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Culturable bacteria associated to the rhizosphere and tissues of *Iris pseudacorus* plants growing in a treatment wetland for winery wastewater discharge

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ABSTRACT

Winery wastewater is of great concern due to its complex composition, characterized by high organic content and high amounts of readily and slowly biodegradable and recalcitrant compounds. In the present study, the culturable bacteria from rhizo (interstitial water and substrate) and endosphere (inside roots and shoots) of *Iris pseudacorus* plants inhabiting a treatment wetland mesocosm receiving winery wastewater, were isolated and identified. The innovative approach combined the use of these plants with a substrate based on used cork stoppers, as a support.

Of the 53 bacterial isolates retrieved from the rhizo (interstitial water and substrate) and endosphere (root and shoot) of *I. pseudacorus* plants, the class γ-*Proteobacteria* was predominant in the shoot and root tissues (72%), but it was also present in the interstitial water and substrate (28%). In total, 13 different genera were found. *Pseudomonas* and *Bacillus* were the most represented genera in the rhizosphere while *Rahnella* and *Pseudomonas* were dominant in the endosphere of *Iris* plants. Plant tissues and the water-substrate shared 31% of the genera. Used cork stoppers supported plant growth and can be valorized as substrate in constructed vegetated systems for wastewater treatment and future bioremediation developments may be assisted by the use of resilient bacteria retrieved from such harsh environments.

1. Introduction

Winery wastewater (WW) production is not regular along a year round, it varies in duration, quantity and composition, having a peak at the harvest time (Kumar et al., 2006; Shepherd et al., 2011). Oliveira and Duarte (2010) reported a ratio of wastewater/wine of 0.5 L/14 L, while Fernández et al. (2007) reported a ratio of about 0.03 L/1.9 L. The main sources of WW come from washing the floor, open areas, equipment, bottles and storage tanks (Masi et al., 2015).

Wineries often use their wastewater for irrigation or discharge them into surface water bodies or sewage. These actions, when planned and controlled, may be useful to agriculture contributing to save water and recycle nutrients (Oliveira and Duarte, 2010; Kumar et al., 2006). The higher toxicity of WW on aquatic ecosystems was associated to a certain stage of wine production and to small wineries that did not employ adequate treatment to wastewater (Kumar et al., 2006). Concerning terrestrial ecosystems, the adverse impact of WW has been seen at the level of the soil physicochemical properties, with significant impacts on soil microbial community structure after long-term application (Mosse et al., 2012).

Winery wastewater is characterized by high organic loadings, up to 5000 g COD/m^2 d (COD: chemical oxygen demand) (Masi et al., 2015). The organic content comprises sugars, alcohols, acids and other recalcitrant compounds such as polyphenols. In addition, WW has a low pH and unfavorable C/N, besides other factors that lead to caution in discharging it in the environment (Masi et al., 2015; Arienzo et al., 2009; Shepherd et al., 2011). Constructed wetlands stands out as an attractive alternative to WW treatment due to their advantages in terms of efficiency, energy savings and low maintenance (Masi et al., 2015; Shepherd et al., 2011). Plants constitute one of the main components of constructed wetlands being of utmost importance to define the organic loading limits, since WW may cause phytotoxicity at certain

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concentrations, thus decreasing system performance (Masi et al., 2015). For instance, in a phytotoxic trial with a WW presenting chemical oxygen demand of 17,000 mg/L and total phenol content of 10.6 mg/L, macrophytes fed with water streams containing more than 25% of this WW in their composition did not survive. As such, the choice of plant species is very important, since they should tolerate high organic loads and at same time be able to promote effluent decontamination (Arienzo et al., 2009). In these treatment technologies, microorganisms, like rhizo- and endophytic bacteria, also pose important roles in the ecosystem (Calheiros et al., 2010, 2017a,b) and may improve plant resilience and contaminant removal (Dimitroula et al., 2015; Syranidou et al., 2016). Endophytes inhabit plant tissues without causing negative effects in the host plant and are known to possess plant growth promoting traits that help plants to tackle several environmental stresses (Pereira and Castro, 2014; Pereira et al., 2016; Syranidou et al., 2016). You et al. (2016) highlighted the biotechnological potential for the application of endophytes from hydrophytes in order to improve growth or salt tolerance of plants in water polluted environments. This is in alignment with Dunne et al. (2012) that supported the phytoremediation potential of *Miscanthus giganteus* and *I. pseudacorus* through the use of endophytic bacteria for xenobiotics wastewater treatment, since several bacterial isolates from *Iris* were able to use naphthalene, toluene or biphenyl as a sole carbon source. Due to the harsh conditions that plants are subject when exposed to WW the exploitation of related rhizo and endosphere bacterial communities have been an issue of increasing interest since they may support a strategy towards enhanced performance of plants, although very little work is done in this area (Ramond et al., 2012).

In subsurface flow constructed wetlands, a substrate is frequently used as a support for the plants and the biota, such as different types of gravel (Masi et al., 2015) or expanded clay (Calheiros et al., 2017a). There are indications that the substrate has a relevant effect on the dynamics and diversity of the bacterial community within constructed wetlands and play an active role in the organic matter degradation (Calheiros et al., 2009a,b).

Cork has being gaining interest as a substrate due to the sorbent capacity towards different compounds, such as pharmaceuticals (Dordio et al., 2011), pesticides (Jové et al., 2017) and mercury (Lopes et al., 2014), although the full potential is not yet unveiled.

The present work aimed at assessing the culturable bacteria colonizing the rhizosphere and the inside tissues of *Iris pseudacorus* inhabiting a treatment wetland receiving WW, with used cork stoppers as substrate. The intention is to gain knowledge on the type of bacteria that can be found in these harsh environments and to retrieve isolates that may be of interest to support future bioremediation approaches.

2. Material and methods

2.1. Treatment wetland site

A treatment wetland was placed after a winery with a production of 6000 bottles a year, in a farm located in the north of Portugal. This is a family winery that has its own production of vinho verde (typical from Minho region) and a tourism facility. Climate conditions at Minho region are considered temperate with rainy winters and dry summers with mild temperatures, classified as Csb according to Köppen classification (Kottek et al., 2006).

The structure of the mesocosm mimic a wetland discharge site and was fitted at the soil level, made of propylene with a surface area 1.2 m^2 and an effective depth of the substrate of 0.60 m (Fig. 1). The mesocosm was filled with “used cork stoppers” with a granulometry ranging from 3 to 7 mm, provided by Corticeira Amorim SGPS, SA – Portugal, as substrate. The vegetation planted was *I. pseudacorus* retrieved from the farm surroundings, in a range of 8 plants per m^2.

The system was thought to receive once a year the WW and is expected to be sustainable in terms of plant survival along the year till the next feeding time occurs. At the setup the system was filled with water and after three weeks the WW was connected, which was aligned with the time of the grape harvest. After grape harvest the mesocosm was not fed and was left to the real climate conditions. This cycle was repeated in the following year: at the grape harvest time, with the mesocosm receiving again the WW during approximately a week.

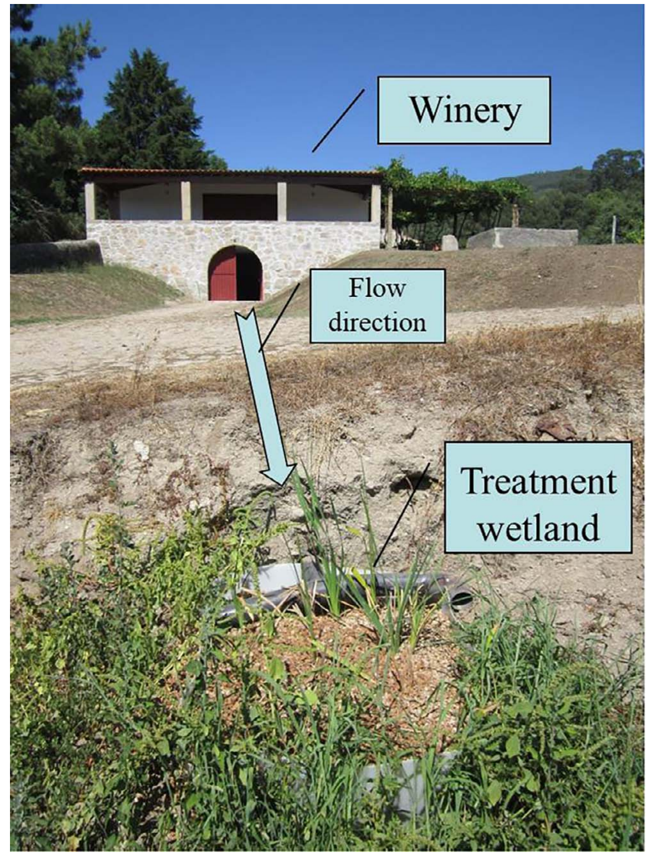

Fig. 1. Treatment wetland discharge site receiving winery wastewater.

2.2. Chemical analysis of water and substrate

The substrate and the interstitial water matrices were analyzed for different parameters in the second year of operation, after three months of the feeding time with WW. The interstitial water was evaluated concerning COD, biochemical oxygen demand (BOD_5), total suspended solids (TSS), pH and conductivity, based on Standard Methods protocols (APHA, 1998). The concentration of PO_4^{3-}-P, NH_4^+-N and NO_3^--N was determined with Photometric test kits (Spectroquant®). Five subsamples (from center and each side of the mesocosm) were pooled together to form a composite water sample.

The substrate was analyzed, before use, for pH and conductivity (Houba et al., 1995), porosity and bulk density (Tan, 1995), and water holding capacity (European Standard EN 1097-6:2000).

2.3. Enumeration and isolation of culturable bacteria

The enumeration and isolation of culturable bacteria was carried out, considering samples from the interstitial water (W) and substrate (ST) of the rhizosphere of *I. pseudacorus* plants inhabiting the mesocosm, and from the endosphere – inside their roots (IR) and shoots (IS). For that, the plant endosphere was considered as that retrieved from the shoot and root interior, and the rhizosphere the soil/substrate close to the root surface, as in other reports (Vandenkoornhuyse et al., 2015; Turner et al., 2013).