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Enhancement of carbon and nitrogen removal by helophytes along subsurface water flowpaths receiving treated wastewater

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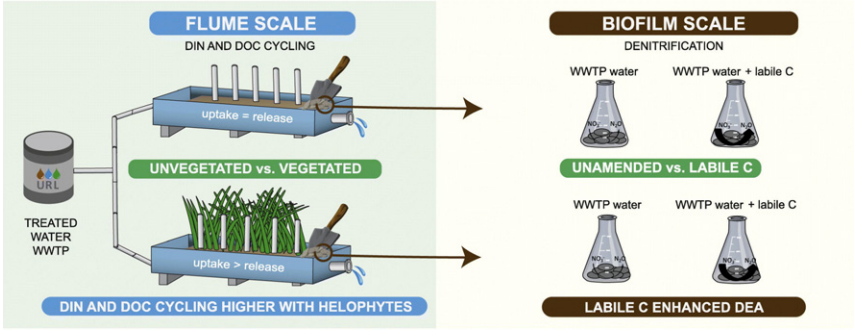
HIGHLIGHTS

- Presence of helophytes enhanced DIN and DOC removal from the WWTP effluent.
- Denitrification of biofilms was limited by DOC quality of the WWTP effluent.
- Helophytes: bioremediation tools to improve water quality in WWTP-impacted systems.

GRAPHICAL ABSTRACT



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ABSTRACT

Wastewater treatment plant (WWTP) effluents are sources of dissolved organic carbon (DOC) and inorganic nitrogen (DIN) to receiving streams, which can eventually become saturated by excess of DIN. Aquatic plants (i.e., helophytes) can modify subsurface water flowpaths as well as assimilate nutrients and enhance microbial activity in the rhizosphere, yet their ability to increase DIN transformation and removal in WWTP-influenced streams is poorly understood. We examined the influence of helophytes on DIN removal along subsurface water flowpaths and how this was associated with DOC removal and labile C availability. To do so, we used a set of 12 flow-through flumes fed with water from a WWTP effluent. The flumes contained solely sediments or sediments with helophytes. Presence of helophytes in the flumes enhanced both DIN and DOC removal. Experimental addition of a labile C source into the flumes resulted in a high removal of the added C within the first meter of the flumes. Yet, no concomitant increases in DIN removal were observed. Moreover, results from laboratory assays showed significant increases in the potential denitrifying enzyme activity of sediment biofilms from the flumes when labile C was added; suggesting denitrification was limited by C quality. Together these results suggest that lack of DIN removal response to the labile C addition in flumes was likely because potential increases in denitrification by biofilms from sediments were counterbalanced by high rates of mineralization of dissolved

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organic matter. Our results highlight that helophytes can enhance DIN removal in streams receiving inputs from WWTP effluents; and thus, they can become a relevant bioremediation tool in WWTP-influenced streams. However, results also suggest that the quality of DOC from the WWTP effluent can influence the N removal capacity of these systems.

1. Introduction

During the last decades, implementation of wastewater treatment plants (WWTP) in developed countries has contributed to reduce the inputs of organic matter and nutrients derived from urban activity to natural streams (i.e., EEA – European Environmental Agency, 2010). However, despite the relevant technological advances achieved in wastewater treatments (Metcalf and Eddy, 2014) so far, the effluents from WWTPs are still important sources of dissolved organic carbon (DOC) and inorganic nutrients, such as nitrogen (N) and phosphorus (P). This excess of organic matter and nutrients can eventually cause deterioration of the water quality and ecological status of the receiving aquatic ecosystems (EEA – European Environmental Agency, 2010; Smith et al., 1999). This problem is particularly relevant in regions with water scarcity, where inputs from WWTP effluents can account for 100% of stream flow, especially during summer (Martí et al., 2010). The process of nutrient removal within WWTP facilities has important energetic and economic constraints (Carey and Migliaccio, 2009). Therefore, it is important to explore alternative management strategies involving less cost and higher sustainability to reduce nutrient loads, such as those focused on increasing the nutrient retention capacity of receiving aquatic ecosystems (Newcomer Johnson et al., 2016).

Streams receiving WWTP effluents have a remarkable capacity to biogeochemically process the excess of dissolved inorganic nitrogen (DIN) loads from WWTP effluents (Haggard et al., 2005; Lofton et al., 2007; Merseburger et al., 2005). In streams, DIN processing (i.e., uptake, transformation and removal) occurs along water flowpaths in both surface stream channels (Ribot et al., 2012) and subsurface hyporheic zones (Lawrence et al., 2013). These processes are mostly driven by microbial assemblages (i.e. biofilms) that develop on different streambed substrata as well as on hyporheic sediments (Battin et al., 2003; Pusch et al., 1998). In addition, aquatic plants (e.g., helophytes) developing on the streambed and stream-riparian margins can also contribute to decrease DIN concentration from surface (Pastor et al., 2013; Peipoch et al., 2013) and subsurface water (Schade et al., 2001) because they rely on DIN to meet their N demand for assimilatory uptake. Furthermore, the root system of helophytes (i.e., rhizosphere) provide favorable habitat for biofilm development and activity in hyporheic sediments (Andrews and Harris, 2000; Ramey et al., 2004) because it can provide oxygen and labile DOC (Maltais-Landry et al., 2009; Stottmeister et al., 2003). Thus, helophytes can directly or indirectly enhance DIN uptake, transformation and removal, especially along subsurface water flowpaths and thus, they have been used as tertiary treatments of WWTP effluents (Gottschall et al., 2007; Toscano et al., 2015). More recently, helophytes have been used as bioengineering tools in river restoration projects to stabilize river margins and reduce channel erosion (Evette et al., 2009; Li and Eddleman, 2002). However, in these restoration projects, the potential role of helophytes on DIN processing has been largely overlooked.

Denitrification is a microbial process that leads to the removal of DIN from the stream water into the atmosphere. Under low oxygen conditions, denitrifying bacteria oxidize DOC using NO_3^- as an electron acceptor, which is reduced to N_2O, NO, and N_2 (Lin et al., 2009; Seitzinger et al., 2006). However, because other biogeochemical processes associated with DIN processing co-occur in streams, the DIN removal capacity of the stream ultimately depends not only on the rate at which denitrification occurs, but also on the balance between processes contributing to DIN uptake from the water column (i.e., DIN assimilation, denitrification) and DIN release to the water column (i.e., mineralization of dissolved organic nitrogen). In this sense, studies conducted in WWTP-influenced streams have shown small downstream changes in DIN concentration, suggesting either that release and uptake processes counterbalance each other as it occurs in pristine streams (Bernal et al., 2015; von Schiller et al., 2015) or that rates of uptake processes, in particular denitrification, are low due to some limiting factor (Lofton et al., 2007; Merseburger et al., 2005; Ribot et al., 2012). Among other factors, such as redox and oxygen concentration, denitrification can also be limited by the availability of DOC as observed in pristine ecosystems (Hill et al., 2000) or among streams subjected to different human pressures (Mulholland et al., 2008). Given that WWTP effluents are relevant sources of DOC to receiving streams; DOC availability may not be a limiting factor for denitrification in WWTP-influenced streams (Meng et al., 2013; Saadi et al., 2006). However, the quality of DOC (i.e., the structural complexity of molecules and its lability) can also affect denitrification rates, as shown by decreases in the uptake rates when DOC sources are recalcitrant (Fernandez-Nava et al., 2010; Hagman et al., 2008; Pulou et al., 2012). Previous studies indicated that the quality of DOC from WWTP effluents mostly depends on the wastewater treatment process within the WWTP facility (Imai et al., 2002; Krasner et al., 2009). For instance, the higher the nitrification efficiency during the aerobic phase of the treatment, the lower the quality of DOC at the effluent of the WWTP (Krasner et al., 2009), thus releasing more complex and less bioavailable molecules of DOC to recipient streams. Therefore, the processes occurring within the WWTP could influence denitrification rates, and ultimately the extent of DIN removal in receiving streams, because they influence the quality of DOC in these ecosystems.

In this study, we experimentally examined the influence of helophytes on DIN removal along subsurface water flowpaths and how this removal was associated with DOC removal and the availability of labile C sources. To this aim, we examined longitudinal profiles in DIN and DOC concentrations along 9 flumes containing three different species of helophytes and compared them with those observed in 3 unvegetated flumes. All flumes were continuously fed with water from a WWTP effluent and had only subsurface water flowing through. We additionally tested the effect of adding a labile C source on DOC and DIN removal in this experimental setting and on the potential denitrifying enzyme activity (DEA) associated with the microbial assemblages developed on the flume sediments. We expected that DIN removal along subsurface water flowpaths will be higher in flumes with helophytes since they assimilate DIN and their roots can release labile DOC compounds that may enhance rates of microbial denitrification. We also expected that the addition of a labile C source would further enhance DIN removal along the flumes due to the stimulation of denitrification rates associated with microbial assemblages in sediments. This study contributes to elucidate how the presence of helophytes can influence subsurface water DIN removal in stream ecosystems impacted by WWTP effluents, and provides insights on the role of DOC quality on DIN removal in these ecosystems.

2. Material and methods

2.1. Description of the experimental flumes

The study was performed at the "Urban River Lab" outdoor research facility located in the municipality of Montornès del Vallès (NE Barcelona, Spain; www.urbanriverlab.com). This facility has 12 flow-through mesocosms (flumes). Each flume consists of a cross sectional U-

shaped concrete channel (length: 12 m, width: 0.6 m, depth: 0.4 m) filled to a depth of 25 cm with commercially available sediments (i.e., gravel), used in river restoration. Flumes are fed with water from the effluent of the WWTP of Montornès del Vallès, which is neither additionally treated nor diluted with any other source of water. A fraction of water from the effluent is pumped from the WWTP outlet into a tank and then distributed to all the flumes by gravity. Inflow discharge at each flume is 5 L min^{-1} and water flow along the flumes is maintained at subsurface levels. Inflow water is characterized by high electrical conductivity (EC, 2.5 ± 0.02 mS cm^{-1}), low dissolved oxygen (DO, 4.5 ± 2% saturation), and high DOC and DIN concentrations (9.7 ± 0.6 mg C L^{-1} and 5.2 ± 0.6 mg N L^{-1}, respectively). DIN is mostly composed by NO_3^- (89 ± 3%), whereas NH_4^+ and NO_2^- represent the 13 ± 3% and 1.3 ± 0.2% of DIN, respectively.

For this study, the experimental set up included 3 flumes only with sediments (i.e., unvegetated) and 9 flumes with sediments and 3 species of helophytes (3 flumes per species): *Iris pseudacorus L.*, *Scirpus lacustris L.* (common bulrush) and *Phragmites australis L.* (common reed). These species are autochthonous of the region and are typically used in constructed wetlands and stream restoration actions (Evette et al., 2009; Larned et al., 2006; Toscano et al., 2015). The plant density was set at 6.7 shoots m^{-2} in each flume, which were planted in early march 2015. The 3 replicates for each treatment (i.e., no helophytes and 3 flume sets with different helophyte species) were randomly distributed across the 12 flumes. To sample subsurface water along each flume, we installed 5 PVC tubes, 50 cm long and 2,5 cm diameter, that were screened 15 cm over the bottom. PVC tubes were placed at 1, 3, 5, 7, 9 m from the inlet.

2.2. Field experiment in the flumes

The experiment was carried out during 4 consecutive weeks from July 29th to August 27th of 2015, when helophytes were fully developed. During this period, the weather was sunny and hot, without major rain events. The mean daily temperature was 24.1 °C, ranging from 19.4 to 27.7 °C. The mean daily relative humidity (%) was 63.4%, ranging from 52.0 to 72.0%. During the whole study period (n = 30 days), there were 7 rain events with an average of 8.6 mm per event. In any case, we sampled under raining conditions. Meteorological data was provided by the Servei Meteorològic de Catalunya (www.meteo.cat) from a meteorological station located 2.9 km from the experimental facility.

The experiment consisted of measuring longitudinal profiles of DIN and DOC concentrations as a proxy of removal capacity and then assessing the biogeochemical response of the flumes to an addition of a labile organic C source. For this purpose, we characterized longitudinal changes in DOC and DIN concentrations in the flumes previous to and during the addition of the labile organic C source (hereafter referred as the PRE and +C samplings, respectively). During each week, the experiment was conducted on a set of 3 randomly selected flumes where we collected water samples from the PVC tubes and from the inlet (0 m) and outlet (12 m) of each flume before and during the C addition (7 sampling points in total along each flume, Fig. 1). Subsurface water samples from the PVC tubes were collected using a plastic syringe connected to a silicone tube. Samples from the inlet and outlet were directly collected using a plastic syringe. Moreover, we measured EC and DO concentration in the 7 sampling points of each flume using a WTW portable conductivity meter and an YSI portable oxygen meter, respectively. We also conducted slug additions of a conservative tracer (i.e., NaCl) into the flumes (n = 3 per flume) to estimate the mean water residence time (WRT) in each treatment using a mass balance approach (Gordon et al., 2004).

The labile organic C source that was added into the flumes consisted of a by-product of the brewing process, which is rich in monosaccharides and oligosaccharides (see Table 1 of the Supplementary Material). We prepared a stock solution of this product (30:1 dilution in tap water) and we injected it to the inlet of the flumes at a constant rate (50 mL min^{-1}) during 72 h to achieve an increase in DOC concentration of 4 mg L^{-1} above that measured as ambient level (see Section 2.1). The +C sampling along the flumes was done 1 h prior to stopping the C addition.

All water samples were immediately filtered through ashed Whatman GF/F glass fiber filters (0.7 μm pore size). A 10 mL aliquot was placed in a Falcon tube and stored frozen until the analysis of the different forms of DIN. A 25 mL aliquot was stored in acid washed glass vials with pH adjusted to 5.5–6 to analyze DOC and total dissolved N (TDN). We analyzed water samples for NO_3^-, NO_2^- and NH_4^+ with standard colorimetric methods (Apha and WPCF, 1995) on an Automatic Continuous Flow Futura-Alliance Analyzer at the Nutrient Analysis Service of the CEAB-CSIC. The detection limits for the used method were 13.2, 0.6 and 13.4 μg for NO_3^-, NO_2^- and NH_4^+, respectively. The DIN concentration was estimated as the sum of the concentrations of the three forms of DIN. The concentration of DOC and TDN was analyzed on a Shimadzu (Tokyo, Japan) TOC-VCSH analyzer. The detection limits for the used method were 0.3 mg L^{-1} for DOC and TDN, respectively. Dissolved organic nitrogen (DON) was estimated by subtracting DIN from TDN concentrations.

2.3. Laboratory experiments with sediments from the flumes

We further assessed the influence of the labile organic C source, used in the flume experiment, on the potential denitrification enzyme activity (DEA) of biofilms that naturally grow on the sediments of the flumes. We used the acetylene (C_2H_2) block technique to measure the DEA as the production rate of N_2O in incubation bottles following the procedure by Holmes et al. (1996). To do so, in the laboratory we incubated sediments naturally colonized in the flumes with either treated water alone or treated water plus the labile C (i.e., the by-product of the

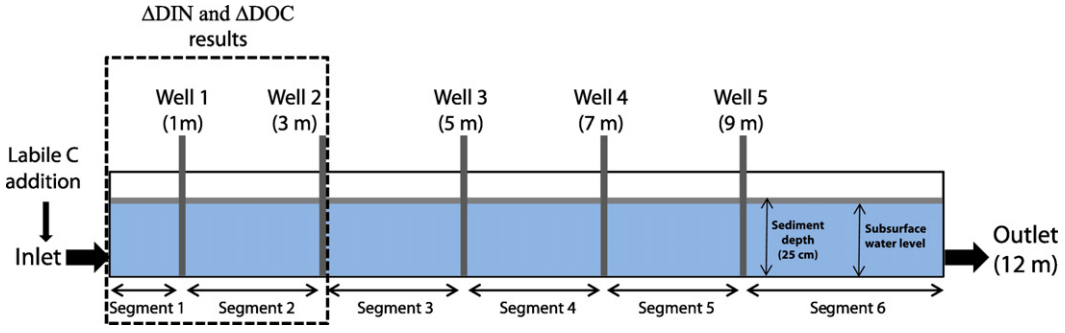

Fig. 1. Scheme of the flume setting used to experimentally approach the objectives of the study. Sediment depth was 25 cm and water was set at subsurface level. We installed 5 wells at 1, 3, 5, 7 and 9 m from the inlet point. The outlet was situated at the end of the flume (12 m). We sampled the 7 points before and during labile C additions referred as PRE sampling and +C sampling, respectively. We then calculated the relative change of either DIN or DOC concentration (ΔDIN and ΔDOC respectively, both in %) between each consecutive pair of sampling points. Since the majority of DOC was consumed within the first 3 m of the flumes, we only showed ΔDIN and ΔDOC results within segment 1 and 2 (flume section framed in bold).

brewing process). Sediments were collected from a three different treatments (i.e., unvegetated, *Iris* and *Phragmites*) after the labile C addition. We collected ca. 300 g of sediments exposed to subsurface water flow (5–10 cm depth) at random locations along each flume (3 flumes per treatment; unvegetated, *Iris*, and *Phragmites*) and placed in a plastic bag as a composite sample for each flume Sediment samples were transported to the laboratory at 4 °C in coolers. Once in the laboratory, ca. 100 g of sediment from each flume was placed into two 250 mL glass bottles. We added 150 mL of flume water to each bottle and left the biofilm to acclimate for 12 h. After acclimation, one of the two bottles was amended with the C source (i.e., + C treatment) while the other one remained unamended (i.e., control treatment).

Incubations amended with labile C were targeted to increase DOC concentration by 4 mg L^{-1} above background concentration as we did for the flume additions. The same procedure was followed for each pair of bottles for each flume treatment. The water in the incubation bottles was then made anoxic by purging helium for 10 min. Bottles were then sealed tight with septa-fitted screw-top lids. We added 10 mL of acetylene (C_2H_2) with a syringe to each incubation bottle. In DEA assays, C_2H_2 is used to block the transformation of nitrous oxide (N_2O) to nitrogen gas (N_2), thus the accumulation of N_2O in the headspace of the incubation bottles is used to estimate denitrification rates (Holmes et al., 1996). Bottles were gently shaken for several minutes to ensure that C_2H_2 mixed well with the water, and were incubated in the dark at ambient laboratory temperature. Gas samples from the headspace were collected using a double needle in 10 mL vacutainers (DB Vacutainer©), after 10 min and 18 h of the C_2H_2 addition. After collecting each gas sample, we added the same volume of C_2H_2 (i.e., 10 mL) to each bottle to maintain the gas volume constant and avoid pressure changes. The analysis of N_2O concentration was conducted in the Serveis cientifico-tecnics of the University of Vic on an Agilent 7890A gas chromatography system (Agilent Technologies, Santa Clara, USA) equipped with electron-capture (ECD) and flame-ionization (FID) + methanizer detectors and three valves to obtain separately carbon dioxide (CO_2), methane (CH_4,) and N_2O for every gas injection. An HP-Plot Q column (30 m × 0.32 mm × 20 μm) was used with a pre-column of the same characteristics but it was 15 m long. The injector and the oven temperature were set to 50 °C. The temperature of the FID and the ECD detectors were set to 250 and 300 °C, respectively. The methanizer temperature was set to 375 °C. For the FID detector, H_2 was used as a carrier gas and N_2 as a make-up gas at 35 and 25 mL min^{-1}, respectively. In the case of the ECD detector, 5% CH_4 in argon was used as a make-up gas at 30 mL min^{-1}. The detection limits for the used method was 0.02 mg L^{-1} of N_2O.

The headspace of each bottle was measured after collection of the final N_2O samples to scale up the concentrations obtained in the 10 mL vacutainers to the total N_2O production in the bottle. The biomass of the biofilm in each bottle was measured as the ash-free dry mass (AFDM). Sediment in each bottle was dried at 60 °C for 12 h, cooled at room temperature and weighed to determine the dry mass. Sediment was then heated at 550 °C in a muffle furnace for 4 h and reweighed. AFDM was estimated as the difference between the weight of the dry mass and the weight of the mass after being muffled.

2.4. Data analysis

We used linear regression analysis with data from PRE-samplings to estimate DIN and DOC removal along the flumes based on the longitudinal variation of DIN and DOC concentrations for each flume treatment (i.e., unvegetated, *Iris*, *Scirpus* and *Phragmites*) before the C addition. Given that there were no additional water inputs along the flumes, we considered that longitudinal changes in DIN and DOC concentration were the result of the net balance between uptake (assimilation by biota and denitrification) and release (organic matter mineralization) processes within the flumes, and thus, could provide a good estimate of the net removal capacity of each flume. For DOC, we considered that microbial respiration was the main responsible process that contributed to the declines in concentration along the flumes (Berggren and del Giorgio, 2015; Wiegner et al., 2015). When DIN concentration increased along the flumes, we assumed that mineralization of organic matter was the main responsible of these increases (Teissier et al., 2007), although direct rates of mineralization were not directly measured. We also considered that root and microbial exudates could contribute to longitudinal increases in DOC concentration (Stottmeister et al., 2003), although this source was assumed to be low compared to DOC inputs from the effluent water which were high. Finally, we assumed no increases in DIN and DOC concentration along the flumes associated with evapotranspiration because longitudinal changes in EC (here used as a hydrological tracer) were minimal during the study period (i.e., <5%; see values in Section 2.3).

Despite longitudinal changes in concentration do not provide specific information about the magnitude of a particular biogeochemical process, this type of data analysis is useful for understanding whether the study mesocosms act either as net sinks (i.e., nutrient removal) or net sources (i.e., nutrient increase) of solutes. Longitudinal decreases in concentration for either DIN or DOC indicate that uptake processes predominate over release and thus, that the flume is acting as a net sink of these compounds and that nutrients are effectively removed along the flumes. Longitudinal increases in concentration indicate the opposite, so that release dominate over uptake processes; and thus, that the flume is acting as a net source of DIN and/or DOC along the flumes. No clear longitudinal pattern of DIN and/or DOC concentration was interpreted as an indication that uptake and release processes counterbalance each other. A similar conceptual approach has been successfully applied for inferring net nutrient uptake (i.e., nutrient removal) in more complex systems such as headwater stream reaches (Bernal et al., 2015; von Schiller et al., 2015).

To further explore the biogeochemical processes associated with longitudinal patterns of DIN concentration, we investigated longitudinal changes in the relative contribution of NO_3^-, NO_2^- and NH_4^+ to total DIN. We assumed that longitudinal decreases in the proportion of NH_4^+ coupled to the increase in the proportion of either NO_3^- or NO_2^- were an indication of the occurrence of nitrification (i.e., oxidation of NH_4^+ to NO_3^-) along the flume. Notice that in this study nitrification was considered a DIN transformation process because do not influence DIN concentration and thus, the overall removal DIN capacity of each flume.

To investigate whether the addition of labile C induced changes in DIN and DOC removal along the flumes, we compared the relative change of either DIN or DOC concentration (ΔDIN and ΔDOC respectively, both in %) between data from PRE and + C samplings. Along each flume, we defined 6 segments comprised within two consecutive pair of sampling points as outlined in Fig. 1. We calculated ΔDIN and ΔDOC for each segment as follows:

$$\Delta Concx = \left(\frac{Conc_{x-1} - Conc_x}{Conc_{x-1}}\right) \times 100 \quad (1)$$

where $Conc_x$ and $Conc_{x-1}$ are the concentrations at a given sampling point and at the previous sampling point, respectively (mg L^{-1} of either DIN or DOC). Similar to longitudinal changes in concentration, ΔDIN and ΔDOC cannot be associated to a particular biogeochemical process, but to the dominant process (i.e., uptake or release) that is characterizing the longitudinal profile of DIN and DOC at each segment. Thus, $\Delta Conc > 0$ denotes a decrease in concentration within the segment indicating uptake > release of either DIN on DOC (i.e., a removal), while $\Delta Conc < 0$ denotes the opposite. The ΔDIN and ΔDOC from the PRE- and + C samplings estimated in the different flume segments were compared using a two-way ANOVA (C addition and segments as fixed factors). The ANOVA test was run separately for each flume treatment (i.e., unvegetated, *Iris*, *Scirpus* and *Phragmites*). Post-hoc Tukey HSD tests followed significant ANOVA ($p < 0.05$).

In the laboratory assays, we focused on how the source of labile C influenced denitrification in biofilms from the flumes, which was expected to be the main biogeochemical process responsible for the permanent removal of DIN from the water column. We used data from the laboratory assays to calculate potential rates of DEA (in g N_2O g $AFDM^{-1}$ h^{-1}) as follows:

$$DEA = \frac{M_f - M_i}{t \; x \; biomass} \quad (2)$$

where M_f and M_i are the N_2O mass in the incubation bottle at the end and at the beginning of the incubation, respectively, t is the incubation time (17.8 h), and *biomass* is the biofilm biomass in the sediments measured as AFDM (in g). Total mass of N_2O in the incubation bottle was calculated using the volume of the headspace and its N_2O concentration and the volume of water corrected for N_2O solubility in the liquid phase with an appropriate temperature-dependent Bunsen coefficient (Knowles, 1979). We used a two-way ANOVA model to explore differences in DEA among sediments from different flume treatments (i.e., unvegetated, *Iris* and *Phragmites*) and between unamended (i.e., control) and C amended (i.e., +C treatment) incubations. Post-hoc Tukey HSD tests followed significant ANOVA ($p < 0.05$).

We ran all statistical tests with R 2.15.0 (R Foundation for Statistical Computing, Vienna, Austria, http://www.R-project.org/.). When necessary, data were log-transformed before analysis to meet assumptions of homogeneity of variance and normality (Zar, 1996).

3. Results

3.1. Characterization of subsurface water within the flumes during PRE samplings

Physicochemical conditions in subsurface water were similar for all flumes (Table 1). EC was high in all cases, ranging from 1.8–2.8 mS cm^{-1}. There were no changes in EC along the unvegetated flumes (<1% increase), while EC tended to increase by 3.1, 4.6 and 2.0% along flumes with *Iris*, *Scirpus* and *Phragmites*, respectively. Oxygen saturation (in %) was consistently low in all the flumes, ranging from 3.6 to 4.5%. DIN was dominated by NO_3^- and represented the 55% of TDN, whereas DON represented the remaining 45%. DOC concentration was high in all flumes, ranging from 6.8 to 11 mg C L^{-1}.

3.2. Longitudinal patterns of DIN and DOC concentrations during PRE samplings

On average, EC increased by 0.4, 3.1, 4.6 and 2.0% between the inlet and outlet of unvegetated flumes and flumes with *Iris, Scirpus* and *Phragmites*, respectively. These results indicated that evapotranspiration had a low effect on longitudinal profiles of DIN and DOC concentrations. Mean WRT (±SE) was 3.9 ± 0.1, 5.4 ± 0.3, 7.7 ± 0.7 and 9.2 ± 0.4 h for unvegetated flumes and flumes with *Iris*, *Scirpus* and *Phragmites*, respectively.

Unvegetated flumes showed no longitudinal changes in DIN concentration, whereas DIN concentration significantly decreased along flumes with helophytes (Fig. 2). On average, DIN concentration decreased by 16.5, 12.0 and 37% between the inlet and outlet of the flumes with *Iris*, *Scirpus* and *Phragmites* respectively.

The relative contribution of NO_3^-, NO_2^- and NH_4^+ to total DIN remained constant along the unvegetated flumes (Fig. 2A). In contrast, in flumes with helophytes the relative contribution of NH_4^+ decreased while the relative contribution of NO_3^- increased along the flumes (Fig. 2B, C and D). This longitudinal shift in the relative contribution of different DIN species was more evident in the flumes with *Scirpus* and *Phragmites* than in those with *Iris*.

Longitudinal changes in DOC concentration varied among the flume treatments. Unvegetated flumes showed no longitudinal changes in DOC concentrations, whereas DOC concentration significantly declined in flumes with helophytes (Fig. 3). On average, DOC concentration decreased by 5.3, 6.3 and 6.9% between the inlet and outlet of flumes for *Iris, Scirpus* and *Phragmites*, respectively.

3.3. Effect of labile DOC addition on DIN and DOC concentrations

On average, the DOC addition increased DOC concentration (mean ± SE) at the inlet from 10.3 ± 0.6 to 11.5 ± 0.6 mg C L^{-1}, which is a 10.4% increase of background DOC concentration. Most of the DOC added was removed within the first meter of the flumes (84 ± 11%). This pattern was consistent among all flumes for all treatments. Thus, the comparison of Δ*Conc* between PRE and +C samplings was conducted for the first two segments of the flumes (i.e., 0–1 m and 1–3 m; Fig. 1).

During PRE sampling conditions, there were small changes in DIN concentration for the first two segments of the flumes (Fig. 4). Mean (±SE) ΔDIN was 0.4 ± 4.0 and 3.3 ± 4.0% for the first and the second segment, respectively, with no statistically significant differences between them (two-way ANOVA, factor flume segment, $df = 1$, $F < 1.2$, p-value ≥0.31). This trend was consistent among unvegetated and vegetated flumes. Similar to DIN, during PRE sampling conditions, there were small differences in DOC concentration between the two segments of the flumes (Fig.5). Mean (±SE) ΔDOC was 0.5 ± 0.6 and 1.8 ± 0.7% for the first and the second segment respectively with no statistically significant differences between them (two-way ANOVA, factor flume segment, $df = 1$, $F < 3.4$, p-value ≥0.11). This trend was consistent among unvegetated and vegetated flumes (Fig. 5).

During +C sampling conditions, changes in DIN concentration for the first two segments were greater than those during the PRE samplings (Fig. 4). Mean (±SE) ΔDIN was 11.0 ± 7.4 and 8.2 ± 4.7% for the first and the second segment respectively with no statistically significant differences between them (two-way ANOVA, factor flume segment, $df = 1$, $F \leq 4.4$, p-value ≥0.07). During +C sampling conditions, changes in DOC concentration for the first two segments were greater than those during the PRE samplings (Fig. 5). Mean (±SE) ΔDOC was 13.1 ± 1.7 and 2.0 ± 1.1% for the first and the second segment respectively. ΔDOC was significantly higher in the first than in the second segment in all flumes except in the *Phragmites* treatment (Tukey test; p-value ≤0.04).

3.4. Effect of labile DOC amendment on DEA in flume sediments

The rates of N_2O production in DEA assays from unamended incubations significantly differed among sediments from different flume treatments (Fig. 6). N_2O production rates of sediments from flumes with *Phragmites* were higher than those from flume sediments with *Iris* (Tukey test; p-value = 0.003) but similar to those of sediments from unvegetated flumes (Tukey test; p-value = 0.46). DEA assays incubated

Table 1
Electrical conductivity (EC), dissolved oxygen saturation (DO sat) and nutrient concentrations of the subsurface water within the sediments in the unvegetated flumes and in flumes containing *Iris pseudachorus* (Iris), *Scirpus lacustris* (Scirpus) and *Phragmites australis* (Phragmites). Data shown correspond to the PRE samplings (i.e., before C addition). Values given are means ± SE. In all cases $n = 21$ (3 flumes per treatment and 7 sampling points per flume).

Variable	Unvegetated	Iris	Scirpus	Phragmites
EC (mS cm^{-1})	2.8 ± 0.002	2.6 ± 0.01	2.8 ± 0.02	1.8 ± 0.01
DO sat (%)	4.5 ± 1.5	5.6 ± 2.5	3.6 ± 1.8	4.3 ± 2.0
NO_3^- (mg N L^{-1})	6.0 ± 0.6	4.3 ± 0.2	5.6 ± 0.5	2.0 ± 0.3
NO_2^- (mg N L^{-1})	0.1 ± 0.02	0.02 ± 0.001	0.08 ± 0.02	0.02 ± 0.01
NH_4^+ (mg N L^{-1})	0.65 ± 0.4	0.12 ± 0.03	0.73 ± 0.3	0.09 ± 0.03
DIN (mg N L^{-1})	6.7 ± 0.4	4.5 ± 0.2	6.4 ± 0.4	2.1 ± 0.3
DON (mg N L^{-1})	4.5 ± 1.8	5.2 ± 0.9	4.4 ± 1.4	1.6 ± 0.3
TDN (mg N L^{-1})	9.5 ± 0.6	8.8 ± 0.1	9.5 ± 0.5	3.7 ± 0.2
DOC (mg C L^{-1})	11.3 ± 0.06	11.0 ± 0.05	10.9 ± 0.12	6.8 ± 0.04

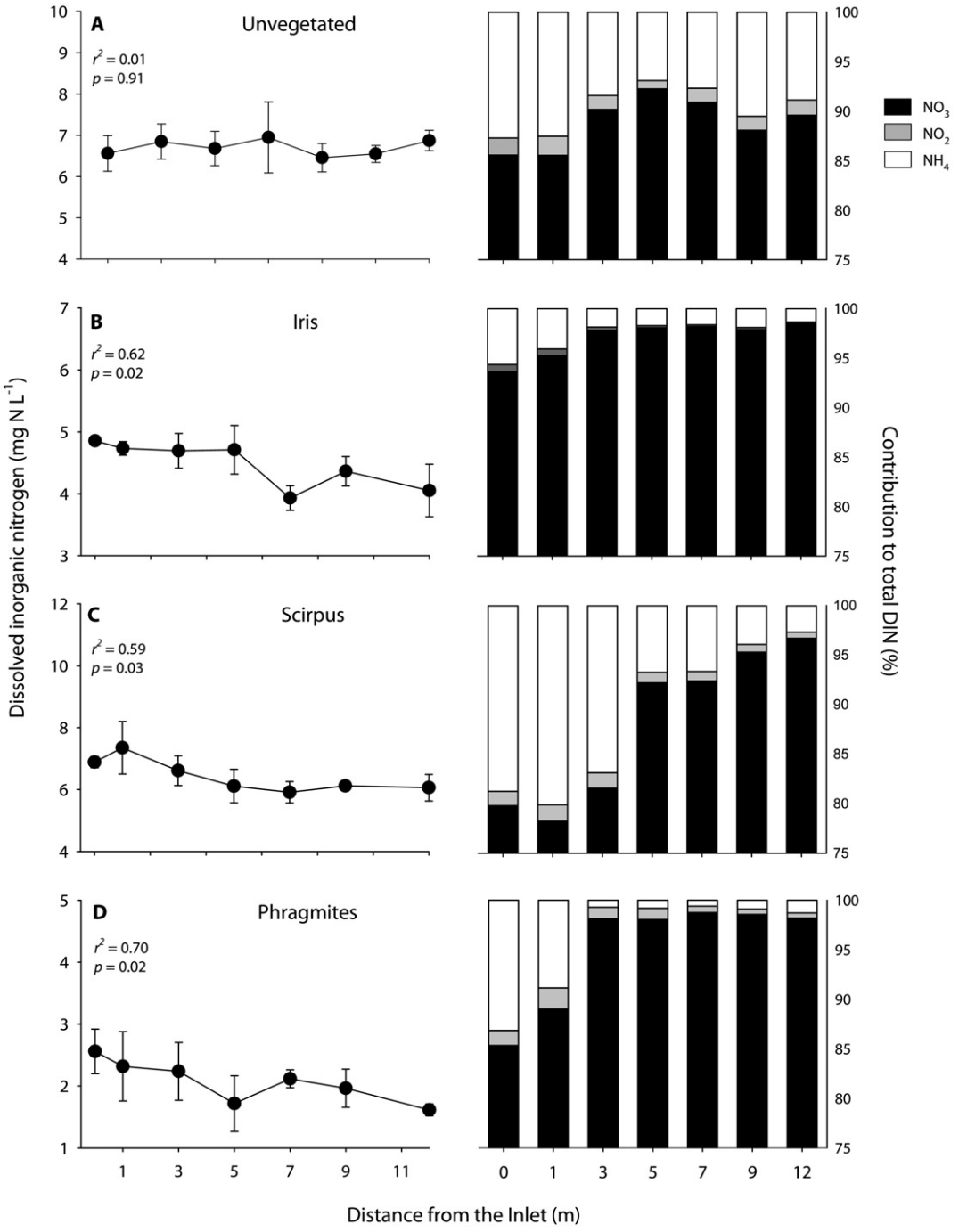

Fig. 2. Longitudinal gradients of dissolved inorganic nitrogen (DIN) concentrations along the flumes (left column) and the relative contribution of nitrate (NO_3^-), nitrite (NO_2^-) and ammonium (NH_4^+) to total DIN concentrations (right column) before C additions (i.e., PRE samplings) in unvegetated flumes (A) and in flumes containing *Iris pseudachorus* (B; Iris), *Scirpus lacustris* (C; Scirpus) and *Phragmites australis* (D; Phragmites). For the longitudinal DIN gradients, the adjusted R-squared (r^2) and *p*-value (*p*) from the linear regression analysis are shown. For each treatment, data given are the mean ± SE of the 3 flumes.

with amendment of labile C showed N_2O production rates 2 orders of magnitude higher than those from unamended DEA assays (Table 2, Fig. 6), and N_2O production rates were similar among sediment treatments (Tukey test; *p*-value ≥ 0.73). The interaction between the flume treatment (i.e., unvegetated, *Iris* and *Phragmites*) and the C treatment (unamended vs amended) was not significant (Table 2).

4. Discussion

Results from this study showed that WWTP effluent-receiving flumes with helophytes had a remarkable capacity to biogeochemically transform and remove DIN from the effluent. This finding was supported by two different observations: (i) the change in the contribution of different N forms to total DIN mostly along the vegetated flumes, and (ii) the significantly decrease in total DIN concentration along the flumes with helophytes. These results agreed with our expectations and were consistent with previous studies showing that aquatic plants (i.e., helophytes) promote DIN transformation and removal along subsurface water flowpaths (Nivala et al., 2007; Schade et al., 2001). The longitudinal shift in the relative contribution of NH_4^+ towards NO_3^- observed in the flumes with helophytes, suggested that activity of nitrifying bacteria was enhanced in these flumes. This finding was in

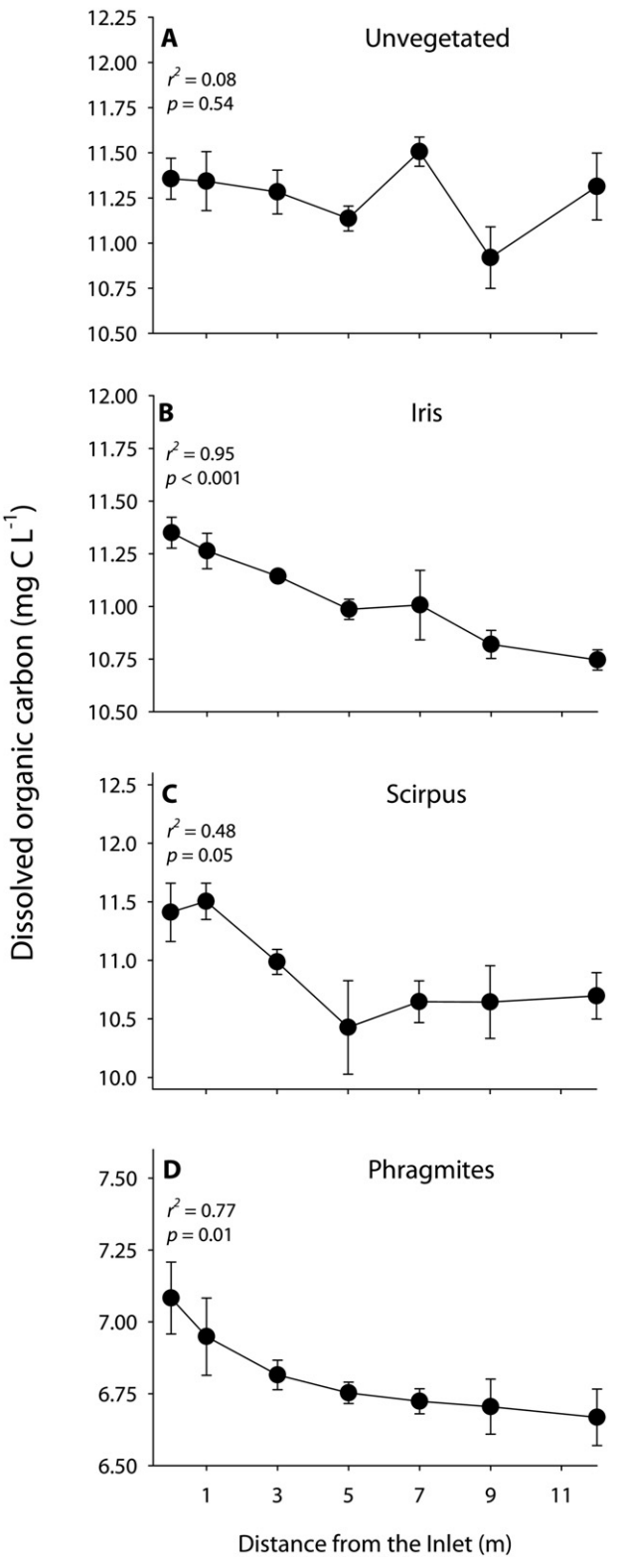

Fig. 3. Longitudinal gradients of dissolved organic carbon (DOC) along the flumes before labile C addition (i.e., PRE samplings) in unvegetated flumes (A) and in flumes containing *Iris pseudachorus* (B; Iris), *Scirpus lacustris* (C; Scirpus) and *Phragmites australis* (D; Phragmites). The adjusted R-squared (r^2) and *p*-value (*p*) for the linear regression analysis are showed. For each treatment, data given are the mean ± SE of the 3 flumes.

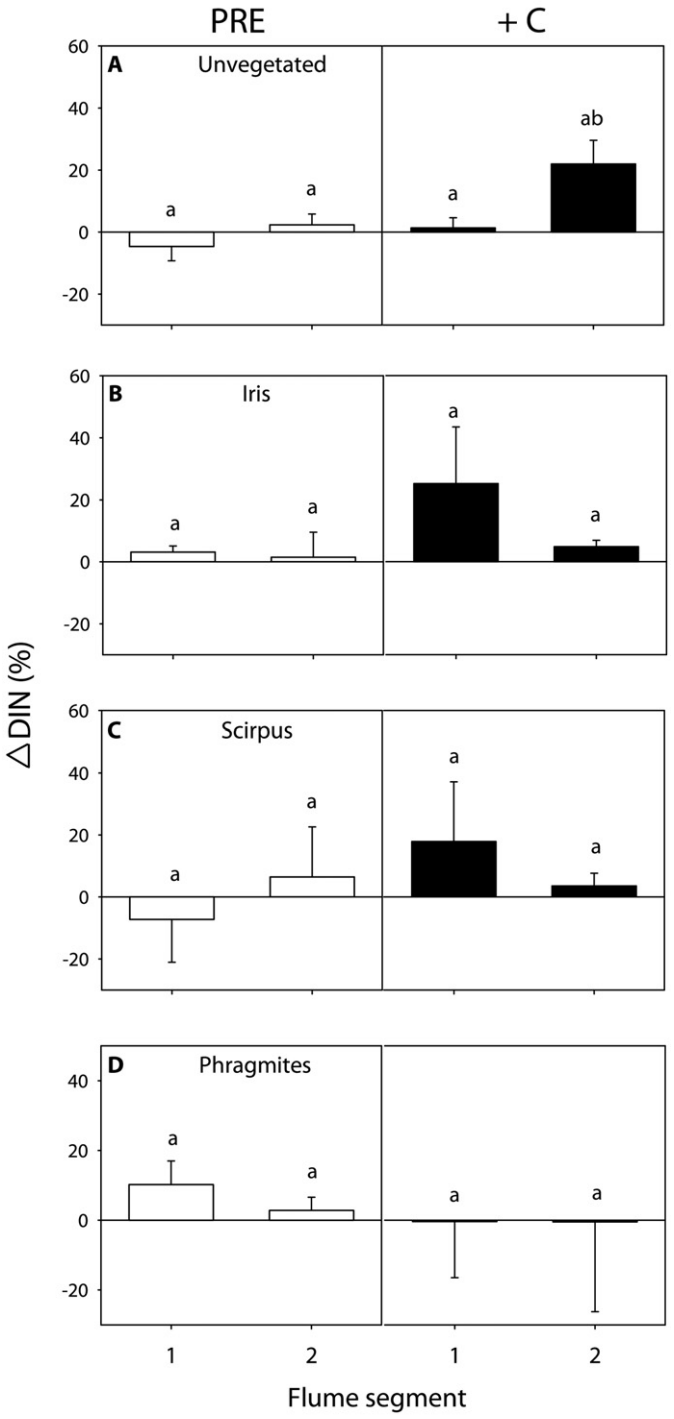

Fig. 4. Percentage variation of dissolved inorganic nitrogen (ΔDIN) concentration at flume segment 1 (i.e., 0–1 m) and 2 (i.e, 1–3 m) in unvegetated flumes (A) and in flumes containing *Iris pseudachorus* (B; Iris), *Scirpus lacustris* (C; Scirpus) and *Phragmites australis* (D; Phragmites). For each treatment, data given are the mean ± SE of the 3 flumes. Different letters denotes statistically significant differences ($p < 0.05$) in ΔDIN based on results from a two-way ANOVA model with flume segment (i.e., 1 and 2) and C addition (i.e., PRE in left column and +C sampling in right column) as a fixed factors and post-hoc Tukey HSD tests.

agreement with previous studies reporting that helophytes stimulated bacterial nitrification within the sediments by generating aerobic microenvironments during the translocation of O_2 from the shoots to the roots (Gersberg et al., 1986; Reddy et al., 1989; Williams et al., 1994). Given the hypoxic environment in the flumes (dissolved oxygen saturation < 10%), the translocation of O_2 by helophytes may be essential for ensuring the occurrence of nitrification at the microsite scale. Nitrification involves the oxidation of NH_4^+ into NO_3^-; thus this process does not directly contribute to the removal of DIN. However, in

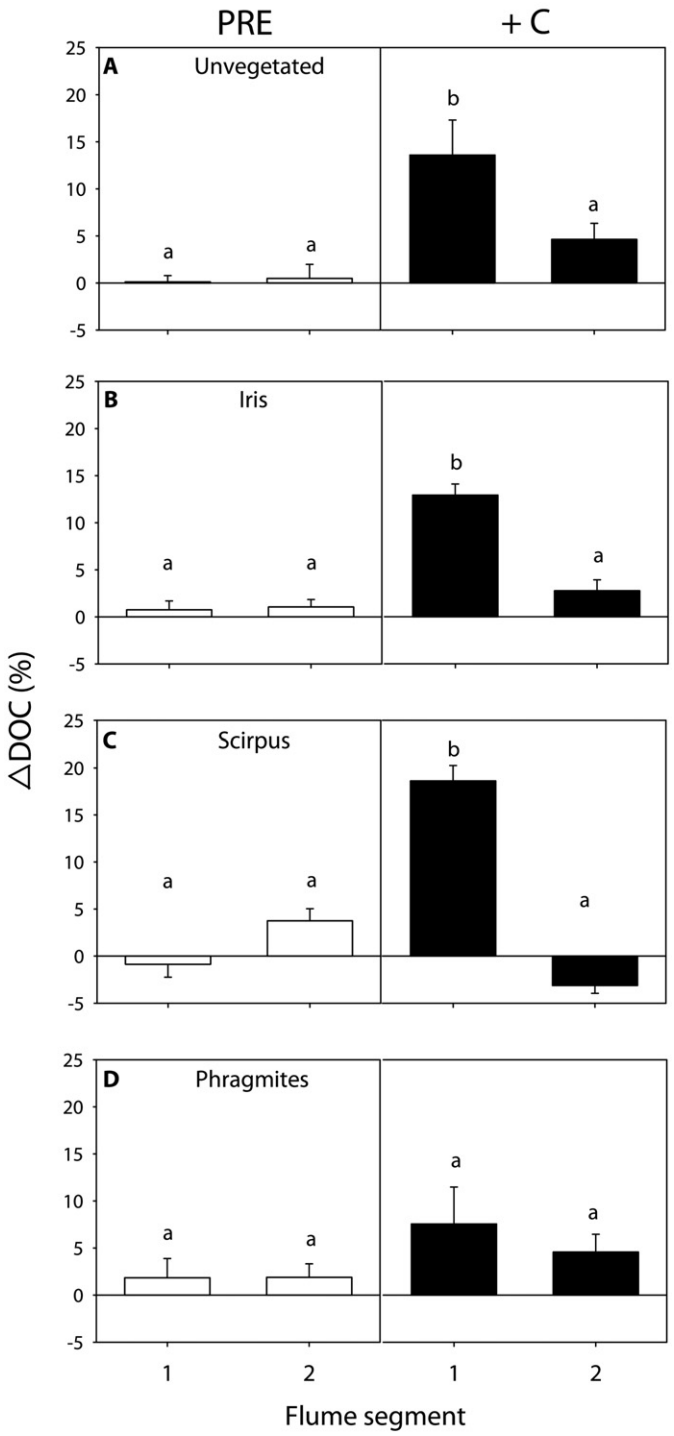

Fig. 5. Percentage variation of dissolved organic carbon (ΔDOC) concentration at flume segment 1 (i.e., 0–1 m) and 2 (i.e., 1–3 m) in unvegetated flumes (A) and in flumes containing *Iris pseudachorus* (B; Iris), *Scirpus lacustris* (C; Scirpus) and *Phragmites australis* (D; Phragmites). For each treatment, data given are the mean ± SE of the 3 flumes. Different letters denotes significant differences ($p < 0.05$) in ΔDOC based on results from a two-way ANOVA model with flume segment (i.e., 1 and 2) and C addition (i.e., PRE in left column and +C sampling in right column) as a fixed factors and post-hoc Tukey HSD tests.

WWTP effluent-receiving systems, this is a relevant biogeochemical process that contributes to decrease high ambient NH_4^+ concentration and thus, decrease the harmful effects of high levels of NH_4^+ on aquatic biota (Camargo and Alonso, 2006; Lambert and Davy, 2011). In addition, the resulting NO_3^- from nitrification can eventually be transformed into N gas by denitrifying bacteria and thus, eventually become permanently removed from the system.

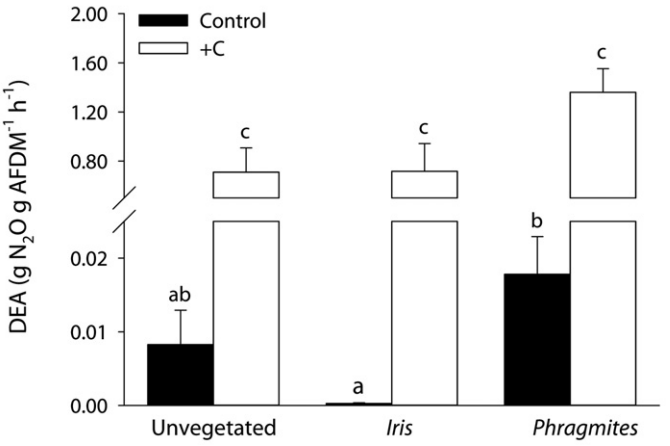

Fig. 6. Comparison of potential denitrification enzyme activity (DEA; mg N_2O g $AFDM^{-1}$ h^{-1}) between unamended (i.e., control) and C amended (+C) incubations of biofilm developed on gravels from the unvegetated flumes (A) and the flumes containing *Iris pseudachorus* (B; Iris) and *Phragmites australis* (C; Phragmites). For each treatment, data given are the mean (±SE) from the 3 different flumes. Different letters denotes significant differences ($p < 0.05$) based on results from a two-way ANOVA model with flume treatment (i.e., unvegetated, *Iris* and *Phragmites*) and C amendment (i.e., control and +C) as fixed factors and post-hoc Tukey HSD tests.

The longitudinal decrease in DIN concentration observed in vegetated flumes suggests that presence of helophytes contributed to increase removal of DIN from the water column, and further, that the magnitude of the processes responsible for DIN removal (i.e., assimilatory N uptake and denitrification) was larger than DIN production through organic matter mineralization. Previous studies have shown that helophytes rely on DIN to meet their N requirements, thus assimilatory uptake of both NO_3^- and NH_4^+ by these plants could contribute to explain the observed pattern (Levi et al., 2015; Pastor et al., 2013; Peipoch et al., 2013). In addition, helophytes provide favorable environments for biofilm development around their root systems (Ramey et al., 2004; Stottmeister et al., 2003). Therefore, bacterial assemblages associated with the rhizosphere could additionally contribute to DIN removal through microbial assimilation. Furthermore, helophytes contributed to increase the subsurface water residence time within the flumes as suggested in previous studies (Chazarenc et al., 2007; Knowles et al., 2010). This physical factor could additionally favor the interaction between DIN and biota within the flumes which may enhance biological DIN removal (Drummond et al., 2016; Hall et al., 2002).

In addition, microbial denitrification within the rhizosphere could also contribute to the observed decrease in DIN concentrations along vegetated flumes. This is especially feasible since dissolved oxygen concentration in subsurface water was low along the flumes throughout the whole study period. However, hypoxic conditions were not sufficient to explain the decline in DIN concentration, because unvegetated flumes also showed low dissolved oxygen concentration. Previous studies have proposed that helophytes burst denitrification because their root exudates are an important local source of labile DOC (Kofoed et al., 2012; Williams et al., 1994). However, the DEA assays in control

Table 2
Results from the two-way ANOVA model on N_2O production rates from the potential denitrification enzyme activity (DEA) assays conducted with biofilms developed on sediments from the flumes, with flume treatment (i.e., unvegetated, *Iris* and *Phragmites*) and C amendment (i.e., control and +C) as fixed factors. Values highlighted in bold indicate significant effects ($p < 0.05$).

Variable	df	F	*p*-Value
N_2O production			
Flume treatment	2	10.33	**0.002**
C amendment	1	288	<**0.001**
Treatment * C amendment	2	3.86	0.05

treatments (without addition of labile C) showed that biofilms growing in vegetated flumes had the same potential for denitrification than those in unvegetated flumes. In addition, DEA assays highlighted that denitrification in sediment biofilms from the flumes was dramatically increased with the addition of the labile C source regardless of flume treatment. These results indicated that quality of DOC was a limiting factor for microbial denitrification, suggesting that quality of DOC from the WWTP effluent could constrain denitrification in the flumes (Fernandez-Nava et al., 2010; Pulou et al., 2012).

At the flume scale, addition of labile C leads to the conclusion that microbial activity was limited by the availability of labile DOC because 84% of the added C was removed within the first meters in all the flumes. Therefore, our results highlight that the study system was strongly limited by C, despite high DOC concentrations (9.7 ± 0.6 mg C L^{-1}) in the WWTP effluent. Nevertheless, the high demand for labile C observed in the flumes and the concomitant high rates of DOC removal was not accompanied by declines in DIN concentration, as it would be expected if DOC would have been used as electron donor by denitrifying bacteria (Lin et al., 2009; Seitzinger, 1988). This result does not necessarily mean that denitrification was not enhanced by the addition of labile DOC, in fact this burst in denitrification was suggested by the laboratory DEA experiments. Thus, we proposed that increases in the rate of denitrification may not be large enough to counterbalance DIN produced by mineralization of dissolved organic matter (Teissier et al., 2007). This result highlights that the occurrence of concomitant declines in DIN and DOC concentrations, as observed along the vegetated flumes in the PRE sampling conditions, may not necessarily be coupled to each other, and could respond to different biogeochemical processes such as assimilatory uptake (for DIN) and microbial respiration (for DOC). Despite the approach used in this study has limited power to identify particular biogeochemical processes associated with net changes in ambient nutrient concentrations, our results show that this is a helpful tool to identify contrasting patterns in DOC and DIN concentrations that emerged with the presence of helophytes in the flumes. Future studies using ^{15}N or ^{14}C additions would be helpful to disentangle the different biogeochemical processes that contribute to the observed longitudinal patterns of DIN and DOC concentration as well as to quantify the relative contribution of each biological compartment (i.e., helophytes and microbial communities) to N and C removal.

In conclusion, results from this study showed that presence of helophytes contributed to (i) remove the excess of DIN from WWTP effluents, and (ii) enhance nitrification along subsurface water flowpaths. Therefore, helophytes contribute to the transformation and removal of DIN from the WWTP effluent. Nevertheless, removal of DOC and its response to addition of labile DOC addition did not seem to be related to DIN removal at the flume scale. In contrast, laboratory DEA assays indicated that the availability of labile DOC in the WWTP effluent was a limiting factor for microbial denitrification. Considering results at both flume and sediment scale together, it seems that labile DOC additions in flumes mostly contribute to increase respiration rather than denitrification. Alternatively, considering that oxygen availability was low, results suggest that potential increases in denitrification associated with the addition of a labile C source were counterbalanced by high rates of mineralization. Altogether, this study highlights the potential role of helophytes as relevant bioremediation tool to improve water quality in WWTP effluent-influenced aquatic ecosystems. However, it also provides insights on the relevance of DOC quality from WWTP effluent and how it can contribute to deal with DIN removal in streams receiving WWTP effluents.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2017.05.114.

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