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Feasibility of using rural waste products to increase the denitrification efficiency in a surface flow constructed wetland

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ABSTRACT

A surface flow constructed wetland (CW) was set in the Lerma gully to decrease nitrate (NO_3^-) pollution from agricultural runoff water. The water flow rate and NO_3^- concentration were monitored at the inlet and the outlet, and sampling campaigns were performed which consisted of collecting six water samples along the CW flow line. After two years of operation, the NO_3^- attenuation was limited at a flow rate of ~ 2.5 L/s and became negligible at ~ 5.5 L/s. The present work aimed to assess the feasibility of using rural waste products (wheat hay, corn stubble, and animal compost) to induce denitrification in the CW, to assess the effect of temperature on this process, and to trace the efficiency of the treatment by using isotopic tools. In the first stage, microcosm experiments were performed. Afterwards, the selected waste material was applied in the CW, and the treatment efficiency was evaluated by means of a chemical and isotopic characterization and using the isotopic fractionation (ϵ) values calculated from laboratory experiments to avoid field-scale interference. The microcosms results showed that the stubble was the most appropriate material for application in the CW, but the denitrification rate was found to decrease with temperature. In the CW, biostimulation in autumn-winter promoted NO_3^- attenuation between two weeks and one month (a reduction in NO_3^- between 1.2 and 1.5 mM was achieved). After the biostimulation in spring-summer, the attenuation was maintained for approximately three months (NO_3^- reduction between 0.1 and 1.5 mM). The $\epsilon^{15}\text{N}_{\text{NO}_3/\text{N}_2}$ and $\epsilon^{18}\text{O}_{\text{NO}_3/\text{N}_2}$ values obtained from the laboratory experiments allowed to estimate the induced denitrification percentage. At an approximate average flow rate of 16 L/s, at least 60% of NO_3^- attenuation was achieved in the CW. The field samples exhibited a slope of 1.0 for $\delta^{18}\text{O}\text{-NO}_3^-$ versus $\delta^{15}\text{N}\text{-NO}_3^-$, similar to those of the laboratory experiments (0.9–1.2). Plant uptake seemed to play a minor role in NO_3^- attenuation in the CW. Hence, the application of stubble in the CW allowed the removal of large amounts of NO_3^- from the Lerma gully, especially when applied during the warm months, but its efficacy was limited to a short time period (up to three months).

1. Introduction

Since nitrate (NO_3^-) is known to cause ecological and human health problems (Vitousek et al., 1997; Ward et al., 2005), the presence of this nutrient in water bodies worldwide is a matter of concern. The extensive application of synthetic and organic fertilizers is a major source of NO_3^- pollution. Therefore, agricultural runoff water should be treated before it is drained into larger water bodies such as aquifers, rivers, and/or lakes. Constructed wetlands (CWs) are considered

promising, low cost systems for the remediation of diverse water pollutants, are simple to operate, and have low energy requirements (Wu et al., 2015). Hence, directing agricultural runoff water through a CW could be useful for removing NO_3^- to minimize pollution.

The surface flow CWs consists of free surface water flowing horizontally through an artificial pond containing floating and/or emergent rooted vegetation and a high diversity of microorganisms (Ilyas and Masih, 2017; Sirivedhin and Gray, 2006; Vymazal, 2007). The main processes that might contribute to NO_3^- pollution mitigation in surface

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flow CWs are plant uptake, assimilation by microorganisms, and denitrification (Rogers et al., 1991). The latter refers to the reduction of NO_3^- by microorganisms through a series of enzymatic reactions involving the intermediates NO_2^- , NO , and N_2O , before finally being reduced to N_2 (Knowles, 1982). Parameters such as temperature, dissolved oxygen (O_2), NO_3^- loading, the source and amount of organic carbon (C), microbial species, the type and density of macrophytes, wetland age, and hydraulic conditions play key roles in the NO_3^- removal efficiency (Bachand and Horne, 1999; Beutel et al., 2009; Kong et al., 2009; Sirivedhin and Gray, 2006). Different approaches can be implemented to enhance water remediation, but strategies directed toward the induction of bacterial NO_3^- respiration are preferred since denitrification is an authentic N sink in water, unlike biomass sequestration (Scott et al., 2008). N storage by plants is generally considered temporary, because organic N returns to the system after the death and decay of plants if they are not harvested (Cooper and Cooke, 1984; Gumbrecht, 1993).

In CWs, macrophytes are able not only to assimilate NO_3^- , but also to promote denitrification efficiency. Plants exert an influence on the diversity of microbial species and their enzymatic activities by releasing exudates and oxygen to the rhizosphere (Kong et al., 2009, and references therein), and decomposed plant material can be used by microbes as a source of organic C. For this reason, increased NO_3^- removal is usually found in vegetated CWs relative to that in non-vegetated systems (Jacobs and Harrison, 2014; Soana et al., 2017). If the CW cannot provide enough organic C to support complete denitrification (e.g., from inlet water, soil, plant root exudates, and decomposed vegetal material), the addition of an external organic C source as an electron donor could enhance the heterotrophic denitrification efficiency (Lu et al., 2009; Si et al., 2018). Since the use of pure reagents such as glucose, acetate, or ethanol may be expensive in long-term treatments, the use of industrial or agricultural residues that are rich in organic C could represent a more sustainable solution. Solid products such as animal or vegetal waste (Grau-Martínez et al., 2017; Si et al., 2018; Trois et al., 2010), as well as industrial liquid by-products (Carrey et al., 2018; Margalef-Marti et al., 2019), have already been reported as being useful for promoting denitrification.

The pollutant removal efficiency in CWs can be estimated by monitoring the inlet and outlet concentrations of the pollutant (Kovacic et al., 2000; Tanner et al., 2005; Uusheimo et al., 2018). However, this method does not reveal the specific processes involved in the attenuation, making it challenging to focus on the improvement of the wetland design and operation. Stable isotope analyses can provide information on the NO_3^- transformation pathways. In the course of denitrification, the unreacted residual NO_3^- becomes enriched in the heavy isotopes ^{15}N and ^{18}O , permitting the distinction between biological attenuation and other processes such as dilution which could also lead to decreases in concentration without influencing the isotopic signature (Böttcher et al., 1990; Fukada et al., 2003; Mariotti et al., 1981; Aravena and Robertson, 1998). In plants, significant enrichment in both ^{15}N and ^{18}O

is observed in the NO_3^- extracted from leaves after uptake relative to the NO_3^- from water, but the changes in the NO_3^- isotopic composition in the water are minor (Estrada et al., 2017; Spoelstra et al., 2010). Therefore, the NO_3^- isotopic characterization of water samples collected at the CW might improve the understanding and support the evaluation of the performance of the remediation strategy.

In this context, the present work was developed to assess the feasibility of using rural waste products (wheat hay, corn stubble, and animal compost) to induce denitrification in a surface flow CW, and to trace the treatment efficiency in the autumn–winter and spring–summer seasons. In the first stage, lab-scale experiments were performed to identify the most appropriate electron donor to be applied in the CW, and to evaluate the effect of temperature on NO_3^- reduction. The isotopic fractionations (ϵ) of N and O of dissolved NO_3^- under each condition were also determined. In the second stage, the selected material was applied in the CW and the treatment efficiency was evaluated by means of a chemical and isotopic characterization using the ϵ values calculated from the laboratory experiments.

2. Methods

2.1. Laboratory experiments

Six types of batch experiments were performed in 150 mL crystal Pyrex® bottles crimp-sealed with butyl rubber stoppers under an argon (Ar) headspace. Each microcosm contained 100 mL of water (2 mM NO_3^-) collected from the inlet of the studied CW (see Section 2.2) and a specific C source: corn stubble (1 g); wheat hay (1 g); or animal compost (0.25 g). The six series of parallel experiments were determined according to the waste product employed and the incubation temperature. Series I (C-24) and II (H-24) contained animal compost and wheat hay, respectively, and were incubated at 24 °C; series III (S-24), IV (S-16), and V (S-8) contained corn stubble and were incubated at 24 °C, 16 °C, and 8 °C, respectively; series VI (DS-24) contained partially decomposed corn stubble and was incubated at 24 °C. The partially decomposed stubble was obtained from the CW 7.5 months after its application on September 25, 2017 (see Section 2.2). All series included at least eight replicates of the biostimulated microcosms. Control microcosms for each tested material were prepared using deionized water (DIW) to discard the potential supply of N from the waste products. The detailed content of each microcosm is described in Table 1. During incubation, all microcosms were maintained in darkness and under constant vibratory shaking. The biostimulated microcosms were sacrificed at time intervals depending on the denitrification dynamics until complete NO_3^- and NO_2^- removals were achieved. The control microcosms were sacrificed at the end of the biostimulation experiments. Water samples from batch experiments were analyzed for major anions (NO_3^- , NO_2^- , Cl^- , and SO_4^{2-}), ammonium (NH_4^+), non-purgeable dissolved organic C (NPDOC), dissolved inorganic C (DIC), major cations, trace elements, $\delta^{15}\text{N}\text{-NO}_3^-$, $\delta^{18}\text{O}\text{-NO}_3^-$, and $\delta^{13}\text{C}\text{-DIC}$. Samples from control microcosms

Table 1
Series of experiments. Tested conditions and composition of microcosms. DIW = deionized water.

Series	Condition	Code	C source	Material (g)	C (g/L)	Water source (100 mL)	Temperature (°C)
I	Biostimulated	C-24	Animal compost	0.25	0.8	Wetland	24
	Control	C-24-blank	Animal compost	0.25	0.8	DIW	24
II	Biostimulated	H-24	Wheat hay	1	4.2	Wetland	24
	Control	H-24-blank	Wheat hay	1	4.2	DIW	24
III	Biostimulated	S-24	Corn stubble	1	3.6	Wetland	24
	Control	S-24-blank	Corn stubble	1	3.6	DIW	24
IV	Biostimulated	S-16	Corn stubble	1	3.6	Wetland	16
	Control	S-16-blank	Corn stubble	1	3.6	DIW	16
V	Biostimulated	S-8	Corn stubble	1	3.6	Wetland	8
	Control	S-8-blank	Corn stubble	1	3.6	DIW	8
VI	Biostimulated	DS-24	Decomposed stubble	1	3.6	Wetland	24
	Control	DS-24-blank	Decomposed stubble	1	3.6	DIW	24

were analyzed for major anions and NPDOC. The gas accumulated in the headspace of the vials was collected and analyzed for nitrous oxide (N_2O) concentration. The three organic C sources were analyzed for C and N concentrations and $\delta^{13}\text{C}\text{-C}_{\text{bulk}}$ and $\delta^{15}\text{N}\text{-N}_{\text{bulk}}$.

2.2. CW test

In the 2000s, approximately 20,000 ha of rainfed croplands were transformed into irrigated agricultural land in the Arba River Basin (Zaragoza, Spain). A small watershed representative of the area (Lerma basin, 733 ha) was monitored to assess the effects of this transformation on the water balance and the salt and NO_3^- -N exports (Merchán et al., 2015, 2014, 2013). In general, the implementation of irrigation implied a three-fold increase in N export to the receiving water bodies, in this case the Arba River, which was the first surface water body in the Ebro River Basin to be declared affected by NO_3^- pollution according to the Nitrates Directive 91/676/EEC. In order to diminish the release of NO_3^- from the Lerma Basin to the Arba River, a surface flow CW was constructed in October 2015, initially covering an area of $\sim 1500\text{ m}^2$, and was enlarged in June 2017, covering a final area of $\sim 2500\text{ m}^2$ with a depth of $\sim 40\text{ cm}$. The surface water of the Lerma gully can be partially diverted towards the CW. Water flow in the Lerma gully varies between 15 and 60 L/s. Temperature and precipitation data collected monthly in the area are reported in Supporting information (Table S1).

The CW is fully automated, with high-frequency monitoring (every 10 min) of the water flow rate and NO_3^- concentration at both the inlet and the outlet. Emergent macrophytes (*Typha* and *Phragmites*) started growing since its construction, and occupied approximately 75% of the CW surface at the time the present study began, since the enlarged part was still unvegetated. The field survey was performed in three periods and involved 13 sampling campaigns, each consisting of the collection of six water samples (H1 to H6) from along the wetland flow line (Fig. 1). In the first period (June to September 2017), two different operating conditions were tested before the biostimulation by modifying the inlet opening; three sampling campaigns were performed at two different flow rates ($\sim 5.5\text{ L/s}$ and $\sim 2.5\text{ L/s}$). The second period involved the application of corn stubble obtained from the surrounding crops ($\sim 8000\text{ kg}$) on September 25, 2017, and the evaluation of treatment efficiency by performing two sampling campaigns 7 and 14 d after the application. The third period involved a second application of corn stubble ($\sim 6000\text{ kg}$) on May 11, 2018, and the evaluation of treatment efficiency by performing eight sampling campaigns from May 2018 to October 2018. In the two biostimulation periods, the corn stubble was applied over all the CW surface between H1 and H3. Throughout these second and third periods, the CW was operated at a higher flow rate ($\sim 16\text{ L/s}$). The given flow rate for the CW test periods is that measured at the outlet. The calculated residence time of NO_3^- in the CW was 21,

51 and 112 h for the tested flow rates of 16, 5.5 and 2.5 L/s, respectively. Detailed information about the sampling campaigns is shown in Table 2. Water samples collected at the CW were analyzed for major anions (NO_3^- , NO_2^- , Cl^- , and SO_4^{2-}), NH_4^+ , NPDOC, DIC, major cations, trace elements, $\delta^{15}\text{N}\text{-NO}_3^-$, $\delta^{18}\text{O}\text{-NO}_3^-$, $\delta^{34}\text{S}\text{-SO}_4^{2-}$, $\delta^{18}\text{O}\text{-SO}_4^{2-}$, and $\delta^{13}\text{C}\text{-DIC}$.

2.3. Analytical methods

Water samples for the field and laboratory batch experiments were immediately filtered through $0.2\text{ }\mu\text{m}$ Millipore® filters after being collected, and were stored at $4\text{ }^\circ\text{C}$ until analysis. The aliquots for NH_4^+ , $\delta^{15}\text{N}\text{-NO}_3^-$, and $\delta^{18}\text{O}\text{-NO}_3^-$ analysis were frozen, and the aliquots for the DIC and $\delta^{13}\text{C}\text{-DIC}$ analyses were left with no headspace and stored at $4\text{ }^\circ\text{C}$.

Anions (Cl^- , NO_2^- , NO_3^- , and SO_4^{2-}) were analyzed by high-performance liquid chromatography (HPLC) (Waters 515 pump and Waters IC-Pak anion column with Waters 432 and KONTRON UV/Vis detectors). NH_4^+ was analyzed by three techniques due to equipment availability issues: I) spectrophotometry using the indophenol blue method (CARY 1E UV-visible), II) ion chromatography or III) ammonia ion selective electrode (ORION, Thermo Scientific). DIC was analyzed by titration (METROHM 702 SM Titrino), NPDOC by organic matter combustion (TOC 500 SHIMADZU), and major cations and trace elements by ICP-OES (Perkin Elmer Optima 8300). The concentration of N_2O accumulated at the headspace of the vials was analyzed by gas chromatography (GC) (Thermo Scientific Trace 1300 with ECD detector), and C and N concentrations in the waste materials employed as C sources were analyzed with an elemental analyzer (EA) (Carlo Erba1108 CHNS-O EA). The $\delta^{15}\text{N}\text{-NO}_3^-$ and $\delta^{18}\text{O}\text{-NO}_3^-$ were determined following the cadmium and azide reduction method (McIlvin and Altabet, 2005; Ryabenko et al., 2009). The isotopic composition of the N_2O obtained from the NO_3^- reduction was analyzed using a Pre-Con coupled to a Finnigan MAT 253 isotope ratio mass spectrometer (IRMS) (Thermo Scientific). For the SO_4^{2-} isotopic analysis, the dissolved SO_4^{2-} was precipitated as BaSO_4 by adding $\text{BaCl}_2\cdot 2\text{H}_2\text{O}$ after acidifying the sample with HCl and boiling it in order to prevent precipitation of BaCO_3 (Dogramaci et al., 2001). The $\delta^{34}\text{S}\text{-SO}_4^{2-}$ was analyzed with a Carlo Erba EA coupled in continuous flow to a Finnigan Delta XP Plus IRMS, whereas the $\delta^{18}\text{O}\text{-SO}_4^{2-}$ was analyzed with a ThermoQuest high-temperature conversion analyzer (TC/EA) coupled in continuous flow to a Finnigan Matt Delta XP Plus IRMS. The $\delta^{13}\text{C}\text{-DIC}$ was analyzed via carbonate conversion to CO_2 gas by adding a phosphoric acid solution and measuring the gas evolved with a Gas-Bench II coupled to a MAT-253 IRMS (Thermo Scientific). The $\delta^{13}\text{C}\text{-C}_{\text{bulk}}$ and $\delta^{15}\text{N}\text{-N}_{\text{bulk}}$ of the waste materials employed as C sources were determined with a Carlo Erba EA coupled to a Finnigan Delta C IRMS.



Fig. 1. CW design. Photograph of the surface flow CW with emergent macrophytes. The sampling points are depicted with white squares (H1 to H6), and the water flow within the CW with striped arrows. Non-treated water flow discharging to the Lerma gully is depicted with black arrows, and that of treated water with a white arrow.

Table 2
Sampling campaigns. Sampling dates and operation mode of the CW for all sampling campaigns (six samples each).

Test period	Date	Days since stubble addition	Operation mode	Observations
I	14/06/2017	–	5.5 L/s	No external organic C addition
	05/09/2017	–	5.5 L/s	
	12/09/2017	–	2.5 L/s	
II	02/10/2017	7	16 L/s	First organic C source addition on 25/09/2017
	10/10/2017	14		
III	11/05/2018	0	16 L/s	Second organic C source addition on 11/05/2018
	18/05/2018	7		
	25/05/2018	14		
	12/06/2018	32		
	13/07/2018	63		
	19/08/2018	100		
	13/09/2018	125		
19/10/2018	161			

The isotopic notation is expressed in terms of δ (‰) relative to the international standards: atmospheric N_2 (AIR) for $\delta^{15}N$, Vienna Standard Mean Oceanic Water (V-SMOW) for $\delta^{18}O$, Vienna Pee Dee Belemnite (V-PDB) for $\delta^{13}C$, and Vienna Canyon Diablo Troilite (V-CDT) for $\delta^{34}S$. Hence, $\delta = ((R_{\text{sample}} - R_{\text{standard}}) / R_{\text{standard}})$, where R is the ratio between the heavy and the light isotopes. Following Coplen (2011), several international and laboratory (CCiT) standards were interspersed among the samples for normalization of the results (Supporting Information Table S2). The reproducibilities (1 σ) of the samples, calculated from the standards systematically interspersed in the analytical batches, were $\pm 1.0\text{‰}$ for $\delta^{15}N\text{-NO}_3^-$, $\pm 1.5\text{‰}$ for $\delta^{18}O\text{-NO}_3^-$, $\pm 0.2\text{‰}$ for $\delta^{15}N\text{-N}_{\text{bulk}}$, $\pm 0.2\text{‰}$ for $\delta^{13}C\text{-C}_{\text{bulk}}$, $\pm 0.2\text{‰}$ for $\delta^{13}C\text{-DIC}$, $\pm 0.2\text{‰}$ for $\delta^{34}S\text{-SO}_4^{2-}$, and $\pm 0.5\text{‰}$ for $\delta^{18}O\text{-SO}_4^{2-}$. Samples for chemical and isotopic analyses were prepared at the laboratory of the MAiMA-UB research group, and analyzed at the Centres Científics i Tecnològics of the Universitat de Barcelona (CCiT-UB).

3. Results and discussion

3.1. Lab-scale evaluation of the nitrate removal capacities of compost, hay, and stubble at 24 °C

The chemical and isotopic characterization of the samples obtained from the laboratory experiments are presented in the Supporting Information (Table S3). Although the intrinsic N content measured in the three waste products (stubble, hay, and compost) were low (Table 3), it was not possible to disregard a certain supply of N from these materials throughout the incubations. The control microcosms containing the three C sources and DIW showed NO_3^- , NO_2^- , and NH_4^+ concentrations below 0.09 mM, 0.02 mM, and 0.12 mM, respectively.

The biostimulation experiments showed that the three tested C sources were able to promote NO_3^- removal. Complete denitrification (total NO_3^- and NO_2^- removal) was reached in approximately 40 h in the microcosms containing stubble and hay, and in approximately 95 h in that containing compost (Fig. 2A). NH_4^+ was detected in some of the samples (in concentrations of up to 1 mM), but with no clear pattern, suggesting the possible coexistence of denitrification and dissimilatory NO_3^- reduction to NH_4^+ (DNRA) and/or the input of NH_4^+ -N supplied from the C sources tested. Transient NO_2^- accumulation of up to 1.5 mM

Table 3
Waste products composition. C and N concentrations and isotopic composition of the corn stubble, wheat hay, and animal compost employed to promote denitrification.

Source	C (%)	N (%)	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)
Animal compost	32.1	3.1	–25.4	10.8
Wheat hay	40.9	0.4	–27.8	3.0
Corn stubble	36.1	1.0	–13.6	6.7

(stubble and hay) and 0.7 mM (compost) were observed. The highest concentration of NO_2^- in the stubble and hay microcosms were detected after complete NO_3^- reduction, and subsequently decreased to below the detectable limit in less than 40 h from the beginning of the experiment. Contrarily, in the microcosms containing compost, the highest NO_2^- concentration was observed after 40 h, and thereafter decreased along with NO_3^- concentration until both compounds were below the detectable limits, after 96 h. The differences in NO_2^- accumulation between the compost, stubble, and hay experiments were likely related to the rate of NO_3^- reduction. NO_2^- accumulation has been reported to depend on the relative rates of NO_3^- and NO_2^- reduction (Betlach and Tiedje, 1981), as well as on the type of C source and C/N ratios employed (Akunna et al., 1993; Ge et al., 2012). The slower reduction observed with compost could be due to the lower amount of material used in the experiments (0.25 g instead of the 1 g used for stubble and hay). Although the intrinsic C concentrations of the three sources were similar (Table 3), the C bioavailability could differ between each product, and even between replicates, due to heterogeneity in the materials (Breulmann et al., 2014; Sobczak and Findlay, 2002; Warneke et al., 2011). Consequently, the NPDOC concentration did not show a clear correlation with NO_3^- reduction, but provided an approximation of the amount of added C present in dissolved form. Although the quantity of compost in the microcosms was only one-quarter of the quantity of vegetal materials used, the measured NPDOC concentrations in the three types of microcosms were similar (13.2–27.3 mM for stubble, 11.8–16.8 mM for hay, and 5.3–14.3 mM for compost). The $\delta^{13}C\text{-DIC}$ provided information about the transformation of organic C from the waste materials to inorganic C; a brief discussion is presented in the Supporting Information (Section S1).

Concerning the safety of the materials, the ICP-OES analyses showed that there was no release of toxic trace elements from any of the tested compounds (Supporting Information Table S4). Hay and stubble seemed to be more feasible than compost for application in the CW. Compost resulted in a lower denitrification rate, the NO_2^- accumulation lasted longer, and it was highly soluble and could be rapidly removed from the CW via the water flow. In this study, stubble was selected for application in the studied CW due to a higher availability in the area. Therefore, further experiments were only performed with stubble.

3.2. Lab-scale evaluation of the effect of temperature on denitrification induction by stubble

The denitrification activity of microorganisms is usually increased with higher temperatures, and therefore higher NO_3^- attenuation from water can be observed during warm periods (Rivett et al., 2008; Spieles and Mitsch, 1999). To assess the effect of temperature on the induced denitrification strategy, additional experiments were performed. A comparison between different incubation temperatures in corn stubble experiments showed that denitrification reached completion across the

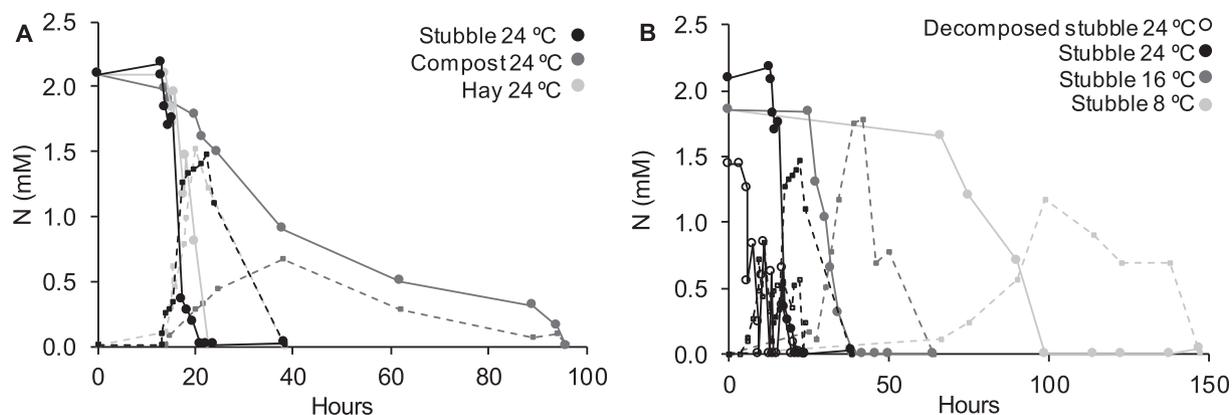


Fig. 2. Evolution of denitrification in the biostimulated microcosms. NO_3^- (circles joined by a continuous line) and NO_2^- (squares joined by a dashed line) measured in (A) the batch experiments employing different C sources and (B) the experiments testing the effects of temperature and lifespan of the stubble.

whole temperature range studied (from 8 to 24 °C), but with different lag periods and NO_3^- reduction rates. Complete denitrification was achieved after 40 h at 24 °C, 65 h at 16 °C, and 140 h at 8 °C (Fig. 2B). The decrease in NO_3^- began after 10 h at 24 °C, whereas at 16 °C and 8 °C lag periods of 45 h and 79 h, respectively, were observed. A decrease in NO_3^- reduction rate associated with lower temperatures following the Arrhenius relationship has been well documented (Dawson and Murphy, 1972). Therefore, the denitrification efficiency might decrease during the winter months or low-temperature periods in comparison to that during the summer months, and thus application of the carbon source throughout the spring months might be advantageous. Significant transient NO_2^- accumulation (up to 1.5 mM at 24 °C, 1.8 mM at 16 °C, and 1.0 mM at 8 °C) was observed in all the experiments. As discussed in the previous section, NO_2^- accumulation was less significant in the experiment with a lower denitrification rate (8 °C).

3.3. Lab-scale assessment of the lifespan of the denitrification induced by stubble

One of the main issues associated with biostimulation strategies is their effectiveness during long-term treatments. It is thus important to consider the lifespan of the material to be employed in the CW. In another laboratory experiment with vegetable materials (palm leaves and compost), induced NO_3^- degradation was shown to be maintained for more than 220 d (Grau-Martínez et al., 2017). In this context, microcosms containing partially decomposed stubble (sampled in the CW 7.5 months after its application) were incubated and compared to microcosms containing fresh stubble. The denitrification induced by the partially decomposed stubble proceeded at a higher rate than that induced by the fresh stubble; complete NO_3^- reduction was achieved in less than 25 h with the former, instead of 40 h with the latter (Fig. 2B). In the partially decomposed stubble microcosms, transient NO_2^- accumulation was below 0.8 mM. Due to the increased heterogeneity of the material after being in the field and in contact with water for months, high variabilities in both NO_3^- and NO_2^- concentrations were observed between replicates. Therefore, the reduction rates obtained from these experiments must be considered approximations. These results showed that the intrinsic capacity of the stubble to promote denitrification after 7.5 months being in contact with water was still important, at least at lab-scale. However, the NPDOC content in the microcosms containing partially decomposed stubble (1.7–8.8 mM) were lower than those in the microcosms with fresh stubble incubated at 24 °C (13.2–27.3 mM), pointing to a decreased availability of the electron donor over time. In the CW, the specific lifespan of the treatment might be shorter, since the organic C also typically consumes O_2 before using NO_3^- as an electron donor. The N_2O accumulated in the headspace of the microcosms containing partially decomposed stubble incubated at 24 °C (as well as

that in the microcosms containing fresh stubble incubated at 16 and 8 °C) was also measured since the release of greenhouse gases during N transformation processes is a matter of concern. The maximum N_2O concentration detected accounted for 0.015% of the initial N-NO_3^- content of the microcosms (Supporting Information Table S3)).

3.4. Lab-scale: NO_3^- isotopic fractionation calculation.

Under closed-system conditions, the isotopic fractionation (ϵ) for a determined element (e.g., N and O from dissolved NO_3^-) can be calculated by means of a Rayleigh distillation equation (Equation (1)). Thus, ϵ can be obtained from the slope of the linear correlation between the natural logarithm of the remaining substrate fraction ($\text{Ln}(C_{\text{residual}}/C_{\text{initial}})$, where C refers to analyte concentration) and the determined isotope ratios ($\text{Ln}(R_{\text{residual}}/R_{\text{initial}})$, where $R = \delta + 1$). These $\epsilon^{15}\text{N}_{\text{NO}_3/\text{N}_2}$ and $\epsilon^{18}\text{O}_{\text{NO}_3/\text{N}_2}$ values, determined at lab-scale under controlled conditions, can be later applied at field-scale to estimate the contribution of denitrification to the NO_3^- attenuation, while avoiding field-scale interference such as dilution due to rainfall (Böttcher et al., 1990; Mariotti et al., 1988). We calculated $\epsilon^{15}\text{N}_{\text{NO}_3/\text{N}_2}$ and $\epsilon^{18}\text{O}_{\text{NO}_3/\text{N}_2}$ under all tested conditions at lab-scale (Fig. 3) to appropriately evaluate the efficacy of the induced denitrification strategy tested at the CW. A summary of the calculated $\epsilon^{15}\text{N}_{\text{NO}_3/\text{N}_2}$, $\epsilon^{18}\text{O}_{\text{NO}_3/\text{N}_2}$, and $\epsilon^{15}\text{N}/\epsilon^{18}\text{O}$ values is shown in (Table 4); $\epsilon^{15}\text{N}_{\text{NO}_3/\text{N}_2}$ ranged from -31.9 to -10.5% , $\epsilon^{18}\text{O}_{\text{NO}_3/\text{N}_2}$ from -30.4 to -9.7% , and $\epsilon^{15}\text{N}/\epsilon^{18}\text{O}$ from 0.8 to 1.8. These values fall within the reported range for heterotrophic denitrification (see Table 4; Grau-Martínez et al., 2017). The lowest $\epsilon^{15}\text{N}_{\text{NO}_3/\text{N}_2}$ and $\epsilon^{18}\text{O}_{\text{NO}_3/\text{N}_2}$ values were found for the microcosms containing compost incubated at 24 °C and stubble incubated at 8 °C, which were the two experiments that presented lower NO_3^- reduction rates. Apart from the microcosms containing stubble incubated at 8 °C, the other microcosms containing stubble (both fresh and partially decomposed and incubated at 16 or 24 °C) presented narrower ranges of $\epsilon^{15}\text{N}_{\text{NO}_3/\text{N}_2}$ (from -28.3 to -22.5%), $\epsilon^{18}\text{O}_{\text{NO}_3/\text{N}_2}$ (from -30.4 to -21.2%) and $\epsilon^{15}\text{N}/\epsilon^{18}\text{O}$ (from 0.8 to 1.1). These values were employed to assess the efficiency of the biostimulation strategy at the studied CW.

$$\text{Ln} \left(\frac{R_{\text{residual}}}{R_{\text{initial}}} \right) = \epsilon \times \text{Ln} \left(\frac{C_{\text{residual}}}{C_{\text{initial}}} \right) \quad (1)$$

3.5. Performance of the CW before application of stubble

The chemical and isotopic characterization of the samples obtained from the CW both before and after application of the electron donor are presented in Supporting Information (Table S5). Three sampling campaigns were performed at the CW before stubble application. While

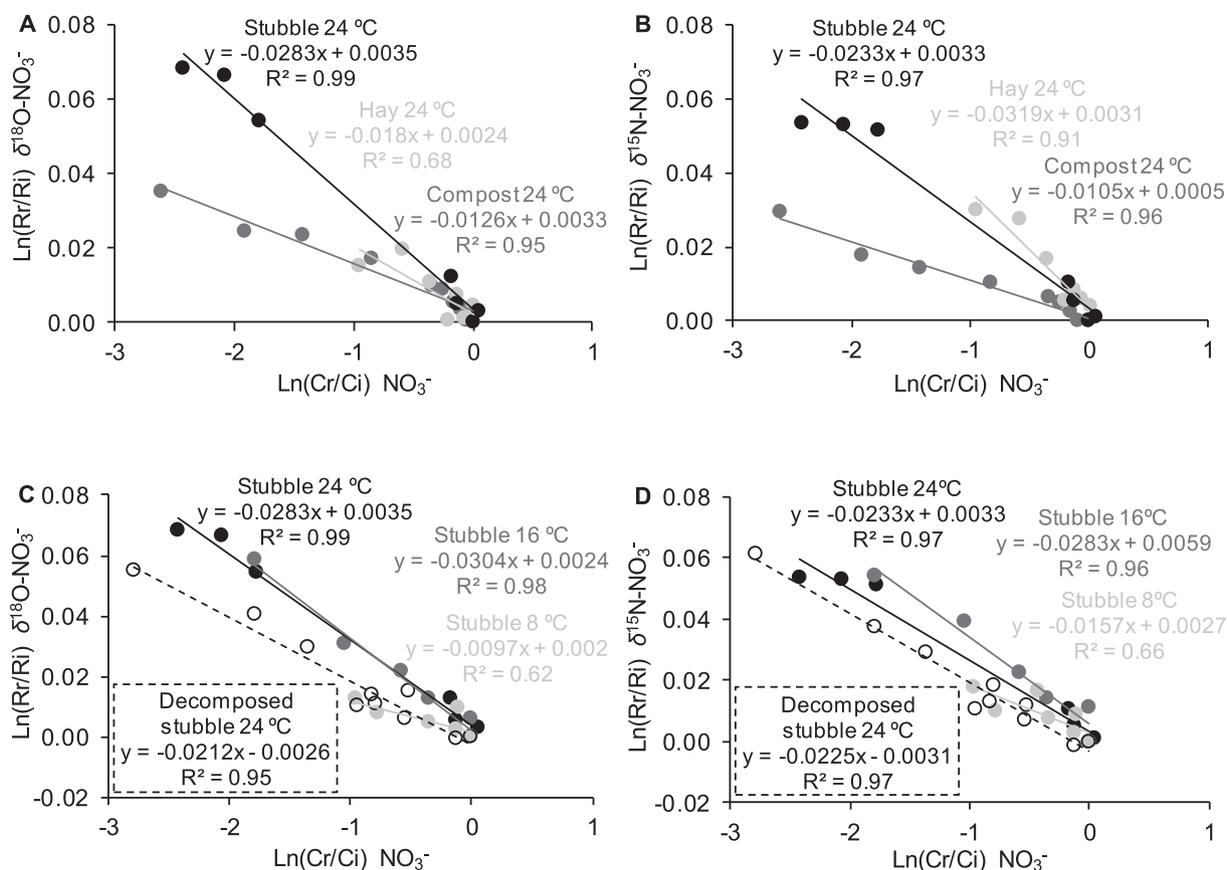


Fig. 3. NO_3^- -O and NO_3^- -N isotopic fractionation throughout denitrification in the biostimulated microcosms. Results from the batch experiments testing (A, B) different C sources and (C, D) the effects of temperature and lifespan of the stubble.

Table 4

Calculated ϵ values from the laboratory microcosms. $\epsilon^{18}\text{O}_{\text{NO}_3/\text{N}_2}$, $\epsilon^{15}\text{N}_{\text{NO}_3/\text{N}_2}$, and $\epsilon^{15}\text{N}_{\text{NO}_3/\text{N}_2}/\epsilon^{18}\text{O}_{\text{NO}_3/\text{N}_2}$ for each condition tested at laboratory-scale.

Experiment	$\epsilon^{18}\text{O}_{\text{NO}_3/\text{N}_2}$	$\epsilon^{15}\text{N}_{\text{NO}_3/\text{N}_2}$	$\epsilon^{15}\text{N}/\epsilon^{18}\text{O}$
Compost 24 °C	-12.6	-10.5	0.8
Hay 24 °C	-18.0	-31.9	1.8
Stubble 24 °C	-28.3	-23.3	0.8
Stubble 16 °C	-30.4	-28.3	0.9
Stubble 8 °C	-9.7	-15.7	1.6
Decomposed stubble 24 °C	-21.2	-22.5	1.1

NO_3^- was not significantly reduced during the two sampling campaigns performed at a ~ 5.5 L/s flow rate (June and September 2017), a slight attenuation (from 1.3 to 0.8 mM) occurred under operation at ~ 2.5 L/s (September 2017) (Fig. 4A). In all samples, NO_2^- was below the detection limit and NH_4^+ was below 0.01 mM, suggesting that NO_3^- had been either transformed to gaseous N products through denitrification or assimilated by plants or microorganisms (Supporting information Table S5). Whereas no increase in $\delta^{15}\text{N}-\text{NO}_3^-$ nor $\delta^{18}\text{O}-\text{NO}_3^-$ was observed in the samples collected during the first campaign at ~ 5.5 L/s (June 14, 2017), an increase was observed at the middle section of the CW (H3) during the second survey at ~ 5.5 L/s (September 5, 2017). In this latter campaign, the inlet (H1) presented $\delta^{15}\text{N}-\text{NO}_3^-$ and $\delta^{18}\text{O}-\text{NO}_3^-$ values of +11.6‰ and +8.7‰, respectively, which increased at the middle point (H3) up to +19.2‰ and +18.2‰, respectively, and decreased at the outlet (H6) to +9.6‰ and +7.1‰, respectively (Fig. 4B). A proposed explanation is that the occurrence of preferential flows within the wetland (e.g., heterogeneous flow rate between surface and bottom water or between lateral and central water) could have led to an increased hydraulic retention time and/or stagnant water at the

H3 sampling site. In the campaign performed at a ~ 2.5 L/s flow rate (September 12, 2017), the decrease in NO_3^- concentration was coupled to increases in $\delta^{15}\text{N}-\text{NO}_3^-$ and $\delta^{18}\text{O}-\text{NO}_3^-$ from the inlet (+7.0‰ and +4.7‰, respectively, at H1) to the outlet (+17.1‰ and +13.0‰, respectively, at H6) of the CW (Fig. 4B). The slope of the relation between $\delta^{18}\text{O}-\text{NO}_3^-$ and $\delta^{15}\text{N}-\text{NO}_3^-$ for the samples collected in these three campaigns was 0.8 ($r^2 = 0.91$) (Fig. 4B), which is indicative of denitrification activity (Aravena and Robertson, 1998). These results are in agreement with previous results reporting the occurrence of denitrification in CWs even in the presence of dissolved O_2 (Sirivedhin and Gray, 2006). The intrinsic denitrification activity in the CW did not support complete denitrification, likely due to the low NPDOC content of the water (0.4–0.6 mM). Therefore, it was decided to evaluate the feasibility of adding an external electron donor source to promote NO_3^- attenuation when operating at a ~ 16 L/s flow rate.

3.6. Performance of the CW after application of stubble

Application of stubble in autumn (September 25, 2017) induced denitrification in the CW approximately 2 d after the application (Fig. 5B). Denitrification was almost complete at the outlet (H6) by 14 d following the application (0.2 mM NO_3^- remaining of the initial 1.4–1.7 mM) (Fig. 5A). In the two sampling campaigns, NO_2^- accumulated beginning at H2 and reached a concentration of 0.2 mM by the outlet (H6) by 7 d after treatment, but decreased to 0.1 mM by 14 d. Such a decrease in NO_2^- accumulation over time has been previously observed in laboratory experiments and other denitrification studies (Carrey et al., 2014; Margalef-Marti et al., 2019; Vidal-Gavilan et al., 2013). The maximum NH_4^+ concentration of 0.02 mM was measured at H3 after 7 d, while it decreased by the outlet (H6) to 0.01 mM in both campaigns, pointing to a non-significant contribution of DNRA. Due to

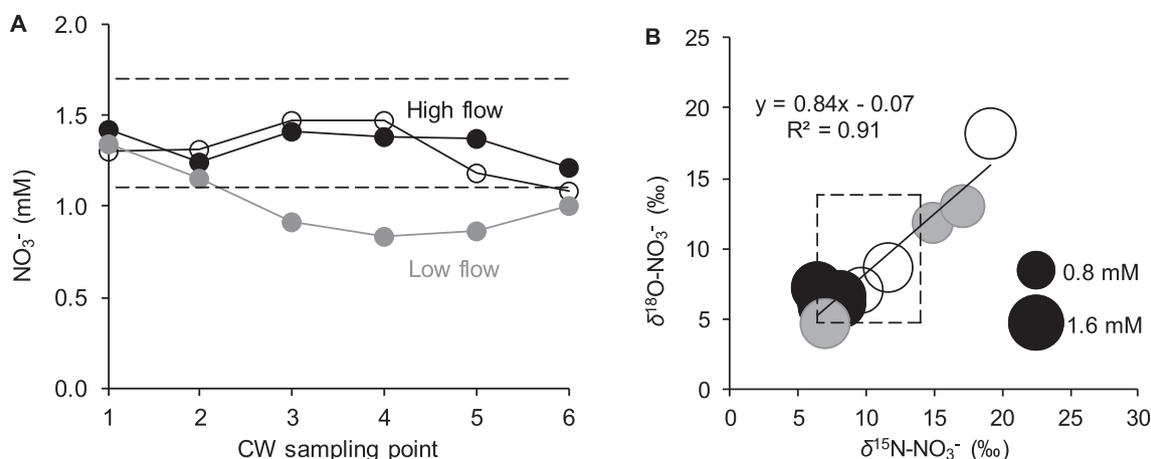


Fig. 4. NO₃⁻ attenuation in the CW before the application of stubble. Black circles depict the sampling campaigns performed at a ~5.5 L/s flow rate (full symbols for the campaign of June 14, 2017 and empty symbols for that of September 5, 2017), and grey circles depict the sampling campaigns performed at a ~2.5 L/s flow rate (September 12, 2017). (A) NO₃⁻ concentration along the CW flow direction, where dashed lines represent the range of NO₃⁻ concentrations measured at the inlet of the CW throughout the study period. (B) Isotopic characterization including the regression line, where the dashed square represents the range of isotopic compositions measured at the inlet of the CW throughout the study period.

the application of stubble, the outlet flow rate decreased until the system became partially blocked, leaving the monitoring probes exposed to the air. When the problem was solved (October 17, 2017) and the outlet flow was stabilized at approximately 16 L/s, the NO₃⁻ concentration monitored at the outlet showed fluctuations, pointing to a slight denitrification activity until October 24, 2017 (Fig. 5C). Thus, the lifetime of the treatment in autumn (recorded temperatures in October 2017 ranged from 10.3 °C to 20.4 °C, averaging 16.0 °C) was estimated to be between 2 weeks and 1 month.

Application of stubble in spring (May 5, 2018) also induced denitrification and underwent a shorter acclimation time (1 d) with respect to the first application, likely due to faster acclimation by the previously stimulated bacterial community (Fig. 6A). By 7 d after the stubble application, the NO₃⁻ concentration at the outlet (H6) was 0.2 mM, and denitrification was complete after 14 d. The NO₃⁻ concentration in the outlet then began to increase progressively until reaching a level similar to that at the inlet by approximately 100 d after treatment (Fig. 6B). A lower NO₃⁻ concentration measured in H3 during the last sampling campaign (+161 d) was attributed to stagnant water near the sampling point due to the accumulation of partially decomposed stubble. Thus, the treatment in spring-summer (temperatures recorded from May to October 2018 presented monthly minimums from 9.6 to 19.9 °C, monthly maximums from 20.0 to 28.4 °C, and monthly averages from 15.8 to 24.6 °C) induced significant denitrification for approximately three months, which is three times longer than that induced by the treatment in autumn. The NO₃⁻ concentration decrease at the outlet compared to inlet during these three months ranged from 0.1 to 1.5 mM (highest attenuation corresponded to the first month after stubble application). All the monitored NO₃⁻ concentrations at the inlet and outlet of the CW during this study period are presented in the Supporting Information Figure S1. The increased efficiency of the treatment in spring-summer compared to that of the treatment in autumn is in accordance with laboratory results (incubation at 8, 16, and 24 °C) and with previous wetland studies reporting increased denitrification rates at higher temperatures (Bachand and Horne, 1999; Christensen and Srensen, 1986; Si et al., 2018). The faster acclimation by the previously stimulated bacterial community could have been also responsible for this increased attenuation activity.

After the second stubble application, 0.1 mM of NO₂⁻ was detected at the outlet (H6) for 63 d. Afterwards, it was no longer detected (except at the aforementioned point H3 where water stagnated), confirming a decreased NO₂⁻ accumulation with time as observed during the previous treatment period. The maximum NH₄⁺ concentration

detected, 0.3 mM, was recorded at H4 7 d after application, while at the outlet (H6), the concentration was 0.05 mM. At 14 d and one month after application, NH₄⁺ at the outlet decreased to 0.02 mM and 0.01 mM, respectively. These results suggest that transient DNRA activity could have arisen between H2 and H4 following the stubble application. NO₃⁻ is reduced to NH₄⁺ through DNRA, depending on parameters such as microbial growth rate, NO₂⁻ accumulation, and the C:N ratio (Kraft et al., 2014). It is generally accepted that DNRA is favored at high C:N ratios, when NO₃⁻ is limited (rather than the electron donor being limited), or when high NO₃⁻ levels inhibit NO₂⁻ reductase (Giles et al., 2012; Kelso et al., 1997). This hypothesis is consistent with the higher degree of NH₄⁺ accumulation observed in laboratory experiments compared to that observed in the field, since higher C:N ratios with a more homogeneous distribution were found in the batch experiments. It is also necessary to account for the possible input of N from the applied stubble. NO₂⁻ and NH₄⁺ have a lower threshold for human consumption (0.01 and 0.03 mM, respectively) with respect to that of NO₃⁻ (0.8 mM) (98/83/EC, 1998). However, since NH₄⁺ accumulation decreased before the outlet and fell to insignificant levels by 14 d after treatment, and the NO₂⁻ accumulation also decreased over time, stubble was considered effective in removing N compounds from agricultural runoff water.

A few authors have previously attempted to calculate the denitrification efficiency in CWs by means of isotopic assessment, but using ϵ values available in the literature and only for N-NO₃⁻ (Lund et al., 1999; Søvik and Mørkved, 2008). The $\epsilon^{15}\text{N}_{\text{NO}_3/\text{N}_2}$ and $\epsilon^{18}\text{O}_{\text{NO}_3/\text{N}_2}$ values obtained from our lab-scale experiments in which fresh stubble was incubated at 24 and 16 °C, and partially decomposed stubble was incubated at 24 °C, were used to calculate three denitrification % lines (Eq. (2), derived from Eq. (1)) that were plotted on a graph of $\delta^{18}\text{O}\text{-NO}_3^-$ versus $\delta^{15}\text{N}\text{-NO}_3^-$ along with the isotopic results for the CW samples (Fig. 7). These three laboratory conditions encompass the average temperatures recorded during the biostimulation periods tested at the CW. The slope of $\delta^{18}\text{O}\text{-NO}_3^-$ versus $\delta^{15}\text{N}\text{-NO}_3^-$ for the field samples collected after the biostimulation treatment was 1.0 ($r^2 = 0.98$) (Fig. 7), which is slightly higher than that obtained for the intrinsic denitrification before the stubble addition (0.8 ($r^2 = 0.91$)), which is likely due to the use of a different C source and the promotion of a different bacterial community. Also, the slope obtained after the biostimulation (1.0) was similar to the slopes obtained in the lab-scale experiments using partially decomposed stubble incubated at 24 °C (0.9) and those using fresh stubble incubated at 24 and 16 °C (1.25 and 1.1, respectively). This is consistent with the temperatures registered in

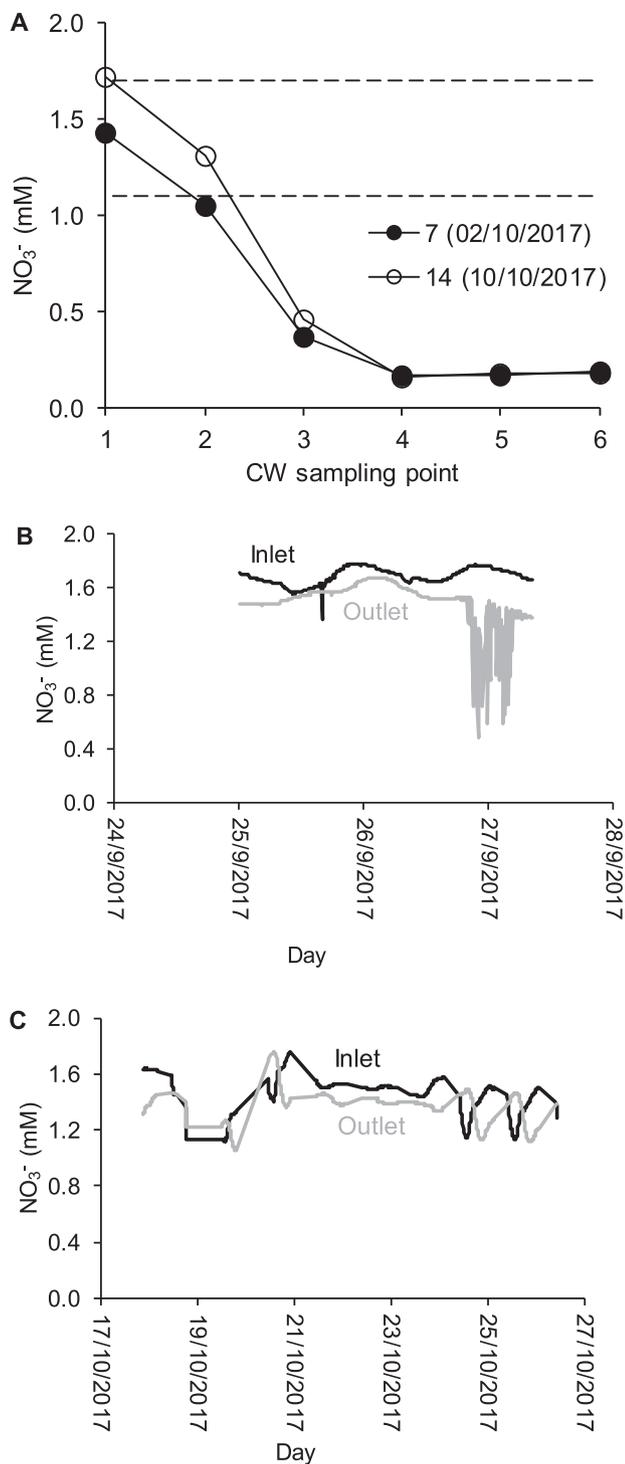


Fig. 5. NO_3^- attenuation in the CW after the first application of stubble in autumn. (A) NO_3^- concentration along the CW, where dashed lines represent the range of NO_3^- concentrations measured at the inlet of the CW throughout the study period. Full symbols depict the sampling campaign conducted on October 2, 2017, and empty symbols depict that conducted on October 10, 2017 (seven and fourteen days after the application of the stubble, respectively). (B, C) NO_3^- concentrations monitored at the inlet (black) and outlet (grey) in the days before and after the sampling campaigns, respectively.

the area throughout the test period, and with the stubble being in contact with water. This similarity between the field-scale and lab-scale slopes, together with the observed isotopic fractionation in the CW suggested that plant uptake did not likely contribute significantly to the NO_3^- removal.

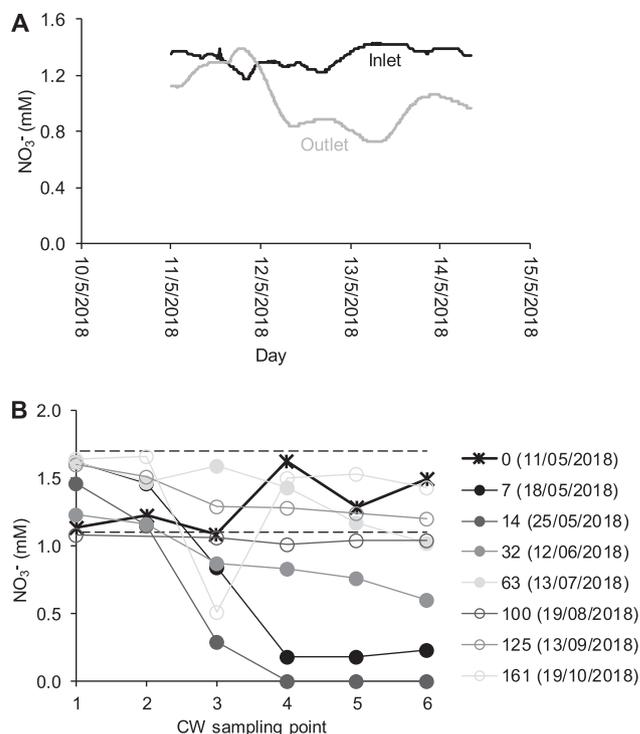


Fig. 6. NO_3^- attenuation in the CW after the second application of stubble in spring. (A) NO_3^- concentrations monitored at the inlet (black) and outlet (grey) of the CW throughout the first days of treatment. (B) NO_3^- concentrations along the CW flow direction, for each sampling campaign. Dashed lines represent the range of NO_3^- concentrations measured at the inlet of the CW throughout the study period. The 7 sampling campaigns performed throughout the 100 days after stubble application are represented by shades of grey (from darker to lighter as time progressed), with the last represented by the empty symbols. The campaign performed before application is represented by asterisks.

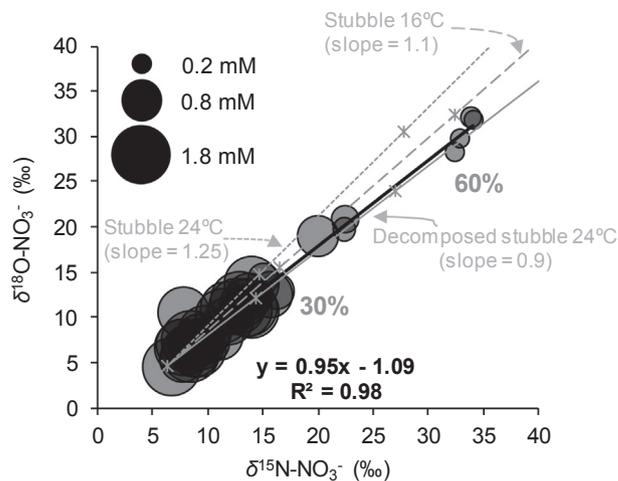


Fig. 7. Denitrification efficiency in the CW determined from the laboratory-obtained ϵ values. Isotopic values obtained from the samples collected at the CW, including the regression line (black). The three denitrification % lines (grey) presented correspond to the three conditions tested in the laboratory that were closest to the CW conditions throughout the field-scale test.

$$\text{Denitrification\%} = \left[1 - \left(\frac{C_{\text{residual}}}{C_{\text{initial}}} \right) \right] \times 100$$

$$= \left[1 - e^{\left(\frac{\delta_{\text{residual}} - \delta_{\text{initial}}}{\epsilon} \right)} \right] \times 100 \quad (2)$$

The results showed that at least 60% of NO_3^- attenuation was

achieved in the CW due to the induced denitrification, although this value was obtained from the less-favorable situation (where the denitrification % was calculated from the experiments using stubble incubated at 24 °C). If the denitrification % is instead calculated based on the experiment using partially decomposed stubble incubated at 24 °C, which presents the slope most similar to that of the field samples (0.9 compared to 1.0), then denitrification accounted for a 70% NO_3^- removal. The largest contribution of denitrification, as determined by isotopic data, was observed in the outlet samples (H6) taken 7 and 14 d after the first stubble application, and 7 d after the second stubble application. By 14 d after the second stubble application, NO_3^- concentration in some samples was below the level required for the isotopic analysis. Therefore, the induced denitrification allowed a NO_3^- attenuation close to 100%. After two weeks of treatment, the contribution of the induced denitrification at the outlet (H6) began to decrease, from 30% in June 2018 to 5% in September 2018, and slightly increased to 20% by the last survey in October 2018. Considering an average flow rate of 16 L/s (the application of stubble led to fluctuations in the flow rate due to partial blockages at some points caused by stubble accumulation) and these results, in the studied CW, at least 80 kg of NO_3^- were removed per day over the first two weeks after the stubble application in May 2018 and 30 kg/d were removed from 14 to 63 d after the supply, after which time the removal decreased. A comparison between the denitrification percentages calculated using chemical and isotopic data revealed that using concentration values always resulted in a higher value. Since the contribution calculated from the isotopic data was considered to be linked to NO_3^- attenuation due to denitrification, the difference could be due to a decrease in NO_3^- concentration provoked by dilution due to precipitation, or the contribution of plant uptake to NO_3^- attenuation.

3.7. Effect and evolution of NPDOC in the CW

Since the amount of organic C released from the CW to the Lerma gully, and from there to the Arba River was a matter of concern, the NPDOC concentration in the field samples were measured. Only the results obtained from the second stubble application are discussed (the first application gave similar results). The NPDOC concentration at the outlet increased with respect to the background values by 7 d after application, and the increased level was maintained for 14 d in total (Fig. 8). The increase began at H3, reached a maximum between H4 and H5 (1.6–1.7 mM), and decreased to approximately 1.0–1.1 mM by the outlet (H6), indicating a release of organic C to the gully (background NPDOC concentrations ranged from 0.5 to 0.8 mM). Because the gully contained NO_3^- -polluted water, it was considered that the surplus organic C could lead to NO_3^- attenuation downstream. The high NPDOC concentration detected between H3 and H5 could have provoked a

decrease in water quality within the CW due to the promotion of processes such as DNRA (previously discussed) and bacterial SO_4^{2-} reduction (BSR), through which NH_4^+ and H_2S are produced, respectively. Although BSR is usually induced when NO_3^- is completely removed from the environment, the coexistence of denitrification and BSR in the presence of an abundance of an electron donor has also been demonstrated (Laverman et al., 2012). The isotopic characterization of SO_4^{2-} can provide information to assess its transformation processes because the decrease in SO_4^{2-} concentration is coupled to increases in $\delta^{34}\text{S}\text{-SO}_4^{2-}$ and $\delta^{18}\text{O}\text{-SO}_4^{2-}$ through the BSR (Strebel et al., 1990). Correlation between SO_4^{2-} concentration and isotopic composition was not identified in the samples collected at the CW, suggesting that BSR was not occurring in the CW. Therefore, the possibility of a decrease in water quality in the CW due to H_2S production as a result of excess organic C was discarded.

3.8. Suitability of the strategy and future improvements

The remediation strategy tested in the CW allowed the induction of the removal of NO_3^- from agricultural runoff water. The NO_3^- attenuation was primarily related to denitrification. Previous studies also found that denitrification was an important N sink in CWs in comparison to plant uptake (Lin et al., 2002; Soana et al., 2017). It has to be considered that denitrification can only be considered a N sink if intermediate products such as NO_2^- or N_2O are not accumulated during the NO_3^- reduction. The added stubble could have enhanced denitrification not only by increasing the organic C content of the water but also by inhibiting O_2 production through photosynthesis by shading the water column, as previously hypothesized by Jacobs and Harrison (2014) for floating vegetation in CWs. However, the denitrification efficiency was limited. The most likely explanation involves the high O_2 content of the inlet water (near saturation, approximately 0.28 and 0.34 mM in summer and winter, respectively) (Merchán et al., 2014) and the vast surface available for O_2 diffusion. Other parameters that could have also contributed to the limited denitrification efficiency include the high water flow rate tested in the CW (~16 L/s), and the possible generation of preferential flows within the CW (e.g., due to stubble accumulation in some points) that could lead to a low degree of interaction between water and stubble.

Although application of solid residues such as maize stubble in surface flow CWs might have advantages over the application of liquid organic C sources, which face the problems of greater loss by bacterial oxidation (Lin et al., 2002) and greater release with the water flow, new strategies for increasing the lifespan and efficacy of the induced denitrification must be investigated. In addition, increased intrinsic denitrification capacity of the CW is expected after plant growth covers the entire surface. Previous studies have reported increased denitrification activity in vegetated CWs relative to the levels in non-vegetated systems (Lin et al., 2002; Soana et al., 2017), with efficacy varying among plants of different species or age (Lin et al., 2002; Lund et al., 1999). The organic C pool released after plant senescence has also been demonstrated to increase the bacterial activity, as this C can also be used as electron donor (Peralta et al., 2012; Soana et al., 2017). In this direction, Kang et al. (2018) proposed the use of plants whose growth season is winter. Therefore, the organic C supply from senescence would occur throughout the summer months, when temperatures are higher and more appropriate conditions are established for the promotion of significant denitrification activity.

N_2O production was not assessed in our field-scale tests. At lab-scale, limited N_2O production was observed. However, at field-scale, higher N_2O emissions could occur as a result of denitrification induced by the stubble addition because the high O_2 content of the inlet water and the free surface water flow might allow more extensive O_2 diffusion in water. Since N_2O emissions are detrimental for air quality, the production of this greenhouse gas should also be monitored in treatments aiming to induce denitrification. Isotopic characterization of the N_2O

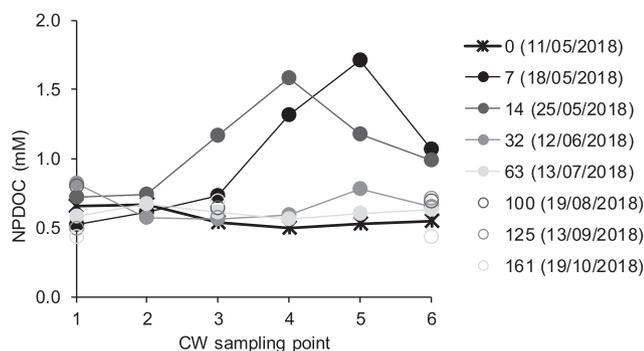


Fig. 8. Dissolved organic C in the CW before and after application of stubble. NPDOC concentration along the CW flow. The 5 sampling campaigns conducted within the 63 days after stubble application are presented in shades of grey, from darkest to lightest as time progressed. The campaign performed before application is represented by asterisks.

emitted from a given CW could also provide further information about the N transformation processes that led to the decrease in NO_3^- concentration.

4. Conclusions

At laboratory-scale, maize stubble, wheat hay, and animal compost were able to induce complete denitrification. Stubble was selected for field-scale application due to its better local availability. In the following incubations, stubble sampled from the CW 7.5 months after its application was still able to support complete NO_3^- attenuation. Complete NO_3^- attenuation was achieved over the temperature range of 8 to 24 °C, although lower temperatures resulted in lower reduction rates. The $\epsilon^{15}\text{N}_{\text{NO}_3/\text{N}_2}$ and $\epsilon^{18}\text{O}_{\text{NO}_3/\text{N}_2}$ values obtained from the laboratory experiments allowed evaluation of the performance of the remediation strategy at the CW.

Before the application of the stubble, NO_3^- attenuation at the CW (from 1.3 to 0.8 mM) was only observed when the flow was decreased from approximately 5.5 to 2.5 L/s. The biostimulation in autumn–winter promoted NO_3^- attenuation lasting between 2 weeks and one month, while in spring–summer the attenuation capacity remained for approximately three months (~16 L/s flow rate). The isotopic characterization of the CW samples indicated that at least 60% of the initial NO_3^- was removed in the CW due to the induced denitrification. However, since in a few samples NO_3^- was below the limit necessary for isotopic analysis, the contribution could have been higher. The slope of $\delta^{18}\text{O}\text{-NO}_3^-$ versus $\delta^{15}\text{N}\text{-NO}_3^-$ obtained in the CW after the stubble application (1.0) was close to that obtained in the experiments involving partially decomposed stubble incubated at 24 °C (1.1). Plant uptake seemed to play only a minor role in NO_3^- attenuation in the CW. The treatment was considered safe because NO_2^- and NH_4^+ accumulation (below 0.2 and 0.1 mM, respectively) decreased over time, surplus NPDOC (below 2.3 mM) released from the CW could maintain NO_3^- attenuation downstream, and because the occurrence of BSR was discarded. However, the longevity and effectivity of the treatment were limited due to the high O_2 content of the inlet water, high water flow, and the possible generation of preferential flows within the CW.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jhydrol.2019.124035>.

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