Nitrates removal in vertical flow constructed wetland planted with *Vetiveria zizanioides*: Effect of hydraulic load

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Abstract

A vertical flow constructed wetland (VFCW) planted with *Vetiveria zizanioides* (0.24 m² x 0.70 m) intended to receive a synthetic wastewater with high nitrate concentration was used to study the influence of the hydraulic load (Hl) on nitrate load removal, keeping a low carbon nitrogen ratio (C/N). The inlet nitrate concentration was kept constant ([NO₃] = 83 ± 7 mg L⁻¹) and four different levels of Hl were used, from 148 ± 5 to 473 ± 5 L m⁻² d⁻¹; accordingly, nitrate load increased from 12.4 ± 0.7 g m⁻² d⁻¹ to 39.9 ± 0.7 g m⁻² d⁻¹. The nitrate load removal occurred in all trials, increasing proportionally to Hl and nitrate load applied, reaching a maximum value of 11.9 ± 0.7 g m⁻² d⁻¹ at a nitrate load applied of 20.4 ± 1.2 g m⁻² d⁻¹ and Hl of 239 ± 7 L m⁻² d⁻¹. Beyond that maximum, a further increase in Hl or nitrate load applied led to a considerable decrease in nitrate load removal. Anaerobic conditions were not detected and denitrification was observed even at 5.2 ± 1.2 mg L⁻¹ O₂ in influent. Toxicity signs in *Vetiveria zizanioides* leaves were never detected and the results obtained suggest that the used plant absorbs and assimilates nitrogen from wastewater. Although *Vetiveria zizanioides* is not commonly used in constructed wetland, it proved to be very a robust, efficient and promoting system, encouraging further studies.

1. Introduction

Increasing nitrogen levels in environment and their subsequent impacts on aquatic ecosystems has attracted growing attention. The excessive accumulation of nitrate discharged into water bodies can cause serious ecological problems, namely, the eutrophication of lakes, rivers and groundwater. Eutrophication can result in a serious degradation of aquatic ecosystems and impair the quality of water for drinking, industry, agriculture, recreation, or other purposes (Kadlec and Wallace, 2009). Highly contaminated wastewater with nitrogen is supposed to be treated before discharging. Biological treatment technologies such as activated sludge processes associated with the modified Ludzack-Ettinger process (MLE), and membrane bioreactor have been widely used to treat nitrogen from polluted wastewaters, achieving nitrate removal to some extent (Metcalf and Eddy, 2003; Li et al., 2014).

Consistent efficiencies can be achieved with other technologies, for example, ion exchange, reverse osmosis, electrodialysis and adsorption (Chatterjee and Woo, 2009). However, these processes require high operation and maintenance costs, as well as energy consumption.

Constructed wetlands (CWs) are an efficient and environment-friendly treatment technology with low cost of construction, operation and maintenance (Vymazal, 2011). In constructed wetlands, nitrate is removed from the wastewater mainly by microbial denitrification and plant uptake (Lin et al., 2008). Denitrification is an anoxic process where bacteria use nitrate (NO₃⁻) or nitrite (NO₂⁻) as an electron acceptor and organic carbon as an electron donor to obtain energy for growth and maintenance, and produces nitrogen gas (N₂), nitrous oxide (N₂O) or nitric oxide (NO) (Saeed and Sun, 2012). There are some factors that influence denitrification, namely, availability of carbon and carbon/nitrogen ratio (C/N), dissolved oxygen (DO), hydraulic load (Hl) and species of the wetland plants (Sirivedhin and Gray, 2006; Vymazal, 2007).

Carbon source in CWs usually comes from wastewater, soil and plant root exudates (Songlu et al., 2009; Vymazal and Kropfelova, 2008). The external carbon addition may be needed when influents contain low C/N ratios. The most frequently used carbon sources are glucose, sodium acetate, fructose, starch, cellulose, etc. (Saeed and Sun, 2012; Sirivedhin and Gray, 2006). Plants are considered an indispensable component in constructed wetlands. They not only take up the nitrate as nutrient, but also provide energy and carbon to fuel denitrification either from decaying biomass or root release. Numerous studies have reported that plants stimulate nitrate removal in CWs, however the effects are often divergent with regard to different plant species (Lin et al., 2002; Kadlec and Wallace, 2009; Songlu et al., 2009). Plants may also influence soil microenvironment by excreting enzymes, exudates and oxygen, which can affect microbial diversity or indirectly disturb the rhizosphere enzymatic activity (Kong et al., 2009). Therefore, the plants selection is an important issue in CWs, as they must survive the potential toxic effects of the wastewater and its variability. In Portugal, the main macrophyte species used in CWs are *P. australis*, *Iris pseudacorus* (yellow iris) and *Cyperus* spp. In some systems, *Juncus effusus* (soft rush), other *Juncus* spp., and *Scirpus* spp. (bulrushes) are also found to establish spontaneously (Calheiros et al., 2007). In this experimental study, *Vetiveria zizanioides* was selected due to some evidences, for instance, this plant has been used successfully in VFCW for wastewater treatment. Additionally, *Vetiveria zizanioides* presents a great ability to remove total nitrogen, ammonia and nitrate (Kantawamichkul et al., 1999, 2013; Almeida, 2012). This plant was first recognized in 1995 for wastewater treatment in Australia (Truong, 2000). *Vetiveria zizanioides* is a perennial grass species belonging to the Poaceae family, with short rhizomes and a massive, finely structured root system that grows very quickly. In this sense, it can be referred that in some applications the root depth reaches 3-4 m during the first year. This deep root system makes the vetiver plant an extremely drought tolerant species and very difficult to dislodge when exposed to a strong water flow, presenting a good performance for wastewater treatment (Truong, 2002).

Many researchers or studies have been conducted to study the impact of different C/N ratios on nitrogen removal in CWs. For example, Lin et al. (2002) observed that nitrate removal efficiency depending on the C/N ratio used, increasing until 95%, when C/N exhibited a value of 6.2. In addition, Yan et al. (2012) investigated the influence of influent C/N ratios on greenhouse gas emission in VFCW. Their results indicated that low and middle C/N ratios (2.5 and 5.0) were most beneficial for reducing the greenhouse gas emissions. It is important to mention that although adding external carbon to the influent improves the nitrate removal, a significant fraction of the added carbon can be lost via other microbial processes (e.g., oxidation) in the wetlands, and the costs increase (Wu et al., 2015).

Hydraulic load and consequently hydraulic retention time (HRT) are some of the most important factors that control the performance of subsurface flow wetland systems (SSFW). High Hl promotes a quicker passage of wastewater through the substrate, reducing the contact time and increasing the nitrate load received by the denitrifying bacteria. However, it may have a negative impact on the nitrate removal, e.g. through oxygenation of the surface sediments and resuspension of organic material, possibly resulting in substrate limitations for the denitrifying bacteria (Spies and Mitsch, 1999). However, Bastviken et al. (2009) observed no significant difference in nitrate removal in CWs, receiving Hl of 130 and 390 L m⁻² d⁻¹. The effect of Hl may differ between CWs depending on the dominant plant community and the shape of the basin, as these factors can affect the hydraulic efficiency of wetlands according to Kjellin et al. (2007) and Persson and Wittgren (2003).
The objective of this study was to evaluate the effect of the hydraulic load increase on nitrate load removal from synthetic wastewater, maintaining low C/N ratio.

2. Material and methods

2.1. Vertical flow constructed wetland and operational condition

The experimental work was carried out in a pilot-scale vertical flow constructed wetland (0.24 m$^2$ x 0.70 m), planted with *Vetiveria zizanioides* (Fig. 1), filled with light expanded clay aggregates (Leca® NR 10/20), with a bottom slope of 2%, applied in order to enable the hydraulic collection of the effluent. Equidistant sprinklers on the top of the bed distributed continuously the influent. A layer of gravel (diameter 10-50 mm) was placed around the outlet valve to prevent clogging by fine particles. The flooding levels were maintained at 14% through a siphon in the outlet.

A synthetic wastewater was prepared dissolving, in one reservoir, potassium nitrate in water (34 g of KNO$_3$, from Merck KGaA, Darmstadt, Germany in 125 L of tap water) and in another reservoir, fructose in water (7 g of fructose, from Merck KGaA, Darmstadt, Germany, in 125 L of tap water. The first acted as a nitrogen source and the second as a carbon source, as described in Table 1. A minimum mineral medium was also added to the potassium nitrate reservoir, prepared using reagents purchased from Merck KGaA, Darmstadt, Germany, and composed of 28 mg L$^{-1}$ CaCl$_2$, 52mgL$^{-1}$ MgSO$_4$-7H$_2$O, 17.40mgL$^{-1}$ K$_2$HPO$_4$, 11 mgL$^{-1}$ K$_2$SO$_4$, 0.03mgL$^{-1}$ CuCl$_2$-2H$_2$O, 0.18mgL$^{-1}$ MnCl$_2$-4H$_2$O, 0.08mgL$^{-1}$ ZnCl$_2$, 1.7mgL$^{-1}$ FeSO$_4$-7H$_2$O (Hunter et al., 2001). 125 L batches of the above mentioned solutions were prepared and mixed immediately (1:1), before feeding the VFCW. This VFCW was fed in continuous mode, through network sprinklers, equidistantly located over the whole VFCW using a submersible pump (Eheim- 1250, Deizisan, Germany) in each of the feeding tank.

The VFCW was acclimatized during one month before the beginning of trials by the application of 83 mg L$^{-1}$ of nitrate and 90 mg L$^{-1}$ of COD at the lowest H$_L$ used, until variation in nitrate and COD concentration were not observed. The experiment was delineated to apply increasing H$_L$ in order to rise nitrate and COD load mass applied. We worked during approximately one month for each H$_L$. Each new trial was initiated by increasing the flow rate until the desired H$_L$. This proceed was maintained until the end of the each trial. Every day, samples were collected at inlet and outlet of the VFCW. The beginning of each trial was considered only when the results performance of nitrate and COD removal was stable. The experimental conditions applied to the VFCW during each trial are presented in Table 1.

2.2. Monitoring of climatic conditions and plant growth

These trials were carried out in a well-established 8-year-old VFCW, with a plant density higher than 120 plants m$^{-2}$, installed in the campus of 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. The maximum annual medium temperature in 2015 presented a value of 22 $^\circ$C, and the average temperature from May until September exhibited a value of 30 $^\circ$C. Generally, the average total rainfall is 558 mm per year. The year 2015 was particularly dry in Beja, specifically, from May to September was 90 mmm.

Air (T$_{air}$) and soil (T$_{soil}$) temperatures at 10 cm depth (Table 1) were monitored from Monday to Friday, at 10:00 a.m., using two thermometers, one inserted into the soil and other suspended in the wall near the VFCW. Rainfall was excluded by covering the bed with a tunnel of transparent fine plastic, when it was raining.
At the start of trials, the plant leaves were cut to a height around 30 cm. From the beginning until the end of this work, the plants were visually inspected to detect signs of toxicity, such as chlorosis, leaf curl, early senescence stages and plant rot. Ten plants were randomly selected, and the height of the leaves monitored during the study.

2.3. Wastewater and plant analysis

Wastewater samples were collected at the inlet and outlet of the VFCW from Monday to Friday, at 10:00 a.m (May-September). The electrical conductivity (EC), pH, redox potential (Eh) and dissolved oxygen (DO) were immediately measured. Aliquots were frozen at a temperature of -20°C to determine the remaining parameters. EC was measured using a Jenway 4510 conductivity Meter. A WTW integrated pH and redox inoLab pH Level 1 electrode were used for pH and Eh determination. DO was determined by the Winkler method. Chemical oxygen demand (COD), ammonium (NH₄⁺), nitrite (NO₂⁻) and nitrate (NO₃⁻) were determined according to Standard Methods (APHA, 2013). COD was determined by closed reflux colorimetric method. The samples were digested during 2 hours at 150°C in a Velp Scientific Thermoreactor. The formed chromic ion (Cr³⁺) was analyzed by spectrophotometer method at 600 nm, using a Pharmacia Ultrospec 2000 UV/VIS spectrophotometer. NH₄⁺ was determined after a distillation step using a Distillation Unit Buchi K-350, followed by titrimetric method with sulphuric acid, 0.02 N. The NO₂⁻ was determined through a modification of a reddish purple azo dye produced from pH 2.0 to 2.5 by coupling diazotized sulfanilamide with N-(1- naphthyl)-ethylenediamine dihydrochloride (NED), at 543 nm using a Pharmacia Ultrospec 2000 UV/VIS spectrophotometer. The NO₃⁻ concentrations were analysed using the Cd-reduction technique. So, nitrate was reduced almost quantitatively to NO₂⁻ in the presence of cadmium (Cd). After, produced NO₂⁻ was determined according to the same method used for nitrite, which was previously mentioned.

In the aboveground plant tissue, the concentration of the essential elements and nutrients, namely, Total Kjeldahl nitrogen (TKN), phosphorus (P), calcium (Ca), magnesium (Mg), sodium (Na), potassium (K), and dried matter were determined. *Vetiveria zizanioides* leaves were cut into small pieces, well mixed and oven dried to constant weight at 70°C for 48 h to moisture determination, using a Memmert U10 25 L oven. The remaining residue was ashed in a Nabertherm controller B-170 muffle furnace at temperatures of 500-550°C for 4-8 h (Campbell and Plank, 1998; Miller, 1998). Then, the ash was dissolved using 10 mL of 3 M hydrochloric acid to determine the concentration of Ca, Mg, K, Na and P. Concentrations of Ca and Mg were determined by atomic absorption spectrophotometry Spectra20 and 220FS, while K and Na contents were analysed using the Cd-reduction technique. So, nitrate was reduced almost quantitatively to NO₂⁻ in the presence of cadmium (Cd). After, produced NO₂⁻ was determined according to the same method used for nitrite, which was previously mentioned.

2.4. Hydraulic retention time and removal efficiency calculations

The hydraulic retention time was established from the Eq. (1) (Crites et al., 2006):

\[
\text{HRT} = \frac{V}{Q_i} = \frac{\text{A}}{\text{Q}_i} \quad (1)
\]

where Q_i (L d⁻¹) is the influent flow rate (measured every day at inlet of the bed), A is the surface area of the bed (m²), V is the bed volume (m³), y is the flow depth (m), and p is the porosity (which expresses the space available for the water to flow through the media, roots and other solids in the SSSF constructed wetland system) (Crites et al., 2006).

The volume of the voids (Vv) was measured experimentally at the start of this work and, as the bed volume is known, the porosity can be calculated by Eq. (2): p = (Vv/V)100(2)

The removal efficiency (n) of pollutants passing through the VFCW was calculated according to the Eq. (3): 

\[
n = \frac{(L_i - L_e)}{L_i} \times 100 \quad (3)
\]

where L_i is the pollutant load in the influent (g m⁻² d⁻¹) and L_e is the pollutant load (g m⁻² d⁻¹) in the effluent. The flow rate was measured in inlet and outlet of the VFCW. L_i and L_e were calculated according to Eqs. (4) and (5), where C_i is the influent concentration (mg L⁻¹), C_e is the effluent concentration (mg L⁻¹), Q is the influent flow rate, Q_e is the effluent flow rate, and A is the surface area of the bed (m²):

\[
L_i = C_i Q_i A \quad (4)
\]

\[
L_e = C_e Q_e A \quad (5)
\]

Pollutant mass load removal (Lr) was calculated according to the Eq. (6):

\[
L_r = L_i - L_e \quad (6)
\]

2.5. Statistical data analysis

A statistical data analysis was performed using the software “Statistica 8.0” (StatSoft, Inc., USA). Differences in the wastewater quality between influent and effluent of the VFCW were tested using ANOVA at the significance level of p < 0.05. Difference in the treatment performance between each assay was tested by one-way ANOVA. Post hoc comparison of treatment means was performed using Tukey’s test at a 95% confidence level. All data are presented as means ± standard deviation (SD), calculated for n > 10.

3. Results and discussion

3.1. Influence of the exposure to nutrient on plant development

Visual inspection revealed that all the wetland plants grew well without obvious symptoms of toxicity signals, such as chlorosis, leaf curl, early senescence stages, and deficit of nutrients or even plants death. From Fig. 2, it can be noticed that plant leaves grew...
Fig. 2. *Vetiveria zizanioides* growth curve (A) and nitrate load applied (•). Values are means ± SD, (n > 10).

Faster after cutting, practically exponential, until 3 cm d⁻¹. Then, a slower plants growth was observed, attaining a maximum height of 130 ± 9 cm (Fig. 2). Dudai et al. (2006) observed that *Vetiveria zizanioides* heights increased rapidly in the first 6 weeks of growth (from 20 cm to 150 cm), after which they increased gradually to a maximum value (130 ± 9 cm). Yet, according to these authors, temperature between 29 and 21 °C and day-length contributed to the growth observed. However, in our study, the *Vetiveria zizanioides* growth may have been due not only to their life cycle, but also to the increase of nitrate loads applied. The plants height reached a value that was practically kept constant until the end of the trial (130 ± 9 cm), when nitrate load was 39.9 ± 0.7 g m⁻² d⁻¹ (last trial). As, we did not work with VFCW without feeding with nitrate, it was not possible to conclude if the growth stop of *Vetiveria zizanioides* was due to the increase of nitrate load or the life cycle of the plant.

The *Vetiveria zizanioides* exposure to increasing nitrate load resulted in a total nitrogen rise in the plant leaves tissue, once the nitrogen content increased from the beginning until the end of the trials (p < 0.05) (Fig. 3). Thus, some nitrogen was removed through the nutrient uptake by plants from the effluent. Cation content in leaves showed a tendency to fall except for magnesium (Mg), which rose slowly in the end of the trial, but the results were not significantly different (p < 0.05) (Fig. 3).

3.2. Electrical conductivity, redox potential, dissolved oxygen and pH changes under different hydraulic and nitrate load applied

Electrical conductivity (EC) values for each trial can be observed in Fig. 4a. The EC reduced in VFCW influent by increasing Hl due to the EC decrease of the tap water in the city of Beja. The city is supplied during most of the year by surface water with high CE. In the summer months, owing to water scarcity, surface water is mixed with groundwater, in variable proportions. This causes a decrease in EC as observed in our tests. There were no significant differences (p > 0.05) between inlet and outlet in VFCW despite the slight increase in EC from inlet to outlet, probably due to the evapotranspiration. Total dissolved solids (TDS) were determined according to Metcalf and Eddy, (2003). It can observe 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 > 0.05). These results indicate that there was not retention of salts into the VFCW.

The EC is often associated with both soil salinity and plant growth reduction (Deifel et al., 2006). According to Truong (2000), *Vetiveria zizanioides* plants survive in soils with high salinity, but decrease their productivity when EC is higher than 8 dS m⁻¹. However, *Vetiveria*
A synthetic wastewater was used and influent pH varied within the neutrality range (7.6 ± 0.3) (Fig. 4b). Effluent pH showed a slowly tendency to decrease, although without significant differences compared with influent pH (Fig. 4b). Usually, nitrate removal and denitrification process lead to the alkalinity production, and consequently the pH increases (Songliu et al., 2009). In this study, although nitrate removal has been observed (Fig. 6), pH mean values in effluent never increased relatively to the influent. This effect can be caused by microbial oxidation of some carbon present, added in the fructose form, with production of CO2 and consequently lowering both pH (Songliu et al., 2009) and carbon available to the denitrification process. Wu et al. (2012) observed that Vetiveria zizanioides has the capacity to release some organic acids, namely oxalic, tartaric, formic, acetic, citric and succinic. As a result, a pH drop can be obtained.

Eh average in influent varied from 120 ± 45 to 189 ± 63 mV (Fig. 4c). In this sense, it was observed a decrease in Eh when Hl = 239 L m⁻² d⁻¹ was applied. This result can be due to the variation of the tap water composition, as mentioned before (mixing of various water types: surface water and groundwater, which have different levels of Eh and DO). A slight redox potential (Eh) decrease was detected in the effluent compared to the influent, although no significant differences were found (p > 0.05). Masscheleyn et al. (1993) and Wlodarczyk et al. (2007) showed that denitrification rate increases by decreasing Eh. In this work, it was observed that denitrification (Fig. 6) occurred irrespective of redox potential that showed positive values in the influent and effluent. VFCW was fed using an influent with a DO content of 4.7 ± 2.1 up to 5.2 ± 1.2 mg L⁻¹ O₂ (Fig. 4d). The DO was not significantly different between the influent and effluent of the VFCW (p > 0.05). However, effluent DO content showed a tendency for a slight decrease, probably due to the organic matter oxidation. Although the effluent never showed anaerobic conditions, nitrate load removal occurred for all assays (Fig. 6).
Denitrification is referred as a strictly anaerobic process in which the synthesis of the enzymes involved in each step and corresponding denitrification rates are greatly repressed by the presence of dissolved oxygen (DO) (Sirvedhin and Gray, 2006). However, denitrification has been observed in presence of DO. This effect was observed by Metcalf and Eddy (2003) in systems with DO lower than 1 mg L$^{-1}$ O$_2$ and Kadlec and Wallace (2009) when DO presented values in the range of 0.3-1.5 mg L$^{-1}$ O$_2$. Unfortunately, in this study, DO was not determined into the VFCW, whereby it is difficult to establish the mechanisms involved in nitrate removal when DO was present.

3.3. Organic matter removal and C/N ratio variation in influent and effluent

In general, carbon can already be present in wastewater to be treated, come from the decay of plant biomass or plant root exudates (Songliu et al., 2009; Wu et al., 2012) or added as external carbon source (Songliu et al., 2009). In this work, synthetic wastewater was used, consequently fructose was added to promote denitrification efficiency. The amount of fructose was quantified by COD (Fig. 5a). The COD load applied increased from 13.3 ± 1.7 to 45.2 ± 2.1 g m$^{-2}$ d$^{-1}$. There was removal in all trials (Fig. 5a), apparently following a first order kinetics (with a correlation of $R^2$= 0.9517).

The removal efficiency decreased from 53.1 ± 2.4 to 35.3 ± 3.1%, probably due to the organic load increase, simultaneously with the HRT decrease. The relation between carbon and nitrogen applied in each assay is presented in Table 1. There was no significant difference ($p > 0.05$). The C/N ratios used in this study were smaller than those usually used (Zhao et al., 2010), but within the range referred by Ding et al. (2012) (0.20 ± 0.15 < C/N < 0.10 ± 0.70). Nitrate removal occurred in all assays (Fig. 6) and some carbon still remained in effluent (Fig. 5b). The differences in the C/N ratio between the inlet and the outlet were not significant ($p > 0.05$).

**Fig. 6.** Variation of nitrate load removal (•) and nitrate removal efficiency (-) with hydraulic load (H_L) and nitrate load (NO$_3$-inf.) applied. Values are means ± SD, (n>10).

Nonetheless, it was evident a slight tendency to decrease the C/N ratio, probably because the carbon was used for the denitrification process. Additionally, carbon can be oxidized by aerobic microorganisms with consumption of DO (Fig. 4c) (Zhu et al., 2014).

3.4. Nitrogen removal performance

The effects of H_L on nitrate load removal and nitrate removal efficiency are shown in Fig. 6. Nitrate load removal increased with H_L and nitrate load applied, reaching a maximum value, beyond which a further increase in H_L or nitrate load applied led to a considerable decrease in the nitrate load removal. The maximum nitrate load removal was 11.9 ± 0.7 g m$^{-2}$ d$^{-1}$, while the nitrate load removal efficiency was 60 ± 3% for a H_L of 239 ± 7 L m$^{-2}$ d$^{-1}$ using a nitrate load applied of 20.4 ± 1.2 g m$^{-2}$ d$^{-1}$ (Fig. 6). C/N of 0.33 ± 0.09 (Table 1) and hydraulic retention time of 140.1 ± 0.4 min. So, in the first two trials (H_L of 148 ± 5 and 239 ± 7 L m$^{-2}$ d$^{-1}$, respectively), removal efficiency was 44 ± 3% and 60 ± 3%. Nitrate concentration in effluent complies with the wastewater Portuguese legislation (<50 mg L$^{-1}$ NO$_3$) (Decreto-Lei n.º 236/98, de 1 de Agosto). In the remaining assays, nitrate concentration was higher than 50 mg L$^{-1}$ NO$_3$ when nitrate removal efficiencies decreased to 36 ± 2% and 15 ± 2%.

It is well accepted that both nitrate and organic matter concentrations may limit the nitrate removal rate of a biological denitrification process. Although wetland denitrification possesses much more complexities, these two substrates can be considered essential factors, limiting denitrification and nitrate removal in constructed wetlands according to Lin et al. (2008).

From the results above referred and also because the C/N ratio in influent was identical in all trials ($p < 0.05$) (Table 1), it is evident that the nitrate load removal rate was mainly controlled by nitrate load and/or H_L applied. Additionally, nitrate load removal rate is not limited by the carbon in the influent, since the carbon was not completely consumed during the processes (Fig. 5a and b), although these C/N ratios were smaller than the values usually used (Zhao et al., 2010).

Nitrate load applied seems to be a rate-limiting factor here. The results presented in Fig. 6 apparently follow a well-known Haldane or Andrews kinetics (Grady et al., 1999), which imply that inhibition may occur when nitrate load applied is higher than 20.4 ± 1.2 g m$^{-2}$ d$^{-1}$ and H_L rose from 239 ± 7 L m$^{-2}$ d$^{-1}$. In this work, due to the H_L increase between the trials, the HRT decreased significantly from 226.5 ± 0.8 min (in first trial) to 70.9 ± 0.1 min (last trial), ($p < 0.05$). Thus, the contact time between synthetic wastewater, plants and microorganisms also decreased. This decrease may also have contributed to the results observed. But this does not fully explain the removal inhibition. It is important to mention that all these HRT were much smaller than those values reported by some researchers, from 2 up to 10 d (Saeed and Sun, 2012).

There are other equally important factors for the nitrate removal in CWs, namely species, type and density of plants used, because they can contribute through release of root exudate and dead biomass to the denitrification increase (Sirvedhin and Gray, 2006). The rate of root exudation is affected by several factors, including photosynthesis, temperature, light-regime, nutrients availability, plant species and anaerobic conditions (Chen et al., 2011; Jones et al., 2009; Zhai et al., 2013).

According to Wu et al. (2012), Vetiveria zizanioides plants release dissolved organic carbon in root exudates, and the root exudate production is higher when the nitrogen concentration is low. Thus, the increase in nitrogen load applied may have contributed to the decrease of root exudates, and consequently nitrate removal rate.
Furthermore, the mature degree of a treatment wetland could affect the organic content of wetland sediments that may in turn influence the nitrate removal of the wetland. *Vetiveria zizanioides* has a root biomass that grows generally in vertically, and its depth reaches 3-4 m (Truong, 2002), contributing positively to the production of carbon via root mucilage, sloughed root cells, root hair and dead roots. This study was made in a wetland field VFCW. Several studies have shown that wetland denitrification rate and nitrate removal rate can be greatly promoted by plant tissue degradation and the supply of carbon. This possibly explains the results obtained with low C/N ratios used in this work.

The great extension of *Vetiveria zizanioides* roots also may have contributed to the nitrate removal in wastewater even in the presence of DO. So, inside the CWs there are complicated spatial zonations, with microzones near the plant roots at different oxidation state (anoxic and anaerobic), where denitrification may occur (Brix,1987; Kadlec and Wallace, 2009), and that could explain no inhibition of denitrification when DO is present in the influent and effluent. However, it must be stressed out that in presence of DO, the dissolved organic carbon obtained from plant litter decomposition is smaller than that for anaerobic conditions (Chen et al., 2011). However, it was not found any relationship between DO presence and nitrate load removal inhibition observed.

Nitrite content in influent and effluent was also monitored. It was detected in small amounts in influent (nitrite mass load <0.3 ± 0.1 g m⁻² d⁻¹). Trace amounts of NH₄⁺ ion were not found, suggesting that dissimilatory nitrogen reduction to ammonia did not play a significant role in the nitrate removal of constructed wetlands.

Temperature was not perceptible because soil and air temperatures (ranged from 18.6°C to 30.5°C) during the whole experiment period. In this study, both H. and nitrate load applied at a ratio of 1:1.5, and simultaneously HRT decreased, it is unclear how and which of these parameters contributed to nitrate removal inhibition. Thus, this point needs further investigation.

### 4. Conclusions

The effects of H. on nitrate removal were investigated. A VFCW planted with *Vetiveria zizanioides* in light expanded clay aggregates was fed with a NO₃⁻-N concentration of 83 ± 7 mg L⁻¹ (nitrate load applied from 11.9 ± 0.7 g m⁻² d⁻¹ to 39.9 ± 0.7 g m⁻² d⁻¹) and C/N ratio from 0.30 ± 0.08 to 0.34 ± 0.06. The system demonstrated to be able to attain nitrate removal efficiencies of 60 ± 3%. Nitrogen load removal occurred according to Haldane/Andrews kinetic, as it increased proportionally with hydraulic load and nitrate load applied, reaching a maximum value. After this value, further increase in Ht led to a considerable decrease in nitrate removal rate. The inhibition of removal seems to depend on the Ht (and the HRT) and nitrate load. The maximum nitrate load removal was 11.9 ± 0.7 g m⁻² d⁻¹, occurring at a nitrate load applied of 20.4 ± 1.2 g m⁻² d⁻¹ and HRT of 140.1 ± 0.4 min.

Further investigation is needed to clarify the role of the *Vetiveria zizanioides* in nitrate removal mechanisms, as well as nitrate removal inhibition.

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