Ecological Engineering 82 (2015) 49–56

Contents lists available at ScienceDirect

Ecological Engineering

journal homepage: www.elsevier.com/locate/ecoleng

Bacteria viability and decay in water and soil of vertical subsurface flow constructed wetlands

P. Foladori [a,*], L. Bruni [b], S. Tamburini [c]

[a] Department of Civil, Environmental and Mechanical Engineering, University of Trento, via Mesiano 77, 38123 Trento, Italy
[b] Agenzia per la Depurazione, Autonomous Province of Trento, via Gilli 3, 38122 Trento, Italy
[c] Centre for Integrative Biology (CIBIO), University of Trento, Via delle Regole 101, Mattarello, 38123 Trento, Italy

ARTICLE INFO

Article history:
Received 29 April 2014
Received in revised form 4 March 2015
Accepted 6 April 2015
Available online 29 April 2015

Keywords:
Wastewater
Vertical subsurface flow constructed wetland
Flow cytometry
Bacteria
Viability
Decay

ABSTRACT

In this study the functional status of bacterial biomass within a vertical subsurface flow (VSSF) constructed wetland was examined with the aim to understand the relationship between viable and dead bacteria in soil and influent/effluent wastewater and elucidate the large amount of dead cells in the soil which may affect the long-term behavior of the system. The quantification of viable and dead bacteria in influent and effluent wastewater and in the soil of a VSSF was performed at single-cell level by flow cytometry (FCM). An optimised pre-treatment was applied to soil samples using sodium pyrophosphate and ultrasonication at a specific energy of 80 kJ/L. Viable and dead cells were detected on the basis of cellular membrane integrity coupling SYBR-Green I and Propidium Iodide. The bacteria profile in the VSSF soil depends on the depth and the material grain size. In the upper 0–10 cm sand layer the number of total bacteria per gram of dry weight (DW) was higher (1.82×10^9 cells/gDW) than in the deeper 40–50 cm (4.8×10^8 cells/gDW) probably due to the vertical feeding and a sieving effect of influent in the top layers. Bacterial biomass in the entire VSSF depth was 0.082 mgVSS/gDW or 144 gVSS/m^3 (per cubic meter of VSSF bed). Size of viable bacteria in the VSSF was smaller (0.16 μm^3/cell) than typical size of activated sludge (0.23 μm^3/cell), due to lower nutrient conditions and a longer retention time of viable bacteria in the bed, estimated at around 130 days by mass balance. Dead bacteria were prevalent in the VSSF soil with a viable/dead bacteria ratio (V/D) of 0.52. The content of dead bacteria might be higher in the soil due to the presence of unsaturated zones not reached by fresh influent wastewater ("dead-zones"), where moisture and substrate are not so available and bacteria may die. Conversely, the higher V/D ratio (3.3) in the effluent reflects the enrichment of wastewater with viable bacteria during the passage through the VSSF bed and along preferential water flow, with higher water content and substrate availability, where the bacterial growth is favored.

1. Introduction

The removal of pollutants in Constructed Wetlands (CW) is mostly driven by microorganisms which are closely tied to the cycling of carbon, nitrogen and sulfur (Faulwetter et al., 2009). Organic matter removal is carried out by heterotrophic bacteria living under both aerobic and anaerobic conditions, while nitrogen removal in CWs is the result of the combination of nitrification and denitrification, even though the significant role of anaerobic oxidation of ammonium was demonstrated in the last years (Truu et al., 2009). Although the high relevance of bacteria communities that live in CW and in vertical subsurface flow CWs (VSSF) is widely recognised and well documented (Faulwetter et al., 2009; Truu et al., 2009; Kadlec and Wallace, 2009), the quantification and the distribution of bacteria in such systems (both in water and in soil) have not been investigated sufficiently and some microbiological aspects remain unknown (Tietz et al., 2008; Langergraber, 2011).

Conventional cultivation-based methods produce heavily biased results and strong underestimations when applied to quantifying bacteria in CWs due to the uncultivability of a large fraction of bacteria (Decamp and Warren, 2001; Alexandrino et al., 2007).

Molecular and biochemical approaches can lead to more in-depth microbiological investigations, but they are not yet completely exploited in the CWs field (Faulwetter et al., 2009). Among them, fluorescent staining of microbial nucleic acids, fluorescent in-situ hybridisation of 16S rRNA gene sequences (FISH), polymerase chain reaction (PCR or PCR-DGGE) or new emerging metagenomic approach have been proposed in the literature to investigate microbial diversity and abundance in

* Corresponding author. Tel.: +39 0461 282683.
E-mail address: paola.foladori@ing.unitn.it (P. Foladori).

http://dx.doi.org/10.1016/j.ecoleng.2015.04.058
0925-8574/

wastewater treatment and CWs (Decamp and Warren, 2001; Sawaittayothin and Polprasert, 2007; Alexandrino et al., 2007; Tietz et al., 2008; Krasnits et al., 2009; Faulwetter et al., 2009; Zhao et al., 2010; Zhang et al., 2012; Adrados et al., 2014; Ligi et al., 2014; Lv et al., 2014). In these microbiological investigations, bacteria viability and death have rarely been quantified (Decamp and Warren, 2001). When the absolute quantification of bacteria is done under epifluorescence or confocal microscope, the analyses result as time-consuming and labor-intensive, requiring several hours to analyse few samples.

Flow cytometry (FCM) has been proposed for the absolute quantification of bacteria in environmental samples at a rate of thousands of cells in few minutes, with high accuracy and precision (Porter et al., 1997; Steen, 2000; Vives-Rego et al., 2000; Bergquist et al., 2009; Foladori et al., 2010). FCM is a multi-parametric, single-cell and rapid analysis which acquires, simultaneously, fluorescent signals (related to cell properties) and light scattering signals (related to cell size and biovolume) for each bacteria cell. In the field of CWs, FCM has rarely been exploited to investigate bacteria abundance or to distinguish specific properties of bacteria such as viability, activity or decay and only rare publications are available in the field (Scholz et al., 2001; Gagnon et al., 2007; Chazarenc et al., 2009).

The objective of this study was to explore the functional status and the depth profiles of bacterial biomass within a VSSF CW, and to compare them with the biomass in the influent and effluent wastewater. The ultimate objective is to provide a better understanding of which portion of the bacterial biomass is responsible for the turnover of elements (living or viable biomass) and which portion is inactive in the soil (dead biomass, which might have a role in the long-term clogging mechanisms).

In this study, viability and death of bacteria were investigated applying FCM with the aim of evaluating: (1) the time-profiles of viable and dead bacteria in influent and effluent wastewater during typical cycles; (2) the depth-profiles of viable and dead bacteria in gravel and sand layers; (3) some aspects affecting the physiological status of bacteria cells when removed from wastewater and retained in the soil.

To our knowledge, the FCM analysis of viability and decay of bacteria in VSSF CW systems, comparing simultaneously influent wastewater, effluent wastewater and bacteria attached to soil inside the bed, has not been reported yet in the literature, while Decamp and Warren (2001) analysed viable and dead bacteria only in influent and effluent wastewater. This paper aims to contribute to advancing knowledge in microbiological aspects of VSSF CWs by considering viable and dead bacteria both in water and soil and by providing a bacteria biomass balance and some aspects about the retention of bacteria in the VSSF system.

2. Materials and methods

2.1. The VSSF CW plant

The outdoor above-ground VSSF pilot plant was located at 739 m a.s.l. (Province of Trento, Italy). The screened municipal wastewater underwent settlement in an Imhoff tank before being pumped up into the VSSF wetland. The VSSF wetland had a surface area of 2.25 m^2 and was characterised by the following layers, starting from the bottom: (1) 0.2 m coarse gravel Ø15–30 mm, (2) 0.05 m medium gravel Ø7–15 mm, (3) 0.5 m sand Ø1–3 mm, (4) 0.05 m medium gravel Ø7–15 mm (layers are graphically indicated in Supplementary material SPM1). Porosity of both sand and gravel was 0.30.

The VSSF was a conventional down-flow system with a single feeding per cycle applied on top of the bed (6.6 h/cycle, 3.6 cycles/day) and wastewater was discharged by free drainage for the entire cycle, resulting in a time-variable flow rate effluent during the cycle. The hydraulic loading rate applied to the VSSF was 63 L m^{-2} d^{-1} on average during the 1-year monitoring period, while the applied organic load was correspondent to 3.2 m^2/PE. The plant has been in operation since 2009 and this research was carried out after three years of operation. The VSSF CW was unplanted, but Tietz et al. (2007) showed no statistically significant difference in bacterial biomass in all the layers of planted and unplanted VSSF systems.

2.2. Sampling and chemical analyses in influent and effluent wastewater

Samples of influent and effluent wastewater were collected weekly. Effluent concentrations were measured in 6.6-h-composite-samples obtained by sampling aliquots proportional to effluent flow rate. Intensive monitoring campaigns were conducted monthly during the typical VSSF cycle to obtain time-profiles of effluent concentrations in the intervals 0–5 min, 5–10 min, 10–20 min, 20–30 min, 30 min–1 h, 1–2 h, 2–4 h, 4–6 h and 6 h.

Routine analyses were: total Chemical Oxygen Demand (COD), Total Suspended Solids (TSS), Total Kjeldhal Nitrogen (TKN), NH_4-N, NO_3-N and total P (APHA, 2005). Filtered COD was measured after filtration on a 0.45-μm-membrane. The mean concentrations in influent and effluent wastewater are indicated in Table 1 and they are in accordance with typical performances expected from VSSF systems. The VSSF wetland demonstrated high efficiency in TSS removal (>74%), total COD and filtered COD removal (>81%). Nitrification occurred in the VSSF wetland due to the aerobic conditions originated by the intermittent loading mode. Influent wastewater temperature varied in range from 7 to 20 °C during one year of monitoring.

2.3. Plate counts

Total Coliforms, *Escherichia coli* and Faecal Streptococci, which are common microbiological indicators in wastewater, were measured for a comparison in influent and effluent wastewater by Membrane Filter Techniques. The original samples were diluted to 10^{-2} to 10^{-6} and volumes of 10–100 mL (in triplicate) were filtered on 0.45-μm membrane filters to obtain plates with 20–100CFUs.

Total Coliforms were measured after growth on Coliforms-*E. coli* agar (C-EC agar, Biolife, Italy) for 24 ± 2 h at 36 ± 1 °C (Italian Standards APAT CNR IRSA Manual 29/2003 Methods 7010C). *E. coli* were measured after growth on Tryptone Bile X-glucuronide medium (TBX, Oxoid, UK) after 21 ± 3 h at 44 ± 1 °C according to Italian Standards (APAT CNR-IRSA, 2003a). Faecal Streptococci were measured by growth on Slanetz and Bartley medium (Oxoid, UK) for 44 ± 4 h at 36 ± 1 °C according to Italian Standards (APAT CNR-IRSA, 2003b).

Table 1
Concentrations of the main parameters in influent and effluent wastewater in the VSSF CW.

	Influent concentration (mg/L)	Effluent concentration (mg/L)	Removal rate (g m^{-2} d^{-1})
COD	544	89	28.9
Filtered COD	282	51	14.7
TSS	161	41	7.6
TKN	72.7	17.1	3.5
NH_4-N	59.7	12.9	3.0
NO_3-N	2.4	32.6	–
Total P	8.6	5.4	0.2
No. samples	55–84	55–85	–