



Organic load and hydraulic regime influence over the bacterial community responsible for the nitrogen cycling in bed media of vertical subsurface flow constructed wetland



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ABSTRACT

The aim of this study was to evaluate the influence of the organic loading rate (OLR) and the hydraulic regime on the nitrifying and denitrifying bacterial community present in the bed media of vertical flow constructed wetland (VFCW) employed as urban wastewater treatment solution. Two microcosms filled with sand ($d_{10} = 0.3$ mm and uniformity coefficient = 6.2) simulated the vertical profile of VFCW and were monitored for 360 days. Microcosm 1 was operated with a constant OLR of $41 \text{ g COD m}^{-2} \text{ d}^{-1}$ and with an average hydraulic loading rate (HLR) of 72 mm d^{-1} . Microcosm 2 was operated with a constant HLR of 170.5 mm d^{-1} and with an average OLR of $104 \text{ g COD m}^{-2} \text{ d}^{-1}$. After 180 days feeding weekly, an intermittent regime with 30 days of feeding followed by 30 days of rest period was adopted. In total, 7 periods were monitored (4 periods of feeding and 3 periods of rest). At the end of each period, the total, nitrifying and denitrifying bacterial communities were characterized using FISH technique. At the end of the experiment, genome sequencing of the bacterial community was performed at the surface and bottom of the two microcosms. The OLR and the HLR significantly affected microcosms' performance and bacterial community structure. Rest periods did not affect Bacteria domain abundance. However, they presented an influence on nitrifying bacteria abundance. Ammonia oxidizing bacteria abundance (*Nitrosomas* sp and AOB β -proteobacteria) was higher in microcosm 1 in feeding (15%) and rest (3%), periods when compared to microcosm 2 (9% and 1% for feeding and rest periods). The same dynamics were observed for nitrite oxidizing bacteria (*Nitrobacter* sp, *Nitrospirae* and *Nitrospina gracilis*), with relative abundance of 18% and 10% for microcosm 1, and 8% and 4% for microcosm 2, in feeding and rest periods, respectively. Differently from nitrifying bacteria dynamics, a higher abundance of denitrifying bacteria (*Pseudomonas* spp.) was observed in rest periods (12% for microcosm 1 and 18% for microcosm 2) than in feeding periods (2% for microcosm 1 and 10% for microcosm 2). The identification of nitrifying and denitrifying bacteria in both microcosms can indicate the process of simultaneous nitrification and denitrification.

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1. Introduction

Vertical flow constructed wetlands (VFCW) are an ecotechnology widely employed as treatment solution to different influent

wastewater compositions. These units are designed to promote advanced treatment performance, in terms of carbonaceous organic matter and suspended solids removal as well in nitrogen transformations, markedly the nitrification (Kadlec and Wallace, 2009).

VFCW sizing is dependent on the hydraulic loading rates (HLR) and the organic loading rates (OLR) applied on its superficial area (Sezerino et al., 2012; Hoffmann et al., 2011; Platzer, 1999). Besides HLR and OLR, the hydraulic regime is also a fundamental param-

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eter to maximise treatment performance in constructed wetlands (CW). Intermittence of feeding and rest periods allows the control of biomass in the bed media (Molle et al., 2008).

These design and operational variables are directly related with the nitrogen transformation processes that happen in CW (Saeed and Sun, 2012). Besides that, they also affect the microbial community inside the bed media, since the microbial diversity, density and stability are directly related with the treatment performance of such units (Faulwetter et al., 2009).

Nitrogen removal in CW can occur through well-known mechanisms as nitrification followed by denitrification, microbial and vegetal uptake, and adsorption on bed media. Not enough, this phenomenon can also happen due to recently discovered mechanisms reliant on microorganisms only (Saed and Sun, 2012). Several studies report the existence of others microbial metabolic pathways to nitrogen transformations than nitrification and denitrification, such as the anaerobic ammonium oxidation (ANAMMOX) (Wang and Li, 2011; Dong and Sun, 2007), completely autotrophic nitrogen removal over nitrite (CANON) (Sun and Austin 2007; Hu et al., 2014), heterotrophic nitrification and aerobic denitrification (Austin et al., 2006)

Nowadays, the microbial interactions and their role in treatment performance are being increasingly main subjects of many studies conducted on VFCW. Hu et al. (2016) elucidated through the identification of functional gene expressions, the microbial nitrogen removal pathways in those systems. The relation of viable and dead bacteria in different depths along the VFCW profile was addressed by Foladori et al. (2015). Guan et al. (2015) evaluated the influence of substrate composition on the microbial community. The impact of different macrophyte species on the bacteria community was evaluated by Zhang et al. (2010). Salomo et al. (2009) evaluated the metabolic diversity of the microbial community in different VFCW layers. Tietz et al. (2007a) assessed the ammonia oxidizing bacteria (AOB) community in a VFCW.

Even though there is a high number of studies that investigate microbial dynamics in CW, there is not a clear understanding of how intermittent feeding and alternating operational periods affect the structure of the bacterial community engaged in nitrogen transformation in VFCW. In that way, a detailed knowledge about the structure and physiology of the microbial community involved in nitrogen transformations within this operational outline is essential. Firstly, because this hydraulic regime is widely employed in real systems in Brazil (Trein et al., 2015) and, secondly, due to the possible optimization of nitrogen removal pathways, and therefore, unit's performance, once these mechanisms are better understood. Furthermore, the relations of this group of microorganisms with the total microbial community and the design and operation variables can also enhance nitrogen removal efficiencies.

Given that, the aim of this study was to evaluate the effect of the OLR and the hydraulic regime over the nitrifying and denitrifying bacterial community present in the bed media of vertical flow constructed wetland (VFCW) employed as wastewater treatment solution.

2. Materials and methods

2.1. Experimental units

This study was conducted in bench scale using two microcosms (microcosm 1 and microcosm 2), which represent a vertical profile of bed media from VFCW. The microcosms had superficial area equal to 0.0176 m², depth of 0.30 m and coarse sand ($d_{10} = 0.3$ mm and uniformity coefficient = 6.2) as bed media. Both microcosms were operated under an average temperature of 20 °C. The treatment units were unplanted, Nevertheless, Tietz et al. (2007b)

showed no statistically significant difference in bacterial biomass in all the layers of planted and unplanted VFCW systems.

Microcosm 1 was operated with a constant OLR of 41 g COD m⁻² d⁻¹, according to Sezerino et al. (2012) recommendations, while the HLR was variable presenting an average value of 72 mm d⁻¹. Microcosm 2 was operated with variable OLR around 104 g COD m⁻² d⁻¹ and constant HLR of 170.5 mm d⁻¹. The latter HLR value refers to calculations based on Platzer (1999) oxygen transfer model. The calculations provide an oxygen positive balance considering air drag through the superficial area and oxygen consumption for carbon and nitrogen removal.

The two microcosms were fed with urban wastewater, from the sewerage system of the municipality of the Florianópolis, southern Brazil. The feeding of the two microcosms was based on two strategies. During the start-up period (first 180 days), feeding was performed every week, three days per week (Tuesdays, Wednesdays and Thursdays) and three times per day (9:00 AM, 1:00 PM and 5:00 PM). This strategy was adopted to assure the development of microbial community inside the bed media. After 180 days of start-up, feeding was done based on a hydraulic regime that alternates periods of 30 days of feed and 30 days of rest. This strategy of feeding and rest periods was implemented to allow the evaluation of the effect of rest and operation periods on bacterial communities in the filtering media. Thus, seven periods were alternated, which 4 were operation periods (periods 1, 3, 5 and 7) and three were rest periods (2, 4 and 6), totalling 360 days of operating and monitoring (Fig. 1).

The physical-chemical characterization of the influent and treated effluent during operational periods was done every week throughout the 360 days. The evaluated parameters were: pH, alkalinity, total suspended solids (TSS), chemical oxygen demand (COD), ammonia nitrogen (NH₄-N), nitrite nitrogen (NO₂-N) and nitrate nitrogen (NO₃-N). All parameters analyses followed APHA (2005) recommendations, exception being made to the parameter NH₄-N, which followed Vogel (1981) recommendations.

2.2. Bacterial analysis

2.2.1. Technique of fluorescence in situ hybridization

The total, nitrifying and denitrifying bacterial communities were monitored in both microcosms using the technique of Fluorescence in situ hybridization (FISH). It was used specific probes to each taxon (Table 1), according to Amann (1995) recommendations.

Aliquots of 20 g from the surface of both microcosms were collected after each operational and rest period, throughout the 360 days of monitoring. For the analysis procedure, 50 mL of deionized water was added to the aliquot, followed by mixing at 3000x G for 5 min. Afterwards, the samples were sonicated with ultrasound for 5 min, aiming to detach the biofilm from the grains of sand. Finally, the supernatant was again centrifuged at 1500 G for 5 min.

In order to quantify the bacterial population, at least 10 images were recorded for each sample, and examined with a microscope (Olympus BX41, Tokyo, Japan). For the estimation of the abundance of hybridized cells using the probe EUB mix, the cells stained with 4',6-diamidino-2-phenylindole (DAPI) were considered as representatives of 100% of all microorganisms determined by digital image. For the rest of the probes, 10 fields were randomly singled, and cells stained with the probe EUB mix were considered as being 100% of all bacteria determined by digital image.

2.2.2. Total bacterial community gene sequencing

At the end of the 360 days of monitoring, the microcosms were deactivated and samples from the bed media were collected. Altogether, two different depths of the vertical profile were evaluated, at 0–0.05 m (surface) and 0.15–0.30 m (bottom).

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