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Application of bioinoculation to enhance rhizocompetence of horizontal subsurface flow 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* using biotechnology. The bioinoculation of denitrifier promoting rhizobacteria fluorescent *Pseudomonas* spp. has been used to confer higher rhizosphere competence by environmentally friendly biological approaches. In order to study the behaviour or the denitrifying potential of the inoculated bacteria *ex situ* and *in situ*, the models of Chick–Watson and Series-Event were used with modifications. The comparison of kinetic parameters: (*k*): the denitrifying coefficient (h^{-1}) and the denitrifying rate at the first contact with a determined concentration of nitrate (*A*) for different bioassays has shown that the application of the bioinoculation increases remarkably the efficiency of the water treatment system for the reduction of nitrates even by an individual bioinoculated bacterium.

Keywords: Bioinoculation; Constructed Wetland; Denitrification; *Pseudomonas* spp.; Rhizosphere

1. Introduction

Constructed wetlands (CWs) are engineered systems, designed to use the natural functions of wetland vegetation, soils and their microbial populations to treat contaminants in surface water, groundwater or waste streams [1]. These wastewater treatment technologies are simple and low cost that use natural processes in the shallow basins filled by substrates (soil, sand and gravels) and involving microorganism's activities [2]. In addition, CWs offer several additional advantages compared to natural wetlands (site selection, design, control of hydraulic and retention time, etc.) [3,4]. CWs have proven to be a good ecological technology, ensuring appropriate performances for wastewater treatment, especially in villages and small communities [5–7]. Several studies show that CW processes for the remediation of wastewaters polluted with harmful organic chemicals are an emerging field [8,9].

Various types of CWs differ in their main design characteristics as well as in the processes, which are responsible for pollutant removal. The main CWs types

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are: (i) subsurface flow (SFCW) that can be operated with vertical or horizontal flow (VSFCW or horizontal subsurface flow constructed wetland (HSFCW)) and (ii) free-water surface flow constructed wetland (FWSFCW) [10]. For the purpose of this paper, only an SFHCW planted by *Phragmites Australis* is considered.

The soil around the plant roots (rhizosphere) contains bacteria that can live symbiotically with plants to help them get the nutrients they need. CW may provide an effective area of contaminant degradation by stimulating microbial activity in the rhizosphere; and it is considered a good ecosystem for nitrogen recycling [10], such as NH_4^+ and NO_3^- [11,12]. Thus, plant growth promoting rhizobacteria (PGPR) has been shown beneficial to plant growth and health by stimulating their activity on nitrogen fixation, the production of phytohormones and antifungal compounds and induced systemic resistance [13]. In this perspective, PGPR fluorescent pseudomonads offer an attractive choice as bioinoculants and biocontrol agents. Metabolic versatility and its capabilities in producing a large range of antimicrobial metabolites render PGPR *Pseudomonas* as prominent bioinoculants in a horizontal subsurface flow constructed wetland.

A number of plant-associated bacteria, including *Pseudomonas* spp. and *Azospirillum brasilense*, are able to use nitrogen oxides as alternative electron acceptors under oxygen limiting conditions (denitrification). This process has been shown to play a role in rhizosphere colonization since mutants of *P. fluorescents* impaired in nitrate or nitrite reductases are deficient in the colonization of the rhizosphere. Furthermore, it has been shown that denitrification is statistically associated with rhizosphere competence in rhizosphere-isolated fluorescent *Pseudomonas* [14].

Based on the importance of rhizosphere competence or root colonization in beneficial plant–microbe interactions, the main goal of the present study was to enhance the denitrification rates in HSFCW planted by *Phragmites australis* using denitrified bacteria (fluorescent *Pseudomonas* spp.). The bioinoculation of denitrifier promoting rhizobacteria (DPR) is used to enhance rhizocompetence.

2. Methods

2.1. Sampling and isolation of *fluorescent* Pseudomonas *spp.*

Fluorescent *Pseudomonas* spp. were isolated from different environmental sites: wastewater, soil, *Phragmites australis* roots and leaves of (CW) from the Technical Demonstration Center (TDC) that treats sewage from a student house located at the Agronomic Institute of Tunis (INAT). Other samples were collected from Technopark Borj Cedria.

Rhizosphere samples were collected from each CW at the entrance, middle and exit at a depth of approximately 30 cm under the gravel surface. All samples were analysed in the laboratory.

To isolate *Pseudomonas* spp. strains 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 15 min in order to release the bacteria attached to roots into the solution. Decimal dilutions were carried out starting from this suspension in a sterile saline solution and plating on selective medium King's B agar (KB).

The same protocol was followed to isolate bacteria from the leaves of CW. Concerning the wastewater samples, these samples underwent decimal dilutions in sterile saline solution and spread out over selective medium King's B agar (KB) culture.

The identification of selected bacteria was based on the phenotypic aspect of colonies, the microscopic examination and biochemical tests [15].

2.2. Screening of denitrifying *fluorescent* Pseudomonas *spp.-isolated strains*

Growth test in anaerobic conditions of fluorescent *Pseudomonas* spp.-isolated strains in the presence or absence of KNO_3 as electron acceptor [16] was performed. Each isolated strain was cultivated in duplicate tubes containing 6 mL nutrient broth supplemented with 2 g/L KNO_3. To trap nitrogen gas produced through denitrification, the inverted Durham tubes were used. The cultures were incubated for 7 d at 24°C. The presence of nitrate in tubes after incubation was evaluated using ionic chromatography (Metrohm).

2.3. Antagonism test between isolated bacterial strains

Antagonism test had been performed between isolated bacteria 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 50 µL of filtered supernatant of a putative antagonist strain, collected after centrifugation at 4,000 rpm for 15 min. The diffusion of the antimicrobial agents was enhanced by incubation at 24°C for 24 h. The antagonist activity was revealed by the appearance of inhibition zone around the discs deposited on the culture medium and drenched with 50 µL of filtered supernatant of a putative antagonist strain.

2.4. Conception and construction of the pilot system

The pilot system includes two small identical HSFCW basins having the same properties and composition and working in the same conditions. Both were filled with gravel and planted with reed (*Phragmites australis*). The first one served as a control and the second served for the different experimental assays, which were done *ex situ* and *in situ* with a synthetic solution containing different nitrate concentrations. The size of each CW bed was $0.3 \times 0.44 \times 0.28$. The treatment area was packed with 8–12 mm diameter pea gravel, while bigger and larger gravel of 30–80 mm diameter was used at the inlet and outlet areas in order to prevent clogging of the filter media. The relative porosity has been calculated as 0.26 (n = Vv/Vt where Vv is the void volume and Vt is the total volume [8]). A laboratory-scale unit simulating HSFCW system had a bottom slope of 1% to facilitate the flow of water by gravity. The plants were allowed to grow and multiply over 3 months. There was a periodic application of wastewater to serve as a source of nutrients for the plants.

The main characteristics of the experimental system are summarized in Table 1. Figs. 1 and 2 show the conception of HSFCW pilot adopted in this study.

2.5. Monitoring of the nitrate assimilation

The bioassays were done *ex situ* and *in situ* with a synthetic solution containing different nitrate concentrations. Indeed, the selected bacteria were put into physiological water solution. After 18 h, a nitrate concentration was discharged into the solution (9 and 90 mg/L of KNO_3). Denitrifying activity was monitored over time. The experiment *in situ* was done as follows: at the beginning, a selected bacterium was injected, directly and once, into the rhizosphere of the inoculated basin (*F*). After an adaptation period, synthetic nitrate solution alimentation was carried out. The nitrate concentration was monitored by ionic chromatography.

Table 1
Main characteristics of the experimental system simulating HSFCW system

Parameters	Unit	Value
Surface area	dm^2	13.2
Hydraulic residence time (HRT) (theoretical)	Day	0.385
Gravel depth	dm	2
Average starting reed heights	Cm	57

Notes: Values are given as a mean of three replications; HRT = pond volume/average flow rate.

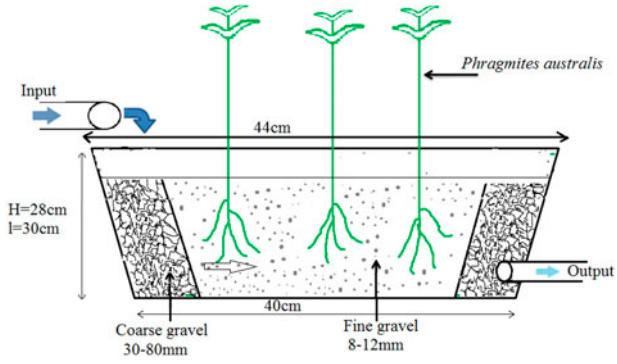

Fig. 1. Layout of the HSFCW pilot.

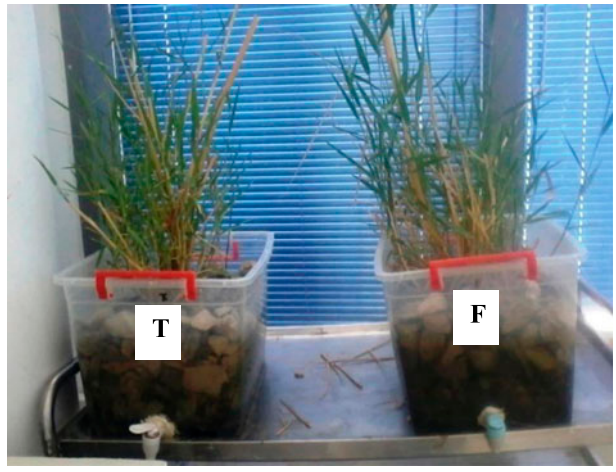

Fig. 2. Conception of the HSFCW pilot (*F*: with bacteria inoculation, *T*: without inoculation).

3. Results and discussion

3.1. Isolation and Screening of fluorescents Pseudomonas spp. strains

The 19 strains of fluorescent *Pseudomonas* spp. isolated and purified from different environmental sites are ranged in Table 2. The isolates were characterized by Gram staining, motility test, plating on selective medium. The bacterial colonies, cultivated on King B medium, are round, smooth, cream-white, with diameter from 1 to 2 mm, with a curved surface and a fluorescent pigmentation under UV (365 nm). The aromatic odour is fairly typical of all the isolates. All strains studied are oxydase$^+$ and catalase$^+$.

3.2. Denitrification test

The denitrification is a microbial process in which nitrogen oxides are utilized as an electron acceptor to

Table 2
Fluorescents *Pseudomonas* spp. isolated from different origins

Isolates	Origin	Sampling	Sites of sampling
PFS	Septic tank	Water	The TDC that treats sewage from the university home located at the agronomic institute in Tunis
PEV	Input of VSFCW		
PEH	Input of HSFCW		
PS	Output of HSFCW		
PFV_1	Plant foliage of the VSFCW1	*Phragmites australis* leaves	
PFH_1	Plant foliage of the HFCW1		
PFH_2	Plant foliage of the HSFCW2		
PFV_2	Plant foliage of the VSFCW2		
PRV	The rhizosphere of VSFCW	*Phragmites australis* roots	
PRH	The rhizosphere of HSFCW		
PREH	Rhizosphere input of HSFCW		
PRMH	Rhizosphere in the middle of HSFCW		
PRSV	Rhizosphere output of VSFCW		
PRSH	Rhizosphere output of HSFCW		
PRMV	Rhizosphere in the middle of VSFCW		
PR1	The rhizosphere 1 of EL-Oued		El-Oued next to the Technopark Borj Cedria
PR2	The rhizosphere 2 of EL-Oued		
PSol	Soil	Soil of EL-Oued	

produce energy in the absence of oxygen. In this reaction, the nitrates are reduced to nitrites [17]. Among the tested strains, 10 strains were revealed as denitrifying bacteria (PEV, PEH, PFS, PRV, PFH_1, PR1, PR_2, PRMH, PFH_2 and P_{Sol}). The strain isolated from the plant foliage of SHFCW (PFH_1) produces the most important quantity of nitrogen gas (N_2). This strain seems to be considered as the most denitrifying.

3.3. Monitoring of the nitrate assimilation

3.3.1. Monitoring of the nitrates assimilation ex situ

The bioassays were done *ex situ* and with two synthetic solutions Figs. 3 and 4 showed a kinetic of nitrate assimilation *ex situ* with initial nitrate concentration 9 and 90 mg/L, respectively. All data are averages of three experiments.

In order to study the denitrifying potential of inoculated bacteria, the Chick–Watson model was used with modification [18] as follows.

$$C/C_0 = A\ \exp(-K^n t) \quad (1)$$

with C_0 and C: initial nitrate concentration and at time t, respectively; t: time (h); K: denitrifying coefficient (h^{-1}); n: is the order of Chick–Watson model; A:

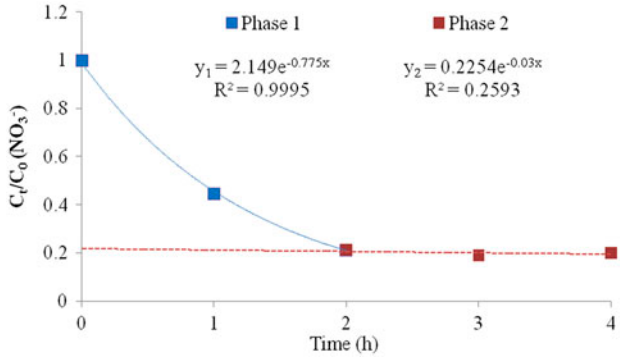

Fig. 3. Monitoring of the nitrate assimilation *ex situ* by fluorescent *Pseudomonas* spp. (PFH1) with an initial concentration of nitrate $[NO_3^-]_i$ equal to 9 mg/L.

denitrifying rate at the first contact with a determined concentration of nitrate.

Chick–Watson model was modified to consider the denitrifying rate at the first contact with a determined concentration of nitrate.

According to the kinetic Figs. 5 and 6, two kinetic stages of nitrate assimilation could be distinguished. The first stage during which the nitrate assimilation rate is important followed by a stage showing no nitrate assimilation (or very low) correlated with the

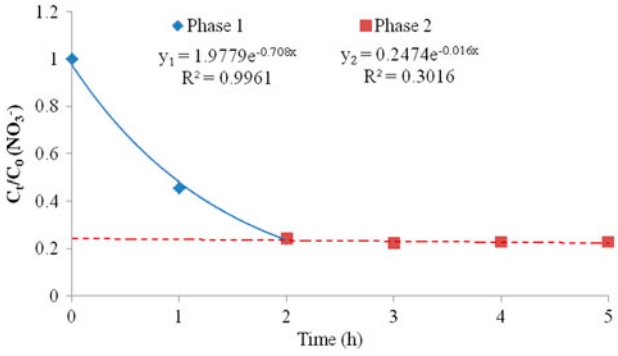

Fig. 4. Nitrate assimilation *ex situ* by fluorescent *Pseudomonas* spp. (PFH_1); initial concentration [NO_3^-] 90 mg/L.

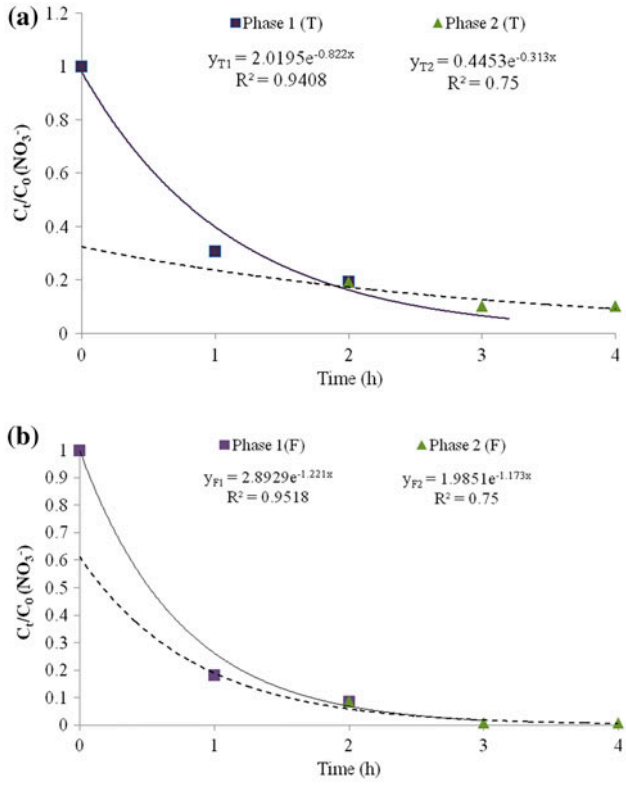

Fig. 5. (a) Monitoring of the nitrate assimilation *in situ* by fluorescent *Pseudomonas* spp. (PFH1) with an initial concentration of nitrate [NO_3^-]$_i$ equal to 12 mg/L in the tested CW. (b) Monitoring of the nitrate assimilation *in situ* by fluorescent *Pseudomonas* spp. (PFH1) with an initial concentration of nitrate [NO_3^-]$_i$ equal to 12 mg/L in the inoculated CW.

stagnancy of nitrate concentration in the medium. The first phase is characterized by a greater denitrification coefficient (K) than determined by the second denitrifying stage. During the first phase, the denitrifying

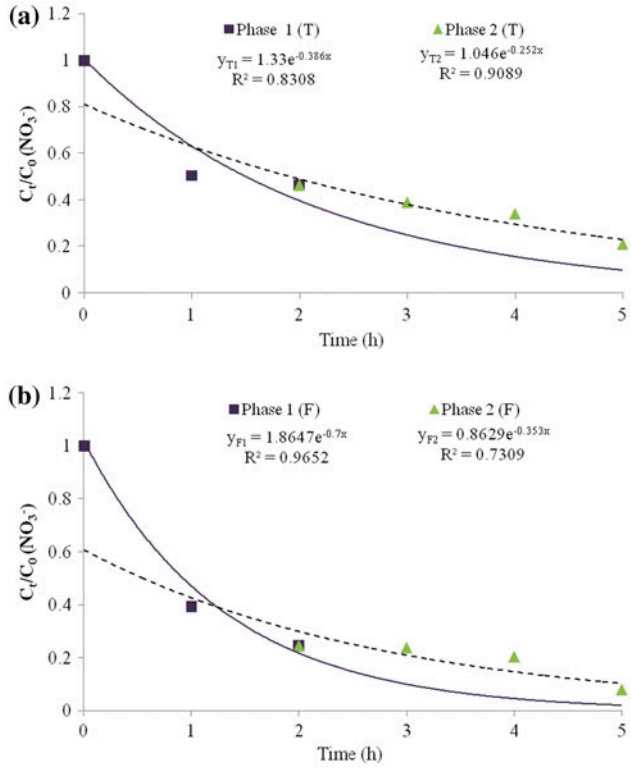

Fig. 6. (a) Monitoring of the nitrate assimilation *in situ* by fluorescent *Pseudomonas* spp. (PFH1) with an initial concentration of nitrate [NO_3^-]$_i$ equal to 90 mg/L in the tested CW. (b) Monitoring of the nitrate assimilation *in situ* by fluorescent *Pseudomonas* spp. (PFH1) with an initial concentration of nitrate [NO_3^-]$_i$ equal to 90 mg/L.

rate at the first contact with a determined for two used concentrations of nitrate, 9 and 90 mg/L, (A) is greater than determined during the second denitrifying kinetic stage. The constants (A) for the two nitrate concentrations (9 and 90 mg/L) during the first phase are high (1.708 and 1.688, respectively), while for the second phase, the coefficient loses more than 90% of his value. Consequently, the nitrate assimilation determined by the first denitrifying kinetic stage *ex situ* ranges between 86 and 89%, respectively, from 9 to 90 mg/L nitrate concentrations.

The difference between denitrifying rate determined in the first and second phase of nitrate assimilation represents the potential of inoculated bacteria (PFH_1) to enhance rhizocompetence in denitrification process.

$$\mu_d = A_1 - A_2 \quad (2)$$

Based on these results, we can consequently modify the kinetic model of the denitrification process for bioinoculated case as follows:

$$C/C_0 = A'\exp(-K'^n t) \quad (3)$$

with,

$$A' = A + \mu_d \quad (4)$$

$$K' = K^{\mu d} \quad (5)$$

3.4. *Monitoring of the nitrate assimilation* in situ

By analysing Figs. 6 and 7, we can note the same shape of the nitrate assimilation curve established in the case of the reduction of nitrates *ex situ*. Indeed, we distinguish two kinetic phases of nitrate assimilation: The first kinetic phase is characterized by a rapid denitrification rate. In the contrast of the first denitrifying kinetic stage, the second one is defined by a slow denitrification rate that tends to be constant over time. All data are averages of three experiments.

However, this studied case is different from the first one (bioinoculation of fluorescent *Pseudomonas* spp. *ex situ*). Indeed, the application of bioinoculation into the rhizosphere of *Phragmites australis* already colonized with an autochthon rhizobacterium is completely different from the monitoring of nitrate assimilation by single bacteria *in vitro*.

For this reason, we are adopting another mathematical model; series-event kinetic model (Severin et al. 1984) with modification to report the bioassimilation of nitrate in two filter pilots (*T* and *F*) The bacterial denitrification kinetic was assumed to be at a threshold level of assimilation, where each step is characterized by first-order kinetics (Chick–Watson Kinetic). Each denitrification level D_i has a kinetic constant K_i and n is the threshold level of bacterial denitrification.

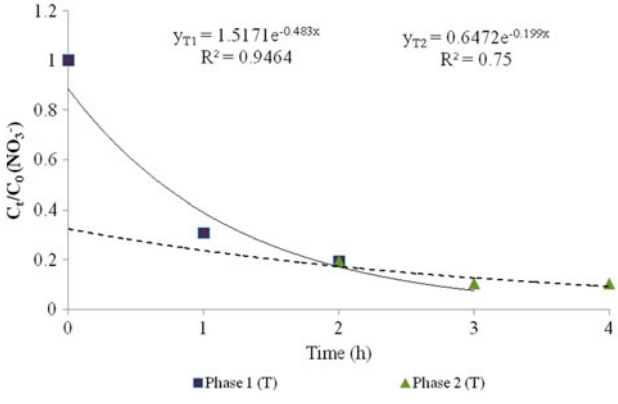

Fig. 7. Monitoring of the nitrate assimilation *in situ* by fluorescent *Pseudomonas* spp. (PFH1) with an initial concentration of nitrate $[NO_3^-]_i$ equal to 12 mg/L in control minifilter pilot (T) at the threshold level of bacterial denitrification; $n = 3$.

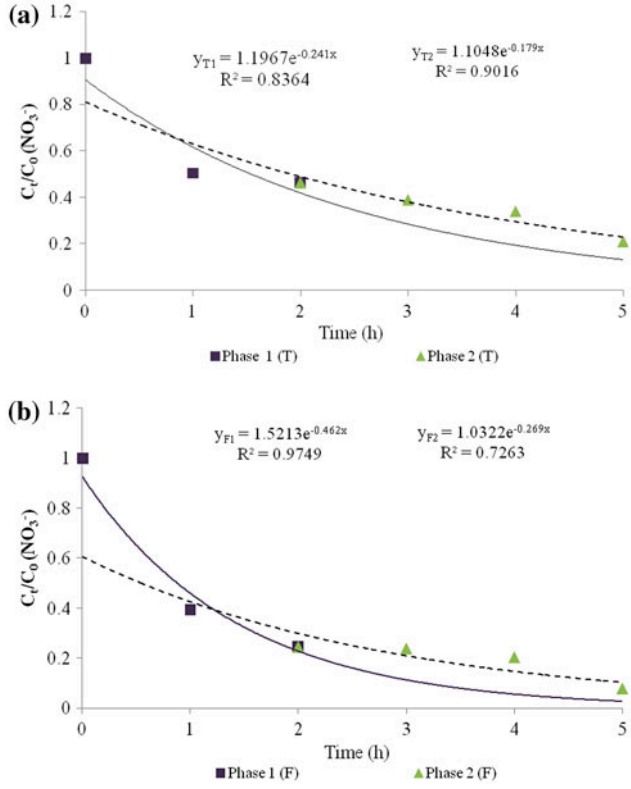

Fig. 8. (a) Monitoring of the nitrate assimilation *in situ* by fluorescent *Pseudomonas* spp. (PFH_1) with an initial concentration of nitrate $[NO_3^-]_i$ equal to 90 mg/L (T) at the threshold level of bacterial denitrification; $n = 2$ (tested CW). (b) Monitoring of the nitrate assimilation *in situ* by fluorescent *Pseudomonas* spp. (PFH_1) with an initial concentration of nitrate $[NO_3^-]_i$ equal to 90 mg/L (T) at the threshold level of bacterial denitrification; $n = 2$ (inoculated CW).

$$D_0 \xrightarrow{k_1} D_1 \xrightarrow{k_2} D_2 \xrightarrow{k_i} D_i \ldots D_{n-1} \xrightarrow{k_n} D_n \quad (6)$$

By assuming that the kinetic constant is the same at each level, the following generalized expression can be derived from the series-event model.

$$\frac{N}{N0} = A\exp(-K \times C^n t^m) + \ln\frac{(1 + \sum_{i=0}^{n-1} \times C^n t^m)^i}{i!} \quad (7)$$

Figs. 7 and 8 illustrate the ability of the series-event model to report bacterial denitrification behaviour into the rhizosphere. All data are averages of three experiments.

We can note no change in the kinetic shape, but the use of this model can report the series-events undergone by rhizobacterium supplemented or not with denitrified bacteria (PFH_1). For example, for an initial concentration of nitrate equal to 12 mg/L, underwent a series of nitrate bioassimilation D_3 in the control minifilter pilot (T) vs. n equal to 1 (D_1) in the bioinoculated filter (F). The inoculation by denitrifying bacteria in minifilter (F) enhances the nitrate assimilation rate in one event instead of three events, the control test (T) for the same $[NO_3]_I$ and incubation time.

According to used kinetic models, the reduction kinetics of nitrate is faster at filter (F) comparing to the control test at the filter (T). This result highlights the beneficial effect of bioinoculation with fluorescent *Pseudomonas* spp. (PFH_1) and improves the potentiality of the interest bacteria to enhance the rhizocompetence in the denitrification process by 47.54 and 21.36% for an initial nitrate concentration of 12 and 90 mg/L in inoculated filter (F) determined by the first kinetic stage.

The bioinoculation of denitrifying strain (PFH_1) increases remarkably the efficiency of the water treatment system for the reduction of nitrates *ex situ* and *in situ*.

4. Conclusion

The application of bioinoculation, even by an individual DPR, has a beneficial impact on denitrification process. Indeed, the preliminary results show the capacity of bioinoculated strain, fluorescent *Pseudomonas* spp. (PFH_1), to enhance rhizobacterium potential in HSFCW system planted by *Phragmites australis*.

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