Impacts of willow and miscanthus bioenergy buffers on biogeochemical N removal processes along the soil–groundwater continuum

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Abstract

In this article, the belowground and aboveground biomass production in bioenergy buffers and biogeochemical N removal processes along the soil–groundwater continuum was assessed. In a sandy loam soil with shallow groundwater, bioenergy buffers of miscanthus and willow (5 and 10 m wide) were planted along a ditch of an agricultural field (AF) located in the Po valley (Italy). Mineral N forms and dissolved organic C (DOC) were monitored monthly over an 18-month period in groundwater before and after the bioenergy buffers. Soil samples were measured for inorganic N, DOC, microbial biomass C (MBC) and N (MBN), and potential nitrate reductase activity (NRA). The results indicated that bioenergy buffers are able to efficiently remove from groundwater the incoming NO_3-N (62% – 5 m and 80% – 10 m). NO_3-N removal rate was higher when nitrate input from AF increased due to N fertilization. Willow performed better than miscanthus in terms of biomass production (17 Mg DM ha^{-1} yr^{-1}), fine root biomass (5.3 Mg ha^{-1}) and N removal via harvesting (73 kg N ha^{-1}). The negative nonlinear relationship found between NO_3-N and DOC along the soil–groundwater continuum from AF to bioenergy buffers indicates that DOC:NO_3-N ratio is an important controlling factor for promoting denitrification in bioenergy buffers. Bioenergy buffers promoted soil microbial functioning as they stimulated plant–microbial linkages by increasing the easily available C sources for microorganisms (as DOC). First, willow and miscanthus promoted high rates of biological removal of nitrate (NRA) along the soil profile. Second, rhizosphere processes activated the soil microbial community leading to significant increases in MBC and microbial N immobilization. Herbaceous and woody bioenergy crops have been confirmed as providing good environmental performances when cultivated as bioenergy buffers by mitigating the disservices of agricultural activities such as groundwater N pollution.

Keywords: bioenergy buffers, biomass production, dissolved organic C, ecological stoichiometry, fine root biomass, groundwater quality, miscanthus, nitrate removal, soil microbial biomass, willow

Introduction

In the last decade, it has become increasingly important to identify which proportion of the landscape should be occupied by bioenergy cropping systems (Gelfand et al., 2013; Manning et al., 2015). The key question is which land-use strategy can be implemented to avoid land-use conflicts while maximizing yields and ecosystem services provision (Fritsche et al., 2010; Payne, 2010; Dale et al., 2011; Popp et al., 2011; Anderson-Teixeira et al., 2012). To solve the so called 'food, energy and environ-

ment trilemma' (Tilman et al., 2009), several scenarios in which food and bioenergy cropping systems are spatially mixed within farmlands have been recently proposed (Asbjornsen et al., 2012; Gopalakrishnan et al., 2012; Christen & Dalgaard, 2013; Manning et al., 2015).

Positive impacts on the regulation of climate, water and biodiversity ecosystem services have been reviewed during the transition of cropland to the production of bioenergy feedstock with perennial herbaceous and woody crops (Holland et al., 2015; Milner et al., 2015). The application of spatial multicriteria analysis revealed that a careful allocation of perennial cropping systems into the landscape would foster multiple ecosystem services and mitigate ecosystem disservices from

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current annual food cropping systems (Powers *et al.*, 2011; Parish *et al.*, 2012; Meehan *et al.*, 2013). Nevertheless, it emerged that the links of bioenergy crops with the provision of ecosystem services are strictly dependent on the spatial allocation of the crops relative to the adjacent land uses as revealed for pest regulation and pollination (Meehan *et al.*, 2012; Werling *et al.*, 2013; Bourke *et al.*, 2014) and for water quality regulation (Meehan *et al.*, 2013).

Within this framework, an excellent case study area in which to explore the possibility to optimize land use for food, energy and ecosystem services is the agricultural landscape of Po valley (northern Italy). In the last decades, this area experienced an intensification of the conventional farming systems with the result that several areas suffer from problems of nitrate contamination of surface and groundwater (Capri *et al.*, 2009). At the EU level, buffer strips have become a widely adopted measure to mitigate such problems of nonpoint source agricultural pollution. The efficiency in removing NO_3-N from groundwater is widely reported in the literature for riparian areas (Sabater *et al.*, 2003; Hickey & Doran, 2004; Mayer *et al.*, 2007) and for filter strips (van Beek *et al.*, 2007; Zhou *et al.*, 2010). For this reason, buffer strips were made mandatory among member states in order to fulfil the obligations to maintain and improve Good Ecological Status under the EU Water Framework Directive (EC 2000/60). In Italy, 5-m-wide buffer strips are mandatory along watercourses where water quality status is scarce or bad (Italian Ministerial Decree DM 27417 of 22 December 2011). Within the 2014–2020 Rural Development Programmes (RDP) of the Emilia-Romagna and Lombardy regions, in Italy, two voluntary measures that provide money to farmer to install and maintain herbaceous buffers or woodland buffer strips have been introduced. Nevertheless, some operating spaces are left by these RDP measures for including bioenergy crops in buffer strips. For this reason, the water quality issue seems to offer an opportunity to redesign bioenergy landscapes with buffers for biomass production.

In this manuscript, bioenergy buffers have been proposed as an alternative land-use scenario for bioenergy production within the intensively managed agricultural landscape of the Po valley. Bioenergy buffers, in our view, are perennial landscape elements consisting of linear narrow bands placed along watercourses and cultivated with perennial herbaceous or woody bioenergy crops. Although extensive knowledge on the ecological functioning of buffer strips with natural vegetation is available for the case study area (Balestrini *et al.*, 2008, 2011), several research questions on bioenergy buffers relative to their productive performances still have to be explored, as do their role in providing ecosystem

services and sustaining soil functioning (such as mitigation of groundwater N pollution and soil microbial C- and N cycling). To date, the only literature available on the effectiveness of bioenergy buffers in removing N is modelling studies (Gopalakrishnan *et al.*, 2012; Meehan *et al.*, 2013; Ssegane *et al.*, 2015). Furthermore, there have been no specific studies for bioenergy crops on the role of dissolved organic C (DOC) and belowground biomass as indicators for the activation of the soil microbial community and its implications on N removal processes from soil (e.g. denitrification and microbial N immobilization). To be adopted under different climatic and pedological conditions, there needs to more evidence on the biogeochemical processes involved in N removal in the plant–soil–groundwater system under bioenergy buffers. Within the case study area, an experimental field trial of bioenergy buffers with miscanthus and willow was set up in a sandy loam soil with shallow groundwater. The main objectives of the experiment were as follows: (i) to evaluate bioenergy buffers effectiveness (BSE) in removing N from shallow groundwater; (ii) to identify the main biogeochemical processes and key factors governing N removal along the soil–groundwater continuum; and (iii) to quantify root fine biomass, biomass production and plant N removal in bioenergy buffers.

Materials and methods

Site description and experimental design of bioenergy buffers

The experiment was located in a typical farm in the north-west of Italy (Fig. 1a) (45°3′37.87″N, 9°47′30.19″E altitude 43 m a.s.l.), where the climate is continental with an average annual rainfall of 980 mm and rainfall peaks in autumn and spring. The average temperatures during the experiment were 5.5, 15.5, 15, 24.4 °C, respectively, for winter, autumn, spring and summer. The field was flat, rectangular and bordered at one side by a ditch (Fig. 1b). It was 200 m wide with a 180 m long 2% slope downward to a 3-m-wide ditch. The water level in the ditch fluctuated from 0.2 to 0.9 m below soil surface (-bss). The field was characterized by a deep sandy aquifer interrupted by a silty clay aquitard (Fig. 1c). The local groundwater system showed a prevalent SW-NE direction, and it was perpendicular to the ditch. The agricultural field was cultivated following a common crop rotation for the area: maize (2013), soybean (2014) and tomato (2015). Maize was fertilized with KNO_3 (170 kg N ha^{-1}). Soybean was irrigated twice in June 2014 (total 60 mm of water) but not fertilized. In May 2015, there was a preplanting fertilization (70, 110 and 170 kg ha^{-1}, respectively for N, P and K), and after planting the tomatoes, there was a biweekly fertirrigation from June to August (18 events; total 210 mm water and 50, 40 and 100 kg ha^{-1}, respectively, of N, P and K). According to the USDA Soil Taxonomy (Soil Survey Staff, 2014), the soil is Udifluventic Haplustept, the texture is

sandy loam, and the content of soil organic C and total N is low. The main soil physical and chemical characteristics of the soil profile are reported in Table S1.

The bioenergy buffers were installed in April 2013, with two buffer widths: the mandatory 5 m width (as requested by Italian Ministerial Decree DM 27417 of 22 December, 2011) and 10 m width. No pest management, irrigation and fertilization were applied. Soil was ploughed at 30 cm depth before the experiment started. The experiment was organized following a randomized block design (RBD) with three replicates (Fig. 1b). Bioenergy buffers consisted of miscanthus (*Miscanthus x giganteus* L.) and willow (*Salix matsudana* Koidz (hybrid)). The plots hosting the control treatment (hereinafter referred to as 'spontaneous species') were not planted to enable natural revegetation. The control treatment has been considered as an unsown field margin strip (De Cauwer *et al.*, 2005) Spontaneous species recorded were (%) as follows: *Echinochloa crus-galli* (L.) Beauv. (30%), *Sorghum halepense* (L.) Pers. (30%), *Amaranthus retroflexus* L. (10%), *Convolvulus arvensis* L. (10%), *Cynodon dactylon* (L.) Pers. (10%) and other species (10%). Willow bioenergy buffers were planted by stem transplantation (up to 40 cm depth). Plant density was 13.000 plants ha^{-1} (0.6 × 1.5 m spacing). The failure of the transplants was nearly zero after establishment. Miscanthus buffers were planted with rhizomes (0.1 m depth) with a density of 4 rhizomes m^2 (0.36 × 0.7 m spacing). Emergence rates for rhizomes in May 2013 ranged from 15 to

20% due to a severe waterlogging event. New rhizomes were planted in June 2013 to reduce patchiness (in February 2015 patchiness reached values <5%).

Groundwater, soil, root and aboveground biomass measurements

Before bioenergy buffer establishment, a whole soil profile was opened to describe the soil horizons (Table S1) and a geological survey was carried out in order to characterize the local aquifer system. Some preliminary piezometers were installed at 2 and 5 m depth at random intervals to get information on groundwater hydraulic head and the groundwater table dynamics. This was carried out to spatially design the RBD experimental design. After having fully characterized the aquifer, the experimental site was equipped in May 2014 with piezometers installed along a series of perpendicular transects from the agricultural field to the ditch (Fig. 1b). Each of the transects consisted of three sampling piezometers. Two piezometers were installed within the agricultural field upgradient of each group of experimental blocks, and one was installed immediately downgradient of each buffer plot to study the effects of bioenergy buffers on groundwater N coming from the agricultural field (AF). The PVC piezometers were installed at a depth of 1.5–2 m. Piezometers were 2.5-m-long, 5-cm-diameter PVC pipe and were screened at 1–2 m -bss. Piezometers were
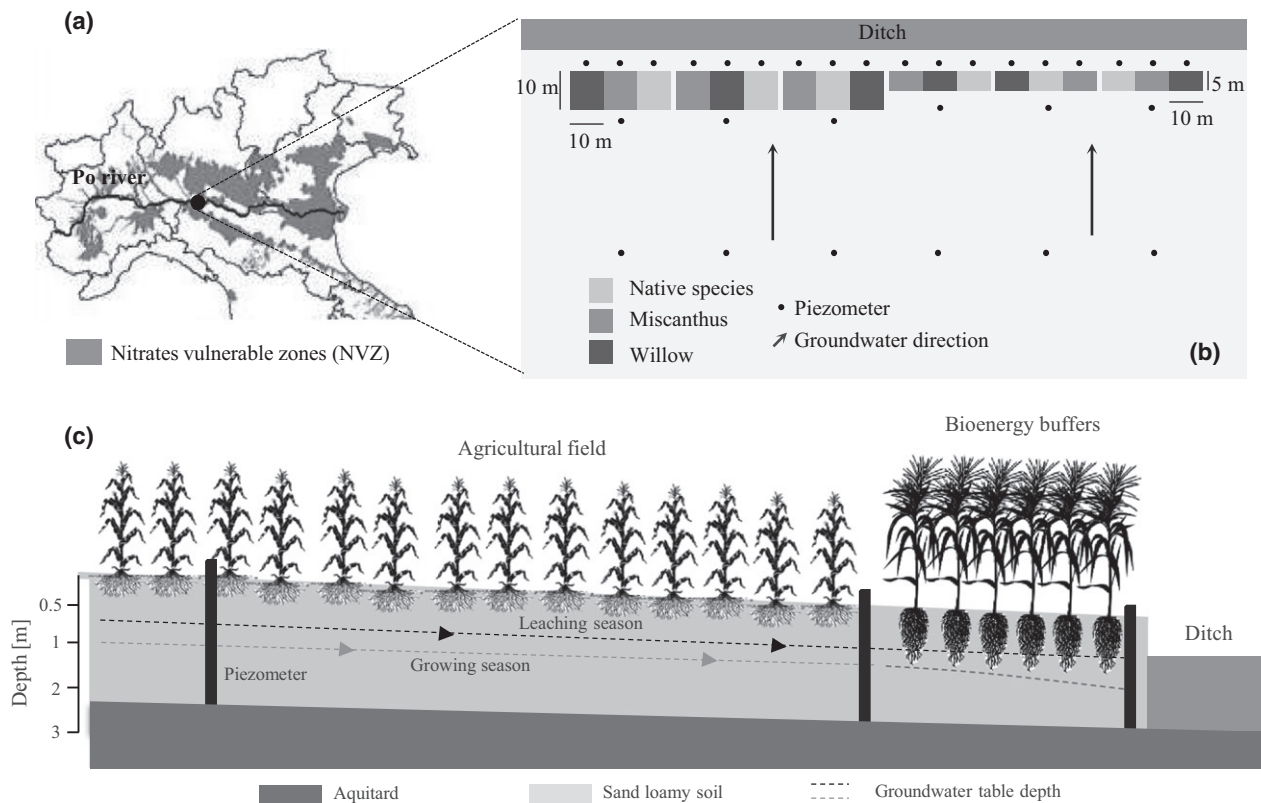
Fig. 1 Localization of the field trial in NW Italy (a) and distribution of the nitrate-vulnerable zones (source: ISPRA – Institute for Environmental Protection and Research), field experimental design for bioenergy buffers (b) and vertical-cross section of the field trial representing the shallow groundwater system (c).

installed by driving into the soil twice a steel corer with an inner removable PVC pipe (5 cm diameter, 1 m long) using a hydraulic jackhammer and extracted using a tripod ratchet. The final piezometer was then manually inserted into the soil. This procedure was also used to obtain soil samples.

Groundwater samples were collected from May 2014 until August 2015 approximately with a monthly sampling frequency during the 2014 and 2015 growing seasons. Hereinafter, the monitoring season is divided as follows: 2014 growing season (T1: 30 May 2014, T2: 28 June 2014, T3: 1 August 2014, T4: 10 September 2014), 2014 leaching season (T5: 18 December 2014, T6: 4 February 2015) and 2015 growing season (T7: 24 April 2015, T8: 6 May 2015, T9: 2 June 2015, T10: 15 July 2015 and T11: 1 August 2015). Groundwater table depth was measured using a sounding probe during each sampling event. Differences in groundwater table depth in total heads along the piezometer transects were used to determine dominant flow paths of groundwater. Before sampling, the wells were pumped empty and allowed to settle again. Dissolved O_2 (ppm), groundwater total dissolved solids (ppm), conductivity ($\mu s\ cm^{-1}$), pH and water temperature (°C) were measured within each piezometer by inserting a specific multiparameter probe (HI 98196; Hanna Instruments, Padova, Italy). Groundwater was sampled with a slow pumping technique, 0.5–1 L was collected from each piezometer, and samples were kept refrigerated during the transport to the laboratory. Samples were then immediately filtered (0.45 μm cellulose acetate) and kept at 4 °C until analysis. Samples were analysed for NO_3-N, NH_4-N, NO_2-N, TDN (Total Dissolved N), DOC (dissolved organic C), TDP (total dissolved P), PO_4-P and chlorides (Cl⁻). The sum of NO_3-N, NH_4-N and NO_2-N forms the dissolved inorganic N (DIN), and the difference between TDN and DIN is the dissolved organic N (DON). NO_3-N was analysed with dual wavelength UV spectroscopy (275, 220 nm) on acidified (HCL 1M) samples and pipetted into 96-well quartz microplates. NH_4-N, NO_2-N and PO_4-P were measured through colorimetric reactions based on a 96-well microplate format and read with a microplate reader (Biotek Synergy 2, Winooski, VT, USA). NH_4-N was measured with Berthelot reaction (Rhine *et al.*, 1998), NO_2-N with Griess reaction (Griess Reagent Kit G-7921; Molecular Probes Inc., Life Technologies, Monza, Italy) and PO_4-P with the green malachite method (D'Angelo *et al.*, 2001). TDN and DOC were measured using a TOC–TN analyser (TOC-VCSN Shimadzu). TDP was measured by an inductively coupled plasma atomic emission spectrometry. Chlorides were analysed by ion chromatography using a Dionex DX-120 equipped with an AS22A column and $Na_2CO_3+NaHCO_3$ as eluent. Chlorides were used as a conservative tracer in groundwater to separate between dilution and N removal (Altman & Parizek, 1995). TDP and PO_4-P in most of the groundwater samples were lower than the detection limit, and the data were therefore not included in this manuscript. Buffer strip effectiveness (BSE) in removing N forms in shallow groundwater was calculated using the formula:

$$\text{Buffer strip effectiveness (BSE)}_i = \frac{1 - C_{igw,\ BUFFER}}{C_{igw,avg,AF}} \times 100 \quad (1)$$

where i is the N_i form for which BSE was calculated (NO_3-N, NH_4-N, NO_2-N, DIN, TDN and their respective i/Cl⁻ ratios), $C_{igw,\ BUFFER}$ is the concentration of the N_i form in groundwater after buffer plots, and $C_{igw,\ avg,\ AGR}$ is the average concentration of the N_i form in piezometers installed in the agricultural field (AF).

Soil was sampled four times in 10-m-wide buffers with the same procedure used for piezometer installation. There were two soil samplings in the first growing season after buffer establishment (1 July 2013 and 10 February 2014), one at the end of second (4 February 2015) and one in the third season (1 August 2015). At each sampling time, three soil cores were taken from each plot to a depth of 60 cm. For miscanthus and willow four soil, cores were taken in two different sampling positions: two cores in the middle of the plant row and two in the inter-row centre. Four random cores were taken from the spontaneous species plots and from the agricultural field. Each soil core was then divided into four sections (0–10, 10–20, 20–30 and 30–60 cm depth). The divided soil cores from each plot were immediately bulked in one composite sample in plastic bags according to the respectively depth, stored at −18 °C and analysed within a month. Soil samples were analysed for extractable NO_3-N, NO_2-N, NH_4-N, DOC, TDN, microbial biomass C (MBC) and for the two microbial N removal processes in soils: microbial N immobilization (MBN) and potential nitrate reductase activity (NAR), the latter as marker for denitrification. Microbial biomass was determined by the fumigation–extraction technique in fresh soil (Vance *et al.*, 1987). The unfumigated soil extracts were used to measured DOC, TDN, extractable NO_3-N, NH_4-N and NO_2-N. As for groundwater samples, DIN and DON were calculated. Extractable mineral N pools were measured with the same microplate-based colorimetric methods adopted for groundwater analysis. For the entire set of soil C and N pools analysed, the values are reported on a stock basis (kg ha^{-1}). Soil nitrate reductase activity (NRA) was measured by soil anaerobic incubation following the modifications of the protocol of Abdelmagid & Tabatabai (1987) introduced by Chèneby *et al.* (2010). NRA were calculated as μg of NO_2-N produced per g of dry soil per day ($\mu g\ NO_2$-N $g_{soil}^{-1}\ day^{-1}$). See Supporting Information (Appendix S1) for a detailed description of the procedure adopted for NRA.

Soil cores for fine root biomass were collected during the last soil sampling (1 August 2015). During this soil sampling, three additionally soil cores were collected for fine root biomass quantification. For miscanthus and willow, soil cores were taken in three different sampling positions following the scheme proposed by Zatta *et al.* (2014): one next to the plants, one in the middle of the plant row and one in the inter-row centre. From the spontaneous species plots, three cores were taken randomly. All cores were divided into the same four sections as for soil cores (0–10, 10–20, 20–30 and 30–60 cm depth). Before root extraction, soil samples were stored at −18 °C. To extract fine roots (<2 mm), soil samples were immersed in oxalic acid (2%) for 2 h and then washed in a hydraulic sieving-centrifuge device (Chimento & Amaducci, 2015). Once cleaned, roots were recovered by hand picking from the water using a 2-mm-mesh sieve, oven dried at 65 °C for 48 h and weighed.

Some samples of miscanthus included rhizomes, which were not included in the root biomass sample. The dry root weight was divided by the whole volume of soil samples and reported as Mg of fine roots per hectare (Mg ha^{-1}). After weighing, the three replicates were combined by depth for each plot and ground to 1 mm. The samples were then analysed for N using a CN analyzer (Vario Max CN Analyzer; Elementar Americas, Inc., Hanau, Germany).

Harvestable biomass from bioenergy buffers was collected in late winter periods every year for miscanthus (10 February 2014 and 15 February 2015) and at the end of 2nd growing season for willow (15 February 2015). Aboveground biomass samples were collected cutting each row of plants along a transect in each plot. Each plant row was weighed in the field and a subsample was taken for fresh weight to dry matter (DM) conversion and CN analysis. Calculations for harvestable biomass (Mg DM ha^{-1}) and N exportations by harvesting (kg N ha^{-1}) were performed for each plot as a whole (by averaging the DM values of all plant rows along the buffer transect) and on a plant row basis (DM and kg N ha^{-1} plant row^{-1}).

Statistical analysis

All the data were analysed using the 'nlme' package (Pinheiro *et al.*, 2015) of RStudio 0.99.484. For groundwater data (concentration and BSE), a mixed model of repeated-measures ANOVA was used with crop type (CROP), buffer width (WIDTH) and monitoring season (SEASON) as fixed effects, whereas piezometers (PIEZ) and sampling times (TIME) were crossed within the random effects structure of the model. Significance of the fixed effects was assessed with F and P values. Model residuals were checked for normality by the Kolmogorov–Smirnov test and for homogeneity of variances by the Levene's test for each of the fixed factors. The temporal autoregressive structure (based on moving average residual) was used as covariance matrix within the mixed model. This structure obtained the lowest Akaike's information criteria (AIC) values than those obtained for other structure tested (autoregressive temporal structure and block diagonal). Significant differences among levels of the fixed factors were identified at the 0.05 probability level of significance constructing specific contrast matrices based on Tukey's contrasts carried out using the *multcomp* package of R software (Hothorn *et al.*, 2015).

Similar mixed models of repeated ANOVA and *post hoc* analysis were applied to soil variables. Crop type (CROP), soil depths (DEPTH) and sampling seasons (SEASON) were used as fixed effects, whereas experimental blocks (BLOCK) and SEASON were defined as random effects. For belowground measurements, only CROP and DEPTH as fixed effects were studied, being root biomass sampled only once during the 2015 growing season. To assess differences in harvestable biomass and N exportation, one-way ANOVA comparisons for RBD designs were run, with CROP and BLOCK as fixed factors. For these parameters, to assess their differences among plant rows along buffer transect, one-way ANOVA comparisons were made separately for miscanthus and willow buffers, with PLANT ROW ($n_{rows} = 13$ for miscanthus, $n_{rows} = 7$ for willow) and BLOCK as main factors. For all these one-way ANOVAs, means were compared by the Tukey's test ($\alpha = 0.05$), after confirma-

tion that data were normally distributed and variance was homogeneous.

Additional regression analyses were then performed on soil, root and groundwater data using *nlme* package of R software. The relationship between the concentration (mg L^{-1}) of DOC and NO$_3$-N in groundwater samples and soil extracts was calculated applying a nonlinear regression model ($y = a + be^{-k(x)}$) (Taylor & Townsend, 2010). The relationship between groundwater nitrate input entering the buffers and buffer strips effectiveness (BSE) in removing N was calculated by a power function: $y = ax^b$ (Mayer *et al.*, 2007). BSE (%) in removing NO$_3$-N was also plotted against buffer width. A nonlinear regression model ($y = ax^b$) was used here to obtain information on the optimal buffer width necessary to obtain a given value of BSE (50%, 75%, 90% and 100%).

Results

N concentration patterns in groundwater

The concentrations of NO$_3$-N, NH$_4$-N, DIN and TDN in groundwater were significantly lower after the bioenergy buffers by comparison with the concentration in the agricultural field (AF) (Table 1). In particular, groundwater nitrate had the highest reduction compared to AF ($F = 77.1$, $P < 0.0001$). For TDN, the values were $F = 40.1$, $P < 0.0001$. The only mineral N that resulted slightly increased was NO$_2$-N that after bioenergy buffers increased up to 0.2 mg NO$_2$-N L^{-1}. No significant differences ($F = 1.9$, $P = 0.55$) were found in Cl$^-$ concentration after bioenergy buffers suggesting that no input of Cl$^-$ occurred in the local aquifer system and thus no dilution effects were observed in groundwater before and after bioenergy buffers (Table S2). Cl$^-$/NO$_3$-N and Cl$^-$/TDN ratios increased in groundwater after bioenergy buffers (data not shown), indicating that for the entire period of monitoring, all N forms were effectively removed from the shallow groundwater. On average, 70% and 85% of groundwater TDN was mineral N (DIN), respectively, in bioenergy buffer and AF. On average, groundwater DIN in AF was formed by NO$_3$-N (60%), NH$_4$-N (29%) and NO$_2$-N (10%). After the bioenergy buffers, NO$_3$-N (47%) was still the main component of groundwater DIN, but the proportion of NO$_2$-N (14%) and NH$_4$-N (38%) increased significantly.

The NO$_3$-N concentration in groundwater after the bioenergy buffers ranged from 0.32 mg NO$_3$-N L^{-1} (or 1.4 mg NO$_3^-$ L^{-1}) to 1.27 mg NO$_3$-N L^{-1} (5.6 mg NO$_3^-$ L^{-1}). TDN ranged from 1.54 to 2.77 mg L^{-1}. The mean input of NO$_3$-N and TDN from the AF was different when soybean (2014) and tomato (2015) were cultivated. N fertilization during the fertirrigation of tomato affected the concentration of NO$_3$-N in groundwater; it was on average 4.73 mg NO$_3$-N L^{-1} (20.9 mg NO$_3^-$ L^{-1}) during the tomato growing season. The maximum NO$_3$

Table 1 Average concentrations of the N forms measured in shallow groundwater after bioenergy buffers (BS- crop) of two different widths and in the agricultural field (AF-crop). Values with different letters in superscript show statistically different means among crop types across growing seasons (Tukey's HSD test, P < 0.05) and within N forms

			NO$_3$-N		NO$_2$-N		NH$_4$-N		DIN		DON		TDN	
		CROP	5 m	10 m	5 m	10 m	5 m	10 m	5 m	10 m	5 m	10 m	5 m	10 m
2014 growing season	BS	Spontaneous spp.	0.66^A	0.59^A	0.15^A	0.20^A	0.69^A	0.66^A	1.44^A	1.51^A	1.00^A	0.33^B	2.44^A	1.84^{BC}
		Miscanthus	0.58^A	0.49^B	0.15^A	0.21^A	0.80^C	0.67^{AB}	1.44^A	1.47^A	0.57^B	0.38^B	2.01^B	1.85^C
		Willow	0.45^A	0.56^A	0.14^A	0.20^A	0.80^C	0.74^{BC}	1.57^A	1.33^A	0.54^B	0.34^B	2.11^B	1.67^C
	AF	Soybean	1.49^{C*}		0.11^B		1.37^D		3.01^B		0.99^A		4.11^D	
2014 leaching season	BS	Spontaneous spp.	0.43^B	0.42^B	0.15^A	0.20^A	0.23^E	0.23^E	0.85^C	0.84^C	1.29^C	0.80^A	2.10^A	1.65^C
		Miscanthus	0.44^B	0.32^B	0.15^A	0.21^A	0.12^F	0.30^E	0.84^C	0.71^C	0.84^A	0.82^A	1.77^C	1.48^E
		Willow	0.41^B	0.32^B	0.16^A	0.20^A	0.41^G	0.30^E	1.03^C	0.77^C	0.55^A	0.77^A	1.58^{AC}	1.54^{AC}
	AF	Bare soil	1.90^C		0.12^A		0.53^D		2.62^B		0.83^A		3.45^D	
2015 growing season	BS	Spontaneous spp.	1.27^D	1.19^d	0.15^A	0.20^A	0.54^A	0.49^{AG}	1.96^D	1.86^D	0.82^A	0.66^A	2.77^A	2.52^A
		Miscanthus	1.14^D	0.95^E	0.15^A	0.18^A	0.57^A	0.51^A	1.86^D	1.65^{AD}	0.88^A	0.53^{AB}	2.74^A	2.18^B
		Willow	1.38^D	0.90^E	0.20^A	0.13^A	0.55^A	0.54^A	2.14^B	1.58^A	0.36^B	0.50^B	2.48^{AB}	2.08^B
	AF	Tomato	4.73^F		0.14^A		0.89^C		5.84^E		0.43^B		6.27^F	
All seasons	BS	Spontaneous spp.	0.87^A†	0.85^A	0.15^{AB}	0.19^A	0.54^A	0.50^A	1.56^A	1.55^A	0.97^A	0.56^B	2.53^A	2.11^B
		Miscanthus	0.78^A	0.70^A	0.15^{AB}	0.20^A	0.57^A	0.53^A	1.50^A	1.44^A	0.80^A	0.50^B	2.30^{AB}	1.93^{BC}
		Willow	0.91^A	0.63^B	0.16^A	0.21^A	0.62^B	0.57^A	1.73^B	1.34^A	0.46^B	0.49^B	2.18^B	1.83^C
	AF	Food crops	3.04^C		0.12^B		1.01^B		4.27^C		0.71^{AB}		4.87^D	

*Average concentration of all the piezometers installed in AF along the perpendicular transects towards bioenergy buffers (see Fig. 1c, d).

†Values with different letters in superscript show statistically different means among crop types (Tukey's LSD test, $P < 0.05$) within averaged values for all seasons.

level of 11.3 mg N L^{-1} (50 mg NO$_3^-$ L^{-1}) indicated in the EU nitrate directive (91/676/EEC) was not exceeded.

Buffer strip effectiveness (BSE) in removing N from shallow groundwater

Similar F and P values for BSE in removing N forms and their respective Cl$^-$/N ratios were observed among the ANOVA factors tested (Table 2). This similarity indicates that Cl$^-$ concentration patterns in groundwater did not affected N removal dynamics.

Figure 2 shows the temporal dynamics of the BSE in removing NO$_3$-N (Fig. 2a, b) and TDN (Fig. 2c, d). No effect of crop types on BSE in removing any of the N forms analysed in shallow groundwater was found (Table 2). However, buffer width had a significant effect on NO$_3$-N and TDN removal rates. Ten-metre-wide buffers (Fig. 2a, c) removed significantly more nitrate ($F = 31.7$, $P < 0.0001$) and TDN ($F = 5.2$, $P = 0.012$) compared to 5-m-wide buffers (Fig. 2b, d). The results of nonlinear regression model (Table S3) confirmed that a significant percentage of variance of BSE in removing

Table 2 Results of the mixed model of repeated measures ANOVA used to investigate the effect of crop (C), buffer width (W) and season (S) on buffer strip effectiveness (BSE) in removing from shallow groundwater the different N forms. The table presents the F and P values of the main fixed effect terms and their interactions. All mixed models showed values of adjusted R^2 (including both fixed and random effects) higher than 0.87 (expect for NH$_4$ and NO$_2$ that were respectively 0.56 and 0.45)

	Crop		Width		Season		CxW		CxS		WxS		CxWxS	
N form	F	P	F	P	F	P	F	P	F	P	F	P	F	P
NO$_3$	1.8	ns	31	***	14	***	1.6	ns	1.4	ns	10	***	1.2	ns
Cl/NO$_3$	1.5	ns	17	***	13	***	1.1	ns	1.4	ns	7.8	***	1.1	ns
NO$_2$	0.8	ns	1.4	ns	0.2	ns	0.2	ns	0.1	ns	0.1	ns	0.1	ns
Cl/NO$_2$	0.2	ns	1.2	ns	0.6	ns	0.1	ns	0.1	ns	0.2	ns	0.1	ns
NH$_4$	0.6	ns	0.1	ns	7.4	***	0.4	ns	1.9	*	0.6	ns	0.7	ns
Cl/NH$_4$	0.7	ns	0.1	ns	7.3	***	0.5	ns	1.2	*	0.2	ns	0.8	ns
DIN	1.4	ns	1.4	ns	11	***	0.6	ns	0.6	ns	1.7	ns	0.4	ns
Cl/DIN	1.1	ns	1.2	ns	9.8	***	0.7	ns	0.2	ns	1.6	ns	0.2	ns
TDN	1.9	ns	5.4	*	7.0	***	0.1	ns	0.6	ns	3.1	**	1.8	*
Cl/TDN	1.7	ns	5.2	*	6.8	***	0.1	ns	0.5	ns	2.4	*	1.5	*

*Denotes significance at $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$

NO_3-N was explained by buffer width. For the entire period of monitoring, NO_3-N removal rate indicates that 50, 75, 90 and 100% of BSE could potentially be reached by creating bioenergy buffers, respectively, 3, 9, 15 and 20 m wide ($R^2 = 0.18$, $P = 0.031$) (Table S3). The highest percentages of variance of BSE explained by buffer width were found in the 2014 leaching season ($R^2 = 0.83$, $P < 0.001$) and 2014 growing season ($R^2 = 0.29$, $P = 0.008$).

A relevant seasonal pattern of the BSE in removing nitrate was observed (Table 2, Table S3 and Fig. 2). During the 2015 growing season, nitrate removal rates of bioenergy buffers were significantly higher than in 2014 (Table S3). A significant positive relationship between groundwater NO_3 input (mg NO_3^- L^{-1}) and buffer strip effectiveness in removing NO_3^- (BSE %) was found (Fig. 3). Bioenergy buffers exponentially increase their NO_3 removal rates when they started to receive more NO_3 in May 2015 after the beginning of NPK fertirrigation of tomato in the adjacent AF. Five-metre-wide buffers (Fig. 3a) were found to be more correlated with NO_3^- input than wider buffers that, on the other hand, showed to have reached their maximum buffering capacity (Fig. 3b).

As result of the influence of NO_3^- input on N removal rate, a significant interaction between buffer width and season was found for NO_3-N ($F = 10.3$, $P < 0.0001$) and TDN ($F = 3.1$, $P = 0.023$). The most significant effects of buffer width on nitrate removal were observed during the 2014 growing season ($F = 12.45$, $P = 0.001$) and in the 2014 leaching season ($F = 16.2$, $P < 0.0001$). Based on the model $y = ax^b$, 50% and 75% of the BSE in removing NO_3 were estimated to occur during the 2015 growing season in 1-m and 4-m-wide bioenergy buffers, respectively (Table S3).

Among the other mineral N forms, NH_4-N and NO_2-N removal rates were not affected as much as NO_3-N and TDN by buffer width, season and by their interaction. NH_4-N and NO_2-N had large variances explained by the random factor in mixed model of ANOVA (data not
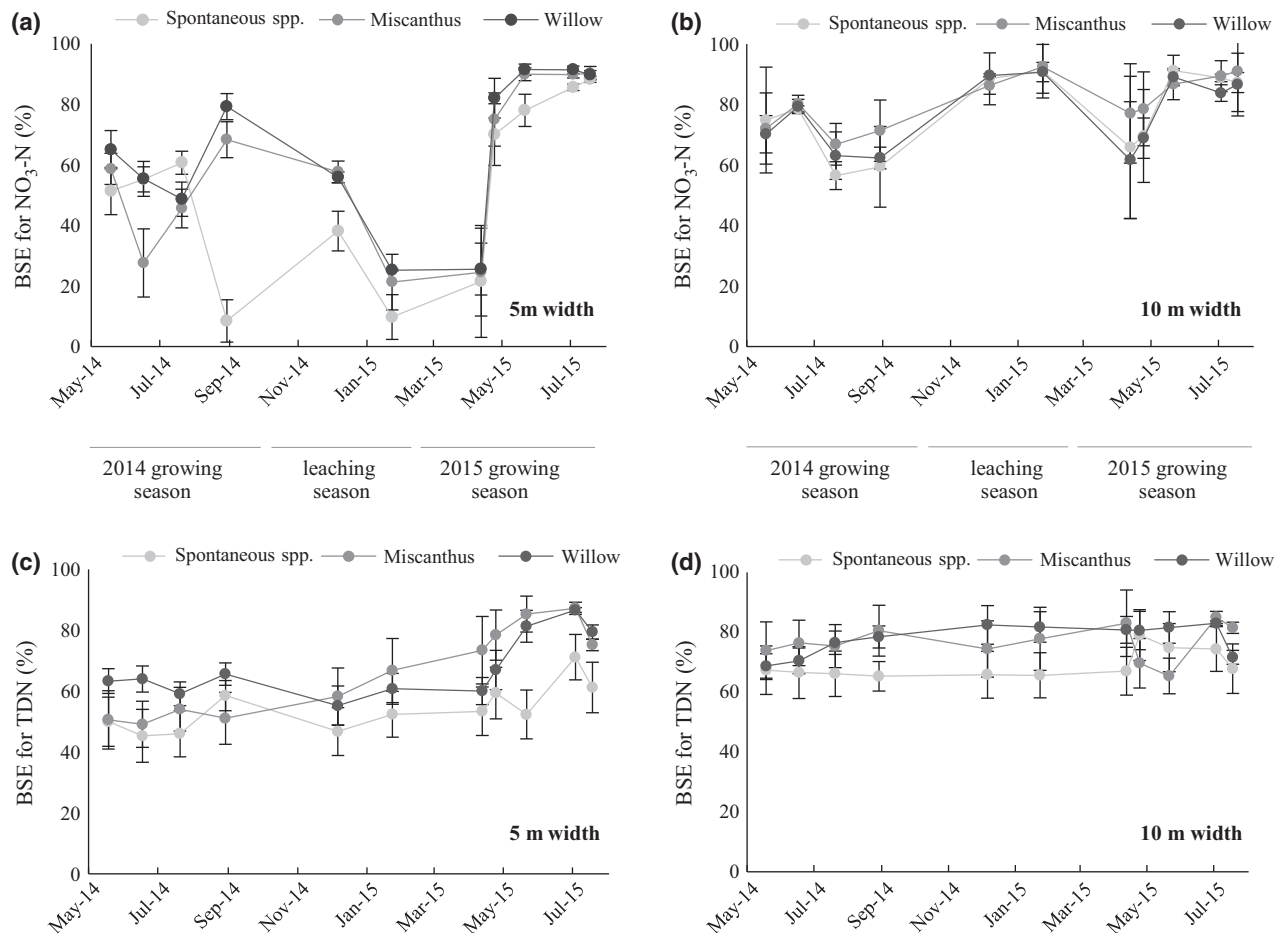
Fig. 2 Temporal dynamics of bioenergy buffers effectiveness (BSE – %) in removing NO_3−N (a–b) and TDN (c–d) for buffers 5 m wide (a–c) and 10 m wide (b–d). Error bars show standard error of the mean ($n = 3$).
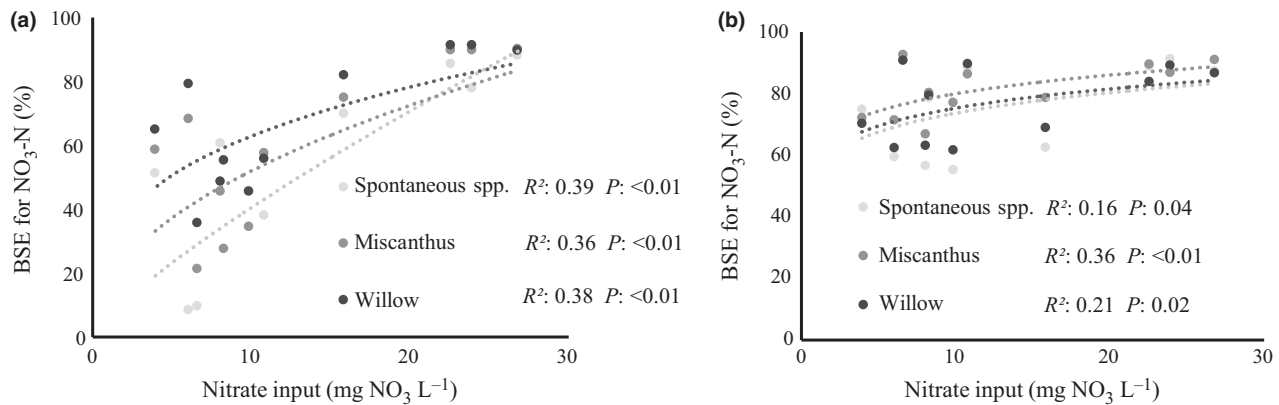
Fig. 3 Relationship between lateral NO_3^- inputs and buffer strip effectiveness (BSE) in removing NO_3^- for bioenergy buffers 5 m wide (a) and 10 m wide (b). Data points represent mean values ($n = 3$) of the eleven groundwater sampling events.
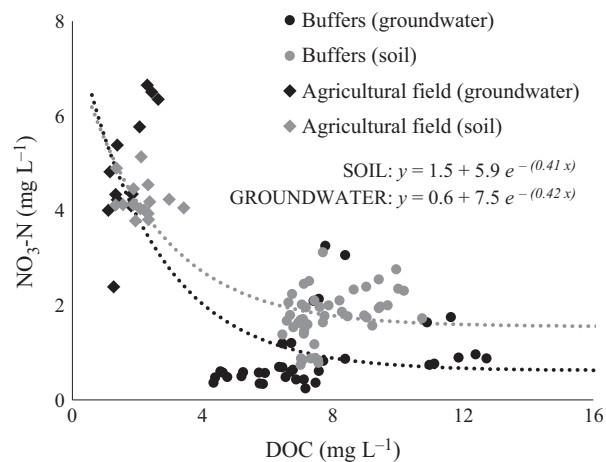
Fig. 4 Relationship between the concentration of DOC and NO_3^-N in groundwater (black) and soil (gray). The data are grouped for bioenergy buffers (points) and agricultural field (diamonds). The soil dataset (unfumigated samples of soil microbial biomass extraction) was created using the data of the last two sampling seasons ($n = 64$) that represent the temporal window where groundwater was monitored (samples of 10 m wide buffers, $n = 44$, where soil samples were collected). The results of the regressions model ($y = a + be^{-k(x)}$) were significant for both dataset: groundwater (R^2: 0.58, P: 0.025) and soil (R^2: 0.74, P: 0.012).

shown). On average, NH_4-N removal rates were 44% for bioenergy buffers. Nitrite instead showed in 92% of the cases negative values of BSE indicating that release of nitrite in groundwater by bioenergy buffers prevailed over removal (Table 2). As consequence of the contrasting patterns revealed by NO_2-N (release) and NH_4-N (high variability among replicates), DIN resulted not significantly affected by crop type, buffer width and by the interactions of these factors (Table 2). DIN removal by bioenergy buffers ranged from 56% in 2014 growing season to 69% in 2015 growing season.

Groundwater geochemistry and hydrology

Water table fluctuated along the measuring period following the precipitations pattern (Fig. S1). On average, water table depth ranged between 0.95 m − bss in winter and autumn and 0.62 m − bss during spring and summer. Water table depth did not differ significantly in AF and bioenergy buffers ($F = 0.626$, $P = 0.6082$). Dissolved oxygen in AF resulted significantly higher ($F = 5.2$, $P = 0.034$) than under bioenergy buffers. Dissolved oxygen in AF was on average 2.74 and 2.25 mg L^{-1} in bioenergy buffers (Table S2). No statistical differences were found instead for dissolved O_2 among bioenergy buffers types. DOC concentration showed an increase along the transect of piezometers towards the ditch (Table S2). Agricultural field showed significant lower DOC levels (on average 1.71 mg DOC L^{-1}) compared to groundwater after bioenergy buffers ($F = 11.2$, $P = 0.004$). Willow showed the highest groundwater DOC values (on average 7.76 mg DOC L^{-1}), while no significant differences were found for the same parameter between spontaneous species and miscanthus. Moreover, a significant negative nonlinear relationship was found between groundwater DOC and NO_3-N ($P = 0.025$ $R^2 = 0.58$). Overall, groundwater after bioenergy buffers resulted more C rich and more N depleted in NO_3-N compared to groundwater coming from AF (Fig. 4). A potential decrease in elemental DOC:NO_3-N ratio in groundwater under bioenergy buffer was found (Table S2). Under bioenergy buffers, starting from the 2014 leaching season until the 2015 growing season, elemental DOC:NO_3-N was below 3 in 95% of the cases. A significant inverse linear relationship between BSE (%) in removing nitrate and elemental DOC:NO_3-N was found (Fig. 4). Elemental DOC:NO_3-N ratio was also seen to be a significant factor in determining BSE of 5-m-wide buffers more

than in 10-m-wide buffers (Fig. S2a). Only during the 2015 growing season was DOC:NO$_3$-N ratio significantly correlated with BSE (Fig. S2b) because of the increase in N input from AF.

Impacts of bioenergy buffers on soil C and N cycling

Bioenergy buffers had a significant impact on the stock of several soil N and C pools compared to AF (Table S4 and Fig. 5a–d). Considering the dissolved mineral N forms that were analysed, AF showed lower dissolved inorganic N (DIN) and NH$_4$-N in the soil compared to the bioenergy buffers. No effects of crop type and season were found for TDN (Table S4). Only in the third growing season (2015) was a significantly higher TDN

stock found in the AF ($F = 6.65$, $P < 0.0001$). Under the tomato cultivation, potential leachable NO$_3$-N was highest ($F = 6.05$, $P < 0.0001$) at all soil depths (Fig. 5a), and consequently, TDN was increased along the soil profile. Three months after willow buffer establishment, a significant increase in potential leachable NO$_3$-N along the soil profile was found compared to the other bioenergy buffer types (Fig. 5a). No other significant potential leaching phenomena were found for willow in the following years compared to other bioenergy buffers.

On average, the proportion of NO$_3$-N, NH$_4$-N and NO$_2$-N in soil DIN of AF was 92%, 6%, 2%, respectively. In comparison with AF, the proportions of NO$_2$-N (9%) and of NH$_4$-N (14%) in soil DIN of bioenergy buffers were significantly increased and NO$_3$-N was signifi-
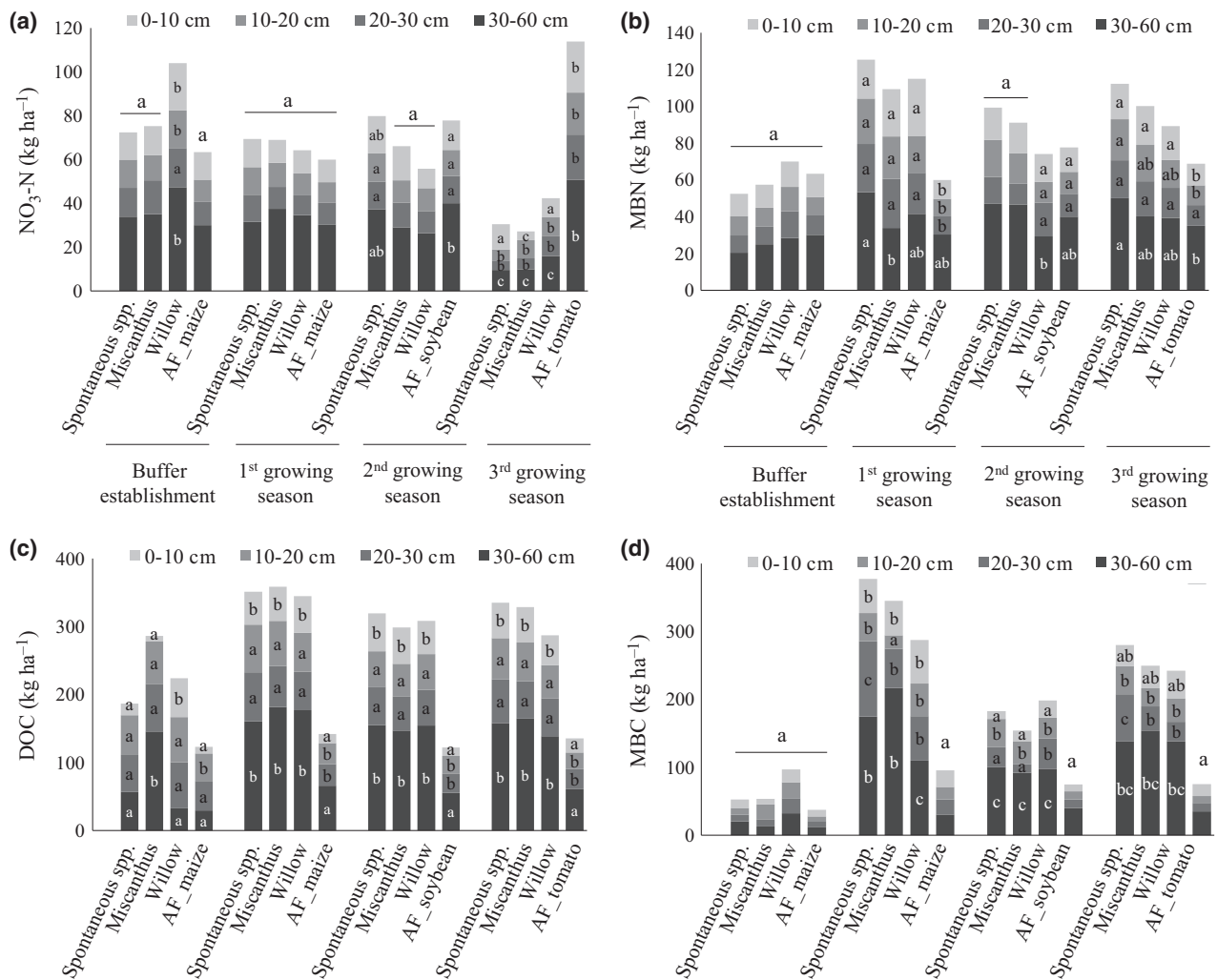
Fig. 5 Average values of potentially leachable NO$_3$–N (a), microbial biomass nitrogen – MBN (b), dissolved organic C – DOC (c) and microbial biomass C – MBC (d) in bioenergy buffers and in agricultural field (AF) at different soil depths across different growing seasons. Different letters within staked columns show statistically different means among crop types (Tukey's test, P: 0.05) within the same soil depth. Horizontal lines above column(s) indicate that the letter is the same for all the soil depths.

cantly reduced (77%). Soil TDN pool in bioenergy buffers consisted of a great percentage of N in a dissolved organic form (DON). DON was significantly higher in bioenergy buffers that in AF in the top soil layers (0–10, 10–20 and 20–30 cm).

Three years after bioenergy buffer establishment, DOC resulted the soil C pool mostly affected (in terms of positive stocking) by the crop types (Fig. 5c). Significant effects for crop type ($F = 7.40$, $P = 0.006$), soil depth ($F = 5.40$, $P = 0.002$), growing season ($F = 5.97$, $P = 0.003$) and their interactions were found for DOC (Table S4). Bioenergy buffers soils showed a significant increase in DOC stock compared to AF at all soil depths and for each of the first three growing seasons (Fig. 5c). No differences in these parameters were found among bioenergy buffers indicating a similar trend of increase in DOC stock along the soil profile. The most significant increases in DOC under bioenergy buffers were observed in the 20–30 and the 30–60 cm soil layers. Similarly to what observed in groundwater, a significant negative nonlinear relationship ($P = 0.012$ $R^2 = 0.74$) was found in soil between the concentrations of DOC and NO_3-N (Fig. 4).

The increase in DOC in bioenergy buffers also contributed to an increased C availability for microorganisms. Figure 5d clearly shows how microbial biomass C (MBC) significantly increased along the soil profile in bioenergy buffers compared to AF ($F = 5.92$, $P = 0.004$). After the first period of buffers establishment, significant interactions between crop and soil depths ($F = 3.91$, $P = 0.029$) and between crop and growing seasons ($F = 3.38$, $P = 0.013$) were observed for MBC. Under bioenergy buffers, the 30–60 cm soil layer showed the greatest increase in MBC stock ($P = 0.013$) compared to AF. A significant increase in microbial biomass N (MBN) stock was also observed in bioenergy buffers compared to AF ($F = 3.99$, $P = 0.023$) (Fig. 5b).
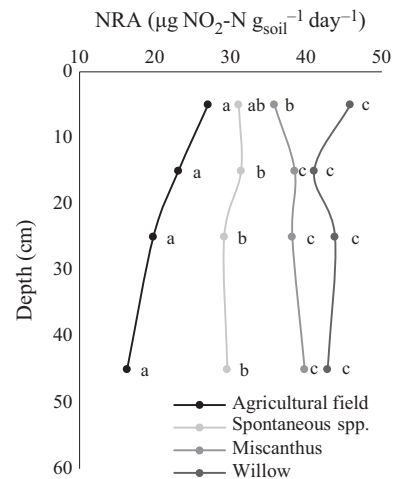
Fig. 6 Soil nitrate reductase activity (NRA) in bioenergy buffers and in agricultural field at different soil depths. Values are reported as average values of the first three growing seasons. Different letters show statistically different means (Tukey's test, P: 0.05) within soil depths.

Among bioenergy buffers, spontaneous species was seen the treatment with the highest ability to immobilize N in soil microbial biomass at different depths compared to miscanthus ($P = 0.028$) and willow ($P = 0.003$). Elemental C:N ratio of microbial biomass was found significantly higher ($F = 2.11$, $P = 0.047$) in bioenergy buffers (6.01) compared to the AF (4.45).

The rate of biological reduction of nitrate to nitrite (nitrate reductase activity – NRA) was found to be strongly affected by the crop types, soil depths and across different growing seasons (Fig. 6 and Table S4). Bioenergy buffers, in particular willow, supported a soil microbial community able to remove nitrate at higher rates compared to AF since the first periods after crop establishment (Fig. S3). On average, NRA values along the soil profile were 38.1 and 43.4 μg N-
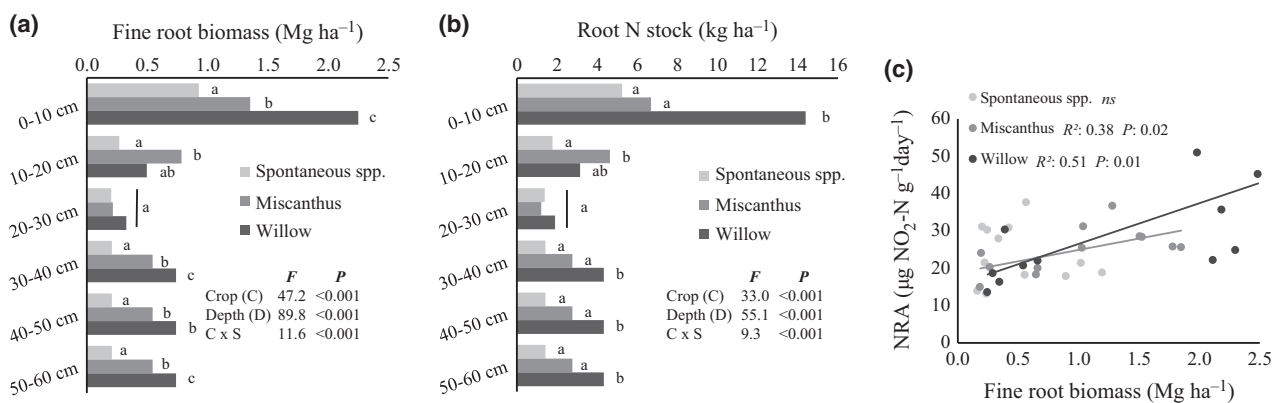
Fig. 7 Fine root biomass (a) and root N stocks (b) in bioenergy buffers at different soil depths. Different letters show statistically different means (Tukey's test, P: 0.05) among crop types within the same soil depth interval. (c) Linear relationship between fine root biomass and soil nitrate reductase activity (NRA) in bioenergy buffers in third growing season (2015).

NO_2 g_{soil}^{-1} day^{-1}, respectively, for miscanthus and willow. These values were significantly higher ($F = 56.50$, $P < 0.0001$) than those observed for the spontaneous species (30.3 μg $N-NO_2$ g_{soil}^{-1} day^{-1}) and for the AF (21.5 μg $N-NO_2$ g_{soil}^{-1} day^{-1}).

Belowground and aboveground biomass production and N stocks

After 3 years from the establishment of bioenergy buffers, fine root biomass (<2 mm) was significantly affected by crop type, soil depths and by the interaction of both factors (Fig. 7a). In the whole soil profile (0–60 cm), willow showed the significantly highest fine root biomass (5.30 Mg ha^{-1}) compared to miscanthus (3.99 Mg ha^{-1}), while the lowest value was found for the spontaneous species (2.03 Mg ha^{-1}). On average, 59% of fine roots in willow and miscanthus were found in the top soil layer (0–30 cm) and 41% in bottom soil layer (30–60 cm). In the spontaneous species, the greatest proportion of fine root biomass (70%) was found in the top layer. Significant linear relationships were found between fine root biomass and soil NRA for miscanthus and willow (Fig. 7c). The crop ranking for fine root biomass (willow > miscanthus > spontaneous species) was the same for soil NRA.

N root content (g kg^{-1}) did not vary significantly among crops ($F = 1.67$, $P = 0.211$) and along the soil profile ($F = 0.15$, $P = 0.926$). On average, at 0–10, 10–20, 20–30 cm and 30–60 cm depth root N content was, respectively, 5.8, 6.1, 6.1 and 5.9 kg N g_{root}^{-1}. N stock in fine roots was significantly affected by crop types, soil depths and by the interaction of both factors (Fig. 7b). Willow showed a higher root N stock (32.40 kg N ha^{-1}) along the whole soil profile (0–60 cm) compared to miscanthus (20.79 kg N ha^{-1}). Spontaneous species instead showed the lowest root N stock (12.67 kg N ha^{-1}).

Harvestable biomass in bioenergy buffers for miscanthus, after winter killing frost (February), was 3.2 ± 0.6 Mg DM ha^{-1} in the establishment year (2013) and 10.76 ± 0.51 Mg DM ha^{-1} at the second year (2014). Willow, after the first 2 years rotation cycle, produced significantly more than miscanthus ($F = 99.55$, $P < 0.0001$) with a harvestable biomass of 34.15 ± 1.71 Mg DM ha^{-1}. N exportations via harvesting were, respectively, 5.9 kg N ha^{-1} in 2013 and 16.1 kg N ha^{-1} in 2014 for miscanthus and 73.7 kg N ha^{-1} for willow in 2014. By analysing the biomass data of each single row in each plot, it was found that there was an exponential decrease in biomass yield along the buffer transect (Fig. S4). The plant rows closer to the AF showed the highest values in harvestable biomass and N

removal in comparison with the plant rows near to the ditch. Harvestable biomass for the 10-m-wide willow buffers (Fig. S4a) ranged from 47.4 Mg DM ha^{-1} in the plant rows adjacent to the AF to 26.6 Mg DM ha^{-1} in the plant rows near to the ditch. Similarly, N removal was highest in plant rows adjacent to the AF (120.6 kg N ha^{-1}) and lowest near the ditch (51.78 kg N ha^{-1}). The same effect was less evident in miscanthus, and it was limited to the first two rows adjacent to the AF (Fig. S4b).

Discussion

Bioenergy buffers effectiveness (BSE) in removing N and key factors governing BSE

Our results clearly indicate bioenergy buffers effectiveness in removing NO_3-N and TDN from shallow groundwater (Fig. 2 and Table S3). BSE in removing NO_3-N was 70% for miscanthus and 71% for willow, respectively (Table S3). These values are in accordance with the 60–70% range reported at landscape level by Ssegane *et al.* (2015) and Gopalakrishnan *et al.* (2012) for buffer strips cultivated with switchgrass, miscanthus and willow. Similar findings were reported also in riparian buffers of *Salix* spp. (Young & Briggs, 2005). No differences between herbaceous and woody crops and between bioenergy crops and spontaneous species on N removal rate were observed (Table 2). This indicates that vegetation types in narrow buffer strips do not remove N from subsurface water flow with significant differences (Sabater *et al.*, 2003; Mayer *et al.*, 2007). Mayer *et al.* (2007) conducted a meta-analysis over 45 published studies on nitrate removal by riparian buffers and found that the mean mass of NO_3-N removed per unit length was not statistically different between forested and herbaceous buffers. Similarly, our results confirmed that DIN was dominantly present as NO_3 and it was removed 9.38% m^{-1} by spontaneous species, 10.12% m^{-1} by miscanthus and 9.43% m^{-1} by willow, respectively. These values are in accordance with the mean values found in 14 riparian buffers across Europe (Sabater *et al.*, 2003). Yet, it is confirmed that from the first periods after establishment bioenergy crops can remove N from groundwater as much as buffers strips with spontaneous species.

Buffer width had a significant effect on NO_3-N and TDN removal rates from shallow groundwater, with 10-m-wide buffers being more effective. Nonetheless, bioenergy buffers that are as wide as national recommendations (5 m) suffice to remove more than 50% of the incoming nitrate in most cases (Table S3). The effect of buffer width in this study was unexpected as in literature reports have shown significant differences where

buffer widths differed by more than 10–20 m (Hickey & Doran, 2004; Mayer *et al.*, 2007; Sweeney & Newbold, 2014). In addition, nitrate removal rate was seen to be even higher when nitrate input from AF increased (Fig. 3). The results of nonlinear regression (Table S3) suggested that, in linear and straightforward hydrological conditions similar to our case study, a 3-m-wide buffer, made of miscanthus or willow, can remove up to 75% of nitrate during a high N input season. This indicates that no N saturation effects occurred in our 3-year-old bioenergy buffers, although clear symptoms of N saturation have been reported in situations with long-term N loadings (Aber, 1992; Hanson *et al.*, 1994; Sabater *et al.*, 2003; Hefting *et al.*, 2006).

Biomass production and plant N removal in bioenergy buffers

The reasons for which no evidence of N saturation was observed in this study can be found in the aboveground and belowground biomass dynamics. Biomass production and plant N uptake have been shown to be important N removal processes in forested (Hefting *et al.*, 2005) and herbaceous buffers (van Beek *et al.*, 2007; Balestrini *et al.*, 2011). In this study, willow buffers performed very well in terms of biomass production in the first 2-year cycle (34.2 Mg DM ha^{-1}). This value of biomass yield is higher than the mean values reported for *Salix* spp. in Canada and the United States (Amichev *et al.*, 2014), Europe (Zegada-Lizarazu *et al.*, 2010) and in northern Italy (Rosso *et al.*, 2013). The tolerance of willow to saturated soils and oxygen shortage at deeper soil layers is widely reported (Krasny *et al.*, 1988; Jackson & Attwood, 1996; Aronsson & Perttu, 2001). Furthermore, lateral N loadings by enriched groundwater significantly affected biomass production along the buffer transect (Fig. S4a) with the first two rows (adjacent to the AF) being the most productive (up to 48 Mg DM ha^{-1} plant row^{-1}) and the ones that contributed most to N removal via uptake and harvesting (Fig. S4).

Miscanthus biomass production in the first 2 years was 3.2 and 10.8 Mg DM ha^{-1}. These values are lower than those found in field trials with similar stand age in temperate regions; from 15 to 20 Mg DM ha^{-1} (Lewandowski & Heinz, 2003; Angelini *et al.*, 2009). For miscanthus, the low yields might have been affected by the presence of shallow groundwater (Lewandowski *et al.*, 2003) and by the high soil hydraulic conductivity and sandy loam texture (Table S1). The latter two factors may increase the soil moisture deficit in upper soil layers for relatively long periods during the summer season; previous studies (Heaton *et al.*, 2004; Monti & Zatta, 2009; Mann *et al.*, 2012) have shown miscanthus

to be highly productive where water is not limiting, but very sensitive to water shortage.

By comparison with spontaneous species, both willow and miscanthus had deeper fine root systems (Fig. 7a) and higher root N stocks (Fig. 7b). The ability of perennial bioenergy crops to penetrate deep-rooting zones (to access nutrients more efficiently) is widely recognized (Rytter, 2001; Glover *et al.*, 2010; Ens *et al.*, 2013; Owens *et al.*, 2013; Amichev *et al.*, 2014). The total belowground biomass found in willow can be placed at the highest ranking positions among the willow hybrids studied in Stadnyk (2010) and reviewed in Amichev *et al.* (2014). After 2 years from planting, miscanthus had a mean belowground biomass of 4 Mg ha^{-1} between 0 and 60 cm in depth. At this depth interval, this value is in line with those reported in previous studies carried out on mature stands (>3–4 years) in Italy (Monti & Zatta, 2009; Chimento & Amaducci, 2015), Europe and USA (Heaton *et al.*, 2004; Amougou *et al.*, 2010; Dohleman *et al.*, 2012; Anderson-Teixeira *et al.*, 2013; Zatta *et al.*, 2014).

With regard to root biomass distribution along soil profile, it was observed that willow with 2.2 Mg ha^{-1} and miscanthus with 1.6 Mg ha^{-1} are characterized by an high contribution of fine roots (41%) to whole root biomass at deeper layers (30–60 cm). In a 6-year-old multispecies experiment, Chimento & Amaducci, 2015 found that only 0.9 Mg ha^{-1} (17%) and 2 Mg ha^{-1} (23%) of the whole root mass was allocated respectively by willow and miscanthus at 30–60 cm depth. These results on rooting patterns clearly indicate how cultivating bioenergy crops along the field margins offers the opportunity to intercept N loads from surrounding agricultural fields at deeper soil layers compared to buffers with spontaneous species. This would ultimately increase the environmental performance of bioenergy buffers in term of plant N removal from soil. Furthermore, as root biomass was shown to be a good indicator of soil organic C sequestration (Chimento *et al.*, 2016; Chimento & Amaducci, 2015), our results suggest how bioenergy buffers have a higher potential compared to patches of adventitious plants to contribute to C storage and GHG savings in the deep soil layers.

Biogeochemical processes governing N removal in plant–soil–groundwater system

In addition to the role of vegetation, a series of biogeochemical processes in soil and groundwater are recognized as being important in determining N removal in bioenergy 3 buffers. The patterns of dissolved O_2, pH, NO_2-N, NO_3-N and DOC in groundwater (Table 2, Table S2 and Fig. 4) suggest that denitrification plays a predominant role in the nitrate depletion observed in

bioenergy buffers. Suboxic conditions were found in groundwater after the bioenergy buffers (Table S2); such conditions are optimal for denitrification (Vidon & Hill, 2005). There was also a significant increase in the contribution of NO_2-N to DIN at the expense of NO_3-N which indicates that a rapid nitrate reduction occurred (Giles et al., 2012; Butterbach-Bahl et al., 2013). The alkaline pH of groundwater (Table S2) and of soil (Table S1) and the average depth of the groundwater table (Fig. S1) denote the presence of ideal conditions for soil denitrifying communities (Groffman et al., 1991; Weier et al., 1993; Rich & Myrold, 2004). Moreover, an increase in the stock of DOC along the soil profiles of bioenergy buffers (Fig. 5c) might have promoted the observed enrichment of DOC in groundwater after the bioenergy buffers (Table S2). DOC levels in groundwater after the bioenergy buffers (>5 mg DOC L^{-1}) indicated that incoming groundwater found suitable conditions for denitrification under the bioenergy buffers (Cosandey et al., 2003; Gumiero et al., 2011; Senbayram et al., 2012). In comparison with spontaneous species, willow and miscanthus, indeed, promoted an active zone of biological removal of nitrate along the whole soil profile because of their deep and dense root systems as revealed by the positive relation between NRA and fine root biomass (Fig. 7c). High soil moisture in sandy loam soils has been shown to stimulate root exudation of easily available C sources (DOC) for microorganisms, thus triggering microbial activity (Dijkstra & Cheng, 2007). On this regard, the use of DOC and the incoming nitrate, respectively, as donor and electron acceptor by denitrifying microbial communities plays a key role in the nitrate depletion observed in groundwater. A significant exponential negative relationship between DOC and NO_3-N was found along the groundwater soil continuum from the AF to the bioenergy buffers (Fig. 4). This indicate that the shift in elemental stoichiometry (DOC:NO_3-N ratio) promoted the microbial N removal by denitrification in bioenergy buffers by constraining N accrual in groundwater. The presence of a confining layer at a shallow depth (Fig. 1c) forces most of the incoming oxic and enriched nitrate groundwater to flow through the subsurface, DOC rich, soil layer of the bioenergy buffers (Gold et al., 2002). As consequence, the DOC:NO_3-N ratio dropped below the range of 3–6 (Table S2) and triggered NO_3-N removal by denitrification (Taylor & Townsend, 2010), which is in agreement with results available in literature (Groffman et al., 1992; Hedin et al., 1998; Hill & Cardaci, 2000; Gold et al., 2002; Cosandey et al., 2003; Senbayram et al., 2012).

The results discussed above indicate that the N removal processes are strictly linked to the increase in DOC in bioenergy buffers. Dissolved organic C compounds are important drivers of denitrification in ripar-

ian soils (Hill et al., 2000). Easily available C for microorganisms measured as DOC has been also thought to be the main source of subsoil organic matter (Rumpel & Kögel-Knabner, 2011) and under bioenergy crops could be of relevance due to the their deep-root systems (Agostini et al., 2015). In fact, the observed increase in soil DOC in willow and miscanthus buffers was found to be significantly correlated to fine root biomass ($R^2 = 0.35$, $P = 0.04$). Through the release of exudates of low molecular weight (the main source of DOC), the root environment (the so called rhizosphere) increases microbial activity through MB utilization of new easily available C sources (Kuzyakov, 2002; Zhu et al., 2014). The dual increase in DOC and MBC observed along the soil profile in our bioenergy buffers as compared to the AF (Fig. 5c, d) revealed that establishment of bioenergy crops with such dense and deep-rooting systems triggered the soil microbial community. The activities of soil C, N and P-acquiring enzymes such as β-glucosidase, leucine aminopeptidase and alkaline phosphatase have been observed to significantly increase under bioenergy buffers at 0–30 cm depth (A. Ferrarini & F. Fornasier, unpublished data). The rhizosphere priming effect promotes N mining from SOM and the mineralized N is retained by the microbial community through rapid immobilization (Kuzyakov, 2002; Dijkstra et al., 2013; Kuzyakov & Xu, 2013; Blagodatskaya et al., 2014; Chen et al., 2014; Zhu et al., 2014). Microbial biomass N, indeed, significantly increased in the top soil layers under the bioenergy buffers by comparison with the AF (Fig. 5b). Microbial N retention was also observed in other perennial agroecosystems (Hargreaves & Hofmockel, 2013). However, elemental CN ratio of microbial biomass (MB) along the soil profile did not decrease because of MBN increase. A MB CN ratio around 6 is close to that of the SOM that would be decomposed (SOM CN of 8 at 0–60 cm) and this highlights how soil microbial biomass should not undergo adjustments of microbial element use efficiency (Mooshammer et al., 2014). As the stoichiometry of the soil resource was balanced with that of the microbial biomass, soil microbes could not excrete N in excess and thus soil N is retained and N losses should have been prevented (e.g. during winter period with potential N leaching) (Manzoni et al., 2012; de Vries & Bardgett, 2012). Indeed, potentially leachable nitrate did not increase significantly along the soil profile under the bioenergy buffers compared to the AF after the beginning of the second growing season (2014) (Fig. 5a). Overall, the increase in easily available C for microorganism (DOC), MBC and MBN confirmed the results of Bengtson et al. (2012) and Paterson (2003) of a strong coupling of root C release, SOM cycling and microbial N cycling.

In conclusion, herbaceous and woody bioenergy crops have been confirmed as being effective in mitigating shallow groundwater N pollution when cultivated as bioenergy buffers. Up to 50, 70 and 90% buffer strip effectiveness in removing NO₃-N could be reached by creating bioenergy buffers 3, 9 and 15 m wide, respectively. The use of ecological stoichiometry (DOC:NO₃-N) revealed that denitrification plays a key role in the nitrate removal observed along the soil–groundwater continuum. Deep-rooting systems of bioenergy crops promoted the activation of soil microbial processes involved in N removal from soil. Our findings also suggest that biomass production and N removal through multiple harvests further contributes to N retention in bioenergy buffers compared to unmanaged buffer strips with spontaneous species. Bioenergy crops placed along watercourses in sandy loam soils with shallow groundwater enhance ecosystem services and sustain soil functioning such as water quality regulation and soil microbial C and N cycling.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Hydrological features of the field trial.

Figure S2. Relationship between elemental DOC:NO_3-N ratio under bioenergy buffers and buffer strip effectiveness (BSE %) in removing NO_3-N from groundwater.

Figure S3. Soil NO3 reductase activity (NRA) under bioenergy buffers and agricultural field at different soil depths across the four sampling seasons: (a) after buffers establishment (July 2013); (b) end of 1st growing season (Feb 2013); (c) end of 2nd growing season (Feb 2014); (b) middle of 3rd growing season (Aug 2015).

Figure S4. Harvestable biomass (black lines) and N removal via harvesting (grey bars) of willow (a) and miscanthus (b) for different plant rows along the 10 m wide buffer transects.

Appendix S1. Lab protocol adopted for potential soil nitrate reductase activity (NRA).

Table S1. Main soil physical and chemical characteristics of the soil horizons.

Table S2. Average concentrations of groundwater chemical species after bioenergy buffers (BS- crop) and in agricultural field (AF-crop).

Table S3. Mean values of NO3 removal rate as BSE (%), BSE per unit length (% m^{-1}) and mean mass of N removed per unit length (mg NO3-N L^{-1} m^{-1}) for bioenergy buffers across the growing seasons.

Table S4. Results of the mixed model of repeated measures ANOVA used to investigate the effect of crop (C), depth (D) and growing seasons (S) on the stock (kg ha^{-1}) of soil inorganic N forms, C and N pools of dissolved organic matters (DOM) and microbial biomass (MB) and the effects on potential soil nitrate reductase activity (NRA – µg NO_2-N g_{soil}^{-1} day^{-1}).