



Microbial assisted phytodepuration for water reclamation: Environmental benefits and threats

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HIGHLIGHTS

- Constructed wetlands can improve water reuse, especially in the least developed countries.
- Plant growth promoting bacteria can improve plant services and phytodepuration.
- Horizontal Gene Transfer is promoted in ecological niches pivotal for water cleanup.
- Antibiotic resistance diffusion is a challenging aspect of wastewater treatment.

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ABSTRACT

Climate changes push for water reuse as a priority to counteract water scarcity and minimize water footprint especially in agriculture, one of the highest water consuming human activities. Phytodepuration is indicated as a promising technology for water reclamation, also in the light of its economic and ecological sustainability, and the use of specific bacterial inocula for microbial assisted phytodepuration has been proposed as a further advance for its implementation. Here we provided an overview on the selection and use of plant growth promoting bacteria in Constructed Wetland (CW) systems, showing their advantages in terms of plant growth support and pollutant degradation abilities. Moreover, CWs are also proposed for the removal of emerging organic pollutants like antibiotics from urban wastewaters. We focused on this issue, still debated in the literature, revealing the necessity to deepen the knowledge on the antibiotic resistance spread into the environment in relation to treated wastewater release and reuse. In addition, given the presence in the plant system of microhabitats (e.g. rhizosphere) that are hot spot for Horizontal Gene Transfer, we highlighted the importance of gene exchange to understand if these events can promote the diffusion of antibiotic resistance genes and antibiotic resistant bacteria, possibly entering in the food production chain when treated wastewater is used for irrigation. Ideally, this new knowledge will lead to improve the design of phytodepuration systems to maximize the quality and safety of the treated effluents in compliance with the 'One Health' concept.

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1. Introduction

Water scarcity is a highly relevant and still unresolved global issue. In 2018, the World Economic Forum ranked the water crisis among the top 3 global risks for the third consecutive year (WEF, 2018) and, given the continuous world population growth and the impact of climate change, global water demand is predicted to increase of 55% by 2050 (UN, 2015; Gain et al., 2016; Erclin and

Hoekstra, 2014). Among anthropogenic activities, agriculture is the most water-consuming one, using for irrigation purposes about 75% of the world's freshwater (FAO, 2015; Sato et al., 2013; Falkenmark, 2013). The consequences of water scarcity on agriculture are particularly relevant in countries currently facing an intense growth of population and economy and characterized by harsh environmental conditions, such as those located in the Middle East and North Africa (MENA) region that is among the most arid areas of the world. Here, the economic growth is forecast to determine an increase in water demand of 47% by 2035 (Frascardi et al., 2018), intensifying the competition among different water-

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consuming sectors. This may negatively affect the allocation of freshwater to agriculture and, eventually, threaten food security. Furthermore, climate changes have already intensified phenomena like inconstant rainfall distribution and drought in Europe (Lehner et al., 2006; NOAA, 2017), exacerbating their consequences on agriculture and promoting the development of alternative solutions also on those territories once considered safe in terms of water availability (Rolli et al., 2015).

In this context, pursuing an improved water reuse is a priority to fight water crisis and minimize water footprint in agriculture (Van der Hoek et al., 2002) and, accordingly, the reuse of treated wastewaters is a priority for the European water management policy (SCHEER, 2017). In fact, non-conventional water resources (e.g. wastewaters) can offer complementary supplies to alleviate water shortage in areas where renewable water sources are particularly limited (Qadir et al., 2007) and it is estimated that at least twenty million hectares of cultivated lands worldwide are irrigated with wastewaters (Bouaroudj et al., 2019). Unfortunately, this approach frequently implies the release of untreated wastewaters in agriculture fields, and eventually in the food chain (Garner et al., 2016), representing an issue particularly in the least developed countries (LDCs) and in the MENA region.

Among wastewater treatment technologies, constructed wetlands (CWs) are engineering systems based on the purifying processes that occur in natural wetlands, i.e. the ability to remove pollutants and nutrients present in the water that flows through, thus enhancing water quality (Carvalho et al., 2017). Compared to the conventional wastewater treatment processes, CW systems need low building and maintenance costs, require less external energy and can be implemented at both small and large scales: these features make them highly suitable for wastewater treatment in the LDCs (Mahmood et al., 2013). CWs can be used as unique treatment to cleanup wastewaters produced by small communities, being effective in decreasing chemical and biochemical oxygen demand (COD and BOD) and removing total suspended solids (TSS) and nutrients (i.e. ammonia and phosphorus) (Vymazal, 2010; Wang et al., 2018). Moreover, CWs can be used as tertiary treatment for the final cleaning step in conventional wastewater treatment plants, which are not specifically designed for the removal of micropollutants (e.g. emerging organic contaminants – EOCs-like pharmaceuticals and personal care products) (Verlicchi and Zambello, 2014; Li et al., 2014). The CW system is indeed suitable for the improvement of water quality of different types of wastewaters including domestic (Wang et al., 2018) and industrial (Wu et al., 2015) ones.

As in natural wetlands, in CW systems several physicochemical and biological processes take place spontaneously at the same time, allowing the removal of organic and inorganic compounds by soil/sediment sorption and sedimentation, phytodegradation and plant uptake. In addition, the soil/sediment matrices and the plant root apparatus provide a niche for the development of microbial communities that are pivotal for the nutrient and pollutant removal from wastewaters and can at the same time sustain plant growth (Faulwetter et al., 2009) (Fig. 1). Recently, different studies have demonstrated that the addition of specific bacteria to the plant species used in CWs can be a promising strategy to boost the phytodegradation performances (Prum et al., 2018; Rehman et al., 2018; Syranidou et al., 2016), given their capacity to promote plant growth and remove different pollutants (see chapter 2 and 3 of this review).

CWs have been proposed as a solution to reduce contaminants of emerging concern like antibiotics, antibiotic resistance genes (ARGs) and antibiotic resistant bacteria (ARB), typically present in wastewaters containing human and animal stools (Chen and Zhang, 2013). Nevertheless, contrasting results are reported in the

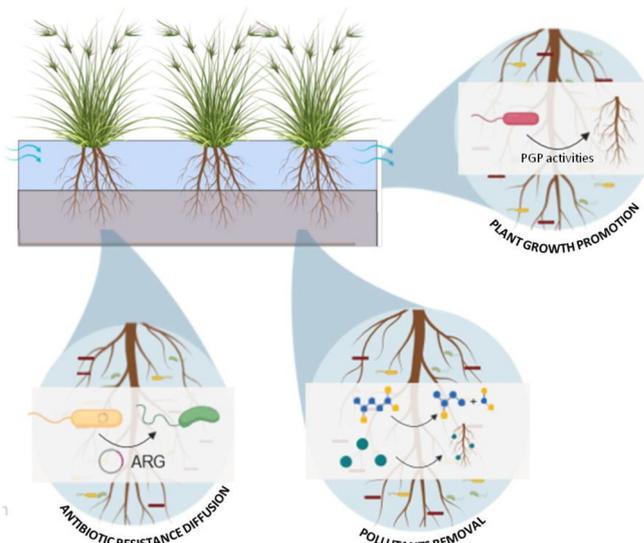


Fig. 1. Bacteria mediated processes in phytodepuration system. Bacterial populations can play a beneficial effect in phytodepuration systems acting as Plant Growth Promoting strains and contributing to the degradation of micropollutants of emerging concerns (e.g. pharmaceuticals). On the other side, bacteria are involved in the phenomenon of antibiotic resistance diffusion into the environment through Horizontal Gene Transfer events, which allow the spread of antibiotic resistance genes.

literature on this topic, as shown in the chapter 4 of this review. The possible occurrence of horizontal gene transfer (HGT), the mechanism supporting the spread of ARGs (Jechalke et al., 2014) is an issue in phytodepuration technology (Fig. 1), considering that it can be enhanced in the bacterial community inhabiting plant rhizosphere (Chen et al., 2019). This critical aspect urgently needs to be addressed, since the spread of antibiotic resistance through treated wastewater used in agriculture could increase the presence of environmental ARB that can potentially be recruited by the plant and eventually enter the food production chain.

In this review, we propose some considerations on the advantages and the possible risks correlated with the use of selected bacterial inocula in CWs for microbial assisted phytodepuration to improve nutrient and micropollutant removal, focusing specifically on the threat of antibiotic resistance diffusion into the environment amplified by HGT events.

2. PGP bacteria support to plant growth under adverse conditions

When growing in adverse environments plants face a number of growth-limiting factors, such as water scarcity, high salinity, reduced nutrients bioavailability and toxicity of polluted water and soil (Soussi et al., 2016). These conditions determine oxidative stress, reduced photosynthetic rate and plant growth, thereby affecting negatively plant productivity and related ecosystem services, including soil and water depuration (Khan et al., 2015). Plant growth-promoting (PGP) bacteria are known to establish an intimate association with the plant rhizosphere and endosphere, where they can sustain plant growth and development by counteracting the effects of stress conditions through a variety of mechanisms that have been extensively reviewed and can be categorized as direct and indirect (Abilash et al., 2016; de Bashan et al., 2012; Riva et al., 2019). PGP bacteria can directly promote plant growth acting as biofertilizers by increasing the bioavailability of key nutrients including nitrogen, phosphorus and iron. Moreover, PGP bacteria sustain the growth of associated plants by

mechanisms that interfere with the production and regulation of phytohormones, such as auxins and ethylene, orchestrating root development and plant biotic and abiotic stress response (Backer et al., 2018).

2.1. Mechanisms relevant for plant growth promotion in CWs

The adverse conditions encountered by plants growing in constructed wetland (CW) ecosystems seem to be mostly related to the toxicity of the pollutants contaminating the wastewaters, including pharmaceuticals, azo-dyes and metals in high concentrations. These compounds are often slightly hydrophobic and can easily enter the roots and be translocated through the xylem to the plant tissues, where they determine an increase of the reactive oxygen species (ROS) and the activation of the plant stress response supported by an increase of the respiration rate (Ferreira et al., 2014; Christou et al., 2016; Mesa-Marín et al., 2018). Such physiological changes alter the carbon balance against plant biomass production, eventually inducing a reduction of the depuration rate in the CW system (Ferreira et al., 2016). PGP bacteria ability to counteract abiotic stress and to enhance the development of the root system represents thereby a valid biotechnological resource to sustain plant development and enhance the detoxification process (Vergani et al., 2017). The ability of indole-3-acetic acid (IAA) producing bacteria to promote the development of the root apparatus represents a significant advantage for the plant that can explore an increased volume of soil for water and nutrient uptake (Backer et al., 2018), but can be also exploited to increase the phytodepuration rate of wastewaters. Plant associated bacteria also interfere with the production of stress and senescence related hormone, i.e. ethylene, through the expression of the enzyme 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase (Nascimento et al., 2018). This enzyme hydrolyses the immediate precursor of ethylene in plants, thereby lowering the concentration of endogenous ethylene related to stress response in polluted waters and indirectly promoting plant growth. However, the mechanisms underlying microbial-assisted phytoremediation remain widely unclear and variable, depending on the bacteria and plant species and on the type of contamination. Different studies report that plant stress tolerance and phytoremediation performances benefit from the enhancement of antioxidant enzymes activity (e.g. catalase, superoxide dismutase, glutathione reductase) triggered by the inoculated bacteria (Rajkumar et al., 2012; Backer et al., 2018). This type of mechanism assumes particular interest when the target pollutants in the wastewaters are hydrophilic organic contaminants and metals that can be taken up by the roots, translocated and eventually metabolized within the plant tissues. Alternatively, PGP bacteria can exert a protective effect towards the plant, decreasing roots respiration rate and reducing the energy consumption for antioxidant enzymes related to the stress response, hence leading to higher root biomass production and increased phytoremediation potential (Mesa-Marín et al., 2018).

3. PGP bacteria application in phytodepuration

As previously mentioned, in phytodepuration systems like CWs the pollutants present in wastewaters can impair plant growth and performance. PGP bacteria have the potential to improve the efficiency of CW plants by i) decreasing stress response and in turn increasing plant growth and services and ii) degrading phytotoxic compounds (Faulwetter et al., 2009). The chemical pollutants occurring in wastewaters are indeed hardly degradable compounds and the supplementation in CWs of microbes owing specific metabolic pathways able to degrade chemical compounds and to promote plant growth has been indicated as a strategy to accelerate

and enhance wastewaters depuration (Shehzadi et al., 2014; Wu et al., 2015).

3.1. Methods for PGP bacteria selection, application in CWs and testing

The successful exploitation of PGP bacteria in CW systems foresees different research steps, including the isolation of the proper bacterial strains, their taxonomic identification in order to omit potential pathogens, the functional characterization to select the most promising ones. The whole procedure must also include the assessment of bacterial effects on the mesocosm-scale CW system and the quality of the treated wastewater effluent. A survey of the peer-reviewed articles published in the last 10 years on this topic showed that different approaches can be adopted in the pipeline that leads from bacteria selection (Table 1) to bacteria inoculum (Table 2) and performance validation (Table 3).

Different authors performed the PGP bacteria selection establishing a collection of strains from the endosphere or rhizosphere of wetland plant species commonly used in phytodepuration systems (Kabra et al., 2013; Saleem et al., 2018; Salgado et al., 2018, Table 1). PGP bacteria were isolated from the same plant species used in the CW to be bioaugmented (Ijaz et al., 2015) or from a different plant species grown in CWs treating the wastewater type of interest (Syranidou et al., 2016). Alternatively, PGP bacteria used in CWs were isolated from a site polluted with the target contaminant that had to be removed from the wastewater (Lingua et al., 2015; Prum et al., 2018; Rehman et al., 2018). The selection of the most promising strains suitable for assisted phytodepuration was then performed by analyzing the isolates for the potential capacity to tolerate/degrade the target contaminants and for the plant growth promotion ability both *in vitro* and *in vivo* (Table 1).

Once the best candidates were selected, pilot scale experiments were implemented to validate their possible application in CWs. As summarized in Table 2, most of the studies applied a consortium of two or more bacteria rather than single strain inocula to CW system, and the bioaugmentation was usually performed once, at the beginning of the experiment. The bacteria inoculation was performed by adding a cell suspension of determined concentration directly into the wastewater (Salgado et al., 2018; Shehzadi et al., 2014) or supplied around the plant root apparatus (Lingua et al., 2015; et al., Syranidou et al., 2016). As an alternative, before placing the plant in the CWs, it was possible to dip the root apparatus in the bacterial cells suspension as proposed by Prum et al. (2018).

Different chemical parameters were measured to verify the efficacy of the PGP bacteria inoculation in enhancing the CW depuration performance (Table 3). Obviously, the pollutant removal in the effluent must be evaluated, comparing wastewaters treated with inoculated and non-inoculated plants in CWs. Chemical and biological oxygen demands (COD and BOD) were routinely measured as general parameters that provide an indication of effluent quality. Moreover, the target pollutants in CWs, varying according to the wastewater origin, were quantified. For instance, heavy metals and emerging organic contaminants (EOCs) such as pharmaceuticals and personal care products were monitored in industrial and domestic wastewater effluents (Carvalho et al., 2014; Syranidou et al., 2016; Zhang et al., 2018). In addition, the quality of the treated wastewater was assessed through ecotoxicological tests using model microbes, plants or fishes (Table 3).

To complete the analysis of the bacterial inocula effect on a CW system, plant growth promotion was also evaluated, generally measuring plant dry biomass, root and shoot lengths and ROS concentration (Table 3). Furthermore, a key aspect for the validation of the PGP bacteria to be used in assisted phytodepuration was

Table 1
Overview of the scientific literature reporting *in vivo* bioaugmentation experiments in Constructed Wetlands (CWs). The table includes CW type (FTW = floating treatment wetland; VF = Vertical flow; SF = subsurface flow), wastewater type, plant species and the criteria applied for the selection bacteria in each study. ACC = 1-Aminocyclopropane-1-Carboxylate deaminase activity, P sol. = phosphate solubilization, sid. = siderophore production, IAA = Indole-3-acetic acid production.

References	CW type	Wastewater type	Plant species	Bacteria applied for bioaugmentation in CW		
				Bacterial isolation site	Characterization of pollutant tolerance/degradation	Characterization of PGP potential
Ijaz et al. (2015)	microcosm (FTW)	sewage effluent	<i>Brachiaria mutica</i>	<i>Brachiaria mutica</i> shoot	COD/BOD removal	ACC, P sol., sid.
Kabra et al. (2013)	mesocosm	textile effluent	<i>Glandularia pulchella</i>	<i>Glandularia pulchella</i> root	dye decolorization	–
Lingua et al. (2015)	mesocosm	nitrate polluted water	<i>Phragmites australis</i>	forest soil	–	IAA, sid., P sol., <i>in vivo</i> P. <i>australis</i> promotion
Prum et al. (2018)	mesocosm (VF)	arsenic polluted water	<i>Echinodorus cordifolius</i>	arsenic contaminated soil	arsenic tolerance	–
Rehman et al. (2018)	microcosm (FTW)	oil field wastewater	<i>Brachiaria mutica</i> / <i>Phragmites australis</i>	crude oil-contaminated site	hydrocarbon degradation	IAA, ACC, sid., P sol.
Saleem et al. (2018)	microcosm (FTW)	phenol polluted water	<i>Typha domingensis</i>	Plant rhizosphere and shoot	phenol degradation	ACC
Salgado et al. (2018)	mesocosm	domestic wastewater	<i>Typha latifolia</i>	<i>Typha domingensis</i> plant	COD/nitrogen/phosphorus removal	–
Shehzadi et al. (2014)	mesocosm (VF)	textile effluent	<i>Typha domingensis</i>	wetland plants roots	textile effluent degradation	ACC
Syranidou et al. (2016)	microcosm (static)	EOCs and metals polluted water	<i>Juncus acutus</i>	<i>Juncus acutus</i> endosphere	BPA/antibiotic/metals tolerance	IAA, ACC, P sol., sid., organic acid production
Watharkar et al. (2015)	mesocosm (static)	textile effluent	<i>Pogonatherum crinitum</i>	<i>Petunia grandiflora</i> rhizosphere	dye decolorization	–
Zhao et al. (2016)	mesocosm (SF)	sewage effluent	<i>Acorus calamus</i>	CW/commercial bacteria	nitrogen removal	–

Table 2
List of the different methods applied in the literature for the inoculation of bacteria in a CW system. The table reports for each reference the type of inoculum used, the way and the frequency of the microbe addition to CW.

References	Bacteria inoculation methods in CW		
	Inoculum type	Inoculation method	Frequency of inoculation
Ijaz et al. (2015)	consortium	wastewater inoculation	once
Kabra et al. (2013)	single strain	soil inoculation	daily (for 15 days)
Lingua et al. (2015)	single strain	bacterial suspension around root apparatus	once
Prum et al. (2018)	consortium	root dipping	once
Rehman et al. (2018)	consortium	wastewater inoculation	once
Saleem et al. (2018)	consortium	wastewater inoculation	once
Salgado et al. (2018)	consortium	wastewater inoculation	once
Shehzadi et al. (2014)	consortium	wastewater inoculation	once
Syranidou et al. (2016)	consortium	bacterial suspension around plant base	once
Watharkar et al. (2015)	single strain	cell immobilization	once
Zhao et al. (2016)	consortium	bacterial suspension around plant base	thrice

the persistence of the inoculum into the system and its capacity to establish a tight bacteria-plant interaction. Such analyses can be performed using different molecular methods as reviewed by Rilling et al., (2018) and showed in Table 3.

3.2. PGP bacteria contribution to increase plant performances

PGP bacteria can improve the fitness of plants used in CW systems. As reported by Shehzadi et al., 2014, the inoculation of the wetland plant *Typha domingensis* with the endophytic strains *Microbacterium arborescens* TYSI04 and *Bacillus pumilus* PIRI30 in a vertical flow CW reactor promoted plant growth besides improving the depuration of the effluent. In the system, textile effluent negatively influenced the development of *T. domingensis*, however the endophytes inoculation reduced the toxic effects of textiles, restoring the plant biomass production up to the values of *T. domingensis* plants irrigated with clean tap water and used as control in the study. Likewise, the addition of a bacterial consortium to a floating treatment wetland enhanced *Brachiaria mutica* and *Phragmites australis* growth that was normally inhibited by the

presence of oil-contaminated water (Rehman et al., 2018). In the study, bacterial inoculation restored plant health and increased root (36–46%) and shoot (35–36%) biomass and root (15–29%) and shoot (4–21%) length, enabling these two plant species to cope with the oil-induced stress.

The influence of PGP bacteria on wetland plants can be also indirectly evaluated by measuring the decrease in plant content of antioxidative enzymatic activity, which is linked to the oxidative stress induced in plants (Prum et al., 2018; Syranidou et al., 2016). It is known that PGP bacteria stimulate enzymatic and non-enzymatic antioxidant responses that scavenge ROS compounds related to stress condition (Marasco et al., 2013; Wang et al., 2012). The monitoring of antioxidative enzymatic activity has been used by Syranidou et al. (2016) in a microcosm scale experiment designed for the depuration of water artificially contaminated with metals, bisphenol-A and antibiotics to simulate urban wastewaters. The authors showed a significant reduction of activity of several enzymes involved in anti-oxidant defense in *Juncus acutus* plants inoculated with a consortium of endophytic PGP bacteria.

Table 3

List of the parameters measured in the literature to assess the effect of bacteria inoculation in CW systems. The table refers to different bacteria-mediated effects possibly enhancing CW performance (i.e. pollutant removal, effluent toxicity, plant growth promotion) besides inoculum persistence.

References	Bacterial effect assessment			
	Pollutant removal	Effluent toxicity	Plant growth promotion	Inoculum persistence
Ijaz et al. (2015)	BOC, COD, TS, TDS, TSS, DO, oil and grass, SO ₄ , Cl, total N, PO ₄ , Na, K, metals	Fish toxicity test	–	Plating + RFLP analyses
Kabra et al. (2013)	dyes, COD, BOD, TOC	Plant toxicity test	–	–
Lingua et al. (2015)	nitrogen, ammonium	–	N ^o of stems/sprouts/leaves; stem length; shoot and root fresh/dry biomass and weights	Plating
Prum et al. (2018)	arsenic	–	ROS and IAA levels	–
Rehman et al. (2018)	COD, BOD, Na, K, metals, residual oil	Fish toxicity test	fresh and dry biomass, root and shoot length	Plating + RFLP analyses
Saleem et al. (2018)	phenol, COD, BOD, TOC	–	fresh and dry biomass	Plating + RFLP analyses
Salgado et al. (2018)	COD, NH ₄ , PO ₄	–	–	–
Shehzadi et al. (2014)	dyes, COD, BOD, TOC, TDS, TSS	Ames test	fresh and dry biomass	Plating + RFLP analyses
Syranidou et al. (2016)	metals, BPA, antibiotics	–	dry biomass	ARISA
Watharkar et al. (2015)	dyes, BOD, COD	Plant and fish toxicity test	–	–
Zhao et al. (2016)	nitrogen, COD, BOD	–	–	high-throughput sequencing

3.3. PGP bacteria contribution to nutrient removal

CWs are often used as secondary treatment systems for municipal wastewater cleanup in rural communities where wastewater treatment facilities are not present due to the high cost of treatment processes (Ghrabi et al., 2011; Fountoulakis et al., 2017). Phytodepuration is also used as tertiary treatment when the common treatment plant is not efficient enough in the achievement of legal standards for organic matter and nutrient removal to allow the safe discharging of treated water in the environment (Ijaz et al., 2015). Wetland plants, such as *Phragmites* and *Typha* spp., are able to uptake nitrogen and phosphorus from wastewaters (Vymazal, 2010; Fountoulakis et al., 2017). However the addition of bacteria in CWs can help plants in the removal of these contaminants and contribute to organic matter decrease by the indigenous microbial communities, improving the quality of treated wastewaters (Ijaz et al., 2015; Salgado et al., 2018).

Salgado et al., (2018) investigated the effects of the application of four indigenous rhizobacteria to *Typha latifolia* on the performance of a vertical flow CW system treating domestic wastewaters. The study showed that the system removed over 75% of the organic matter, ammonium and phosphate and it had better performance than i) *T. latifolia* CW mesocosms without bacteria inoculation and ii) CW mesocosms established using plant with sterilized root surface supplemented by the four selected rhizobacteria. This interesting experiment suggests that the inoculation of the selected indigenous rhizobacterial strains activated the overall microbiome associated to *T. latifolia* in the bioaugmented plants, resulting in a synergic effect that significantly improved the effluent quality. Another study analyzed the removal of nitrogen from nitrate polluted and urban wastewaters using pilot scale-CWs enriched with non-indigenous bacteria aiming to achieve the legal standards of nitrogen emission in surface waters and to prevent the risk of eutrophication in lakes and rivers (Zhao et al., 2016). The addition of the selected PGP bacteria improved the performance of the system and ameliorated the efficiency of denitrification up to 10% during the wastewater phytodepuration process.

3.4. PGP bacteria contribution to micropollutant removal

Wastewaters contain several categories of micropollutants, including metals and organic contaminants of emerging concern (e.g. pharmaceuticals, personal care products, pesticides), which pose serious risks for human health if released into the environment (Li et al., 2017; Pruden et al., 2012; Tezel and Pavlostathis, 2015; Zuccato et al., 2000). In this review, we focused on EOCs that are the prevalent class of micropollutants occurring in urban wastewater and show a relationship with the antibiotic resistance diffusion in the environment, as illustrated in chapter 4. EOCs that are frequently present in wastewater at trace concentrations and are hardly removed by common wastewater treatment plants, which are not tailored for their elimination (Gorito et al., 2017). CWs are proposed as tertiary treatment for the removal of micropollutants from industrial and municipal effluents (Verlicchi and Zambello, 2014; Garcia-Rodríguez et al., 2014; Wu et al., 2015), basing on the capacity of certain plant species to accumulate organic and inorganic compounds from water and considering their potential association in the rhizosphere and endosphere with pollutant degrading bacteria (Borruso et al., 2017). Bacterial degradation has been demonstrated to be the main process involved in micropollutant removal in CWs treating urban wastewaters (Carvalho et al., 2014; Li et al., 2016; Zhang et al., 2018), hence through the addition of microbial inocula having a specific degrading pathway it is in principle possible to achieve treated effluents at reuse grade. In a recent study, the endophytic bacteria *Rhizobium radiobacter* and *Diaphanobacter nitroreducens*, isolated from reed plants and characterized for plant growth promotion and carbamazepine degradation capacities, were inoculated to hairy root cultures of *Armoracia rusticana*. The inoculated hairy root cultures showed higher carbamazepine removal rate (21% and 10% for the cultures inoculated with *Rhizobium radiobacter* and *Diaphanobacter nitroreducens* respectively) compared to the non-inoculated one (Sauvêtre et al., 2018) and, even though the plant-endophyte synergy needs to be further investigated, these data clearly indicated that plant associated microbes could be successfully exploited for municipal wastewater treatment in

phytodepuration system. A CW mesocosm experiment for the depuration of urban wastewaters was recently described by Saleem et al., (2018): in a floating treatment wetland, *T. domingensis* was able to remove a small amount of phenol from the polluted water, however the addition of three phenol-degrading bacterial strains to the system significantly improved the phenol removal, which increased from 0,146 g/m²/day to 0,166 g/m²/day. Other studies have demonstrated that the addition of bacteria inocula to plants can be also effective for the treatment of textile wastewaters, contaminated by toxic dyes. In two different pilot scale experiments, Kabra et al. (2013) and Watharkar et al. (2015) inoculated respectively plants of *Glandularia pulchella* and *Pogonatherum crinitum* with two isolates belonging to the species *Pseudomonas monteilii* and *Bacillus pumilus*. The results highlighted a synergic effect of the plant and bacteria components, showing that inoculated plants were more efficient in dye removal and BOD and COD decrease compared to i) non-inoculated plants and ii) reactor systems with the presence of the sole bacterium without the plant.

An additional advantage of assisted phytodepuration is that the combined use of plants and bacteria is useful to deal with mixed pollution. Syranidou et al. (2016) investigated the potential of endophytic bacteria to sustain the removal of metals and emerging organic pollutants, such as bisphenol-A and antibiotics, by *Juncus acutus* plants in CWs. The results showed that beneficial effect of the bacteria in phytodepuration was especially evident when high concentration of contaminants were used, significantly improving the removal of organics and metals in shorter time by inoculated plants compared to the non-inoculated ones.

4. Antibiotic resistance dimension in phytodepuration

The majority of antibiotics are not completely metabolized and absorbed by animal and human's bodies, thus they are discharged in municipal and hospital wastes in their original or transformed chemical species (Huang et al., 2017; Berglund et al., 2014). As other EOCs, antibiotics escape wastewater treatment systems, which are not specifically designed to remove these compounds, thus representing micropollutants in treated effluents (Luo et al., 2014). Antibiotics are considered "pseudo-persistent" contaminants because of their constant addition through municipal wastewaters at low concentrations into the environment (Gorito et al., 2017) and can reach biologically active amounts due to accumulation as reported for the waters and sediments of Italian rivers where they have been measured at ppm and ppb concentrations respectively (Zuccato et al., 2000). The principal concern for their release is related to the selection and spread of antibiotic resistance bacteria (ARB) and antibiotic resistance genes (ARGs) (Rizzo et al., 2013; Liang et al., 2018; Yan et al., 2019), as already hypothesized by Sir Alexander Fleming, the discoverer of the first antibiotic, during his Nobel Lecture in 1945 (Fleming, 1964). Indeed, antibiotic resistance is nowadays one major concern threatening human and animal health due to the emergence of multi-drug resistant pathogens, which cannot be eliminated by pharmacological therapy with serious consequences for the overall society. The genetic determinants of antibiotic resistance (i.e. ARGs) are typically present in mobile genetic elements (MGE) and are considered micropollutants themselves since, if acquired by a receiving sensitive cell, can produce a new antibiotic resistant mutant.

The study of the antibiotic resistance dimension in the environment has to include the processes occurring in wastewater treatment plants (WWTPs), identified as one of the main sources of ARB and ARGs (Amos et al., 2015; Czekalski et al., 2014). The inadequacy of WWTPs in antibiotic removal from wastewaters has been identified as a major cause for the ubiquitous occurrence of ARB and ARGs in different environmental compartments (Amos

et al., 2015; Tang et al., 2016; LaPara et al., 2011; Ju F et al., 2019). For instance, recent findings suggest that the use of struvite as crop fertilizer can increase the diversity and the concentration of ARGs in the microbiome inhabiting soil and plant microhabitats, i.e. roots and leaves (Chen et al., 2017). Moreover, WWTPs are a suitable environment for antibiotic resistance selection and spread due to the continuous contact between the bacteria involved in the biological treatment processes and the antibiotics present in the wastewaters at sub-inhibitory concentrations (Rizzo et al., 2013). The presence of sub-lethal concentrations of antibiotics in WWTPs and in their effluents is a peculiar ecological driver favoring the ARB selection and the outbreak of antibiotic resistance (Andersson and Hughes, 2014). Other EOCs (e.g. biocides) and metals are typical stressors in WWTPs that might exert selective pressure contributing to the enrichment of ARGs and ARB through selection or co-selection events (Berendonk et al., 2015; Di Cesare et al., 2016). Indeed, the numerous bacteria thriving in wastewater, WWTPs and related habitats (e.g. biofilm, sludge) can host a myriad of ARGs and a better wastewater treatment technology is required to limit the risk of antibiotic resistance spread.

4.1. ARB & ARG threat in CWs

Phytodepuration of wastewater has gained increasing attention as low-cost and eco-friendly system for water reclamation and reuse, producing effluent of high quality for irrigation purposes (Petrosselli et al., 2015, 2017). Several works have highlighted the efficacy of CWs in limiting the diffusion of antibiotics, ARGs and ARB (Hijosa-Valsero et al., 2011; Chen et al., 2015) by removing antibiotics from wastewaters with different mechanisms such as biodegradation, plant uptake, substrate absorption, photolysis and volatilization (Liu et al., 2013; Chen et al., 2016a). Chen and Zhang (2013) demonstrated that CWs used as tertiary treatment of WWTP were more effective than biological filter and UV disinfection for ARG removal. Chen et al. (2016a) proved that different mesocosm-scale CWs treating raw domestic wastewaters reached a removal efficiencies of total antibiotics from the aqueous phase comprised between 75.8 and 98.6%, and those of total ARGs varied between 63.9 and 84%. Berglund and coworkers (2014) assessed the effluent quality of surface-flow experimental wetlands exposed to antibiotics at concentrations commonly found in wastewaters, showing that the CW system did not promote the dispersal of ARGs and leading to hypothesize that the low ARG concentration detected was probably due to a background genetic resistance present in the wetlands.

The fate of ARGs in CWs and their effluents is extremely influenced by the operating conditions of the CW (e.g. plant species, substrate type, flow type and velocity, system configuration) and environmental factors (e.g. temperature) (Liu et al., 2013; Chen and Zhang, 2013; Chen et al., 2016a; Fang et al., 2017; Huang et al., 2017). For instance, the relative abundance of tetracycline resistance genes (*tet*) seemed to be higher in the CW effluents of an up-flow system compared to down-flow treatment (Huang et al., 2017). Likewise, the use of different substrates can influence the removal of *tet* genes, as shown by Liu et al., (2013) in CWs established using volcanic (CW1) or zeolite (CW2) substrates. The results of the study indicate that the absolute abundance of *tet* and the universal bacterial 16S rRNA genes decreased in both CW1 and CW2 effluents, while the relative abundance of *tet* genes increased in CW1, pointing out an increase of the resistant populations over the total bacterial community.

During the summer, an increase of ARGs concentration was registered in wastewater effluent compared to the winter period (Fang et al., 2017). Indeed, temperature has a key role for the regulation of several microbial processes and metabolisms in CWs,

eventually influencing the microbial community structure (Truu et al., 2009). High temperature and solar irradiation positively influenced the degradation of different pharmaceuticals such as diclofenac, ibuprofen, ketoprofen, naproxen, salicylic acid, triclosan, and carbamazepine (Li et al., 2014) and antibiotics possibly share the same fate. On the other side, higher concentrations of ARGs were reported in natural and constructed wetlands during the summer period compared to winter (Li et al., 2019). Nevertheless, the influence of temperature on ARG fate is under debate and a different study revealed that low temperatures promote antibiotic resistance and horizontal gene transfer (HGT) of integron-associated ARGs (Miller et al., 2014).

A further issue on the efficacy of CWs in restricting ARGs and ARB diffusion in the environment has been overlooked until recently. The natural microbial community associated to CW plants could become itself a reservoir for specific ARGs due to the continuous exposition to antibiotics and ARB present in the raw or partially treated wastewater. In a ten years-experiment, Fang et al. (2017) showed that CW system could promote the increase of ARG level in the effluent mostly because of the accumulation of these genes into CW sediment. Similar results were obtained by Song et al., (2018) using synthetic wastewater, with negligible ARG abundance, as CW inflow. The effluent presented a lower abundance of ARGs compared to the CW sediments, however the ARG abundance was higher in the effluent compared to the inflow, unveiling the role of the natural bacterial community associated to *Oenanthe javanica* plants in the antibiotic resistance diffusion.

4.2. Role of horizontal gene transfer (HGT) in the ARG diffusion into the environment and within the plant microbiome

HGT is a crucial mechanism involved in evolution that consists in the movement of genetic material across different organisms. In bacteria it is generally divided in three main mechanisms, i.e. transformation, transduction and conjugation (Aminov 2011; Van Elsas et al., 2003; Baltrus, 2013). In the last few years HGT has been increasingly investigated in relation to the spread of ARGs that are frequently linked to MGE (Petrovich et al., 2018) and could be finally internalized by pathogenic bacteria representing a risk for human health (Song et al., 2018; Headd and Bradford, 2018).

HGT events have been examined in many different studies mimicking environmental conditions (Qiu et al., 2018; Baur et al., 1996; Hasegawa et al., 2018). For instance, Klümper et al., (2015) showed the possible spread of different plasmids in a soil bacterial community highlighting the importance of HGT in this environment. In a recent study, MGEs have been discovered in metal contaminated soil amended with pig manure (Garbisu et al., 2018), in agreement with the previous knowledge that manure's utilization as fertilizer in crops may enhance the abundance of ARGs in the soil microbiota by HGT (Chen et al., 2016b; Gotz and Smalla, 1997). The frequency of HGT events depends on many different factors, beside the specific molecular nature of the mobile element and donor/receiving strains. As a general factor, HGT could be enhanced by selective pressure due to anthropogenic activities that release into the environment different classes of pollutants, e.g. metal or antibiotics under the minimal inhibitory concentration (MIC) (Santos and Ramos, 2018). Furthermore, HGT events are enhanced in environments with high cell density and metabolic activity (Ulrich et al., 2015). Conjugation, for example, may depend on the ratio of donor to recipient strains and on the dimension and the conformation of the plasmid (Zeaiter et al., 2018). The HGT promoting conditions can occur in specific hot spots in natural and engineered ecosystems, mostly characterized by the presence of dense aggregates of colonies where cell-to-cell contact is boosted. Notably, even low concentrations of antibiotics were proved to

determine the aggregation of bacterial cells in aquatic ecosystems, thus creating conditions prone to cell-cell DNA transfer (Corno et al., 2014; Eckert et al., 2019).

Biofilm and mycosphere are two examples of environmental hot spots for HGT events. Qui et al., (2018) visualized ARGs transfer through conjugation in an activated sludge bacterial biofilm, and several studies underlined that conjugation and transformation events happen at a higher frequency when bacterial cultures are in biofilm rather than in the planktonic state (Madsen et al., 2012). Another HGT hot spot is the mycelia produced by Fungi that provide a high amount of nutrients and a wide surface to which bacterial cells can adhere, enhancing the frequency of the conjugation events (Berthold et al., 2016). High concentrations of MGEs have been detected in water ecosystems like WWTPs (Petrovich et al., 2018), where the frequency of HGT events can be increased due to the presence of a dense microbial population and sublethal concentrations of antibiotics acting as selective pressure toward the resistance acquisition. WWTP effluents can influence the spread of ARGs through MGEs in aquatic sediments (Chu et al., 2018) and it has been demonstrated that the release of tertiary treated urban wastewater can increase ARG concentration in the surface layers of the receiving water bodies (LaPara et al., 2011). Moreover, Di Cesare et al., (2016) demonstrated that chemical disinfection of urban wastewaters, treated in different WWTPs of Northern Italy, could favour the survival of bacterial populations resistant to antibiotics, able to form cell aggregates and harbouring different ARGs.

Major HGT hot spots related to the plant niche are rhizosphere, phyllosphere and spermosphere (Aminov 2011; Chen et al., 2019; Van Elsas et al., 2003) and, accordingly, HGT events in the plant microbiome have been extensively reported. Conjugation between exogenous and indigenous bacteria have been detected in bacterial communities of poplar plants (Ulrich et al., 2015) while plasmid transfer from *Pseudomonas putida* to indigenous bacteria have been studied on the surface of alfalfa sprout (Mølbak et al., 2003). Likewise, Björklöf et al. (1995) demonstrated that conjugative plasmid transfer occur in the phyllosphere of bean plants. Microcosm scale experiments showed that conjugation events can occur in barley seedling spermosphere, where the transfer of plasmid RP4 from a *Pseudomonas* strain donor to the indigenous bacteria has been detected (Sørensen and Jensen, 1998).

In the frame of phytodepuration, the rhizosphere is the most crucial hot spot of HGT events. In phytodepuration systems such as CWs, the root apparatus and its associated microbial community are continuously exposed to substances (i.e. pharmaceutical, biocides, metals) promoting selection and co-selection events. Moreover, in this habitat bacterial populations are metabolically active due to the high nutrient content, live in high concentration and tight contact and harbour abundant MGEs (Chen et al., 2019). Plasmid transfer has been studied in barley and pea rhizosphere (Mølbak et al., 2003; Sørensen and Jensen, 1998) and a higher relative abundance of IncP-1 plasmid has been detected in lettuce rhizosphere in comparison with bulk soil (Jechalke et al., 2014). Accordingly, a recent study suggests that plasmid transfer in rhizosphere soil of maize seedlings occurs with higher abundance than in bulk soil (Zhu et al., 2018). The chemical composition of root exudates, including sugars, organic acids and amino acids, enhances HGT and plasmid transfer events in the rhizosphere (Zhu et al., 2018), as specifically shown for nodulation-inducing flavonoid (Ling et al., 2016). The investigation of HGT events in the rhizosphere of plants in phytodepuration systems is still neglected, revealing a gap of knowledge that should be taken into account for the overall evaluation of this wastewater treatment technology, especially in the frame of ARG spread into the environment.

Even if there is massive evidence on HGT occurrence and ARGs presence in the environment, limited information is still available

on their relationship in both natural and human impacted ecosystems, although previous studies suggest that HGT events, such as conjugation, happen in nature at several orders of magnitude higher than *in vitro* studies (Davies and Davies, 2010). According to the available literature, HGT events in plant microbiome are influenced by different abiotic factors such as pH, temperature, micro- and macro-nutrients, oxygen and moisture content, and by biotic agents such as the presence of antagonistic, syntrophic or competing organisms (Van Elsas et al., 2003). Unfortunately, the high variability of such parameters over time and space makes extremely difficult the comprehension of the spread and dynamics of antibiotic resistance in the environmental niches where HGT can take place, hampering the modelling of antibiotic resistance fate that could be useful for successful water reuse management strategies.

5. Concluding remarks and research perspectives

In this review, we presented the advantages related to the implementation of microbial assisted phytodepuration. Bacterial inocula have indeed the potential to contribute to the biological wastewater cleanup in phytodepuration systems that nowadays are proposed as a low maintenance solution for water reclamation at suitable quality for water reuse in agriculture. Thanks to different PGP mechanisms, bacteria are able to support the growth of wetland plants improving their service in term of nutrient and pollutant removal, through the action of a more developed root apparatus and higher biomass. Microorganisms, enriched in plant rhizosphere, can also degrade recalcitrant micropollutants, such as pharmaceuticals, that are not targeted by common wastewater treatment plants, directly improving the effluent quality.

On the other side, we evidenced the possible peculiar risk of this technology in terms of antibiotic resistance diffusion in the environment. Designed wetlands seem to have a high efficiency in the removal of pollutants of emerging concerns, such as antibiotics, ARGs and ARB, although this is still debated given some contrasting results present in the literature. We highlighted that the pollutant removal processes in phytodepuration are performed in the rhizosphere, which is reported as a hot spot for HGT that could drive the diffusion of ARGs and the ARB. Although the root apparatus of plants used in phytodepuration of urban wastewaters is constantly exposed to ARB and low concentration of biocides and antibiotics, we point out that HGT of ARGs is still not sufficiently investigated in this type of wastewater treatment facilities. In particular, we recommend the need of a deep characterization of the antibiotic resistance profile and the related genetic determinants for the bacteria selected as promising inocula for assisted CW phytodepuration, characterization that is lacking in all the literature works discussed in this review. Some authors demonstrated a role of the natural community associated to plants in CWs in increasing the ARG concentration in the effluent, making important to clarify the dynamics of antibiotic resistance in response to the addition of bacterial inocula in such systems. The investigation of such aspects is pivotal in the frame of the 'One-Health' approach, especially when the treated wastewater is reused for irrigation purposes, entering the food production chain and eventually contributing to the diffusion of ARGs that could be acquired by pathogenic bacteria.

Even though further studies are necessary to deepen our knowledge on the above-mentioned phenomena, assisted phytodepuration can be considered a valuable approach able to enhance the quality of treated wastewater and it can be part of a wider strategy to boost water reuse worldwide.

Conflict of interest

The authors declare that they have no conflict of interest.

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