Microbial nitrate removal in groundwater polluted from agricultural activities with hybrid cork treatment wetlands

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Abstract

Agricultural practices have raised the level of nutrients reaching aquifers. In Europe, nitrate pollution is considered as one of the main threats for the quality of groundwater in agricultural areas. Treatment wetlands (TWs), also known as Constructed Wetlands, are used for groundwater treatment in areas with an important concentrations of nitrogen compounds; total nitrogen removal depends on the type and operation scheme. Cork by-product from the industry has shown clear adsorbent properties to remove organic pollutants. The work is focused on the characterization of microbial communities involved in the nitrate-nitrogen removal process in groundwater polluted from agricultural activities. The experimental design allowed the comparison of nitrate removal efficiency depending on the filter media material, cork by-product or gravel, used in two hybrid TWs (a vertical flow cell followed by a horizontal subsurface flow cell), installed in areas close to two irrigated agricultural plots at the Lleida plain area (Spain). Both physicochemical and microbial results were consistent and confirm the nitrate removal efficiency using cork as a filter media. A significant ($p=0.0025$) higher removal in Bellvís TW using cork compared with the Vilanova de la Barca gravel system was observed, achieving a removal rate from 80 to 99% compared to the 5-46%, respectively. Regarding the community composition of the two different TWs, microorganisms were mainly related to the phylum Proteobacteria, and included members found to be key players in the nitrogen cycle, such as ammonia and nitrite oxidizers, as well as denitrifiers. Also, the group Bacteroidetes turn to be another abundant phylum from our bacterial dataset, whose members are suggested to be strongly involved in denitrification processes. Some groups showed to prevail depending on the type of media (cork or gravel); Firmicutes and Delta and Epsilonproteobacteria had a significant higher abundance in the TW with cork, while Acidobacteria and Planctomyces were prevalent in gravel. Therefore, cork could be an alternative material used by treatment wetlands to minimize the impact in the environment caused by nitrogen pollution in groundwater bodies.

Keywords

Hybrid treatment wetland, nitrate pollution, cork by-product, microbial communities, denitrifiers, high-throughput sequencing.

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1. INTRODUCTION

Since the 50s, agricultural practices have been developed applying large amounts of chemical fertilizers and pesticides to sustain the increasingly higher yields and productivity in crops (Novotny, 1999). These activities raised the level of nutrients reaching aquifers -specially for nitrogen and phosphorus-, therefore polluting surface and groundwater sources and consequently affecting water quality.

The quantity of nitrogen compounds discharge to subsurface and groundwater by agriculture activities are conditioned by many factors including transformations and transport processes in the nitrogen cycle in agricultural soils, the type of activities carried out on the surface ground, the kind and depth of the non-saturated area and/or the irrigation methods used (Fernández, 2007).

In Europe, the Nitrates Directive (Directive 91/676/EU) considers the agricultural use of nitrates in organic and chemical fertilisers as the major source of water pollution. The Nitrates Directive, and other EU policies, such as the Water Framework Directive (Directive 2000/60/EC) and the Groundwater Directive (Directive 2006/118/EC), aims to protect water quality by preventing the discharge of nitrates from agricultural sources (European Unión, 2010). Isermann (as cited in Delgado, 2007) stated that in the European Union, 50 to 80% of the nitrogen present in water bodies is due to agricultural activities. In Spain, 80% of the groundwater has nitrate concentrations above 25 mg L^{-1} and 13% of the national territory has been declared vulnerable to nitrate water pollution (Fernández, 2007), where, its concentration exceeds 50 mg L^{-1}. Therefore, water quality monitoring, as well as intensive restoring practices to improve river basins are urgently required (Menció et al., 2011).

Treatment Wetlands (TWs) are engineered systems that simulate processes from natural wetlands, with low external energy requirements, to improve water quality by means of a combination of physical, chemical and biological processes (Brix, 1993; Vymazal, 2010; Wu et al., 2014). TWs. For example, plant roots absorb nutrients and establish a symbiotic relation with microorganisms, oxygen supply and particle filtration (Brix, 1987). TWs are used as wastewater treatment in places with an important amount of nitrogen compounds. The two most important nitrate removal mechanisms, nitrification followed by denitrification, takes place simultaneously in the filter media of TWs. When oxygen transport and availability in the wetland is limited, nitrification will be limited, affecting the overall total nitrogen removal as well. However, denitrification can be very efficient even with low carbon levels (Platzer, 1999).

TWs can be successfully used for nitrogen removal from secondary effluents, with efficiencies higher than 90% (Xiong et al., 2011). According to Vymazal (2013 and 2014), Horizontal Subsurface Flow Treatment Wetlands (HSSF) which have saturated beds, and thus, a limited capacity for nitrification due to the absence of available oxygen are not effective for ammonia removal. Therefore, Vertical Flow Treatment Wetland (VF) followed by a HSSF TW, a hybrid system, with higher ammonia removal efficiency, for example, an experimental hybrid treatment wetland system showed a 71% removal of total nitrogen, (Ghrabi et al., 2011). In fact, Vymazal (2007) reported that total nitrogen removal varied in TWs between 40 to 50%, depending on the type and operation scheme, with loading removal rates ranging between 250 and 630 gr N m^2 y^{-1}, showing good potential for total nitrogen removal.

The filter layer used in TWs is a key element for pollutants removal from wastewater. Depending on vegetation and flow regime, conventional TWs can remove N in the range of 30 to 80% of nitrates from domestic wastewater (Ayaz, 2003). However, recycled materials have been tested as granular media for wastewater treatment. García-Pérez (2016) reported removal efficiencies of 87% for Ammonia-N, 57% for Total Kjeldahl Nitrogen and 56% for Nitrate-Nitrogen using recycled shredded-tire chips as filter media. Recently, studies have focused on alternative adsorbents to remove organic pollutants (Estevinho et al., 2006). In that sense, cork waste showed a clear adsorbent ability related to its chemical composition. Suberin is the major component of cork cell walls and is the responsible for most of their properties related to its adsorption capacity of organic pollutants (Domingues et al., 2007; Zhou et al., 1995).

Several methods have been used to study the microbial communities attached to the granular media in TW. However, molecular techniques are the most applied method in the study of environmental samples. The use of these techniques leads to a progress in the determination, characterization and counting of microbial communities (Ferrera and Sánchez, 2016b; Sánchez, 2017).

In this work, the nitrate-nitrogen removal in groundwater polluted from agricultural activities using a cork or gravel hybrid (vertical and horizontal) subsurface flow Treatment wetland was studied along 12 months. The project aimed at using TWs to treat groundwater polluted by nitrates from agricultural activities to mitigate the environmental impact generated, focusing in the characterization of the microbial communities involved in the process. Microbial communities were further investigated by applying Illumina sequencing of the 16S rRNA gene, a method that provides thousands of sequence reads. Additionally, the presence of denitrifiers was quantified using a quantitative molecular approach (qPCR).

The project called for the establishment of treatment wetland built under the framework of the REAGRITECH LIFE project ("Regeneration and reuse of runoff and drainage water in agricultural plots by combined natural water treatment systems"; LIFE+11 ENV/ES/579).

2. MATERIALS AND METHODS

2.1 Site Description

Nitrate vulnerable areas were identified. The site is located in the regions of Urgell and Segarra-Garrigues channels (Lleida, Catalonia). Water was characterized to compare to select the best locations. Additionally, parameters studied to select the sites included physical characteristics of the sites, slope.

From the characterizations, two sites were selected, one at Vilanova de la Barca and the other in Bellvís, municipalities at the Lleida plain area, where two hybrid TWs were established in areas close to irrigated agricultural plots, where ground water extraction was used for irrigation.

2.2 Treatment System

The Hybrid Treatment Wetland used in the study was a combination of a VF followed by a HSSF treatment wetland. The sizing of both prototypes was done with the first order model PKC*, according to Kadlec and Wallace (2009). The goal for water treatment was established to treat a maximum of 750 L d^{-1} influent, and to obtain effluents with Nitrate-Nitrogen (NO_3-N)

concentrations below 10 mg L^{-1}. This value was established by Ayers and Westcot (1985) as a standard for water used in agricultural irrigation

The system was designed as a compact, modular and mobile system in two 20 ft. shipping containers that could be transported and installed at different sites. The modularity enabled the treatment of higher loadings if needed, by adding more modules (Gallegos et al., 2016).

The TWs were built using Open Top shipping containers to host the filter media. A close container was used as a control rooms, where the components of the hydraulic, electric and automation equipment were installed. The walls and the roof of the control unit were externally coated with cork plates and planted with autochthonous vegetation to improve thermal insulation on the field.

The system is fitted with hydraulic controls and electronic modules that enabled the remote operation and control via website, which allowed flexible control of the operation of the system, including loading, recirculation of water among all treatment stages at different loading rates, to evaluate various loading operational schemes and their removal performance.

The open container was divided in two sections by welding a reinforced steel structure inside the container to fit the vertical/horizontal treatment wetlands, creating two compartments that were calculated to withstand the pressure from water and filter media. The system was impermeabilized with a HDPE (high-density polyethylene) covered with a geomembrane to protect against damages. For the VF, on the bottom of the bed to evacuate treated waters, a collection manifold embedded in a 20 cm coarse gravel (10-20 mm) layer and built from 100 mm Ø perforated high-density PVC pipe network was present. The distribution system consisted of a 50 mm perforated pipes distributed on the top of the bed. For the HFFS the distribution system was built from 100 mm pipes located in one end while the collection system, built from 100 mm pipes is located on the opposite and bottom.

Cork byproduct, rejected from the cork industry, was used as filter media for the Bellvís hybrid system and washed granitic gravel and sand for the Vilanova hybrid system (Table 1). An insulating top gravel layer was placed on the filter media to prevent cork from floating at the Bellvis prototype. All treatment wetlands were planted with *Phragmites australis*, with 4 plants per m^2 density.

Table 1. Granular media used at the Bellvis and Vilanova de la Barca treatment wetlands.

Cell	Area (m^2)	Layer	Depth (m)	BELLVIS		VILANOVA	
				Media	Ø (mm)	Media	Ø (mm)
VF	5.5	Drainage	0.2	Gravel	25 - 40	Gravel	25 - 40
		Filter	1.0	Cork	16	Sand	5-7
		Insulating	0.2	Gravel	25 - 40	Cork	16
HSSF	8.2	Filter	0.8	Cork	3 - 7	Gravel	25 - 40
		Insulating	0.2	Gravel	25 - 40	Cork	16

Groundwater is supplied was by means of submersible pumps installed at a depth of 5 m to a two-chambered sedimentation tank (ST) as pre-treatment. Once water is sedimentated, the pre-treated water was loaded to the VF and after, to the HSSF. After the water was treated it was discharged as irrigation of a vegetated buffer strip (Figure 1).

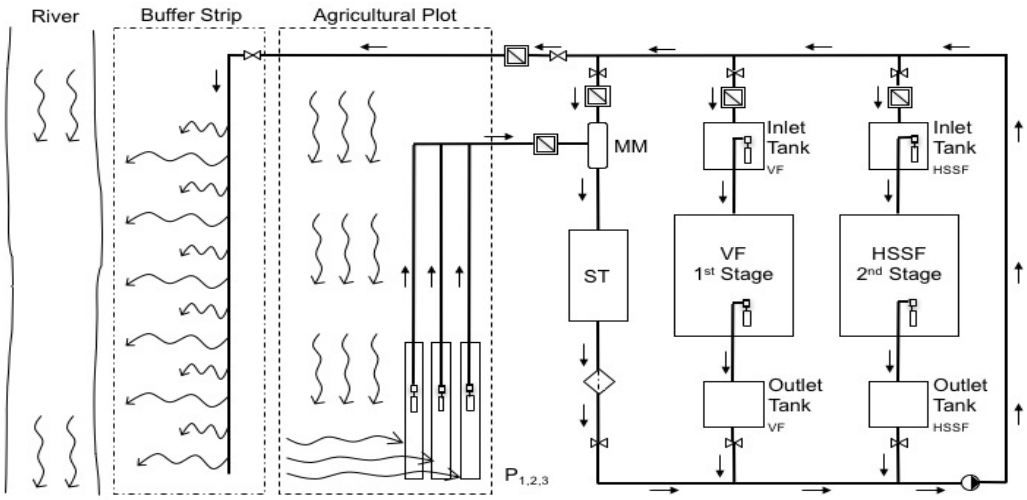

Figure 1. Functional diagram of the Bellvís and Vilanova treatment wetlands, with the groundwater extraction pumps (P), the sedimentation tank (ST) and the VF system in the first stage and the HSSF in the second stage.

2.3 Sample Collection

Grab samples were taken from the groundwater (influent water) and from the effluent of each treatment wetland (Vertical Wetland: VF_Out; Horizontal Subsurface Flow Wetland: HSFF_Out). The samples were taken on monthly basis, from July to December 2016, three consecutive days campaigns according to the for groundwater sampling procedures established by the Catalan Water Agency (2005) and the UNE-EN ISO 5667-1 and 3 (2007, 2004). The water samples were collected in 1 L sterile plastic bottles and transported under refrigeration (4°C) to the laboratory for water analysis.

Cork and gravel samples of were collected from the bed media of the vertical and horizontal wetlands from Vilanova and Bellvis. Filter media sampling was carried out from October 2016 to January 2017. A total of 24 samples were collected (n=24). Samples were taken from three points along the length of the horizontal wetland depending namely the beginning of the TW (BEG), the middle (MID) and at the end of the bed (END). In contrast the vertical wetland was sampled along the depth, namely Top (0 to 0.2 m depth), Middle (0.25 to 0.8 m depth) and the Bottom of the wetland.

Approximately 200 g of gravel and 40 g of Cork were sampled in 500 mL sterile glass bottles containing 250 mL of PBS 1X (Phosphate Buffer Saline, 130 mM NaCl, 10 mM NaH_2PO_4/Na_2HPO_4, pH 7.2). The bottles were stored at 4°C to avoid drying and cellular lysis.

2.4 Physico-Chemical Analyses

The water quality parameters measured included *in situ* measurements of water temperature, oxygen saturation and electric conductivity by means of calibrated electrodes. Samples were immediately transported under refrigeration to the LEITAT laboratory for further analysis. Additional water quality parameters were evaluated following Standard Methods included COD (APHA 5200 B), BOD_5 (APHA 5210 B), total nitrogen (Kjeldhal method), nitrates (APHA 4500-NO_3 F), nitrites (APHA 4500 NO_2 B), ammonia nitrogen (APHA 4500-NH_3 D), phosphorus (APHA 4500-

P B), total suspended solids (APHA 2540 D), turbidity (APHA 2130 B), conductivity (APHA 2510 B), pH (APHA 4500-H^+ B) and alkalinity (APHA) (APHA, 2012).

2.5 Microbial community analyses

For microbial community analyses, tag sequencing of the 16S rRNA gene and real-time PCR assays from DNA attached to filter media were performed in order to assess the bacteria population structure and identify the main microorganisms involved, and to quantify two of the key functional genes for denitrification: *nirS* and *nosZ*.

2.5.1 DNA extraction

To obtain the biofilm DNA, filter media samples were sonicated for 3 minutes in an ultrasonic bath (Selecta Group). The supernatant was centrifuged at 4000 rpm for 8 minutes in a Medifriger Centrifuge (Selecta Group) to concentrate the detached biofilm sample (Adrados et al., 2014). DNA extraction from biofilm samples was performed using the DNeasy Power Soil Kit (Qiagen) according to the manufacturer's instructions. DNA concentration and purity were measured using a Nanodrop spectrophotometer at 260 nm and 260/280 nm, respectively. DNA extracts were conserved at -20°C until further analyses.

2.5.2 Amplicon Sequencing

Illumina sequencing was performed in 17 out of the 24 original samples by the Research and Testing Laboratory (Lubbock, TX, USA; www.researchandtesting.com). Two primers were used to amplify bacterial 16S rRNA gene: (1) 341F (5'-CCTACGGGNGGCWGCAG-3') and (2) 805R (5'-GACTACHVGGGTATCTAATCC-3') (Herlemann et al., 2011). Illumina MiSeq 2 x 250 flow cells were used following protocols described elsewhere (Cúcio et al., 2016). Sequence data was processed as described in Ferrera et al. (2016a). Briefly, pair-end sequence reads underwent a quality filter and were merged using PEAR (Zhang et al., 2014). Then, sequences were clustered into operational taxonomic units (OTUs) at 97% cutoff using USEARCH (Edgar, 2013). De novo chimera were done using the UCHIME algorithm (Edgar et al., 2011). Chimeric sequences and singleton OTUs (those represented by a single sequence) were removed. Taxonomic assignment of bacterial OTUs was performed using the RDP Classifier (Cole et al., 2014). Sequence data has been submitted to the Genbank Sequence Read Archive under BioProject ID number PRJNA449332.

2.5.2 qPCR

Three bacterial strains with the studied genes were selected for qPCR standard curves determination: *Escherichia coli* NCTC 9001, *Pseudomonas aeruginosa* CECT110, and *Ralstonia eutropha* (*Cupriavidus necator* DSM 545°) (Chon et al., 2011). Bacteria were cultivated in TSB medium at 37°C. The DNA was extracted from a culture of each strain with the v-DNA reagent (GenIUL). DNA absorbances at 260 and 280 nm were measured with a spectrophotometer to determine DNA concentration for each sample as well as DNA purity, respectively.

The next step was a conventional PCR with the Horse-PowerTM Taq DNA Polymerase mix (Canvax Biotech, S.L.). The set of primers used are specified in Table 2. The final volume was of 20 µL, 1 µL for each primer, 0.2-10 µL of template DNA depending on sample concentration, 0.2 µL of Taq polymerase, and 2 µL of both 25 mM MgCl2 and 8 mM dNTPs. The cycling program used was: 94°C for 5 min followed by 29 cycles at 95°C for 30 sec, the Tm for 30 sec and 72°C for 1 min, a final step at 72°C for 10min and 4°C ∞.

The PCR was followed by an agarose gel electrophoresis of the PCR product. The gel was dyed with ethidium bromide for half an hour and the amplicon band was visualized, cut off and

purified with the Illustra GFX PCR DNA and Gel Band purification kit (GE Healthcare). Finally, the absorbance at 260nm for the amplicon of the gene of interest was measured, and the number of copies was calculated.

To elaborate the standard curves, a series of dilutions was performed for each sample. The dilutions were from 10^{10} to 10^{1}.

Table 2. Primers used for conventional 16S r RNA gene PCR and qPCR of *nirS* and *nosZ*.

Gene	Forward primer	Reverse primer	Tm	Amplicon length	References
16S rRNA	ATG GCT GTC GTC AGC T	ACG GGC GGT GTG TAC	52°C	352 bp	(Chon et al., 2011)
nirS	TAC CAC CCS GAR CCG CGC GT	GCC GCC GTC RTG VAG GAA	64°C	164 bp	(Chon et al., 2011)
nosZ	AGA ACG ACC AGC TGA TCG ACA	TCC ATG GTG ACG CCG TGG TTG	63°C	474 bp	(Scala & Kerkhof, 1998)

Real-Time PCR assays were carried out in order to quantify the key functional genes *nirS* and *nosZ* using primers nirS2F/ nirS3R and nosZF/nosZR, respectively. Reactions were performed in a Light Cycler 1.5 (Roche-Applied) according to the manufacturer's instructions using Eva Green (5x HOT FIREPol® EvaGreen®qPCR Mix Plus/Solis BioDyne, Estonia) based detection.

A final volume of the reaction was 20 µL, 0.3 µL for each primer were added, 4 µL of the HOT FIREPol® EvaGreen® mix and 5 µL of DNA template, the rest was PCR water. The cycling programme was: 95°C for 12 min followed by 45 cycles at 95°C for 15 sec, the Tm for 20 sec, 72°C for 20 sec and a last step at 85°C for 15 sec. All reactions were finished with a melting curve and a final step at 40°C for 20sec.

2.5 Statistical Analyses

Analysis of variance (ANOVA) was performed to compare the nitrate removal and the number of gene copies versus the material (cork or gravel) variable. Student T tests were performed to compare the averages of the variables versus material. Statistical analyses were performed using the Minitab® 18 software. Before further analyses, the original data of the three gene abundances was logarithmically transformed; hence it was approximated to a normal distribution necessary to apply a parametrical test. On the other side, a Pearson correlation coefficient was performed to compare and to define if a correlation existed between the removal % variable versus the number of gene copies.

Sequence statistical analyses were performed using the R statistical software (R Developement Core Team, 2015) and the packages *vegan* and *venneuler*. Alpha- and betadiversity analyses were performed using an OTU abundance table that was previously subsampled down to the minimum number of reads in order to avoid artifacts due to an uneven sequencing effort among samples. For alphadiversity analyses, we calculated the Chao1 index as a measure of richness and the Shannon index as diversity metrics. Differences in microbial composition (betadiversity) were assessed using hierarchical clustering of Bray-Curtis dissimilarity matrices and the Unweighted Pair Group Method with Arithmetic Mean algorithm (UPGMA), as well as non-metric multidimensional scaling (nMDS) plots.

3. RESULTS AND DISCUSSION

3.1 Nitrogen Removal Efficiency

Physico-chemical results from the groundwater (Table 3) showed low concentration of organic matter for both pilot locations, with the exception of November in Bellvís, where values of 72 mg L^{-1} and 30.5 mg L^{-1} were measured for COD and BOD_5 respectively. TN and NO_3-N in groundwater were higher in Vilanova (7.1-18.9-mg L^{-1}, where gravel was used as filter medium) than in Bellvis (1.8-11.9-mg L^{-1}, cork as filter medium). NO_2-N and NH_4-N concentrations for both locations were lower than 0.1 mg L^{-1} and 1.9 mg L^{-1}, respectively. During first months of groundwater quality monitoring, nitrate-nitrogen values were higher than 10 mg L^{-1}, limit suggested by Ayers and Westcot (1985). The values clearly decreased after August for both locations.

Table 3. Hydraulic loading rate (HLR) (litres per day) and physico-chemical results from groundwater (Influent) analysed at different pilot plant locations (Mean ± SD, n=19 for HLR and n=3 for physic-chemical results).

Pilot location	Parameter	Month					
		Jul	Aug	Sep	Oct	Nov	Dec
Vilanova	HLR (l d^{-1})	400 ± 16	400 ± 7	600 ± 12	600 ± 19	600 ± 23	700 ± 14
Gravel	COD (mg l^{-1})	< 30	< 30	31 ± 0.2	< 30.0	33 ± 2.3	< 30
(Vg)	BOD_5 (mg l^{-1})	< 30.0	< 30.0	< 30.0	< 30.0	< 30.0	< 30.0
	TN (mg l^{-1})	23 ± 0.1	19 ± 0.4	14 ± 0.2	12 ± 0.3	9.7 ± 0.4	9.4 ± 1.1
	NO_3-N (mg l^{-1})	19 ± 0.1	16.9 ± 0.8	9.8 ± 0.1	7.6 ± 0.2	8.4 ± 0.5	7.1 ± 1.1
	NO_2-N (mg l^{-1})	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	0.4 ± 0.1
	NH_4-N (mg l^{-1})	< 1.9	< 1.9	< 1.9	< 1.9	< 1.9	< 1.9
	P (mg l^{-1})	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0
	TSS (mg l^{-1})	< 5.0	< 5.0	< 5.0	6.7 ± 0.9	< 5.0	< 5.0
	pH	7.4 ± 0.1	8.1 ± 0.1	---	7.6 ± 0.1	7.9 ± 0.0	7.7 ± 0.1
	Conductivity (S/cm)	3.1 ± 0.0	2.8 ± 0.1	---	2.6 ± 0.0	2.6 ± 0.1	0.9 ± 0.0
	Alkalinity (mmol h+l^{-1})	---	3.6 ± 0.7	5.0 ± 0.0	7.4 ± 0.2	6.0 ± 0.2	8.2 ± 0.1
	Turbidity (NTU)	12 ± 2.6	2.7 ± 0.4	3.4 ± 0.7	3.8 ± 0.8	1.2 ± 0.5	2.5 ± 0.8
Bellvis	HLR (l d^{-1})	400 ± 22	500 ± 36	700 ± 41	600 ± 11	700 ± 13	700 ± 23
Cork	COD (mg l^{-1})	< 30.0	< 30.0	< 30.0	33.5 ± 3.1	72.0 ± 36.0	< 30.0
(Bc)	BOD_5 (mg l^{-1})	< 30.0	< 30.0	< 30.0	< 30.0	30.5 ± 0.3	< 30.0
	TN (mg l^{-1})	15 ± 1.0	10 ± 0.1	6.7 ± 0.7	< 5.0	< 5.0	5.3 ± 0.2
	NO_3-N (mg l^{-1})	12 ± 0.5	7.9 ± 0.2	5.2 ± 0.1	2.3 ± 0.2	1.8 ± 1.6	2.9 ± 1.4
	NO_2-N (mg l^{-1})	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
	NH_4-N (mg l^{-1})	< 1.9	< 1.9	< 1.9	< 1.9	< 1.9	< 1.9
	P (mg l^{-1})	< 1.0	< 1.0	< 1.0	< 1.0	1.5 ± 0.4	< 1.0
	TSS (mg l^{-1})	< 5.0	< 5.0	< 5.0	5.7 ± 0.3	9.5 ± 3.5	8.8 ± 1.0
	pH	7.6 ± 0.10	8.0 ± 0.2	7.5 ± 0.0	7.2 ± 0.3	7.9 ± 0.1	7.7 ± 0.1
	Conductivity (S/cm)	2.3 ± 0.1	2.2 ± 0.1	2.2 ± 0.0	3.8 ± 0.6	3.7 ± 1.0	1.6 ± 0.2
	Alkalinity (mmol h+l^{-1})	---	5.5 ± .01	5.5 ± 0.1	6.6 ± 0.4	6.6 ± 0.8	6.9 ± 0.2
	Turbidity (NTU)	18 ± 8.9	2.2 ± 0.4	5.6 ± 1.2	1.6 ± 0.3	25 ± 18.3	2.3 ± 0.9

A stabilisation period from April to June was carried out with a HLR of 500 L d^{-1} per pilot hybrid treatment wetland. For both treatment wetlands 5 pulses of 100 L per day of groundwater were pumped to the VF cell with resting periods of 10 minutes between each pulse. The Hydraulic Retention Time (HRT) for the HSSF cell was two days.

After the stabilisation period and due to low organic matter contents in the influent groundwater, the HLR was increased during next months. These operational parameters were maintained from July to December 2016, using "n" pulses of 100 L d^{-1}, according to the HLR. The Hydraulic Retention Time (HRT) for the HSSF cell was maintained for two days.

In the first month (July), groundwater was only treated in the HSSF, to enhance the microbiological activity in this treatment bed. From August to October, the pilots were operated in hybrid mode, with a VF bed followed by the HSSF bed. With this configuration, an increase of TN and NO_3-N was reported at Vilanova's VF effluent. On the contrary, all reported parameters showed a consistent decrease in Bellvís for each stage of the treatment. During the following months (November and December), the influent was treated only using the HSSF bed (Figure 2).

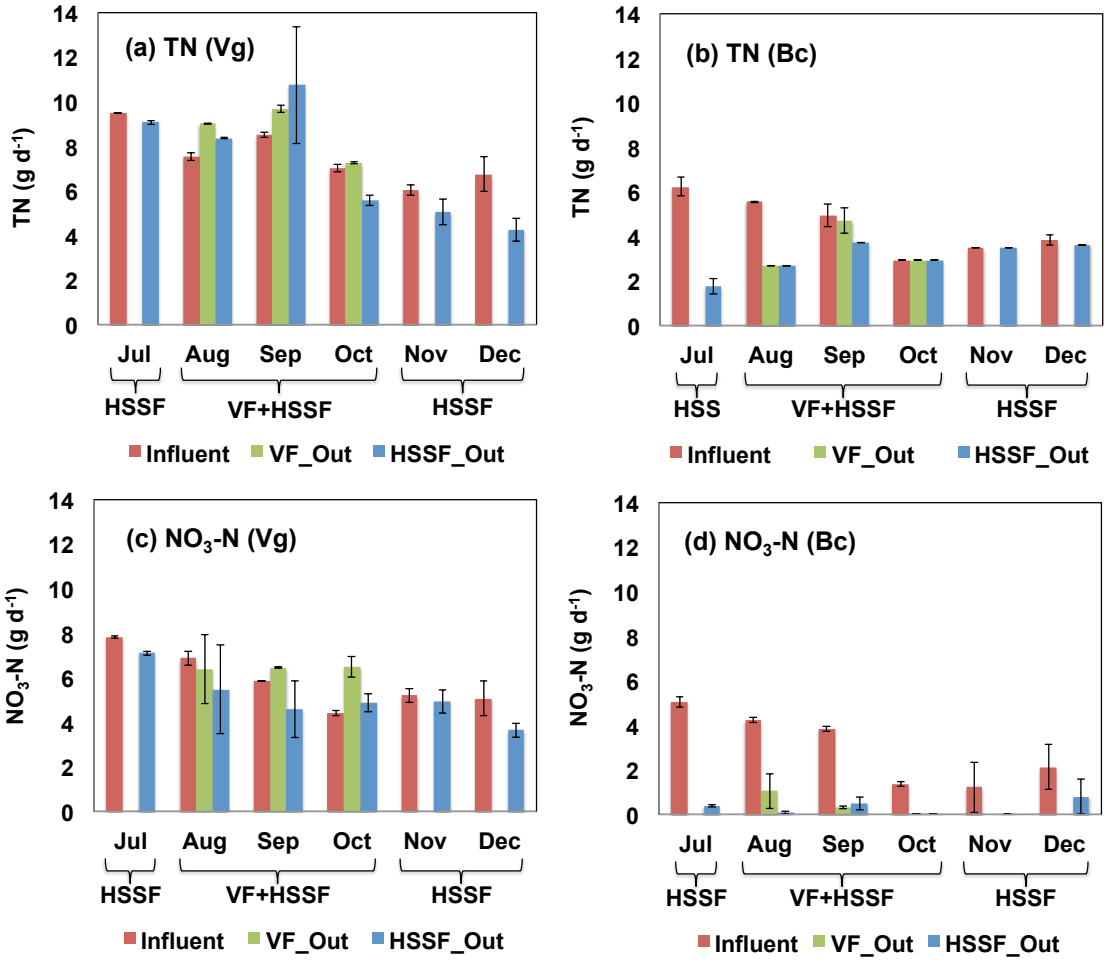

Figure 2. Evolution of TN and NO_3-N in Bellvís using cork (b,d) and Vilanova de la Barca using gravel (a,c).

During the first months of operation, an increase of organic matter and a brown water colour was reported in the effluent at each stage on Bellvis treatment wetland, due to the washing of thecork process. COD and BOD_5 values and the intensity of water colour started to decrease at the end of the experiment (Figure 3). At Vilanova, using gravel as filter medium, COD and DBO_5 values were lower than the detection limit.

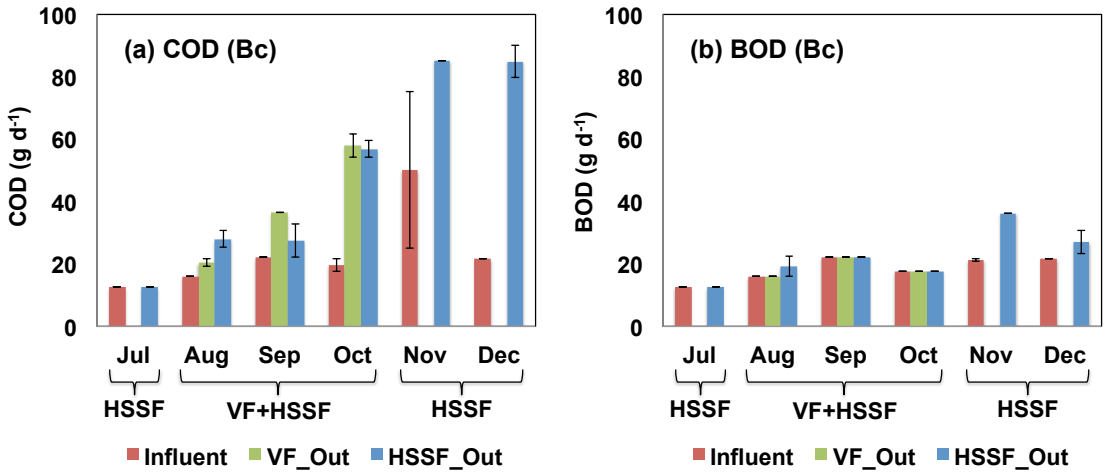

Figure 3. Evolution of COD (a) and BOD_5 (b) in Bellvís treatment wetland using cork; the results show the effect of organic release from cork particles.

The experimental design allowed the comparison of nitrate removal efficiency depending on the filter media material (Figure 4). A significant (p=0.0025) higher removal in Bellvís TW using cork (Bc) compared with the Vilanova de la Barca gravel system (Vg) was observed, achieving a removal rate from 80 to 99% compared to the 5-46%, respectively (Figure 5). The NO_3-N concentrations obtained from Vilanova and Bellvis effluents were always below 10 mg L^{-1}.

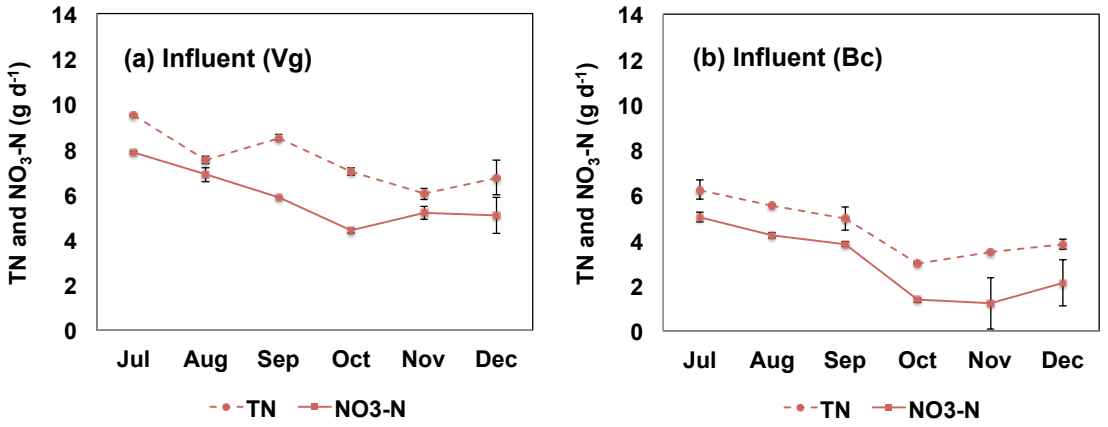

Figure 4. Influent and effluent (HSSF_Out) results of TN and NO_3-N at the Bellvis (b,d) and Vilanova (a,c) Hybrid TWs filled with different granular media, cork (Bc) and gravel (Vg) respectively.

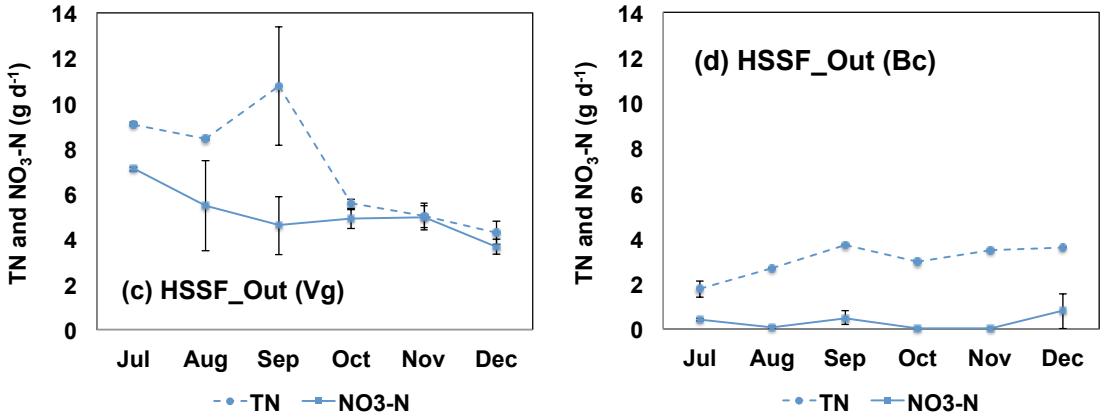

Figure 4.Cont. **Influent and effluent (HSSF_Out) results of TN and NO_3-N at the Bellvis (b,d) and Vilanova (a,c) Hybrid TWs filled with different granular media, cork (Bc) and gravel (Vg) respectively.**

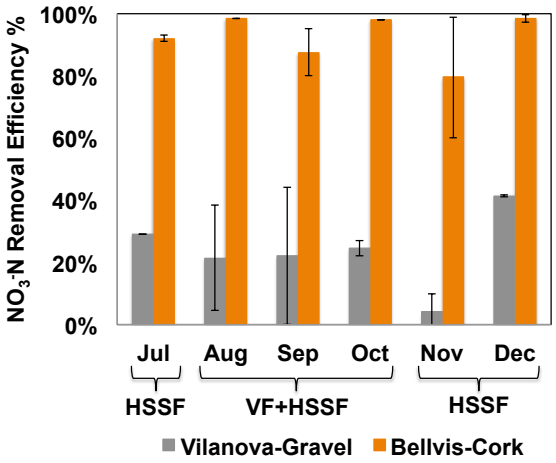

Figure 5. **Removal efficiency of nitrate-nitrogen in Vilanova (gravel) and Bellvis (cork) pilots.**

The operation of the system, as hybrid wetland (vertical followed by a horizontal wetland) or as horizontal wetland, the type of the filter medium used (gravel or cork) and the nitrates load were the most important parameters that affected the performance of the systems as well as the water quality. The treated water was used to irrigate the vegetation of buffer strips, which had been used as a complementary system for the control and improvement of groundwater.

3.2 Microbial Community Analyses

3.2.1 Community Structure

Differences in microbial composition (betadiversity) between samples over time and at different positions of the TWs were assessed for the two locations (Vilanova de la Barca and Bellvís), which differed in the composition of the filter material (gravel and cork, respectively). To infer the variation of bacterial assemblages, the Bray-Curtis dissimilarity index was used on community composition. Dissimilarity matrices were constructed based on the relative abundance of each OTU. Representation of hierarchical clustering revealed that the communities mainly grouped according to the filter material, with the exception of three samples, one of them corresponding to Vilanova de la Barca (gHMIXOCt) and the other two to Bellvís (CVMIDNov, CVMIDJan), which clearly separated from the rest (Figure 6).

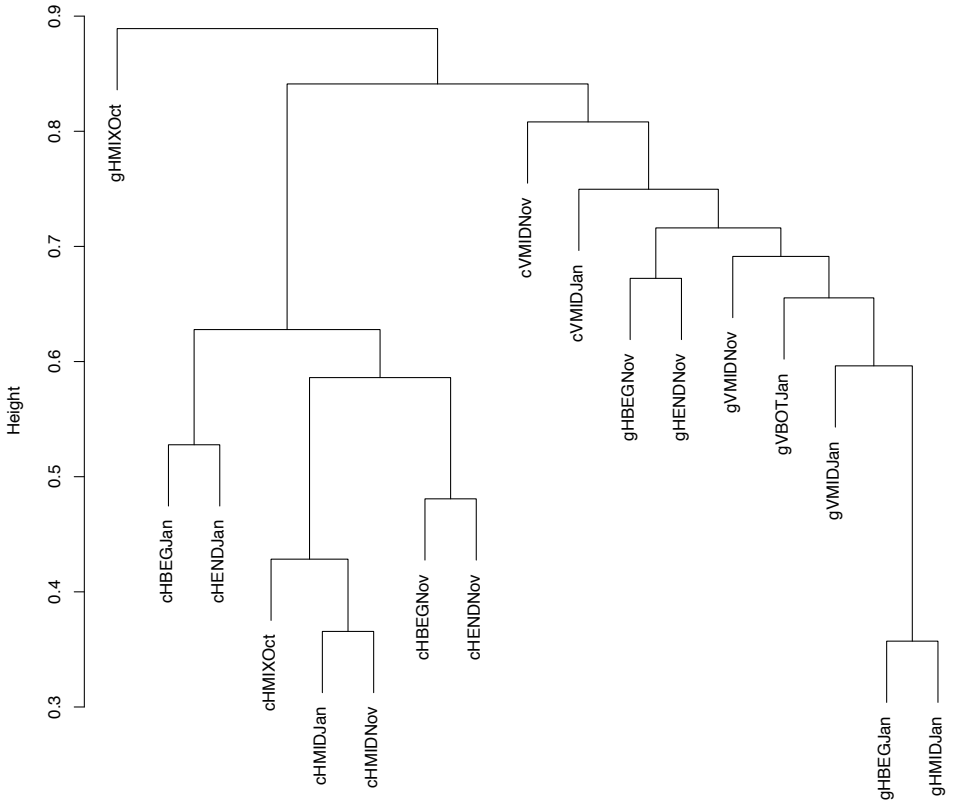

Figure 6. Hierarchical clustering of biofilm samples in the Vilanova de la Barca TW (using gravel) and the Bellvís TW (using cork) along time. c: cork, g: gravel, H: horizontal TW, V: vertical TW, BEG: sample taken at the beginning of the TW, MID: sample taken at the middle of the TW, END: sample taken at the end of the TW, MIX: mixture of samples from different positions of the TW, BOT: sample taken at the bottom of the TW, Oct: October 2016, Nov: November 2016, Jan: January 2017.

Interestingly, all the samples that grouped together corresponding to cork were collected from the HSSF TW, while those samples with this filter material collected from the VF TW separated in another cluster and contained different communities. On the other hand, those samples with gravel as filter material (Vilanova de la Barca TWs) grouped together regardless of the type of TW (horizontal or vertical), excluding sample gHMIXOct, completely separated from the rest. Visualization of Bray-Curtis dissimilarities between samples using nMDS plots clearly showed again that, with the exception of gHMIXOct, samples grouped together by filter material, indicating that it was key in selecting the community that develops in the biofilms of TWs (Figure S1).

3.2.2 Community Diversity and Taxonomy

A total of 755,785 high-quality sequences were obtained, with an average of 44,458 sequences per sample (minimum 31,262, maximum 88,960). Curated sequences were clustered into 8,962 different operational taxonomic units (OTUs; 1017-3084 per sample, average 1806) using a 97% cutoff, which is the standard value for clustering related phylotypes of bacterial 16S rRNA gene sequences (Gevers et al., 2005). These data suggest that thousands of bacterial species can colonize these surfaces. From those, 30.4% of OTUs were shared between samples, which differed in the filter material (cork and gravel) (Figure 7). However, the proportion of shared OTUs (2,722 out of 8,962) represented 70% of the reads.

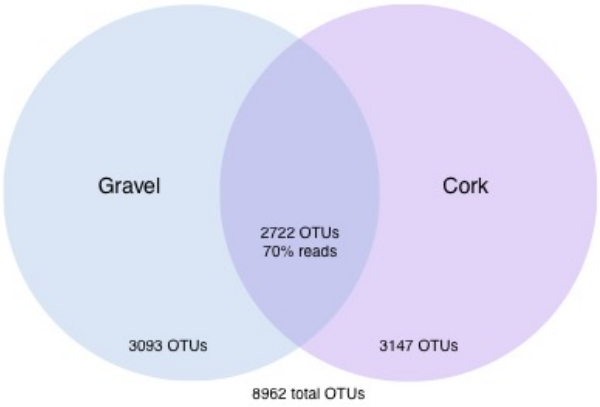

Figure 7. Venn diagram of shared OTUs between biofilm samples in the Vilanova de la Barca TW (using gravel) and the Bellvís TW (using cork).

Most bacterial sequences were related to the phylum Proteobacteria (average of all bacterial dataset, 54%), particularly to the classes Alpha- (22.3%), Beta- (10.4%) and Gammaproteobacteria (15.5%). Delta- and Epsilonproteobacteria were also present, but at lower relative abundances (average of 3.6 and 2.1% respectively for all bacterial dataset). Members of the phyla Acidobacteria, Actinobacteria, Bacteroidetes, Chloroflexi, Firmicutes, Planctomycetes and Verrucomicrobia were also abundant (>1%) (Figure 8), while other groups such as the Aquificae, Armatimonadetes, Candidatus Parcubacteria, Candidatus Saccharibacteria, Chlamidiae, Chlorobi, Cyanobacteria, Deferribacteres, Deinococcus-Thermus, Elusimicrobia, Fibrobacteres, Fusobacteria, Gemmatimonadetes, Ignavibacteriae, Lentisplaerae, Nitrospirae, Omnitrophica, Oligoflexia, Spirochaetes, Synergistetes, Tenericutes and Thermotogae were represented mainly by rare OTUs (<1%), and are grouped as 'Other groups' in Figure 8 to ease visualization. At a broad taxonomic level, all groups shown in Figure 8 developed in either filter media (cork and gravel), although at different proportions and with different compositions at the OTU level.

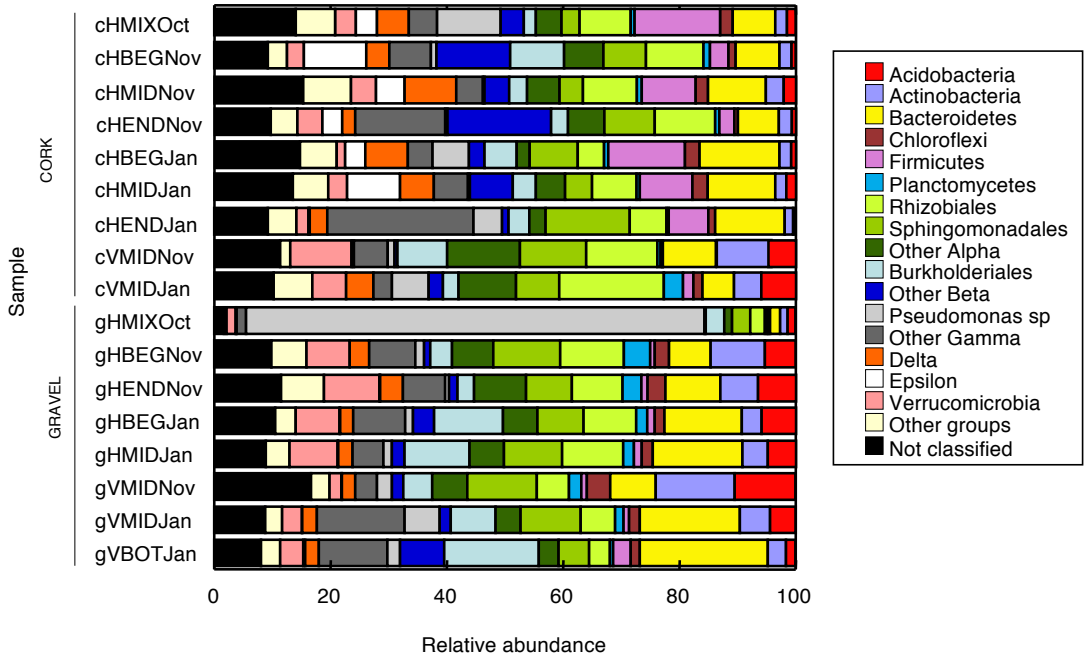

Figure 8. Bar graphs showing the proportions of the major taxonomic groups (>1% frequency in at least one sample) based on the relative abundance of the Illumina sequences of biofilm samples in the Vilanova de la Barca TW (using gravel) and the Bellvís TW (using cork) along time; c: cork, g: gravel, H: horizontal TW, V: vertical TW, BEG: sample taken at the beginning of the TW, MID: sample taken at the middle of the TW, END: sample taken at the end of the TW, MIX: mixture of samples from different positions of the TW, BOT: sample taken at the bottom of the TW, Oct: October 2016, Nov: November 2016, Jan: January 2017.

Remarkably, one of the samples, corresponding to Vilanova de la Barca (gHMIXOct), exhibited a large amount of sequences belonging to the genus *Pseudomonas* sp. (79%), a well-known denitrifier from the Gammaproteobacteria. In fact, denitrification is a widespread ability in diverse phylogenetic lineages, and different phototrophic, lithoautotrophic, and chemoorganotrophic microorganisms can perform this process (Zumft, 1997). Numerous genera of bacteria, like *Alcaligenes, Pseudomonas, Methylobacterium, Bacillus, Paracoccus, Hyphomicrobium, Ralstonia, Azospirillum, Magnetospirillum, Halomonas, Roseobacter, Thiobacillus, Azoarcus, Comamonas, Aquitalea, Rhodobacter, Aeromonas, Vibrio*, as well as members of the order Rhodocyclales among others, are able to carry out denitrification (Hosselhoe et al., 2009; Wagner et al., 2002; Zumft, 1997) and they were present in the different samples of this study.

Furthermore, it was detected the presence of sequences belonging to *Anaeromyxobacter dehalogenans*, a bacterium that could catalyze the reduction of N2O to N2 using an atypical nitrous oxide reductase (Sandford et al., 2012), or the occurrence of *Nitrosomonas* sp., a proteobacterial ammonia oxidizer which can denitrify when grown under oxygen limitation (Bock et al., 1995). These results highlight the major role of microbes on the removal of nitrate in the hybrid TWs of this work.

In general, these findings are in agreement with previous studies reporting the population composition of different samples from TWs, such as soil or sediment (Ansola et al., 2014; Ligi et al., 2008; Zhao et al., 2015), rhizosphere (Bai et al., 2014; Lünsmann et al., 2016), lagoon water (Elsayed et al., 2014; Ibekwe et al., 2016), inlet and outlet water (Abed et al., 2014), manure influent (Ibekwe et al., 2016), or biofilms from substrate particles (He et al., 2016; Wang et al., 2016; Zhao et al., 2015) and vegetation (Zhang et al., 2016), which showed a permanent dominance of the phylum Proteobacteria, including members of the classes Alpha-, Beta-, Gamma-, Delta- or Epsilonproteobacteria, although in different proportions depending on the conditions (Sánchez, 2017). Within this group, different microorganisms have been found to be key players in the nitrogen cycle of TWs, including the Betaproteobacteria *Nitrosomonas* and *Nitrosospira*, and the gammaproteobacterium *Nitrosococcus* (aside from *Nitrosococcus mobilis*, a betaproteobacterium), which are ammonia oxidizers (Schmidt, 2003) and have also been retrieved in this work.

Other microorganisms playing a role in the nitrogen cycle, such as nitrite oxidizing bacteria like the genera *Nitrobacter* (Alphaproteobacteria), *Nitrococcus* (Gammaproteobacteria) and *Nitrospira* (Nitrospirae) have been well documented in different wastewater treatment systems (Wagner et al., 2002; Wang et al., 2016). Remarkably, sequences of *Nitrobacter* and *Nitrospira* have been recovered from our dataset. However, little is known about the diversity and ecological role of these bacteria involved in nitrification processes in complex communities. Recent metagenomic studies reported the existence of a complete set of nitrification genes

(*amo*, *hao*) in both soil and water samples of a TW, mainly associated to *Nitrosomonas eutropha* (Bai et al., 2014).

On the other hand, the phylum Bacteroidetes is likewise often reported to be abundant in TWs (Wang et al., 2016; Sánchez, 2017). On average, it constituted 10.5% of all bacterial dataset of this study. Their members are known by their ability to degrade complex organic matter, and they are suggested to be strongly involved in denitrification processes from different TWs (Adrados et al., 2014). The most abundant genera retrieved in this study were *Bacteroides*, *Algoriphagus*, *Flavobacterium, Vitellibacter* and *Mucilaginibacter*.

When comparing the relative contribution of the biofilms in both filter media, some interesting trends could be observed. For example, there was a significant difference within the groups Acidobacteria, Firmicutes, Planctomycetes, and Delta- and Epsilonproteobacteria between both type of media (ANOVA, $p < 0.05$), being the relative abundance of Firmicutes, and Delta- and Epsilonproteobacteria significantly higher in the TW with cork, while the contribution of Acidobacteria and Planctomyces was superior in the TW with gravel. These findings suggest a remarkable role of filter material on the composition of microbial communities. The remaining groups did not show significant differences between both filter media ($p > 0.05$).

Actually, the influence of substrate type on TW microbial communities has already been reported in several works. Thus, Vacca et al. (2005) showed differences on rhizospheral microbial populations depending on filter material (expanded clay and sand), while Calheiros et al. (2009) observed that bacterial richness and community structure was affected by the use of different types of expanded clay aggregates and fine gravel. Using high-throughput sequencing methods, Guan et al. (2015) also demonstrated a clear effect of soil material on the different bacterial groups detected, and Li et al. (2010), comparing the microbial assemblages of eight types of substrate (steel lag, bio-ceramic, ceramic, gravel, vermiculite, shale, anthracite and zeolites), concluded that phospholipid fatty acid (PFLA) profiles exhibited significant differences among the diverse materials. Neverthless, other authors (Gorra et al., 2007) did not detect a clear effect of substrate (soil with marble sand, zeolite, magnetite, ceramic wastes, and gravel) on ammonia oxidizing bacteria populations.

On the other hand, when comparison was made contrasting the type of TW (horizontal or vertical), significant differences in the relative contribution of the different groups could be observed in Acidobacteria and Actinobacteria, being higher in the vertical TW ($p < 0.05$); conversely, only minor differences were found between the biofilms developed in horizontal and vertical TW for the remaining taxa. Thus, the design of the TW is also a key factor that influences the composition of microbial assemblages, at least for some groups. Arroyo et al. (2013) also observed that, besides plant presence, the type of flow (free water, FW, vs subsurface flow, SSF) seemed to be the main design parameter that increased efficiency to remove arsenic and zinc, being the removal of metals better in FW flow TWs. In this work, the Proteobacteria phylum, characterized by 16S rRNA gene amplification and cloning, was once again the most abundant group under all conditions tested. Furthermore, Sidrach-Cardona et al. (2015) also demonstrated that hydraulic configuration was crucial in shaping microbial communities in FW and SSF TWs. In contrast, Lin et al. (2008) concluded that there was no significant difference between both types of TWs concerning nitrogen removal.

3.2.3 Diversity Indices

In order to investigate whether the filter material had an influence on bacterial diversity, Chao1 and Shannon indices were determined, the Chao 1 index for richness, and the Shannon index for diversity estimation (Hill, 1973; Magurran, 1988; Chao and Lee, 1992) (Figure 9). Nevertheless, analysis of variance showed no significant differences between systems for any of the indices tested. Shannon index varied between 5.9 to 6.9 for gravel samples (with the exception of sample gHMIXOct, with a value of 2.3), and values for cork samples ranged between 5 and 6.4. In general, the Shannon index for bacteria typically vary in wastewater treatment systems between 2.8 (aerated lagoons, Mehmood et al., 2009) to 7.8 (Treatment wetlands; Wang et al., 2016). The values obtained in this work were quite constant and fell within this range. On the other hand, Chao 1 fluctuated between 1092 and 3645 for gravel, and between 1,383 and 2,511 for cork.

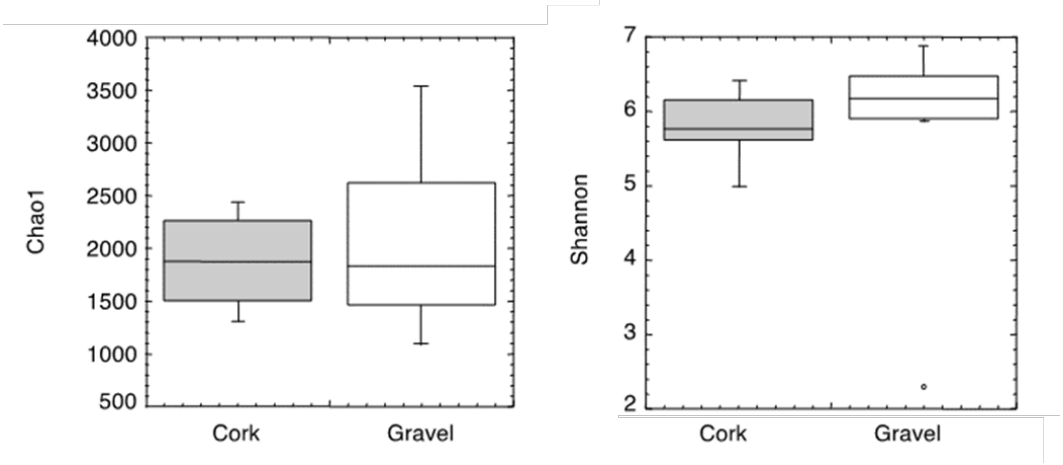

Figure 9. Box plots showing two estimates of alphadiversity (Shannon, Chao1) depending on the material of the filter media (Vilanova de la Barca TW - gravel and Bellvís TW – cork).

Rarefaction curves were also computed (Chao 1 richness estimate), normalizing the dataset at the minimum sequencing depth for comparative purposes (Figure S2). They were not saturated, indicating that the real diversity in the samples was likely higher.

3.3 Real-time PCR

3.3.1 Standar Curves

Standard curves were used as the reference to extrapolate and calculate the concentrations of environmental DNA samples. Standard curves for real-time PCR were established using diluted amplicon of 16S rDNA, *nirS* and *nosZ* genes resulting from PCR. All standard curves showed high correlation efficiencies and similar slopes (Figure S3).

3.3.2 Quantification of denitrifying genes: *nirS* and *nosZ* genes

The copy numbers for denitrifying *nirS* and *nosZ* genes in the two media, cork and gravel, were determined by real time-PCR (Figure 10). The results exhibited significant amounts for both denitrifying communities. *nosZ* showed higher significant levels (p=0.05) for cork system in Bellvis, whereas for *nirS* genes, differences were not significant (p=0.18). The results did not reveal significant differences from different treatments, HSSF and VF.

Cork is a natural product with a complex chemical composition, mainly composed of suberin, lignin, waxes and polysaccharides (cellulose and hemicellulose), which are structural components, but also includes other extractables such as tannins (Machado et al. 2017). From

our results, it seems clear that the available carbon sources from cork which can promote the denitrifying bacterial growth, could positively affect the presence of *nosZ* and *nirS* genes. In fact, with its anaerobic conditions, horizontal TW (HSSF) could favour the development and the growth of the denitrifying community (Vymazal 2013).

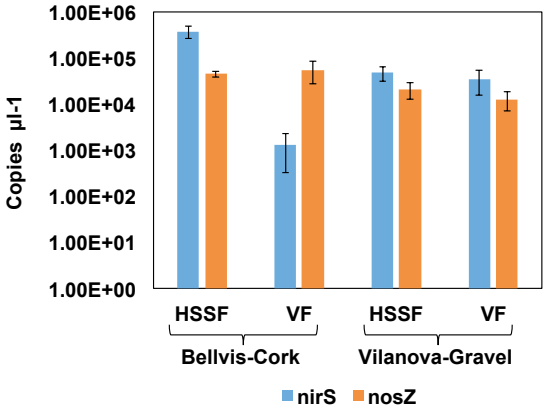

Figure 10. *NirS* and *nosZ* copy numbers for the different treatment wetlands in Bellvís (cork) and Vilanova (gravel).

The comparison between two filter media, cork and gravel, showed that cork could be a good granular media for treatment wetlands for nitrate removal. İn fact, both results, physicochemical and microbial analysis were consistent and confirm the nitrate removal efficiency using cork as a filter media.

4. CONCLUSIONS

Bellvís' TW with cork as filter media showed higher nitrate removal than Vilanova's TW filled with gravel suggesting that cork could be an alternative material to remove TN and minimize the impact in the environment caused by nitrogen contamination in groundwater bodies.

Regarding the community composition of the two different TWs, microorganisms were mainly related to the phylum Proteobacteria, and included members found to be key players in the nitrogen cycle, such as ammonia and nitrite oxidizers, as well as denitrifiers. These results are in agreement with previous studies reporting the population analysis o f different samples of TWs. Also, the group Bacteroidetes turned to be another abundant phylum from our bacterial dataset, whose members are suggested to be strongly involved in denitrification processes. Nonetheless, some groups showed to prevail depending on the type of media (cork or gravel); Firmicutes and Delta and Epsilonproteobacteria had a significant higher abundance in the TW with cork, while Acidobacteria and Planctomyces were prevalent in gravel. Besides the filter material, the type of TW (horizontal or vertical) also played a role in structuring microbial assemblages.

The results from our work show that cork filled treatment wetlands could be an appropriate technology to treat and/or remediate nitrate polluted groundwater from agricultural activities. As a result, a new approach using natural technologies for diffuse pollution remediation can be efficiently used in river basin areas, improving at the same time the circular economy of agricultural activities, increasing water and nitrogen fertilizers reuse, and, finally, improving the ecological quality of river basin.

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SUPPLEMENTARY FIGURES

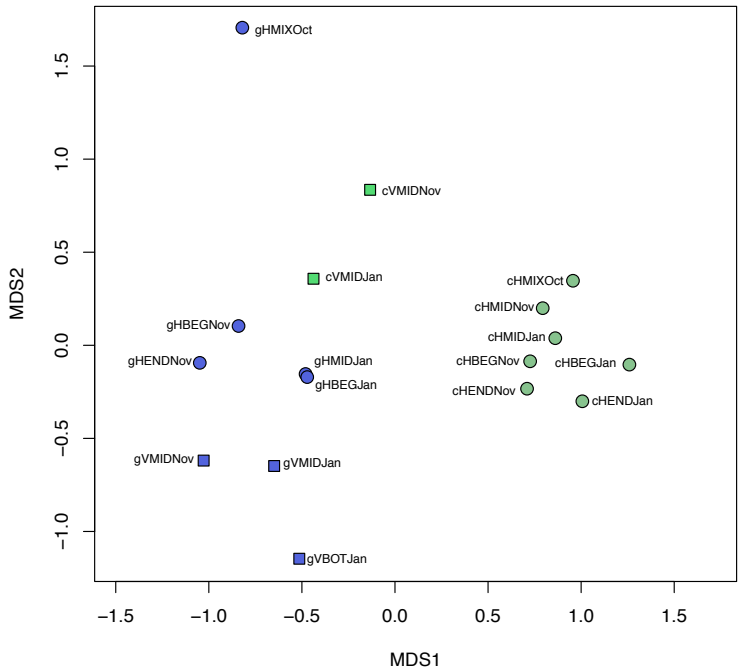

Figure S1. Non-metric multidimensional scaling (nMDS) plot of biofilm samples in the Vilanova de la Barca TW (using gravel) and the Bellvís TW (using cork) over time. The different colors indicate the two locations (blue: Vilanova de la Barca, green: Bellvís), while the different shapes refer to the type of TW (squares: VF, circles: HSSF); c: cork, g: gravel, H: horizontal TW,

V: vertical TW, BEG: sample taken at the beginning of the TW, MID: sample taken at the middle of the TW, END: sample taken at the end of the TW, MIX: mixture of samples from different positions of the TW, BOT: sample taken at the bottom of the TW, Oct: October 2016, Nov: November 2016, Jan: January 2017.

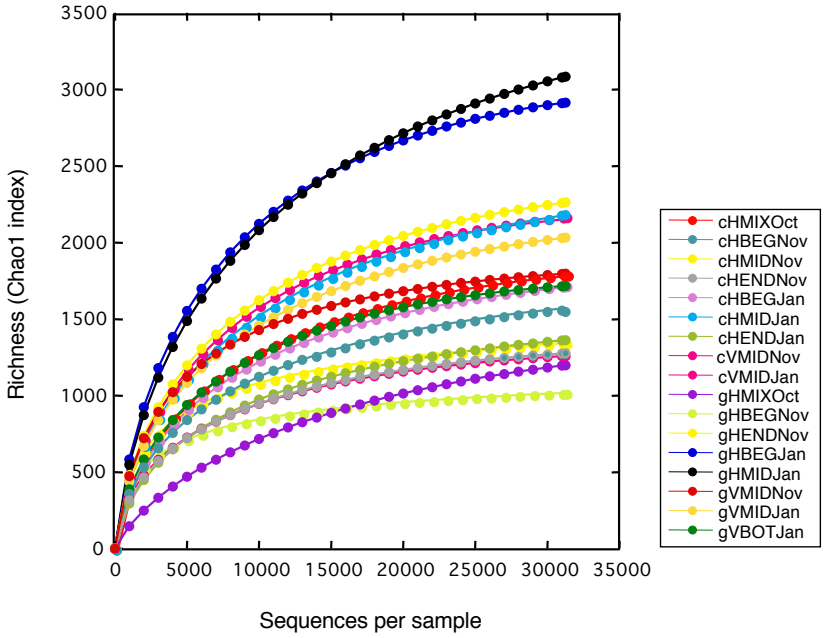

Figure S2. Rarefaction curves of 16S rRNA OTUs defined by 3% sequence variation of biofilm samples in the Vilanova de la Barca TW (using gravel) and the Bellvís TW (using cork); c: cork, g: gravel, H: horizontal TW, V: vertical TW, BEG: sample taken at the beginning of the TW, MID: sample taken at the middle of the TW, END: sample taken at the end of the TW, MIX: mixture of samples from different positions of the TW, BOT: sample taken at the bottom of the TW, Oct: October 2016, Nov: November 2016, Jan: January 2017.

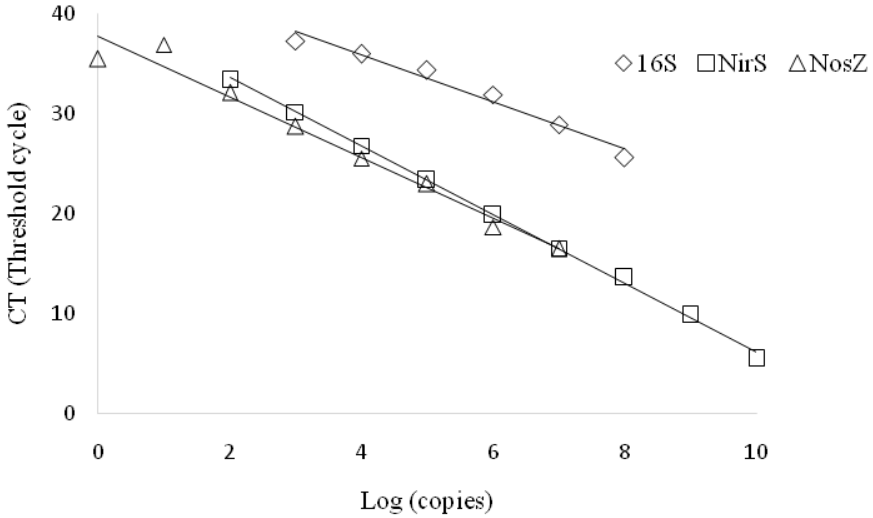

Figure S3. Standard curves of 16S rDNA, *nirS*, and *nosZ* assays obtained by calculated gene copy numbers versus threshold cycle. *nirS*: y=-3,43x + 40,45, R2 : 0,9987; *nosZ*: y=-3,03x + 37,70, R2 : 0,972 16S: y=-2,35x + 45,25, R2 : 0,9703.