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# Benthic nitrogen metabolism in a macrophyte meadow (*Vallisneria spiralis* L.) under increasing sedimentary organic matter loads

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**Abstract** Organic enrichment may deeply affect benthic nitrogen (N) cycling in macrophyte meadows, either promoting N loss or its recycling. This depends upon the plasticity of plants and of the associated microbial communities, as those surrounding the rhizosphere. Rates of denitrification, dissolved inorganic N fluxes and N uptake were measured in sediments vegetated by the submerged macrophyte *Vallisneria spiralis* L. under increasing organic matter loads. The aim was to investigate how the combined N assimilation and denitrification, which subtract N via temporary retention and permanent removal, respectively, do vary along the gradient. Results showed that *V. spiralis* meadows act as regulators of benthic N cycling even in organic enriched sediments, with negative feedbacks for eutrophication. A moderate organic load stimulates N uptake and denitrification coupled to nitrification in the rhizosphere. This is due to a combination of weakened competition between macrophytes and N cycling bacteria and enhanced radial oxygen loss by roots. An elevated organic enrichment affects N uptake due to hostile conditions in pore water and plant stress and impairs N mineralisation and its removal via denitrification coupled to nitrification. However, the loss of plant performance is almost completely compensated by increased denitrification of water column nitrate, resulting in a shift between the relative relevance of temporary and permanent N removal processes.

**Keywords** Organic enrichment · *Vallisneria spiralis* · Radial oxygen loss · N fluxes · Denitrification · N uptake

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## Introduction

Organic loadings from agricultural runoff, urban sewage effluents, and fish farming waste have become a widespread problem to aquatic ecosystems in human-impacted watersheds (Holmer et al. 2005; Nixon 2009; Raun et al. 2010). Labile organic matter (OM) enrichment in surface sediments leads to severe changes in chemical and physical features, and biogeochemical dynamics, such as higher microbial activity and oxygen

exhaustion, shift to anoxic degradation pathways, and accumulation of potentially phytotoxic compounds (e.g. organic acids and reduced ions as $Fe^{2+}$, $Mn^{2+}$, $NH_4^+$ and $S^{2-}$). From the benthic perspective, such changes determine also a loss of biodiversity, as sensitive plants and associated micro-, meio- and macrofauna may not tolerate hostile chemical environments (Terrados et al. 1999; Smolders et al. 2002; Sand-Jensen et al. 2008). The decline of benthic vegetation suppresses relevant ecosystem functions mediated by macrophytes, such as the control of nutrient recycling and the stimulation of N removal via denitrification coupled to nitrification (Risgaard-Petersen and Jensen 1997; McGlathery et al. 2007; Boerema et al. 2014). This generates a positive feedback to eutrophication as inefficient or slower mineralization rates result in a net OM accumulation. Some freshwater species tolerate nutrient-rich waters and organic substrates (Wu et al. 2009; Pulido et al. 2010; Soana et al. 2012). The survival of rooted plants and the persistence of the connected ecosystem services depend on plant plasticity, i.e. rapid physiological and morphological adaptations to counteract hostile environmental conditions. Radial oxygen loss (ROL) by roots and associated oxidation processes in the rhizosphere can be viewed as key functions determining meadows persistence in organic enriched sediments (Vartapetian and Jackson 1997; Pezeshki 2001). Rooted macrophytes have the potential to exhaust the pore water inorganic nitrogen pool, coupling ammonification and uptake and setting to zero inorganic nitrogen regeneration. ROL promotes simultaneously N loss via denitrification coupled to nitrification, which may remove in eutrophic systems mineralized nitrogen in excess to plants N requirements (Racchetti et al. 2010; Soana and Bartoli 2014).

As organic enrichment and N pollution are common issues in aquatic environments of agricultural basins, an interesting question is to investigate how the short-term plasticity and the strategies adopted by plants to tolerate hostile pore water conditions do affect the microbial-mediated N processes in sediments. Specifically, a central point is to analyse how plant N uptake and denitrification do co-vary along progressively more enriched conditions.

In bare marine sediments, nitrification and denitrification generally reach their maximum with a moderate organic load, but then collapse under higher enrichment (Caffrey et al. 1993; Sloth et al. 1995; Holmer et al. 2005). Much less is known about the effect of organic accumulation in freshwater environments where tolerant plants survive. In organic-poor sediments and nutrient-limiting conditions, benthic vegetation competes with N cycling bacteria and may inhibit denitrification, which removes this essential nutrient from the sytem (Risgaard-Petersen et al. 1998; Ottosen et al. 1999). In OM enriched sediments, a greater availability of mineralized N may promote its removal through a combination of uptake and denitrification coupled to nitrification. Indeed, enhanced ROL may stimulate the oxidation of ammonium, while attenuated N limitation and plant-bacteria competition may enhance denitrification. We hypothesize that a moderate OM enrichment would probably maximise this combined ecosystem function, while an extreme enrichment would suppress both direct (uptake) and indirect effects (stimulation of nitrification by ROL and associated denitrification) of rooted plants on net N removal.

The objective of this research was to test whether and to what extent the submerged macrophyte *Vallisneria spiralis* L. (Hydrocharitaceae family) affects sediment N dynamics in response to organic enrichment. *V. spiralis* is a freshwater stoloniferous species having basal rosettes of flexible ribbon-like leaves, widespread in the tropical and subtropical areas of both hemispheres and also in southern Europe (Hussner and Lösch 2005). This plant is abundant in the high-plain sections of Northern Italy rivers, in the irrigation canal network, and in the littoral zones of the Alpine lakes (Pinardi et al. 2009; Bresciani et al. 2012; Bolpagni et al. 2013). Multiple evidences indicate that site-specific or seasonal-specific oxygen release by roots can explain its common occurrence in eutrophic environments (Ribaudo et al. 2011; Soana and Bartoli 2013). To verify our hypothesis, assimilative and dissimilative benthic N paths were measured in sediments devoid of plants and in sediments colonized by *V. spiralis*, along an OM gradient simulating different eutrophication conditions.

## Materials and methods

### Sampling procedure and microcosm setup

Sediment, water and specimens of the rooted macrophyte *V. spiralis* were collected from the upper Mincio River in a shallow-water eutrophic site (Massimbona

location, Northern Italy, 45°16′43″N, 10°42′32″E, and ~1.5 m depth). The sediment was muddy, with an average porosity of ~0.78, an organic matter content of ~8.3 % (as loss on ignition, LOI) and a C/N of ~23. Dissolved inorganic nitrogen in water (DIN, ~100 µM) was mostly accounted for nitrate (78 %), followed by ammonium (18 %) and nitrite (4 %). Sampling and experimental activities were carried out in July, during the biomass peak of the macrophyte meadows (Pinardi et al. 2009). An approach based on microcosm incubation under controlled conditions after an in situ acclimatization period was adopted (Ribaudo et al. 2011).

Over 70 l of sediment from the upper 10 cm depth horizon were collected and sieved with a 2 mm mesh in order to remove coarse plant debris and macrofauna, and then homogenized. Thereafter, the sediment was divided and transferred into 12 l buckets. Five treatments were created, and sediment was amended with increasing amounts of organic matter. Added OM was in the form of commercially available fish feed pellets (~90 % OM, of which 42 % organic C, and 6 % organic N), previously dried at 70 °C and ground to a powder in a mortar. Treatments had 0 (A), 1 (B), 2.5 (C), 5 (D) and 10 g (E) of ground pellets per liter of sediment added and homogenized. OM content differed by nearly 13 % between the control (~8.3 % as LOI) and the most enriched sediment (~9.4 % as LOI). Such enrichment may appear low but it consists of extremely labile and reactive organic matter, with C/N ratio (~7) similar to those of algal communities, whilst the sedimentary OM pool includes black, recalcitrant and scarcely reactive carbon. Previous laboratory studies confirmed a large stimulation of microbial metabolism and significant alterations of pore water chemistry where sediment was amended with comparable amounts of fish feed (Mascaró et al. 2009; Valdemarsen et al. 2009; Soana et al. 2012).

Simultaneously, ~500 shoots of *V. spiralis* were carefully collected by hand to preserve intact the root systems and washed with river water. Plants similar in size were selected for the subsequent transplant. The sediment of each OM level was transferred into cylindrical Plexiglass microcosms of three different diameters and same height (10 cm) for the measurements of benthic metabolism (Fig. 1). For each level, 6 microcosms with the outer diameter of 4 cm were left unvegetated, and randomly selected individuals of *V. spiralis* similar in size were transplanted into

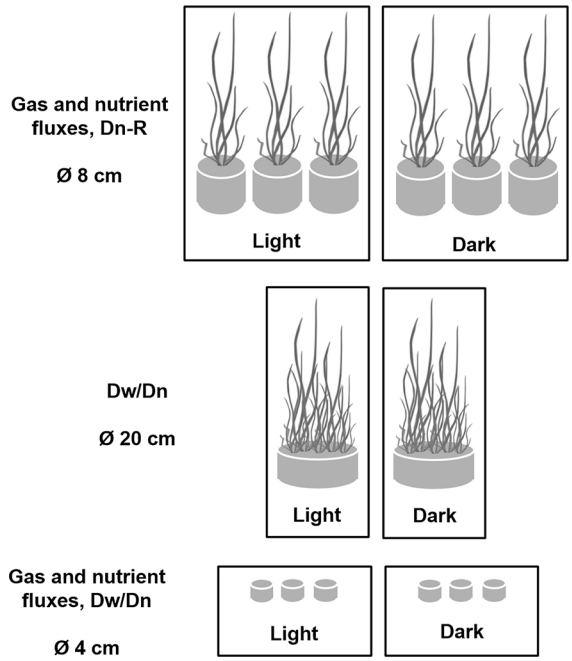


**Fig. 1** Experimental design. Different types of microcosms and relative number of replicates set up for each organic matter level for incubations purpose (fluxes of gases and nutrients, denitrification in the rhizosphere—Dn–R, denitrification of water column nitrate—Dw, and surface denitrification coupled to nitrification—Dn)

microcosms of two dimensions, i.e. 6 microcosms with an outer diameter of 8 cm (3 shoots in each) and 2 microcosms with an outer diameter of 20 cm (20 shoots in each). Plant biomass in microcosms reflected that previously measured in summer months at the investigated site (400–500 g of dry weight $m^{-2}$; Ribaudo et al. 2011). All the microcosms were located on the river bottom, within vegetated and unvegetated patches, and left in situ for 10 days under natural conditions of temperature, irradiance, water chemistry, and flow. Thereafter, all microcosms were transferred underwater in transparent Plexiglass liners with internal diameter perfectly fitting microcosm outer diameter. Simultaneously to the microcosm recovery, ~200 l of river water were collected for pre-incubation and incubation procedures. Within 2 h from recovery, all liners were brought fully submerged to the laboratory for further processing.

Once in the laboratory, microcosms were kept submerged by river water continuously aerated with aquarium pumps and maintained at field temperature (~24 °C). Microcosms were subject to a 16/8 h light/dark cycle at an irradiance of ~400 µmol

photons $m^{-2}$ $s^{-1}$ (Photosynthetically Active Radiation, PAR) by means of 1000 Watt halogen lamps. The chosen light intensity reflected the average in situ irradiance at the sediment level and was also set for incubations. Water temperature was measured with an YSI Multiple Probe (mod 556, Yellow Springs, OH, USA) and PAR intensity with a luxmeter (LI-192 Underwater Quantum Sensor) and a LI-250A Light Meter (Li-Cor, Lincoln, NE, U.S.A.). All microcosms were stored in the same tank and water was regularly replenished to avoid extensive nutrient accumulation and to minimize algal growth.

## Measurements of gas and nutrient fluxes

Microcosms were incubated at in situ temperature according to the procedures for flux measurements of oxygen (SOD, Sediment Oxygen Demand), methane ($CH_4$), and dissolved inorganic nitrogen forms ($NO_3^-$, $NO_2^-$, $NH_4^+$) described in Dalsgaard et al. (2000). For each OM level, 3 vegetated (Ø 8 cm) and 3 unvegetated (Ø 4 cm) microcosms were used for the light treatment, and the same number for the dark treatment (Fig. 1). Microcosms were transferred to transparent Plexiglass liners (Ø 8 cm and height 30 cm for vegetated microcosms, Ø 4 cm and height 20 cm for unvegetated microcosms). Microcosms of different dimensions were used according to the standard for measuring biogeochemical processes in benthic systems. Homogeneous stirring of the water column without sediment resuspension or damage to plant fronds was ensured by magnetic bars positioned in the upper portion of each liner that were driven by an external motor (40 rpm). Incubations lasted ~2 h and when they started, each core was sealed with a transparent Plexiglass lid with a water sampling port. Water samples (~60 ml, corresponding to ~6 % of the water volume in the core) for gas and nutrient determinations were collected 3 times (initial, intermediate, final) at regular time intervals from each liner. An equivalent amount of the sampled water was replaced with water from the incubation tank through a one-way valve in the core lid. Samples for gas determinations were transferred to gas-tight vials (12 ml Exetainer, Labco, High Wycombe, UK). For oxygen analyses Winkler reagents were immediately added (A.P.H.A. et al. 1981), while for $CH_4$ analyses saturated mercuric chloride ($HgCl_2$) solution was added to stop biological activity (100 µl for a sample volume of 12 ml). Samples for nutrient determinations were filtered through Whatman GF/F glass fiber filters, transferred to polyethylene vials ($NO_3^-$, $NO_2^-$ and $NH_4^+$) and frozen for later analysis. $O_2$ was measured with Winkler titration (detection limit 5 µM, precision ±5 %). Gas samples for dissolved $CH_4$ determinations were extracted from water according to the headspace equilibration technique (McAuliffe 1971). Methane analyses were performed with a Fisons 9000 series gas chromatograph equipped with a flame ionization detector (detection limit 0.2 nM, precision ±1 %). Ammonium was determined on a double beam Jasco V-550 spectrophotometer (Bower and Holm-Hansen 1980). Nitrite and nitrate were measured on a Technicon Auto Analyser II (Armstrong et al. 1967). Detection limits were 0.5, 0.1, and 0.4 µM for $NH_4^+$, $NO_2^-$, and $NO_3^-$, respectively. Precision ranged between ±3 and ±5 % for the three nutrient analyses. Hourly fluxes of gas and nutrients (µmol $m^{-2}$ $h^{-1}$) were calculated after linear regression of concentration versus time, multiplied by the average number of light (16) and dark (8) h in the sampling period and summed to obtain daily values (mmol $m^{-2}$ $h^{-1}$).

## Denitrification coupled to nitrification in *V. spiralis* rhizosphere

Following measurements of dissolved gas and nutrient fluxes, vegetated microcosms (Ø 8 cm) were incubated to estimate coupled nitrification–denitrification rates in the rhizosphere of *V. spiralis* (Dn-R) by means of the $^{15}NH_4^+$ injection technique (Caffrey and Kemp 1992). During the injection procedure the microcosms were removed from the transparent Plexiglass liners. Each microcosm was provided with four series of vertical holes filled with silicon glue spaced in 1 cm intervals. Anoxic 10 mM $^{15}NH_4Cl$ solution (98 at.% $^{15}N$ enrichment) was injected into the pore water by means of glass syringes (Hamilton 725RN 250 µl) through the side ports of each microcosm. The whole 10 cm vertical sediment horizon was labelled by means of 40 injections per microcosm. Interstitial ammonium concentrations were measured on sediment samples of the five OM levels after the in situ acclimatization period. The added volume of labelled solution was set to increase pore water ammonium concentrations by at least 30 %. After the injection of the labelled solution, microcosms were transferred back to the transparent Plexiglass liners and bottom and top lids positioned.

For each OM level, three vegetated (Ø 8 cm) microcosms were used for the light treatment (~400 µmol photons $m^{-2}$ $s^{-1}$) and the same number for the dark treatment (Fig. 1). After ~2 h, sediment and water phase of each microcosm were gently mixed and an aliquot of the slurry was transferred to a 12 ml gas-tight vial (Exetainer, Labco, High Wycombe, UK). Immediately afterwards, 200 µl of zinc chloride solution (7 M) was added to each sample to stop microbial activity. Samples were stored upside down and refrigerated until later analysis. Isotopic composition of $N_2$ was determined by GC-IRMS (Delta V Advantage, Thermo Scientific; detection limit 0.1 µM, precision ±0.1 %) at the Department of Geological Sciences, Stockholm University, Sweden. Denitrification coupled to nitrification was calculated as the sum of $Dn_{15}$ and $Dn_{14}$, namely the rates of denitrification of $^{15}NO_3^-$ and $^{14}NO_3^-$ produced within the sediment via $^{15}NH_4^+$ and $^{14}NH_4^+$ oxidation, respectively (Risgaard-Petersen and Jensen 1997; Risgaard-Petersen et al. 1998). As described for gas and nutrient fluxes, light and dark Dn-R rates were combined to obtain daily fluxes.

The limit of the adopted method could be the not-homogeneous pore water labelling, compared to the perfusion technique already used for vegetated sediments, but not suitable for muddy substrates. Moreover, the presence of multiple hotspots of nitrification and denitrification in the rhizosphere may determine variable ratios of $^{14}NO_3^-$ and $^{15}NO_3^-$, violating the technique assumptions and causing underestimation of Dn-R rates. However, the whole set of vegetated microcosms was treated in the same way, so we are confident that the differences along the organic gradient are reasonably robust and reliable.

### Surface-associated denitrification in bare and vegetated sediments

Following measurements of dissolved gas and nutrient fluxes, total denitrification rates were estimated with the Isotope Pairing Technique (IPT, Nielsen 1992). In sediments where denitrification and anammox (anaerobic oxidation of ammonium) coexist the assumptions of the IPT are invalidated (Risgaard-Petersen et al. 2003). We therefore performed pilot tests in anoxic slurries (Risgaard-Petersen et al. 2005) collected in vegetated and plant-free sediments to measure potential denitrification rates and the contribution of anammox to total $N_2$ fluxes. Our results suggest that in the Mincio River sediments anammox accounts on average for <2 % of the total $N_2$ production. This is in agreement with similar measurements in eutrophic freshwater environments where anammox represents a negligible fraction of $N_2$ fluxes (Trimmer et al. 2003; Schubert et al. 2006; Zhou et al. 2014). We thus considered the IPT as an accurate method for denitrification measurement in the sediments employed in this study. Total denitrification rates were split into denitrification of nitrate diffusing from the water column to the anoxic sediment (Dw) and denitrification of nitrate produced by nitrification within the sediment (Dn). Dark rates were measured in bare sediments (3 microcosms for each OM level). Moreover, 2 vegetated microcosms (Ø 20 cm) for each OM level were incubated, one in light and one in dark condition. For the incubation, bare and vegetated microcosms were transferred to transparent Plexiglass liners. At the beginning of the incubation, labelled nitrate (15 mM $Na^{15}NO_3$ solution, 98 at.% enrichment) was added to the water column to have a final $^{15}N$ at.% of ~30 % (Dalsgaard et al. 2000). The same incubation conditions as for the $^{15}N$–$NH_4^+$ incubations were used. At the end of incubations, 3 sub-cores were sampled in each vegetated microcosm and slurry samples were collected and analyzed as previously reported for measurement of denitrification coupled to nitrification in the rhizosphere. Denitrification rates were calculated according to the equations and assumptions of Nielsen (1992). Daily rates were obtained as already described for gas and nutrient fluxes.

### Theoretical nitrogen assimilation by *V. spiralis* and estimation of microbial DNRA (Dissimilative Nitrate Reduction to Ammonium)

N uptake by *V. spiralis* was calculated from net production rates and average C/O and C/N ratios (Racchetti et al. 2010; Soana and Bartoli 2013) in photosynthetic tissues. At the end of all incubations, plants were collected from each microcosm by sediment sieving with a 2 mm mesh. *V. spiralis* specimens were rinsed to remove epiphytes and sediment residues. Above and below-ground tissues of plants from each microcosm were separately desiccated at 70 °C and expressed in term of dry weight (gDW).

DNRA (Dissimilative Nitrate Reduction to Ammonium) was not measured in the present study but its

role was double checked by comparing nitrate consumption rates ($NO_3^-$ flux) to Dw rates, and measured $NH_4^+$ fluxes to the expected $NH_4^+$ release during OM oxidation. Theoretical $NH_4^+$ production was calculated from the main respiration paths (oxygen- and nitrate- based) and the C/N stoichiometry of degraded organic carbon. Two values of C/N were considered, namely 23 for the in situ sediment and 7 for the added fish feed, in order to obtain a reliable range of ammonification rates. The calculation was performed for bare and vegetated sediments in dark condition.

### Statistical analyses

The effects of factors *organic level* and *light condition* (*light/dark*) on dependent variables (gas and nutrient fluxes and denitrification rates) were tested by means of two-way ANOVA. Data from bare and vegetated microcosms were analysed separately to simplify the model and exclude any predictable significance due to plant activity. Previous studies have demonstrated that benthic metabolism is significantly affected by the presence of *V. spiralis* (Pinardi et al. 2009; Ribaudo et al. 2011). Normality (Shapiro–Wilk test) and homoscedasticity (Levene's test) were previously examined and Box-Cox transformation was used when necessary. Differences were not considered significant if $p > 0.05$. All statistical analyses were performed with SigmaPlot 11.0 (Systat Software, Inc., CA, USA), and R (R Core Team 2013). In the graphs, average values are reported with associated standard deviation (std. dev.).

## Results

### Microcosm features after acclimatization

A visual check across the transparent liner walls revealed that microcosms developed differently according to their OM enrichment. All *V. spiralis* specimens were alive after the in situ acclimatization period, but they displayed marked differences in biomass reflecting either stimulated growth or impact of the organic enrichment. Average biomass (above + belowground) of the transplanted plants was minimum at the maximum organic enrichment ($252 \pm 78$ gDW $m^{-2}$), and maximum at level B ($631 \pm 89$ gDW $m^{-2}$). Control microcosms (level A, no OM addition) had light brown-reddish sediment, an indication of oxidized iron species. Otherwise, all artificially enriched microcosms had dark brown-blackish sediments with the exception of a surface layer (<5 mm) that appeared light brown in sediments of levels B and C. At the last two levels, the oxidized portion was restricted to the uppermost ~1 mm-thick layer. At levels A and B *V. spiralis* roots were surrounded by an oxidized 1–2 mm-thick layer of light brown sediment, suggesting oxidized conditions. Otherwise, no oxidized halos were evident around roots at levels C, D and E. High chemical and microbial oxygen consumption probably minimized the thickness of oxic layers in the high OM enrichments.

Once recovered from microcosms of levels D and E, *V. spiralis* specimens appeared to be anchored with just the primary root and shedding of all the lateral fine roots was evident. Below-ground tissues were blackish and seemed to be rotting (Fig. 2). Red-colored iron plaques were detected only on root surfaces of plants recovered from microcosms of levels A and B. In the remaining levels, the chemically reduced pore water probably explained the absence of oxidized metals coating on the below-ground tissues.

### Gas fluxes

Organic enrichment affected all measured gas and nutrient fluxes (Table 1). Bare sediments were oxygen sinks both in the light and in the dark, suggesting the absence of significant microphytobenthos activity. SOD increased along with OM addition and was on average 2.5 times higher in level

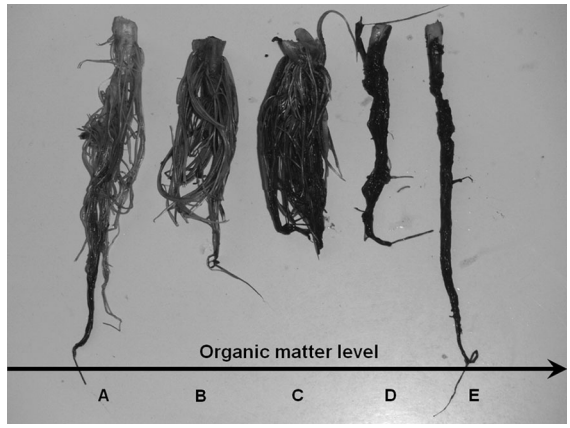


**Fig. 2** Root systems of plants recovered from the five organic matter levels

**Table 1** Results of the two–way ANOVA performed to test the effect of factors *organic level* and *light condition* (*light/dark*) on gas and nutrient fluxes measured in bare and vegetated sediments

| Variable | Factor | Df | Bare sediments | | | Vegetated sediments | | |
|---|---|---|---|---|---|---|---|---|
| | | | MS | F | *P* | MS | F | *P* |
| $O_2$ flux | Organic level | 4 | 12.613 | 52.007 | *** | 363.784 | 6.714 | *** |
| | Light/dark | 1 | 0.541 | 2.23 | NS | 28,152.525 | 519.57 | *** |
| | Organic level × light/dark | 4 | 0.078 | 0.322 | NS | 702.653 | 12.968 | *** |
| | Residual | 20 | 0.243 | | | 54.184 | | |
| $CH_4$ flux | Organic level | 4 | 714,851.477 | 82.532 | *** | 9,074,306.317 | 17.951 | *** |
| | Light/dark | 1 | 4831.207 | 0.558 | NS | 6536.445 | 0.013 | NS |
| | Organic level × light/dark | 4 | 100,623.894 | 11.617 | *** | 321,332.311 | 0.636 | NS |
| | Residual | 20 | 8661.506 | | | 505512.831 | | |
| $NH_4^+$ flux | Organic level | 4 | 1,548,716.021 | 25.269 | *** | 19,103,300.183 | 26.524 | *** |
| | Light/dark | 1 | 1,357,992.553 | 22.157 | *** | 31,114,793.008 | 43.201 | *** |
| | Organic level × light/dark | 4 | 349,947.364 | 5.71 | ** | 2,779,505.311 | 3.859 | * |
| | Residual | 20 | 61,289.665 | | | 720,233.128 | | |
| $NO_3^-$ flux | Organic level | 4 | 700,351.642 | 24.621 | *** | 22,174,182.176 | 29.268 | *** |
| | Light/dark | 1 | 48,616.995 | 1.709 | NS | 4,959,160.934 | 6.546 | * |
| | Organic level × light/dark | 4 | 21,529.827 | 0.757 | NS | 11,552,379.737 | 15.248 | *** |
| | Residual | 20 | 28,445.813 | | | 757,638.973 | | |
| $NO_2^-$ flux | Organic level | 4 | 885.803 | 8.789 | *** | 91,542.723 | 5.494 | ** |
| | Light/dark | 1 | 4965.605 | 49.267 | *** | 66,477.150 | 3.99 | NS |
| | Organic level × light/dark | 4 | 1021.082 | 10.131 | *** | 19,926.489 | 1.196 | NS |
| | Residual | 20 | 100.789 | | | 16,660.808 | | |

*** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$; *NS* not significant

E ($6.7 \pm 0.8$ mmol $O_2$ $m^{-2}$ $h^{-1}$) than in control sediments ($2.8 \pm 0.3$ mmol $O_2$ $m^{-2}$ $h^{-1}$) (Fig. 3a). Oxygen fluxes were about one order of magnitude higher in vegetated compared to bare sediments (Fig. 3a, b). They ranged between $-20.6 \pm 6.5$ and $-31.7 \pm 5.1$ mmol $O_2$ $m^{-2}$ $h^{-1}$ and between $18.5 \pm 8.2$ and $65.9 \pm 15.9$ mmol $O_2$ $m^{-2}$ $h^{-1}$, in dark and light conditions, respectively. Oxygen production increased markedly from level A to level B and then it decreased in the following levels. Similarly, $O_2$ production rates normalised for the above-ground biomass peaked in level B (Table 2).

Methane fluxes ranged between $9 \pm 1$ and $1128 \pm 132$ μmol $CH_4$ $m^{-2}$ $h^{-1}$, and between $-142 \pm 89$ and $2350 \pm 1008$ μmol $CH_4$ $m^{-2}$ $h^{-1}$, in bare and vegetated microcosms, respectively (Fig. 3c, d). In unvegetated sediments, $CH_4$ effluxes were extremely low in A, while they increased progressively with the OM level. Plant presence affected both the direction and the magnitude of benthic methane exchanges. Under no and low OM addition (A and B), methane fluxes were directed from the water to the sediment with higher rates in light. From C to E, $CH_4$ fluxes reversed both in the light and dark conditions, with emission rates always greater than 1000 μmol $CH_4$ $m^{-2}$ $h^{-1}$.

## Inorganic nitrogen fluxes and theoretical N uptake by *V. spiralis*

Ammonium fluxes ranged between $167 \pm 133$ and $1623 \pm 205$ μmol N $m^{-2}$ $h^{-1}$, and between $-2564 \pm 1197$ and $4576 \pm 593$ μmol N $m^{-2}$ $h^{-1}$ in bare and vegetated microcosms, respectively, and depended on both organic matter level and light/dark condition (Table 1; Fig. 4a, b). As for oxygen, the organic enrichment stimulated $NH_4^+$ fluxes. Bare sediments were always ammonium sources (Fig. 4a), whereas sediments with *V. spiralis* displayed both ammonium release and uptake. Plant presence increased dark ammonium release compared to the corresponding plant-free treatment. In the light,

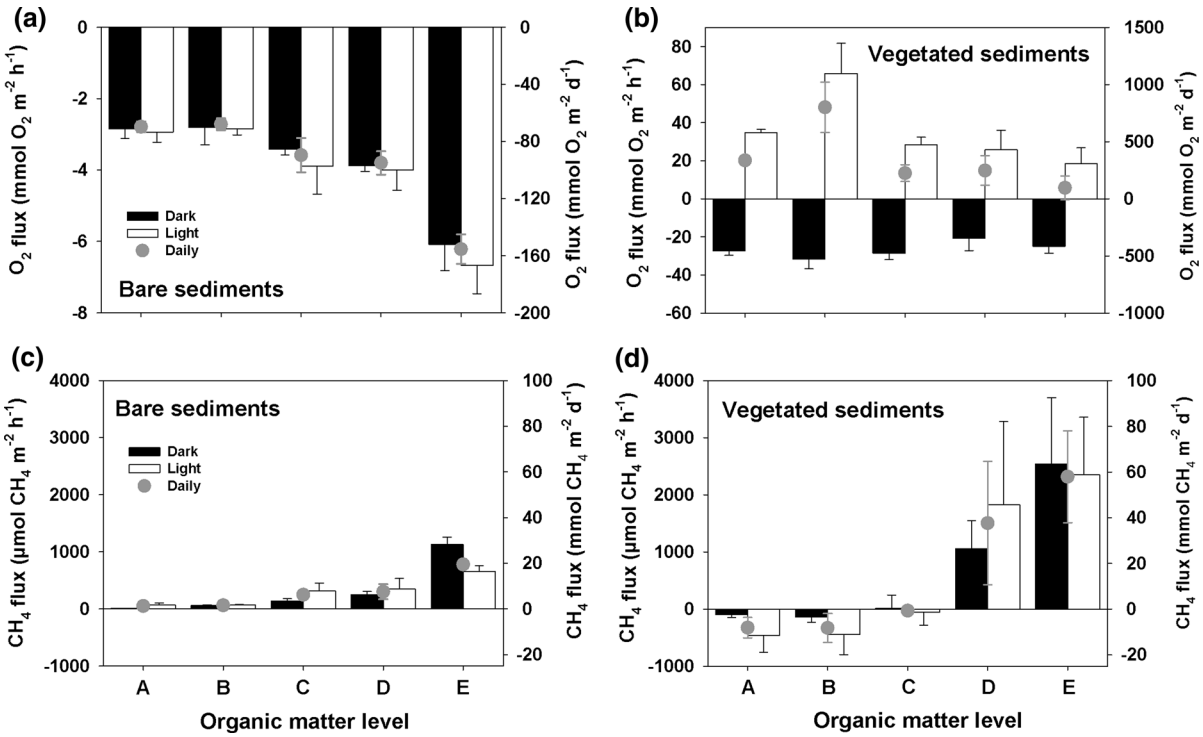


**Fig. 3** Light, dark and daily fluxes of $O_2$ and $CH_4$ measured in bare (*panels a*, *c*) and *V. spiralis* vegetated microcosms (*panels b*, *d*) of the five organic matter levels. Average values ± std. dev. are reported (n = 3). Light and dark fluxes are expressed as mmol $O_2$ $m^{-2}$ $h^{-1}$ and µmol $CH_4$ $m^{-2}$ $h^{-1}$ (*left axis*), while daily fluxes for both gases as mmol $m^{-2}$ $d^{-1}$ (*right axis*). Note the different ranges of values used in the two *panels* reporting $O_2$ fluxes in bare and vegetated microcosms

vegetated sediments assimilated ammonium at A, B (peaking with $\sim -2600$ µmol N $m^{-2}$ $h^{-1}$) and C, while at D and E ammonium was regenerated but with lower rates than in the dark.

Unvegetated sediments were always a nitrate sink ($-1089 \pm 126 < x < -186 \pm 69$ µmol N $m^{-2}$ $h^{-1}$), with nitrate uptake at level E doubling that measured in A (Fig. 4c). In sediments with plants, nitrate fluxes ($-5718 \pm 1445 < x < 1206 \pm 296$ µmol N $m^{-2}$ $h^{-1}$) were negative in the dark with higher consumption than in bare sediments (Fig. 4c, d). In the light nitrate production was measured in A, B and C.

Nitrite fluxes ranged between $-15 \pm 4$ and $58 \pm 16$ µmol N $m^{-2}$ $h^{-1}$, and between $97 \pm 58$ and $482 \pm 154$ µmol N $m^{-2}$ $h^{-1}$, in bare and vegetated microcosms, respectively (Fig. 4e, f). $NO_2^-$ release was up to one order of magnitude higher in the presence of *V. spiralis*. Ammonium, nitrate and nitrite fluxes normalised for the above-ground biomass followed the same patterns of the correspondent areal rates (Table 2).

In unvegetated microcosms, only control sediments were a net DIN (dissolved inorganic nitrogen) sink on a daily basis ($\sim -3$ mmol N $m^{-2}$ $day^{-1}$), while organic matter addition stimulated DIN regeneration, with a peak measured at level C ($\sim$ 17 mmol N $m^{-2}$ $day^{-1}$) (Fig. 4g). In vegetated sediments, net DIN fluxes were always directed from the water to the sediment ($-31 < x < -5$ mmol N $m^{-2}$ $day^{-1}$), with level C as only exception (Fig. 4h).

The inorganic nitrogen necessary to sustain *V. spiralis* primary production was calculated from net oxygen fluxes, assuming a conservative photosynthetic quotient of 0.69 and a C/N ratio of 12 for photosynthetic tissues. Values ranged between $\sim$3000 and 8300 µmol N $m^{-2}$ $h^{-1}$, with the same trend of net oxygen fluxes and a maximum calculated for level B.

## Denitrification rates

Rates of coupled nitrification–denitrification in the rhizosphere of *V. spiralis* (Dn-R) followed two

**Table 2** Oxygen and inorganic nitrogen fluxes normalized by above-ground biomass and denitrification rates in the rhizosphere normalized by below-ground biomass

| Organic matter level | $O_2$ flux (μmol $O_2$ g $DW^{-1}$ $h^{-1}$) | | $NH_4^+$ flux (μmol N g $DW^{-1}$ $h^{-1}$) | | $NO_3^-$ flux (μmol N g $DW^{-1}$ $h^{-1}$) | | $NO_2^-$ flux (μmol N g $DW^{-1}$ $h^{-1}$) | | Dn-R (μmol N g $DW^{-1}$ $h^{-1}$) | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Dark | Light | Dark | Light | Dark | Light | Dark | Light | Dark | Light |
| A | −61.18 (0.97) | 78.11 (7.29) | 0.36 (1.73) | −2.44 (1.46) | −4.60 (0.96) | 1.32 (0.36) | 0.98 (0.23) | 0.68 (0.26) | 0.33 (0.04) | 0.44 (0.03) |
| B | −53.19 (8.33) | 110.35 (22.95) | 2.41 (0.47) | −3.17 (0.62) | −2.62 (1.42) | 3.31 (2.13) | 0.79 (0.11) | 0.35 (0.05) | 0.11 (0.04) | 0.85 (0.12) |
| C | −66.67 (7.56) | 66.05 (6.59) | 3.23 (2.15) | −0.88 (1.54) | −3.22 (1.45) | 2.81 (0.64) | 1.11 (0.35) | 0.91 (0.21) | 0.05 (0.04) | 0.39 (0.41) |
| D | −80.53 (18.57) | 101.22 (32.68) | 10.25 (4.84) | 8.11 (5.86) | −12.02 (8.09) | −19.64 (17.40) | 0.80 (0.15) | 0.98 (0.29) | 0.09 (0.04) | 0.08 (0.05) |
| E | −121.70 (15.06) | 84.93 (21.14) | 23.48 (9.86) | 9.68 (6.02) | −12.38 (2.53) | −25.36 (1.83) | 0.79 (0.94) | 0.44 (0.18) | 0.14 (0.08) | 0.09 (0.07) |

Average values and standard deviation (in brackets) are reported

different patterns along the organic gradient in light and dark conditions (Fig. 5). In the dark, Dn-R decreased progressively with increasing organic level, from 28 ± 10 (level A) to 5 ± 1 μmol N $m^{-2}$ $h^{-1}$ (level E), whereas light rates peaked at level B (62 ± 10 μmol N $m^{-2}$ $h^{-1}$) and progressively decreased to 4 ± 1 μmol N $m^{-2}$ $h^{-1}$ at E. Dn-R rates normalized for the below-ground biomass followed the same patterns along the organic gradient of the correspondent areal rates (Table 2).

In bare and vegetated sediments, the water column was the dominant nitrate source for denitrification (Fig. 6a, b). In the dark, both in presence and absence of the plant, Dw was similar from level A to level D, but it was on average four times higher at level E. In the light, Dw increased with the organic matter level in vegetated sediment. At level B, in the light and in the dark and in vegetated and unvegetated sediments, Dn represented nearly 30 % of total rates. At level E, in vegetated sediments, denitrification was sustained exclusively by Dw, both in light and dark conditions.

## Discussion

Results from the present study confirm the service provided by rooted aquatic plants as benthic filters of nitrogen in eutrophic conditions (Sousa et al. 2012; Nizzoli et al. 2014). Sediments with *V. spiralis* removed nearly one order of magnitude more N compared to bare sediments, regardless the level of organic enrichment. Most of this difference was due to direct uptake that represented up to >90 % of the total N removal by the benthic system (Table 3). OM addition stimulated in both bare and vegetated sediments the denitrification of water column nitrate, as reported in a number of previous studies (Karjalainen et al. 2001; Forshay and Dodson 2011). The availability of water column nitrate and sedimentary OM determined quantitatively high denitrification rates, similar in the two conditions and increasing likewise along the gradient. As hypothesized, high OM impacted the plants, resulting in a significant reduction of N uptake. Decreased nitrogen assimilation was compensated by increased N loss via denitrification, that in the most enriched level accounted for >30 % of nitrogen removal (Table 3).

In the rooted sediments of the Mincio River, the smallest OM addition stimulated simultaneously sub-

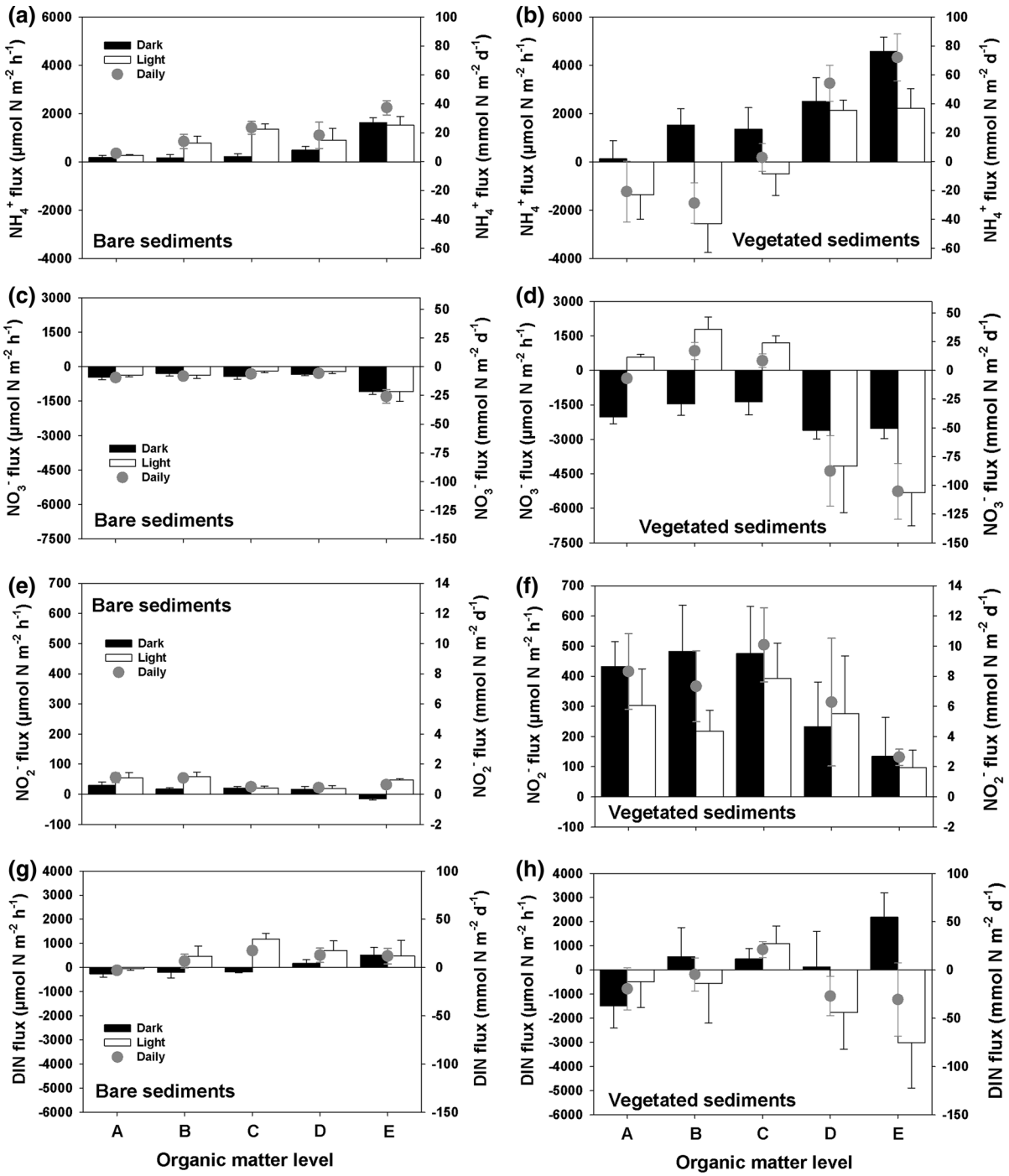


**Fig. 4** Light, dark and daily fluxes of nitrogen ($NH_4^+$, $NO_3^-$, $NO_2^-$, DIN–Dissolved Inorganic Nitrogen) measured in bare (*panels a*, *c*, *e*, *g*) and *V. spiralis* vegetated microcosms (*panels b*, *d*, *f*, *h*) of the five organic matter levels. Average values ± std. dev. are reported (n = 3). Light and dark fluxes are expressed as µmol N $m^{-2}$ $h^{-1}$ (*left axis*), while daily fluxes as mmol N $m^{-2}$ $d^{-1}$ (*right axis*)

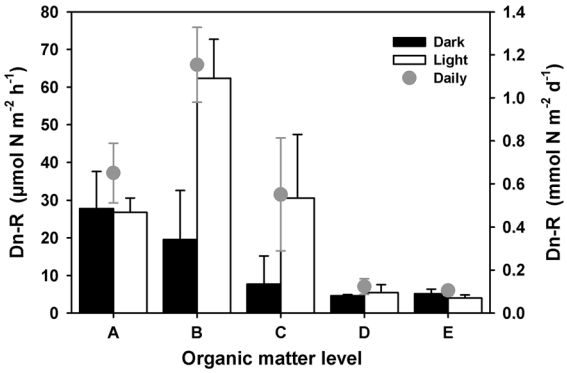


**Fig. 5** Light, dark and daily denitrification rates associated with the rizosphere in *V. spiralis* vegetated microcosms of the five organic matter levels (Dn–R). Average values ± std. dev. are reported (n = 3). Light and dark rates are expressed as µmol N $m^{-2}$ $h^{-1}$ (*left axis*), while daily rates as mmol N $m^{-2}$ $d^{-1}$ (*right axis*)

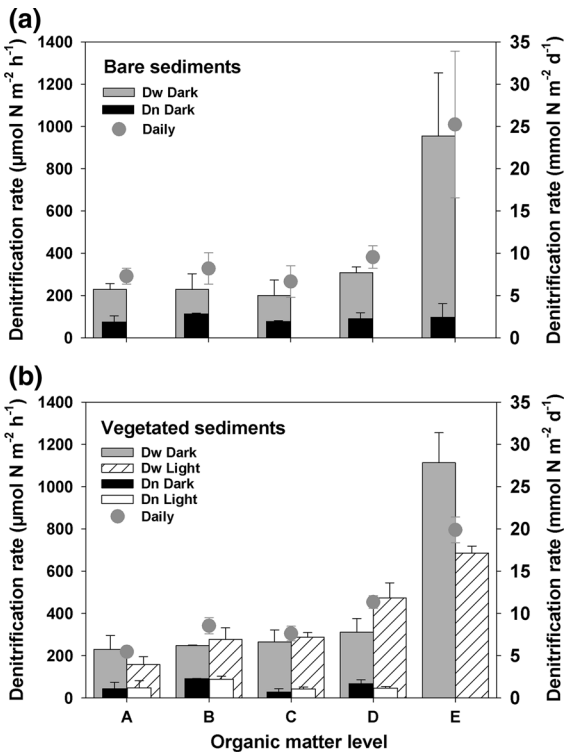


**Fig. 6** Denitrification rates splitted in the contribution of Dw (denitrification of water column nitrate) and Dn (surface denitrification coupled to nitrification) in bare (*panel a*) and *V. spiralis* vegetated microcosms (*panel b*) of the five organic matter levels. In vegetated sediments, Dn rates were not detectable in level E. Average values ± std. dev. are reported (n = 3). Light and dark rates are expressed as µmol N $m^{-2}$ $h^{-1}$ (*left axis*), while daily rates as mmol N $m^{-2}$ $d^{-1}$ (*right axis*)

surface N loss via denitrification coupled to nitrification and *V. spiralis* primary production, thus having a positive effect for microbial and plant N-related ecosystem services. The mechanisms underlying increased rates of Dn-R are likely complex and involve higher ammonification, short-term plant response to organic enrichment and increased ROL within ammonium-rich pore waters. Increased primary production was likely due to mobilization of limiting micronutrients, as labile organic matter may have had a primer effect on the recycling of trace elements/compounds. On the contrary, high OM suppressed Dn-R likely due to oxygen deficit or hostile chemical environment for bacteria. Future research should address processes as DNRA that may be quantitatively relevant in $NO_3^-$-rich eutrophic sediments (McGlathery et al. 2007; Nizzoli et al. 2010). Variable fractions of the recycled ammonium do not match the expected ammonification rates, providing indirect evidences for such hypothesis.

## Benthic metabolism along the OM gradient: bare versus vegetated sediments

In plant-free condition, mineralization rates increased along the gradient, as shown by the progressively higher consumption of oxygen and nitrate, and the concurrent release of methane and ammonium. SOD was similar in the dark and light conditions suggesting that benthic metabolism was driven by heterotrophic activity. Oxygen fluxes detected in the most enriched level were comparable to those measured during summer in naturally organic-rich sediments of temperate freshwater bodies (Longhi et al. 2008; Racchetti et al. 2011). Bare sediments were always an ammonium source, with progressively greater release along the OM gradient, and higher in the light than the dark, likely due to higher oxygen penetration in the sediment stimulating ammonification. Similar oxygen fluxes measured in the light and in the dark do not allow to calculate net primary production by benthic microalgae, even if photosynthesis at the interface cannot be excluded. Any oxygen flux directed downward may amplify the oxic microlayer at the sediment–water interface, resulting in higher aerobic respiration and likely higher mineralization.

Among anaerobic reactions, denitrification and methanogenesis likely dominated in OM degradation. Denitrification was sustained by the high nitrate

**Table 3** Benthic N exchanges (mmol N $m^{-2}$ $d^{-1}$) in bare and vegetated sediments along the organic matter gradient

| Organic matter level | DIN | Denitrification | | | DE (%) | Uptake | Total N removal |
|---|---|---|---|---|---|---|---|
| | | Dw | Dn | Dn–R | | | |
| Bare sediment | | | | | | | |
| A | –2.86 (1.45) | 5.50 (0.65) | 1.79 (0.69) | | 100 | | 7.28 (0.93) |
| B | 6.62 (7.28) | 5.49 (1.79) | 2.71 (0.11) | | 66 (28) | | 8.20 (1.86) |
| C | 17.50 (3.99) | 4.79 (1.76) | 1.87 (0.10) | | 30 (12) | | 6.66 (1.85) |
| D | 12.78 (7.45) | 7.38 (0.66) | 2.16 (0.68) | | 38 (16) | | 9.54 (1.34) |
| E | 11.92 (8.08) | 22.92 (7.16) | 2.32 (1.56) | | 69 (19) | | 25.24 (8.68) |
| Vegetated sediment | | | | | | | |
| A | –19.53 (21.75) | 4.37 (0.08) | 1.11 (0.38) | 0.65 (0.14) | 100 | 72.49 (3.33) | 78.61 (3.36) |
| B | –4.50 (17.15) | 6.41 (0.88) | 2.13 (0.24) | 1.15 (0.17) | 100 | 132.75 (30.67) | 142.44 (30.68) |
| C | 21.25 (8.39) | 6.72 (0.75) | 0.89 (0.18) | 0.55 (0.26) | 30 (11) | 61.31 (7.90) | 69.47 (7.94) |
| D | –27.06 (20.39) | 10.07 (0.70) | 1.27 (0.10) | 0.12 (0.04) | 100 | 57.41 (19.62) | 68.87 (19.63) |
| E | –30.62 (38.02) | 21.17 (1.45) | n.d. | 0.11 (0.02) | 100 | 47.53 (15.90) | 68.80 (15.97) |

*DIN* Dissolved Inorganic Nitrogen fluxes, Dw (denitrification of water column nitrate); Dn (surface denitrification coupled to nitrification); Dn–R (deep denitrification coupled to nitrification in the rhizosphere); denitrification efficiency, *DE* (Dw + Dn + Dn-R)/(Dw + Dn + Dn-R + DIN) if DIN > 0, *DE* 100 % if DIN < 0; *V. spiralis* uptake; total N removal = Dw + Dn + Dn-R + uptake

Average values ± std. dev. are reported. *n.d.* not detectable

availability in the water column (~78 μM), and the release of gas bubbles during microcosm handling, especially from the sediment of the two highest organic levels, suggested stimulation of methanogenesis or inhibition of methanotrophy (Roden and Wetzel 1996). Iron and manganese reduction probably did not play a significant role in anaerobic decomposition, as the magnitude of oxidized metal pools in the Mincio sediment is low (Soana et al. 2012).

Rates of *V. spiralis* primary production were not affected by the organic content and were comparable to those previously measured in summer at the same riverine site (Pinardi et al. 2009; Ribaudo et al. 2011). However, high labile OM additions caused a dramatic reduction of root biomass and clear signs of stress. Even if active, the root system was probably damaged by the oxygen deficit and high concentrations of phytotoxins in pore water due to the organic enrichments. Contrary to what is described for other plants inhabiting reduced sediments (Colmer et al. 1998; Kotula et al. 2009), there is evidence that *V. spiralis* does not form diffusive barriers (i.e. layers of suberin or lignin below the root surface) that prevent oxygen release into the sediment (Lemoine et al. 2012). It is likely that this macrophyte can overcome the risk of root damage in anoxic sediments by reducing the biomass, minimizing root surface exposure to the hostile interstitial environment and maintaining a sufficient oxygen supply to the root apex.

*Vallisneria spiralis* tended to increase the magnitude of solute fluxes and switched the benthic metabolism from heterotrophic to autotrophic. This capacity, previously demonstrated in less organic sediments (Ribaudo et al. 2011), was also maintained in the present conditions of OM enrichment. Up to level C, *V. spiralis* was not only able to buffer methane evasion but also to reverse its fluxes. Net methane consumption, measured both in light and dark conditions, may be a consequence of methanotrophy by epiphytic organisms growing on the canopy (Heilman and Carlton 2001). However, a net methane influx could also be related to oxic conditions in the sediment promoting both biotic and abiotic $CH_4$ oxidation. ROL can stimulate deep aerobic respiration, far away from the uppermost oxic sediment layer, and catalyze the oxidation of anaerobic metabolic end products. Indeed, greater rates of methane consumption were detected in light conditions, when ROL rates are the highest due to photosynthetic activity (Soana and Bartoli 2013). By contrast, vegetated sediments at the two most enriched levels became a methane source greater than the corresponding bare ones, probably

**Table 4** Theoretical ammonium production calculated from oxygen and nitrate respiration, measured ammonium fluxes, and contribution of Dw (denitrification of water column nitrate) to the total nitrate consumption along the organic matter gradient

| Organic matter level | Theoretical $NH_4^+$ production from $O_2$ and $NO_3^-$ respiration (μmol N $m^{-2}$ $h^{-1}$) | | Measured $NH_4^+$ flux (μmol N $m^{-2}$ $h^{-1}$) | Dw/nitrate consumption (%) |
|---|---|---|---|---|
| | C/N 23 | C/N 7 | | |
| Bare sediment | | | | |
| A | 130 (10) | 449 (34) | 178 (85) | 60 (14) |
| B | 137 (19) | 450 (63) | 167 (133) | 82 (32) |
| C | 160 (9) | 527 (28) | 220 (118) | 56 (17) |
| D | 186 (9) | 611 (29) | 489 (145) | 89 (9) |
| E | 310 (41) | 1020 (133) | 1622 (205) | 87 (20) |
| Vegetated sediment | | | | |
| A | 1198 (110) | 3936 (361) | 124 (100) | 12 (4) |
| B | 1393 (222) | 4577 (728) | 1530 (667) | 19 (8) |
| C | 1251 (150) | 4112 (494) | 1354 (893) | 22 (8) |
| D | 916 (286) | 3009 (941) | 2511 (976) | 12 (4) |
| E | 1134 (153) | 3726 (502) | 4576 (593) | 47 (13) |

Two values of C/N of the degraded OM were considered (23 for the background sediment and 7 for fish feed). Dark average values ± std. dev. are reported

because of gas transport conveyed by the aerenchymatous plant tissues. Aerenchyma can provide a conduit for $CH_4$ from the rhizosphere to the water column, bypassing the oxidizing sediment layers (Beckett et al. 2001; Colmer 2003). This pathway can result in greater $CH_4$ emissions from areas colonized by aerenchymatous plants relative to bare sediments. Moreover, rooted macrophytes can also provide litter and root exudates as a carbon source for methanogenic bacteria (Joabsson et al. 1999).

*Vallisneria spiralis* was able to maintain vegetated sediment as a net ammonium sink up to level C. Ammonium release by microbial ammonification was more than compensated by plant uptake and nitrification. The latter was likely stimulated by ROL, through the growth of nitrifiers in the proximity of *V. spiralis* roots (Soana and Bartoli 2014). Biofilms of ammonium oxidizers may grow as well on the plant leaves. At the last two levels, ammonium production apparently exceeded the plant N requirements and the oxidation capacity of the benthic system, resulting in a net release to the water column. In the dark, vegetated sediments acted as a greater nitrate sink compared to the plant-free condition. A higher diversity of microbial communities of nitrate reducers was recently demonstrated in vegetated compared to bare sediments, as a consequence of oxygen and labile carbon root release (Kofoed et al. 2012). By contrast, in the light, nitrate release from the sediment (up to level C) was likely a consequence of nitrification rates occurring in surface and subsurface sediments as well as associated to the plant canopy, as also proven by elevated ammonium consumption. Higher nitrite effluxes from vegetated sediment, compared to bare sediment, may be a consequence of ammonium oxidation to nitrite by epiphytic organisms growing on the dense canopy. Ammonia-oxidizing bacteria may colonize the leaves of different species of submerged macrophytes and in ammonium-rich environments the role of epiphytic nitrification must be taken into account (Eriksson and Weisner 1999; Coci et al. 2010).

## Do plants promote denitrification coupled to nitrification in eutrophic settings?

Rates of denitrification coupled to nitrification in the rhizosphere may be dependent upon the relative influences of oxygen and organic exudates released by roots, and competition between plants and N cycling bacteria. This issue was investigated in several studies, but almost exclusively in N and OM-poor

systems. When nitrogen is limiting, lower rates detected in light compared to dark suggest that the competition for N between roots and bacteria dominates over the potential stimulation of nitrification by ROL (Risgaard-Petersen and Jensen 1997). Moreover, N dissipation via denitrification coupled to nitrification is generally low if compared to plant uptake (Risgaard-Petersen et al. 1998; Ottosen et al. 1999; Welsh et al. 2000). The present results are distinct from those from earlier studies, because a moderate organic enrichment (levels B and C) stimulated Dn-R in the light. We speculate that mineralization of the added OM results in high inorganic nitrogen availability that smooths the competition between roots and bacteria in the rhizosphere. The same effect is also determined by direct assimilation of inorganic nitrogen from the canopy that reduces pore water DIN consumption via root uptake. An increase of nitrate reductase enzymatic activity in leaves was demonstrated for freshwater plants exposed to increasing levels of nitrate in water (Cedergreen and Madsen 2003; Takayanagi et al. 2012). The highest Dn-R measured in level B may be due to a combination of higher ammonium availability (greater ammonium regeneration from OM mineralization) and higher ROL by the plant, to counteract the more hostile condition in the sediment. Enhanced anaerobiosis may in fact promote aerenchyma formation to facilitate gas transport mechanisms (Colmer 2003). Lemoine et al. (2012) have measured an increase in root porosity and oxygen release potential of *V. spiralis* specimens grown in anoxic compared to more oxygenated sediments. During the acclimatization period, the plants of level B could have increased their root porosity to allow below-ground tissue respiration and survival in more OM-impacted substrates. However, the time needed to develop such a morphological adaptation is still to be investigated. The decrease in Dn-R above level C suggests that further OM enrichment stimulated benthic respiration and ammonium production via ammonification, but simultaneously limited nitrification, resulting in higher $NH_4^+$ efflux and lower N dissipation via denitrification coupled to nitrification. From level C, the oxygen amount injected directly by roots in the deep sediment appeared not to be enough to maintain oxic micro-niches for nitrification and sustain ammonium oxidation. In sediments with high oxygen demand, nitrification is usually hampered because aerobic heterotrophs and other chemoautotrophic bacteria have a higher affinity for oxygen, which outcompetes ammonia-oxidizing bacteria (Henriksen and Kemp 1988; Bonaglia et al. 2014). Moreover, accumulation of reduced species, such as sulphide, can have an inhibitory effect, especially to nitrifiers (Strauss and Lamberti 2000; Sears et al. 2004). Stimulation of degradation processes also results in increased stressful condition for the macrophytes (van Wijck et al. 1992; Britto and Kronzucker 2002; Wu et al. 2009). Plants in the most enriched microcosms could have been impacted by the hostile pore water conditions, resulting in a progressive loss of oxygen release capacity and assimilative functions, as already reported for the less tolerant Isoetids (Sand-Jensen et al. 2008; Raun et al. 2010). Signs of stress were evident in the below-ground tissues of *V. spiralis* specimens from the most OM-impacted substrates (Fig. 2).

### Bare and vegetated sediments as N sink: the effect of organic enrichment

In bare sediments, OM addition stimulated ammonification and $NH_4^+$ release more than $NO_3^-$ uptake, resulting in net DIN regeneration peaking at level C (Fig. 4; Table 3). Denitrification efficiency (DE, Eyre and Ferguson 2002), evaluated as the ratio between total denitrification rates (Dtot) and inorganic nitrogen effluxes across the sediment–water interface (DIN + Dtot) was 100 % only at level A, suggesting net inorganic nitrogen loss. DE decreased in all the other treatments, down to a minimum of ~30 % (level C), meaning that labile OM addition stimulated denitrification but made simultaneously this process less efficient. In bare sediments, pooling data from all OM levels, nitrate consumption rates ($NO_3^-$ flux) were reasonably comparable to rates of denitrification of water column nitrate (Dw). The equation of the linear regression between the two processes [$Dw = (-89 \pm 73) + (0.93 \pm 0.1)*NO_3^-flux$, $n = 15$] suggests that, if present, DNRA was responsible for a minor fraction of total nitrate consumption. Looking at the data in more detail, DNRA cannot be excluded as a relevant process in level E, where Dw represented on average nearly 87 % of nitrate consumption (Table 4). The unaccounted $NO_3^-$ demand may sustain a fraction of the measured ammonium

recycling via DNRA. The latter is higher and does not match the theoretical ammonium production calculated from the ratio between the combined oxygen and nitrate demand and the C/N stoichiometry of the degraded OM.

Sediments with *V. spiralis* were net DIN sinks in 4 out of 5 treatments, with level C as the only source. This intermediate level was critical also in sediments alone as it coincided with the peak of DIN release (Table 3). DE was 100 % in four out of five treatments, with level C as the only exception ($\sim$30 %). Here, the OM addition resulted in a combination of increased ammonification, decreased plant uptake and very limited stimulation of denitrification (Fig. 4–6; Table 3). In vegetated sediments, OM addition produced a drastic effect at levels D and E, where ammonium release and nitrogen loss via denitrification of water column nitrate were greatly stimulated.

Theoretical ammonium production calculated from dark oxygen and nitrate respiration, and OM stoichiometry underestimated the ammonium effluxes measured in vegetated sediments at the more enriched level (Table 4). Moreover, dark nitrate demand of all OM levels exceeds denitrification of water column nitrate, suggesting the presence of other nitrate sinks. For a minimum of $\sim$600 to a maximum of $\sim$2000 μmoles of consumed nitrate $m^{-2}$ $h^{-1}$ there is not an equivalent amount accounted for by denitrification, leaving the possibility for large ammonium recycling via DNRA. These speculations should be considered with caution as dark uptake by plants and associated epiphytes may occur and cannot be excluded (Nelson et al. 1981; Hansen et al. 2000; Dudley et al. 2001). Previous studies have demonstrated that reduced chemical conditions as those established in OM enriched levels do favor DNRA over denitrification (Gardner and McCarthy 2009; Nizzoli et al. 2010; Bonaglia et al. 2014). However, little is known about the occurrence of this process in freshwater sediments with rooted plants (Smyth et al. 2013).

In conclusion, vegetated sediments were a significantly greater N sink (as sum of permanent N removal via denitrification and temporary storage in biomass) compared to bare sediments along the whole organic gradient (Table 3). Plant uptake explained from $\sim$70 (E) to >90 % (B) of total N removal. Under moderate organic enrichment (B), ROL promoted deep sediment nitrification and denitrification. However, under progressively OM enriched conditions, the relevance of Dn-R decreased, due to a combination of reduced interstitial status, plant stress and nitrification inhibition. For the same reason, also *V. spiralis* N uptake was minimum at levels D and E. However, total N removal at the most enriched levels only slightly decreased compared to level C because a distinct decrease in plant performance was almost completely compensated for by increased denitrification of water column nitrate, from 7 to 30 % of total N removal in levels B and E, respectively. This means that an important ecosystem service is maintained by the N cycling bacteria when the plants cannot cope with hostile pore water conditions. Future researches should address the long term tolerance of *V. spiralis* and its physiological and morphological adaptations to organic enrichment, as well as the ecosystem consequences of enhanced DNRA that promotes inorganic N recycling versus dissipation.

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