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Assessment of culturable bacterial endophytic communities colonizing *Canna flaccida* inhabiting a wastewater treatment constructed wetland

Cristina Sousa Coutinho Calheiros[a,*], Sofia Isabel Almeida Pereira[a], Hans Brix[b], António Osmaro Santos Silva Rangel[a], Paula Maria Lima Castro[a]

[a] Universidade Católica Portuguesa, CBQF—Centro de Biotecnologia e Química Fina—Laboratório Associado, Escola Superior de Biotecnologia, Rua Arquiteto Lobão Vital, Apartado 2511, 4202-401 Porto, Portugal
[b] Department of Bioscience, Aarhus University, Ole Worms Allé 1, 8000 Aarhus C., Denmark

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ABSTRACT

Microorganisms, including the bacterial populations living inside plant tissues (endophytes), and vegetation are components of constructed wetland (CW) systems playing pivotal roles in the water treatment process and in the ecosystem establishment. The present study focuses on the assessment of the culturable endophytic bacterial communities of *Canna flaccida* plants, the dominant plant species in a polycultured CW treating wastewater from a tourism facility. Bacterial endophytes were grouped by random amplified polymorphic DNA and identified by 16S ribosomal RNA gene sequencing. From the bacterial isolates, 103 were considered for phylogenetic analysis, falling in 25 genera within the γ-, β-, α-*Proteobacteria*, *Flavobacteria*, *Sphingobacteria*, *Actinobacteria* and *Bacilli* classes. Forty-nine percent of the isolates belonged to the *Enterobacteriaceae* family, suggesting that the plants in CW systems may act as a sink of potential human pathogenic microorganisms; nevertheless their abundance on the plant tissues was reduced from the inlet (62%) to the outlet zone (38%). High diversity in terms of genera was found in *C. flaccida* tissues, although the number of genera common to both sampling zones was low, which suggests that the processes occurring within the CW, including the water characteristics gradient from the inlet to the outlet (e.g. total suspended solids, organics and fecal loads), had the ability to shape the diversity of the endophytic communities.

1. Introduction

Constructed wetlands (CWs) are man-made biological treatment systems that are widely used for depuration of water from different origins (Iasur-Kruh et al., 2010; Wu et al., 2015). Domestic wastewater contains fecal and potentially human pathogenic bacteria and its treatment to satisfactory water quality levels for discharge and reuse constitutes a challenge. Several studies have addressed the issue of bacteria and enteric pathogens removal from wastewater in CWs contributing to the understanding of these systems dynamics (Neralla et al., 2000; Karim et al., 2004; Vymazal, 2005; Reinoso et al., 2008; Headley et al., 2013; Wu et al., 2016). For instance, the hydraulic loading rate and the presence of vegetation primarily influence the removal of enteric microbes (Vymazal, 2005). On the other hand, the type of CW flow has also an impact on the removal of indicator bacteria (total coliforms, *Escherichia coli*, fecal streptococci and *Clostridium perfringens*) (Reinoso et al., 2008). Within the CW, natural die-off due to starvation or predation, sedimentation and filtration, and adsorption, are the most frequent and well-validated removal mechanisms of fecal indicator bacteria and pathogens (Wu et al., 2016). Karim et al. (2004) have concluded that significant concentrations of pathogens accumulate in the substrate of CWs. Hence, it is of major importance to understand the pathways taken by bacteria through the substrate and how they interact with the vegetation in the CWs.

There is evidence that plants are also hosts or reservoirs for human pathogenic enterobacteria, and the plant tissue invasion may be extended beyond roots, depending on the bacteria-plant interaction (Tyler and Triplett, 2008; Holden et al., 2009). This issue stands out when plants used to treat domestic wastewaters in CWs

* Corresponding author.
E-mail addresses: cristina@calheiros.org (C.S.C. Calheiros), siapereira@portugalmail.com (S.I.A. Pereira), hans.brix@bios.au.dk (H. Brix), arangel@porto.ucp.pt (A.O.S.S. Rangel), plcastro@porto.ucp.pt (P.M.L. Castro).

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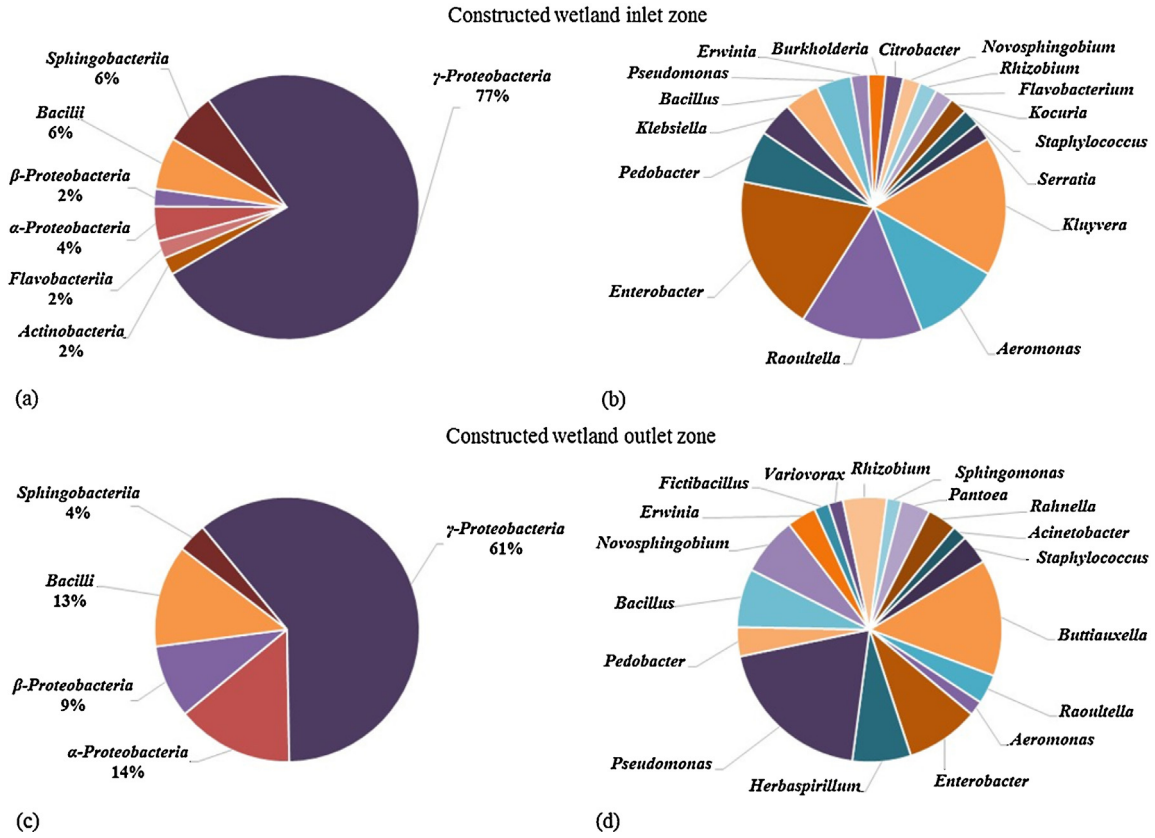

Fig. 1. Distribution of the bacterial endophytes recovered from tissues of *Canna flaccida* plants from the constructed wetland inlet and outlet zones, in the different classes (a, b) and genera (c, d).

may be used for ornamental purposes in households (Calheiros et al., 2015) or considered for horticultural production (Monnet et al., 2002).

Endophytic bacteria can be defined as those bacteria that inhabit the interior of healthy plant tissues without inducing symptoms of disease or injuries on their host (Schulz and Boyle, 2006). Studies associated with bacterial endophytes have gained interest since the knowledge acquired may be related to plant growth promotion potential (Pereira and Castro, 2014; Shehzadi et al., 2015), pollutant degradation and phytoremediation enhancement (Li et al., 2010; Chen et al., 2012; Afzal et al., 2014; Shehzadi et al., 2015) and plant-endophyte partnerships (Tyler and Triplett, 2008; Holden et al., 2009; Afzal et al., 2014). However, much is still unrevealed and research is needed to deepen our knowledge on the abundance and composition of endophytic populations in wetland plants, since endophytic bacterial communities in plants inhabiting CWs have been scarcely reported. Li et al. (2010) studied the endophytic populations in the roots of *Phragmites australis* while Chen et al. (2012) compared populations in *P. australis*, *Potamogeton crispus*, *Nymphae tetragona* and *Najas marina*. On the other hand, Shehzadi et al. (2015) isolated endophytes from the roots and shoots of *Typha domingensis*, *Pistia stratiotes* and *Eichhornia crassipes* inhabiting a CW treating textile effluent, in order to search for bacteria with effluent degrading and plant growth promoting abilities.

In this study, the culturable endophytic bacterial communities from *Canna flaccida,* the dominant ornamental plant in a polycultured CW treating wastewater from a tourism facility, were analysed. The aim was to evaluate the composition and the phylogenetic diversity of the endophytic communities colonising *C. flaccida* plants growing in the inlet and outlet zone of the CW and to identify potential pathogenic bacteria inside the plant tissues, which to our knowledge has not been addressed before in CWs.

2. Material and methods

2.1. Constructed wetland design and physico-chemical and microbial water analysis

A horizontal subsurface flow CW (area = 40.5 m^2) was set-up in 2010 after a septic tank for the treatment of wastewater from a tourism unit in the North of Portugal. The system comprised a substrate of expanded clay – Leca®M (Saint-Gobain Weber Portugal, S.A.) and was planted with *C. flaccida, Canna indica, Zantedeschia aethiopica, Watsonia borbonica* and *Agapanthus africanus*. The setup conditions and detailed description of the CW has been reported by Calheiros et al. (2015).

In order to complement previous wastewater monitoring campaigns (Calheiros et al., 2015), samples (n = 3) were collected at the inlet and outlet of the CW for water quality characterization during spring 2014. In terms of overnight stays a trend similar to that presented in Calheiros et al. (2015) was verified. Determinations were based on Standard Methods (APHA, 1998): chemical oxygen demand (COD), biochemical oxygen demand (BOD_5), total suspended solids (TSS) pH and conductivity. Selective media, ChromoCult® Coliform Agar (Merck) and DIFCO™ mFC Agar, were used for enumeration of *E. coli* and total coliforms, by plate counting according to manufacturer instructions.

2.2. Enumeration and isolation of culturable bacterial endophytes from C. flaccida

C. flaccida was selected as the plant species for this study since it is the most abundant and dominant in the CW polyculture. Endophytic culturable bacteria were isolated and enumerated from roots and shoots of *C. flaccida* plants. For that, four healthy plants were