Enhancement of rhizocompetence in pathogenic bacteria removal of constructed wetland system

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Abstract

The main goal of the present study was to enhance the rhizobacterium potential in horizontal subsurface flow constructed wetland system planted by *Phragmites australis* through environmentally friendly biological approaches. The bioinoculation of antagonist bacteria has been used to promote higher rhizosphere competence in pathogenic bacteria removal. The experience was performed with once and with sequential bio-inoculation. The results show that the strain PFH_1 played an active role in pathogenic bacteria removal. In fact, the individual bioinoculated improves remarkably the inactivation kinetics of a pathogenic tested bacteria; S. *typhi* in plant rizosphere by, 0.8 U-Log_{10} with once bio-injection and approximately, 2.5 U-Log_{10} with sequential bio-injections.These results suggested that this strain represents a promising candidate to improve the water purification by constructed wetland.

Key words: Antagonism, Bioinoculation, Constructed Wetland, Rhizosphere.

Introduction

Constructed wetlands (CWs) have been used as a green technology to treat various wastewaters for several decades. They offer a land-intensive, low-energy, and less operational-requirements alternative to conventional treatment systems, especially for small communities and remote locations(Ghrabi et al. 2011; Shen et al. 2015; Tee et al. 2016) (Ghrabi et al. 2011; S. Wu et al. 2015) These engineered systems are designed to treat contaminants in surface water, groundwater or waste streams by using natural functions of

wetland vegetation, soils and their microbial populations (Vymazal 2014). They have a great potential for the treatment of wastewater of different origin (Wang et al. 2015; Zaytsev et al. 2011) such as domestic sewage, agricultural wastewater, industrial effluent, mine drainage, landfill leachate, urban runoff, and polluted river water (Liu et al. 2015). CWs have been successfully used to mitigate environmental pollution by removing of a wide variety of pollutants from wastewater such as organic compounds, suspended solids, pathogens, metals, and nutrients (Zhang et al. 2014). 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 (Wang et al. 2015) .

CWs are typically classified into two types according to the wetland hydrology: 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, and based on the flow direction, SSF CWs could be further divided into vertical flow (VF) and horizontal flow (HF) CWs. A hybrid CW, a combination of various wetland systems, was also introduced for the treatment of wastewater (Wu et al. 2015). For the purpose of this paper, only a subsurface flow constructed wetland and especially the horizontal subsurface flow constructed wetland planted by *Phragmites australis* is considered.

Microorganisms play a vital role in degradation of multiple pollutants in CWs. It has been recognized that the removal of most pollutants in CWs is due primarily to microbial activity (Meng et al. 2014). Removal of a particular pollutant is typically 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 performance (Faulwetter et al. 2009).

It has long been renowned that many naturally occurring rhizosphere bacteria and fungi may offer a viable substitute for the use of chemicals and are antagonistic towards crop pathogens. Thus, Plant growth promoting rhizobacteria (PGPR) has been shown beneficial to plant growth and health by emancipating their activity on nitrogen fixation, the production of phytohormones and antifungal compounds, and induced systemic resistance (Sindhu, Rakshiya, and Sahu 2009).

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 rates in horizontal subsurface flow constructed wetland system planted by *Phragmites australis* using antagonistic bacteria. This work aimed to demonstrate the beneficial application of biotechnology to confer higher rhizosphere competence in the removal of pathogenic bacteria; *S. typhi* ATCC 560 by environment friendly biological approaches.

Methods

1. Sampling and isolation of bacterial strains from different environments

Bacterial strains were isolated from different ecological niches: wastewater, soil, *Phragmites australis* roots and sheets from the Technical Demonstration Center (TDC) that treats sewage from the university home located at the Agronomic Institute of Tunis (INAT). Rhizosphere samples were collected from each wetland at the entrance, middle, and exit at a depth of approximately 30 cm under the gravel surface. All samples were processed in the laboratory.

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 vortexes 15min in order to release the bacteria attached to roots into the solution.

The same protocol was followed to isolate bacteria from the sets of reeds. Concerning the wastewater samples, these samples underwent decimal dilutions in sterile saline solution and spread out over selective medium.

2. Identification of the strain and detection of siderophores production

The identification of selected bacteria was based on the phenotypical aspect of colonies, the microscopic examination standard microbiological and biochemical tests. Siderophore was detected by the method of Jalal and Vander 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.

3. Antagonism test between isolated bacterial strains and against pathogenic bacteria *S. typhi* ATCC 560

Antagonism test had been performed between isolated bacterial to avoid negative interaction between them after their bioinoculation. The Petri dish surface was seeded by an indicative strain and then the blank discs deposited on the culture medium had been drenched with 50µL of filtered supernatant of a putative antagonist strain, collected after centrifugation at 4000 r.p.m for 15 min. The diffusion of the antimicrobial agents was enhanced by incubation at 37°C for 24hr. Antagonist activity was revealed by the appearance of inhibition zone around the discs.

4. Study of motility of isolated bacteria and biofilm production.

The different types of mobility (*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 and al. (1989), consisted of plating the test strains on solid medium contained brain heart infusion broth 37 g/L, sucrose 50 g/L, agar 10 g/L and Congo Red indicator 8 g/L (Sujatha N. 2013). After incubation at 30°C for 24hr,

black colonies indicate biofilm production. After this qualitative study, we proceeded in a quantitative study describes by O'Toole (1998). This method uses the dye crystal violet (CV). biofilm production was estimated by spectrophotometric measurement of the OD 600nm.

5. Molecular identification of the selected strain

Bacterial DNA 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 260nm. DNA purity was estimated from the A260/A280 ratio. The complete 16S rRNA gene was amplified using 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 PCR reaction with a final volume of 25 µl contained: 2µl of template DNA, 0.5 µl of each deoxynucleoside triphosphate at a concentration of 10 µM, 0.75 µl of MgCl2 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 Biorad thermocycler using the following program: initial denaturation at 94ºC for 5 min, followed by 30 cycles of 1 min at 94ºC, 1 min at 55 ºC and 2 min at 72 ºC, and a final extension step of 10 min at 72ºC. 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 was performed by Genoscreen (Lille, France) with primers 27F and 1492Rmod.

6. Conception and construction of the pilot-scale systems

The experimental system designed for the bio-inoculation, included two small parallel identical horizontal subsurface flow constructed wetlands. Both basins were filled with gravel and planted with reed. The first one served as a control and the second served for the different

bio-assays. The size of each constructed wetland bed was 0.3 x 0.44 x 0.28. The treatment area was packed with 8-12mm diameter pea gravel while bigger and larger gravel of 30-80mm diameter was used at the inlet and outlet areas in order to prevent clogging of the filter media. The relative porosity has been calculated in 0.26 (n = Vv/Vt where Vv is the void volume and Vt is the total volume (Meng et al. 2014)). The pilote constructed wetlands had bottom slope of 1% to facilate the flow of water by gravity. The plants were allowed to grow and multiply over three months. There was a peridioc application of wastewater to serve as a source of nutrients for the plants. The main characteristics of the experimental system are as follows: Surface area: 13.2dm^2, Hydraulic Residence Time HRT (theoretical): 0.385, Gravel depth: 2dm, Average starting reed heights: 57cm.The figures 1 and 2 show the conception of horizontal subsurface flow constructed wetland systems adopted in this study.

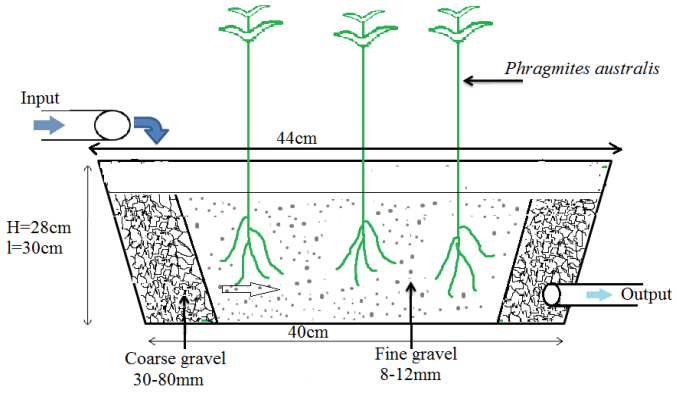

Figure1. The representative figure of the pilot-scale of subsurface flow constructed wetland

7. Monitoring of bacteria removal

To optimize the experience, the kinetic growth of the selected strain was determined using a spectrophotometer to estimate absorbance of cell suspensions (DO_{600}). Series tests were conducted to determine the lag time that made the selected bacteria to adapt to new conditions.

The experience *in situ* was done as follows: Phase I start from the sowing of interest bacteria into the rizosphere environment. The main events are activation of the antagonist inoculum

and establishment of an antagonist population in the plant rizosphere. Phase II is the process of the introduced antagonist and native root-associated microbes to establish a population density and persist in the rhizoplane, rhizosphere or inside the root.

Therefore, the antagonist bacterium was inoculated into the rhizosphere of the pilot-scale (F). After an adaptation period (depends on growth kinetic parameters), a contaminated effluent by 10^4 UFC/ml of an indicator bacteria; *S. typhi* ATCC 560 was added for both pilot- scales (F and T).

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

The monitoring of pathogenic bacteria removal after bioinoculation was determined by culture on selective medium (SS: Selmonella Shigella agar).

Results and discussion

1. Isolation and Screening of bacterial strains

After sampling, isolation and purification stages, 19 bacterial strains were isolated from different ecological niches from the Technical Demonstration Center (TDC) that treats sewage from university home located at the Agronomic Institute of Tunis (INAT). The isolats strains were selected and screened for general functional properties of plant promoting rhizobacteria; namely, siderophore production and antagonist activity against pathogenic bacteria in addition of the bacterial motility (swimming, swarming and twitching motilities) and biofilm production.

Bacterial biofilm formation is important for root colonization. Indeed, root-associated bacteria have been studied extensively, and many of these promote the growth of host plants or are used as biocontrol agents (Dekkers et al. 1998). The plant-growth-promoting bacteria have been reported to discontinuously colonize the root surface, developing as small biofilms along epidermal fissures.

Among the isolates, we were screened PFH_1 strain to apply as an interest to be inoculated into the rizospheric zone to enhance the reduction of pathogenic bacteria. Indeed, the antagonism test had revealed the strain PFH_1 as the most antagonist bacteria against *S. typhi*. Indeed, 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 (W.-Y. Liu et al. 2013).

2. Molecular identification of the selected strain

The 16S rRNA gene sequences of the selected bacteria were determined. The sequence analysis revealed that this bacteria has 99% of similarity with *Enterobacter cloaceae*.

Enterobacter cloacae are a gram-negative Proteobacterium belonging to the *Enterobacter*iaceae family. Within this family, *Enterobacter* is most closely related to, and is grouped in a sub-clade with, *Klebsiella*. The *E. cloacae* species comprises an extremely diverse group of bacteria that has been found in diverse environments, ranging from plants to soil to humans (Liu et al., 2013).

Enterobacter species have been reported as both plant pathogens and human opportunistic pathogens (Nishijima et al., 2007), and also 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. In this sense, we are used the

selected bacteria to control the pathogenic density and its application to improve the water treatment by constructed wetland.

3. Application bioinoculation of selected bacteria.

To optimize the experience and before inoculation into the rhizosphere, the kinetic growth of PFH_1 strain was investigated in saline water 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}), the bacterium generation time (t_G) (Figure 2).

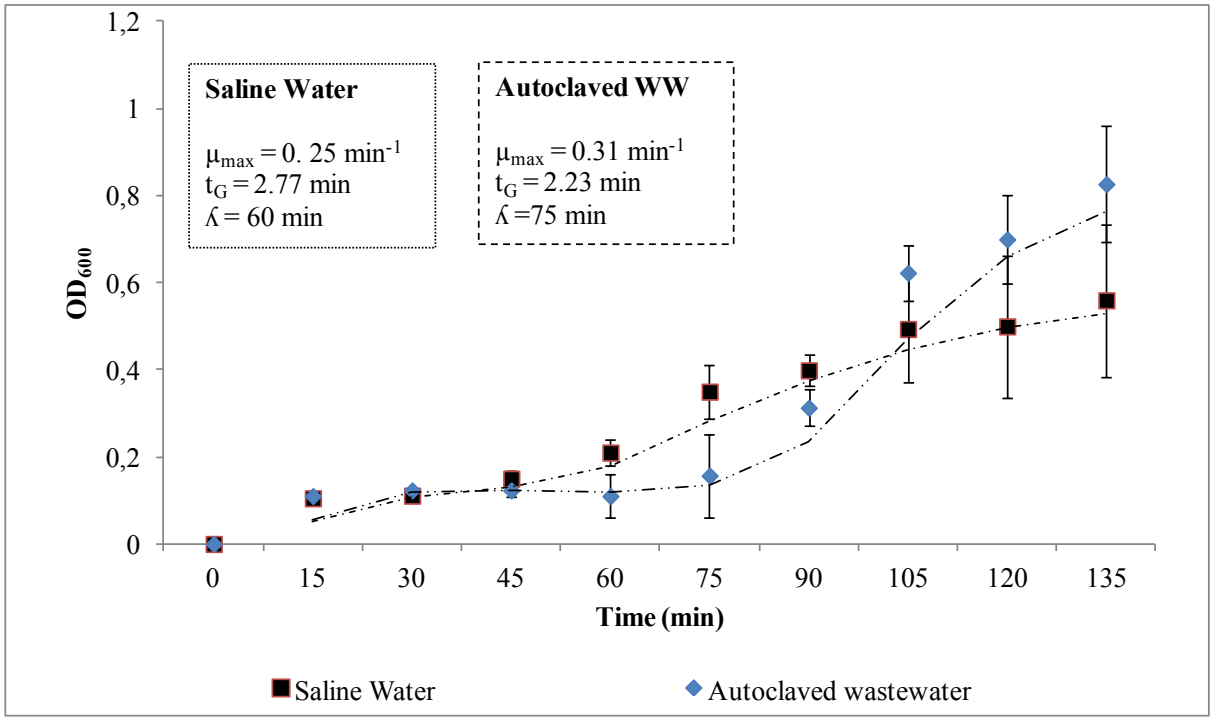

Figure 2. Kinetics growth of PFH_1 in different growth temperature conditions.

Data are averages of three experiments

3.1. Removal of pathogenic bacteria with single bioinoculation

The figure 3 shows the kinetic of bacteria removal with an initial concentration of indictor bacteria (*S. typhi*) equal to 10^6 UFC/ml in presence of PFH_1.

After a retention time equal to 3 hours, we were noticed a reduction in the number of pathogenic bacteria (*S. typhi*) in both filters: inoculated and non inoculated one (F and T pilot-scale filters).

The kinetics of *S. typhi* inactivation turns in perfect agreement with the model of Chick-Watson (CW) model with modification:

$$N/N_o = A \exp(-k^n.t) \quad (1)$$

With ; N/N_0: is the reduction in the indicator bacterial concentration, N: 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: The bacterial reduction rate; n: threshold inactivation or threshold events suffered by the bacterium after inactivation series, $n = 1$ for first degree model.

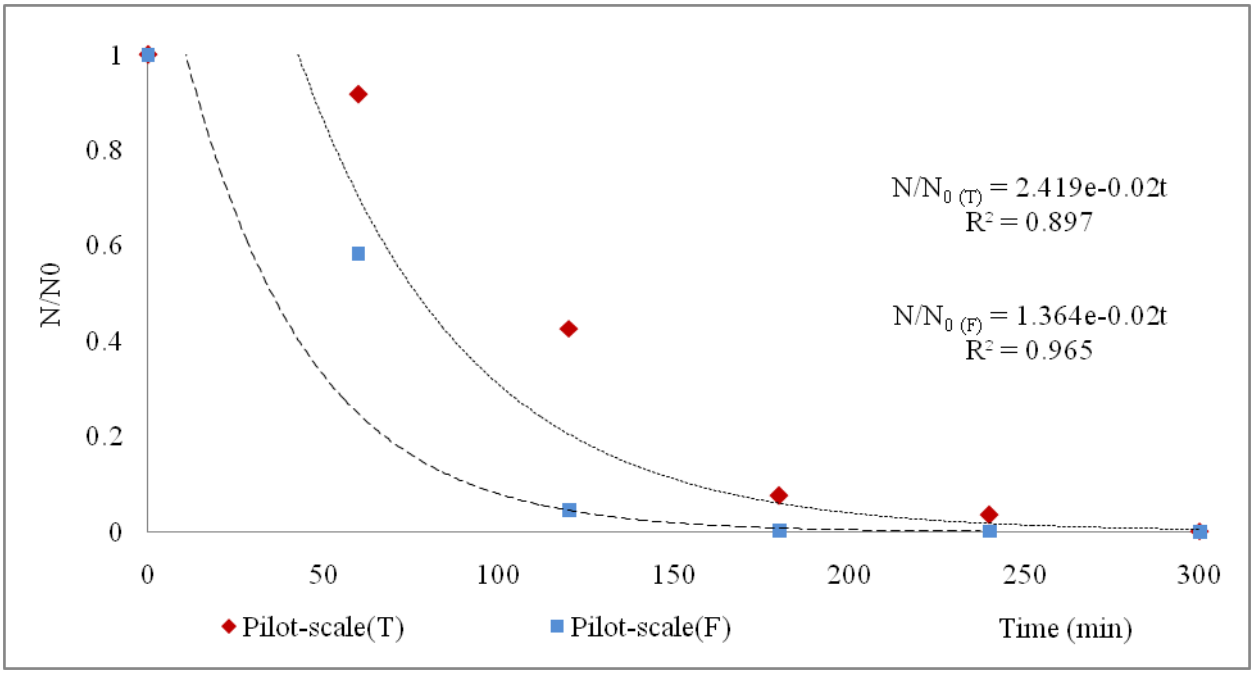

Figure 3. The kinetic of pathogen bacteria removal in the two filters (T) and (F) with single bioinoculation.
Data are averages of three experiments.

By analysis of bacterial inactivation curves, we can note an increase in bacterial reduction over time in the both pilot scales filters with a difference in the bacteria inhibition rate.

The injection of selected bacteria into the rhizosphere of *Phragmites australis* improves the kinetics of *S. typhi* inactivation by approximately 1U-Log_{10} (N/N_0) compared to the control mini- filter (T).

The enhancement of 0.8 U-Log_{10} of bacteria inactivation in inoculated filter (F) during a short retention time (5 hrs) is probably related to a good colonization ability of the rhizosphere and to the antagonist activity of the selected inoculated strain.

Several studies demonstrate the effectiveness of macrophytes systems in the elimination of pathogenic bacteria strain (Hill et Sobsey 2001). Other study showed that the reduction of the pathogenic bacterium *S. typhi* is equal to 2.3 U-Log_{10} ($N/_{N0}$) for 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).

The exploitation of the results of bacterial reduction by modified kinetic models CW has allowed us to determine different kinetics parameters. The most important values are: the coefficient of inactivation (k) and the bacterial reduction rate in the contact with autochtone rizobacterium with and without bioinoculation (A).

The analysis of kinetic parameters shows an increase of the inactivation coefficient (k) that represents the slope of inactivation curve; determined for inoculated pilot-scale (F) with an antagonistic bacteria (PFH_1). The increase of this coefficient confirms the effectiveness of inoculated bacteria to strengthen the rhizospheric effect and increase the reduction of pathogenic bacteria. .

For the bacterial reduction rate (A), this parameter shows a small decrease for inoculated mini-filter compared to the control mini-filter (T). This parameter revel the inactivation of target bacteria at the first contact with autochtone rhizospheric biomass with and without Bio-helper (bio-inoculation). The stabilization of this parameter indicated directly the need of acclimation

time for the inoculated bacteria to the *in situ* environment. Therefore, the first inactivation effect was govern by autochthone biomass by various interactions such as antibiosis, biological antagonism, the competition for nutrients and parasitism (Di Francesco, Martini, and Mari 2016).

By a single inoculation of antagonistic bacteria, we can increase pathogenic bacteria removal by 1.6 U-Log_{10} of initial indicator bacteria. This result affirms well the use of bioinoculation for biocontrol.

The enhancement of the rhizobacterium potential in mini-filter planted by *Phragmites australis* is strongly related to antagonist bacteria growth parameters, namely, the lag time (λ_t) the maximum specific growth rate (μ_{max}), the bacterium generation time (t_G).

In the control minifilter (T), the inhibition of pathogenic bacteria (*S. typhi*) is carried out by the autochthon bacterial colonization of the rhizoplane.

The bacterial inactivation kinetic is in perfect agreement with the first order model of Chick-Watson (Equation1).

However, in the mini-filter (F), after bioinoculation of interested bacteria (PFH_1), we cannot apply the first order model of Chick-Watson to report the effect of bioinoculation on indicator bacteria inactivation. Indeed, in the inoculated pilot-scale, we must consider several parameters. The most important are: the growth parameters of inoculated bacteria (the lag time, (λ_t) the maximum specific growth rate (μ_{max}), the bacterium generation time (t_G), the adaptable time, etc.

For example the optimal growth rate of inoculate bacteria (μ_{opt}) is determined where all environmental conditions are optimal such as temperature (t_{opt}), pH (pH_{opt}), the water activity (aw_{opt}), etc.

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:

$$\gamma = \mu / \mu_{opt} \text{ (Comprise between 0 and 1)} \quad (3)$$

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

Where γ: represents a function taking into account the factor influencing μ_{opt}; **θ**: temperature (°C)

In the inoculated mini-filter (F) we cannot overlook the contribution of autochthon rizobacterium in pathogen bacteria removal. Indeed, the antagonist activity of inoculated bacteria set up after a lag time (λ_t):

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

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

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), we can model the inactivation kinetics of indicator bacteria as following:

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

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: The bacterial reduction rate; k': coefficient of bacteria inactivation related by the presence of injected bacteria; A': The bacterial removal rate related by the presence of injected bacteria, m: threshold inactivation or threshold events undergo by the bacterium after bioinoculation and α: A acclimation time of inoculated bacteria.

2.2. Removes 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 min and 180 min. The choice of the injection time was based on the bacterium lag time that equal to 60 min (Figure 4).

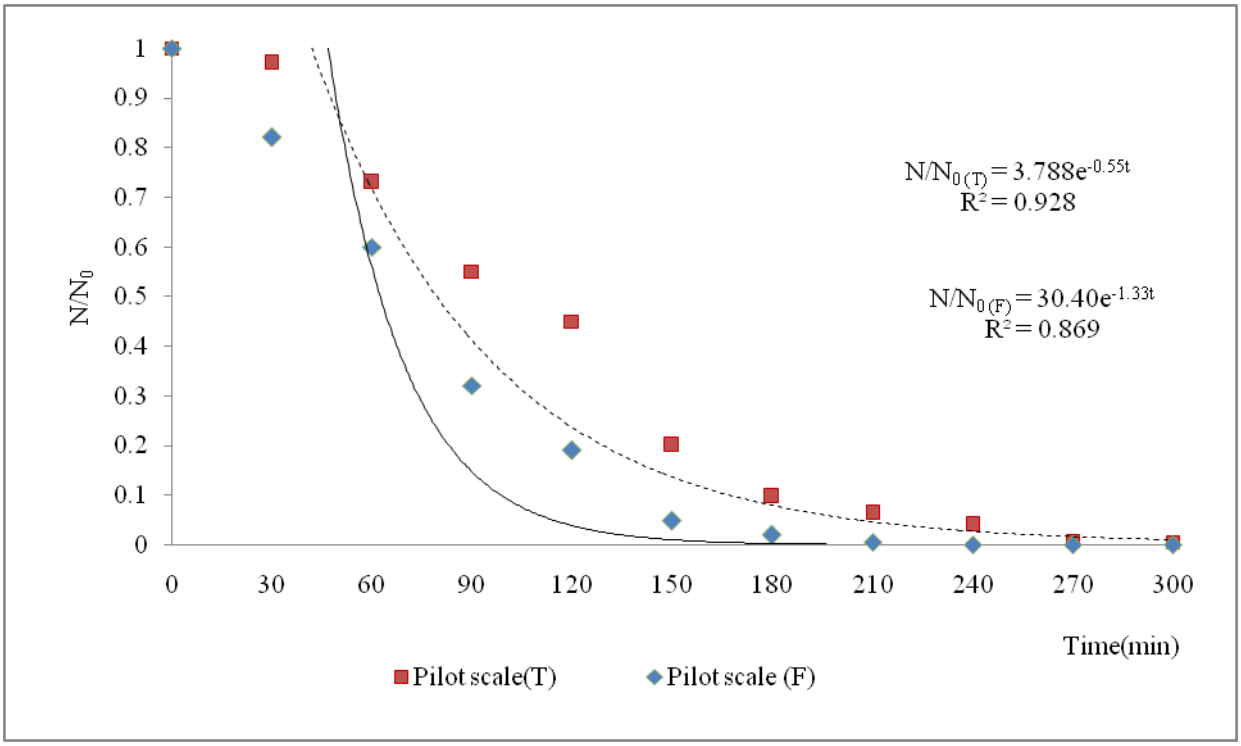

Figure 4. The kinetic of pathogen bacteria removal in mini-filter (F) with sequential bioinoculation of antagonistic bacteria.

By a series of multi-bioinoculation, we can see the enhancement of pathogen bacteria reduction compared to the inhibition rate in control pilot-scale (T) by 2 U-$Log_{10}(N/N_0)$.

The accumulative effect of sequential bio-injection and the keeping of the exponential growth phase of interest bacteria (based on the growth curve of PFH_1 strain) allowed us to increase the coefficient of indicator bacteria inactivation (k) to 1.33 min^{-1} determined after three bio-injection in mini-filter (F) versus a value of k equal to 0.35 min^{-1} determined in the control mini-filter (T) without bio-injection.

We can note the increase of inactivation rate (A) determined for inoculated pilot-scale (F) compared to control scale.

The difference in kinetic parameter (k and A) determined for both mini-filter T and F is proportional of growth bacteria factors (λ_t) the maximum specific growth rate (μ_{max}), the bacterium generation time (t_G)).

The enhancement of pathogen inactivation rate is positively correlated with the growth bio-inoculum factors (A' and k') and the number of injection.

We can modulate this result as following:

$$N/N_o = [A \times (1+ \mu_{max})_{\alpha} \exp [(- k \times (1+\mu_{max})^{n+m}] t]^b \quad (8)$$

With, b: the number of inoculation.

To resume, after the accumulation effect of three sequential bio-injections into a rizosphere environment of *Phragmites australis*, the rizocompetence in bacterial removal was increased by k' (1.33 min^{-1}) and A' (30.4) relative to the antagonist activity of interest bacteria with a reduction in contact time.

The bioinoculation of antagonist showed positive results for most of the evaluated traits single and multisequential injections), demonstrating the great potential of this practice use in order to increase the quality of sanitary. The results of the present study reaffirm the possibility of

developing a commercial bioinoculant to be applied in biological water treatment process to improve the treated water quality.

CONCLUSION

From the present research, we can conclude that application of bioinoculation has a potential to enhance the pathogenic bacteria removal process. Indeed, the preliminary results show 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.

This study has contributed with an eco-friendly strategy to improve water treatment process by constructed wetland, and highlighted the fact that better yields can be obtained thought bio-inoculation.

As a perspective of this study, the application of this strategy in field conditions with multi-inoculation of antagonists substances protected by natural polymers to inactivate pathogenic bacteria in treated water without chemical addition, extension in the retention time or addition of a complement water treatment stages.

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