Isolation and characterization of microbial communities from a constructed wetlands system: A case study in Tunisia

Nasr Houda¹, Cherif Hanene², Mehri Ines¹, Ben said Myriam¹* Daly Imen¹ and Hassen Abdennaceur¹

¹Centre des Recherches et Technologies des Eaux, Laboratoire Traitements des Eaux Usées, BP 273, 8020, Soliman, Tunisia.
²Laboratoire Microorganismes et Biomolécules Actives, Faculté des Sciences de Tunis, Campus Universitaire, 2092, Tunis, Tunisie.

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Constructed wetlands (CWs) have received increasing attention in the last decade due to their high potential for wastewater treatment. The nitrogen removal performance is always the main focus when evaluating the treatment ability of CWs. In this work, we aimed to isolate and identify the heterotrophic bacteria using the 16S rRNA gene sequencing method and to characterize the microbial communities removing nitrogen from wastewater and rhizosphere of constructed wetlands and quantified the heterotrophic nitrification on a microtiter plate by applying a spectrofluorometric assay. A number of 35 isolates within the obtained bacterial collection (380 isolates), were selected for molecular classification based on their genetic patterns after amplification of ITS fragments. Results obtained from the PCR amplification of the 16S-23S rDNA gene followed by the partial sequencing of the 16S rDNA genes confirmed the affiliation of the isolates to γ-Proteobacteria, Firmicutes, α-Proteobacteria, Actinobacteria and δ-Proteobacteria. Batch tests were carried out to investigate the capacity for heterotrophic nitrification in the pure culture. The batch test results indicate that nitrifying bacteria can utilize the organic carbon as the source of assimilation when it grows on glucose and ammonium chloride medium accompanying the formation of NO₂⁻ (nitrified products > 80%). Quantification of bacterial biofilms grown on microtiter plate showed that six strains were strongly adhesive to polystyrene microtiter plate with an OD₅₉₅ ranging between 3.031 and 4.0368 and only one strain was weak biofilm forming. These bacteria might be used to activate nitrifying bioreactor for a tertiary domestic wastewaters treatment.

Key words: Wastewater treatment, 16S-23S intergenic spacer, nitrifying heterotrophic bacteria, Biofilm.

INTRODUCTION

Biological process is widely used in wastewater treatment plants for its ease of implementation, high-efficiency, and cost benefit. Nitrogen removal, as an integral component of wastewater treatment in general involves nitrification process carried out by autotrophic nitrifiers under aerobic conditions and denitrification process by heterotrophic denitrifiers under anoxic conditions.

Constructed wetlands (CWs) have been proposed for the reduction of pollutants in wastewaters, (Kayranli et al., 2010; Konnerup et al., 2009; Villasenor et al., 2011). In general, this reduction is due to physical (the sedimentation processes, filtration, absorption/adsorption) chemical
Figure 1. Schematic representation of the constructed wetlands system.

(chemical precipitation) and biological processes (microbial interactions, biofilm formation, bacteria/plant interactions) (Vymazal, 2009).

The CWs are designed to take advantage of many wastewater treatment processes. This natural treatment process is classified according to the life form of the dominating macrophyte into systems with free-floating, rooted emergent and submerged macrophytes (Jan Vymazal, 2010). This technologies can be divided into surface and subsurface flow systems.

The Surface flow constructed wetlands (SFCW) are most similar to natural environments due to the permanent standing water and conditions favorable for wetland plant species (Scholz and Lee, 2005).

In recent years, plant roots and rhizomes are important for the microbial transformation processes and subsequently to wastewater purification process (Münch et al., 2007; Stottmeister et al., 2003). The removal of complex organics present in wastewater can be achieved partially by plant uptake, absorption on intersystem surface and by the physical, chemical and biological processes which might alter the chemical nature of the organic pollutants present in wastewater (Venkata et al., 2010).

Microbial assemblages can be found as a biofilm on substrate and root surfaces (Gagnon et al., 2007). Thus, in the rhizomes of oxygen-releasing plants, the availability of substrates (organic substrates and ammonia) for nitrifying and heterotrophic bacteria will also influence the interactions of these organisms under oxygen-limiting conditions. Heterotrophic bacteria may either be suspended in water or attached on sediment, plants and litters. Attached growth is thought to play a major role in improving the water quality of CWs (Brix, 1994).

The constructed wetlands have been introduced as a cost-efficient alternative to conventional technologies for treatment of surface and groundwater polluted with organics chemicals (Braeckeveldt et al., 2007; Matamoros et al., 2007; Schroder et al., 2007).

We propose in this study (i) to isolate heterotrophic nitrifying bacteria from constructed wetlands (CWs); (ii) to characterize the isolates bacteria by molecular analysis tools mainly, ribosomal intergenic spacer analysis and 16S rDNA sequencing; (iii) to examine the ability of the isolated bacteria to remove ammonium from the medium containing high concentration ammonium and finally, (iv) to evaluate the potential of heterotrophic nitrifying bacteria to produce biofilms.

MATERIALS AND METHODS

Constructed wetlands site description

The constructed wetland system is located at a domestic wastewater treatment plant in Tunisia. The site of the CW system is located at latitude 36°15'29.34"N, longitude 9°56'55.44"E in a small rural community. It serves a population of almost 1000 inhabitants. The climatic type of the zone is semi-arid, characterized by annual mean precipitation of 470 ± 160 mm and an annual mean air temperature which varies from 10.4 to 27.8°C. The influent enters the treatment plant under a batch flow regime and with a mean hydraulic rate of approximately 24 m3/d. The influent wastewater is distributed evenly to a vertical water flow basin (Surface area 121 m²) and finally to a horizontal flow basin (Surface area 207 m²). Two types of emergent macrophytes, reeds and cattails, were planted on the wetlands at a density of 4 plants/m². The series were planted with Typha latifolia and Phragmites australis in expanded clay aggregates (Figure 1).

Enumeration of nitrifying strains (MPN)

The numbers of autotrophic ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB) were determined by the most probable number (MPN) method used (Schmidt and Belser, 1982) from the plant roots and Enter Station (ES), Substrate Vertical Basin (SVB), Substrate Horizontal Basin (SBH). Briefly, two sets
of three subsamples were pooled to form one composite sample (10 g) of plant roots and substrate (from a depth between 10 and 15 cm) of each CWs Typha Vertical Basin (TVB), Phragmites australis Vertical Basin (RVB), Typha Horizontal Basin (THB), Phragmites australis Horizontal Basin (RHB) placed separately in sterile tubes with 10 ml of 10 mM phosphate buffer solution (PBS) and vortexed for 1 min at room temperature. The compositions of ammonia-oxidizing bacteria (AOB) medium was: 0.04 g/L MgSO\(_4\cdot7\)H\(_2\)O, 0.5 g/L (NH\(_4\))\(_2\)SO\(_4\), 0.2 g/L KH\(_2\)PO\(_4\), 0.04 g/L CaCl\(_2\) 2H\(_2\)O, 0.0005 g/L Chelated iron, 0.0005 g/L Phenol red in 1 L of distilled water. The compositions of Nitrite-oxidizing bacteria (NOB) medium was: 0.30 g/L KNO\(_2\), 0.1875 g/L MgSO\(_4\cdot7\)H\(_2\)O, 1.5 g/L KHC\(_2\)O, 0.5 g/L K\(_2\)HPO\(_4\), 0.5 g/L KH\(_2\)PO\(_4\), 0.1875 g/L NaCl, 0.0125 g/L CaCl\(_2\cdot2\)H\(_2\)O, 0.01 g/L FeSO\(_4\cdot7\)H\(_2\)O in 1 L of distilled water (Nancy et al., 2001). Spot tests for total oxidized-N (nitrite and nitrate) were carried out on approximately 2 mL medium using the Griess-llosvay method (Keeney and Nelson, 1982) every week.

**Isolation of heterotrophic nitrifying bacteria**

Different types of bacterial colonies were obtained after spreading 0.5 mL of water or rhizosphere suspensions (Rhizosphere samples were collected from each wetland at the entrance, middle, and exit at a depth of approximately 30 cm under the gravel surface). These roots were separated from the rhizomes and cut into 1 cm fragments after hand-shaking in order to separate off sludge particles not adhering tightly to the roots. The obtained fragments were introduced into a phosphate buffer solution (pH 7; 1:10, W/V) and then mechanically shaken at a speed of 350 r/min during 2 h at room temperature (20°C) to release the roots’ adsorbed biomass) on trypticase-soy-agar (TSA) plates and incubated at 25°C for 24–48 h. This organic medium was used without adding autotrophic nitrification inhibitor N-serve (2-chloro-6-trichloromethyl pyridine) (Goring, 1962).

**DNA extraction and PCR amplification of 16S–23S rDNA genes**

Freshly grown single colony from each strain was transferred to 1 ml of standard Luria Broth (LB) medium (difico, Detroit, MI) and incubated overnight in shake culture at 25°C. Bacterial DNA was extracted according to the protocols developed by Chen and Kuo (1993) for Gram-negative bacteria and by Sohail (1998) for Gram-positive bacteria. The ribosomal intergenic spacer analysis was carried out using nucleotide single universal strand primers S (5'-AGAGTTTGTATCCTGGCTACG-3') and L (5'-CTAGGCTACTTGGTTGACGC-3') (Alm et al., 1996). PCR was performed in a PCR thermocycler (UNOII Biometra) with an initial denaturation of DNA template at 94°C for 4 min, followed by 40 cycles at 94°C for 30 s, 45°C for 30 s, and 72°C for 45 s, followed by a final extension performed at 72°C for 7 min. The PCR products were separated on 1.5% agarose gel. The 1.5 kb rDNA PCR products were purified and partially sequenced. Partial 16S rDNA sequences of the isolates were compared with 16S rDNA sequences available by the BLAST search (Altschul et al., 1990) in the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov).

**Pre-selection of heterotrophic strains**

Bacterial colonies were sub-cultured in trypticase-soy-broth (TSB) at 25°C for 24–48 h with shaking at 250 rpm/min. Then, a volume of 0.5 ml of each bacterial culture was inoculated into Erlenmeyer flasks containing 50 ml of a heterotrophic medium composed by the Yan et al. (2007) mineral medium supplemented with acetic acid according to the following composition: 0.5 g/L (NH\(_4\))\(_2\)SO\(_4\), 10 g/L NaNO\(_3\), 0.04 g/L MgSO\(_4\cdot7\)H\(_2\)O, 0.203 g/L KH\(_2\)PO\(_4\), 0.013 g/L CaCl\(_2\cdot2H_2O\), 0.01 g/L iron chelate, 0.01 g/L trace elements 0.01 and 1 g/L acetic acid. Acetic acid was added as an organic carbon source. The pH of the culture medium was adjusted to 7.2 using 1 mol/L NaOH. This heterotrophic medium reproduces the characteristics of the effluent of the CWs plant, which is overloaded with ammonia-nitrogen and contains residual organic carbon. Incubation was carried out at 25°C during 30 days in the dark with mechanical agitation at 200 rpm. This step of pre-culture involves the adaptation of previously heterotrophically isolated bacteria to the heterotrophic medium.

**Nitrification activity**

The bacterial cells were recovered by 4000 rpm/min centrifugation for 10 min and then transferred back into the same heterotrophic medium under the same culture conditions mentioned in nitrification activity. This medium, containing 51.3 mg/L of ammonium as the sole nitrogen source, was used for detection of the bacterial capacity to remove ammonium under aerobic conditions. Subsequently, aliquots from each culture were used for a residual ammonium assay. For this, 2 mL of Seignette’s salt solution (potassium sodium tartrate) and 2 mL of Nessler reagent were incorporated with 1 ml of bacterial culture. The standard solution was prepared from a concentrated NH₄Cl solution (10 mmol/L). Ammonium rates were assessed spectrophotometrically at a wavelength of 420 nm (Jibin et al., 2011).

At the end of incubation period, aliquots from each culture were used for determination of biomass total nitrogen, ammonium and nitrite concentrations. Biomass total nitrogen measurement was accomplished using the Kjeldahl method modified by Hiller et al. (1948) and expressed as μg/g dry weight (dw). The dw was evaluated applying the method described by Wade (1952). Ammonium content was quantified as described above. Produced nitrite was assessed using the diazotization method. For this, 0.1 ml of the diazotization reagent (sulfanilic acid: 0.26 g; N1-Naphthyl-ethylene-diamine dihydrochloride: 0.6 g) was incorporated with 5 mL of bacterial culture. The standard solution was prepared from a concentrated NaNO₂ solution (10 mmol/L). Nitrite concentrations were assessed spectrophotometrically at 543 nm. Then, ammonium removal efficiency (ARE, %) was calculated by the following equation:

\[
\text{ARE} = \left(\frac{[\text{C}_0 - \text{C}_a]}{[\text{C}_0]}\right) \times 100\% \quad \text{Where, C}_0 \text{ (mg/L) and C}_a \text{ (mg/L) are the initial and the final ammonium concentrations, respectively.}
\]

**Quantitative estimation of biofilm formation by isolated bacterial strains**

The ability of isolates to form a biofilm on abiotic surface was quantified using the protocol described by Toledo-Arana et al. (2001).
All strains were grown overnight in Brain Heart Infusion Broth (12.5 g/L Brain infusion solids, 5 g/L Beef heart infusion solids, 10 g/L Pepto-complex, 2 g/L Glucose, 5 g/L Sodium chloride) at 37°C. The culture was diluted to 1:20 in fresh BHI plus (0.25%) glucose. A volume of 200 μl of tested suspension was used to inoculate sterile 96-well polystyrene microtiter plates (Nunc, Roskilde, Denmark). The plates were incubated at 37°C for 24 h. The cultures were removed and the microtiter wells were washed twice with phosphate-saline buffer (PBS) (7 mM NaH₂PO₄, 3 mM NaH₂PO₃ and 130 mM NaCl at pH 7.4) to remove non-adherent cells and were dried in an inverted position. Adherent bacteria were stained with 1% Crystal violet (Merck, France) for 15 min. The wells were rinsed once more and the Crystal violet was solubilised in 200 μl of ethanol-acetone (80:20 v/v). The optical density (OD595nm) was measured in spectrophotometer (Amadéo Bibby, Sterlin France). The following values were assigned for biofilm determination: (-) non biofilm forming OD595 ≤1; (+) weak biofilm forming 1<OD595 ≤2; (++) medium biofilm forming 2<OD595 ≤3; (+++) strong biofilm forming OD595 ≥3. Each essay was performed in triplicate (Figure 4).

**Statistical procedures**

Student-Newman-Keuls was used to show significant difference between all the parameters data using Statistical Package for the Social Sciences (SPSS), version 10, software (SPSS for Windows, SPSS Inc. Chicago, Il, USA).

**RESULTS**

**Enumeration of nitrifying strains (MPN)**

The enumeration of nitrifying bacteria was determined up to the 10⁹ of serial dilution for both ammonia and nitrite oxidation. The distribution of nitrifying bacteria in the macrophytes and wastewater of the constructed wetland is depicted in Figure 2. The differences in number of ammonia oxidizing bacteria followed by the macrophyte treatments were significant (p=0.05 for all variables): RHB> THB> RVB> TVB (Figures 2A). Furthermore, a comparison of number of nitrite oxidizing bacteria followed the content of *phragmite* and *Typha* of horizontal and vertical basin, respectively (Figures 2B); significant differences were not detected.

Though the rates of the number of ammonia oxidizing bacteria were higher in ES, SBH, SVB respectively, one-way anova did not detect significant differences between the number of constructed wetland (Figure 2C) but the number of nitrite oxidizing bacteria present in the wastewater at the inlet and outlet of the CWs was significantly different: p=0.05 (ES) > (SVB) > (SBH) in ((Figure 2D).

**16S-23S rDNA intergenic spacer analysis**

Thirty-five bacterial isolates were selected for molecular classification based on their genetic patterns after amplification of ITS fragments. The amplification of the intergenic region of isolates with conservative primers from the 16S and 23S rDNA gene region yielded PCR products ranging between 250 to 800 bp in size (Figure 3). Intergenic transcribed spacers (ITS) 16S - 23S rDNA genes were selected as a first discriminating tool to classify the isolates into putative different taxonomic units. The comparisons between the different profiles revealed several differences in band positions and number. Different ITS haplotypes were obtained and isolated strains were accordingly differentiated into 10 groups (Figure 3). Bacteria was classified into 10 different phylogenetic groups. We noted also that the 10 isolates were each classified in a different group: isolates 42C, 37A and 25D formed the groups G1, G4 and G8, respectively; G76G, 70C, 67D, 70D, 115B, 42A and 37H belonged to G2, G3, G5, G6, G7, G9 and G10, respectively. Since they represent each group, the 10 bacterial isolates were selected for assessment of their heterotrophic growth and ammonia oxidation ability and then for sequencing.

**Sequencing results and phylogenetic analysis**

The application of molecular techniques, in particular analysis of 16S rRNA genes, provides new opportunities for the assessment of ammonia-oxidizing populations in natural environments. Genotyping of the dominant bacterial was done by 16S, and isolates displaying distinct ITS profiles were characterized through the determination of the nucleotide sequence of the 16S rRNA encoding gene. According to BLAST results (Table 1), four strains were affiliated to α-Proteobacteria (67D), to β-Proteobacteria (42C), to γ-Proteobacteria (25D), *Actinobacteria* (70D, 42A, 37H) and to *Firmicutes* (37A, 70C, 76G, 115B). These strains were isolated from plant roots (TVB, RVB, THB and RHB), and (SVB and SHB).

**Nitrifying activity**

10 bacterial strains which belong to different group were selected based on their capacity to remove ammonia-nitrogen in the presence of acetate (Table 3).

Table 2 presents the nitrification performance within the isolated nitrifying bacteria strains. The results show a difference of nitrifying activity between tested bacterial strains due to a variation in bacterial growth rates. Meanwhile, the monitoring of OD₆₆₀ of isolates 37A, 115B and 76G showed close shapes over the time. As well as for 42A, 70D and 37H bacterial strains at the end of incubation period, we were detected important concentrations of nitrite, versus low residual ammonium contents.

**Quantitative estimation of biofilm production by isolated strains**

The experiments performed in our study enabled us to measure the rate of adherence and subsequent biofilm formation of the tested bacteria. Summarised results of the microtiter-plate tests are presented in Figure 4. Six strains were strongly adhesive to polystyrene microtitre plate *Arthrobacter* sp. (70D and 42A) *Ochrobactrum* anthropi (67D) *Arthrobacter aurescens* (37H) *Bordetella petri* (42C) and *Pseudomonas putida* (25D). Only one strain was weak biofilm, *Exiguobacterium sibiricum* (70C) which formed...
Figure 2. Average spatial distribution of nitrifying bacteria in Enter Station, Substrate Vertical Basin, Substrate Horizontal Basin and on roots of *Typha latifolia* and *Phragmites australis* in a pilot constructed wetland.

Table 1. Phylogenetic affiliation of bacterial strains isolated from *Thypha* and *Phragmites* root (R) and substrate (S).

<table>
<thead>
<tr>
<th>Strain number</th>
<th>Accession number</th>
<th>Most related organisms</th>
<th>Phylogenetic group</th>
<th>Sequence similarity (%)</th>
<th>Sequence bp</th>
<th>Samples assigned origins</th>
</tr>
</thead>
<tbody>
<tr>
<td>42A</td>
<td>NC_008541.1</td>
<td><em>Arthrobacter</em> sp.</td>
<td>Actinobacteria, <em>Arthrobacter</em></td>
<td>97%</td>
<td>703</td>
<td>RHB</td>
</tr>
<tr>
<td>25D</td>
<td>NC_010322.1</td>
<td><em>Pseudomonas putida</em></td>
<td>Proteobacteria; Pseudomonas</td>
<td>97%</td>
<td>471</td>
<td>SVB</td>
</tr>
<tr>
<td>37A</td>
<td>NC_009848.1</td>
<td><em>Bacillus pumilus</em></td>
<td>Firmicutes, <em>Bacillus</em></td>
<td>100%</td>
<td>711</td>
<td>THB</td>
</tr>
<tr>
<td>37H</td>
<td>NC_008711.1</td>
<td><em>Arthrobacter aurescens</em></td>
<td>Actinobacteria, <em>Arthrobacter</em></td>
<td>88%</td>
<td>689</td>
<td>THB</td>
</tr>
<tr>
<td>42C</td>
<td>NC_010170.1</td>
<td><em>Bordetella petrii</em></td>
<td>Proteobacteria; Bordetella</td>
<td>81%</td>
<td>517</td>
<td>RHB</td>
</tr>
<tr>
<td>67D</td>
<td>NC_009668.1</td>
<td><em>Exiguobacterium</em></td>
<td>Firmicutes, <em>Bacillus</em></td>
<td>99%</td>
<td>611</td>
<td>TVB</td>
</tr>
<tr>
<td>70C</td>
<td>NC_010556.1</td>
<td><em>Arthrobacter</em> sp.</td>
<td>Actinobacteria <em>Arthrobacter</em></td>
<td>96%</td>
<td>742</td>
<td>TVB</td>
</tr>
<tr>
<td>76G</td>
<td>NC_004604.1</td>
<td><em>Bacillus megaterium</em></td>
<td>Firmicutes, <em>Bacillus</em></td>
<td>87%</td>
<td>683</td>
<td>SHB</td>
</tr>
<tr>
<td>115B</td>
<td>NC_009848.1</td>
<td><em>Bacillus pumilus</em></td>
<td>Firmicutes, <em>Bacillus</em></td>
<td>98%</td>
<td>597</td>
<td>RVB</td>
</tr>
</tbody>
</table>

Table 2. Nitrification performance within the isolated nitrifying bacteria strains.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>OD 660 nm</th>
<th>5 days</th>
<th>15 days</th>
<th>25 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>37A</td>
<td>0.16 ± 0.02</td>
<td>1.4 ± 0.33</td>
<td>2.10 ± 0.18</td>
<td></td>
</tr>
<tr>
<td>115B</td>
<td>0.21 ± 0.07</td>
<td>1.26 ± 0.12</td>
<td>1.93 ± 0.09</td>
<td></td>
</tr>
<tr>
<td>76G</td>
<td>0.18 ± 0.04</td>
<td>1.12 ± 0.20</td>
<td>2.16 ± 0.22</td>
<td></td>
</tr>
<tr>
<td>42A</td>
<td>0.05 ± 0.01</td>
<td>0.15 ± 0.04</td>
<td>0.23 ± 0.07</td>
<td></td>
</tr>
<tr>
<td>70D</td>
<td>0.06 ± 0.00</td>
<td>0.16 ± 0.02</td>
<td>0.24 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>37H</td>
<td>0.06 ± 0.02</td>
<td>0.13 ± 0.01</td>
<td>0.22 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>67D</td>
<td>0.41 ± 0.11</td>
<td>1.36 ± 0.22</td>
<td>2.09 ± 0.10</td>
<td></td>
</tr>
<tr>
<td>70C</td>
<td>0.12 ± 0.05</td>
<td>0.17 ± 0.06</td>
<td>0.19 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>25D</td>
<td>1.15 ± 0.2</td>
<td>1.89 ± 0.19</td>
<td>1.93 ± 0.83</td>
<td></td>
</tr>
<tr>
<td>42C</td>
<td>0.48 ± 0.13</td>
<td>2.51 ± 0.23</td>
<td>3.60 ± 0.52</td>
<td></td>
</tr>
</tbody>
</table>

Table 3. Nitrogen balance of ammonium removal in shaking culture experiment by bacteria.

<table>
<thead>
<tr>
<th>Run number</th>
<th>NH4+-N Concentration</th>
<th>Nitrification Production</th>
<th>Intracellular N (μg/g dw)</th>
<th>Nitrified products (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial NH4+-N</td>
<td>Final NH4+-N</td>
<td>NO2 --N (mg/L)</td>
<td></td>
</tr>
<tr>
<td>37A</td>
<td>4.2±0.3</td>
<td>0 (25d)</td>
<td>42.2±0.6</td>
<td>4.9±0.4</td>
</tr>
<tr>
<td>115B</td>
<td>4.8±0.5</td>
<td>0 (25d)</td>
<td>42.8±0.2</td>
<td>3.7±0.2</td>
</tr>
<tr>
<td>76G</td>
<td>4.1±0.2</td>
<td>0 (25d)</td>
<td>42.4±0.4</td>
<td>4.8±0.3</td>
</tr>
<tr>
<td>42A</td>
<td>1.5±0.4</td>
<td>0 (25d)</td>
<td>41.7±0.7</td>
<td>8.1±0.2</td>
</tr>
<tr>
<td>70D</td>
<td>2.9±0.3</td>
<td>0 (25d)</td>
<td>41.3±0.4</td>
<td>7.1±0.2</td>
</tr>
<tr>
<td>37H</td>
<td>2.1±0.2</td>
<td>0 (25d)</td>
<td>40.4±0.5</td>
<td>8.8±0.2</td>
</tr>
<tr>
<td>67D</td>
<td>1.9±0.6</td>
<td>0 (25d)</td>
<td>44.4±0.3</td>
<td>5.0±0.4</td>
</tr>
<tr>
<td>70C</td>
<td>2.8±0.5</td>
<td>0 (25d)</td>
<td>44.2±0.2</td>
<td>4.3±0.1</td>
</tr>
<tr>
<td>25D</td>
<td>1.7±0.4</td>
<td>0 (25d)</td>
<td>42.8±0.3</td>
<td>6.8±0.4</td>
</tr>
<tr>
<td>42C</td>
<td>1.2±0.1</td>
<td>0 (25d)</td>
<td>41.0±0.7</td>
<td>9.1±0.3</td>
</tr>
</tbody>
</table>

OD660: optical density at 660 nm.

with an OD595 ≤ 2 of the three strains able to adhere moderate to polystyrene surfaces.

DISCUSSION

In this study, MPN has been applied in order to evaluate the abundance of some functional groups such as ammonia-nitrifying and nitrifying bacteria in a constructed wetland. These results show that the numbers of autotrophic bacteria determined in phragmites were superior to the numbers of this category of bacteria determined in Typha.

Bahlo and Wach (1993) were found more intensive to biological degradation of ammonium to nitrate close to the rhizomes. Thereby, treatment systems can be sub-classified by the flow direction of effluent (vertical or horizontal), the plant species or type of soil.

P. australis plant was established the most successfully in the CWs; Phragmites is known to be the most frequently used plant worldwide in subsurface flow constructed wetlands (Kadlec et al., 2000). Plant tissue provides a large amount of surface areas for microbial growth. Their arguments, confirms our results demonstrating that the number of fixed nitrifying bacteria on phragmites was superior to that of the number determined at the outlet basin. Therefore, we can explain these results by the attachment of bacteria to phragmites. This attachment of microorganisms to submersed solid surfaces, such as sediment and macrophytes, leads to the formation of biofilms (Peterson and Teal, 1996).

Different surfaces can affect the presence of nitrifiers and denitrifiers by being more or less suitable as attachment surfaces. Surfaces in the water phase, where oxygen and ammonium concentrations are high would favour nitrification. But the number of Nitrobacter at vertical basin is greater than that of horizontal basin.

The treatment of wetlands is generally considered to be a highly aerobic system, since wastewater drains vertically through the planted matrix, allowing for unsaturated conditions and excellent oxygen transfer (IWA, 2000). We found no information on direct redox measurements in the literature, but circumstantial evidence strongly suggests that vertical flow wetlands have high redox potentials that favor aerobic microbial processes. For example, BOD removal and nitrification were significantly higher in
vertical flow compared to surface and subsurface flow wetlands, but denitrification was low (Cooper et al., 1996; Vymazal, 2007; Li et al., 2008). Furthermore, direct microbial measurement has shown that the microbial density and activity were maximized in the first 5–10 cm of the vertical flow filter (Ragusa et al., 2004; Tietz et al., 2007b). This distribution of microorganisms is presumed to be caused by the high nutrient content and the ample oxygen supply in the upper zone of the vertical flow wetlands (Tietz et al., 2007b).

Then, a collection of the heterotrophic bacterial isolates retrieved from the roots and substrate samples of the CWs were closely related to environmental isolates reported from sources such as wastewater and roots. Heterotrophic ammonia oxidation test aimed to simulate the nutritional conditions of the constructed wetlands wastewater, containing inorganic nitrogen (ammonium) and organic carbon. These bacterial strains were characterized by various ammonium removal efficiency (ARE) and diverse haplotypes belonging to different phylotype groups. Among these bacteria, 10 isolates were able to achieve the highest ARE exceeding 80% in the heterotrophic culture medium. To better understand the differences in bacterial diversity among samples, we excised discriminable bands and sequenced these to determine the strain associated with each group, its phylogeny classification and nucleotide sequence similarity as well as its relative abundance under different operating conditions.

Based on the presence of their 16S rRNA genes, the heterotrophic nitrifying bacterial community was affiliated with \( \gamma \)-Proteobacteria, Firmicutes, \( \alpha \)-Proteobacteria, Sphingobacteria, Actinobacteria and Bacteroidetes. Another bacterial isolate from the roots of Typha was identified as \( E. \) sibiricum. This is in accordance with the results obtained by Sawayama (2006), who showed that some species of the genus \( Eixguobacterium \) are involved in the ammonium oxidation process. Recently, \( E. \) sibiricum was also isolated from the rhizosphere of plants (Lioussanne et al., 2010). The nitrification ability of \( Arthrobacter \) sp. has been observed amongst isolates from water (Witzel and Overbeck, 1979). Xia (2008) reported that \( O. \) anthrop was found in a pilot-scale bioreactor, to be responsible for the removal of nitrogen compounds. In addition, the pure cultures of bacteria capable of heterotrophic nitrification have been documented as \( Pseudomonas \) putida (Daum et al., 1998). In Korea, It has been known as an advanced wastewater treatment system in which Bacillus strains have predominated. It has been reported that the process is able to remove nitrogen and phosphorus as well as organic matter efficiently (Choi et al., 2000). Bacillus strains have been known to be involved in heterotrophic nitrification (Mevel and Prieur, 2000).

In addition, the analyses of microtiter plate systems for quantifying of biofilm formation have been investigated using different bacterial strains stains. A particular focus has been on the use of the stain crystal violet as an indicator of total attached biomass. Detailed knowledge about the microbial assemblages and interaction is needed to understand and explain the CWs functioning and thus the phytoremediation processes (Christensen et al., 1985; Stepanovic et al., 2000; Deighton and Balkau, 1990; Miyake et al., 1992). In the studied systems, nitrifying bacteria may consume inorganic carbon not only to form cell mass but also to excrete organic carbon (soluble microbial products, SMP), which will support heterotrophic bacterial growth (Rittmann et al., 2002). Many factors contribute to biofilm formation and attachment in \( Pseudomonas \), including exopolysaccharides, nucleic acids, motility functions, dispersal factors and surfactants (Toutain et al., 2004). This high diversity may be attributed to the potential for nitrification, denitrification, nitrate reduction, nitrite reduction, ammonia assimilation, and carbon oxidation processes to occur simultaneously in the system.

**Conclusions**

In this study, the microbial dynamics of root and substrate from two-stage series of CWs was studied. The application of molecular tools, namely 16S allowed deepening
the research in this field since very few studies were undertaken with the specificity of this type of domestic application. The majority of these isolates belonged to the rhizospheres of reeds, likely indicating their capacity to form protective biofilms. This work needs to be continued by studying the nitrification activity and biofilm-forming capacity of these isolates to understand their contribution to the treatment process in constructed wetland systems.

REFERENCES


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