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

Elisa Soana[1,2*], Mariachiara Naldi[2], Stefano Bonaglia[3], Erica Racchetti[2], Giuseppe Castaldelli[1], Volker Bruchert[3], Pierluigi Viaroli[2], Marco Bartoli[2]

[1] Department of Life Sciences and Biotechnology, University of Ferrara, Via L. Borsari 46, 44121, Ferrara, Italy

[2] Department of Life Sciences, University of Parma, Viale G.P. Usberti 33/A, 43124 Parma, Italy

[3] Department of Geological Sciences, Stockholm University, Frescativägen 8 SE 114 18 Stockholm, Sweden

*corresponding author

snolse@unife.it

Abstract

Organic enrichment may deeply affect benthic 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

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 et al., 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 inhibits denitrification, that dissipates this precious nutrient (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, amending sediment 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 50°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 (Mascarò 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 cyclindrical 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 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 gDW 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 two hours 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 1000Watt 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 ~2h 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 (APHA 1981), while for CH_4 analyses saturated mercuric chloride ($HgCl_2$) solution was added to stop biological activity (100µl 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-Halsen 1980). Nitrite and nitrate were measured on a Technicon AutoAnalyser II (Armstrong et al. 1967). Detection limits were 0.5 µM, 0.1 µM, 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) hours 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 atom % ^{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 ~2h, 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 D_{N15} and D_{N14}, 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 the same, 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

214 and anammox (anaerobic oxidation of ammonium) coexist the assumptions of the IPT are invalidated
215 (Risgaard-Petersen et al., 2003). We therefore performed pilot tests in anoxic slurries (Risgaard-
216 Petersen et al. 2005) collected in vegetated and plant-free sediments to measure potential
217 denitrification rates and the contribution of anammox to total N_2 fluxes. Our results suggest that in
218 the Mincio River sediments anammox accounts on average for <2% of the total N_2 production. This
219 is in agreement with similar measurements in eutrophic freshwater environments where anammox
220 represents a negligible fraction of N_2 fluxes (Trimmer et al. 2003; Schubert et al. 2006; Zhou et al.
221 2014). We thus considered the IPT as an accurate method for denitrification measurement in the
222 sediments employed in this study. Total denitrification rates were split into denitrification of nitrate
223 diffusing from the water column to the anoxic sediment (Dw) and denitrification of nitrate produced
224 by nitrification within the sediment (Dn). Dark rates were measured in bare sediments (3 microcosms
225 for each OM level). Moreover, 2 vegetated microcosms (Ø 20 cm) for each OM level were incubated,
226 one in light and one in dark condition. For the incubation, bare and vegetated microcosms were
227 transferred to transparent Plexiglass liners. At the beginning of the incubation, labelled nitrate (15
228 mM $Na^{15}NO_3$ solution, 98 atom% enrichment) was added to the water column to have a final ^{15}N
229 atom% of ~30% (Dalsgaard et al. 2000). The same incubation conditions as for the $^{15}N\text{-}NH_4^+$
230 incubations were used. At the end of incubations, 3 sub-cores were sampled in each vegetated
231 microcosm and slurry samples were collected and analyzed as previously reported for measurement
232 of denitrification coupled to nitrification in the rhizosphere. Denitrification rates were calculated
233 according to the equations and assumptions of Nielsen (1992). Daily rates were obtained as already
234 described for gas and nutrient fluxes.
235
236 *Theoretical nitrogen assimilation by* V. spiralis *and estimation of microbial DNRA (Dissimilative*
237 *Nitrate Reduction to Ammonium)*

238 N uptake by *V. spiralis* was calculated from net production rates and average C/O and C/ N ratios
239 (Racchetti et al. 2010; Soana and Bartoli 2013) in photosynthetic tissues. At the end of all incubations,
240 plants were collected from each microcosm by sediment sieving with a 2 mm mesh. *V. spiralis*
241 specimens were rinsed to remove epiphytes and sediment residues. Above and below ground tissues
242 of plants from each microcosm were separately desiccated at 70 °C until constant weight.
243 DNRA (Dissimilative Nitrate Reduction to Ammonium) was not measured in the present study but
244 its role was double checked by comparing nitrate consumption rates (NO_3^- flux) to Dw rates, and
245 measured NH_4^+ fluxes to the expected NH_4^+ release during OM oxidation. Theoretical NH_4^+
246 production was calculated from the main respiration paths (oxygen- and nitrate- based) and the C/N
247 stoichiometry of degraded organic carbon. Two values of C/N were considered, namely 23 for the *in*
248 *situ* sediment and 7 for the added fish feed, in order to obtain a reliable range of ammonification rates.
249 The calculation was performed for bare and vegetated sediments in dark condition.
250
251 *Statistical analyses*
252 The effects of factors *organic level* and *light condition (light/dark)* on dependent variables (gas and
253 nutrient fluxes and denitrification rates) were tested by means of two-way ANOVA. Data from bare
254 and vegetated microcosms were analysed separately to simplify the model and exclude any
255 predictable significance due to plant activity. Previous studies have demonstrated that benthic
256 metabolism is significantly affected by the presence of *V. spiralis* (Pinardi et al. 2009; Ribaudo et al.
257 2011). Normality (Shapiro-Wilk test) and homoscedasticity (Levene's test) were previously
258 examined and Box-Cox transformation was used when necessary. Differences were not considered
259 significant if $p>0.05$. All statistical analyses were performed with SigmaPlot 11.0 (Systat Software,
260 Inc., CA, USA), and R (R Core Team 2013). In the graphs, average values are reported with
261 associated standard deviation (sd).
262

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±78 gDW m^{-2}), and maximum at level B (631±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 (<5mm) 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.

285

286 *Gas fluxes*

287 Organic enrichment affected all measured gas and nutrient fluxes (Table 1). Bare sediments were
288 oxygen sinks both in the light and in the dark, suggesting the absence of significant
289 microphytobenthos activity. SOD increased along with OM addition and was on average 2.5 times
290 higher in level E (6.7±0.8 mmol m^{-2} h^{-1}) than in control sediments (2.8±0.3 mmol m^{-2} h^{-1})(Figure 3
291 a). Oxygen fluxes were about one order of magnitude higher in vegetated compared to bare sediments
292 (Fig. 3 a,b). They ranged between -20.6±6.5 and -31.7±5.1 mmol O_2 m^{-2} h^{-1} and between 18.5±8.2
293 and 65.9±15.9 mmol O_2 m^{-2} h^{-1}, in dark and light conditions, respectively. Oxygen production
294 increased markedly from level A to level B and then it decreased in the following levels. Similarly,
295 O_2 production rates normalised for the above-ground biomass peaked in level B (Table 2).
296 Methane fluxes ranged between 9±1 and 1128±132 µmol CH_4 m^{-2} h^{-1}, and between -142±89 and
297 2350±1008 µmol CH_4 m^{-2} h^{-1}, in bare and vegetated microcosms, respectively (Fig. 3 c,d). In
298 unvegetated sediments, CH_4 effluxes were extremely low in A, while they increased progressively
299 with the OM level. Plant presence affected both the direction and the magnitude of benthic methane
300 exchanges. Under no and low enrichment (A and B), methane fluxes were directed from the water to
301 the sediment with higher rates in light. From C to E, CH_4 fluxes reversed both in the light and dark
302 conditions, with emission rates always greater than 1000 µmol CH_4 m^{-2} h^{-1}.

303

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

305 Ammonium fluxes ranged between 167±133 and 1623±205 µmol N m^{-2} h^{-1}, and between -2564±1197
306 and 4576±593 µmol N m^{-2} h^{-1} in bare and vegetated microcosms, respectively, and depended on both
307 organic matter level and light/dark condition (Table 1, Fig. 4 a,b). As for oxygen, the organic
308 enrichment stimulated NH_4^+ fluxes. Bare sediments were always ammonium sources (Fig. 4 a),
309 whereas sediments with *V. spiralis* displayed both ammonium release and uptake. Plant presence

310 increased dark ammonium release compared to the corresponding plant-free treatment. In the light,
311 vegetated sediments assimilated ammonium at A, B (peaking with ~-2600 µmol N m^{-2} h^{-1}) and C,
312 while at D and E ammonium was regenerated but with lower rates than in the dark.
313 Unvegetated sediments were always a nitrate sink (-1089±126<x<-186±69 µmol N m^{-2} h^{-1}), with
314 nitrate uptake at level E doubling that measured in A (Fig. 4 c). In sediments with plants, nitrate fluxes
315 (-5718±1445<x<1206±296 µmol N m^{-2} h^{-1}) were negative in the dark with higher consumption than
316 in bare sediments (Fig. 4 c,d). In the light nitrate production was measured in A, B and C.
317 Nitrite fluxes ranged between -15±4 and 58±16 µmol N m^{-2} h^{-1}, and between 97±58 and 482±154
318 µmol N m^{-2} h^{-1}, in bare and vegetated microcosms, respectively (Fig. 4 e,f). NO_2^- release was up to
319 one order of magnitude higher in the presence of *V. spiralis*. Ammonium, nitrate and nitrite fluxes
320 normalised for the above-ground biomass followed the same patterns of the correspondent areal rates
321 (Table 2).
322 In unvegetated microcosms, only control sediments were a net DIN (dissolved inorganic nitrogen)
323 sink on a daily basis (~-3 mmol N m^{-2} d^{-1}), while organic matter addition stimulated DIN regeneration,
324 with a peak measured at level C (~17 mmol N m^{-2} d^{-1}) (Fig. 4 g). In vegetated sediments, net DIN
325 fluxes were always directed from the water to the sediment (-31<x<-5 mmol N m^{-2} d^{-1}), with level C
326 as only exception (Fig. 4 h).
327 The inorganic nitrogen necessary to sustain *V. spiralis* primary production was calculated from net
328 oxygen fluxes, assuming a conservative photosynthetic quotient of 0.69 and a C/N ratio of 12 for
329 photosynthetic tissues. Values ranged between ~3000 and 8300 µmol N m^{-2} h^{-1}, with the same trend
330 of net oxygen fluxes and a maximum calculated for level B.
331
332 *Denitrification rates*
333 Rates of coupled nitrification-denitrification in the rhizosphere of *V. spiralis* (Dn-R) followed two
334 different patterns along the organic gradient in light and dark conditions (Table 1, 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. 6 a,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-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 vs 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

383 oxygen flux directed downward may amplify the oxic microlayer at the sediment-water interface,
384 resulting in higher aerobic respiration and likely higher mineralization.
385 Among anaerobic reactions, denitrification and methanogenesis likely dominated in OM degradation.
386 Denitrification was sustained by the high nitrate availability in the water column (~78 μM), and the
387 release of gas bubbles during microcosm handling, especially from the sediment of the two highest
388 organic levels, suggested stimulation of methanogenesis or inhibition of methanotrophy (Roden and
389 Wetzel 1996). Iron and manganese reduction probably did not play a significant role in anaerobic
390 decomposition, as the magnitude of oxidized metal pools in the Mincio sediment is low (Soana et al.
391 2012).
392 Rates of *V. spiralis* primary production were not affected by the organic content and were comparable
393 to those previously measured in summer at the same riverine site (Pinardi et al. 2009; Ribaudo et al.
394 2011). However, high labile OM additions caused a dramatic reduction of root biomass and clear
395 signs of stress. Even if active, the root system was probably damaged by the oxygen deficit and high
396 concentrations of phyto-toxins in pore water due to the organic enrichments. Contrary to what is
397 described for other plants inhabiting reduced sediments (Colmer et al. 1998; Kotula et al. 2009), there
398 is evidence that *V. spiralis* does not form diffusive barriers (i.e. layers of suberin or lignin below the
399 root surface) that prevent oxygen release into the sediment (Lemoine et al. 2012). It is likely that this
400 macrophyte can overcome the risk of root damage in anoxic sediments by reducing the biomass,
401 minimizing root surface exposure to the hostile interstitial environment and maintaining a sufficient
402 oxygen supply to the root apex.
403 *V. spiralis* tended to increase the magnitude of solute fluxes and switched the benthic metabolism
404 from heterotrophic to autotrophic. This capacity, previously demonstrated in less organic sediments
405 (Ribaudo et al. 2011), was also maintained in the present conditions of OM enrichment. Up to level
406 C, *V. spiralis* was not only able to buffer methane evasion but also to reverse its fluxes. Net methane
407 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 (Radial Oxygen Loss) 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 because of gas transport conveyed by the aerenchimatous 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).

V. 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

433 effluxes from vegetated sediment, compared to bare sediment, may be a consequence of ammonium
434 oxidation to nitrite by epiphytic organisms growing on the dense canopy. Ammonia-oxidizing
435 bacteria may colonize the leaves of different species of submerged macrophytes and in ammonium-
436 rich environments the role of epiphytic nitrification must be taken into account (Eriksson and Weisner
437 1999; Coci et al. 2010).
438
439 *Do plants promote denitrification coupled to nitrification in eutrophic settings?*
440 Rates of denitrification coupled to nitrification in the rhizosphere may be dependent upon the relative
441 influences of oxygen and organic exudates released by roots, and competition between plants and N
442 cycling bacteria. This issue was investigated in several studies, but almost exclusively in N and OM-
443 poor systems. When nitrogen is limiting, lower rates detected in light compared to dark suggest that
444 the competition for N between roots and bacteria dominates over the potential stimulation of
445 nitrification by ROL (Risgaard-Petersen and Jensen 1997). Moreover, N dissipation via
446 denitrification coupled to nitrification is generally low if compared to plant uptake (Risgaard-Petersen
447 et al. 1998; Ottosen et al. 1999; Welsh et al. 2000). The present results are distinct from those earlier
448 studies, because a moderate organic enrichment (levels B and C) stimulated Dn-R in the light. We
449 speculate that mineralization of the added OM results in high inorganic nitrogen availability that
450 smooths the competition between roots and bacteria in the rhizosphere. The same effect is also
451 determined by direct assimilation of inorganic nitrogen from the canopy that reduces pore water DIN
452 consumption via root uptake. An increase of nitrate reductase enzymatic activity in leaves was
453 demonstrated for freshwater plants exposed to increasing levels of nitrate in water (Cedergreen and
454 Madsen 2003; Takayanagi et al. 2012). The highest Dn-R measured in level B may be due to a
455 combination of higher ammonium availability (greater ammonium regeneration from OM
456 mineralization) and higher ROL by the plant, to counteract the more hostile condition in the sediment.
457 Enhanced anaerobiosis may in fact promote aerenchyma formation to facilitate gas transport

458 mechanisms (Colmer 2003). Lemoine et al. (2012) have measured an increase in root porosity and
459 oxygen release potential of *V. spiralis* specimens grown in anoxic compared to more oxygenated
460 sediments. During the acclimatization period, the plants of level B could have increased their root
461 porosity to allow below-ground tissue respiration and survival in more OM-impacted substrates.
462 However, the time needed to develop such a morphological adaptation is still to be investigated. The
463 decrease in Dn-R above level C suggests that further OM enrichment stimulated benthic respiration
464 and ammonium production via ammonification, but simultaneously limited nitrification, resulting in
465 higher NH_4^+ efflux and lower N dissipation via denitrification coupled to nitrification. From level C,
466 the oxygen amount injected directly by roots in the deep sediment appeared not to be enough to
467 maintain oxic micro-niches for nitrification and sustain ammonium oxidation. In sediments with high
468 oxygen demand, nitrification is usually hampered because aerobic heterotrophs and other
469 chemoautotrophic bacteria have a higher affinity for oxygen, which outcompetes ammonia-oxidizing
470 bacteria (Henriksen and Kemp 1988; Bonaglia et al. 2014). Moreover, accumulation of reduced
471 species, such as sulphide, can have an inhibitory effect, especially to nitrifiers (Strauss and Lamberti
472 2000; Sears et al. 2004). Stimulation of degradation processes also results in increased stressful
473 condition for the macrophytes (van Wijck et al. 1992; Britto and Kronzucker 2002; Wu et al. 2009).
474 Plants in the most enriched microcosms could have been impacted by the hostile pore water
475 conditions, resulting in a progressive loss of oxygen release capacity and assimilative functions, as
476 already reported for the less tolerant Isoetids (Sand-Jensen et al. 2008; Raun et al. 2010). Signs of
477 stress were evident in the below-ground tissues of *V. spiralis* specimens from the most OM-impacted
478 substrates (Fig. 2).
479
480 *Bare and vegetated sediments as N sink: the effect of organic enrichment*
481 In bare sediments, OM addition stimulated ammonification and NH_4^+ release more than NO_3^- uptake,
482 resulting in net DIN regeneration peaking at level C (Fig. 4 and 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±73)+(0.93±0.1)*NO_3^-flux, n=15) suggests that, if present, the dissimilative reduction of nitrate to ammonium (DNRA) was responsible for a minor fraction of total nitrate consumption. Looking 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 via DNRA a fraction of the measured ammonium recycling. 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 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 only exception (~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 ~600 to a

maximum of ~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 ~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.

Acknowledgements

532 ES was supported by a doctoral scholarship within the 25th PhD program in Ecology (2010-12),
533 University of Parma. The authors would like to thank Fabio Vincenzi (Department of Life Sciences
534 and Biotechnology, University of Ferrara) for helping with laboratory analyses.
535

536 **References**

537 A.P.H.A., A.W.W.A., W.P.C.F. 1981. *Standard methods for the examination of water and*
538 *wastewater*. A.P.H.A., Washington

539 Armstrong FAJ, Sterus CR, Strickland JDH (1967) The measurement of upwelling and subsequent
540 biological processes by means of the Technicon AutoAnalyzer and associated equipment. Deep-
541 Sea Res 14:381–389

542 Beckett PM, Armstrong W, Armstrong J (2001) Mathematical modelling of methane transport
543 by *Phragmites*: the potential for diffusion within the roots and rhizosphere. Aquat Bot 69:293–
544 312

545 Boerema A, Schoelynck J, Bal K, Vrebos D, Jacobs S, Staes J, Meire P (2014) Economic valuation
546 of ecosystem services, a case study for aquatic vegetation removal in the Nete catchment
547 (Belgium). Ecosystem Services 7: 46–56

548 Bolpagni R, Bartoli M, Viaroli P (2013) Species and functional plant diversity in a heavily impacted
549 riverscape: Implications for threatened hydro-hygrophilous flora conservation. Limnologica 43:
550 230–238

551 Bonaglia S, Deutsch B, Bartoli M, Marchant HK, Brüchert V (2014) Seasonal oxygen, nitrogen and
552 phosphorus benthic cycling along an impacted Baltic Sea estuary: Regulation and spatial patterns.
553 Biogeochemistry 119:139–160

554 Bower CF, Holm–Hansen TT (1980) A salicylate–hypoclorite method for determing ammonia in
555 seawater. Can J Fish Aquat Sci 37:794–798

556 Bresciani M, Bolpagni R, Braga F, Oggioni A, Giardino C (2012) Retrospective assessment of
557 macrophytic communities in southern Lake Garda (Italy) from in situ and MIVIS (Multispectral
558 Infrared and Visible Imaging Spectrometer) data. J Limnol 71:180–190
559 Britto DT, Kronzucker HJ (2002) NH_4^+ toxicity in higher plants: a critical review. J Plant Physiol
560 159:567–584
561 Caffrey JM, Kemp WM (1992) Influence of the submersed plant, *Potamogeton perfoliatus* L., on
562 nitrogen cycling in estuarine sediments. Limnol Oceanogr 37:1483–1495
563 Caffrey JM, Sloth NP, Kaspar HF, Blackburn TH (1993) Effect of organic loading on nitrification
564 and denitrification in a marine sediment microcosm. FEMS Microbiol Ecol 12:159–167
565 Cedergreen N, Madsen TV (2003) Nitrate reductase activity in roots and shoots of aquatic
566 macrophytes. Aquat Bot 76:203–212
567 Coci M, Nicol GW, Pilloni GN, Schmid M, Kamst–van Agterveld MP, Bodelier PLE, Laanbroek HJ
568 (2010) Quantitative assessment of ammonia–oxidizing bacterial communities in the epiphyton of
569 submerged macrophytes in shallow lakes. Appl Environ Microb 76:1813–1821
570 Colmer TD, Gibberd MR, Wiengweera A, Tinh TK (1998) The barrier to radial oxygen loss from
571 roots of rice (Oryza sativa L.) is induced by growth in stagnant solution. J Exp Bot 49:1431–1436
572 Colmer TD (2003) Long–distance transport of gases in plants: a perspective on internal aeration and
573 radial oxygen loss from roots. Plant Cell Environ 26:17–36
574 Dalsgaard T, Nielsen LP, Brotas V, Viaroli P, Underwood GJC, Nedwell DB, Sundbäck K, Rysgaard
575 S, Miles A, Bartoli M, Dong L, Thornton DCO, Ottosen LDM, Castaldelli G, Risgaard–Petersen
576 N (2000) Protocol Handbook for NICE–Nitrogen Cycling in Estuaries: A Project Under the EU
577 Research Programme. Marine Science and Technology (MAST III). National Environmental
578 Research Institute, Silkeborg, 62 pp
579 Dudley BJ, Gahnstrom AM, Walker DI (2001) The role of benthic vegetation as a sink for elevated
580 inputs of ammonium and nitrate in a mesotrophic estuary. Mar Ecol Prog Ser 219: 99–107

581 Eriksson PG, Weisner SEB (1999) An experimental study on effects of submersed macrophytes on
582 nitrification and denitrification in ammonium rich aquatic systems. Limnol Oceanogr 44:1993–
583 1999
584 Eyre BD, Ferguson AJP (2002) Comparison of carbon production and decomposition, benthic
585 nutrient fluxes and denitrification in seagrass, phytoplankton, benthic microalgae and macroalgae
586 dominated warm-temperate Australian lagoons. Mar Ecol Prog Ser 229:43–59
587 Forshay KJ, Dodson SI (2011) Macrophyte presence is an indicator of enhanced denitrification and
588 nitrification in sediments of a temperate restored agricultural stream. Hydrobiologia 668:21–34
589 Gardner WS, McCarthy MJ (2009) Nitrogen dynamics at the sediment–water interface in shallow,
590 sub–tropical Florida Bay: why denitrification efficiency may decrease with increased
591 eutrophication. Biogeochemistry 95:185–198
592 Hansen JW, Pedersen AU, Berntsen J, Rønbøg IS, Hansen LS, Lomstein BA (2000) Photosynthesis,
593 respiration, and nitrogen uptake by different compartments of a Zostera marina community. Aquat
594 Bot 66:281–295
595 Heilman MA, Carlton RG (2001) Methane oxidation associated with submersed vascular
596 macrophytes and its impact on plant diffusive methane flux. Biogeochemistry 52:207–224
597 Henriksen K, Kemp WM (1988) Nitrification in estuarine and coastal marine sediments: methods,
598 patterns and regulating factors. In: Blackburn TH, Sorensen J (eds) Nitrogen cycling in coastal
599 marine environments. John Wiley and Sons, New York, pp 207–250
600 Holmer H, Wildish D, Hargrave B (2005) Organic enrichment from marine finfish aquaculture and
601 effects on sediment biogeochemical processes. In Hargrave B (ed) Environmental effects of
602 marine finfish aquaculture. Hdb Env Chem Vol. 5, Part M, Springer-Verlag Berlin Heidelberg, pp
603 181–206

Hussner A, Lösch R (2005) Alien aquatic plants in a thermally abnormal river and their assembly to neophyte-dominated macrophyte stands (River Erft, Northrhine-Westphalia). Limnologica 35:18–30

Joabsson A, Christensen TR, Wallén B (1999) Vascular plant controls on methane emissions from northern peatforming wetlands. Trends Ecol Evol 14:385–388

Karjalainen H, Stefansdottir G, Tuominen L, Kairesalo T (2001) Do submersed plants enhance microbial activity in sediment? Aquat Bot 69:1–13

Kofoed MV, Stief P, Hauzmayer S, Schramm A, Herrmann M (2012) Higher nitrate-reducer diversity in macrophyte-colonized compared to unvegetated freshwater sediment. Syst Appl Microbiol 35:465–472

Kotula L, Ranathunge K, Schreiber L, Steudle E (2009) Functional and chemical comparison of apoplastic barriers to radial oxygen loss in roots of rice (*Oryza sativa* L.) grown in aerated or deoxygenated solution. J Exp Bot 60:2155–2167

Lemoine DG, Mermillod–Blondin F, Barrat–Segretain M, Massé C, Malet E (2012) The ability of aquatic macrophytes to increase root porosity and radial oxygen loss determines their resistance to sediment anoxia. Aquat Ecol 46:191–200

Longhi D, Bartoli M, Viaroli P (2008) Decomposition of four macrophytes in wetland sediments: Organic matter and nutrient decay and associated benthic processes. Aquat Bot 89:303–310

Mascaró O, Valdemarsen T, Holmer M, Pérez M, Romero J. (2009) Experimental manipulation of sediment organic content and water column aeration reduces *Zostera marina* (eelgrass) growth and survival. J Exp Mar Biol Ecol 373:26–34

McAuliffe C (1971) Gas Chromatographic determination of solutes by multiple phase equilibrium. Chemical Technology 1:46–51

McGlathery KJ, Sundbäck K, Anderson IC (2007) Eutrophication in shallow coastal bays and lagoons: the role of plants in the coastal filter. Mar Ecol-Prog Ser 348:1–18

629 Nelson SG, Smith BD, Best BR (1981) Kinetics of nitrate and ammonium uptake by the tropical
630 freshwater macrophyte *Pistia stratiotes* L. Aquacultre 24:11–19
631 Nielsen LP (1992) Denitrification in sediment determined from nitrogen isotope pairing. Fems
632 Microbiol Ecol 86:357–362
633 Nixon SW (2009) Eutrophication and the macroscope. Hydrobiologia 629: 5–19
634 Nizzoli D, Carraro E, Nigro V, Viaroli P (2010) Effect of organic enrichment and thermal regime on
635 denitrification and dissimilatory nitrate reduction to ammonium (DNRA) in hypolimnetic
636 sediments of two lowland lakes. Water Res 44:2715–2724
637 Nizzoli D, Welsh DT, Longhi D, Viaroli P (2014) Influence of *Potamogeton pectinatus* and
638 microphytobenthos on benthic metabolism, nutrient fluxes and denitrification in a freshwater
639 littoral sediment in an agricultural landscape: N assimilation versus N removal. Hydrobiologia
640 737:183–200
641 Ottosen LDM, Risgaard–Petersen N, Neilsen LP (1999) Direct and indirect measurements of
642 nitrification and denitrification in the rhizosphere of aquatic macrophytes. Aquat Microb Ecol
643 19:81–91
644 Pezeshki SR (2001) Wetland plant responses to soil flooding. Environ Exp Bot 46:299–312
645 Pinardi M, Bartoli M, Longhi D, Marzocchi U, Laini A, Ribaudo C, Viaroli P (2009) Benthic
646 metabolism and denitrification in a river reach: a comparison between vegetated and bare
647 sediments. J Limnol 68:133–145
648 Pulido C, Lucassen E, Pedersen O, Roelofs JGM (2010) Influence of quantity and lability of sediment
649 organic matter on the biomass of two isoetids, *Littorella uniflora* and *Echinodorus repens*. Freshw
650 Biol 56:939–951
651 R Development Core Team (2013) R: A Language and Environment for Statistical Computing. R
652 Foundation for Statistical Computing, Vienna, Austria, ISBN 3–900051–07–0, URL
653 http://www.R–project.org

654 Racchetti E, Bartoli M, Ribaudo C, Longhi D, Brito EQL, Naldi M, Iacumin P, Viaroli P (2010) Short
655 term changes in pore water chemistry in river sediments during the early colonization by
656 *Vallisneria spiralis*. Hydrobiologia 652:127–137
657 Racchetti E, Bartoli M, Soana E, Longhi D, Christian RR, Pinardi M, Viaroli P (2011) Influence of
658 hydrological connectivity of riverine wetlands on nitrogen removal via denitrification.
659 Biogeochemistry 103:335–354
660 Raun AL, Borum J, Sand–Jensen K (2010) Influence of sediment organic enrichment and water
661 alkalinity on growth of aquatic isoetid and elodeid plants. Freshw Biol 55:1891–1904
662 Ribaudo C, Bartoli M, Racchetti, E, Longhi D, Viaroli P (2011) Seasonal fluxes of O_2, DIC and CH_4
663 in sediments with *Vallisneria spiralis*: indications for radial oxygen loss. Aquat Bot 94:134–142
664 Risgaard–Petersen N, Jensen K (1997) Nitrification and denitrification in the rhizosphere of the
665 aquatic macrophyte *Lobelia dortmanna* L. Limnol Oceanogr 42:529–537
666 Risgaard–Petersen N, Dalsgaard T, Rysgaard S, Christensen PB, Borum J, McGlathery K, Nielsen
667 LP (1998) Nitrogen balance of a temperate eelgrass *Zostera marina* bed. Mar Ecol-Prog Ser
668 174:281–291
669 Risgaard-Petersen N, Nielsen LP, Rysgaard S, Dalsgaard T, Meyer RL (2003) Application of the
670 isotope pairing technique in sediments where anammox and denitrification coexist. Limnol
671 Oceanogr Methods 1:63–73
672 Risgaard-Petersen N, Meyer RL, Revsbech NP (2005) Denitrification and anaerobic ammonium
673 oxidation in sediments: effects of microphytobenthos and NO_3^-. Aquat Microb Ecol 40:67–76
674 Roden EE, Wetzel RG (1996) Organic carbon oxidation and suppression of methane production by
675 microbial Fe (III) oxide reduction in vegetated and unvegetated freshwater wetland sediments.
676 Limnol Oceanogr 41:1733–1748
677 Sand–Jensen K, Møller KL, Raun AL (2008) Outstanding *Lobelia dortmanna* in iron armor. Plant
678 Signal Behav 3:882–884

679 Schubert CJ, Durisch-Kaiser E, Wehrli B, Thamdrup B, Lam P, Kuypers MMM (2006) Anaerobic
680 ammonium oxidation in a tropical freshwater system (Lake Tanganyika). Environ Microbiol. 8:
681 1857–1863
682 Sears K, Alleman JE, Barnard JL, Oleszkiewicz JA (2004) Impacts of reduced sulfur components on
683 active and resting ammonia oxidizers. J Ind Microbiol Biot 31:369–378
684 Sloth NP, Blackburn H, Hansen LS, Risgaard–Petersen N, Lomstein BA (1995) Nitrogen cycling in
685 sediments with different organic loading. Mar Ecol-Prog Ser 116:163–170
686 Smyth AR, Thompson SP, Siporin KN, Gardner WS, McCarthy MJ, Piehler MF (2013) Assessing
687 nitrogen dynamics throughout the estuarine landscape. Estuaries Coasts 36:44–55
688 Smolders AJP, Lucassen E, Roelofs JGM (2002) The isoetid environment biogeochemistry and
689 threats. Aquat Bot 73:325–350
690 Soana E, Naldi M, Bartoli M (2012) Effects of increasing organic matter loads on pore water features
691 of vegetated (*Vallisneria spiralis* L.) and plant–free sediments. Ecol Eng 47:141–145
692 Soana E, Bartoli M (2013) Seasonal variation of radial oxygen loss in *Vallisneria spiralis* L.: an
693 adaptation to sediment redox? Aquat Bot 104:228–232
694 Soana E, Bartoli M (2014) Seasonal regulation of nitrification in a rooted macrophyte (*Vallisneria*
695 *spiralis* L.) meadow under eutrophic conditions. Aquat Ecol 48:11–21
696 Sousa AI, Lillebø AI, Risgaard-Petersen N, Pardal MA, Caçador I (2012) Denitrification: an
697 ecosystem service provided by salt marshes. Mar Ecol Prog Ser 448:79–92
698 Strauss EA, Lamberti GA (2000) Regulation of nitrification in aquatic sediments by organic carbon.
699 Limnol Oceanogr 45:1854–1859
700 Takayanagi S, Takagi Y, Shimizu A, Hasegawa H (2012) The shoot is important for high affinity
701 nitrate uptake in Egeria densa, a submerged vascular plant. J Plant Res 125: 669–678.

Terrados J, Duarte CM, Kamp–Nielsen L, Agawin CM, Gacia E, Lacap D, Fortes MD, Borum J, Lubanski M, Grevec T (1999) Are seagrass growth and survival constrained by the reducing conditions of the sediment? Aquat Bot 65:175–197

Trimmer M, Nicholls JC, Deflandre B (2003) Anaerobic ammonium oxidation measured in sediments along the Thames Estuary, United Kingdom. Appl Environ Microbiol 69:6447–6454

van Wijck C, de Groot CJ, Grillas P (1992) The effect of anaerobic sediment on the growth of *Potamogeton pectinatus* L.: the role of organic matter, sulphide and ferrous iron. Aquat Bot 44:31–49

Vartapetian BB, Jackson MB (1997) Plant adaptations to anaerobic stress. Ann Bot-London 79:3–20

Valdemarsen T, Kristensen E, Holmer M (2009) Metabolic threshold and sulfide-buffering in diffusion controlled marine sediments impacted by continuous organic enrichment. Biogeochemistry 95:335–353

Welsh DT, Bartoli M, Nizzoli D, Castaldelli G, Riou SA, Viaroli P (2000) Denitrification, nitrogen fixation, community primary productivity and inorganic–N and oxygen fluxes in an intertidal *Zostera noltii* meadow. Mar Ecol-Prog Ser 208:65–77

Wu J, Cheng S, Liang W, Wu Z (2009) Effects of organic–rich sediment and below–ground sulfide exposure on submerged macrophyte, *Hydrilla verticillata*. B Environ Contam Tox 83:497–501

Zhou S, Borjigin S, Riya S, Terada A, Hosomi M (2014) The relationship between anammox and denitrification in the sediment of an inland river. Sci Total Environ 490:1029–1036

722 **Table 1** Results of the two–way ANOVA performed to test the effect of factors *organic level* and
723 *light condition (light/dark)* on gas and nutrient fluxes measured in bare and vegetated sediments.
724 *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$; NS=not significant
725

			Bare sediments			Vegetated sediments		
Variable	Factor	df	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	28152.53	519.57	***
	Organic level x Light/dark	4	0.078	0.322	NS	702.653	12.968	***
	Residual	20	0.243			54.184		
CH_4 flux	Organic level	4	714851.5	82.532	***	9074306	17.951	***
	Light/dark	1	4831.207	0.558	NS	6536.445	0.0129	NS
	Organic level x Light/dark	4	100623.9	11.617	***	321332.3	0.636	NS
	Residual	20	8661.506			505512.8		
NH_4^+ flux	Organic level	4	1548716	25.269	***	19103300	26.524	***
	Light/dark	1	1357992	22.157	***	31114793	43.201	***
	Organic level x Light/dark	4	349947.4	5.71	**	2779505	3.859	*
	Residual	20	61289.67			720233.1		
NO_3^- flux	Organic level	4	700351.6	24.621	***	22174182	29.268	***
	Light/dark	1	48617	1.709	NS	4959160	6.546	*
	Organic level x Light/dark	4	21529.83	0.757	NS	11552380	15.248	***
	Residual	20	28445.81			757638		
NO_2^- flux	Organic level	4	885.803	8.789	***	91542.72	5.494	**
	Light/dark	1	4965.605	49.267	***	66477.15	3.99	NS
	Organic level x Light/dark	4	1021.082	10.131	***	19926.49	1.196	NS
	Residual	20	100.789			16660.81		

Table 2 Oxygen and inorganic nitrogen fluxes normalized by above-ground biomass and denitrification rates in the rhizosphere normalized by below-ground biomass. Average values and standard deviation (in brackets) are reported.

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)

731 **Table 3** Benthic N exchanges (mmol N m^{-2} d^{-1}) in bare and vegetated sediments along the organic
732 matter gradient. DIN–Dissolved Inorganic Nitrogen fluxes, Dw (denitrification of water column
733 nitrate); Dn (surface denitrification coupled to nitrification); Dn–R (deep denitrification coupled to
734 nitrification in the rhizosphere); denitrification efficiency, DE=(Dw+Dn+Dn-R)/(Dw+Dn+Dn-
735 R+DIN) if DIN>0, DE=100% if DIN<0; *V. spiralis* uptake; total N removal= Dw+Dn+Dn-R+uptake.
736 Average values ± std. dev. are reported. n.d. = not detectable.
737

	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)

738

739

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. 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.

	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)

Figure captions

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)

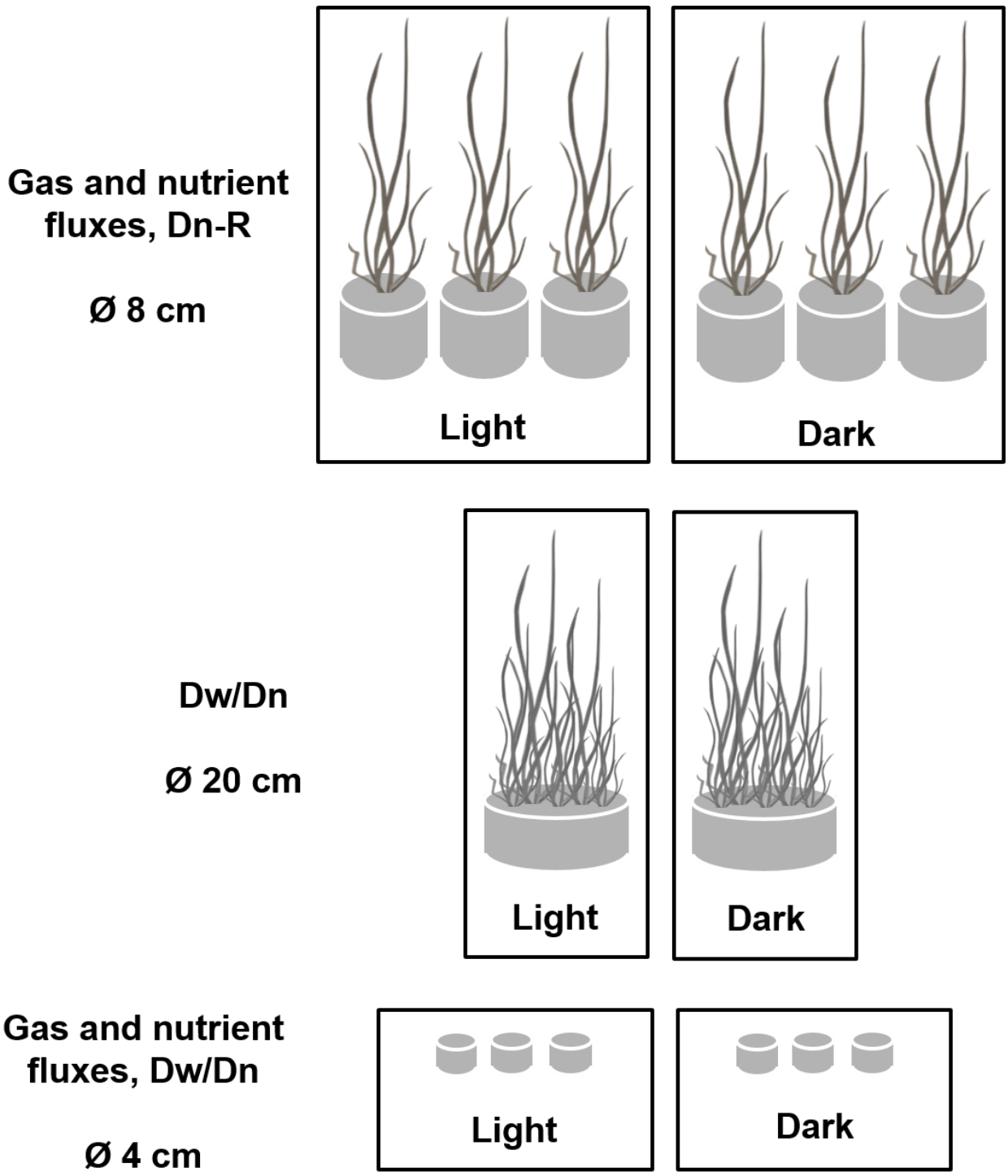
Fig. 2 Root systems of plants recovered from the five organic matter levels

Fig. 3 Light, dark and daily fluxes of O_2 and CH_4 fluxes measured in bare (panels a and c) and *V. spiralis* vegetated microcosms (panels b and 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

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, and g) and *V. spiralis* vegetated microcosms (panels b, d, f, and 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)

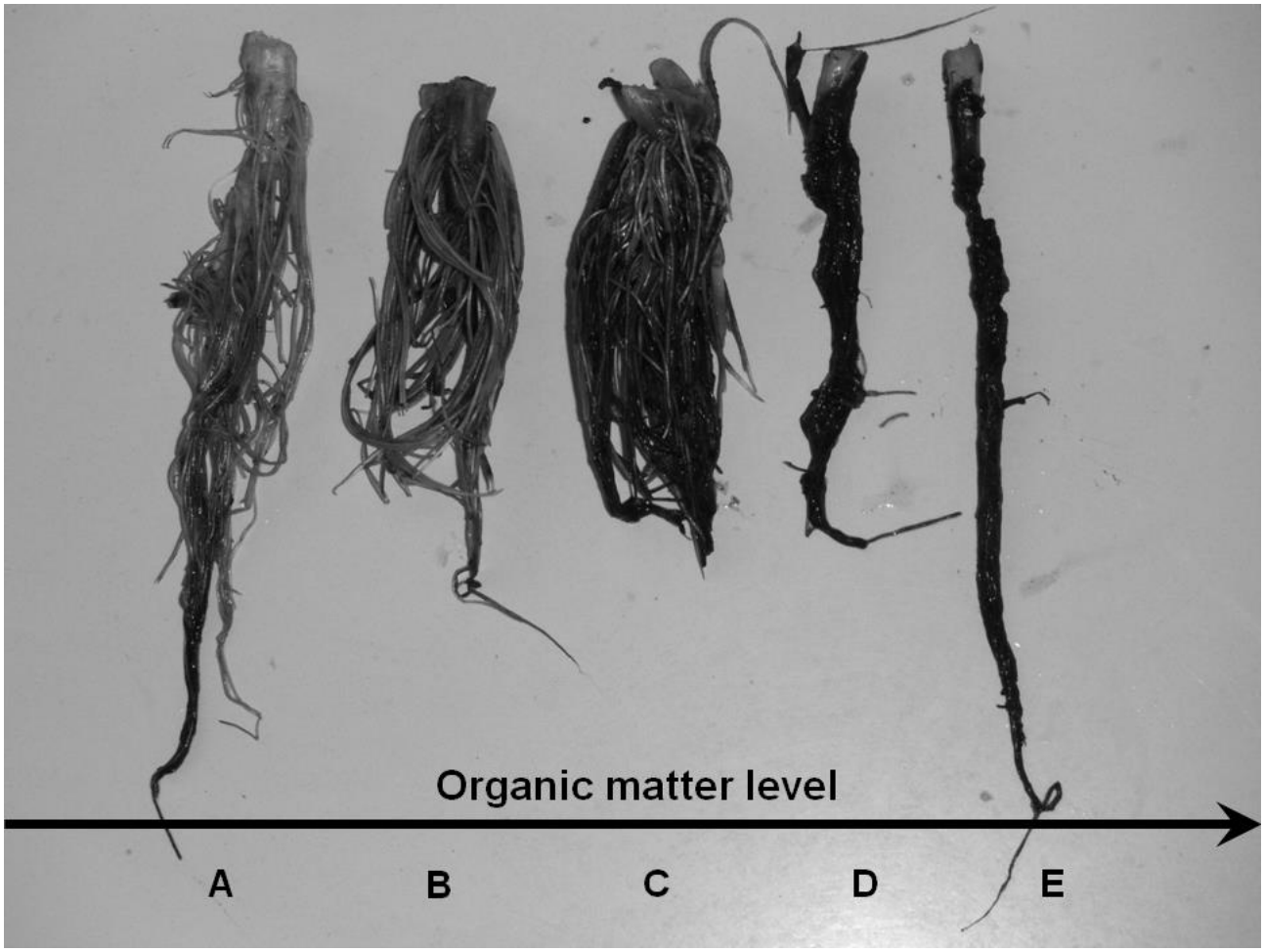
Fig. 5 Light, dark and daily denitrification rates associated with the rizosphere in *V. spiralis* vegetated microcosms of the five organic matter levels (Dn–R). Average values ± std. dev. are reported (n=3). Light and dark rates are expressed as µmol N m^{-2} h^{-1} (left axis), while daily rates as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

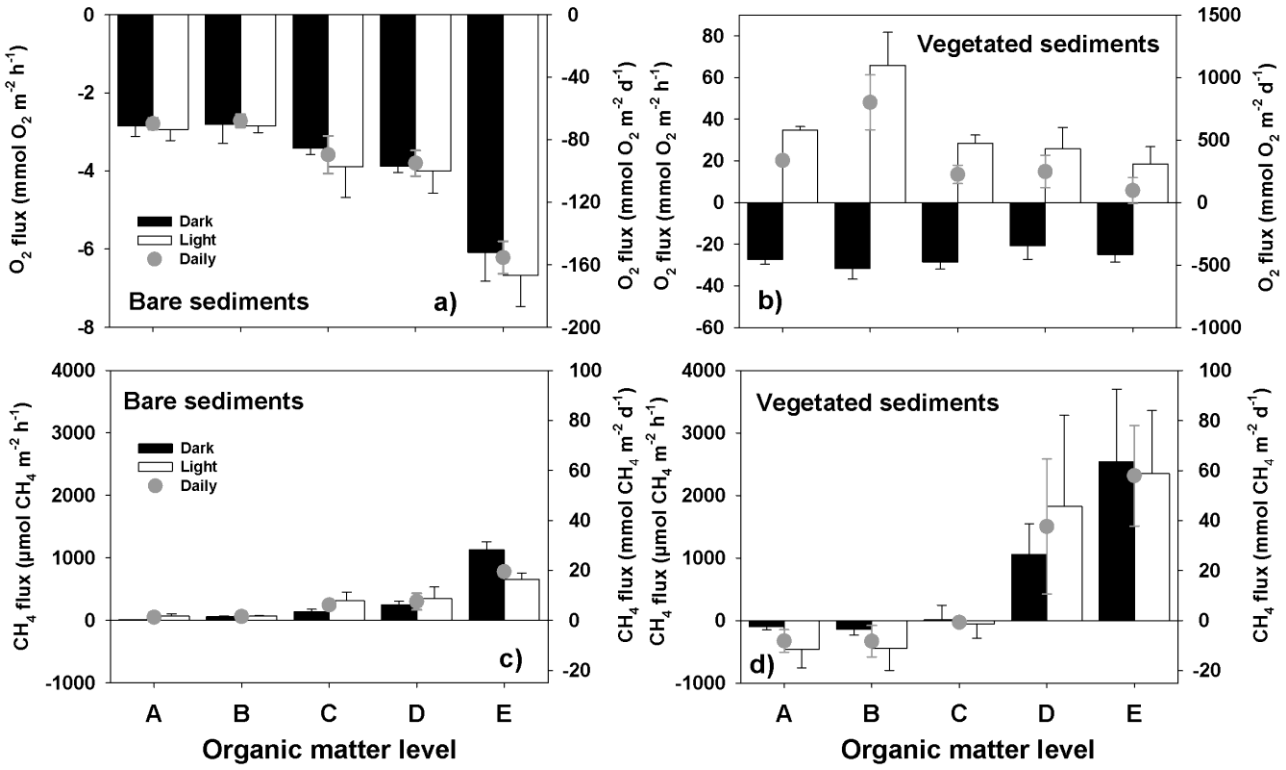

Gas and nutrient fluxes, Dn-R
Ø 8 cm
Light
Dark
Dw/Dn
Ø 20 cm
Light
Dark
Gas and nutrient fluxes, Dw/Dn
Ø 4 cm
Light
Dark

774

775 Fig. 1

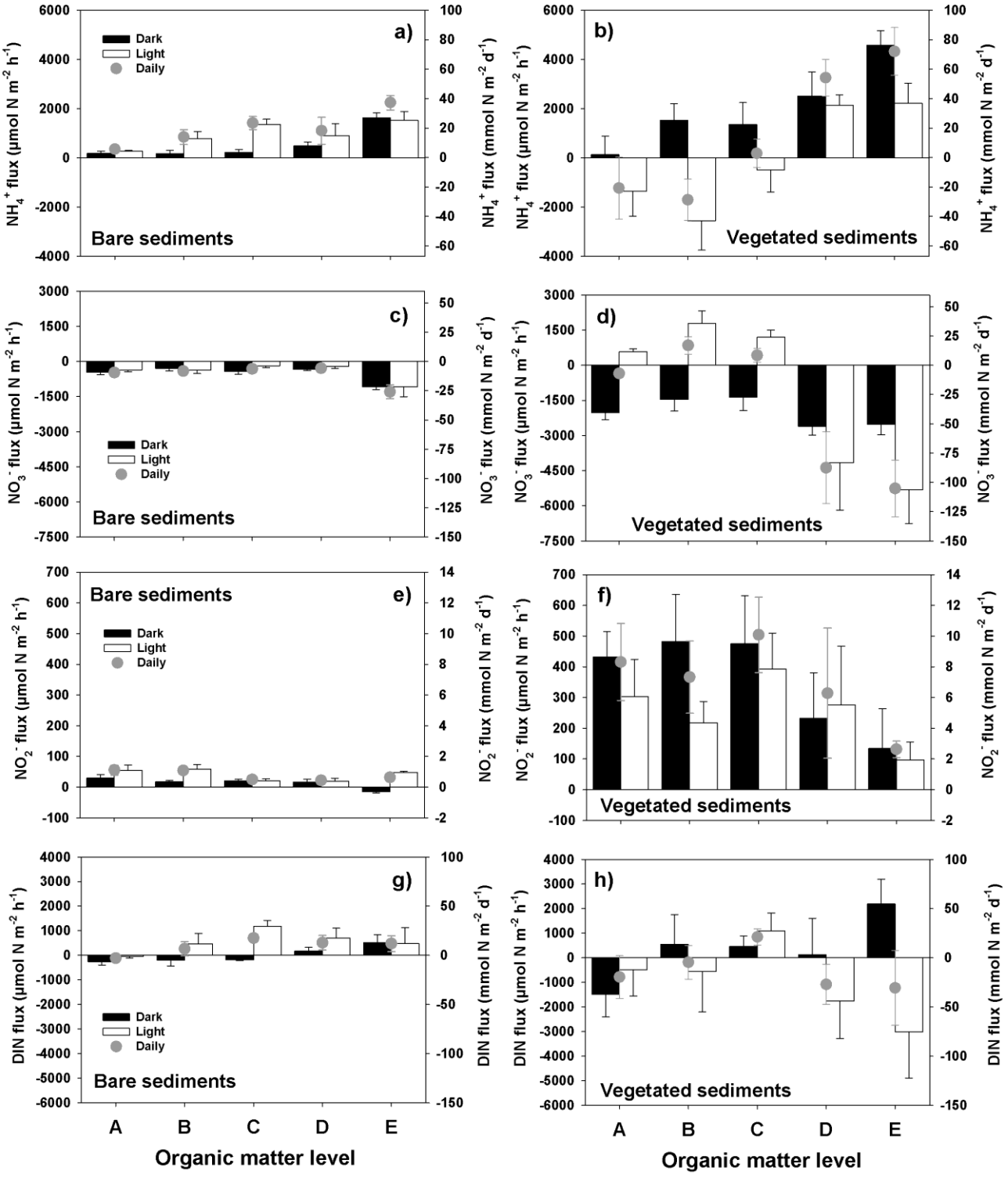


776
777 Fig. 2


Bare sediments
a)
Vegetated sediments
b)
Bare sediments
c)
Vegetated sediments
d)
Dark
Light
Daily
A
B
C
D
E
Organic matter level
O2 flux (mmol O2 m-2 h-1)
O2 flux (mmol O2 m-2 d-1)
CH4 flux (µmol CH4 m-2 h-1)
CH4 flux (mmol CH4 m-2 d-1)

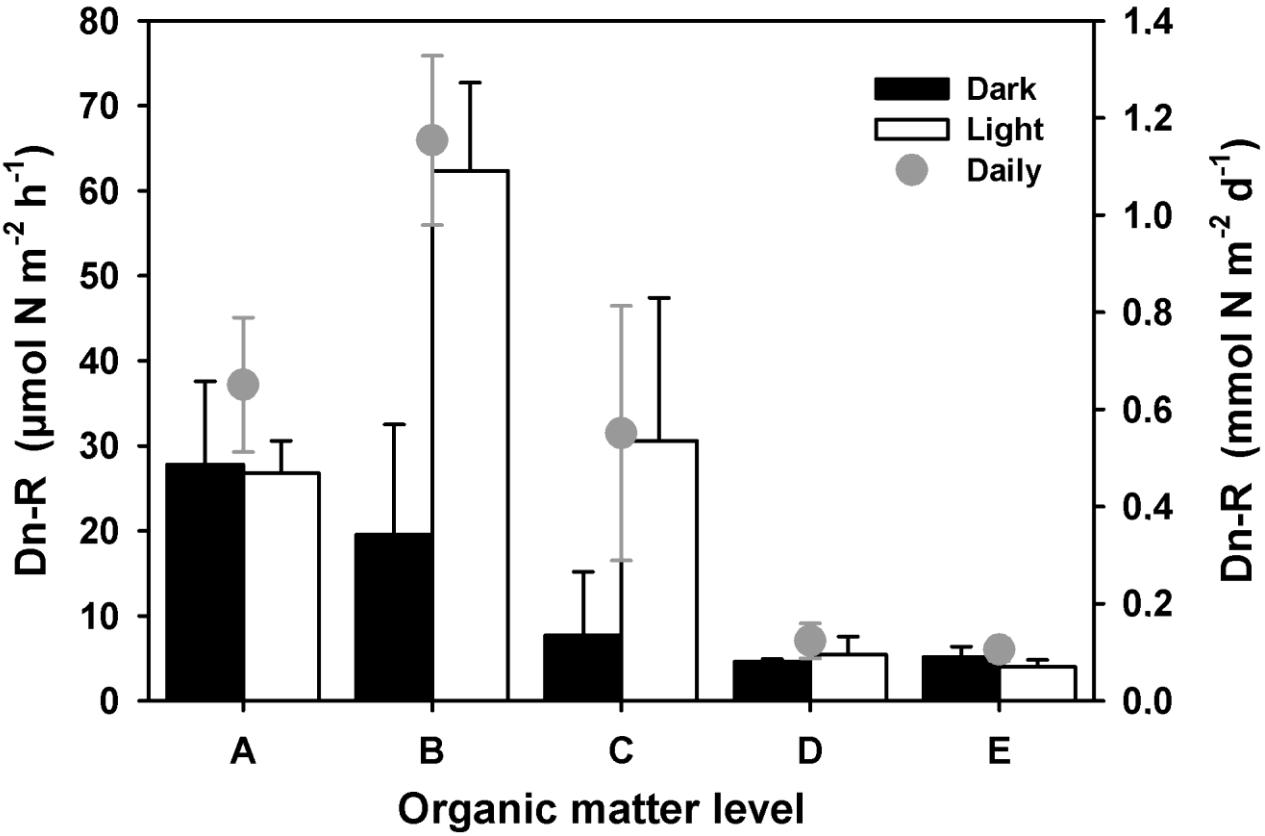
778

779 Fig. 3


a)
b)
c)
d)
e)
f)
g)
h)
Dark
Light
Daily
Bare sediments
Vegetated sediments
NH_4^+ flux (µmol N m^{-2} h^{-1})
NH_4^+ flux (mmol N m^{-2} d^{-1})
NO_3^- flux (µmol N m^{-2} h^{-1})
NO_3^- flux (mmol N m^{-2} d^{-1})
NO_2^- flux (µmol N m^{-2} h^{-1})
NO_2^- flux (mmol N m^{-2} d^{-1})
DIN flux (µmol N m^{-2} h^{-1})
DIN flux (mmol N m^{-2} d^{-1})
A
B
C
D
E
Organic matter level

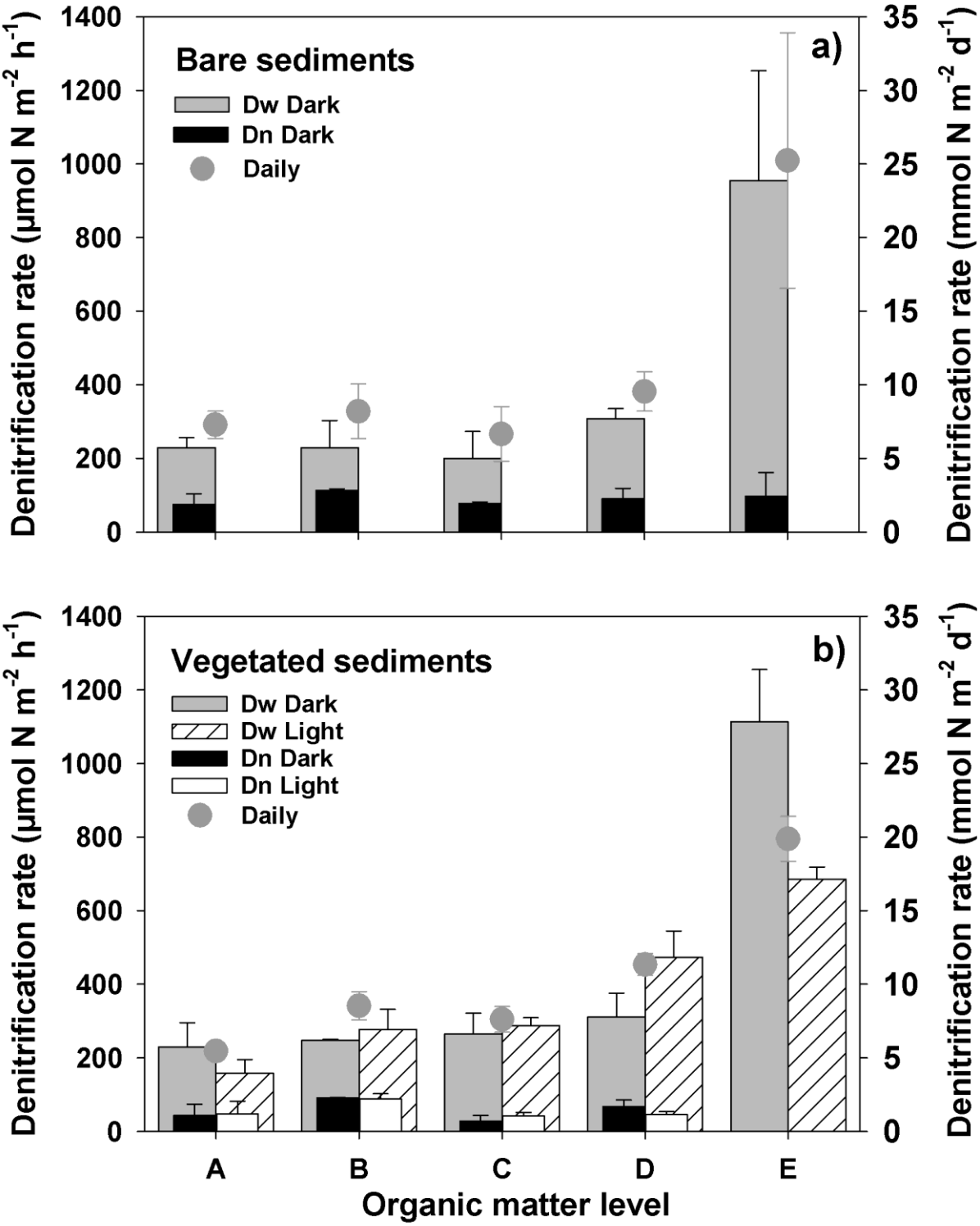
780

781 Fig. 4


Dn-R (μmol N m-2 h-1)
Dn-R (mmol N m-2 d-1)
Dark
Light
Daily
0
10
20
30
40
50
60
70
80
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
A
B
C
D
E
Organic matter level

782

783 Fig. 5


Bare sediments
a)
Dw Dark
Dn Dark
Daily
Vegetated sediments
b)
Dw Light
Dn Light
Denitrification rate (µmol N m-2 h-1)
Denitrification rate (mmol N m-2 d-1)
0
200
400
600
800
1000
1200
1400
0
5
10
15
20
25
30
35
A
B
C
D
E
Organic matter level

784

785 Fig. 6