unsaturated conditions and excellent oxygen transfer.

The DO in VF bed 1 effluent was never lower than in influent, as opposed to VF bed 2, that in the last trial (TNinfl. = 107 ± 1.3 g m^{-2} d^{-1}) showed a lower capacity to replace DO consumed, probably due to the high ammonium nitrogen load applied. As mentioned before, the operational conditions were identical in both VF bed, wastewater affluent drained vertically through the planted substrate, enabling DO to come into the VF bed by diffusion. Thus, VF bed 2 depth was about half of the VF bed 1, DO coming by diffusion to the VF bed 2 was smaller than in VF bed 1, for the same trial.

Yao et al. [54] did a comparative study to assess the potential to release oxygen by the roots of six wetlands plants, found that V. zizanioides possess the highest oxygen release capacity and tolerance to wastewater. Some research suggests that photosynthesis is the source of oxygen released by plant roots, so plants can transfer part of the oxygen produced through
photosynthesis within the aerenchyma to the roots and release it into the wetland substrate [53]. As the above-ground biomass reached different heights, in both VF beds (Figure 2), the photosynthetic activity may have contributed to the DO concentration values in effluent in each VF bed, such as the plants root system, probably smaller in VF bed 2 than in VF bed 1.

However, Ren et al. [22] found that the densities of fresh and dry roots of Canna indica L. showed highest values for lower beds (with 0.1 m depth) in VF and HF bed than in deeper beds (0.3 and 0.6 m).

According to the results already presented in this paper, we can conclude that the VF beds depth, leaf biomass height together with ammonium nitrogen load applied interfered with DO in effluent, although, the average values were different in both VF beds.

3.5. Nitrogen removal

3.5.1.Ammonium

As said before both VF beds were fed from the same reservoir (Figure 1), in parallel and continuous mode, according to operational conditions presented in Table 1. Five increasing ammonium nitrogen load were applied, from 4 ± 0.1 to 103 ± 1.0 g m^{-2} d^{-1} in both VF beds. The results show that ammonium nitrogen removal (Figure 6) occurred in all trials, with an average value from 3 ± 0.2 to 27 ± 1.0 g m^{-2} d^{-1} and from 2 ± 0.3 to 14 ± 1.0 g m^{-2} d^{-1}, in the VF bed 1 and VF bed 2, respectively. In the first trial (TNinfl . =5±0.4 g m^{-2} d^{-1}) there was a little difference between ammonium nitrogen removal in the VF bed 1 and VF bed 2, but

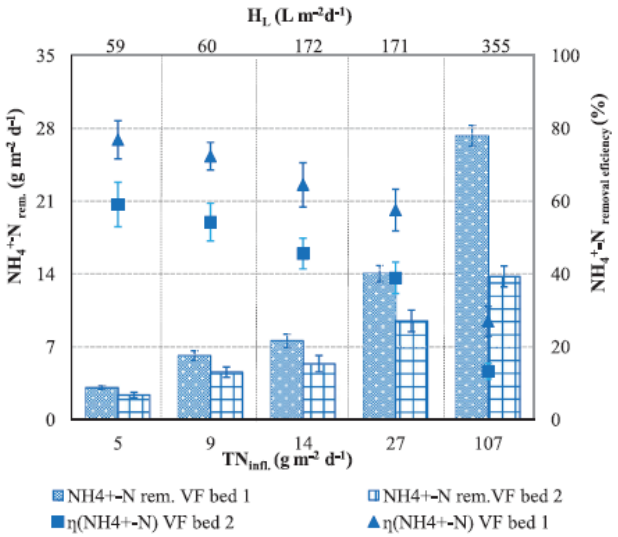


Figure 6. Ammonium nitrogen removed and removal efficiency in VF bed 1 and VF bed 2, with hydraulic load (H_L) and total nitrogen in influent ($TN_{infl.}$). The vertical lines separate the trials.

without a significant difference (ammonium nitrogen removed = 3 ± 0.2 and 2 ± 0.3 g m^{-2} d^{-1}, in the VF bed 1 and VF bed 2, respectively, Figure 6). In the remaining trials, the average values of the ammonium nitrogen removal were significantly different ($p < .05$), in both VF beds. Through the Figure 6, it is possible to observe that the biggest differences between the ammonium nitrogen removed in each VF bed occurred in the last two trials, and this difference was more significant in the last trial when nitrogen load applied was 107 ± 1.3 gm^{-2} d^{-1} and the HRT was 0.7 ± 0.1 and 1.6 ± 0.2 h, respectively, in the VF bed 2 and VF bed 1. The removal efficiencies were always higher in VF bed 1 (Figure 6) and significantly different from those obtained in VF bed 2 ($p < .05$). The ammonium removal efficiencies dropped from 77 ± 3% to 27 ± 3% and from 59 ± 6% to 13 ± 2% in the VF bed 1 and VF bed 2, respectively. As illustrated in Figure 6, ammonium nitrogen removed, and the removal efficiencies were significantly improved by the VF bed depth increasing, although there are many other factors that can contribute to ammonium removal mechanisms.

The mechanisms of NH+-N removal can be influenced by the operational conditions and design parameters, namely temperature, pH, Eh, DO, salinity, ammonium nitrogen concentration, HL, HRT, wetland plants, substrate material and depth, etc. [8,22,55,56]. So, ammonium volatilization depends on wastewater pH, an increase of pH (pH > 9.3) can increase the alkalinity of the wastewater, converting NH_4^+-N ions to NH3 gas followed by the release of gas. It is unlikely because ammonium volatilization played an important role in the ammonium removal processes in both VF bed, as the pH observed was near neutral (7.9 ± 0.3) in the influent and always smaller than 7.5 ± 0.5 in the effluent. The adsorptionphenomenoninTWisgoverned by matrix promoting cation exchange between matrix components and $NH4^+$ ions in the wastewater [8].

The light expanded clay aggregates (LWA) is frequently referred as having a good NH_4^+-N adsorption capacity. Biatowiec et al. [57] used two layers of LWA (upper layer)- gravel (bottom layer) substrates in vertical flow wetland systems and achieved 60% TN removal. Almeida et al. [58] and Almeida [59] used light expanded clay aggregates (Leca ®NR 10/20) as substrate in a VF bed to treat swine and synthetic wastewater and concluded that the NH_4^+-N adsorption by the substrate was insignificant. The same substrate material was used in this study, we can conclude that the ammonium adsorption did not occur.

The presence of plants is essential for wetlands to improve the performance of nitrogen removal. They allow the oxygen to flow to the wetlands, creating zones with different concentrations of oxygen, that is, aerobic, anoxic and anaerobic. Thus, the establishment and development of several communities of microorganisms that perform degradation of organic matter, as well as the process of nitrogen removal, may

occur. Plants also increase and provide carbon from root exudates (due to photosynthetically fixed carbon) and can excrete exogenous enzymes that contribute to the removal of nitrogen [16,60,61]. As shown in Figure 3 the plants growth was higher in the VF bed 1 than in VF bed 2, from trial 2 till the end of the trials and in Figure 4 was observed that NH_4^+-N uptake by plants also occurred ($p < .05$), and was higher in VF bed 1 than in VF bed 2. These results are according to the ones found by Yu et al. [62] and Li et al. [63], who observed a relationship between the plant biomass production and ammonium nitrogen uptake by Vetiveria zizanioides. Perhaps was one of the mechanisms through which the NH_4^+-N removal occurred, and with a higher value in VF bed 1 than in VF bed 2 (Figure 4). Kong et al. [64] correlated the NH_4^+-N removal efficiency with the urease and protease activity in V. zizanioides systems, they observed that the enzymatic activity was higher in the upper layers of the substrate than in the deeper layers. In this study when the ammonium nitrogen load was higher, the deeper VF bed 1 show a better performance concerned to ammonium nitrogen removal. So, it is possible that in the VF bed top layer, the enzymes activity can contribute to NH+-N removed in both VF bed. However, in the deeper zones of VF bed 1 can occur other -processes leading to greater ammonium nitrogen removal.

On the other hand, once V. zizanioides has a big roots system, which grows vertically down, enabling different oxidation states, this can influence positively ammonium oxidation and obtain better ammonium nitrogen oxidation in VF bed 1 than in VF bed 2. Studies carried by Cheng et al. [65] and Li et al. [63] showed a relationship between ammonium nitrogen removal and oxygen transfer and biomass production by V. zizanioides as observed in the Figures 3, 4 and 6(c).

The HL is closely related with nitrogen load, with HRT and this one also depends on the VF bed depth. There is some controversy about the HL ranges employed and which is their effect on VF bed performance. Millot et al. [66] in a VF bed concluded that when HL and nitro- gen load increased the ammonium nitrogen removal efficiencies decreased. Kantawanichkul et al. [67] and Kantawanichkul and Wannasri [68] in VF bed used to treat a swine and urban wastewater, found that when HL increased the ammonium nitrogen and organics matter removal performances was reduced. Ghosh and Gopal [69], in a VF bed, found that HRT greater than 4 days contributes to maximizing ammoniacal nitrogen removal efficiency, since values of 70% were observed in 1 d of HRT and around 100% 4 days of HRT. Akaratos and Tsihrintzis [70], in HF bed, reported an ammonium nitrogen removal efficiency of 15.3% when the HRT was greater than 6 days and above 70% when the HRT was higher to 20 days, the average influent concentration was the 38 mg L^{-1}. Zhang et al. [71] and Stefana- kis and Tsihrintzis [38] verified in a VF bed that the increase of HRT (HL decrease) affected positively the ammonium nitrogen removal due to a longer contact time of the wastewater with plant roots and microorganisms and when the plants were fully matured the aeration supply inside the systems were improved, providing a buffer against HL increment.

In this study two different VF bed depths were used, although the HL applied, and the nitrogen load applied has been identical, in each trial performed in both VF bed, the HRT was different and practically double in VF bed 1 than in VF bed 2. The HRT varied from 10 ± 0.1 h to 1.6 ± 0.2 h and from 4.5 ± 0.5 h to 0.7 ± 0.1 h respectively in VF bed 1 and VF bed 2. The biggest differences in ammonium nitrogen removal between both VF bed, was observed in the two last trials (TNinfl. from 27 ± 1.1 to 107 ± 1.3 g m^{-2} d^{-1}), and in these trials the HRT was small enough, of 3.3 ± 0.1 h in VF bed 1 and 1.5 ± 0.1 h in VF bed 2, trial 4 and 1.6 ± 0.2 h in VF bed 1 and 0.7 ± 0.1 h in VF bed 2, trial 5, that could contribute to a significant reduction of ammonium nitrogen removal when the HL was increased from 171 ± 6 to 355 ± 9 L m^{-2} d^{-1} and nitrogen load from 27 ± 1.1 to 107 ± 1.3 g m^{-2} d^{-1}. The removal efficiency dropped from 58 ± 2 to 27 ± 3% and 39 ± 4 to 13 ± 4%, for VF bed 1 and VF bed 2, respectively. The VF bed 1 exhibited higher removal efficiencies than
VF bed 2. This might be explained by the high ammonium nitrogen load associated with a lower oxygen transfer capacity in the VF bed 2 (Figure 6) and the lower HRT due to the smaller VF bed depth, among others the lower HRT due to less VF bed depth.

3.5.2.Nitrate and nitrite

The results obtained show that nitrate and nitrite load increased in both VF beds effluent (Figure 7(a,b)). So, the nitrate load produced (Figure 7(a)), calculated from subtraction of nitrate load in effluent and the influent, varied from 1.2 ± 0.1 g m^{-2} d^{-1} to 5.9 ± 0.3 g m^{-2} d^{-1} and from 0.4 ± 0.1 g m^{-2} d^{-1} to 3.1 ± 0.8 g m^{-2} d^{-1}, in VF bed 1 and VF bed 2 respectively. As expected, in the first two trials (TNinfl. = 5 ± 0.4 and 9±0.4 g m^{-2} d^{-1}), there were no significant difference ($p > .05$) between nitrate load in effluent and in influent in each VF bed, as it had already been observed with ammonium nitro- gen removal. In the remaining trials 3, 4 and 5 (TNinfl. = 14 ± 0.8; 27 ± 1.1 and 107 ± 1.3 g m^{-2} d^{-1}), nitrate load in effluent increased, showing significant differences with the influent ($p < .05$), as well as between the effluent in both VF beds. The nitrate load produced was higher in VF bed 1 than in VF bed 2, and significantly different ($p < .05$). Nitrite (Figure 7(b)) is an unstable chemical nitrogen form, it is produced in the first part of ammonium oxidation and then in nitrate. On the other hand, as referred by Reddy and DeLaune [72], the nitrate derived through nitrification rapidly diffuses into anaerobic

soil layers, where it is used as an electron acceptor and reduced into dinitrogen via intermediates nitrite, nitric oxide, and nitrous oxide. Therefore, these processes can be broken, resulting in nitrite accumulation.

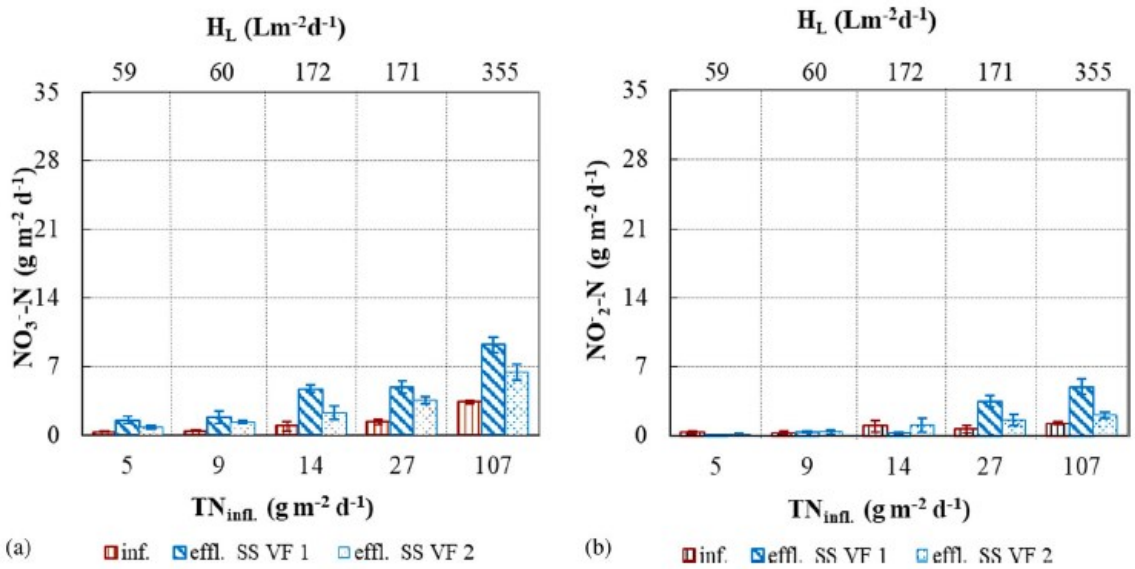


Figure 7. Nitrate (a) and nitrite (b) mass load variation in influent and effluent of the VF bed 1 and VF bed 2, with hydraulic load (H_L) and total nitrogen in influent ($TN_{infl.}$). The vertical lines separate the trials.

Relating to nitrite (Figure 7(b)) in trial 1, 2 and 3, there were no significant differences in the average nitrite load in each VF bed in influent and effluent ($p > .05$), as well as between effluent in each VF bed. However, in the trials 4 and 5, the average nitrite load in effluent raised with significant differences when com- pared with influent ($p < .05$), being also significantly different ($p < .05$) between both VF bed. As nitrite load increased in effluent, with higher values for the VF bed 1, it is possible that nitrification processes were not complete due to the decrease in HRT or the ammonium nitrogen load increase. Zhang et al. [73] found that lower oxygen concentrations favour ammonium oxidation preferentially than nitrite oxidation. However, it does not seem that DO had been a limiting factor to nitrite oxidation (trial 4 and 5), since both VF bed effluent never presented anaerobic conditions, although the VF bed 2 effluent showed a DO decreased comparatively to the influent.

According to Bae et al. [74] temperatures higher than 30°C and pH between 8 and 9 inhibit nitrite oxidation (nitrification). High concentration of free ammonium (0.1-10 mg L^{-1}) also effectively inhibited nitrite oxidation, resulting in its accumulation [73]. In this study, air temperature reached values up to 44°C in the two last trials and pH influent was always near neutral (7.7 ± 0.3) and never decreased in effluent (Figure 4(a)). So, it is unlikely that these parameters had an influence on effluent nitrite accumulation. Free ammonia concentration in VF bed influent was around of 0.002 ± 0.0005 mg L^{-1} suggesting that nitrification as well as nitrification were not inhibited by free ammonium.

If a mass balance was carried out and the ammonium nitrogen removal as well as nitrate and nitrite load produced were considered, it could be seen that there was a difference between them, the ammonium nitrogen removal was superior than the nitrate and nitrite load produced.

Some processes through which ammonium nitrogen removal may occur, have already been analysed. However, it is important to point out the possibility that another process, not yet mentioned, can occur. Short-range nitrification and anaerobic ammonium oxidation can occur [9,10] under low DO. In this study DO increased in all trials, except for the last trial, in VF bed 2. The nitrate load produced in both VF beds, over the whole experimental period, was always higher than it was expected if this process occurred, as main mechanism to ammonium nitrogen removal. Therefore, we can conclude that the nitrification and subsequent denitrification should be the main mechanism to nitrate and nitrite loads removal.

3.5.3.Total nitrogen

The mass balance approach was used to quantify the contributions of different removal pathways in TN removed and evaluating nitrogen transformations in VF bed. TN was considered as the sum of all nitrogenous forms presented, such as NH+-N, NO_3 -N and NO_2 -N.

According to Figure 8, it was found that in both VF beds, total nitrogen removal occurred but, they had a low potential for nitrate removal, thereby the TN removal was smaller than ammonium removal, once in the effluent nitrate and nitrite load remained still. The TN removal varied from 2±1.5 to 19±1.1 g m^{-2} d^{-1}, the removal efficiencies varies from 47±1.5 to 15± 1.1% in VF bed 1 and from 2±0.4 to 11 ±1.3 g m^{-2} d^{-1} with a

removal efficiency from 47±2.9 to 12± 2.1%, in VF bed 2.

It should be noted that there were only significant differences in the ammonium removal, between both VF bed in the last trial, when TNinfl. =107±1.3g m^{-2} d^{-1}. In practical terms it was when depth VF bed effect was more noted in the NH_4^+-N load removed, and also DO in the VF bed 2 effluent was lower than in influent, contributing to NH_4^+-N removed decrease in VF bed 2. So, nitrate derived through nitrification rapidly diffuses into anaerobic soil layers in the deeper VF bed, that was VF bed 1, and was reduced into dinitrogen via inter- mediates nitrite, nitric oxide, and nitrous oxide. More- over, other nitrogen forms can remain in effluent, if denitrification is not complete, e.g. nitroxyl, hyponitrite, etc. [72].

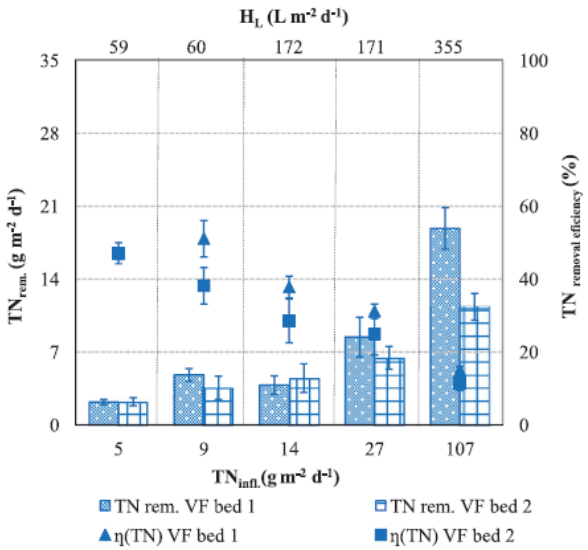


Figure 8. Total nitrogen removed and removal efficiency in VF bed 1 and VF bed 2 with hydraulic load (H_L) and total nitrogen in influent ($TN_{infl.}$). The vertical lines separate the trials.

4. Conclusions

In this study high ammonium nitrogen concentrations ([NH+ -N] from 68 ±3 to 290 ±8 mg L^{-1}) were used without toxicity symptoms in plants, such as chlorosis and death. Significant differences between ammonium removal in each VF bed were obtained to $TN_{infl.}$ > 27 ± 0.8 g $m^{-2}d^{-1}$.The nitrification contributed to ammonium removal and because of that, higher nitrate and nitrite load values in the effluent were observed in VF bed 1 than in VF bed 2. The TN mass balance carried out suggested that the nitrification/ denitrification process occurred together with nitrogen plants uptake.

In conclusion the VF bed depth has an influence on all processes and higher depth of root system promote the creation of zones with different oxidations conditions, which allowed a more intense removal of nitrogen compounds.

The depth of the VF bed is a dimensioning parameter associated with its performance, since when the depth increases the hydraulic retention time increases too. The highest significant differences between the performances of the two VF beds were observed, for all parameters, to very low retention times, 1.6 ± 0.2 h and 0.7 ± 0.1 h, respectively, in VF bed 1 and VF bed 2. Further work is needed to determine how the bed depth influences these processes, mainly in the trials carried out with higher total nitrogen load (TNinfl. > 27 ± 0.8 g m^{-2} d^{-1}).

Disclosure statement

No potential conflict of interest was reported by the authors.

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This is a post-peer-review version of an article published in Environmental Technology, 2020 following peer review. The version of record Almeida, A., Jóźwiakowski, K., Kowalczyk-Juśko, A., Bugajski, P., Kurek, K., Carvalho, F., Durão, A., Ribeiro, C. & Gajewska, M. (2020). Nitrogen removal in vertical flow constructed wetlands: influence of bed depth and high nitrogen loadings. Environmental Technology, 41(17), 2196-2209. https://doi.org/10.1080/09593330.2018.1557749