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

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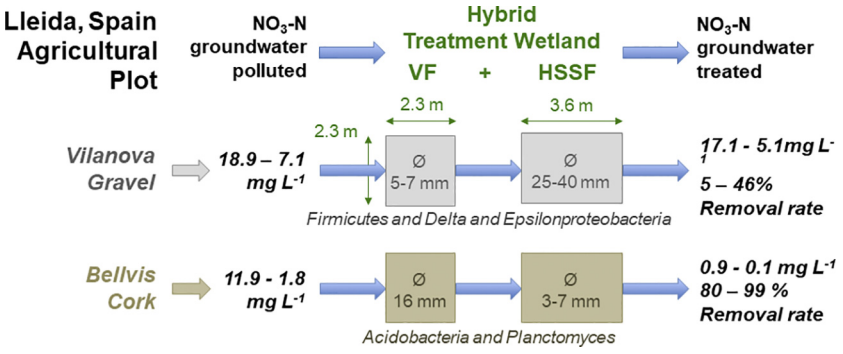
HIGHLIGHTS

- A higher nitrate removal efficiency (80-99%) using cork as substrate was observed.
- Microbial community groups showed to prevail depending on the type of granular filter media.
- The relative abundance of Firmicutes and Delta and Epsilonproteobacteria were significantly higher in the TW with cork.
- The contribution of Acidobacteria and Planctomyces microorganism communities were superior in the TW with gravel
- Cork treatment wetlands could be appropriate to treat nitrate polluted groundwater from agricultural activities.

GRAPHICAL ABSTRACT



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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 concentration 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

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Microbial communities
Denitrifiers
High-throughput sequencing

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* turns 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.

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 -especially 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 fertilizers 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). Microbial processes play a basic role in the removal of different pollutants in treatment wetlands (Truu et al., 2009; Chang et al., 2015). 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 where considerable concentrations of nitrogen compounds are to be removed. Removal of a particular pollutant is typically associated with a specific microbial functional group (Faulwetter et al., 2009), such as nitrogen removal which is primarily facilitated by microorganisms via the nitrification-denitrification processes (Song et al., 2015). The two most important nitrate removal mechanisms, nitrification followed by denitrification, takes place simultaneously in the filter media of TWs if the conditions are adequate. When oxygen transport and availability in the wetland is limited, nitrification will be hindered, affecting the overall total nitrogen removal as well. However, denitrification can be very efficient even with low carbon levels (Platzer, 1999).

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, 2016; Sánchez, 2017).

Several studies on the microbiology of denitrifiers have focused on bacteria, which are generally believed to be the dominant denitrifying microorganisms in most environments (Allenstein et al., 2006). Denitrification consists of four consecutive reaction steps in which nitrate is reduced to dinitrogen gas (Chon et al., 2011a, 2011b), involving four enzymes: nitrate reduction, nitrite reduction, nitric oxide reduction, and nitrous oxide reduction. The genes that codify for the enzymes involved in denitrification have each been used as targets for molecular methods to characterize the composition of denitrifier communities (Allenstein et al., 2006).

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 and subsequent denitrification. 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 and 50%, depending on the type and operation scheme, with loading removal rates ranging between 250 and 630 g 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 et al. (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 shows 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).

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) 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. Nevertheless, 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.

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 et al., 2003). 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).

On the other hand, the phylum *Bacteroidetes* is likewise often reported to be abundant in TWs (Wang et al., 2016; Sánchez, 2017). 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).

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 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 in the Lleida plain (northwest Catalonia, Spain). Two different areas were located near the Urgell and Segarra-Garrigues channels. Taking as reference the concentration of nitrates in the control stations surrounding the site, groundwater was characterized for comparison and select the best locations. Additionally, physical characteristics of the sites, such as available area, slope, accessibility and location were analyzed for final selection of the installation sites.

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 groundwater extraction was used for irrigation.

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.

2.2. Treatment system

The Hybrid Treatment Wetland used in the study was a combination of an unsaturated 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 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 enables the treatment of higher pollutant loadings if needed, by adding more modules (Gallegos et al., 2016).

The TW was built using an open top shipping containers and hosts the filter section. A close container was used as a control room, where the elements from the hydraulic, electric and automation equipment were installed. The containers were externally coated with cork plates and planted with autochthonous vegetation to improve thermal insulation of the TW and the control room for the extreme summer high temperatures.

The system was fitted with hydraulic controls and electronic modules that enabled the remote operation and control via website, which allowed a complete operation of the system, including loading, recirculation of water among all treatment stages at different loading rates. The automation permits the evaluation of various loading operational schemes and the 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 waterproofed with a high-density polyethylene (HDPE) liner covered with a geomembrane to protect against damages.

On the bottom of the VF bed, 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 located to evacuate treated waters. 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 was located on the opposite side and bottom.

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

Groundwater was supplied by means of submersible pumps installed at a 5 m depth, to a two-chambered sedimentation tank (ST) as pre-treatment. The pre-treated water after sedimentation tank was loaded to the VF and after, to the HSSF. Treated water was discharged to irrigate a vegetated buffer strip. In all cases pumps were used for water loading (Fig. 1).

A stabilization 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 min between each pulse. The hydraulic retention time (HRT) for the HSSF cell was two days.

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

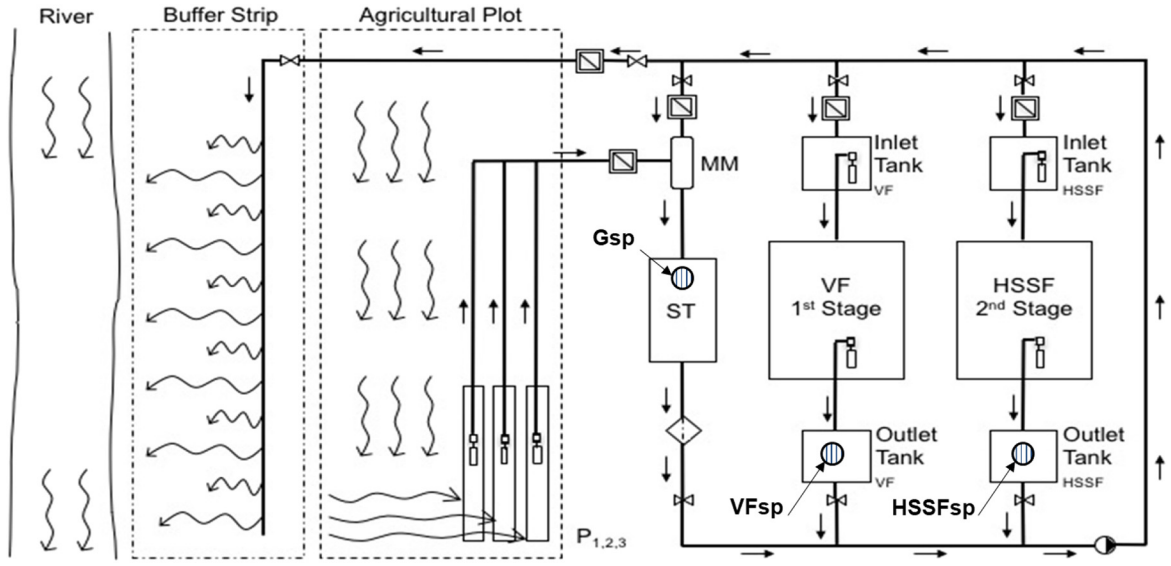

Fig. 1. Functional diagram of the Bellvís and Vilanova treatment wetlands, with the groundwater extraction pumps (P), the sedimentation tank (ST) and the VF system in the first stage and the HSSF in the second stage.

After the stabilization period and due to the 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.

2.3. Sample collection

Grab samples were taken from the groundwater (influent water, Gsp) and from the effluent of each treatment wetland, Vertical Flow Treatment Wetland (VFsp) and Horizontal Subsurface Flow Treatment Wetland (HSSFsp). All sampling points are shown in Fig. 1. Both systems were sampled from July to December 2016, on a monthly basis campaigns, three consecutive days per month (n = 18 per each sampling point) according to the groundwater sampling procedures established by the Catalan Water Agency (2005) and the UNE-EN ISO 5667-1 and 3 (UNE-EN ISO 5667-1, 2007, UNE-EN ISO 5667-3, 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 were collected from the vertical and horizontal wetlands bed media in 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 at 0.2 m depth 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 at 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 1× (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. Additionally, grab water samples were taken and immediately transported under refrigeration to the LEITAT laboratory for further analysis. The water quality parameters 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, 2012).

2.5. Microbia l 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 min in an ultrasonic bath (Selecta Group). The supernatant was centrifuged at 4000 rpm for 8 min 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 × 250 flow cells were used following protocols described elsewhere (Cúcio et al., 2016). Sequence data was processed as described in Ferrera et al. (2016). 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.

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., 2011a, 2011b)
nirS	TAC CAC CCS GAR CCG CGC GT	GCC GCC GTC RTG VAG GAA	64 °C	164 bp	(Chon et al., 2011a, 2011b)
nosZ	AGA ACG ACC AGC TGA TCG ACA	TCC ATG GTG ACG CCG TGG TTG	63 °C	474 bp	(Scala and Kerkhof, 1998)

2.5.3. 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., 2011a, 2011b). 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 s, the Tm for 30 s and 72 °C for 1 min, a final step at 72 °C for 10 min 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 260 nm for the amplicon of the gene of interest was measured, and the number of copies was calculated.

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

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 (5× 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 s, the Tm for 20 s, 72 °C for 20 s and a last step at 85 °C for 15 s. All reactions were finished with a melting curve and a final step at 40 °C for 20 s.

2.6. 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 Development 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

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

Pilot location	Parameter	Month					
		Jul	Aug	Sep	Oct	Nov	Dec
Vilanova gravel (Vg)	HLR (l d^{-1})	400 ± 16	400 ± 7	600 ± 12	600 ± 19	600 ± 23	700 ± 14
	COD (mg l^{-1})	<30	<30	31 ± 0.2	<30.0	33 ± 2.3	<30
	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 cork (Bc)	HLR (l d^{-1})	400 ± 22	500 ± 36	700 ± 41	600 ± 11	700 ± 13	700 ± 23
	COD (mg l^{-1})	<30.0	<30.0	<30.0	33.5 ± 3.1	72.0 ± 36.0	<30.0
	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 ± 0.01	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

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 only 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 the 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.

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 as a 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 (Fig. 2).

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 and leaching form the cork. COD and BOD_5 values and the intensity of water colour started to decrease at the end of the experiment (Fig. 3). At Vilanova, using gravel as filter medium, COD and BOD_5 values were below the detection limit.

The experimental design allowed the comparison of nitrate removal efficiency depending on the filter media material (Fig. 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 (Fig. 5). The NO_3-N concentrations obtained from Vilanova and Bellvis effluents were always below 10 mg L^{-1}.

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 at local scale.

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

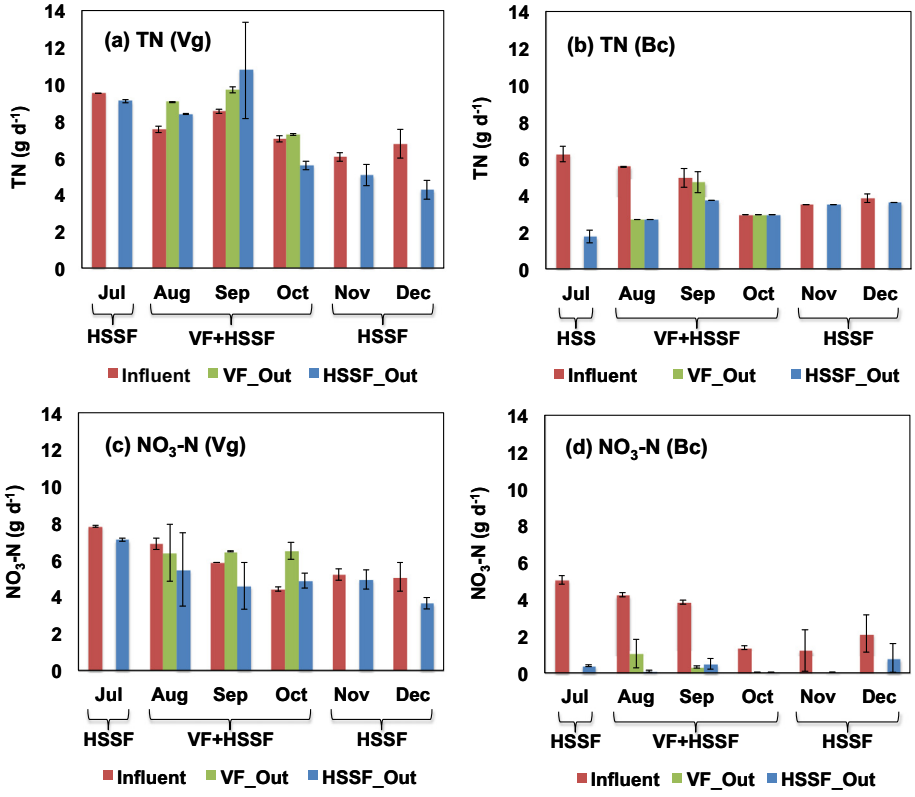

Fig. 2. Evolution of TN and NO_3-N in Bellvís using cork (b,d) and Vilanova de la Barca using gravel (a,c).

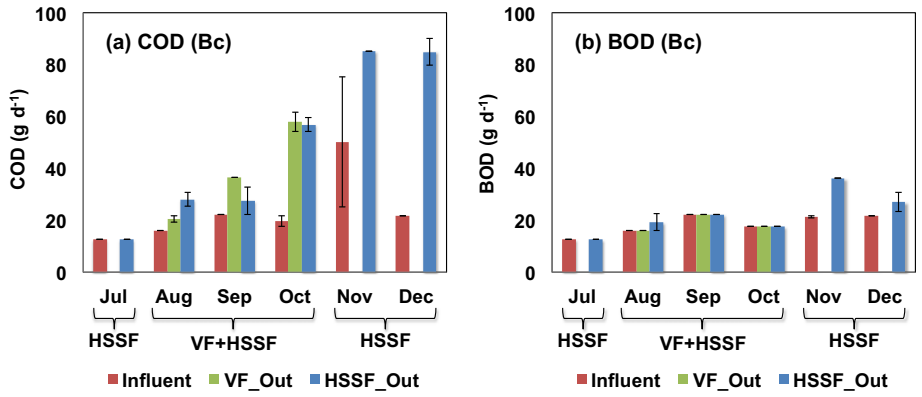

Fig. 3. Evolution of COD (a) and BOD_5 (b) in Bellvís treatment wetland using cork; the results show the effect of organic release from cork particles.

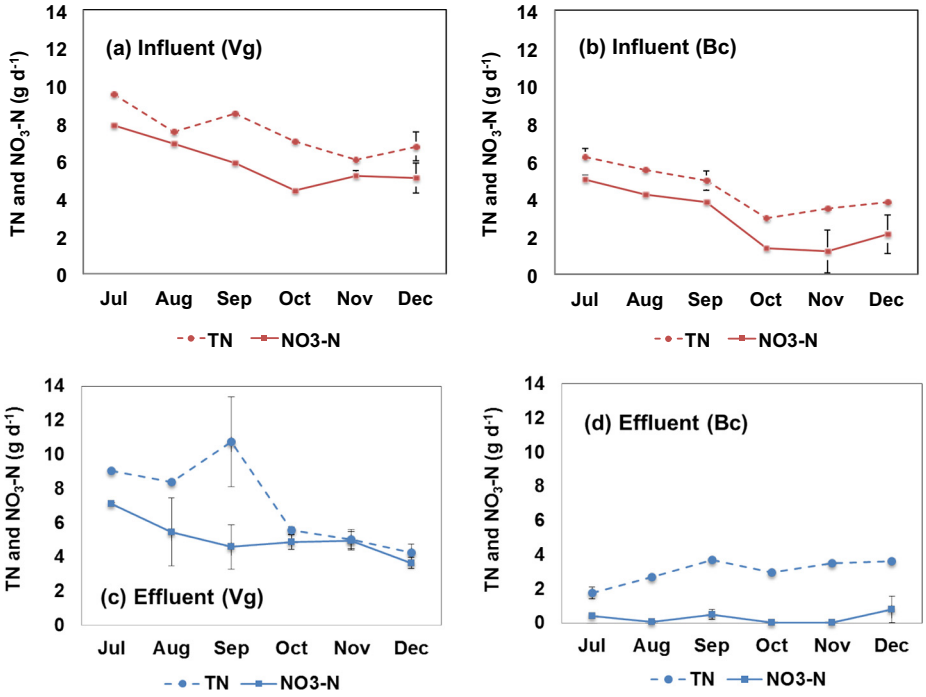

Fig. 4. HSSF Influent (a,b) and effluent (c,d) results of TN and NO_3-N at the Bellvis (b,d) and Vilanova (a,c) TWs filled with different granular media, cork (Bc) and gravel (Vg) respectively.

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 (Fig. 6).

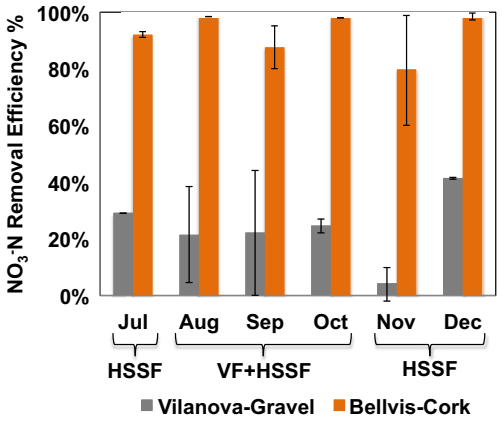

Fig. 5. Removal efficiency of nitrate nitrogen in Vilanova (gravel) and Bellvis (cork) pilots.

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 (Fig. 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 8962 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

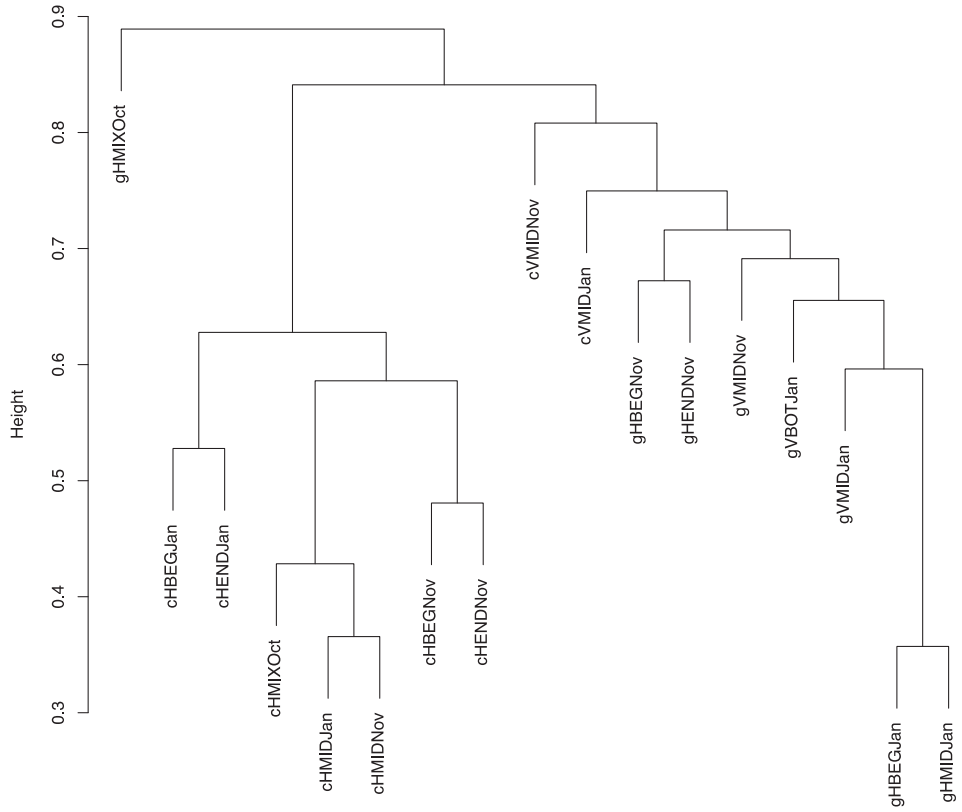

Fig. 6. Hierarchical clustering of biofilm samples in the Vilanova de la Barca TW (using gravel) and the Bellvís TW (using cork) along time. c: cork, g: gravel, H: horizontal TW, V: vertical TW, BEG: sample taken at the beginning of the TW, MID: sample taken at the middle of the TW, END: sample taken 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.

samples, which differed in the filter material (cork and gravel) (Fig. 7). However, the proportion of shared OTUs (2722 out of 8962) represented 70% of the reads.

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%) (Fig. 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 Fig. 8 to ease visualization.

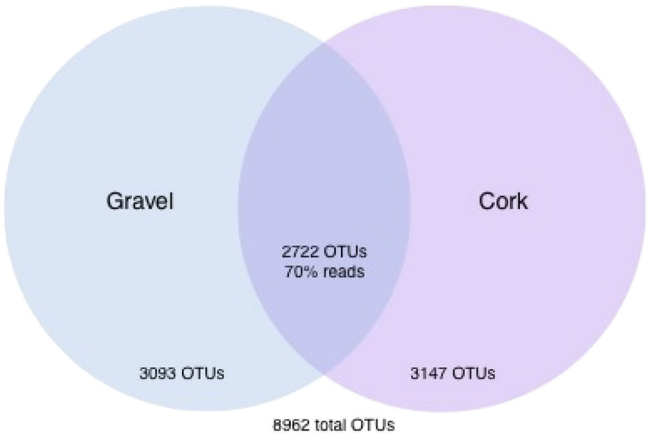

Fig. 7. Venn diagram of shared OTUs between biofilm samples in the Vilanova de la Barca TW (using gravel) and the Bellvís TW (using cork).

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, the presence of sequences belonging to *Anaeromyxobacter dehalogenans* was detected, a bacterium that could catalyze the reduction of N_2O to N_2 using an atypical nitrous oxide reductase (Sanford 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., 2014; Zhao et al., 2015), rhizosphere (Bai et al., 2014; Lünsmann et al., 2016), lagoon

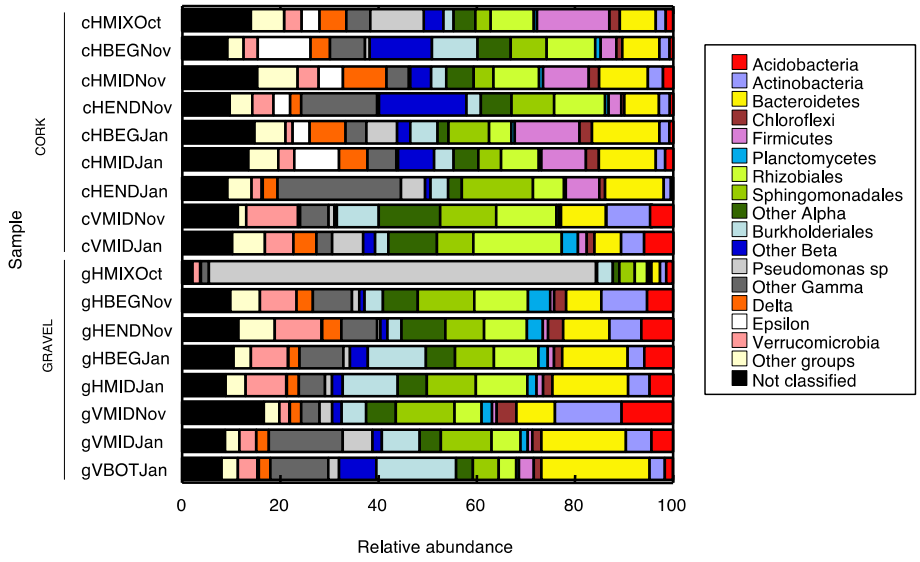

Fig. 8. Bar graphs showing the proportions of the major taxonomic groups (>1% frequency in at least one sample) based on the relative abundance of the Illumina sequences of biofilm samples in the Vilanova de la Barca TW (using gravel) and the Bellvís TW (using cork) along time; c: cork, g: gravel, H: 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.

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 et al., 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 ($p > 0.05$).

Considering the type and configuration of treatment wetland, Sidrach-Cardona et al. (2015) demonstrated that hydraulic configuration was crucial in shaping microbial communities in FW and SSF TWs. In contrast, Lin et al. (2008) concluded that there were no significant differences between both types of TWs concerning nitrogen removal. In our study, 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. 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.

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) (Fig. 9). Nevertheless, analysis of variance showed no significant differences between systems for any of the indices tested. Shannon index varied between 5.9 and 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 1383 and 2511 for cork.

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

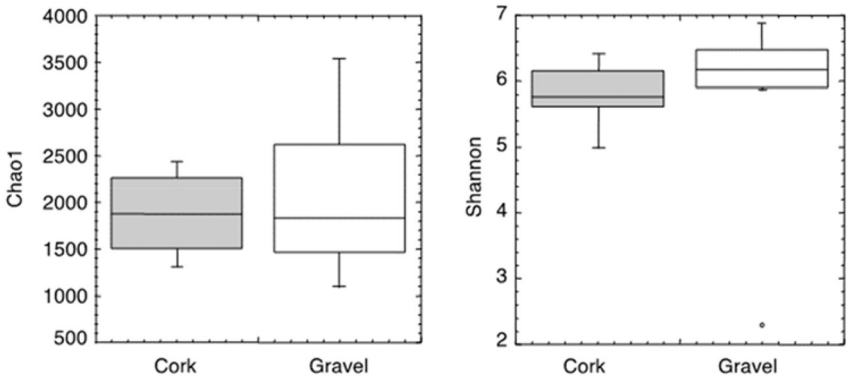

Fig. 9. Box plots showing two estimates of alphadiversity (Shannon, Chao1) depending on the material of the filter media (Vilanova de la Barca TW - gravel and Bellvís TW – cork).

3.3. Real-time PCR

3.3.1. Standard 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 (Fig. 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 (Fig. 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 include other extractable 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).

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.

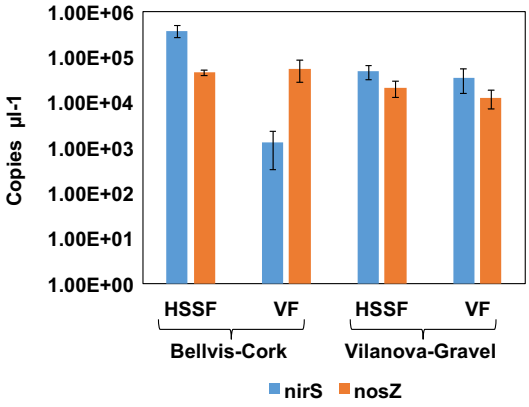

Fig. 10. *NirS* and *nosZ* copy numbers for the different treatment wetlands in Bellvís (cork) and Vilanova (gravel).

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 of 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.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2018.10.426.

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