Enhancement of rhizocompetence in pathogenic bacteria removal of a constructed wetland system

Marwa Ben Saad, Myriam Ben Said, Isabel Sanz-Sáez, Olga Sánchez, Jordi Morató, Latifa Bousselmi and Ahmed Ghrabi

ABSTRACT

The main goal of the present study was to enhance the rhizobacterium potential in a horizontal subsurface flow constructed wetland system planted with *Phragmites australis*, through environmentally friendly biological approaches. The bioinoculation of antagonist bacteria has been used to promote higher rhizosphere competence and improve pathogenic bacteria removal from wastewater. The experiment was performed both with single and sequential bioinoculation. The results showed that strain PFH_1 played an active role in pathogenic bacteria removal, remarkably improving inactivation kinetics of the pathogenic tested bacterium *Salmonella typhi* in the plant rhizosphere. The single bioinoculation of selected bacteria into the rhizosphere of *P. australis* improved the kinetics of *S. typhi* inactivation by approximately 1 U-Log_{10} (N/N_0) (N is the number of viable cultured bacteria at time t, N_0 is the number of viable and cultivable bacteria at time t_0) compared to the control. By a series of multi-bioinoculations, the enhancement of pathogenic bacteria reduction compared to the inhibition rate in the pilot-scale control was of 2 U-$Log_{10}(N/N_0)$. These findings suggested that this strain represents a promising candidate to enhance water purification in constructed wetlands.

Key words | antagonism, bioinoculation, constructed wetland, rhizosphere, wastewater

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INTRODUCTION

For several decades, constructed wetlands (CWs) have been used as an ecological and green technology to treat wastewaters (Wu *et al.* 2015). CWs offer a low-energy, land-intensive, and less operational-requirements alternative to conventional treatment systems, especially for small communities (Ghrabi *et al.* 2011; Shen *et al.* 2015; Tee *et al.* 2016). By using natural functions of wetland vegetation, soils and their microbial populations, these engineered systems are designed to treat contaminants in surface water, groundwater or waste streams (Vymazal 2014). CWs have a great potential for the treatment of wastewater of different origin (Zaytsev *et al.* 2011; Wang *et al.* 2015) such as domestic, agricultural and industrial wastewater (Liu *et al.* 2015). CWs have been successfully used to remove a wide variety of pollutants such as organic compounds, suspended solids, pathogens and metals (Zhang *et al.* 2014; Sánchez 2017). During wastewater treatment in CWs, pollutants are removed through an integrated combination of biological, physical and chemical interactions between the plants, the substrate and the inherent microbial community (Farrar *et al.* 2014). According to the wetland hydrology, CWs are typically classified into two types: free water surface (FWS) CWs and subsurface flow (SSF) CWs. FWS systems are similar to natural wetlands, with a shallow flow of wastewater over a saturated substrate. On SSF systems, wastewater flows through the substrate, which supports the growth of plants. In addition, based on the flow direction, SSF CWs could be further divided into vertical flow

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and horizontal flow CWs. A combination of various wetland systems, or a hybrid CW, was also introduced for the treatment of wastewater (Wu *et al.* 2015).

Microorganisms play a vital role in degradation of different pollutants in CWs. It has been recognized that the removal of most of them in CWs is due primarily to microbial activity (Meng *et al.* 2014). Each pollutant can be associated with a specific microbial functional group; therefore the employment of design and operational methodologies that enhance the activity of that group will better optimize CWs efficiency (Eastman *et al.* 2009; Faulwetter *et al.* 2009). Thus, it has long been thought that many naturally occurring rhizosphere bacteria and fungi may offer a viable substitute for the use of chemicals and are antagonistic towards crop pathogens. For instance, plant-growth-promoting rhizobacteria (PGPR) have been shown to be beneficial to plant growth and health by exhibiting an active role in nitrogen fixation, the production of phytohormones and antifungal compounds, and induced systemic resistance. (Sindhu *et al.* 2009; Dilfuza *et al.* 2015).

Based on the importance of rhizosphere competence or root colonization in beneficial plant–microbe interactions (Ben Saad *et al.* 2016), the main goal of the present study was to enhance the inactivation of pathogenic bacteria in a horizontal subsurface flow CW system planted with *Phragmites australis* using antagonistic bacteria. This work aimed to demonstrate the beneficial application of biotechnology to confer higher rhizosphere competence in the removal of the model pathogenic bacterium *Salmonella typhi* ATCC 560 by means of environment-friendly biological approaches.

METHODS

Sampling and isolation of bacterial strains from different environments

Samplings were conducted at CWs of the Technological Demonstration Center (TDC) located at the Agronomic Institute of Tunisia. The demonstration pilot systems has been developed and erected in the framework of the project 'Sustainable Concepts towards a Zero Outflow Municipality (Zer0-M) (Regelsberger *et al.* 2007). The TDC treated the sewage discharged from the student house. The black water was introduced to a storage tank and was treated in a septic tank followed by horizontal subsurface flow (HSSF) and vertical subsurface flow (VSSF) CWs. The treated water was stored in a tank for green area irrigation. Sampling was conducted at different compartments in the CWs. Water samples were taken from the septic tank, the input of the VSSF and the input and the output of the HSSF. *P. australis* samples (roots and leaves) were collected from each CW. Roots were taken from both HSSF and VSSF at the entrance, middle, and exit at a depth of approximately 30 cm under the gravel surface. Other *P. australis* roots and soil samples were collected from the riverside located at Technopark Borj Cedria (northeast Tunisia). To isolate bacteria from the rhizosphere, the roots were initially separated from the rhizomes, and then small pieces of roots were immersed in sterile saline solution (0.85 g/L NaCl) and vortexed for 15 min in order to release attached bacteria. The same protocol was carried out to isolate bacteria from the sets of reeds. Concerning the wastewater samples, these samples underwent decimal dilutions in sterile saline solution and were spread over selective medium King's B agar (KB) (Ben Saad *et al.* 2016).

Identification of strains and detection of siderophores production

The identification of selected bacteria was based on the phenotypical aspect of colonies, the microscopic examination, and the use of standard microbiological and biochemical tests. Siderophores were detected by the method of Jalal & van der Helm (1990) using a spectrophotometric assay where a peak at 495 nm on the addition of 2% aqueous solution of $FeCl_3$ to 1 mL of supernatant indicated the presence of siderophores.

Antagonism tests

An antagonism test was performed among the isolated bacterial strains to avoid negative interaction between them after their bioinoculation. On the other hand, another antagonism test was performed between bacterial strains and the model pathogenic bacterium *Salmonella thyphi* ATCC 560. The Petri dish surface was seeded with an indicative strain and then blank discs with the putative antagonist strain were deposited on the culture medium. Blank discs were prepared by drenching with a filtered supernatant of a liquid culture of the putative antagonist strain, collected after centrifugation at 4,000 rpm for 15 min. The diffusion of the antimicrobial agents was enhanced by incubation at 37 °C for 24 h. Antagonist activity was revealed by the appearance of an inhibition zone around the discs (Ben Saad *et al.* 2016).

Study of motility of isolated bacteria and biofilm production

The different types of motility (*swimming*, *swarming* and *twitching)* were determined by the method of Reimmann *et al.* (2002). The biofilm production of bacterial isolates was detected by two methods. The first described by Freeman *et al.* (1989), which consisted of plating the test strains on solid medium containing brain heart infusion broth (37 g/L), sucrose (50 g/L), agar (10 g/L) and Congo Red indicator (8 g/L) (Sujatha & Ammani 2013). After incubation at 30 °C for 24 h, biofilm production was indicated by the appearance of black colonies. After this qualitative study, a quantitative study described by O'Toole & Kolter (1998) was carried out, using the dye crystal violet (CV), and estimating biofilm production spectrophotometrically at optical density of 600 nm.

DNA extraction, PCR amplification and sequencing of the selected strain

Bacterial DNA from the strain which showed the highest antagonist activity against *S. typhi* (PFH_1) was extracted and purified using the v-DNA reagent (GenIUL) according to the manufacturer's instructions. The concentration of the extracted DNA was measured using a spectrophotometer at 260 nm. DNA purity was estimated from the A260/A280 ratio. The complete 16S rRNA gene was amplified using universal bacterial primers 27F (5′-TAC GGY TAC CTT GTT AYG ACT T-3′) and 1492Rmod (5′-AGR GTT TGA TCM TGG CTC AG-3′). Each polymerase chain reaction (PCR) reaction with a final volume of 25 µL contained: 2 µL of template DNA, 0.5 µL of each deoxynucleoside triphosphate at a concentration of 10 µM, 0.75 µL of $MgCl_2$ 1.5 mM, 0.5 µL of each primer at a concentration of 10 µM, 0.125 µL of *Taq* DNA polymerase (Invitrogen), 2.5 µL of PCR buffer supplied by the manufacturer (Invitrogen, Paisley, UK) and Milli-Q water up to the final volume. Reactions were carried out in a Bio-Rad thermocycler using the following program: initial denaturation at 94 °C for 5 min, followed by 30 cycles of 1 min at 94 °C, 1 min at 55 °C and 2 min at 72 °C, and a final extension step of 10 min at 72 °C. PCR products were verified and quantified by agarose gel electrophoresis with standard low DNA mass ladder (Invitrogen). Purification and one-shot Sanger sequencing of 16S rRNA gene products were performed by Genoscreen (Lille, France) with primers 27F and 1492Rmod on ABI3730XL to obtain the complete sequence. The gene sequence was deposited in GenBank under accession number MG770377 and it was subject to a BLAST search (Altschul *et al.* 1997) to obtain an indication of the phylogenetic identification.

The pilot-scale systems

Two identical HSSF CWs were used to perform the experiment. Both basins were filled with gravel and planted with *P. australis*. One was used as a control (T) and the other one served for the different bio-assays (F). The size of each CW bed was 0.3 m × 0.44 m × 0.28 m (Ben Saad *et al.* 2016).

Monitoring of bacteria removal

For optimization purposes, the kinetic growth of the selected strain PFH_1 was characterized using a spectrophotometer to estimate absorbance of cell suspensions (OD_{600}). Different growth curves were obtained to determine the lag time for the microorganism to adapt to the new conditions. It was cultivated in two different media: autoclaved wastewater and nutrient broth at room temperature.

Afterward, inocula were prepared by suspending bacteria from nutrient agar plates in nutrient broth to obtain populations of about 10^8 UFC/mL (Bennett & Lynch 1981). The antagonist bacterium was inoculated using a sterile fine pipe directly connected to the rhizosphere. Treatment with contaminated water resulted in the following designations: F, a CW with *P.* inoculated with PFH_1; T, a CW with *P.* but no bacterial inoculation. The water in the basin was kept stagnant for 2 days in order to let microbes settle and attach before starting the experiment. After the adaptation period, 10^6 UFC/mL of the indicator pathogenic bacterium *S. typhi* was added to both pilot-scale CWs (F and T).

Based on growth kinetic parameters of the bacterium of interest (PFH_1), sequential bio-injections were also performed in the pilot-scale CW F to test the spatio-temporal dynamics and microbial ecological processes of root colonization by an antagonist and to explore the impact of the accumulation effect of sequential bioinoculation of antagonist bacteria to promote inhibition of pathogenic bacteria.

The monitoring of pathogenic bacteria removal after bioinoculation in both types of experiments was determined by culture on selective medium (*Salmonella, Shigella* agar).

RESULTS AND DISCUSSION

Isolation and screening of bacterial strains

After sampling, isolation and purification stages, 19 bacterial strains were isolated from different ecological niches. The bacterial colonies, cultivated on KB medium, were round, smooth and cream-white. All strains studied were oxydase+, catalase+ and Gram-negative.

The isolated strains were selected and screened for general functional properties of plant-growth-promoting rhizobacteria; namely, siderophore production and antagonist activity against pathogenic bacteria, in addition to bacterial motility (swimming, swarming and twitching motilities) and biofilm production (Table 1).

Bacterial biofilm formation is important for root colonization. Indeed, root-associated bacteria have been extensively studied, and many of these promote the growth of host plants or are used as biocontrol agents (Dekkers *et al.* 1998; Alizadeh 2011). Plant-growth-promoting bacteria have been reported to discontinuously colonize the root surface, developing as small biofilms along epidermal fissures. Among the isolates, PFH_1 strain could be a possible candidate to be inoculated into the rhizospheric zone to enhance the reduction of pathogenic bacteria. Furthermore, the antagonism test revealed this strain as the most antagonistic bacterium against *S. typhi*. Certainly, the ability of microbes to produce a wide range of antimicrobial compounds, including lytic agents, antibiotics, bacteriocins, protein exotoxins and other secondary metabolites, is critical to their success in antagonistic activities (Liu *et al.* 2013).

Molecular identification of the selected strain

The 16S rRNA sequence analysis of bacterium PFH_1 revealed that this microorganism had 99% of similarity with *Enterobacter cloacae,* a Gram-negative proteobacterium belonging to the *Enterobacteriaceae* family. The PFH_1 strain was classified under *Enterobacter* sp. and its sequencing data were submitted to GenBank NCBI under the accession number MG770377 (https://www.ncbi.nlm.nih.gov/nucleotide/MG770377.1?report=genbank&log$=nucltop&blast_rank=18&RID=FZRT9BXG01R).

Enterobacter is grouped in a subclade with *Klebsiella*, with which it is closely related. The *E. cloacae* species comprise an extremely diverse group of bacteria that have been found in diverse environments, ranging from plants to soil and humans (Liu *et al.* 2013). *Enterobacter* species have been reported as important engineering and plant-growth-promoting bacteria (Nie *et al.* 2002). Some *Enterobacter* strains may play important roles in plant–microbe interactions and hence in biocontrol mechanisms (Taghavi

Table 1 | Physiological and biochemical characteristics of isolated strains

Sampling sites	Samples		Isolates	Siderophore production	Motility			Biofilm production	Lipase production	Antagonism against *S. typhi*
					Swi	Swa	Twi			
Technological Demonstration Center	Water		PFS	+	+	+	+	+	+	–
			PEV	+	+	+	+	–	+	+
			PEH	+	+	+	+	–	–	–
			PSH	+	+	+	+	+	+	–
	P. australis	Plant foliage	PFH_1	+	+	+	+	+	+	+
			PFH_2	+	+	+	+	+	–	+
			PFV_1	+	+	+	+	–	–	–
			PFV_2	+	+	+	–	+	+	–
		Roots	PRV	+	+	+	+	+	–	–
			PRH	+	+	+	+	+	+	–
			PREH	+	+	+	+	+	+	–
			PRMH	–	+	+	+	+	–	–
			PRSH	+	+	+	+	+	+	–
			PREV	–	+	+	+	+	+	–
			PRMV	+	+	+	+	+	+	–
			PRSV	+	+	–	–	+	+	–
Technopark	Riverside	Roots	PR_1	+	–	–	+	+	–	–
			PR_2	+	–	–	+	+	–	–
		Soil	PSol	+	+	+	–	+	+	+

Swi: swimming; Swa: swarming; Twi: twitching.

et al. 2010). Several studies have shown the relevant ability of *Enterobacter* sp. to remove pollutant from wastewater; for example Lu *et al.* (2006) have demonstrated a good metal uptake capacity and high resistance to various heavy metals of a local bacterial isolate *Enterobacter* sp. J1. In this sense, the selected bacterium has been used to control the pathogenic density and has proved its application to improve the water treatment in CWs.

Bioinoculation of selected bacteria

To optimize our experiments and before inoculation into the rhizosphere, the kinetic growth of PFH_1 strain was investigated in nutrient broth and autoclaved wastewater at room temperature in order to determine the specific growth characteristics of PFH_1, namely the lag time (λ_t), the maximum specific growth rate (μ_{max}), and the bacterium generation time (t_G) (Figure 1).

The *in situ* experiment was done as follows. Phase I started with the sowing of the bacterium of interest into the rhizosphere environment. The main events were activation of the antagonist inoculum and establishment of an antagonist population in the plant rhizosphere. Phase II is the process by which the introduced antagonist and native root-associated microbes establish a population density and persist in the rhizoplane, rhizosphere or inside the root.

Removal of pathogenic bacteria with single bioinoculation

Figure 2 shows the kinetics of bacterial removal with an initial concentration of the indicator bacterium (*S. typhi*) equal to 10^6 UFC/mL both in the presence (CW F) and in the absence (CW T) of strain PFH_1. After a retention time equal to 3 hours, a reduction in the number of pathogenic bacteria (*S. typhi*) in both CWs has been noticed. Actually, the kinetics of *S. typhi* inactivation is in perfect agreement with the model of Chick–Watson with one modification:

$$\frac{N}{N_0} = A \exp(-kn.t) \quad (1)$$

where N/N_0 is the reduction in the indicator bacterial concentration, with N the number of viable cultured bacteria at time t, and N_0 the number of viable and cultivable bacteria at time t_0; k is the coefficient of inactivation (min^{-1}), A the bacterial reduction rate, and n the threshold inactivation or threshold events suffered by the bacterium after inactivation series, with $n = 1$ for the first degree model.

The analysis of bacterial inactivation curves showed an increase in bacterial reduction along time in both pilot-scale CWs with a difference in the bacteria inhibition rate. In turn, the injection of selected bacteria into the rhizosphere of *P. australis* improved the kinetics of *S. typhi* inactivation by approximately 1 U-Log_{10} (N/N_0) compared to the control (T). The enhancement of bacteria inactivation in the inoculated CW (F) during a short retention time is probably related to a good colonization ability of the rhizosphere and to the antagonist activity of the selected inoculated strain (Albright & Ode 2011).

Several studies have demonstrated the effectiveness of macrophyte systems in the elimination of pathogenic bacteria (Hill & Sobsey 2001), as well as the reduction of the pathogenic bacterium *S. typhi* by 2.3 U-Log_{10} for the treatment of primary sewage in small communities and rural areas using gravel during a retention time of 23 to 52 hours (Hench *et al.* 2003).

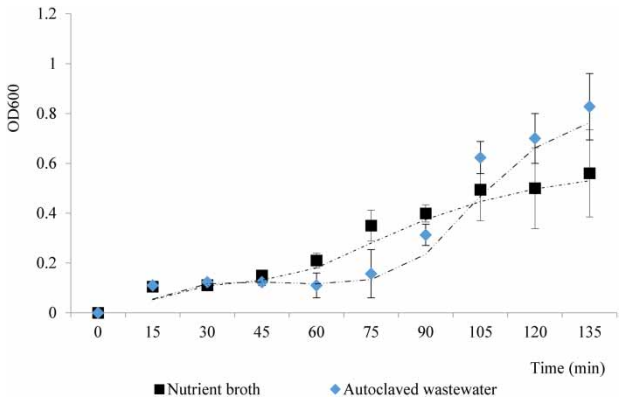

Figure 1 | Growth kinetics of PFH_1 in different media. Data are averages of three experiments. Nutrient broth: $\mu_{max} = 0.25\ min^{-1}$, $t_G = 2.77$ min, $\lambda_t = 60$ min; autoclaved wastewater: $\mu_{max} = 0.31\ min^{-1}$, $t_G = 2.23$ min, $\lambda_t = 75$ min.

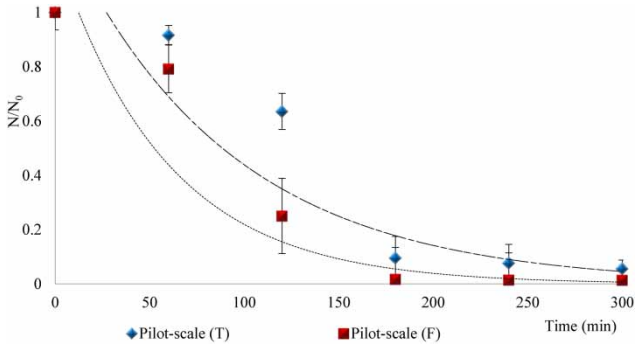

Figure 2 | Kinetics of pathogen bacteria removal in the two CWs (T and F) with single bioinoculation. Data are average of three experiments. $N/N_{0(T)} = 1.644e^{-0.015t}$ ($R^2 = 0.958$); $N/N_{0(F)} = 1.533e^{-0.029t}$ ($R^2 = 0.955$).

Table 2 | Kinetics parameters of *S. typhi* inactivation with single bioinoculation

	k (min^{-1})	A
Pilot-scale T	0.015	1.644
Pilot-scale F	0.029	1.533

The exploitation of the results of bacterial reduction by the modified kinetic model of Chick–Watson has allowed us to determine different kinetics parameters, such as the coefficient of inactivation (k) and the bacterial reduction rate in contact with autochthonous rhizobacteria with and without bioinoculation (A). The analysis of those parameters showed an increase in k, represented by the slope of the inactivation curve, which was more pronounced in the pilot-scale CW with an antagonistic bacteria (PFH_1) (Table 2). The increase of this coefficient confirmed the effectiveness of inoculated bacteria in CW F to strengthen the rhizospheric effect and increase the reduction of pathogenic bacteria.

Concerning the bacterial reduction rate, this parameter showed a small decrease in CW F compared to the control (T). It revealed the inactivation of target bacteria at the first contact with autochthonous rhizospheric biomass with and without bioinoculation. The stabilization of this parameter indicated directly the need of the inoculated bacteria for a period in which to become acclimatized to the *in situ* environment. Therefore, the first inactivation effect was governed by autochthonous biomass by various interactions such as antibiosis, biological antagonism, the competition for nutrients and parasitism (Di Francesco *et al.* 2016).

In summary, by a single inoculation of antagonistic bacteria PFH_1, the pathogenic bacteria removal has been increased by 1 U-Log_{10} of initial indicator bacteria. This result affirms well the use of bioinoculation for biocontrol.

The enhancement of the rhizobacterium potential in the pilot-scale CW planted with *P. australis* is strongly related to antagonist bacteria growth parameters, namely, λ_t, μ_{max} and t_G.

In the control CW (T), the inhibition of pathogenic bacteria (*S. typhi*) was carried out by the autochthonous bacterial colonization of the rhizoplane. The bacterial inactivation kinetics is in perfect agreement with the first order model of Chick–Watson (Equation (1)). However, in CW F, after bioinoculation of bacteria (PFH_1), the first order model of Chick–Watson cannot be applied to report the effect of bioinoculation on indicator bacteria inactivation. Indeed, in the inoculated pilot-scale, other parameters have to be considered such as the growth parameters of inoculated bacteria (λ_t, μ_{max}, t_G, adaptable time, etc.) For example, the optimal growth rate of inoculated bacteria (μ_{opt}) is determined where all environmental conditions are optimal, such as temperature (t_{opt}), pH (pH_{opt}) and water activity (aw_{opt}). The combined effect of several environmental factors is then determined by multiplying the respective gamma factors. The gamma concept was introduced by Zwietering *et al.* (1992) and is based on two principles: (i) all measurable factors that influence the growth rate (μ) are independent and occur multiplicatively:

$$\mu = f(\theta) \times f(pH) \times f(aw) \times \ldots f(others) \quad (2)$$

(ii) the effect of each environmental factor on the growth rate can be represented by a fraction of the maximum growth rate:

$$\gamma = \mu/\mu_{opt} \quad (3)$$

$$\mu_{max} = \mu_{opt}\, \gamma(\theta)\, \gamma(pH)\, \gamma(aw) \ldots \gamma(others) \quad (4)$$

where γ represents a function which takes into account the factor influencing μ_{opt} (γ is between 0 and 1); θ: temperature (°C).

In the inoculated CW (F) the contribution of autochthonous rhizobacteria in pathogen bacteria removal cannot be overlooked, as well as the antagonist activity of inoculated bacteria set up after a lag time (λ_t):

$$\text{If } t < \lambda_t + \alpha;\quad N/N_0 = AT \exp(-k.t) \quad (5)$$

$$\text{If } t \geq \lambda_t + \alpha;\quad N/N_0 = A' \exp(-k'.t) \quad (6)$$

with:

$$A' = A + A\alpha$$

$$A' = A \times (1 + \mu_{max})$$

$$k' = k + (k\,\alpha)$$

$$k' = k(1 + \mu_{max})^{n+m}$$

In the mini-filter (F), the inactivation kinetics of indicator bacteria can be modeled as follows:

$$N/N_0 = A \times (1 + \mu_{max})_\alpha \exp[(-k \times (1 + \mu_{max}^{n+m})]t \quad (7)$$

where N/N_0 is the reduction in the indicator bacterial concentration; N_t: number of viable cultivable bacteria at

time t; N_0: number of viable and cultivable bacteria at time t_0; k: coefficient of inactivation; A: bacterial reduction rate; k': coefficient of bacteria inactivation related to the presence of injected bacteria; A': bacterial removal rate related to the presence of injected bacteria; m: threshold inactivation or threshold events undergone by the bacterium after bioinoculation; and α: acclimation time of inoculated bacteria.

Removal of pathogenic bacteria with sequential bioinoculation

To strengthen the rhizocompetence in pathogen removal bacteria, sequential injections of the selected bacteria were performed at time 0, 60, 120 and 180 min. The choice of the injection time was based on the bacterium lag time, which equals 60 min (Figure 3).

By a series of multi-bioinoculations, the enhancement of pathogenic bacteria reduction compared to the inhibition rate in pilot-scale control (T) was of 2 U-Log$_{10}$ (N/N$_0$).

The accumulative effect of sequential bio-injection and the keeping of the exponential growth phase of the bacteria (based on the growth curve of PFH$_1$ strain) allow an increase k to 1.33 min^{-1}, determined after three bio-injections in CW (F) versus a value of k equal to 0.35 min^{-1} determined in the control CW (T) without bio-injection.

Moreover, the increase of inactivation rate determined for inoculated pilot-scale CW (F) compared to the control one (Table 3) can be observed.

The difference in kinetic parameters (k and A) determined for both CWs T and F is proportional to bacterial growth factors, the maximum specific growth rate, and the bacterium generation time. The enhancement of pathogen

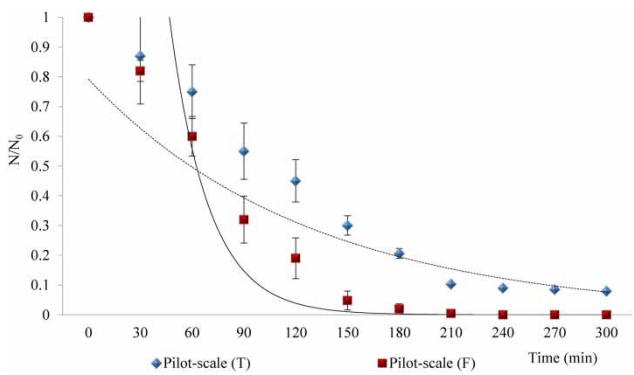

Figure 3 | Kinetics of pathogenic bacteria removal in CWs F and T with sequential bioinoculation of antagonistic bacteria. $N/N_{0(T)} = 1.8054e^{-0351t}$($R^2 = 0.965$). $N/N_{0(F)} = 30.405e^{-1.33t}$ ($R^2 = 0.869$).

Table 3 | Kinetics parameters of *S. typhi* inactivation with sequential bioinoculation

	k (min^{-1})	A
Pilot-scale T	0.351	1.805
Pilot-scale F	1.333	30.405

inactivation rate was positively correlated with the growth bioinoculum factors (A' and k') and the number of injections.

This result can be expressed as the following:

$$N/N_0 = [A \times (1 + \mu_{max})\alpha \exp[(-k \times (1 + \mu_{max})\, n + m]\ \mathrm{t}]\ b$$

with b: the number of inoculations.

In summary, after the accumulation effect of three sequential bio-injections into a rhizosphere environment of *P. australis*, the rhizocompetence in bacterial removal was increased and evidenced by k' (1.33 min^{-1}) and A' (30.4) relative to the antagonist activity of the bacteria with a reduction in contact time. The rhizosphere is a complicated biological system and too many factors affect bacterial inoculum survival (Strigul & Kravchenko 2006).

CONCLUSION

Constructed wetlands have proven to be an effective treatment for the inactivation and removal of pathogens in wastewaters. Several mechanisms are involved in pathogen treatment in CWs such as sedimentation, natural die-off, inactivation or death related to temperature, oxidation, predation, biofilm interaction, mechanical filtration, exposure to biocides and UV radiation (Weber & Legge 2008). Numerous studies have indicated that inoculated microorganisms could enhance the removal of various types of pollutants in CWs, in particular nitrogen and phosphorous (Zaytsev *et al.* 2011; Shao *et al.* 2014; Pei *et al.* 2016).

From the present research, it has been proven that application of bioinoculation has a clear potential to enhance the pathogenic bacteria removal process. Indeed, the preliminary results showed the beneficial effect of the bioinoculated strain (PFH$_1$) in the rhizosphere to increase remarkably the efficiency of the water treatment system for the reduction of pathogenic bacteria with a reduction in contact time. The single bioinoculation of selected bacteria into the rhizosphere of *P. australis* improved the kinetics of *S. typhi* inactivation by approximately 1 U-Log$_{10}$ (N/N$_0$) compared with the control. By a series of multi-bioinoculations, the enhancement of pathogenic bacteria reduction

compared to the inhibition rate in the pilot-scale control (T) was of 2 U-$Log_{10}(N/N_0)$.

The bioinoculation of antagonist showed positive results for most of the evaluated traits (single and multisequential injections), demonstrating the great potential of this practice to increase water quality. Furthermore, the results of the present study confirm the possibility of developing a commercial bioinoculant to be applied in biological water treatment processes to improve the treated water quality.

Screening for strains characterized by a high removal efficiency is a major problem to be solved before bioinoculation is performed in CWs. Therefore, it is better to isolate and screen microbial communities from the polluted environment (Meng *et al.* 2014). Moreover, the effect of intensified treatment of pollutants could only be maintained for a short time because the selected strains often lose the capability that they possess at laboratory pure-culture conditions (Chen *et al.* 2017).

This study has contributed to an eco-friendly strategy to improve the water treatment process by a CW, and highlighted the fact that better pathogen removal efficiency can be obtained through bioinoculation. As a future perspective of this work, the application of this strategy in field conditions, with multi-inoculation of antagonist substances protected by natural polymers to inactivate pathogenic bacteria in treated water without chemical additive, extension in the retention time or addition of complementary water treatment stages, should be taken into consideration.

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