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Effect of aeration on nitrogen removal-associated microbial community in an innovative vertical cork-based constructed wetland for winery wastewater treatment

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ABSTRACT

The wine industry produces large quantities of wastewater that often contains high levels of organic matter and nutrients, like nitrogen and phosphorous. In particular, nitrogen pollution can be harmful, even at low levels, since infants are vulnerable to nitrates in drinking water, and excess nitrogen can harm the health of soils and waterways. Herein, an innovative compact, modular, and mobile treatment wetland (TW) using cork by product as the only granular media was used to treat the final effluents from the Codorniu winery (Sant Sadurní d'Anoia, Spain). The TW was operated during two 5-month periods with or without intermittent induced aeration (40 min cycles, 90 L/min air flow rate). Nitrate (N-NO_3) and total nitrogen (TN) removal were higher (52.8% and 46.8%, respectively, $p < 0.05$) during TW operation under aerated-condition. Additionally, *q*PCR analysis of 16S rRNA, *nir*S and *nos*Z genes revealed that intermittent induced aeration facilitates N-NO_3 reduction by the stimulation of denitrifying bacteria in the TW biofilm (11.4% increase in *nir*S copies number/g cork sample, $p < 0.05$) as well as increasing the number of heterotrophic bacteria adhered to cork (25.5% increase in 16S rRNA copies number/g cork sample, $p < 0.05$). Moreover, SEM images demonstrated the suitability of cork as a resistant filter media for TW after long-term system operation (1.5 years). In conclusion, our results suggest that aeration improved nitrogen compounds removal compared to the non-aerated period, without affecting phosphorous elimination. Additionally, residual cork is presented here in a circular bioeconomy view, as a suitable filling media to treat winery wastewater that can provide additional carbon source to increase C/N rate stimulating denitrification, as well as a reliable organic substrate for biomass growth.

1. Introduction

Southern Mediterranean countries are the world's major cork producers. Currently, >200,000 Tn of cork oak are harvested each year in Portugal, Spain, Morocco and Algeria, were 90% of the cork oak forests are found (Sierra-Pérez et al., 2015). The EU's policy (Waste Framework Directive 1999/31/EC) aims to minimize the amount of cork waste dumped in landfills, while encouraging the reuse and the valorization of cork's by-products. According to the Fourth National Forest Inventory, Catalonia owns 124,132 ha of cork oak forest, representing 5.6% of the world surface. Nevertheless, the exploitable forest area in Catalonia is <30% of the total (Pasalodos-Tato et al., 2018).

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Winery industry is one of the most important industries worldwide, with great economic and social impacts. Although the industry is becoming more effective and environmentally aware, it still produces large amount of byproducts that generate environmental impact and consequently turns into a problem of economic dimensions (Gouvinhas et al., 2019). Wine production demands considerable amount of resources such as energy, fertilizers, organic supplements, and water, as a result of the agro-industrial practices. As a consequence, a considerable amount of wastewater is generated from washing equipment, bottles and purges from cooling towers that entails a significant challenge for the winery industry (Artiga et al., 2004). The effluent produced additionally to typical pollutants such as solids, organic matter a nutrient, contains different pollutants such as ethanol, sugars, organic acids, phenolic compounds, *etc.* (Petruccioli et al., 2002) that must be properly treated and disposed. Constructed wetlands showed to be an environmentally friendly technology, which can help reducing environmental impacts associated with winery wastewater treatment by treating winery waste on-site with low energy and chemicals consumption (Flores et al., 2019).

Treatment wetlands (TW) 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 (Vymazal, 2010; Wu et al., 2014). During system operation, plants uptake nutrients and metals and release organic matter and nutrients through its roots (*e.g.*, oxygen and exudates) that stimulates microbial processes who play a basic role in the removal of different pollutants (Zhu et al., 2014; Chang et al., 2015). TW have been used successfully to treat urban, agricultural, and even industrial wastewaters (Kadlec and Wallace, 2008). Depending on the type of TW selected, hydraulic flow, loading rates depth of the filter and pollutants, will determine the operational conditions in the reactor favoring aerobic and anaerobic environment. Since oxygen (O_2) transfer rates can become a limiting factor that will hinder performance, intensification of TW *via* aeration has the potential of increasing capacity, while reducing the area footprint. Besides, intermittently-aerated TW has the potential for simultaneously host aerobic and anoxic conditions that can improve total nitrogen (TN) removal (Aguilar et al., 2021). Accordingly, the use of an aerated system will increase the performance since O_2 supply up regulates the microbial metabolism bringing a greater biodegradation potential for removing different contaminants (Li et al., 2021).

Nitrogen removal is primarily facilitated by microbial nitrification and denitrification processes (Song and Liu, 2015). Some discrepancies between nutrients plant uptake and water column nutrient levels in TW can be attributed to microbial-mediated nitrogen losses (*e.g.*, denitrification). However, more studies are still needed in order to assess the influence of different factors on the microbiological aspects governing nitrogen removal in TW (*e.g.*, organic and nitrogen load, presence/absence of plants, type of plant used, aeration, *etc.*) (Toro et al., 2019).

Nitrification is often the main mechanism to transform ammonium, therefore dissolved oxygen (DO) availability can increase and optimize biochemical processes resulting in more effective nitrogen elimination in TWs (Dinakar et al., 2020). In a mesocosm study, aeration increased DO but nitrogen and phosphorus removal from the water column decreased compared to unaerated systems (Garcia Chance and White, 2018). Moreover, oxygen transfer rate (OTR) has a significant impact on the design and operation of vertical flow TW intended for simultaneous organic matter removal and nitrification.

On the other hand, denitrification consists of four consecutive reaction steps in which nitrate (NO_3^-) is reduced to dinitrogen gas (N_2) (Chon et al., 2011) involving four enzymatically-mediated processes: nitrate reduction, nitrite (NO_2^-) reduction, nitric oxide (NO) reduction, and nitrous oxide (N_2O) reduction. Several studies on the microbiology of denitrifiers have focused on bacteria, which are generally believed to be the dominant denitrifying microorganisms in most environments including TW (Wallenstein et al., 2006).

Several methods have been used to study the microbial communities attached to the granular media in TW, although molecular techniques are the most suitable and powerful methods to analyzed complex environmental samples. Functional gene expression of nitrifying and denitrifying populations combined with the active microbiome diversity brought new insights on the microbial nitrogen cycling occurring within TW biofilms under different operational conditions (Pelissari et al., 2018). Moreover, 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 (Wallenstein et al., 2006).

In a previous work (Aguilar et al., 2019), using two hybrid TW (a vertical flow cell followed by a horizontal subsurface flow cell), we demonstrated a significant higher nitrate removal efficiency for cork-based treatment system (80–99% removal rate) when compared to a gravel system (5–46% removal rate). Therefore, it is proven that cork could be an alternative filling material used by TW to minimize the environmental impact caused by nitrogen pollution in receiving water bodies. In this work, a new innovative vertical TW using cork as the filtering material was established to treat a real winery wastewater. Additionally, aeration was introduced into the system and nitrogen removal was studied for 2 years focusing on the analysis of the denitrifier communities using a quantitative molecular approach (*q*PCR).

2. Materials and methods

2.1. Site Description

Codorniu is the second oldest wine factory in Spain (1551) and the 10th oldest in the world. Codorniu winery is located at Sant Sadurní d'Anoia, and focused on the production of Cava (sparkling wine) and wines. Different volumes of winery wastewater were generated depending on the period of the year. During the harvest, each variety is collected at its right moment of maturation, beginning with Chardonnay in August and the other varieties in September including Viura, Sauvignon Blanc, and Verdejo. The highest flow of wastewater effluent is generated during harvesting (from August to October) with 250 m^3 of wastewater/d, followed by November and December, when 150 m^3/d wastewater are produced. The lowest flow was generated from January to July, at a flow of 110 m^3/d rate. Codorniu treats its winery effluents in their own wastewater treatment plant (WWTP), consisting of a wastewater accumulation reservoir from which pumping is carried out to three biological and decantation lines that work in parallel. The biological lines consist of an activated sludge supporting by an online system for dosing flocculants and coagulants to accelerate sedimentation.

2.2. Treatment System

An innovative aerated vertical flow subsurface treatment wetland with a 14 m^2 surface was established to treat the final effluents from the Codorniu WWTP. The sizing of the TW was performed using a first order model PKC*, according to Kadlec and Wallace (2008). The system was designed as a compact, modular, and mobile plant in two 20 ft. (6.10 m long x 2.44 m wide x 2.59 m high) shipping containers that could be transported and installed at different sites (Fig. 1A). The modularity enables the treatment of higher pollutant loadings if needed by adding more modules (Gallegos et al., 2016).

The TW was an evolution from a previous design (Aguilar et al., 2019), and was constructed using an open top shipping container, waterproofed with a high-density polyethylene (HDPE) liner, that hosts a 1.4 m depth cork filter layer. The system also included a closed container for the control room, where the elements from the hydraulic, electric, and automation equipment were installed. The filtering media consisted of a 0.2 m drainage layer of 32 mm Ø granite gravel located at the bottom, followed by a 1 m deep layer of Ø 7 mm cork granulates, and

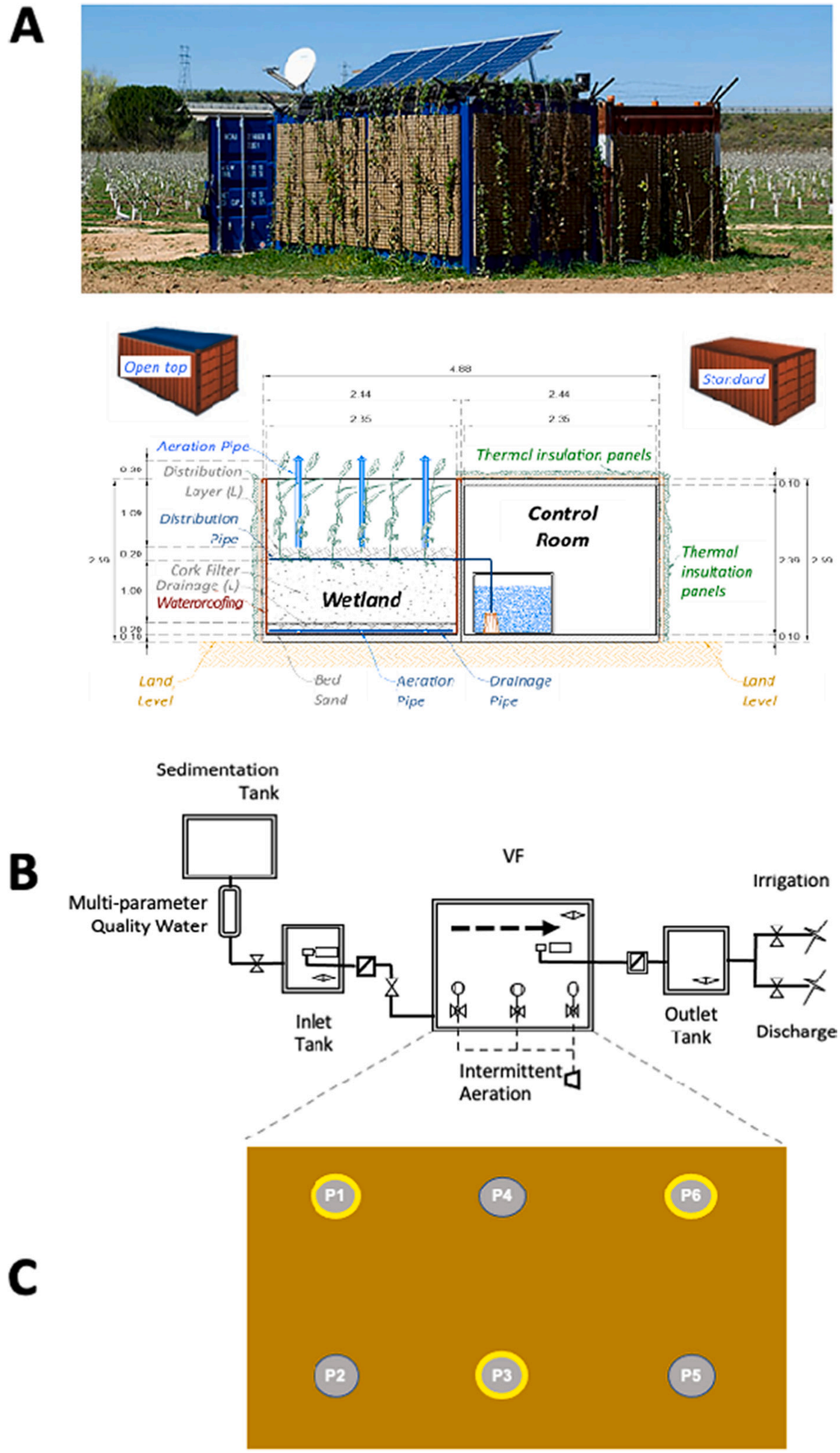

Fig. 1. **A)** Representative photograph and cut section of the innovative design of treatment wetland installed at the Codorniu facilities. **B)** Functional diagram of the hybrid wastewater treatment system. **C)** Location of the six piezometers for cork samples collection.

finally covered with an insulating gravel layer (Ø 32 mm granite) placed on top to prevent cork from floating. The water distribution system was built using 35 mm Ø PVC perforated pipes to achieve a homogeneous distribution on the bed surface. On the bottom of the bed, a collection manifold built from high density polyethylene 100 mm Ø perforated drainage ADS corrugated pipes (Advanced Drainage Systems) was placed to evacuate the treated water. Before loading the bed, the wastewater was pretreated by a two chambered sedimentation tanks to retain suspended solids. Pretreated water was then pumped to the distribution system and discharged homogeneously on the top of the bed.

The system was designed to cope with a hydraulic loading rate (HLR) of 3 m^3/d. The bed was water saturated and loaded sequentially with 2000 L/d at a rate of 4 pulses of 500 L each of winery pretreated wastewater. The treated wastewater was finally pumped to the outlet tank, with the possibility to recirculate a desired flow to the inlet tank (Fig. 1B). A Bibus JDK-S-100 air pump supplied atmospheric air at 90 L/min airflow rate using an intermittent aeration scheme of 40 min aerated, followed by 20 min without air. The aeration system was built using pressurized pipes (0.21 bar) that distributed the air at the bottom of the filter layer. The containers were externally coated with cork plates and planted with autochthonous vegetation to improve thermal insulation of the TW and the control room, in order to mitigate the summer high temperatures. The TW was planted with *Phragmites australis* at a ratio of 4 plants per m^2. A solar panel system provided the necessary energy for pumping and for all the rest of the automation processes.

2.3. Sample collection and analyses

The monitoring of the TW was routinely carried out throughout the operational period (from February 2017 to October 2018). Grab water samples from three points established along the water flow line were collected and analyzed. These sampling points were located at the influent (Codorniu treatment plant's effluent, *i.e.*, before the sedimentation tank), at the inlet tank, and at the effluent of the TW (outlet tank) (Fig. 1B). For the assay, two different operation periods of 5 months each were evaluated (from April to September 2017 when the TW was working in a non-aerated condition, and from May to October 2018 when the TW was working in an aerated condition). The start-up period took place between February to March (2018) were the TW was operated as a non-aerated saturated TW. Once the TW achieved to operate under steady state for both of the studied conditions (*i.e.*, aerated *vs.* non-aerated), samples were monthly collected during three consecutive sampling days. Additionally, during grape harvesting periods (from August to October) the sampling frequency was biweekly since the highest wastewater flow is generated, and for the non-harvesting periods the sampling took place weekly (during the third week of each month).

The water samples were collected in 1 L sterile plastic bottles and transported under refrigeration (4 °C) to the laboratory for physicochemical analyses. Water temperature, pH, O_2 saturation (DO), and electric conductivity were measured *in situ* using calibrated electrodes and a HACH SC1000 Multiparameter Universal Controller. Additional water quality parameters included: COD (APHA 5220 D), total nitrogen (TN) (Kjeldhal method), nitrate (N-NO_3) (APHA 4500-NO_3 F), total phosphorus (TP) (APHA 4500-P B), turbidity (APHA 2130 B), and alkalinity (APHA, 2012). Phosphate content (P-PO_4) was determined by ion chromatography using a Dionex ICS-2100 system (Thermo Fisher Scientific Inc., MA, USA).

For the microbiological analysis cork samples were taken from the bed of the TW (20 cm deep) at three different points using six established piezometers (Fig. 1C). Approximately 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. A total of 5 composite samples were collected for each pilot-scale VFCW. The biofilm DNA was extracted and purify from cork filter media samples according to Aguilar et al. (2019). Real-time *q*PCR assays of the 16S rRNA gene (used as a molecular marker for total bacteria), and the *nir*S and *nos*Z genes (used as molecular markers for denitrifying bacteria) were performed described by Aguilar et al. (2021).

2.4. Scanning electron microscopy

Cork ultrastructure was examined by scanning electron microscopy (SEM). At the end of the last sampling period (1.5 year), wetland cork granulates used as filter media were removed from the TW and fixed by immersion in a 4% formaldehyde solution for 30 min. Then, samples were twice washed with distilled water to remove excess formaldehyde, and soaked for 10 min in pure ethanol. After the dehydration procedure, cork samples were cryofractured by immersion in liquid N_2, mounted on bronze stubs, and coated with a gold layer (15 min, 80 mTorr) before obtaining the SEM images. A Nova NanoSEM™ 450 scanning electron microscope (FEI Europe B.V., Eindhoven, Netherlands) was used to analyze cork surface. All samples were examined using an accelerating voltage of 4 kV and magnifications of 250×, 500× and 1000×.

2.5. Statistical analyses

Statistical analyses were performed using the SigmaStat 3.5 program (Systat Software Inc., USA). Following the assessment of data normality and homogeneity of variances, the ANOVA test was used to compare the collected data. Tukey's *post-hoc* test was applied when the differences in the measured values were different ($p < 0.05$).

3. Results and discussion

3.1. Physicochemical parameters

Table 1 presents the results of the analytical analysis performed during the two phases of TW operation (*i.e.*, no-aerated and aerated). For both sampling campaigns, COD concentration increased significantly ($p < 0,05$). During the start-up period, when the system operated under water saturated conditions and no aeration, COD concentration increased 102 ± 39% mg/L, while in the second camping, when aeration was supplied, the COD concentration increased 31 ± 18% mg/L. The increased in COD concentration can be attributed to the fact that during the first operational stage some of the organic matter from the cork was washed combined with the effect of higher O_2 transfer rates. Even though COD is not completely removed, the remainder organic carbon should be enough to improve the removal of nitrogen compounds since carbon source is the key factor that limits the growth of denitrifying bacteria and therefore denitrification (Aguilar et al., 2021).

On the other hand, DO at the effluent was reduced to around a 50% during both operational periods, regardless of the air input in the reactor. This reduction can be attributed to the consumption of O_2 during microbial growth, organic matter removal, and nitrification. As expected, the ORP (redox potential) and the pH of the treated water were significantly lower ($p < 0,05$). Noteworthy, the observed reduction

Table 1
Physicochemical characteristics of influent and effluent water samples during 5-month of system operation at no-aerated or aerated conditions. Results are presented as mean ± S·D. Different letters at the same row indicate significant statistical difference ($p < 0.05$).

Parameter	no-aerated		aerated	
	inlet	outlet	inlet	outlet
COD (mg/L)	83.0 ± 20.6^b	165.5 ± 43.5^d	69.3 ± 27.8^a	105.2 ± 25.7^c
DO (mg/L)	7.3 ± 1.9^c	3.5 ± 1.2^b	2.8 ± 1.6^b	1.3 ± 1.2^a
Redox potential (mV)	143.1 ± 34.6^c	−116.5 ± 34.9^a	83.5 ± 33.4^b	−106.0 ± 39.4^a
pH	8.2 ± 0.1^c	7.7 ± 0.2^a	8.0 ± 0.1^b	7.7 ± 0.1^a
Alkalinity (mg $CaCO_3$/L)	1243 ± 82^a	1235 ± 90^a	1445 ± 182^a	1421 ± 193^a
Turbidity (NTU)	3.7 ± 0.8^a	4.3 ± 1.2^a	4.3 ± 2.3^a	3.4 ± 1.3^a
Conductivity (μs/cm)	5.1 ± 0.4^c	4.6 ± 0.6^b	3.7 ± 0.3^a	3.6 ± 0.3^a
TN (mg/L)	126.3 ± 25.8^d	80.2 ± 8.9^c	24.2 ± 7.7^b	4.0 ± 1.8^a
N-NO_3 (mg/L)	94.4 ± 23.2^d	54.5 ± 5.8^c	15.7 ± 5.4^b	0.8 ± 0.5^a
TP (mg/L)	12.2 ± 3.3^c	7.1 ± 0.9^b	7.6 ± 1.3^b	3.5 ± 0.3^a
P-PO_4 (mg/L)	9.4 ± 3.0^d	6.4 ± 1.2^c	4.1 ± 1.9^b	2.6 ± 1.7^a

in the pH values of the effluent samples can be attribute to the nitrification process (*i.e.,* the biological oxidation of ammonia, a typical base). Finally, the electric conductivity was rather low regardless of the operation cycle (no-aerated or aerated).

In general, the concentration of phosphorus compounds in the wastewater were rather low, with an average TP influent concentration of 12.2 ± 3.3 mg mg/L of which P-PO_4 were 9.4 ± 3.0 mg/L during the no-aerated operation period, and 7.6 ± 1.3 mg mg/L of which P-PO_4 were 4.1 ± 1.9 mg/L during the aerated operation period. In addition, phosphorus compounds elimination in the wetland under study was not significantly improved by intermittent induced aeration reaching percent removal values around 40–50% for TP and 30–35% for P-PO_4^3 at both operation conditions (Table 1 and Fig. 2).

Accordingly, phosphorus removal along time are issues that have not yet been completely solved when TWs are established (Pascual et al., 2021). In general, plants have an important role in treatment wetlands directly affecting wastewater quality by improving removal processes and consumption of phosphorus, nutrients, and other inorganic compounds including heavy metals (Vymazal, 2010; Zhu et al., 2014).

On the other hand, turbidity at the effluent was always below ~5 NTU and was not affected by the use of aeration. Additionally, the variation of organic matter leached by the cork filling media did not affect the turbidity of the effluent. This result is important, since in a previous works (Aguilar et al., 2021), a clear increase of water color was observed due to the "washing" of the cork during the start-up period of the system. However, we hypothesized the longer the system is under operation, the less effect in regards of color and turbidity will be present.

3.2. Nitrogen compounds

Nitrogen compounds concentration in the influent varied widely along the study, and this behaviour was mainly related with the harvest period. In any sense, total nitrogen (TN) and nitrate nitrogen (N-NO_3) removal showed a significant increase ($p < 0.05$) during the aerated operation period compared to the non-aerated one (Table 1 and Fig. 2). Notably, TN and N-NO_3 percent elimination from water when air was pumped into the reactor reach values as high as 79.1 ± 9.2% and 89.6 ± 9.0%, respectively (Fig. 2).

Therefore, our results agreed with previous reports indicating that nitrification-denitrification process and thus nitrogen removal is enhanced in wetland systems by intermittent induced aeration (Dong et al., 2012; Ilyas and Masih, 2017; Aguilar et al., 2021). For example, Ilyas and Masih (2017) reported that intermittent aeration in vertical flow constructed wetlands (VFCW) facilitates the establishment of aerobic and anaerobic conditions at the same treatment, improving TN removal. More recently, Aguilar et al. (2021) demonstrated a better performance for nitrogen elimination in aerated cork pilot scale VFCW compared to gravel wetlands. However, nitrogen removal in treatment systems is still a key problem, especially for treating low C/N ratio wastewaters (Li et al., 2017). In such case, carbon supplementation may be important for optimizing NO_3^- removal from waters since carbon source is the key factor that limits bacterial denitrification (Saeed and Sun, 2012; Yamashita and Yamamoto-Ikemoto, 2014; Fu et al., 2016; Hang et al., 2016). Cork is a chemically complex natural polymer composed of suberin, lignin, waxes and polysaccharides such as cellulose and hemicellulose, which are structural components, but also include some extractable compounds such as tannins (Machado et al., 2017). Therefore, the use of cork provides an additional carbon source to stimulate denitrification and thus increase TN removal performance in cork wetlands. Further, cork by-products have been proposed as a great adsorbent material for organic pollutants (Domingues et al., 2007; Aguilar et al., 2019, 2021) so it is possible that adsorption of nitrogen-containing organic compounds to cork may also occur, helping to improve TN removal performance in constructed wetlands using cork as the filter media.

3.3. qPCR analysis of denitrifying genes

As we have already mentioned, microbial denitrification consists of several consecutive reaction steps in which NO_3^- is reduced to N_2. The reduction of NO_3^- to NO_2^- can be catalysed by both a membrane-bound nitrate reductase or a periplasmic nitrate reductase encoded by the *nar*G or the *nap*A genes, respectively (Bru et al., 2007). Later, the reduction of NO_2^- to NO is mostly catalysed by a widely distributed cytochrome *cd*1 nitrite reductase (named NiR) encoded by the *nir*S gene (Braker et al., 2000). The last step of the denitrification pathway is catalysed by a nitrous oxide reductase encoded by the *nos*Z gene present in the periplasm of a high number of denitrifying bacteria (Chon et al., 2011).

In the present study, the observed improve in N-NO_3 removal efficiency during the wetland's operation under aerated condition was correlated with a significantly higher ($p < 0.05$) relative abundance of *nir*S and 16S rRNA genes (Fig. 3), as well as lower DO values in both inlet and outlet water (Table 1). However, no differences were observed for the expression of the *nos*Z gene at both operation conditions (Fig. 3). The dissimilatory reduction of NO_3^- to NO_2^- is frequently assume as an

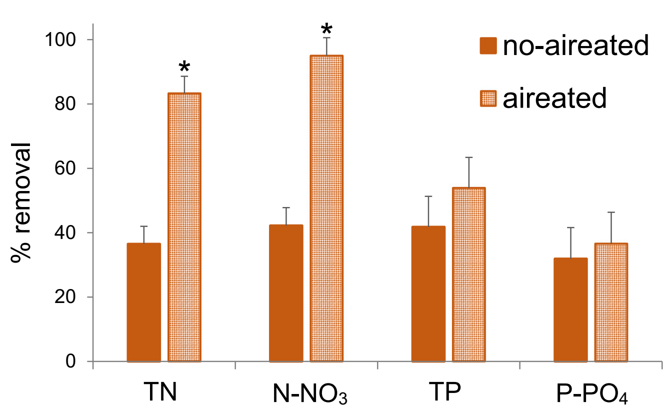

Fig. 2. Removal efficiency of total nitrogen (TN), nitrate-nitrogen (N-NO_3), total phosphorous (TP), and phosphate content (P-PO_4) in the TW installed at the Codorniu facilities during the non-aerated or the aerated operation period. Results are presented as mean ± S.E. *, indicates statistically significant differences ($p < 0.05$).

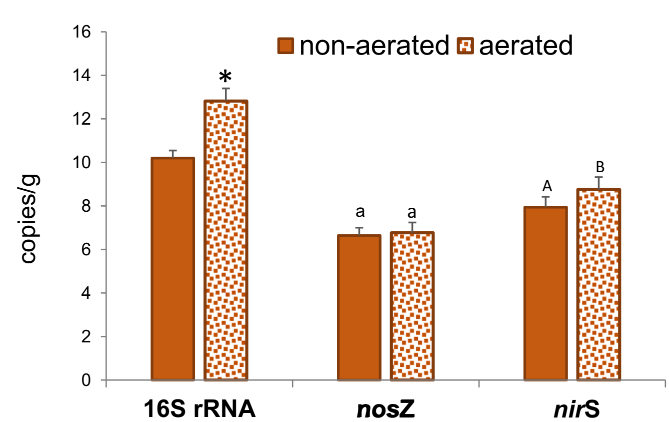

Fig. 3. Copies number of 16S rRNA, *nos*Z and *nir*S genes for the cork treatment wetland during the non-aerated or the aerated operation period. Results are presented as mean ± S.D. at the two last sampling campaign ($n = 6$). Data of sampling system not sharing a common capital letter are statistically different for *nir*S abundance ($p < 0.05$); data of sampling system not sharing a common lowercase letter are statistically different for *nos*Z abundance ($p < 0.05$). *, indicates statistically significant differences between 16S rRNA gene copies number (used to assess bacterial load).

anaerobic process but this first step of denitrification can also occur at low O_2 concentrations (Ji et al., 2015; Roco et al., 2016; Zhang et al., 2016). Aerobic NO_3^- reduction occurs when provided with both NO_3^- and a carbon source, with a concomitant greatest NO_2^- accumulation at the wetlands (Roco et al., 2016). Nitrite is then converted to NO or N_2O through NiR, being the *nir*S gene one of the most widely used molecular biomarker used to characterize the denitrifier communities. In such sense, NiR expression in aerobic environments might be a common phenomenon. Therefore, this result suggest that intermittent induced aeration facilitates NO_3^- reduction by the stimulation of denitrifying bacteria in the wetland biofilm, with a concomitant greatest NO accumulation at the system.

Additionally, the presented result agrees with our previous works describing cork as a suitable and cost-effective filter media for structuring microbial assemblages in constructed wetlands aimed to remove nitrogen compounds from polluted waters (Aguilar et al., 2019, 2021). Related to this, it is important that the filtering material used in constructed wetlands be insoluble and resistant to biodegradation. In such sense, the SEM images showed at Fig. 4 demonstrate the suitability of cork as a resistant filter media since minimal damage to cork's surface and topography could be appraised after long-term system operation (1.5 years). Moreover, in the best of our knowledge, this is the very first report of a cork wetland system long-time operating. Therefore, considering all this, the use of cork by products as filter media in wetland system is a very attractive option due to its low-cost, easy availability, material composition and consistency, and long-term degradation resistance.

4. Conclusions

In this study, we have followed for 1.5 year the functioning of an innovative mobile vertical flow TW using cork by products as the filter media and intended for winery wastewater treatment. Our results showed a ~50% increase in both NO_3-N and TN removal from winery effluents when air was introduced to the system. The observed improve performance in nitrogen removal was correlated with an increase in the relative abundance of the denitrifying *nir*S gene and the development of a higher abundance of heterotrophic bacteria. Therefore, cork proved to be an excellent material to host and support biofilm and microbial communities that increase the performance of TW for nutrients removal, especially when nitrogen is the main target, since carbon requirement is the weakness point for nitrogen elimination *via* biological processes (nitrification-denitrification pathway).

CRediT authorship contribution statement

Lorena Aguilar: Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Leonardo M. Pérez:** Formal analysis, Writing – original draft. **Ángel Gallegos:** Investigation, Methodology. **Eva Fores:** Investigation, Methodology. **Carlos A. Arias:** Conceptualization, Formal analysis, Funding acquisition, Project administration, Supervision, Writing – review & editing. **Carme Bosch:** Funding acquisition, Project administration. **Maria Verdum:** Funding acquisition, Project administration. **Patricia Jove:** Funding acquisition, Project administration. **Joan de Pablo:** Funding acquisition, Project administration. **Jordi Morató:** Conceptualization, Formal analysis, Funding acquisition, Project administration, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

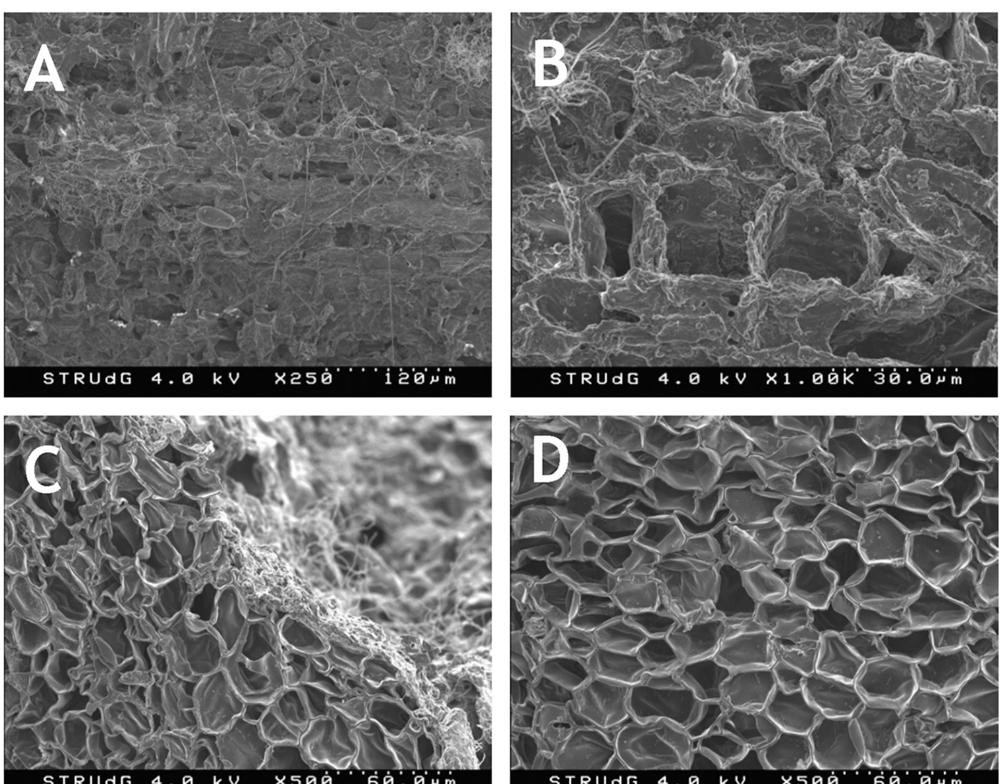

Fig. 4. SEM micrographs of wetland cork granulate after 1.5 year of operation; (A) surface of cork granules in 250 magnifications; (B) surface of cork granules in 1000 magnifications; (C) inner and outer cork granules in 500 magnifications; (D) inner cork granules in 500 magnifications.

Data availability

Data will be made available on request.

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