**ABSTRACT**

Flooding impacts soil microbial communities, but its effect on endophytic communities has rarely been explored. This work addresses the effect of flooding on the abundance and diversity of endophytic diazotrophic communities on rice plants established in a greenhouse experiment. The \(nifH\) gene was significantly more abundant in roots after flooding, whereas the \(nifH\) gene copy numbers in leaves were unaffected and remained low. The PCA (principal component analysis) of T-RFLP (terminal restriction fragment length polymorphism) profiles indicated that root communities of replicate plots were more similar and diverse after flooding than before flooding. The \(nifH\) libraries obtained by cloning and 454 pyrosequencing consistently showed a remarkable shift in the diazotrophic community composition after flooding. **Gammaproteobacteria** (66–98%), mainly of the genus *Stenotrophomonas*, prevailed in roots before flooding, whereas **Betaproteobacteria** was the dominant class (26–34%) after flooding. A wide variety of aerotolerant and anaerobic diazotrophic bacteria (e.g. *Dechloromonas*, *Rhodopseudomonas*, *Desulfovibrio*, *Geobacter*, *Chlorobium*, *Spirochaeta*, *Selenomonas* and *Dehalobacter*) with diverse metabolic traits were retrieved from flooded rice roots. These findings suggest that endophytic communities could be significantly impacted by changes in plant–soil conditions derived from flooding during rice cropping.

**Keywords:** nitrogen fixation; rice tissues; waterlogged condition; high-throughput sequencing

**INTRODUCTION**

Nitrogen fixation is a well-known process (molecular nitrogen reduction to ammonia) that has been extensively studied based on its major role in the sustainability of agricultural systems. Diazotrophic prokaryotes belong to diverse physiological groups of the domains **Bacteria** and **Archaea** (Martínez Romero 2006).

Despite the agricultural and biotechnological relevance of the nitrogen fixation process, the diversity of nitrogen-fixing bacteria has been poorly described, and the physiology and phylogeny of many microorganisms remain unknown according to analyses of a database of \(nifH\) sequences (Gaby and Buckley 2011). Five major clusters have been defined according to a \(nifH\) phylogenetic analysis. Cluster I contains most functional and known \(nifH\) sequences encoding conventional bacterial FeMo nitrogenases. This cluster is dominated by **Proteobacteria**, **Cyanobacteria**, **Firmicutes** and **Actinobacteria** (Zehr et al. 2003). Cluster II, which is smaller than Cluster I, contains sequences from alternative FeV and FeFe nitrogenases and from some **Archaea**. Cluster III mainly...
consists of nifH sequences from anaerobes (Archaea and Bacteria), which are dominated by spirochaetes, sulphate-reducing bacteria, green sulphur bacteria, clostridia, methanogens and acetogens (Zehr et al. 2003). Clusters IV and V consist of nifH paralogues, which apparently are not involved in nitrogen fixation (Young 2005).

Biological nitrogen fixation is an oxygen-sensitive process. The root environment of submerged plants may be particularly suitable for this process due to the formation of aerobic microaerobes in anoxic soil, which could lead to high nitrogen fixation rates in aerobic and anaerobic diazotrophs (Hurek and Reinhold-Hurek 2005). This activity is facilitated by the presence of the aerenchyma, a specialized plant tissue that allows a flooded plant to exchange gas between the roots and atmosphere (Bodelier 2003). Additionally, flooding causes a series of reductive changes in the root environment because of the rapid development of anaerobic conditions (Yoshida 1981).

Flooding has been demonstrated to impact nitrogenase activity in tropical rice soils. Specifically, the diazotrophic activity significantly increased when rice soils were flooded (Das, Bhattacharyya and Adhya 2011). However, the effect of flooding on bacterial communities inhabiting inner plant tissues has rarely been addressed. Under reduced soil microbial complexity conditions (gamma-irradiated soils with artificial community inoculated), Hardoim et al. (2012) found that endophytic communities in the roots and shoots of greenhouse rice plants significantly differed when plants were cultivated for 2 weeks under dissimilar water treatments (flooded and unflooded). Although these authors showed that rice seeds are sources of endophytic bacteria that play an important role during the early rice growth stages, soil is well known to be the major source of bacterial endophytes (Hallmann et al. 1997), and their contribution to the endophytic community composition throughout the rice growth cycle could lead to a different bacterial community composition.

Endophytic nitrogen-fixing bacteria have been extensively studied in rice (Oryza sativa) roots. Most of these studies focused on bacteria associated with rice roots and did not exclusively examine endophytes. The dominant reported genera belonged mostly to the Proteobacteria class. A betaproteobacterial prevalence (50% of the clone library) from the roots of modern rice cultivars was reported by Wu, Ma and Lu (2009), who indicated that nifH sequences were closely related to those of Azorhizus spp. However, other interesting community members were also identified, such as methyloptrophs and methane oxidizers, which may also play an important role in the fixation of nitrogen in rice roots. Ueda et al. (1995) reported that the sulphate-reducing deltaproteobacteria Desulfovibrio gigas was the dominant diazotroph associated with rice roots. Furthermore, sequences of nifH belonging to Alphaproteobacteria (Bradyrhizobium sp. and Methylocystis parvus) were abundant. An nifH microarray analysis revealed that Betaproteobacteria and Gram-positive bacteria were present in wild rice roots (Zhang, Hurek and Reinhold-Hurek 2007), although nifH genes from the latter were mainly expressed. Conversely, the active diazotrophic bacteria in flooded rice were dominated by Deltaproteobacteria, with approximately 70% of the nifH transcripts phylogenetically distant from any sequence reported in databases. Nevertheless, these transcripts clustered with those of Geobacter sulfurreducens (Elbeltagy and Ando 2008).

Thus, considering the importance of nitrogen fixation for agriculture and the diversity of diazotrophic bacteria, this work explores the effect of flooding on the abundance and diversity of rice diazotrophic endophytes in plants growing under greenhouse conditions by using nifH as a functional gene marker.

MATERIALS AND METHODS

Greenhouse experiment and sampling and plant tissues disinfestation

The greenhouse experiment was performed at the National Agricultural Research Institute (Instituto Nacional de Investigación Agropecuaria, Unidad Paso de la Laguna) in Treinta y Tres, southeast Uruguay (32°55’S and 54°50’W). The soil used presented the following characteristics: silty loam in texture, pH 5.2, N-NH₄⁺ 0.3 mmol L⁻¹, 3.0% organic C and 5.0% organic matter.

In Uruguay, rice is planted once per year in late spring over dry soil. After 45–60 days, the plots are flooded at tilling and remain in this condition until 1 week before harvest. Commercial varieties have high yields under these growing conditions. Oryza sativa, type Indica, variety INIA Olimar, obtained from local crossing (inbred variety released in 2002) and relevant for its commercial value was selected for this experiment. The experimental design was completely randomized and consisted of four replicate plots (approximately 0.15 m² each containing 52 kg of soil) established on 25 March 2009.

The usual conditions of the region were used for rice cultivation; fertilization consisting of NPK 120 kg. ha⁻¹ (18-46-0, corresponding to 21 kg of nitrogen) was applied at seeding, and urea 50 kg. ha⁻¹ (corresponding to 23 kg of nitrogen) was applied 34 days after seeding (DAS).

Samples were collected at two different rice plant growth stages, and these samples represented different water regime. The first sample was obtained before flooding (BF), 34 DAS. After this sampling, the plots were flooded with tap water to reach a water column of approximately 7 cm and remained flooded until the end of the experiment. The second sampling was performed 76 DAS, 42 days after flooding (AF). These treatments are henceforth referred to as BF and AF.

For each sampling date, five to ten rice plants were randomly collected from five different points distributed in each replicate plot and pooled.

Pooled plants for each replicate plot from two sampling dates (approximately 7 and 9 g for BF roots and leaves, respectively; and 18 and 10 g for AF roots and leaves, respectively) were disinfected after collecting. The leaves and roots were separated, brushed and washed with tap water and detergent. Distilled water was used for the last rinse. The samples were then disinfected once with 100 mL of 2% sodium hypochlorite for 5 min with agitation, followed by four 200 mL washes with sterilized distilled water (modified from Loaces, Ferrando and Fernández Scavino 2011). Disinfected rice plants were kept at –70°C for further analyses.

DNA extraction and nifH gene amplification

Total DNA was extracted from surface-disinfected rice material. The frozen samples were pulverized in sterilized pre-cooled mortars with liquid nitrogen, transferred to sterilized 1.5 mL microtubes and immediately stored at −70°C. DNA was extracted from 3 g of plant tissue (rice leaves or roots disinfected and pooled) according to Doyle and Doyle (1987). Briefly, pulverized plant material was treated with CTAB buffer (100 mM Tris-HCl pH 8.0; 20 mM EDTA; 1.4 M NaCl, 2% CTAB, 0.2% mercaptoethanol) and then incubated at 65°C for 45 min. After chloroform/isoamyl alcohol (24:1 v/v) extraction, the DNA was precipitated by isopropanol addition. DNA was centrifuged for 10 min at 10 000 rpm, resuspended in TE buffer and subjected to
RNAse treatment (10 μg mL⁻¹ final concentration). The purified DNA was resuspended in 75 μL of milliQ water.

Extracted DNA from leaves and roots was used as a template for nifH gene amplification using primers (PolF and PolR) and PCR conditions described by Poly, Jocuter Monrozier and Bally (2001). All PCRs were carried out in 25 μL (total volume) mixtures containing approximately 100–200 ng of total DNA, 0.1 mM of each primer, 1.5 mM MgCl₂, 75 μM buffer, 0.2 mg mL⁻¹ bovine serum albumin (Roche®), 0.2 mM of each dNTP and 1.2 U of Taq DNA polymerase (Fermentas®). The reactions were performed in a Gradient Palm Cycler (CORBETT Research, model CG1–96 thermocycler, Sidney) using the following programme: initial denaturation step at 94 °C for 4 min, followed by 30 cycles at 94 °C for 60 s, 57 °C for 60 s and 72 °C for 60 s, with a final extension step at 72 °C for 10 min.

Optimization of qPCR for nifH gene and standard preparation

The nifH PCR product from the strain Methyloqua oryzae (formerly methanotroph E10, EU672874) was obtained as explained above and then cloned using a TOPO TA cloning kit (Invitrogen Corp.) following manufacturer’s protocol. The clones were subjected to toothpick PCR (10 clones) using primers T3–T7 provided with the cloning kit. The correct fragment length and the identity of the PCR product were verified by electrophoresis on a 1.5% agarose gel, and the obtained ampiclon was then sequenced by Macrogen Sequencing Service, Korea, using the T7 primer. The plasmid was extracted using the Purelink Quick Plasmid miniPrep kit (Invitrogen ®), and the size of the plasmid fragment was verified by electrophoresis in a 1.5% agarose gel.

PCR amplification was performed in triplicate using the extracted plasmid as a template (primers T3 and T7 provided). The PCR products were pooled, and the ampiclon size was verified by agarose electrophoresis followed by purification with MICROCON®100 columns. The sample was then diluted with sterile MilliQ water to a total volume of 90 μL.

The purified product was quantified with the Qubit dsDNA HS Assay kit in a Qubit 2.0 Fluorometer (Invitrogen ®), and the nifH copy number was calculated based on the total fragment length (partial nifH fragment plus cloning vector fragments flanking the insertion point), the molecular weight of the ampiclon and Avogadro’s number. The nifH standard prepared was divided into 2 μL aliquots and frozen for use as the standard for all qPCR quantifications (3.38 × 10⁻¹⁰ nifH copies μL⁻¹). A standard curve was employed to measure nifH copy numbers in a range between 4 × 10⁰ and 4 × 10⁶ copies of nifH per microlitre of sample. The detection limit was established at 5.3 nifH copies per 10 ng of total DNA extracted from rice tissues.

Abundance of nifH genes in roots and leaves of rice by qPCR

The gene nifH was quantified by real-time PCR (qPCR) using the primers PoIF and PoIR (Poly, Jocuter Monrozier and Bally 2001). All quantifications were carried out on a Rotor-Gene® 6000, model 5-Plex (CORBETT Research, Sidney) using Sybr Green I for detection. The samples were amplified in 10 μL reaction volumes containing 1 μL of concentrated or diluted (one or two 10-fold) template DNA, 1 μM of each primer and 5 μL of Rotor-Gene SYBR Green PCR Mastermix (QIAGEN®, Hilden, Germany). All samples were amplified in duplicate. A standard curve was generated for each qPCR run in triplicate for each standard point (10-fold dilutions from 10⁻² to 10⁻¹⁰). Triplicates of no-template controls were included in each run as a negative control. The thermal cycle followed the manufacturer’s instructions and consisted of an initial step at 95 °C for 5 min followed by 40 cycles of 35 °C for 5 s and 60 °C for 10 s. The fluorescence signal was measured once per cycle after the annealing-elongation step via the addition of one step at 82 °C for 1 s in order to avoid fluorescence signals from primer dimers observed at 60 °C in the melting curve. A melting curve was obtained after each amplification by increasing temperature from 60 °C to 94 °C at a rate of 1 °C s⁻¹ in order to verify the specificity of amplification. The specificity of the qPCR products obtained from leaves and roots was verified by 1.5% agarose gel electrophoresis during the optimization. The reaction efficiencies were calculated by the standard curve method, and all efficiencies were between 85 and 92%. Duplicate qPCR measurements were averaged and corrected by the dilution factor. The results were expressed per ng of total DNA extracted in order to avoid the bias involved in extracting DNA from different type of tissues (leaves and roots) due to different extraction efficiencies.

T-RFLP of nifH genes

The nifH gene amplification product from DNA extracted from leaves was insufficient for T-RFLP. Thus, only the diazotrophic communities inhabiting rice roots were subjected to T-RFLP analysis. Four biological replicates were analysed for each water regime. The same primer set was employed, except the forward primer (PoIF) was 5’ labelled with 6-carboxyfluorescein. The PCR reactions were carried out in a total volume of 25 μL using PCR conditions similar to those described above. A pool of the amplification products from three individual PCR reactions was concentrated and purified with MICROCON®100 columns (Amicon Inc., MA, USA). Approximately 300 ng of labelled PCR products was digested with Alul or HaeIII according to the manufacturer (Fermentas®).

After enzyme inactivation by heating at 65 °C, the samples were desalted and concentrated with MICROCON®10). The samples were sent to MACROGEN Korea for GeneScan Service using the 500 LIZ DNA fragment length standard to assess the length of fluorescently labelled terminal restriction fragments (T-RFs). The raw data were analysed using PeakScanner® Software. T-RF sizes between 45 and 364 bp with peak heights larger than 200 fluorescence units were considered for the analysis. The two data sets obtained (nifH fragments restricted with HaeIII or Alul) were subjected to standardization. Briefly, the total intensity of fluorescence present in each electrophrogram was compared within each data set, and the T-RFLP data were standardized to the lowest quantity, as previously described (Dunbar, Ticknor and Kuske 2001). All profiles were aligned, and T-RFs that presented a defined and separated peak differing by more than 1 bp were considered different. The Shannon diversity index values (H = – ∑ (ni / N) log2 (ni / N)) were calculated from the four replicates using Past 1.42 software (http://folk.uio.no/ohammer/past/). ANOVA and the statistical comparison of diversity indexes were performed using InfoStat/Profesional Version 2.0.

The identity of the dominant observed T-RFs was inferred by in silico analyses from nifH sequences of clones obtained in this work. The sequences were aligned using MEGA version 5 (Tamura et al. 2011), the forward primer and the target site of the enzyme employed (i.e. Alul or HaeIII) were located in the sequence and the terminal fragment size was determined.
Barcoded pyrosequencing analysis of nifH genes

DNA extracted from the roots of two replicates of each water regime was sent to the Mr DNA sequencing facilities (Mr DNA Molecular Research, TX, USA) for nifH barcoded pyrosequencing. The barcoded amplicon sequencing procedure followed under the trademark service (bTEFAP®) was described by Dowd et al. (2008), utilizing nifH specific primers PoIR and PoIF (Poly, Jocteur Monrozier and Bally 2001). A HotStarTaq Plus Master Mix Kit (Qiagen, Valencia, CA) was used and PCR conditions consisted in a first step of 3 min at 94°C, followed by 28 cycles of 94°C for 30 s; 53°C for 40 s and 72°C for 1 min; after which a final elongation step at 72°C for 5 min was performed. Following PCR, all amplicon products from different samples were mixed in equal concentrations and purified using Agencourt Ampure beads (Agencourt Bioscience Corporation, MA, USA). Samples were sequenced utilizing Roche 454 FLX titanium instruments and reagents and following the manufacturer’s guidelines.

Pyrosequencing data processing

The nifH gene sequencing data were initially split in libraries followed by a de novo search for chimeras with userarch61 (Edgar 2010) and QIIME (Caporaso et al. 2010). The split and filtered libraries were then screened for frame shifts using the RDP FunGene (Fish et al. 2013) FrameBot tool (Wang et al. 2013) with a length cutoff of 80 amino acids and an identity cutoff of 0.4. The frame-corrected nucleotide sequences were then clustered (0.03 distance) using uclust (Edgar 2010) within QIIME, and an OTU table was constructed. OTUs with less than 10 counts were filtered from the OTU table. Alpha diversity metrics were also calculated in QIIME. Representative sequences were found for the nifH library and compared with the EMBL/GenBank database using the NCBI Blast algorithm (Altschul et al. 1997). A rarefaction analysis of the two libraries was performed based on the original data set using the free Analytic Rarefaction 1.3 software (http://strata.uga.edu/software/index.html) developed by Steven Holland.

The OTU table was standardized to the sample with the lower number of sequences (1009) and subjected to the following analyses. A phylogenetic analysis of nifH sequences representing more than 1% abundance in the standardized OTU table was performed. Alignments and phylogenetic distance dendrograms were constructed using the neighbour-joining algorithm (Saitou and Nei 1987) as implemented in MEGA version 5 (Tamura et al. 2011). Evolutionary distances were calculated using the Jukes–Cantor method (Jukes and Cantor 1969). The richness estimators Chao1 and ACE (abundance-based coverage estimator) and Shannon indexes for each sample were calculated within QIIME.

Venn diagrams were constructed using the online tool developed by Oliveros (2007–2015).

Cloning and phylogenetic analysis of nifH genes

The nifH gene was cloned by Macrogen® Korea from PCR products obtained from pooled DNA retrieved from each of the biological replicates of each treatment (BF and AF roots) according to Macrogen’s protocol (T-cloning vector). All sequencing reactions were carried out by the Macrogen Sequencing Service, Korea, using an ABI PRISM 3730XL capillary sequencer (Applied Biosystems, Foster City, CA, USA). The clones were sequenced using the M13 vector primer.

A total of 50 clones for BF and 100 clones for AF were analyzed because the rarefaction analyses showed that more intensive sampling was required to reach comparable asymptotic levels in both samples.

All rarefaction analyses of the nifH gene libraries were performed using the free software Analytic Rarefaction 1.3 (http://strata.uga.edu/software/index.html) developed by Steven Holland.

A phylogenetic analysis of the nifH gene, alignments and phylogenetic distance dendrograms were constructed using the neighbour-joining algorithm (Saitou and Nei 1987) as implemented in MEGA version 5 (Tamura et al. 2011). The evolutionary distances were calculated using the Jukes–Cantor (1969) method, followed by a nucleotide phylogenetic analysis.

Statistical and multivariate analysis

The nifH abundance data were log transformed to generate a normal distribution of residues and homogeneity of variance. These data were then subjected to ANOVA, whereas the means were compared using Tukey’s test.

PCA was used to compare the T-RFLP profiles.

All analyses were performed with InfoStat/Profesional Version 2.0.

Nucleotide sequence accession numbers

The nifH sequences obtained by cloning in this study were deposited in the GenBank nucleotide sequence database under the following accession numbers: KF872844–KF872993.

RESULTS

Abundance of nifH gene copies in leaves and roots of rice

The nifH gene copy numbers per ng of total DNA extracted from different plant tissues and treatments were determined (Fig. 1). The results show that the copy numbers of the nifH gene were higher in roots than in leaves by at least 100-fold (p-value 0.0133). Flooding differentially affected the abundance of diazotrophic communities inhabiting rice roots and leaves. Whereas the nifH gene levels in rice roots significantly increased after flooding, the levels of this gene remained low and constant in rice leaves (p-value 0.0396).

Structure of diazotrophic endophytic bacteria in rice roots of replicate plots

The T-RFLP profiles obtained from rice roots with both restriction enzymes indicated diverse diazotrophic communities: 46 and 56 different T-RFs were observed for AluI and HaeII, respectively. Fifty per cent of the total T-RFs showed fluorescence intensities lower than 2% of the total signal, revealing that minor members of the community considerably contributed to the diversity. An average of 19 T-RFs was detected for each sample profile obtained by each restriction enzyme.

According to the PCA of the T-RFLP data (Fig. 2), two distinct groups could be observed in PC1 since the T-RFLP profiles clustered by water regime. Together, PC1 and PC2 explained 47% of the observed variability. Rice roots from replicate plots before flooding (BFR) presented a greater data dispersion than those from rice roots after flooding (AFR), as indicated by the standard deviations (SD) (BFR: SD PC1 ± 2.18, SD PC2 ± 7.19; AFR: SD PC1 ± 0.70, SD PC2 ± 0.27). This dispersion along the PC2 axis is mostly due to the BFR2 sample, which differed from the other replicate
Figure 1. Abundance of nifH gene copies in the leaves and roots of rice. Each bar corresponds to mean values from the four biological replicates. Standard deviation is indicated on each bar. BFR: before flooding roots; BFL: before flooding leaves; AFR: after flooding roots; AFL: after flooding leaves.

Figure 2. PCA of nifH T-RFLP profiles. The proportions of variation explained by first and second ordination axis are indicated in brackets. Open circles: before flooding roots (BFR). Filled squares: after flooding roots (AFR). Different numbers correspond to different biological replicates.

The Shannon index values were obtained for each biological replicate from each treatment. The ANOVA and comparison of means (Tukey’s test, α = 0.05) showed that rice roots after flooding (Shannon index 2.97 ± 0.16) sustained a significantly

plots by the presence of the 152 bp T-RF (4.7% of the total fluorescence) and 10 minor fragments, which were obtained after digestion with HaellII. Furthermore, it also differed by the relative abundance of two major T-RFs (100 bp and 361 bp), which were obtained after Alul digestion.

Some T-RFs were common to most of the samples: the 45, 67, 99, 157 and 185 bp fragments obtained by HaellII restriction and the 100 and 361 bp fragments (unrestricted fragment) obtained by Alul digestion.

The distant grouping of the samples before and after flooding was mainly attributed to changes in the relative abundance of T-RFs that represent at least 2% of the total fluorescence. The 56, 59, 99 and 164 bp T-RFs from the HaellII restriction and 361 bp T-RF from the Alul restriction were the most relevant peaks involved in the clustering samples from roots before flooding. The 70 bp T-RFs (HaellII) and the 63, 127 and 214 bp T-RFs (Alul) were the main T-RFs involved in the clustering samples from the flooding stage.

Although in silico analyses of the nifH sequences retrieved by cloning permitted the T-RF assignment to known sequences, these T-RFs corresponded to the sequences of several different species.

The Shannon index values were obtained for each biological replicate from each treatment. The ANOVA and comparison of means (Tukey’s test, α = 0.05) showed that rice roots after flooding (Shannon index 2.97 ± 0.16) sustained a significantly
Figure 3. Rarefaction curves obtained from nifH pyrosequencing libraries from rice roots before and after flooding. Two biological replicates from each condition were analysed. Before flooding root (BFR); after flooding roots (AFR).

(p-value 0.0048) more diverse diazotrophic community of endophytes than the rice roots before flooding (Shannon index 2.28 ± 0.27).

Community composition and phylogenetic diversity of root endophytic diazotrophs

To further understand the diazotrophic diversity and community composition in replicate plots, the PCR-amplified nifH genes from samples from replicate plots at both stages (BFR7 and BFR8; AFR7 and AFR8) were subjected to 454 pyrosequencing. The raw dataset obtained consisted of a total of 12 655 reads, which were reduced to 7928 sequences: 3402 for BFR7, 1009 for BFR8, 1867 for AFR7 and 1650 for AFR8. After quality checking, i.e. the removal of chimeras and reads that failed to align, 86 OTUs were defined at 90% protein sequence identity.

A rarefaction analysis of the libraries resulted in different saturation profiles (Fig. 3), indicating that the diversity of the nifH gene was different for the analysed samples. The dataset was normalized to the sample that presented the lower number of sequences retrieved (BFR8, 1009 sequences) in order to compare samples. Whereas a total of 61 and 71 OTUs were observed in AFR7 and AFR8 (after flooding), respectively, 6 and 28 were observed in BFR7 and BFR8 (before flooding), respectively (Table 1 and Supporting information Fig. S3). The richness estimators and Shannon diversity index calculated for the standardized dataset showed that diazotrophic communities from flooded rice roots were more diverse than those from unflooded rice roots (Table 1).

Furthermore, Venn diagrams (Fig. 4) show that only 5 (OTU32, OTU121, OTU189, OTU196 and OTU221) of 86 OTUs were present in at least three of four samples analysed. Rice roots before flooding (replicates BFR7 and BFR8) exhibited seven OTUs (OTU18, OTU107, OTU152, OTU197, OTU232, OTU262 and OTU267) exclusive of this condition and shared only one OTU (OTU221). In contrast, 53 OTUs were detected only in flooded rice roots (replicates AFR7 and AFR8), and 34 of these OTUs were shared between replicates.

The major phylogroups retrieved by nifH pyrosequencing of independent replicates were generally the same as those detected via the nifH cloning of pooled samples mentioned below and are shown in Table 2. Replicates BFR7 and BFR8 were dominated by Gammaproteobacteria, comprising 76.1 and 98.3% of diazotrophic communities, respectively. Among the different gammaproteobacterial OTUs, OTU221, which related to Stenotrophomonas, reached 52.5 and 98% abundance in BFR7 and

<table>
<thead>
<tr>
<th>Sample</th>
<th>Estimated OTU richness</th>
</tr>
</thead>
<tbody>
<tr>
<td>BFR7</td>
<td>Chao1: 42, ACE: 36.2, Shannon index: 2.52</td>
</tr>
<tr>
<td>BFR8</td>
<td>Chao1: 6.5, ACE: 7.8, Shannon index: 0.17</td>
</tr>
<tr>
<td>AFR7</td>
<td>Chao1: 73.5, ACE: 72.4, Shannon index: 5.16</td>
</tr>
<tr>
<td>AFR8</td>
<td>Chao1: 65.1, ACE: 69.0, Shannon index: 4.88</td>
</tr>
</tbody>
</table>

Table 1. Richness estimates and diversity index for nifH gene pyrosequencing libraries from rice roots. OTUs, richness estimators and the diversity index were calculated within QIIME (90% similarity cutoff) after the standardization of sequences to the same depth (1009 sequences). ACE: abundance-based coverage estimator; numbers 7 and 8 correspond to different biological replicates analysed. BFR: before flooding roots; AFR: after flooding roots.
**Figure 4.** Venn diagrams obtained from nifH pyrosequencing libraries from rice roots before and after flooding. BFR: before flooding root; AFR: after flooding roots. Different numbers correspond to different biological replicates.

BFR8, respectively. In contrast, this class represented 13.4 and 4.2% for AFR7 and AFR8, and OTU221 was only detected in low abundance in AFR8 (0.4%). OTU228 was also an abundant OTU in replicate BFR7 (17%) and was present at 0.2% in replicate AFR8 after flooding.

The Betaproteobacteria class was dominant in rice roots replicates after flooding, comprising 29.8 and 26.3% of the populations of AFR7 and AFR8, respectively. *Dechloromonas, Dechloromonas/Azospira, Uliginosibacterium* and *Candidatus Accumulibacter* were dominant at this stage. These genera were only detected under submerged conditions. However, *Ideonella* and *Pelomonas* related OTUs were distributed between both conditions (Table 2).

Deltaproteobacteria (*Geobacter* and *Desulfovibrio*) as well as some *Spirochaetes* and *Chlorobi* were exclusively retrieved from flooded conditions (Table 2).

The class Alphaproteobacteria was abundant under both conditions, although differences were observed in the genera detected. OTU related to *Bradyrhizobium/Sphingomonas* was always present, whereas *Rhodospseudomonas*, *Magnetospirillum* and *Azospirillum/Methylosinus* were detected only after flooding.

Among Firmicutes, sequences related to *Dehalobacter* were exclusively detected in samples before flooding, and sequences related to *Selenomonas* were only present after flooding.

Some proteobacterial OTUs were difficult to affiliate accurately because they were related to sequences from different taxa (Table 2).

The nifH gene pools from replicate plots were cloned to assign identity to the T-RFs obtained and explore the phylogenetic affiliation of the main members of the community in plants before and after flooding. The results supported those obtained from the 454 pyrosequencing of the nifH gene from independent replicate samples of each stage. A total of 50 clones from the nifH library of BF rice roots and 100 clones from AF rice roots were sequenced and analysed. Rarefaction analyses indicated an adequate sampling coverage of both nifH gene libraries (Supporting Information, Fig. S1).

The results obtained showed that the composition of the nifH gene clone libraries differed by water regime (Table 3). The clone library of BF rice roots was dominated by *Gammaproteobacteria* (66%), and *Stenotrophomonas* was the main genus of this class, accounting for 50% of the clone library. Furthermore, 16 and 14% of the library consisted of *Alphaproteobacteria* and *Betaproteobacteria*, respectively, and *Bradyrhizobium* sp. and *Pleomorphomonas oryzae* and the poorly affiliated *Ideonella*-like sequences were the most prominent bacteria. *Firmicutes* (4%) were also represented in this pre-flooded condition, with *Paenibacillus* sp. being the only one representative.

Conversely, the clone library from flooded rice roots indicated a completely different and highly diverse community. Alpha-, Beta- and *Gammaproteobacteria* accounted for 20, 37 and 2% of the community, respectively. The abundance of *Gammaproteobacteria*, particularly *Stenotrophomonas* spp., diminished to 1% after flooding, whereas *Alphaproteobacteria* remained important members of the community. The betaproteobacterial nifH sequences doubled after flooding, mainly due to new sequences, such as *Dechloromonas*-like and *Azospira oryzae*-related nifH sequences. In addition, Deltaproteobacteria, *Spirochaetes* and *Chlorobi* (4, 9 and 8% of the clone library, respectively) were detected only under flooded conditions.

*Paenibacillus* sequences and other *Firmicutes* sequences poorly affiliated with *Helibacterium*, *Dehalobacter* and
Table 2. Distribution of OTUs in nifH pyrosequencing libraries. Relative abundance of each OTU (standardized) is shown for each sample. *Consists of OTUs that represent less than 1% abundance in all the nifH pyrosequencing library. **Consists of eight different OTUs.

<table>
<thead>
<tr>
<th>Phylogroup</th>
<th>Main genera detected</th>
<th>OTU</th>
<th>BFR7 (%)</th>
<th>BFR8 (%)</th>
<th>AFR7 (%)</th>
<th>AFR8 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alphaproteobacteria</td>
<td>Rhodopseudomonas</td>
<td>OTU15</td>
<td>11.1</td>
<td>1.2</td>
<td>7.6</td>
<td>11.7</td>
</tr>
<tr>
<td></td>
<td>Pleomorphomonas</td>
<td>OTU102</td>
<td>9.1</td>
<td>0.0</td>
<td>4.4</td>
<td>9.1</td>
</tr>
<tr>
<td></td>
<td>Magnetospirillum</td>
<td>OTU137</td>
<td>0.0</td>
<td>0.0</td>
<td>1.2</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>Sphingomonas-Bradyrhizobium</td>
<td>OTU196</td>
<td>2.0</td>
<td>1.0</td>
<td>1.2</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>Azospirillum-Methylosinus</td>
<td>OTU82</td>
<td>0.0</td>
<td>0.0</td>
<td>0.3</td>
<td>1.2</td>
</tr>
<tr>
<td>Betaproteobacteria</td>
<td>Ideonella</td>
<td>OTU181</td>
<td>0.7</td>
<td>0.0</td>
<td>0.8</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>Dechlorosoma-Azospira</td>
<td>OTU103</td>
<td>0.0</td>
<td>0.0</td>
<td>2.2</td>
<td>1.6</td>
</tr>
<tr>
<td></td>
<td>Dechloromonas</td>
<td>OTU143</td>
<td>0.0</td>
<td>0.0</td>
<td>5.9</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td>Pelomonas</td>
<td>OTU189</td>
<td>0.0</td>
<td>0.0</td>
<td>0.1</td>
<td>1.9</td>
</tr>
<tr>
<td></td>
<td>Ulginosibacterium</td>
<td>OTU117</td>
<td>0.0</td>
<td>0.0</td>
<td>0.4</td>
<td>3.9</td>
</tr>
<tr>
<td></td>
<td>Candidatus Accumulibacter</td>
<td>OTU65</td>
<td>0.0</td>
<td>0.0</td>
<td>2.7</td>
<td>0.2</td>
</tr>
<tr>
<td>Gammaproteobacteria</td>
<td>Stenotrophomonas</td>
<td>OTU197</td>
<td>1.7</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>Geobacter</td>
<td>OTU156</td>
<td>0.0</td>
<td>0.0</td>
<td>3.2</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>Klebsiella</td>
<td>OTU228</td>
<td>0.0</td>
<td>0.0</td>
<td>7.4</td>
<td>2.5</td>
</tr>
<tr>
<td>Deltaproteobacteria</td>
<td>Geobacter</td>
<td>OTU156</td>
<td>0.0</td>
<td>0.0</td>
<td>3.2</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>Desulfuvibrio</td>
<td>OTU150</td>
<td>0.0</td>
<td>0.0</td>
<td>0.3</td>
<td>2.2</td>
</tr>
<tr>
<td>Other Proteobacteria poorly affiliated</td>
<td>**</td>
<td>OTU228</td>
<td>0.0</td>
<td>0.0</td>
<td>7.4</td>
<td>2.5</td>
</tr>
<tr>
<td>Spirochaetes</td>
<td>Spirochaeta</td>
<td>OTU161</td>
<td>0.0</td>
<td>0.0</td>
<td>1.4</td>
<td>0.0</td>
</tr>
<tr>
<td>Firmicutes</td>
<td>Dehalobacter</td>
<td>OTU232</td>
<td>0.0</td>
<td>0.0</td>
<td>0.3</td>
<td>5.1</td>
</tr>
<tr>
<td></td>
<td>Paenibacillus</td>
<td>OTU262</td>
<td>0.0</td>
<td>0.0</td>
<td>2.7</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>Selenomonas</td>
<td>OTU110</td>
<td>0.0</td>
<td>0.0</td>
<td>0.1</td>
<td>1.4</td>
</tr>
<tr>
<td>Chlorobi</td>
<td>Chlorobium</td>
<td>OTU113</td>
<td>0.0</td>
<td>0.0</td>
<td>1.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Others not classified*</td>
<td></td>
<td></td>
<td>2.94</td>
<td>0.4</td>
<td>40.23</td>
<td>17.07</td>
</tr>
</tbody>
</table>

Syntrophobotulus-like sequences were detected in both water regimes.

A phylogenetic analysis was performed from nifH gene sequences (Supporting Information, Fig. S2). The criterion for determining the phylogenetic affiliation of retrieved nifH sequences was selected according to the obtained distance matrix (cutoff 92% similarity).

Within the G1a group (Supporting Information, Fig. S2a), some clusters consisted exclusively of nifH sequences from one water regime. Azospira oryzae, Pelomonas saccharophila and Methylosinus sp. (Alphaproteobacteria) were only detected in flooded rice roots, similarly to the cluster consisting of Rhodopseudomonas, Rhodobacter and Magnetospirillum. Conversely, Sphingomonas azotifigens sp. (Alphaproteobacteria) was detected in
Table 3. Composition of diazotrophic bacteria communities inhabiting rice roots based on the \textit{nifH} gene clone library BFR: before flooding roots; AFR: after flooding roots.

<table>
<thead>
<tr>
<th>Phylogroup</th>
<th>Main genera detected$^b$</th>
<th>Percentage in \textit{nifH} gene libraries (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>BFR</td>
</tr>
<tr>
<td><strong>Alphaproteobacteria</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rhodopseudomonas</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>Pleomorphomonas</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Magnetospirillum</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Azorhizobium-Bradyrhizobium</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8</td>
</tr>
<tr>
<td><strong>Betaproteobacteria</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ideonella</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>Dechlorosoma-Azospira</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>Dechloromonas</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Pelomonas</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td><strong>Gammaproteobacteria</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Stenotrophomonas</td>
<td>66</td>
</tr>
<tr>
<td></td>
<td>Enterobacter</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td></td>
<td>16</td>
</tr>
<tr>
<td><strong>Deltaproteobacteria</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Geobacter</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Desulfovibrio</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td><strong>Spirochaetes</strong></td>
<td>Treponema-Spirochaeta</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td><strong>Firmicutes</strong></td>
<td>Paenibacillus</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Dehalobacter- Syntrophobutus</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td><strong>Chlorobi</strong></td>
<td>Chlorobium</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td><strong>Others$^a$</strong></td>
<td></td>
<td>0</td>
</tr>
</tbody>
</table>

$^a$Includes other minority \textit{nifH} sequences retrieved and sequences poorly related to those from database.

$^b$Corresponded to \textit{nifH} sequences that represented more than 1% of at least one clone library.

low proportions in the pre-flooding stages. The \textit{nifH} sequences related to \textit{P. oryzae} and the \textit{Azorhizobium/Bradyrhizobium} cluster (Alphaproteobacteria) were present in both water regimes.

The sequences of the \textit{nifH} gene distantly related to \textit{Ideonella dechloratans} clustered with the uncultured bacterium clone Pn5 (DQ304853), which has been associated with roots from a perennial grass of the Thar Desert in India (Chowdhury et al. 2009).

The betaproteobacterial \textit{nifH} sequences related to \textit{Dechloromonas} sp. represented 16% of the clone library from flooded rice roots (Cluster G1b, Supporting Information Fig. S2b). \textit{Gammaproteobacteria} predominated the population from unflooded rice roots and were mainly represented by sequences related to \textit{Stenotrophomonas} sp. isolate gx-44, FJ822999. Furthermore, \textit{nifH} sequences related to \textit{Enterobacter} spp. (JQ001785 and FJ593868) were exclusively detected in rice roots before flooding (16%). Similarly, a gammaproteobacterial \textit{Marichromatium/Halorhodospira}-like sequence was detected at low proportions (1%).

\textit{Firmicutes} clustered into three different groups, G2, G4 and G5 (Fig. S2b, Supporting Information). Sequences affiliated with \textit{Paenibacillus} sp. (G2) were detected in both \textit{nifH} gene libraries. However, \textit{Firmicutes} sequences affiliated with \textit{Hellobacterium} spp. and \textit{Dehalobacter/Syntrophobutus} (G4) were exclusively retrieved from flooded samples.

G5, an important cluster consisting only of \textit{nifH} sequences detected in flooded samples (Supporting Information Fig. S2c), consisted of sequences affiliated with \textit{Firmicutes}, \textit{Spirochaetes}, \textit{Chlorobi} and \textit{Deltaproteobacteria}. The dominant species were related to \textit{Selenomonas ruminantium} (AP012292), \textit{Spirochaeta zuelzerae} and \textit{Treponema azotonutricium} (AF325795 and CP001841), \textit{Chlorobium tepidum} (AE006470) and \textit{Desulfovibrio} spp. (AP010904 and CP002298), respectively. This cluster, as well as a Geobacter-like cluster (Deltaproteobacteria, G3), consisted entirely of sequences retrieved from flooded rice roots.

A more distantly and poorly affiliated cluster, G6, included sequences related to \textit{Archaea}, \textit{Firmicutes} and \textit{Bacteroidetes} as well as a subcluster that contained a few clones retrieved from flooded rice roots. Furthermore, sequences of uncultured clones (AY768661 and AY181002) retrieved from \textit{O. longistaminata} tissues from mRNA-based studies clustered together with these sequences.

**DISCUSSION**

Nitrogen fixation in soils is affected by environmental factors, i.e. tillage, water content, oxygen concentration, pH, N and C availability (Hsu and Buckley 2009). However, the impact of these factors on endophytic communities is poorly understood. Oxygen availability is a major factor and an evolutionary force determining the metabolic strategy of bacteria that colonize an environmental niche (Voroney 2007). Although a recent study revealed that some obligate aerobes develop unexpected survival strategies to persist in temporarily oxygen-deprived environments (Berney et al. 2014), succession in microbial communities usually occurs when environmental conditions suddenly change.
The present work addressed the dynamics of endophytic nitrogen fixers in rice tissues at two stages of the natural crop cycle, i.e. before and after the flooding stage. The structure of this diazotrophic community describes the potential of endophytes for nitrogen fixation, but only a fraction of this population may be metabolically active. The active diazotrophs have been explored by several authors through the expression of the nifH gene (Sessitsch et al. 2012; Sampaio Videira et al. 2013).

In this study, the abundance of nifH genes measured by qPCR significantly differed between the pre-flooding and flooding stages. The behaviour of the analysed diazotrophic communities depended on the plant tissue studied (leaves and roots). The nifH gene copy numbers present in rice roots increased 10-fold after flooding (from 5.8 × 10^6 to 5.1 × 10^7 nifH copies per ng of DNA; Fig. 1). Conversely, the nifH gene levels from rice leaves remained unaffected by flooding, and their values were at least two 10-fold lower than those present in roots (1.6 × 10^6 nifH copies per ng DNA; Fig. 1). Diaziotrophs living inside rice leaves seem to comprise a numerically more stable community that is less affected by external factors than that by inhabiting roots. However, the nifH gene has rarely been quantified in inner plant tissues. Juraeva et al. (2006) reported that nifH was more abundant in cucumber roots than in shoots, and its expression positively correlated with the N supply and plant age. Endophytic diazotrophs from roots may be more dependent on the soil characteristics and environmental conditions (soil type, organic matter content, nitrogen input, oxic/anoxic conditions, etc.) than those in leaves due to the relevant and close root-soil interaction, as demonstrated by Hardoim et al. (2012).

A dramatic shift in the community composition was observed with the three approaches employed in this work: T-RFLP, cloning and the pyrosequencing of the nifH gene. The diversity of nifH sequences was appropriately surveyed using techniques applied based on rarefaction results (Fig. 3; Fig. S2, Supporting Information) and the obtained richness estimates (Table 1). ACE and Chao1 estimators showed that the diazotrophic communities established in replicates from AF rice roots were more diverse and similar than replicates from BF rice roots. The Shannon indexes and PCA (Table 1 and Fig. 2) obtained from T-RFLP data for the four replicates of each stage confirmed this trend.

The PCA of T-RFLP data (Fig. 2) showed higher dispersion for replicates of BF rice roots than for communities from AF rice roots. These findings suggest that the plant physiology at the flooding stage and the subsequent environmental changes, such as the decrease in the redox potential, may blur the microniches and erase the differences observed in the diazotrophic communities established in the rice roots during the pre-flooding stages of rice growth. This knowledge could be useful when some agricultural practices, such as inoculation or fertilization, are planned.

A deep pyrosequencing analysis of the nifH gene has recently been employed to study the effect of agricultural practices and the physicochemical properties of the soil (Collavino et al. 2014) on the diazotrophic community or its spatio-temporal variability in different soils (Pereira e Silva et al. 2013).

Venn diagrams of the data retrieved from the 454 pyrosequencing of the nifH gene (Fig. 4) revealed that only a few members of the community were permanently associated with rice roots. Sequences related to Pleomorphomonas, Pelomonas, Bradyrhizobiun Sphingomonas and some Ideonella sp. comprised the core community.

The composition and distribution of sequences from nifH gene libraries (Tables 2 and 3) consistently indicated the main differences between root bacterial communities before and after flooding.

Both a pool of replicate plots (by cloning) and two independent replicate plots analysed more deeply (by 454 pyrosequencing) indicated a remarkable dominance of gammaproteobacterial sequences in BF rice roots. The genus Stenotrophomonas comprised more than 50% of the obtained libraries (Tables 2 and 3) before flooding and less than 1% of libraries obtained from flooded rice roots. Stenotrophomonas sp. isolate gx-44 (FJ322999) was the closest species (Fig. S2, Supporting Information), and according to the GenBank submission information, it was associated with sugarcane tissues from China (unpublished). Bacteria belonging to the Stenotrophomonas genus are strictly aerobic, very versatile and adaptable to different environments and can promote plant growth, as summarized by Ryan et al. (2009). Stenotrophomonas maltophilia and S. rhizophila have been found associated with plants, inhabiting the rhizosphere or vascular tissues of roots and stems. Recently, S. pavanii was described as the first endophytic Stenotrophomonas species able to fix nitrogen in sugarcane (Ramos et al. 2011). Furthermore, bacterial strains related to S. maltophilia were isolated from the rice seeds of two consecutive generations, suggesting that this genus is strongly associated with rice plants and that both dissemination from seeds and colonization from soil may be strategies to sustain this association (Hardoim et al. 2012). Thus, the Stenotrophomonas strains detected in this work may benefit plant growth during the pre-flooding stage, when the soils are aerobic.

Alpha- and betaproteobacterial nifH sequences were also abundantly recovered from the communities colonizing non-submerged rice roots, and these communities primarily consisted of Azorhizobium/Bradyrhizobiun-like sequences and P. oryzae (Alphaproteobacteria) and I. dechrarotans-like sequences (Betaproteobacteria) (Tables 2 and 3).

Conversely, in flooded rice roots, the dominant members of the diazotrophic community belong to the Betaproteobacteria class and account for 26–36% of the nifH libraries. Within this group, the dominant nifH sequences were related to Dechloromonas (4.9–16% of AF libraries), followed by Ideonella (5.2–13.3% of AF libraries) and Azospira (1.6–8% of AF libraries). Within this group, Uliginosibacterium and Candidatus Accumulibacter were also abundant according to the pyrosequencing data (Table 2). Our results agreed with those reported by Wu, Ma and Lu (2009), who also observed that Betaproteobacteria (mainly Azorarcus spp.) dominated the nifH genes that were associated with irrigated roots of modern rice cultivars. According to our results, the betaproteobacteria retrieved are mostly facultative anaerobes or microaerophilic bacteria since many of them have been reported to reduce chloride or perchlorate (Coates et al. 1999). The genus Dechloromonas consists of metabolically diverse bacteria that can reduce nitrate and nitrite, reduce sulphate or contribute to the microbially driven Fe-N redox cycle (Tan and Reinhold-Hurek 2003; Coby et al. 2011). These processes are environmentally relevant, especially in paddy rice ecosystems, and take place not only in the bulk soil but also in the rhizosphere (Kögel-Knabner et al. 2010). According to Scheid, Stubner and Conrad (2004), this genus was also active and dominant in excised rice roots incubated in anoxic conditions with nitrate. Roesch et al. (2008) reported that nifH sequences belonging to Dechloromonas were restricted to maize roots after studying diazotrophs from a maize-cropping ecosystem (rhizosphere, roots and stems).

Notably, a wide metabolic variety of anaerobic nitrogen-fixing bacteria were retrieved from flooded rice roots. The sequences of anaerobes or aerotolerant bacteria belonging to
Spirochaetes, Chlorobi (green sulphur bacteria, Chlorobium spp.), Deltaproteobacteria (Desulfovibrio spp. and Geobacter spp.) and diverse Firmicutes members, i.e. Selenomonas sp., and species related to the anoxygenic phototrophic Heliobacterium and Dehalobacter, were found under flooded conditions. Oxygen released from the roots of submerged plants supports the oxidation of ammonia to nitrate and of sulphide to sulphate in the microchines of the rhizosphere. In addition to fermentative processes that may occur in anoxic conditions, these electron acceptors may become available to the microbial communities associated with roots (Scheid, Stubner and Conrad 2004). Almost 10% of endophytic bacteria detected in Phragmites australis roots, which were obtained from a Chinese constructed wetland, belonged to the sulphate-reducing genera Dechloromonas, Desulfovibrio and Sulfurospirillum (Li et al. 2010). Miletto et al. (2013) observed the emergence of Desulfuvibrioaceae-related sequences exclusively in the rhizosphere of Gliceria maxima, an aerenchymatous helophyte, after flooding with sulphate-rich water, suggesting that this group is better adapted to the conditions created by the plants.

In addition, our findings suggest that anaerobes may play a major role in nitrogen fixation in this ecosystem under anaerobic conditions. Obligate anaerobes (grouped in Cluster III) are reportedly the most diverse of all nifH lineages but also the least explored group to date (Gaby and Buckley 2011). However, a broad spectrum of sequences belonging to anaerobic bacteria was retrieved from flooded rice roots in this work.

To the best of knowledge, this work represents the first report on the effect of flooding on endophytic communities from plants growing in similar conditions to those of paddy fields. Only a few studies have explored the effect of flooding on bacterial communities associated with plants. In addition to bulk and rhizospheric soil, Hardoim et al. (2012) studied shoot and root rice tissues in greenhouse experiments using gamma-sterilized soils, revealing that the endophytic community of rice seems to be largely influenced by soil type, followed by the water regime (flooded and unflooded). Furthermore, Graff and Conrad (2005) reported the impact of flooding on rhizosphere and rhizoplane bacterial communities associated with poplar trees.

Plants adapted to rice cropping in Uruguay require flooding in order to have economical value. Between the two sampled stages selected in this work, the plant physiologically matures. Thus, the observed effects on diazotrophic communities after flooding are likely due to changes in the plant–soil ecosystem after 42 days of flooding, including flooding-induced changes of the plant. The approach used in this work did not individually examine these factors but represents the genuine agronomical production of rice.

In conclusion, this work assessed the impact of flooding during plant growth on diazotrophic endophytic bacteria inhabiting the roots and leaves of rice cultivated under conventional conditions. Exploring the impact of external factors on native endophytic communities established inside rice tissues will likely help to improve inoculants, which must face different community structures throughout the rice growth cycle. Further studies should be performed in order to determine whether the detected diazotrophs are actively fixing nitrogen at both stages of the plant growth.

**ACKNOWLEDGEMENTS**

We thank INIA Treinta y Tres for their support and help in the greenhouse experiments.

**FUNDING**

This work was supported by ANII (Agencia Nacional de Investigación e Innovación) and CSIC (Comisión Sectorial para la Investigación Científica).

**Conflict of interest.** None declared.

**REFERENCES**


**SUPPLEMENTARY DATA**

Supplementary data are available at FEMSEC online.


