UNIVERSITY OF PARMA
DEPARTMENT OF LIFE SCIENCES

PhD in Ecology
XXV Cycle

Radial oxygen loss from roots of *Vallisneria spiralis* L.: biogeochemical implications in eutrophic aquatic ecosystems

Supervisors:

Dr. Marco Bartoli

Dr. Mariachiara Naldi

Prof. Pierluigi Viaroli

Coordinator:

Prof. Paolo Menozzi

Candidate:

Elisa Soana

The present work was funded by the Italian Ministry of University and Research
(Doctoral scholarship, 25th PhD program in Ecology 2010 – 2012, University of Parma).

Abstract

Eutrophication and the accumulation of organic matter have been addressed as the major factors determining the decline of benthic vegetation in impacted water bodies and the consequent loss of key ecosystemic functions. In freshwater environments the literature reports submersed macrophyte die-back events and the switch to free-floating and floating-leaved plants dominated states. Species-specific differences in macrophyte response along organic gradients are evident. Some species have developed adaptations that allow not only their survival along pronounced gradients of sedimentary organic content but also their fast response to short-term variations of pore water chemistry, as those occurring seasonally in freshwater temperate ecosystems. *Vallisneria spiralis* L. (Hydrocharitaceae family), a perennial stoloniferous species, is tolerant to eutrophication and colonizes both lentic and lotic environments. It performs photosynthesis in low light conditions, grows in nutrient-rich waters and on a wide range of substrates, from gravel bottoms to organic-rich muddy sediments.

The aim of this thesis is to investigate the role of a tolerant rooted macrophyte in the regulation of biogeochemical dynamics and the interactions with microbial communities (with a particular focus on nitrogen cycle) in freshwater ecosystems undergoing eutrophication processes. Different methodological approaches are adopted (i.e. hydroponic incubations of plant tissues and intact plants, microcosm incubations, characterization of pore water and measurements of benthic fluxes) and the following aspects are evaluated: I) direct (uptake) and indirect (oxygen release) effects of *V. spiralis* presence on pore water features and redox-dependent processes; II) *V. spiralis* plasticity to colonize substrates with increasing organic content and changes of its influence on sediment chemistry and microbial activity along the gradient; III) relation between assimilative (mediated by vegetation) and dissimilative nitrogen processes (mediated by bacteria) when nitrogen is not limiting.

The key point is the evaluation of the effect of radial oxygen loss by *V. spiralis* on benthic biogeochemical dynamics. Oxygen released by roots has the potential to alter the chemical environment within sediments, with cascade effects on nutrient and gas exchanges at the water-sediment interface. Relevant consequences have been demonstrated for plants growing in oligotrophic systems, while the effects in organic-rich substrates are scantily explored.

The outcomes of the present work show that *V. spiralis* releases a great amount of the photosynthetically produced oxygen to the rhizosphere, affecting significantly the redox-dependent processes. Multiple evidences support the hypothesis that this plant varies seasonally the oxygen quota transported to the below-ground tissues to counteract the changing interstitial chemical conditions. Even if radial oxygen loss represents a small fraction in the plant oxygen economy, it can significantly affect the sediment biogeochemistry of eutrophic sites, representing a relevant amount of the daily benthic oxygen demand.

V. spiralis acts as an *engineer species* controlling actively interstitial features (NH_4^+, NO_x^-, PO_4^{3-}, Fe^{2+} and CH_4) over a wide range of trophic conditions and along its whole vegetative cycle. In sediments with a moderate organic enrichment, radial oxygen loss promotes denitrification coupled to nitrification, thus enhancing nitrogen loss and the ecosystem capacity to control nitrogen contamination. Furthermore, the high nitrogen availability in both pore water and water column weakens the competition between macrophytes and nitrifying and denitrifying bacteria, favoring nitrogen removal through a combination of plant uptake and dissimilative microbial processes. However, at extremely elevated organic enrichment, vegetated sediment lose their role as nitrogen traps due to nitrification inhibition and plant stress induced by very reduced conditions.

In summary, *V. spiralis* has the potential to withstand large perturbations of sedimentary features, being able to colonize organic matter impacted substrates. Even pore water conditions potentially hostile to roots do not affect its function as a benthic metabolism regulator. This macrophyte plays a crucial role in driving water-sediment exchanges of gases and nutrients, partially buffering the negative effects of organic enrichment connected to eutrophication. Moreover, it modifies sedimentary features, with positive feedbacks for water bodies restoration (i.e. regeneration of ferric iron buffer and phosphorus retention in sediment, stimulation of coupled nitrification-denitrification, reduction of internal organic load) which makes this plant an interesting option in programs for improving sediment conditions and favoring ecosystem recovery.

Contents

1. Introduction

1.1. Eutrophication and shift in primary producer community

In the past decades, eutrophication has become one of the major threats to submerged vegetation survival both in coastal and freshwater environments. According to Nixon (1995), the term "eutrophication" can be defined as an increase in the rate of supply of organic matter (OM) to an ecosystem. The OM augment can be due to both allochthonus or autochthonus sources, that is organic loading from adjacent drainage basins (i.e. fertilizers, domestic sewages and aquaculture wastes) or from increased primary production within the system itself. Shallow water bodies can be in two main alternative states, a clear one dominated by benthic vegetation (oligotrophic-pristine condition) and a turbid one where phytoplankton dominates (eutrophic degraded condition). It is well documented that increasing nutrient loads cause a shift in the composition of primary producer community. In brackish and coastal environments seagrass meadows are generally replaced by phytoplankton or floating macroalgae (McGlathery et al., 2007; Viaroli et al., 2008), whereas in freshwater ecosystems also free-floating or floating-leaved communities become a stable state (Scheffer et al., 2001; Scheffer et al., 2003). This represents a shift from benthic primary producers with high light requirements and low nutrient turnover rates to pelagic primary producers with lower light requirements and higher nutrient turnover rates (McGlathery et al., 2007).

Freshwater ecosystems in cultivated and densely populated watersheds have experienced profound changes during the last decades due to increasing rates of nutrient and organic matter supply (Dodds, 2006; Nixon, 2009). Widespread organic enrichment of sediments accompanying higher algal growth or greater terrestrial input has dramatically reduced the distribution of freshwater plants. Macrophyte die-back events have been reported in nutrient-enriched lakes (Sand-Jensen and Borum, 1991; Sand-Jensen et al., 2000; Smolders et al., 2002).

Eutrophication results in changes in ecosystem level dynamics such as productivity, nutrient cycling, trophic structure and energy flow. The shift in the dominant primary producers can produce dramatic perturbations in the structuring and functioning of the benthic system. Submersed plants are peculiar among aquatic vegetation as they connect water column and sediment with their physical structure and this linkage has important implications for nutrient cycling. The eutrophication degree is a key factor in controlling macrophyte-mediated processes.

Sediment physico-chemical properties can be controlled by macrophytes but they can play in turn a critical role in constraining plant colonization and growth (Bornette and Puijalon, 2011). The loss of rooted plants may modify the chemical conditions of surface sediments, which may result in changes in benthic biogeochemical reactions and processes, such as organic matter mineralization, nitrification and denitrification, phosphorus retention, which may discriminate for, or against, the recycling of nutrients. Altered biogeochemical processes can further determine positive feedbacks to eutrophication, which amplifies the perturbation and makes the shift irreversible.

1.2. Submerged macrophyte tolerance to organic-rich sediments

In the literature several examples of macrophyte die-back events are reported, indicating that organic enrichment in sediment and its subsequent effects could be serious stressors on growth and survival of submerged macrophyte species (van Wijck et al., 1992; Terrados et al., 1999). Organic enrichment is usually followed by several chemical and biological processes which accelerate oxygen consumption and build-up of potentially toxic compounds in pore water. This generates a positive feedback as inefficient or slower mineralization rates result in net accumulation and increasing OM levels in sediments. In organic-rich sediments the chemical environment of pore water is generally unfavourable to rooted macrophytes due to strictly anoxic conditions and to the presence of potentially phytotoxic compounds (Pezeshki, 2001; Colmer, 2003). In order to face oxygen shortage, aquatic plants have evolved a lacunar system (aerenchyma) formed by a network of intercellular spaces and aimed at gas transport through the plant body. This air-filled system connects oxygen-producing (leaves) and oxygen-consuming (roots) parts of the plant. Through diffusive transport it allows adequate oxygen supply to the below-ground tissues to support aerobic respiration (Sculthorpe, 1967). Oxygen in excess of the respiratory demand is released into the rhizosphere (ROL, Radial Oxygen Loss) and acts as a buffer against anoxic conditions, ensuring the oxidation of reduced solutes (Cronk and Fennessy, 2001; Colmer, 2003; Gibbs and Greenway, 2003; Lai et al., 2012). The fraction of the total photosynthetically produced oxygen that is transferred to the rhizosphere varies widely among aquatic plants, from nearly 100% in isoetid species to less than 5% in seagrasses (Sand-Jensen et al., 1982). Within certain thresholds, macrophyte meadows are able to buffer perturbations connected to eutrophication events, maintaining oxygen balance, low turbidity and low nutrient concentrations in water column. The root oxygen release and inherent oxidative processes within the rhizosphere, can be viewed as the key function determining the meadow persistence

(Terrados et al., 1999; Bornette and Puijalon, 2011). Nevertheless, above certain OM thresholds in the sediment, ROL may not be sufficient to maintain an oxidized environment and root tissue exposure to phytotoxic compounds, such as organic acids and reduced ions (Fe^{2+}, Mn^{2+}, NH_4^+ and S^{2-}) can occur (van Wijck et al., 1992; Wu et al., 2009).

Organic enrichment of sediments produces several coupled biological, physical and chemical modifications in the benthic system affecting deeply plant growth and survival. The physiological stress induced by widespread anoxia and high levels of reduced species is expected to lead to decrease photosynthetic efficiency and plant growth and can also cause plant mortality (Sand-Jensen et al., 2005; Raun et al., 2010; Møller and Sand-Jensen, 2011). Moreover, shorter roots are usually produced to ensure the oxygen supply to the root apical tips (Sand-Jensen et al., 2008). On the other hand, less developed roots have smaller surface areas for nutrient uptake and are more weakly anchored in the sediments. In addition to adverse chemical effects, organic enrichment makes the sediment softer and less consolidated increasing the risk of uprooting (Raun et al., 2010; Pulido et al., 2010).

Species-specific differences in macrophytes response along chemically reduced pore water (or sedimentary OM) gradients can be found. Isoetid species, for example, have shown high susceptibility to relatively low organic enrichment (OM up to 2-3%) and sediment anoxia, due to their particular morphological adaptations to oligotrophic sites (Sand-Jensen et al., 2005, 2008; Raun et al., 2010). Their high root porosity allows the efficient use of nutrients and CO_2 from the sediment and also the maintenance of a completely oxidized rizosphere, but makes them particularly susceptible to organic enrichment (Smolders et al., 2002). The elevated oxygen consumption of organically enriched substrates leads to the rapid oxygen extraction from the permeable isoetid roots, thereby endangering the proper supply to the apical tips. For example, experiments performed on *Littorella* and *Lobelia* have shown that small additions of labile organic matter to very oligotrophic sediments can stimulate plant growth due to nutrient and CO_2 release from mineralization processes (Raun et al., 2010; Møller and Sand-Jensen, 2011). However, higher organic enrichment reduces root development and photosynthetic performances because of widespread sediment anoxia and accumulation of phytotoxic species. Sand-Jensen et al. (2005, 2008) have demonstrated that *Lobelia dortmanna* reduces its root biomass and loses its oxygen releasing capacity even at low sediment organic contents (2-3%). Similar responses were also detected for other non-isoetid species, such as the genus *Potamogeton* and *Myriophyllum* (Barko and Smart 1986; van Wijck et al., 1992). A negative correlation was found between sediment

organic content and growth rates, due to inadequate oxygen supply to the root system. Reduced oxygen transport to the roots leads to a decreased radial flux of oxygen to the rhizosphere with great impacts on biogeochemical processes in the sediment.

Sensitive species are affected by very small variations of the interstitial chemical environment, while other rooted plants seem to be more tolerant to eutrophication and to the associated organic enrichment in sediments (Wang and Yu, 2007; Wu et al., 2009; Pulido et al., 2010). Whilst in many lakes eutrophication processes have resulted in the macrophyte decline, mainly due to increased turbidity and sedimentation, in riverine environments the mechanical disturbance of turbulent water flow to phytoplankton community establishment has promoted the coexistence of tolerant rooted plant meadows and high nutrient concentrations in the water column. Tolerant species have developed adaptations that allow not only their colonization and survival along a pronounced gradient of sediment organic content but also their fast response to short-term variations of pore water chemistry, as those occurring seasonally in freshwater temperate environments (Ribaudo et al., 2011; Lemoine et al., 2012). Plant species usually found in reduced eutrophic sediments with high oxygen demand have diffusive barriers to ROL in the roots by incorporation of lignin and suberin in the outer epidermis aimed at maintaining sufficient oxygen transport and supply to the apical tips (Visser et al. 2000; Colmer 2003).

1.3. Effects of rooted macrophytes on benthic biogeochemical dynamics

Macrophytes, through their production, consumption, and transport of oxygen, can generate strong oxygen gradients in space and time and be engineers of redox-related biogeochemical “hot spots” (McClain et al., 2003; Caraco et al., 2006). Rooted plant metabolism can deeply affect rhizosphere dynamics. Plant-induced changes in physico-chemical sediment parameters such as oxygen concentration, redox potential and pH alter the structure and the abundance of the microbial community along with benthic biogeochemical processes (Bodelier et al., 1996; Nikolausz et al., 2008; Herrmann et al., 2009; Lamers et al., 2012). Radial oxygen loss is demonstrated to control a number of microbial and chemical reactions occurring in interstitial water, with cascading effects on nutrient and gas fluxes at the water-sediment interface (Colmer, 2003; Philippot et al., 2009). Oxygen diffusing from roots into the sediment creates a mosaic of oxic and anoxic microenvironments. The activity of plants promotes the overlap of reductant and oxidant gradients creating a three-dimensional array of redox processes over a vertical extent of several centimetres instead of a vertical gradient of only a few millimetres that would be expected

at the sediment-water interface in the absence of plants (Brune et al., 2000; Hines, 2006; Hebert et al., 2007). The steep and dynamic oxygen gradients affect the vertical position and rates of redox-sensitive processes such nitrification, denitrification, dissimilative nitrate reduction to ammonium (DNRA), anammox and reduction of manganese, iron and sulphate. Therefore, small patches of oxidized sediment have geochemistry and microbiology quite distinct from the surrounding bulk sediment (Mann and Wetzel, 2000; Karjalainen et al., 2001; Fenchel and Finlay, 2008).

In addition to indirect influence via rhizosphere oxidation, plants may affect sedimentary nutrient pools by direct assimilation from roots. Especially in oligotrophic environments, rooted macrophytes take up most of their nutrients from the sediment since their availability is generally much higher in pore water than in the water column (Barko et al., 1991; Carr and Chambers, 1998).

Several investigations have demonstrated that benthic vegetation can distinctly control sediment biogeochemical dynamics in oligotrophic systems (Jaynes and Carpenter, 1986; Flessa, 1994), but they have hypothesized a negligible influence under eutrophic conditions due to elevated oxygen demand and high levels of reductants in pore water. In fertile substrates the oxygen amount released by roots is thought to be rapidly consumed by chemical and microbial reactions, resulting in a reduction of the thickness of oxic layers around roots and an attenuation of the plants effect on redox-dependent processes. Moreover, at high sedimentary organic matter contents, mineralization rates can saturate the plant uptake capacity, resulting in pore water nutrient accumulation. The outcome from recent laboratory experiments seems to contradict this statement (Racchetti et al., 2010; Ribaudo et al., 2011), but the effects of rooted plants on pore water chemistry of eutrophic sites have been scarcely explored till now.

1.4. Role of benthic vegetation in nitrogen cycling

Submerged macrophytes can strongly affect N cycling in sediments through their activity and their high N-demand for primary production, by several direct and indirect mechanisms. Particularly, in shallow water bodies, N dynamics are modulated by the interactions between benthic vegetation and microbial processes. Rooted macrophytes can control the availability of inorganic N in sediment, both through their assimilation activity and by affecting the balance among ammonification, nitrification and denitrification (Christensen and Sørensen, 1986; Reddy et al., 1989; Caffrey and Kemp, 1992). The indirect effects on N cycling are mediated by photosynthetic

oxygen production and its leakage in the sediment, together with root exudates. Ammonification, the microbial production of NH_4^+ by organic matter degradation, may be enhanced in vegetated sediments by the release of oxygen from the roots and by the availability of allochthonous particulate organic material trapped within the meadows (Caffrey and Kemp 1990; Sand-Jensen, 1998; Hasegawa et al., 2008). Aquatic plants may influence nitrification (chemoautotrophic oxidation of NH_4^+ to nitrite NO_2^- and subsequently to NO_3^-) and denitrification (reduction of NO_2^- or NO_3^- to N_2O or N_2) in different ways. Both nitrification and denitrification are controlled by a variety of environmental parameters, such as substrate availability, redox condition, presence of inhibitors (e.g. S^{2-}), temperature and pH (Fenchel et al., 1998). Oxygen leaking by roots, altering pore water redox status and the thickness of the oxic zone in sediment, impacts both those processes, since nitrifying bacteria are obligate aerobes, whereas denitrifying bacteria are facultative anaerobes. However, oxygen can also favor denitrification by stimulating nitrification and indirectly providing oxidized N source to denitrifiers (Knowles, 1982). In addition to releasing oxygen, plants can also influence directly denitrification by releasing easily degradable organic compounds substrates for heterotrophic denitrifying bacteria (Karjalainen et al., 2001; Sirivedhin and Gray, 2006). By creating heterogeneous oxygen conditions in the root zone (oxic-anoxic interfaces) (Hines, 2006), macrophytes may favor coupled nitrification-denitrification, resulting in a net loss of bioavailable N from the system.

On the other hand, aquatic plants assimilate and incorporate inorganic N into organic matter and they rely primarily on sediments, since benthic mineralization enriches pore water with nutrients (Barko et al., 1991; Carr and Chambers, 1998). Rooted macrophytes therefore compete with nitrifying and denitrifying bacteria for nitrogen, especially when it is limiting in porewater and bottom-water. Macrophytes can assimilate large quantities of inorganic N during the growing season leading to temporary N storage (McGlathery et al., 2007; Kreiling et al., 2011). Especially in oligotrophic conditions, plants have the potential to deplete sediment nutrient pools and competitively exclude some microbial processes in the rhizosphere. Since roots of macrophytes are usually better competitors than nitrifiers for NH_4^+, several studies have reported very low rates of coupled nitrification/denitrification in the rhizosphere (Risgaard-Petersen et al., 1998; Ottosen et al., 1999; Welsh et al., 2000). Thus, whether the presence of rooted plants stimulates or inhibits nitrification-denitrification activity may be system and/or species specific, and may reflect the relative balance between those opposing effects of roots, that is the potential stimulation of

nitrification due to oxygen leakage and the competition with the nitrifying and denitrifying bacteria for ammonium, nitrite and nitrate, respectively.

1.5. Use of tolerant macrophytes in restoration projects

Restoration projects have reduced nutrient levels in the water column resulting in improved light conditions in many previously freshwater eutrophic ecosystems. However, the recovery of submerged vegetation has been poor, possibly caused by the accumulation of OM on the top layer of the sediment produced under eutrophic conditions and the consequent changes in benthic biogeochemical dynamics (Brouwer et al., 2002; Jeppesen et al., 2005).

The re-introduction of tolerant submersed vegetation in OM-impacted aquatic bodies can improve the environmental state by the re-establishment of key ecosystemic functions. Macrophytes contribute significantly to maintain a high physical and biological diversity: they operate as *ecosystem engineers* (Sand-Jensen, 1997) acting as "hot spots" of intense nutrient cycling and providing habitat structure and refugia for aquatic organisms (Wigand et al., 2000; Cronk and Fennessy, 2001; Qiu et al., 2001). Tolerant plants con contribute significantly in accelerating the recovery process by reoxygenating the sediment, intercepting the sediment–water column nutrient flux, and temporarily retaining nutrients in plant biomass. Species with higher growth rates and higher oxygenation capacity can also create suitable sediment conditions for less tolerant plants. Among the features of aquatic plants eligible for restoration programs are their autochthony, their ability to grow in fluffy organic sediments and their capacity to photosynthesize under low light regimes typical of turbid ecosystems (Cronk and Fennessy, 2001). Understanding of how plants withstand and respond to sediment biogeochemistry changes accompanying the organic enrichment is needed to increase the probability of success in restoration actions involving plant transplantation. It is necessary to deepen the knowledge of macrophyte tolerance along OM gradients and detect the threshold of sediment organic load (and oxygen shortage, as a consequence) inducing plant decline. This would allow to chose different plants according to the OM contamination level of the system and their plasticity.

2. Problem statement

2.1. Topic context

Reduction of light availability and pore water environment hostile to roots have been considered as the main causes of benthic vegetation decline, both in coastal and freshwater ecosystems. However, it is not well understood how the sediment conditions (the redox status, in particular) may play a crucial role in constraining rooted macrophyte growth and survival, especially in freshwater environments. More studies are needed to investigate the complex frame of interactions between the plants and the sediment they colonize (i.e. how the sediment affects plant performance and how, in turn, the plant metabolism controls the sedimentary dynamics).

Even if eutrophication processes cause the progressive disappearance of sensitive phanerogams and the switch to phytoplankton or floating plant dominated states, some rooted species are tolerant to nutrient-rich waters and organic substrates. In this context, it is relevant to assess: a) how tolerant plants affect biogeochemical dynamics in environments subject to progressive modifications of water column and sediment chemistry, as those occurring in aquatic systems undergoing eutrophication and b) if within certain perturbation thresholds benthic vegetation can act as a buffer to organic enrichment and can maintain the ecosystemic functions connected to its presence, such as nutrient retention counteracting eutrophication itself.

2.2. Objectives and structure of the thesis

The general aim of my thesis is to evaluate the role of rooted macrophytes in the regulation of benthic biogeochemical dynamics and their interactions with microbial communities (with a particular focus on nitrogen cycle) in freshwater ecosystems undergoing eutrophication processes (i.e. excess nitrogen and organic matter availability). The experimental activities are performed with the submerged macrophyte *Vallisneria spiralis* L. (Hydrocharitaceae family) that is tolerant to eutrophication and shows a high adaptive capacity to grow on a wide range of different substrates. In particular the following aspects are evaluated:

1) *direct (uptake) and indirect (oxygen release) effect of V. spiralis presence on pore water features and redox-dependent processes in the benthic compartment.*
    - Hypothesis – In oligotrophic systems rooted macrophytes have the potential to alter pore water environment with cascade effects on nutrient and gas fluxes at the water-

sediment interface. Under eutrophic conditions, even if scarcely explored, a negligible influence has been hypothesized, due to elevated oxygen consumption and mineralization rates saturating the plant uptake capacity. However, the outcomes from preliminary laboratory experiments performed on *V. spiralis* seem to contradict this statement, suggesting that this plant has a high capacity to detoxify pore water.

- Methodological approach – The potential oxygen release by *V. spiralis* is evaluated by reinterpreting published data on seasonal oxygen and inorganic carbon fluxes measured in vegetated sediments and by laboratory incubations of apical tips and intact plants (Chapter 4). Pore water chemistry and microbial activity are investigated along the vegetative cycle to asses the plant influence (Chapter 5).

Soana E., Bartoli M. 2013. Seasonal variation of radial oxygen loss in *Vallisneria spiralis* L.: an adaptation to sediment redox? Aquatic Botany 104, 228-232.

Soana E., Bartoli M., Viaroli P. Seasonal regulation of nitrification in a rooted macrophyte (*Vallisneria spiralis* L.) meadow under eutrophic conditions. In preparation for Aquatic Sciences.

2) *V. spiralis plasticity to colonize substrates with increasing organic content and changes in its influence on sediment chemistry along the organic gradient.*

- Hypothesis – Rhizosphere is usually strongly inhibited by organic loading. *V. spiralis* has the potential to colonize even substrates theoretically hostile to roots. Therefore, significant differences of pore water features (i.e. less reduced conditions) in vegetated sediments compared to bare ones persisting over a wide range of sedimentary organic content can be hypothesized.
- Methodological approach – The effects of organic enrichment on pore water chemistry are investigated by laboratory incubations of vegetated and plant-free microcosms containing increasing organic matter amounts, simulating an eutrophication gradient (Chapter 6).

Soana E., Naldi M., Bartoli M. 2012. Effects of increasing organic matter loads on pore water features of vegetated (*Vallisneria spiralis* L.) and plant-free sediments. Ecological Engineering 47, 141-145.

3) *relation between assimilative (mediated by vegetation) and dissimilative nitrogen processes (mediated by bacteria) when N is not limiting.*

- Hypothesis – In oligotrophic condition rooted plants appear to suppress denitrification coupled to denitrification in the rhizosphere due to competition for nitrogen with N cycling bacteria. In eutrophic systems, where nutrients are not limiting, the shift in N uptake from roots to leaves can slow down the competition in the rhizosphere between plant and bacteria, promoting N removal from the system via denitrification coupled to denitrification.
- Methodological approach – Nutrient fluxes and denitrification coupled to nitrification rates in vegetated and bare sediments are measured along a gradient of organic matter (nitrogen availability) by laboratory incubations of vegetated and plant-free microcosms (Chapter 7).

Soana E., Bartoli M., Naldi M., Bonaglia S., Racchetti E., Castaldelli G., Viaroli P. Nitrogen benthic metabolism in a macrophyte meadow under increasing sedimentary organic matter loads. In preparation for Freshwater Biology.

3. Study object: *Vallisneria spiralis* L.

Vallisneria spiralis L. (Hydrocharitaceae family) is a dioecious, perennial, submerged angiosperm (Les et al., 2008). It is a freshwater stoloniferous species, capable of rapid clonal extension and possessing basal rosettes of flexible ribbonlike leaves (up to 1 m long) that can form expansive underwater meadows (Fig. 3.1). The species is widespread in the tropical and subtropical areas of both hemispheres and in Europe it is native to the Southern portion (Hussner and Lösch, 2005). *V. spiralis* develops large monospecific meadows in lotic environments and in marginal riverine areas and it is abundant in the high plain sections of the rivers of Northern Italy and in the littoral zones of the Alpine lakes. *Vallisneria* meadows are important components of freshwater ecosystems, as they provide refuge and foraging habitats, promote sedimentation and sediment stability and affect water column and sediment biogeochemistry (Hauxwell et al., 2007 and references therein; Pinardi et al., 2009).

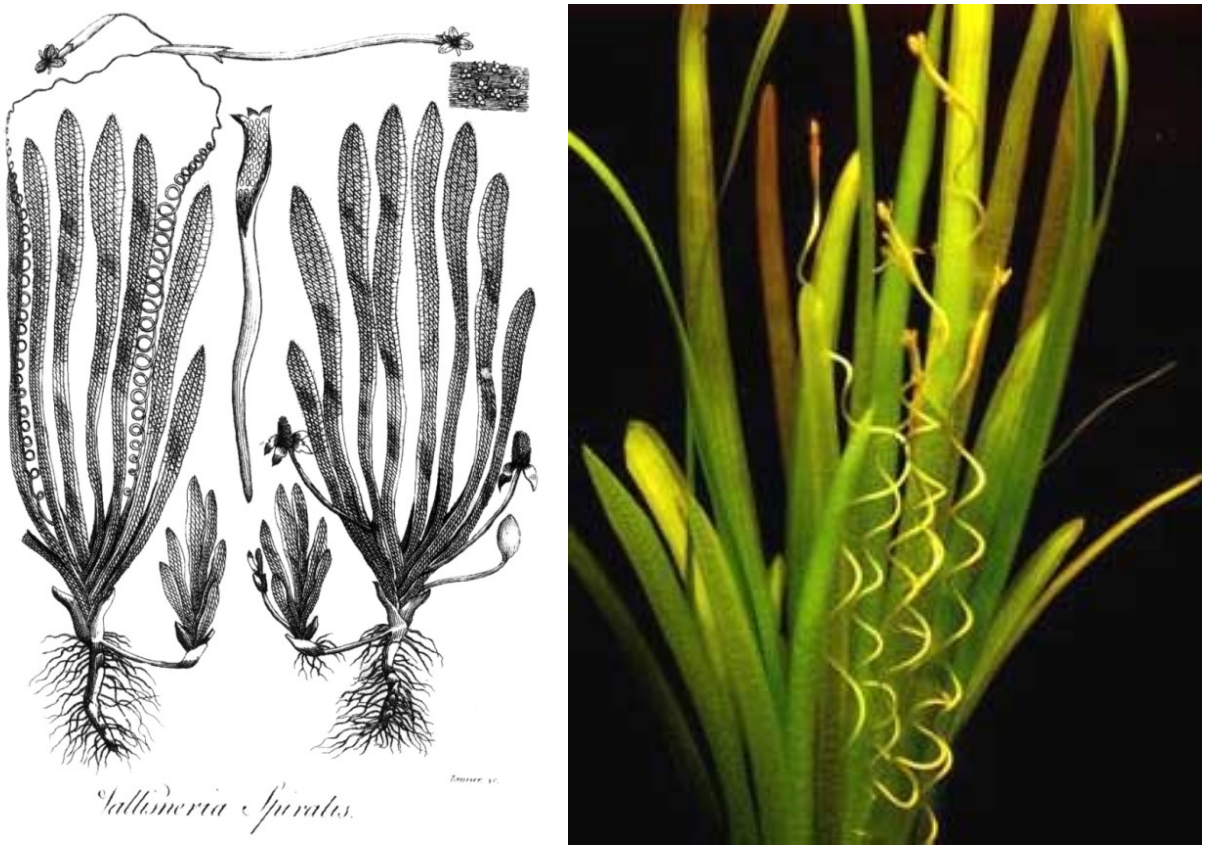

Fig. 3.1. *Vallisneria spiralis* L.

A number of studies suggest that this plant performs photosynthesis in low light conditions (Harley and Findlay, 1994; Blanch et al., 1998), grows in nutrient-rich waters (Xie et al., 2005) and tolerates organic sediments (Xiao et al., 2006; Wang and Yu, 2007). *V. spiralis* can colonize gravel

or sandy bottoms in lotic ecosystems (OM<1%) as well as organic fluffy sediments in eutrophic water bodies (OM>10%). Such plasticity (or tolerance towards different redox conditions) is likely due to its capacity to detoxify pore water. This is suggested by the markedly different color of sediment adjacent to roots compared to that surrounding, the presence of iron plaques on root hairs, and the absence of methane in vegetated sediments (Racchetti et al., 2010; Ribaudo et al., 2011). Its ability to tolerate a wide range of environmental conditions makes it particularly suitable for inclusion in restoration projects. Moreover *V. spiralis* is easy to grow, it reproduces mainly by runners and withstands transplant and handling in laboratory experiments and as a consequence from natural to impacted sites in restoration actions.

V. spiralis specimens used in the experimental procedures of the present work were collected from a shallow eutrophic site of the Mincio River (Massimbona location, Northern Italy, ~1.5 m depth). This river, a tributary of the Po River, originates from the Lake Garda, the largest Italian Alpine Lake. The study site is nutrient enriched from the surrounding agricultural lands and the discharge of the urban wastewater treatment plant of Peschiera del Garda processing over 300,000 equivalent inhabitants. Sediment is muddy and characterized by a soft surface horizon composed of recently deposited fine particles, laying upon a pristine gravel bottom. Riverine marginal areas are suitable for the colonization of benthic vegetation and *V. spiralis* is here the dominant specie, developing high-density mono-specific meadows. In the Mincio River the meadow formation and persistence is supported by plant propagules delivered from the Lake Garda. This plant is expanding mainly due to discharge reduction and development of soft muddy substrates.

4. Direct and indirect evidences of radial oxygen loss

4.1. Aim

Changes of sediment redox geochemistry are connected to temperature variations and conseguently to oxygen solubility, and to variations in inputs of labile organic matter and electron acceptors demand (Schüring et al., 1999). In temperate areas, water overlying sediments of shallow aquatic bodies displays wide seasonal variations of temperature, from nearly 0 to over 25°C and oxygen solubility nearly halves in that temperature range (Racchetti et al., 2011; Pinardi et al., 2011). Benthic respiration is strongly influenced by temperature (Fang and Moncrieff, 2001). Typically, during summer months, increased primary production adds organic matter to a system where the availability of oxygen and nitrate, the main electron acceptors in freshwater sediments, generally drops (Pinardi et al., 2009; Racchetti et al., 2011). High microbial activity and low oxygen availability limit the penetration of this gas to the very upper sediment layer. In such context, a very pronounced seasonal variation of the thickness of oxidized and reduced horizons within sediments is expected, with the latter expanding during summer as a consequence of the above-mentioned co-occurring factors (Hines, 2006; Koretsky et al., 2006).

From the perspective of roots, I hypothesize that phenological plasticity of rooted tolerant plants allows their rapid adaptation to seasonally changing sediment redox. In particular, increasing demand of electron acceptors and accumulation of reducing power within sediments should be counterbalanced by a greater release of oxygen by roots in order to detoxify pore water or as a consequence of increased diffusion gradients across root wall. The literature contains several studies dealing with direct and indirect measurements of ROL in submerged macrophytes but most of them are referred to a single vegetative period and do not provide information on seasonal changes of this parameter (Sand-Jensen et al., 1982; Kemp et al., 1986; Caffrey and Kemp, 1991; Laskov et al., 2006; Lai et al., 2012).

I expect that oxygen evolution to and inorganic carbon uptake from the water column in light by rooted macrophyte meadows are progressively decoupled in the shift winter-summer, as a substantial oxygen amount is driven from the leaves towards the rhizosphere. I thus suggest that the ratio between oxygen and inorganic carbon (PQ, Photosynthetic Quotient) undergoes pronounced seasonal variations because tightly coupled to ROL. As a consequence of variable oxygen leakage, the presence of iron plaques on roots should also be different along the plant

growing season. I tested my hypothesis with the perennial freshwater macrophyte *V. spiralis*. I extrapolated photosynthetic quotients from published data of oxygen and inorganic carbon fluxes measured seasonally in vegetated sediments and I discussed them in the light of the outcomes of laboratory incubations of apical tips and intact plants.

4.2. Material and Methods

4.2.1. Sampling

V. spiralis plants were randomly sampled from the riverine eutrophic site (Mincio River, Massimbona location, Northern Italy) in summer (August 2010). Over 100 shoots were carefully collected by hand to preserve intact plant tissues and brought to the laboratory fully submerged by river water within two hours from the sampling. About 100 l of river water were also collected for pre-incubation and incubation procedures. In the laboratory, healthy plants were carefully washed to remove sediment from the roots and epiphytes from the leaves and kept in pre-incubation tanks free-floating in aereated water maintained at field temperature (~20°C) (Fig. 4.1). Pre-incubation tanks were subjected to a 16/8 h light/dark cycle at an irradiance of about 400 µmol photons $m^{-2}s^{-1}$ (Photosynthetically Active Radiation, PAR) by means of 1000-W halogen lamps. Water temperature was measured with a 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.). Experiments were carried out within a few days from plant collection.

4.2.2. Measure of photosynthetic quotient (PQ) in photosynthetic tissues

Apical tips of *V. spiralis* leaves, each 15 cm long, were cut underwater and maintained in the dark for about 12 hours before the experiment. Tissues were incubated in 250 ml glass bottles (Fig. 4.1); five replicate apical tips from different plants were randomly distributed in each bottle (average biomass ~0.1g_{DW}). The incubation medium was filtered (Whatman GF/F) river water ([NH_4^+] 6 µM; [NO_3^-] 90 µM; [PO_4^{3-}] 4 µM; dissolved inorganic carbon [DIC] 2 mM). To avoid oxygen super-saturation, the water was flushed with high purity N_2 to lower initial oxygen saturation to <50%. Incubation bottles were filled from the bottom with a siphon and allowed to overflow to eliminate air bubbles. Five bottles with plant tissues and 3 control bottles containing only filtered water were incubated fully submersed within a tank filled with site water at controlled temperature (20°C). Incubations were performed at four different irradiance values (20, 70, 140

and 320 µmol photons m^{-2} s^{-1}) and lasted from 1 to 2.5 hours according to light level after an acclimation period of about 20 minutes. Every few minutes submerged bottles were agitated by hand. Water samples for oxygen and DIC determinations were collected at the beginning and at the end of the incubations from each bottle by means of gas-tight syringes and transferred to glass vials (12 ml Exetainer®, Labco, High Wycombe, UK). Oxygen and DIC were measured with Winkler titration and acidimetric titration, respectively (APHA, 1981). At the end of incubations, apical tips of each incubation bottle were dried separately at 70°C until constant weight. Oxygen production and DIC uptake were calculated from the changes in concentration of each bottle during the incubation, corrected for the values in control bottles and normalized for the tissue biomass. Photosynthetic Quotient (PQ) was calculated as the ratio of oxygen to inorganic carbon exchange measured concurrently for the same tissue sample.

4.2.3. Measure of oxygen budget in intact plants

A complete oxygen budget was performed on *V. spiralis* summer plants in hydroponic condition (Sand-Jensen et al., 1982; Kemp and Murray, 1986). Single, intact plants were incubated in transparent Plexiglass cylindrical chambers (inner diameter 8 cm, height 30 cm, wall thickness 5 mm, n=5), each hosting a root compartment (height 10 cm) separated through an opaque, 5 mm thick plastic septum from a leaf compartment (height 20 cm) (Fig. 4.1). The septum was provided with a central hole (diameter 1 cm) through which roots were carefully inserted; thereafter the hole was sealed with dental paste (Lab Silicone Henry Schein-Krugg) in order to hold the plant and to separate the two compartments (Smith et al., 1984; Bal et al., 2009). Pilot experiments revealed no appreciable water exchange between the two chamber sections. The root compartment was double wrapped in aluminium foil to prevent light penetration and filled with filtered (Whatman GF/F) river water previously purged with N_2 to lower oxygen saturation to about 5%. The water within the root compartment was left unstirred during the incubation time, if not just before water sampling for oxygen determination. The leaf compartment was filled with filtered and oxygen undersaturated river water (~70%), continuously stirred during the incubation by a magnetic bar driven by an external motor (40 rpm). Control chambers (n=3) were prepared in an identical fashion, but with no plant material, in order to account for any changes in gas concentrations due to leaking or diffusion through chamber walls. All chambers were closed with Plexiglass transparent lids and placed into an incubation tank. The light intensity was saturating (~400 µmol photons m^{-2} s^{-1}) and the temperature was maintained at 20 °C. Incubations lasted

about 4 hours and variations of oxygen concentrations in leaf and root compartments were used to estimate net production and ROL, respectively. Plants were then recovered from the chambers and maintained in aerated river water for about 12 hours in the dark. The day after, they were incubated with the same experimental set-up to measure ROL in dark conditions. Leaves and roots were then incubated separately in 250 ml glass bottles in the dark. Respiration rates were estimated by oxygen concentration changes in time. Plant material was finally oven-dried at 70°C until constant weight.

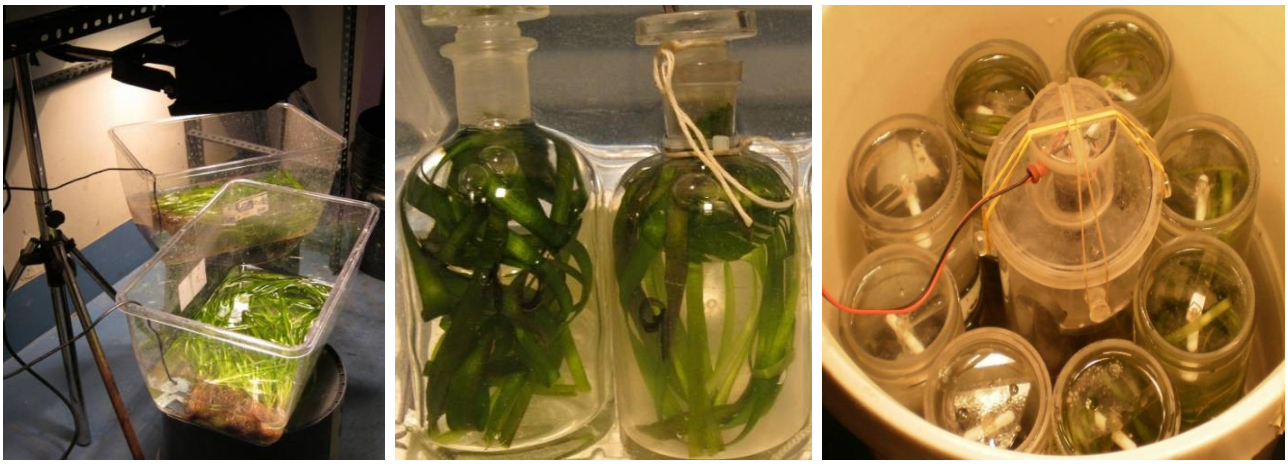

Fig. 4.1. Experimental set-up used for the measurements of PQ (Photosynthetic Quotient) in photosynthetic tissues and oxygen budget in intact plants of *V. spiralis*.

4.2.4. Calculation of seasonal PQ from published data

Pinardi et al. (2009) seasonally measured light and dark oxygen and inorganic carbon fluxes in intact sediment cores vegetated by *V. spiralis* and collected in the same riverine location. For each sampling date the published rates measured during light incubations in vegetated sediment were corrected for the effects of any biological or biogeochemical processes recorded in bare sediment during dark incubations. In doing so, I assumed comparable microbial activities in adjacent patches of bare and vegetated sediments. Calculated rates, due to the plant activity alone, were then normalized for the reported macrophyte biomass: they represent an estimate of seasonal PQs of *V. spiralis* and are readily comparable with the values obtained by laboratory incubation of apical tips.

4.2.5. Analyses of root iron content

Red-brown colour plaques were clearly visible on the root surfaces of plants recovered from intact cores incubated seasonally by Pinardi et al. (2009). Roots were analyzed for total iron via acid digestion. Diluted hydrochloric acid (1 M) was added to the dried tissues after combustion (550°C,

3 hours) and the samples were boiled at 100°C for about 30 minutes (Povidisa et al., 2009). Iron concentrations were analyzed in the supernatant by flame atomic absorption spectroscopy (Varian, model AA240). For each sampling date, six subsamples of roots were analysed.

4.2.6. Statistical analyses

Differences in PQ values among light levels, in PQ values calculated from published data and in root metal contents among sampling dates were tested via one-way ANOVA and pairwise multiple comparison procedure (Holm-Sidak method). Normality (Shapiro-Wilk test) and homoscedasticity (Levene's test) of data were examined. Statistical significance was set at $p \leq 0.05$. Analyses were performed using the R Program (R – Development Core Team, 2011).

4.3. Results

Results from the incubations of intact apical tips showed that, on a molar basis, inorganic carbon assimilation was significantly higher than oxygen production. The relation between oxygen and inorganic carbon fluxes was linear over the whole range of light conditions and their ratio was significantly lower than one (PQ=0.69, slope of the data shown in Fig. 4.2). PQs were independent from irradiance (ANOVA, $p>0.05$).

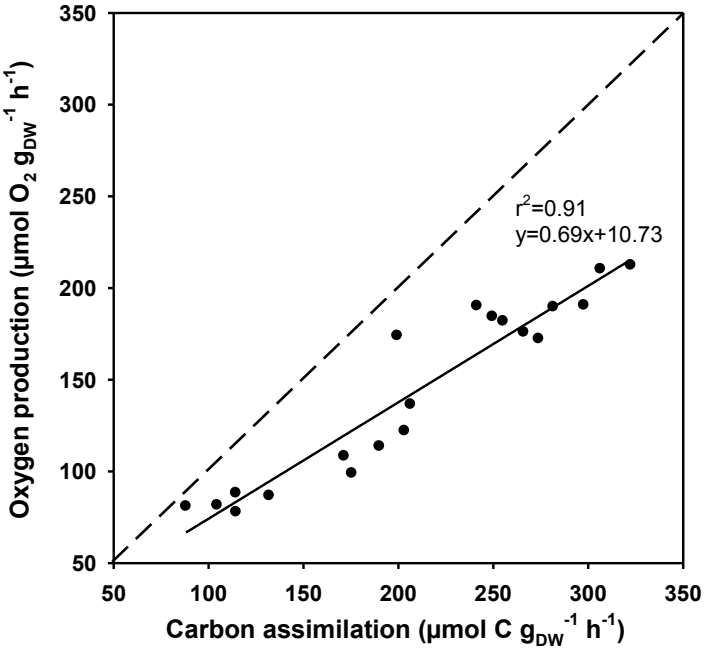

Fig. 4.2. Carbon assimilation versus oxygen production measured in *V. spiralis* apical tips incubated under different light intensities. The slope is statistically significant ($p<0.0001$), whereas the intercept is not ($p=0.33$).

The oxygen budget measured via hydroponic incubations of *V. spiralis* plants in double-compartment chambers is reported in Fig. 4.3. Gross production was estimated in 47.90±3.69 µmol O_2 $plant^{-1}$ h^{-1}, of which 70% and 20% represented by net production and leaves respiration, respectively. The remaining oxygen amount was transferred to the root system, to sustain both belowground tissue respiration (3%) and ROL (7%). In the light the amount of oxygen transferred to belowground tissues was largely in excess to root requirements and the amount of oxygen leaked from roots more than doubled the amount respired by the roots themselves. In the dark the situation was different; ROL was detectable but most of the oxygen transferred to the belowground tissues (~79%) was used for root respiration needs. Average weight specific ROL rates of 38.8±15.5 and 5.13±1.56 µmol O_2 g_{DW} $root^{-1}$ h^{-1}, were obtained in light and dark conditions, respectively.

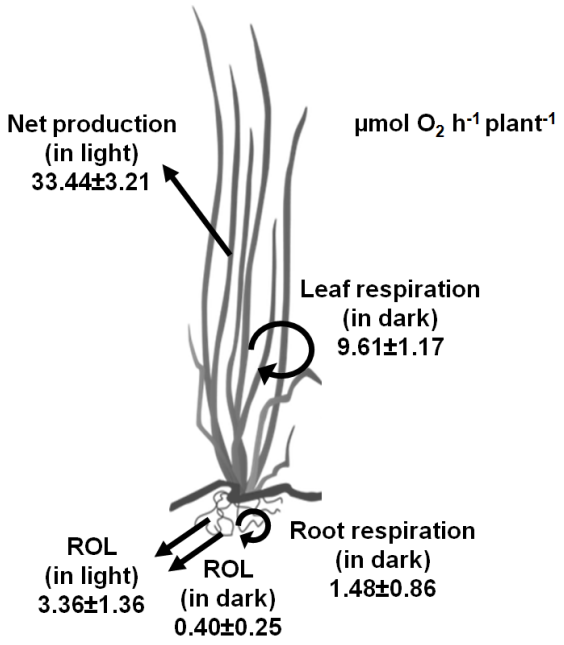

Fig. 4.3. Net production and leaf respiration, root respiration and radial oxygen loss measured in light and dark incubation of single *V. spiralis* plants in double-compartment chambers (reported rates are average±standard deviation, n=5).

PQs calculated from published seasonal data of oxygen and inorganic carbon fluxes in vegetated sediment were significantly different from the unit and constantly below the value measured in the laboratory on intact apical tips (Fig. 4.4, upper panel). They ranged between 0.69 and 0.34 and were significantly different among sampling dates (ANOVA, $p<0.001$). Post-hoc test revealed that PQs calculated in August and October distinguished from each other and also from all the other

sampling dates. PQ was close to the laboratory value in April and then it progressively decreased with a late summer/early autumn minimum. Eventually it increased again in cold season.

Total iron concentrations in roots ranged from 300 up to 640 µmol Fe g_{DW} $root^{-1}$ (Fig. 4.4, lower panel) and were significantly different among sampling dates (ANOVA, $p<0.001$).

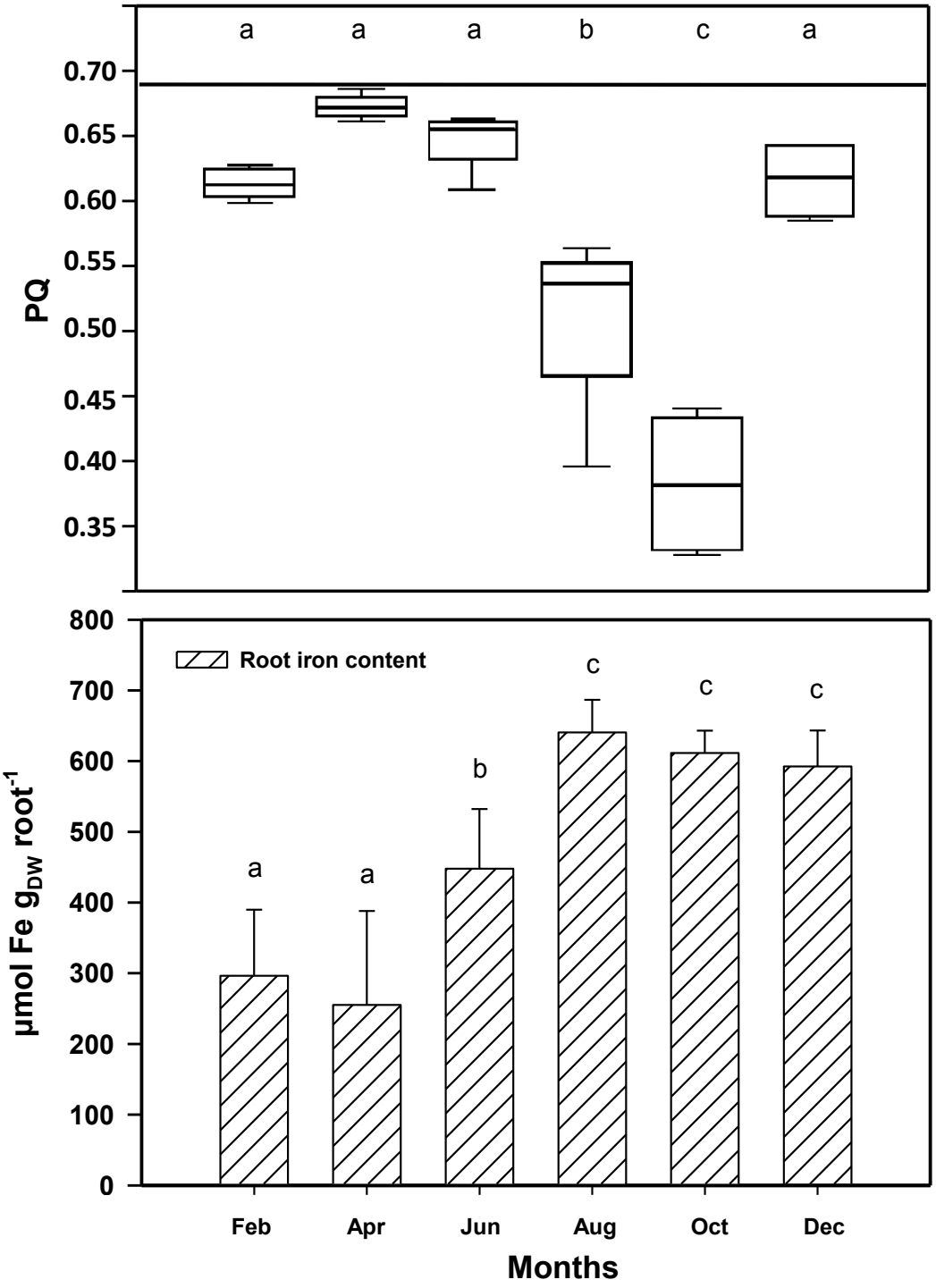

Fig. 4.4. The upper panel reports the seasonal evolution of the ratio between oxygen production and inorganic carbon fixation (PQ) by *V. spiralis* (original data from Pinardi et al., 2009). The lower panel shows the root iron contents (average±standard deviation, n=6).

Considering the experimentally calculated value of 0.69 as the reference PQ of *V. spiralis* photosynthetic tissues, I calculated from data of inorganic carbon uptake reported by Pinardi et al. (2009), the amount of oxygen theoretically produced by the plant in the different sampling seasons. I then subtracted from the calculated rates those measured during core incubations, that were systematically lower. I attributed the difference to the oxygen amount seasonally transferred to belowground plant portions. The main assumptions behind these calculations were the seasonal constancy of the reference PQ value and that DIC uptake is mostly confined to the aboveground tissues. The calculated oxygen amounts, normalized by dry root biomass, ranged between a minimum of 6 (April) and a maximum of 164 µmol O_2 g_{DW} $root^{-1}$ h^{-1} (October) (Fig. 4.5). The pattern of the oxygen amount transferred belowground was specular to that of the *V. spiralis* root:shoot ratio (RSR), calculated from seasonal biomass data reported in Pinardi et al. (2009). Lowest RSR was recorded in October, coinciding with the highest oxygen transport to the rhyzosphere. Oxygen transferred to belowground tissues in August (~144 µmol O_2 g_{DW} $root^{-1}$ h^{-1}) resulted about fourfold higher than the ROL rate experimentally measured with the split-compartment chamber in the light.

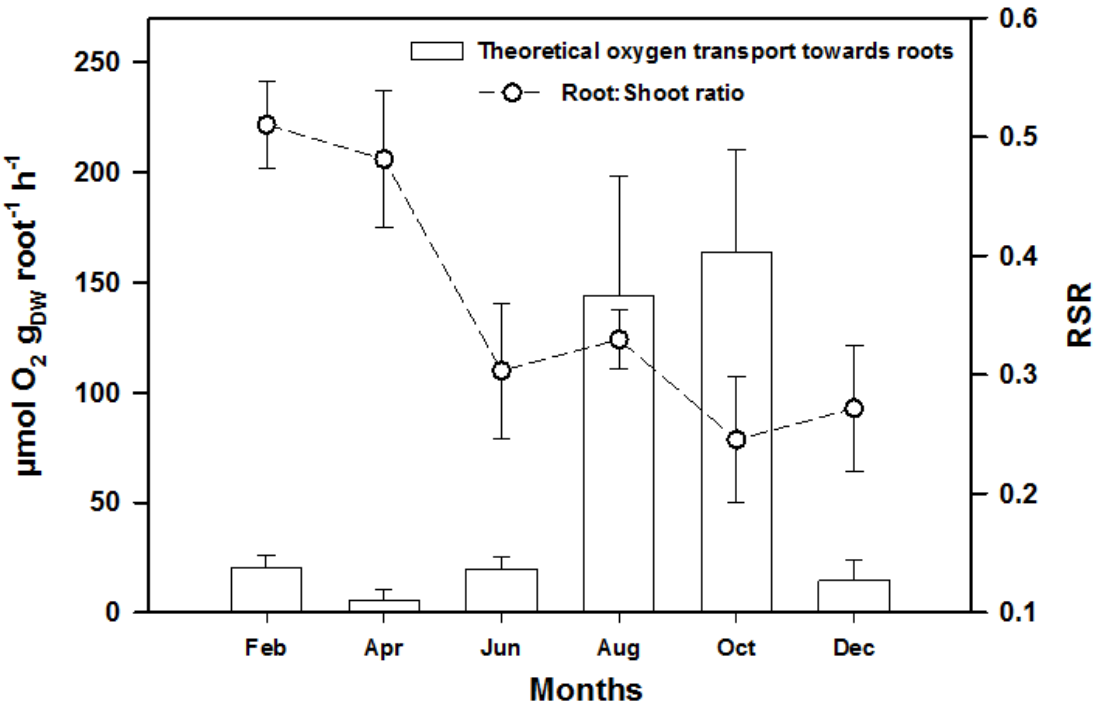

Fig. 4.5. Seasonal rates of oxygen transport towards the rhizosphere by *V. spiralis* and plant's root:shoot ratio. Belowground oxygen transport was calculated from data reported in Fig. 4.4 (see the text for more details). The roots:shoot ratio was calculated from biomass data reported by Pinardi et al. (2009).

4.4. Discussion

4.4.1. Linking *V. spiralis* physiology to seasonal changes of pore water redox

The PQ of a photosynthetic tissue mainly reflects the actual oxygen/carbon stoichiometry in the mixture of photosynthesis's end products, i.e. for oxidized compounds like organic acids PQ<1. The PQ of *V. spiralis* leaves was about 0.69 and values lower than one have also been found in other members of the Hydrocharitaceae family (Pokorny et al., 1989). The type of assimilation products is usually species-specific, but scarce information is available about the macromolecular composition of *V. spiralis* tissues. According to the adopted experimental set-up, the calculated ratio between oxygen and carbon was considered as an upper threshold, reference value. Supposing a negligible gas storage, the whole amount of oxygen produced by photosynthetic process should be in fact released to the water. When intact plants are incubated, variable oxygen amounts can be driven from the above to the below portions of the plant, where they are used for root respiration or can escape to the surrounding environment. This is not the case when only leaf segments are incubated and I considered this statement as a central point of my hypothesis. I believe that the oxygen amount delivered belowground changes seasonally, due to a combination of variable rates of root respiration and radial oxygen loss. Proportionally higher belowground oxygen transport should unbalance the ratio between oxygen evolved to and inorganic carbon removed from the water column, resulting as a consequence in PQ values significantly lower than the reference one. The discrepancy between seasonal PQs and the assumed threshold value could be considered as a proxy of belowground oxygen transport, which includes ROL.

Another element supporting variable ROL in *V. spiralis* is the seasonal pattern of root iron content. Analyses of oxidized metals on root surfaces can provide an indirect evidence for ROL in field situations, since plaque formation is mainly a consequence of oxygen leakage (Colmer, 2003; Li et al., 2011). In *V. spiralis* root iron content increased markedly in the shift spring-summer, with an opposite pattern compared to that of oxygen to inorganic carbon ratios (Fig. 4.4). This is probably due to a combination of higher electron acceptor demand for sediment respiration, leading to increased rates of iron reduction, and of oxygen leakage by roots, ultimately resulting in iron oxidation and precipitation on root surfaces.

Seasonal variation of ROL in submerged macrophytes has been scarcely studied. Caffrey and Kemp (1991) reported for *Potamogeton perfoliatus* a decline in root-rhizome oxygen released from spring to summer, suggesting a decrease in the root permeability and a coupled reduced

transport. Plant species tolerant to eutrophic sediments usually prevent excessive oxygen loss by forming roots with a strong barrier to ROL by incorporation of lignin and suberin in the outer epidermis (Pezeshki, 2001; Colmer, 2003). On the other hand, *V. spiralis* studied in the field situation seemed to have an opposite adaptive mechanism, enhancing oxygen losses.

My calculations from the seasonal measurements reported by Pinardi et al. (2009) either suggest the presence of active, plant-mediated or passive, gradient-mediated mechanisms that modulate seasonally the amount of oxygen leaked by *V. spiralis* roots to the surrounding environment. This result supports the interpretation that ROL is not a constant feature of a given species, but rather a temporary outcome of the interaction between the plant itself and the surrounding environmental conditions (van Bodegom et al., 2005). I speculate that *V. spiralis* ROL is probably related to the oxidation-reduction status of the benthic compartment. In early autumn pore water accumulates reducing power as a consequence of summer anaerobic respiration processes and the meadows begin their senescent phase, which adds further organic matter to the sedimentary pool and keeps elevated microbial activity. At this time *V. spiralis* has the greatest necessity to avoid root damages from reduced compounds and it responds by increasing oxygen amount delivered to the roots and reducing belowground biomass in order to minimize the contact with the harmful pore water environment.

This adaptation can be simply passive as a consequence of increased diffusion gradients across the root wall or also active, that is connected to root morphological modifications. Sediment oxygen demand has been demonstrated to play a crucial role in regulating the oxygen leakage by submerged macrophytes (Sorrell and Armstrong, 1994; Laskov et al., 2006). However, changes in ROL might be also related to alterations of root anatomical structure, for example a change in root porosity or in root wall permeability. Lemoine et al. (2012) have measured an increase in root porosity and potential oxygen release in *V. spiralis* specimens grown in anoxic sediments compared to well-oxygenated ones. An augment in anaerobiosis intensity can promote aerenchyma formation to facilitate gas transport mechanisms and result also in lower root respiration rates on a volume basis (Colmer, 2003). Some studies have shown a significant correlation between ROL and root porosity in submerged freshwater macrophytes (Laskov et al., 2006; Li et al., 2011; Lemoine et al., 2012).

4.4.2. Radial oxygen loss in *V. spiralis* meadows: ecosystem implications in eutrophic sites

The root oxygen loss measured in submerged aquatic plants is highly variable among species. *V. spiralis* ROL rate in light was within the range reported in the literature for submerged non-isoetid freshwater plants (in particular, *Potamogeton* spp. and *Myriophyllum* spp.) and measured via oxygen-depleted solutions (Sand-Jensen et al., 1982; Caffrey and Kemp, 1991; Kemp and Murray, 1986; Kemp et al., 1986). ROL persisted even in darkness, as oxygen diffused from the water column into plant aboveground tissues, with rates among the highest found in submerged plants (Sand-Jensen et al., 1982; Caffrey and Kemp, 1991) (Table 4.1). The marked decrease of ROL in the light-dark shift supports the evidence that ROL is mainly regulated by the activity of the aboveground tissues.

Table 4.1. Radial oxygen loss rates of submerged freshwater macrophytes. Values are reported as average±standard deviation or as ranges (in brackets).

Species	Method	Dark/Light condition	Radial Oxygen Loss ($\mu mol\ O_2\ g_{DW}\ root^{-1} h^{-1}$)	Reference
Vallisneria natans	Ti^{3+}-citrate	L	44.38 ± 0.75	Li et al., 2011
Sparganium simplex	Oxygen-depleted solution	L	11.25 (5.94 – 20.31)	Sand-Jensen et al., 1982
		D	4.69	
Potamogeton frisii	Oxygen-depleted solution	L	35.94 (31.25 – 40.62)	Sand-Jensen et al., 1982
		D	5.00	
Potamogeton crispus	Oxygen-depleted solution	L	33.75 (13.44 – 59.69)	Sand-Jensen et al., 1982
		D	0	
Potamogeton crispus	Oxygen-depleted solution	L	101.88 (90.94 – 112.50)	Sand-Jensen et al., 1982
		D	10.94	
Potamogeton crispus (from organic sediment)	Ti^{3+}-citrate	L	3256 ± 2310	Laskov et al., 2006
Potamogeton crispus (from sandy sediment)	Ti^{3+}-citrate	L	798 ± 546	Laskov et al., 2006
Potamogeton pectinatus	Oxygen-depleted solution	L	19.06 (3.75 – 35.94)	Sand-Jensen et al., 1982
		D	0	
Potamogeton pectinatus	Oxygen-depleted solution	L	38.44 (36.56 – 40.63)	Sand-Jensen et al., 1982
		D	3.44	
Potamogeton perfoliatus	Oxygen-depleted solution	L	2 – 99	Caffrey and Kemp, 1991
Potamogeton perfoliatus	Oxygen-depleted solution	L	0 – 20	Kemp and Murray, 1986
Myriophyllum spicatum (from organic sediment)	Ti^{3+}-citrate	L	153 ± 59	Laskov et al., 2006
Myriophyllum spicatum (from sandy sediment)	Ti^{3+}-citrate	L	148 ± 108	Laskov et al., 2006
Myriophyllum vertillicatum	Oxygen-depleted solution	L	60	Carpenter et al., 1983
Littorella uniflora	Oxygen-depleted solution	L	34.06 (31.56 – 36.88)	Sand-Jensen et al,. 1982
		D	1.56	
Isoetes lacustris	Oxygen-depleted solution	L	45.94 (43.75 – 48.12)	Sand-Jensen et al., 1982
		D	9.68	
Lobelia dortmanna	Oxygen-depleted solution	L	168.75 (125 – 216)	Sand-Jensen et al., 1982
		D	12.19	

Results from hydroponic incubations showed that *V. spiralis* ROL constituted about 7% of the total gross production. Even if this amount can be irrelevant in the plant oxygen economy, it is interesting to discuss the relevance of root-mediated oxygen leakage by a *V. spiralis* meadow in the context of the benthic oxygen balance. The question here is to evaluate whether the injection of oxygen performed by *V. spiralis* roots can have an effect in a eutrophic system where organic sediments can subtract substantial oxygen amounts from the water column. I thus scaled-up the oxygen release rates measured in controlled laboratory conditions at the ecosystem level. The actual oxygen leakage in a natural macrophyte population is very difficult to measure and extremely variable in space and time. However, the potential release calculated on the basis of ROL obtained in laboratory conditions may serve as an indication. I calculated the average oxidation potential in a *V. spiralis* vegetated sediment by multiplying the experimentally determined weight-specific release in light and dark by the mean root density of a meadow in the summer period (24 g_{DW} m^{-2}, Pinardi et al., 2009) and considering a photoperiod of 16 h. Roots cause an oxygen transport of over 15 mmol O_2 m^{-2} d^{-1} within the rhizosphere horizon (about 10 cm depth) and the daily oxygen consumption for the bare sediment of the studied site in summer season may amount to over 77 mmol O_2 m^{-2} d^{-1} (Pinardi et al., 2009). My estimate suggests that oxygen leakage from *V. spiralis* can contribute significantly to the sediment oxygen demand, representing about 20% of the daily benthic consumption. This percentage, which is probably an underestimate (see later in the text), is relevant and can further explain the competitive advantage of *V. spiralis* in colonizing and persisting in soft, reduced organic sediments. Most of the oxygen injection by the roots within sediment occurs in the light at the expenses of an identical amount that would be released to the water column by fronds. Such oxygen probably lowers the pool of reduced compounds that would otherwise accumulate in pore water, or enhances the subsurface organic matter mineralization, with as net result a decreased chemical or biological consumption of water column oxygen. A process with high rates in the light like ROL has as a consequence an overall positive ecosystemic effect also in the dark. A different strategy, based on reduced oxygen leakage from roots and increased oxygen release to the water column, would not bring similar results as this oxygen would be transported downstream or would evade to the atmosphere. In other words, under elevated temperature and sediment oxygen uptake conditions typical of temperate eutrophic environments, the risk of night anoxia in bottom water would be higher.

The estimated percentage of sedimentary oxygen demand supported by root-mediated oxygen leakage could be probably an underestimate as chemical gradients across root walls are much steeper under natural conditions compared to what was simulated in the laboratory. Oxygen-depleted solutions have been extensively used for the quantification of ROL in aquatic plants, and oxygen leakage rates available in the literature for submerged macrophytes have been almost exclusively assessed by means of this technique (Sand-Jensen et al., 1982; Kemp and Murray, 1986; Caffrey and Kemp, 1991), with the exception of some recent researches (Laskov et al., 2006; Li et al., 2011). However, this method has been criticized for not mimicking the *in situ* chemical conditions, in particular the sediment redox potential. Sorrell and Armstrong (1994) have pointed out that the lack of a irreversible oxygen sink in the bathing medium can provide opportunity for oxygen re-adsorption by respiring root tissues and the underestimation of ROL as a consequence. However, light rate of oxygen release measured for *V. spiralis* in the present study was similar to that obtained by Li et al. (2011) for *V. natans* by means of the Ti^{3+}-citrate method (~44 μmol O_2 g_{DW} $root^{-1}h^{-1}$).

ROL by *V. spiralis* can sustain, among others, ammonia oxidation processes and indirectly the removal of nitrogen via denitrification. On the basis of the stoichiometry of oxygen and nitrogen during the nitrification process, the oxygen supplied by the macrophyte in summer can theoretically support rates up to 450 and 60 μmol N m^{-2} h^{-1} in light and dark, respectively, if the process is not limited by ammonia and DIC and if nitrification is the only oxygen consuming process. These potential rates are about ten times higher than summer denitrification coupled to nitrification rates measured by means of $^{15}NH_4^+$ injection in *V. spiralis* vegetated sediment in the study area (Racchetti, 2010). These outcomes suggest the co-occurrence of other oxygen consuming processes: in particular during the summer a major fraction of the oxygen leaked by *V. spiralis* is likely used for the oxidation of toxic reduced compounds or the aerobic degradation of organic matter.

In conclusion, my results provided an indirect evidence of *V. spiralis* plasticity to vary seasonally the oxygen amount released by roots in order to counteract the changing chemical environment in pore water. I speculated that this plant can evolve morphological and physiological features providing a competitive advantage when the interstitial conditions become progressively more reducing. The oxygen injected in the pore water by a *V. spiralis* meadow can significantly influence the biogeochemical dynamics of eutrophic, organic-rich sediments.

5. Seasonal pore water chemistry evolution in vegetated and plant-free sediments

5.1. Aim

Radial oxygen loss in *V. spiralis* has been indirectly demonstrated by photosynthetic quotient estimates (Chapter 4) and pore water monitoring in laboratory conditions (Racchetti et al., 2010). Experiments performed to directly quantify ROL by means of oxygen-free solutions showed that the fraction of photosynthetically produced oxygen released by the roots lies in the upper range reported in the literature for submerged plants. Thus I hypothesize that the presence of this rooted plant may deeply affect pore water chemistry and sediment microbial activity along its annual cycle, promoting more oxidised conditions and lower concentrations of reduced compounds. A vast body of literature has explored a number of single aspects related to how roots can modify the interstitial chemical environment. However, to my knowledge only a few papers have done a comprehensive simultaneous analysis on a large set of pore water parameters as that reported in the present study. Moreover, the role of rooted plants in controlling benthic biogeochemical dynamics in freshwater organic-impacted sites is scarcely investigated. The injection of oxygen by macrophyte roots may have deep biogeochemical consequences in organic-rich sediments as it creates oxic micro-niches in an otherwise strictly anaerobic environment, resulting in the establishment of strong solute gradients between multiple oxic and anoxic interfaces.

The aim of the present study was to evaluate the seasonal evolution in pore water chemistry and microbial activity in adjacent vegetated and bare sediments over the macrophyte annual cycle. I investigated the effect of plant presence and season on the *in situ* dynamics of Eh, pH, gases (DIC, CH_4), nutrients (NH_4^+, NO_x^-, SRP, DRSi, Fe^{2+}, Mn^{2+}) and microbial activity (potential nitrification and denitrification rates) in a riverine ecosystem with organic-rich sediment.[*]

[*] *A characterization of the N cycle microbial populations was performed together with pore water analyses, thanks to a collaboration with Dr. Laura Bortolazzi and Prof. Anna Maria Sanangelantoni (Department of Life Sciences, Section of Genetic and Environmental Biotechnology, University of Parma). DNA-based molecular techniques were used together with classical microbiological methods. This work was a first step towards the understanding of the relationship between macrophytes and N cycle bacteria in eutrophic aquatic ecosystems by the integration of biogeochemical and microbiological approaches.*
Results are reported in: ***Bortolazzi L. 2012. Molecular methods for the detection of microorganisms in food and environment. Doctoral thesis, PhD in Biotechnology, University of Parma*** *(in Italian).*

5.2. Materials and Methods

5.2.1. Sampling program

Sediment samplings were performed in a riverine eutrophic site (Mincio River, Massimbona location, Northern Italy). The experimental schedule was planned to cover three key stages of the vegetative cycle, namely the dormancy period (winter, 02/03/2011), the exponential growth phase (spring, 05/09/2011) and the biomass peak (summer, 08/22/2011).

In each period, 3 macroareas including plant-free and vegetated sediments were chosen within the site. In each macroarea, 6 intact cores (transparent Plexiglass liner, Ø 4 cm, height 20 cm) were sampled randomly by hand, of which 3 within the meadow and 3 in adjacent bare patches. Only the sediment portion corresponding to the rhizosphere maximum extension was considered, that is the first 10 cm surface layer. Sediment samples for nitrification and denitrification assay were collected simultaneously. For bare substrates, aliquots were sampled from the intact cores. Rhizosphere sediment samples were obtained from the root zone by shaking off sediment that was adhering to the root systems (n=9). Samples were immediately transferred to sterile 50-ml Falcon tubes. Intact sediment cores were kept in an upright position to avoid mixing, capped and transported to the laboratory in refrigerated boxes together with rhizosphere sediment samples. Simultaneously to sediment collection, water temperature was measured *in situ* (YSI Multiple Probe, mod. 556) and river water was sampled for the dissolved nutrient (NH_4^+, NO_x^-, SRP and DRSi) analysis (see next paragraph).

5.2.2. Pore water extraction and analyses

Within 2 hours from the collection, water overlying the top of the sediment was pipetted off and the upper 1 cm of each core was discarded. This procedure excluded artifacts due to contamination of the water column during the extraction procedure. Pore water was extracted by mixing and transferring the sediment of each core to 50-ml tubes followed by centrifugation for 10 min at 3000 rpm. Sediment extrusion was performed in a glove-bag under N_2 atmosphere. The supernatant was analyzed for Eh, pH, DIC, CH_4, NH_4^+, NO_x^- (NO_3^-+NO_2^-), DRSi, SRP, Fe^{2+} and Mn^{2+}.

Redox potential and pH were measured with a potentiometric electrode (Radiometer, DK) connected to a high impedance mV-meter (Crison micro pH 2002, ES). Pore water was then returned to the glove bag and sub-sampled for dissolved DIC and CH_4 determinations. Samples for DIC measurements were transferred to glass gas-tight vials (12 ml Exetainer®, Labco, High Wycombe, UK) and DIC was measured with acidimetric titration (Anderson et al., 1986). For CH_4

analyses saturated mercuric chloride solution was added to prevent biological activity. CH_4 analyses were performed with the headspace equilibration method (McAuliffe, 1971; 2 ml headspace in a 6 ml gas-tight vial) by means of a Fisons 9000 series gaschromatograph equipped with a flame ionization detector (FID). The remaining pore water was filtered through glass fibre filters (Whatman GF/F) for nutrient and metal analysis. NH_4^+ was measured using salicylate and hypochlorite in the presence of sodium nitroprussiate (Bower and Holm-Hansen, 1980). NO_3^- was measured after reduction to NO_2^- with activated cadmium; NO_2^- was determined using sulphanilamide and N-(1-naphtyl) ethylendiamine (Golterman et al., 1978). Dissolved reactive silica (DRSi) was measured after reaction with sodium molybdate and sulphuric acid (Golterman et al., 1978). Subsamples for Fe^{2+}, Mn^{2+} and soluble reactive phosphorus (SRP) were acidified immediately after extraction (20 µL of 0.5 M HCl per mL of sample) to avoid precipitation and stored at 5°C until analysis. SRP was measured after reaction with ammonium molybdate and potassium antimonyl tartrate and reduction with ascorbic acid (Valderrama, 1977). Fe^{2+} and Mn^{2+} were determined by flame atomic absorption spectroscopy (A.P.H.A., 1981) on a Varian model AA240 Atomic Absorption Spectrometer.

5.2.3. Potential nitrification and denitrification activity

Potential nitrification activity was measured in dark aerobic incubations at room temperature on a shaker table (Hansen et al., 1981; Caffrey et al., 2007). The measured value gives an estimate of the nitrifying activity in the sediment at saturating substrate conditions (optimum aeration and no ammonium limitation). Denitrification and nitrogen uptake by phototrophs were inhibited by the aerobic dark conditions. A known volume of homogenized sediment (5ml) was added to 50 ml of filtered water from the site, enriched with NH_4Cl to give a final concentration of about 100 µM. After 20 h, an aliquot of the slurry was centrifuged at 3000 rpm for 10 min, filtered through a GF/F filter and analysed for nitrate plus nitrite. NO_3^- was measured after reduction to NO_2^- with activated cadmium. NO_2^- was determined spectrophotometrically using sulphanilamide and N-(1-naphtyl)ethylendiamine (Golterman et al., 1978). Potential nitrification rates were calculated from accumulation of NO_2^-+NO_3^- over time and expressed in terms of sediment dry weight (DW).

Potential denitrification activity was measured in dark anaerobic incubations by using the nitrogen stable isotope ($^{15}NO_3^-$) and measuring the production of single-labelled ($^{14}N^{15}N$) and double-labelled ($^{15}N^{15}N$) di-nitrogen by a mass spectrometer (Thamdrup and Dalsgaard, 2002; Risgaard-Petersen et al., 2005). A known volume of homogenized sediment (1ml) was transferred to glass

gas-tight vials (12 ml Exetainer®, Labco, High Wycombe, UK) together with a glass bead and N_2-purged deionized water. The vials were sealed without headspace and pre-incubated in the dark for over 24 h on an agitator to allow the removal of oxygen and nitrate traces initially present. 200µl of 10mM $Na^{15}NO_3$ solution (98 atom % ^{15}N enrichment) was injected through the septum by means of a glass syringe (Hamilton 725RN 250 µl) into each sample. Incubation was performed in the dark on an agitator and lasted about 20 h. Biological activity was stopped by addition of a saturated zinc chloride solution (200µl, 7M) to incubation vials. Exetainer® were stored upside down until gas analysis. $^{14}N^{15}N$ and $^{15}N^{15}N$ abundance in N_2 were analyzed by mass spectrometry at the National Environmental Research Agency, Silkeborg, Denmark. Potential denitrification rates were calculated from production of $^{29}N_2$ and $^{30}N_2$ over time and expressed in terms of sediment dry weight (DW).

5.2.4. Sediment analyses

For each sampling period aliquots of sediment (from vegetated and bare patches) were analyzed for bulk density (measured as the weight of a volume of 5 ml fresh material) and porosity after drying at 70°C until constant weight. OM content (%) was quantified as loss on ignition (LOI) at 350°C for 3 h on about 0.2 g of dry powdered sediment.

5.2.5. Statistical analyses

I tested the effects of season and plant presence on pore water features and sediment microbial activity by means of a two-way ANOVA. Correlative relationships among pore water variables were identified using the Pearson's Correlation Coefficient (r). Normality and homoscedasticity of data were examined and Box-Cox transformation was used when necessary. Statistical analyses were performed using the R Program (R – Development Core Team, 2011); statistical significance was set at $p \leq 0.05$.

5.3. Results

5.3.1. Water column and sediment characterization

The riverine site exhibited water and sediment features typical of eutrophic environments (Table 5.1 and Table 5.2). Nitrate was the dominant inorganic nitrogen form in water column in all sampled seasons. The ratio between oxidised and reduced N forms ranged between 10 and 40 and

the summer peak of water temperature coincided with a minimum in dissolved inorganic nitrogen concentration. The ratio between dissolved inorganic nitrogen and SRP varied between 18 and 78. The surface sediment (0-10 cm horizon) was muddy with an average bulk density of 1.2 g cm^{-3} and an average porosity of about 0.74 (pooled data) (Table 5.2). Sampling season and presence of plant did not affect significantly those sediment features ($p>0.05$). OM content ranged between 5.30 and 11.03% (as LOI) along the investigated period. Vegetated sediments sampled in summer had an OM value significantly lower than the corresponding bare ones and also lower than sediments collected in all the other seasons.

Table 5.1. Water column features of the sampling site in the three sampled seasons.

Samplig date	**Temperature** (°C)	**[NH_4^+]** (μM)	**[NO_x^-]** (μM)	**[SRP]** (μM)	**[DRSi]** (μM)
02/03/2011	6	2	76	<1	19
05/07/2011	19	5	119	7	25
08/22/2011	25	5	54	2	31

Table 5.2. Density, porosity and organic matter content of the sediment (average ± std. dev., n=9).

Samplig date	**Sediment type**	**Density** (g cm^{-3})	**Porosity**	**OM** (%)
02/03/2011	Vegetated	1.02 (0.06)	0.66 (0.04)	10.54 (0.09)
	Bare	1.09 (0.06)	0.73 (0.04)	11.03 (0.20)
05/07/2011	Vegetated	1.17 (0.09)	0.79 (0.07)	10.13 (0.96)
	Bare	1.24 (0.09)	0.78 (0.08)	9.96 (1.04)
08/22/2011	Vegetated	1.28 (0.04)	0.72 (0.04)	5.30 (0.84)
	Bare	1.14 (0.05)	0.79 (0.04)	8.27 (1.15)

5.3.2. Pore water analyses

Temporal changes in pore water features in the three seasons sampled along the *V. spiralis* annual cycle are reported in Figs. 5.1 and 5.2. All pore water data integrate the uppermost 10 cm sediment layer which includes most of the rhizosphere horizon. As evidenced by the results of the two-way ANOVA, the factor season had a highly significant effect on interstitial water chemistry evolution. Also the presence of plant influenced pore water features, with the exception of pH, SRP, Fe^{2+} and Mn^{2+} (Tab. 5.3). Spatial variability was remarkable, especially in summer, for some redox-sensitive parameters such as SRP, DRSi, Fe^{2+} and Mn^{2+}.

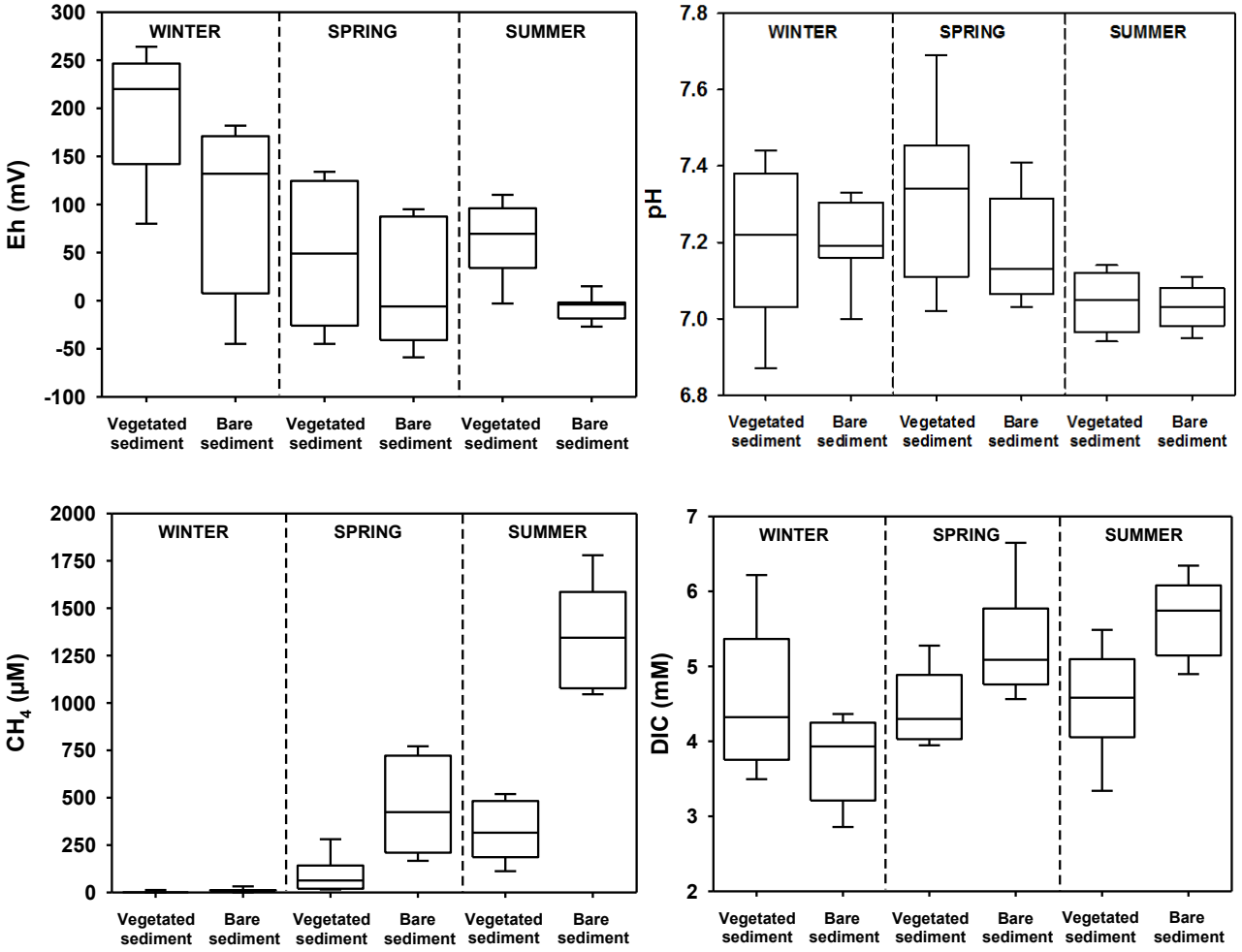

Fig. 5.1. Pore water Eh, pH and gas (CH_4 and DIC) concentrations in vegetated and bare sediments sampled in the three seasons (n=9).

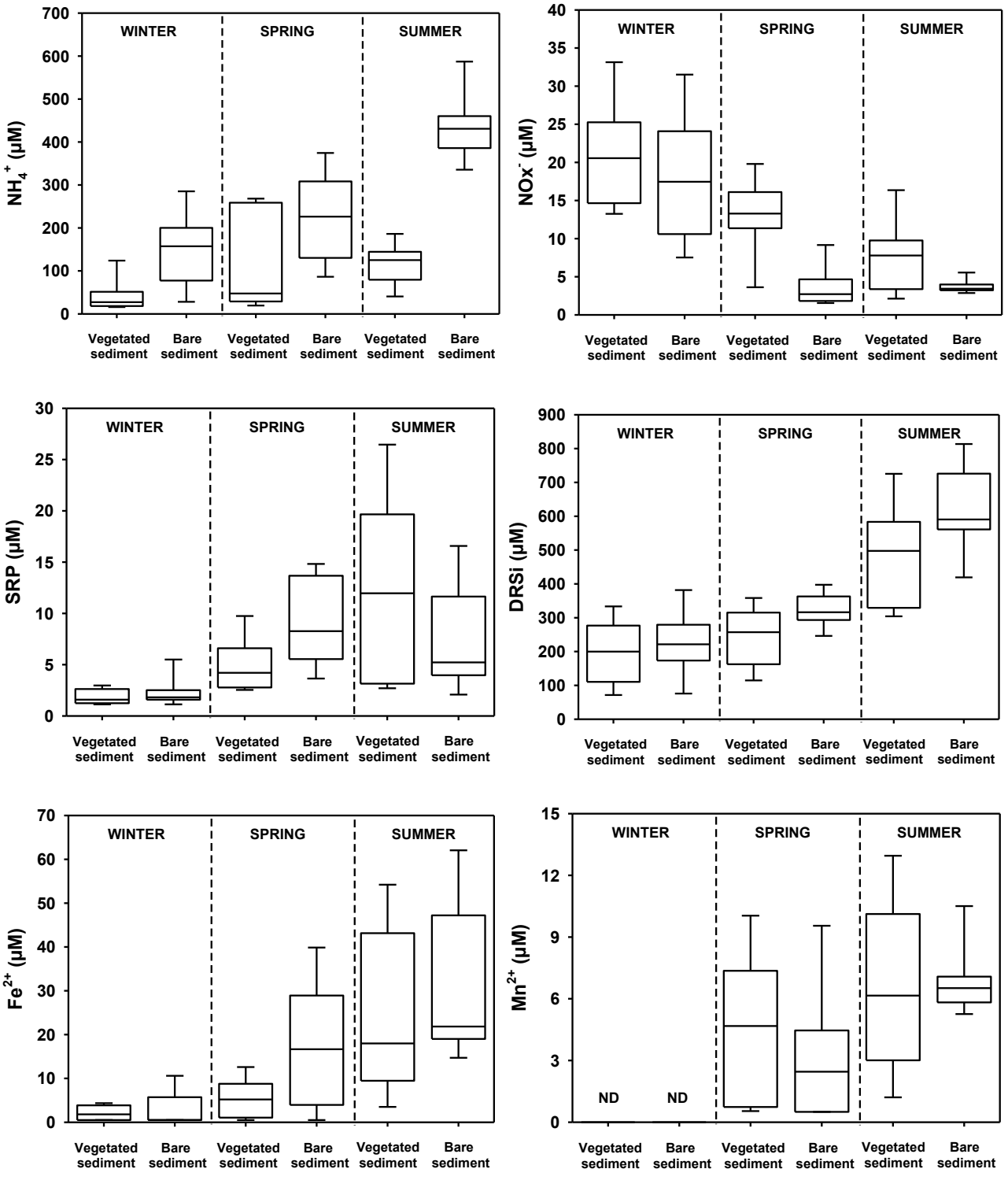

Fig. 5.2. Pore water nutrient (NH_4^+, NO_x^-, SRP and DRSi) and metal (Fe^{2+} and Mn^{2+}) concentrations in vegetated and bare sediments sampled in the three seasons (n=9). ND=concentration below detection limit

Table 5.3. Results of the two-way ANOVA performed to test the effects of season and presence of plant on pore water features (for Mn, winter data were excluded from the statistical analyses, because values were below the detection limit). *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$; NS=not significant

Variable	Factor	*df*	SS	F	*p*
Eh	Season	2	87.419	24.091	***
	Presence of plant	1	100.668	55.484	***
	Season x Presence of plant	2	7.123	1.963	NS
	Error	48	87.090		
pH	Season	2	0.0090640	11.0836	***
	Presence of plant	1	0.0005507	1.3469	NS
	Season x Presence of plant	2	0.0010176	1.2443	NS
	Error	48	0.0196268		
DIC	Season	2	8.7744	10.0923	***
	Presence of plant	1	1.9365	4.4548	*
	Season x Presence of plant	2	10.2678	11.8100	***
	Error		20.8660		
CH_4	Season	2	871.30	226.1957	***
	Presence of plant	1	139.52	72.4429	***
	Season x Presence of plant	2	12.71	3.2999	*
	Error	48	92.45		
NH_4^+	Season	2	137.819	16.7017	***
	Presence of plant	1	199.609	48.3797	***
	Season x Presence of plant	2	12.127	1.4697	NS
	Error	48	198.042		
NO_x^-	Season	2	55.156	36.7444	***
	Presence of plant	1	17.946	23.9110	***
	Season x Presence of plant	2	8.262	5.5038	**
	Error	48	36.026		
SRP	Season	2	11.6878	30.2433	***
	Presence of plant	1	0.2853	1.4765	NS
	Season x Presence of plant	2	0.9532	2.4664	NS
	Error	48	9.2751		
DRSi	Season	2	470.25	45.6167	***
	Presence of plant	1	42.05	8.1575	**
	Season x Presence of plant	2	5.40	0.5239	NS
	Error	48	247.41		
Mn^{2+}	Season	1	7.4864	9.0217	**
	Presence of plant	1	0.0844	0.1017	NS
	Season x Presence of plant	1	1.0573	1.2741	NS
	Error	32	26.5543		
Fe^+	Season	2	98.572	28.1125	***
	Presence of plant	1	2.814	1.6051	NS
	Season x Presence of plant	2	1.677	0.4783	NS
	Error	48	84.153		

Redox potential ranged from -59 mV to 264 mV and a pronounced decline was observed from winter (median value 161 mV, pooled data) to summer (median value 7 mV, pooled data). In all three seasons, plant presence markedly affected sediment redox status, resulting in more oxidized conditions in the rhizosphere compared to the corresponding bare sediments. The presence of roots influenced Eh values particularly in summer, when vegetated sediments were more oxidized than both plant-free and colonized substrates of the previous sampled season, despite the increase in water temperature. Variability in interstitial Eh was maximum in spring.

The range of pore water pH values was quite narrow, that is from 6.87 to 7.69. A slight decrease was evident from winter to summer. A significant influence of plant presence on acidic condition was not detected; pH tended to be lower in bare sediments compared to the adjacent vegetated ones only in spring.

Pore water DIC varied between 3.34 and 6.22 mM and a general increase was observed from winter (median value 4.06 mM, pooled data) to summer (median value 5.14 mM, pooled data). Apart from winter sampling when pore water DIC showed a great spatial heterogeneity, especially in bare sediment, vegetated sediments tended to have lower interstitial DIC content compared to the corresponding bare sediments.

Dissolved CH_4 concentrations exhibited a clear seasonal trend with a summer peak and constantly higher values in bare sediments compared to vegetated ones in all sampled dates. Interstitial methane concentrations varied between <1 and 520 and between <1 and 1,780 µM in vegetated and bare sediments, respectively. The greater build-up of interstitial CH_4 was found in summer, when the greater difference in contents (of over one order of magnitude) between vegetated and bare sediments was also detected. Pore water CH_4 was negatively correlated with redox potential (Tables 5.4 and 5.5).

Ammonium concentrations ranged between 16 and 587 µM with higher values registered in summer. NH_4^+ values were significantly affected by *V. spiralis* presence in all sampling periods. The average ammonium concentration for vegetated and bare sediments was 93 and 267 µM, respectively (pooled data). In summer pore water NH_4^+ contents was, on average, four-fold lower in the presence of *V. spiralis* than in its absence. Pore water NH_4^+ was negatively correlated with redox potential and positively correlated with CH_4 and other reduced species (Tables 5.4 and 5.5).

Pore water NO_x^- concentrations showed a pronounced decrease from winter (median value 20 µM, pooled data) to summer (median value 4 µM, pooled data) and were positively correlated with Eh values (Tables 5.4 and 5.5). Nitrate contributed on average to >95% of the oxidized

nitrogen forms (pooled data). Both in spring and summer, interstitial NO_x^- content was higher in vegetated sediments compared to the adjacent bare ones.

SRP concentrations ranged between <1 and 27 µM and they progressively increased from winter to summer. Both in winter and summer, values were not affected by plant presence while only in spring bare sediments tended to have higher concentrations compared to vegetated ones. In summer a great spatial variability was associated to pore water SRP.

DRSi concentrations increased by a factor of three in the shift winter-summer, from about 200 µM to about 600 µM. With the exception of the cold season, *V. spiralis* presence exerted a significant effect on pore water DRSi concentrations, lowering interstitial silica availability in colonized sediments. Pore water DRSi was highly negatively correlated with redox potential (Tables 5.4 and 5.5).

The seasonal patterns of pore water metals were quite erratic and the effect of plant presence not always clear. Fe^{2+} and Mn^{2+} concentrations ranged between <1 and 62 µM and between <1 and 11µM, respectively. Manganese concentrations in winter were below the detection limit in all the collected cores. Interstitial iron content progressively increased from winter to summer with a general tendency of higher values in bare sediments compared to vegetated ones in each sampled season. On the contrary, there was not a clear seasonal pattern for pore water Mn^{2+} over the study period: only a slight increase was detected from spring (median value 3 µM) to summer (median value 6 µM). The presence of *V. spiralis* did not appreciably affect Mn^{2+} concentrations in summer.

Dissolved iron was generally greater than dissolved manganese content, reflecting the different magnitude of sedimentary metal pools (see Chapter 6). Both pore water Fe^{2+} and Mn^{2+} were negatively correlated with redox potential and positively correlated with SRP (Tables 5.4 and 5.5).

Table 5.4. Correlations between pore water features (Pearson's coefficients) in vegetated sediments. *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$; NS=not significant

	Eh	pH	DIC	CH_4	NH_4^+	NO_x^-	SRP	DRSi	Fe^{2+}	Mn^{2+}
Eh	1									
pH	0.463 *	1								
DIC	-0.360 NS	-0.673 ***	1							
CH_4	-0.632 ***	-0.414 *	0.217 NS	1						
NH_4^+	-0.568 ***	-0.266 NS	0.255 NS	0.536 **	1					
NO_x^-	0.465 *	0.066 NS	0.249 NS	-0.568 **	-0.193 NS	1				
SRP	-0.538 **	-0.333 NS	0.217 NS	0.660 ***	0.354 NS	-0.469 *	1			
DRSi	-0.736 ***	-0.706 ***	0.533 **	0.848 ***	0.416 *	-0.441 *	0.592 **	1		
Fe^{2+}	-0.601 ***	-0.487 **	0.295 NS	0.776 ***	0.371 NS	-0.525 **	0.788 ***	0.798 ***	1	
Mn^{2+}	-0.283 NS	-0.356 NS	0.324 NS	0.558 *	0.662 **	-0.136 NS	0.493 *	0.382 NS	0.434 NS	1

Table 5.5. Correlations between pore water features (Pearson's coefficients) in bare sediments. *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$; NS=not significant

	Eh	pH	DIC	CH_4	NH_4^+	NO_x^-	SRP	DRSi	Fe^{2+}	Mn^{2+}
Eh	1									
pH	0.456 *	1								
DIC	-0.640 ***	-0.499 **	1							
CH_4	-0.426 *	-0.622 ***	0.679 ***	1						
NH_4^+	-0.421 *	-0.598 ***	0.698 ***	0.821 ***	1					
NO_x^-	0.658 ***	0.341 NS	-0.789 ***	-0.553 **	-0.558 **	1				
SRP	-0.445 *	0.133 NS	0.474 *	0.332 NS	0.404 *	-0.467 *	1			
DRSi	-0.432 *	-0.549 **	0.671 ***	0.802 ***	0.817 ***	-0.588 **	0.279 NS	1		
Fe^{2+}	-0.348 NS	-0.237 NS	0.568 **	0.661 ***	0.787 ***	-0.493 **	0.691 ***	0.596 **	1	
Mn^{2+}	0.264 NS	-0.335 NS	0.685 ***	0.532 *	0.556 *	-0.204 NS	-0.008 NS	0.600 **	0.369 NS	1

5.3.2. Potential nitrification and denitrification activity

Along the investigated period, potential nitrification rates ranged between 4 and 171 nmol NO_x^- g_{DW}^{-1} h^{-1} (Fig. 5.3). The measured rates reached a maximum in the cold season ($p<0.001$) and a general decline was observed from winter (median value 78 nmol NO_x^- g_{DW}^{-1} h^{-1}, pooled data) to summer (median value 35 nmol NO_x^- g_{DW}^{-1} h^{-1}, pooled data). In all sampled seasons, nitrification rates in vegetated sediments were significantly higher than those measured in the corresponding bare ones ($p<0.001$). However, the discrepancy between median rates in colonised and bare sediments decreased in the shift winter-summer.

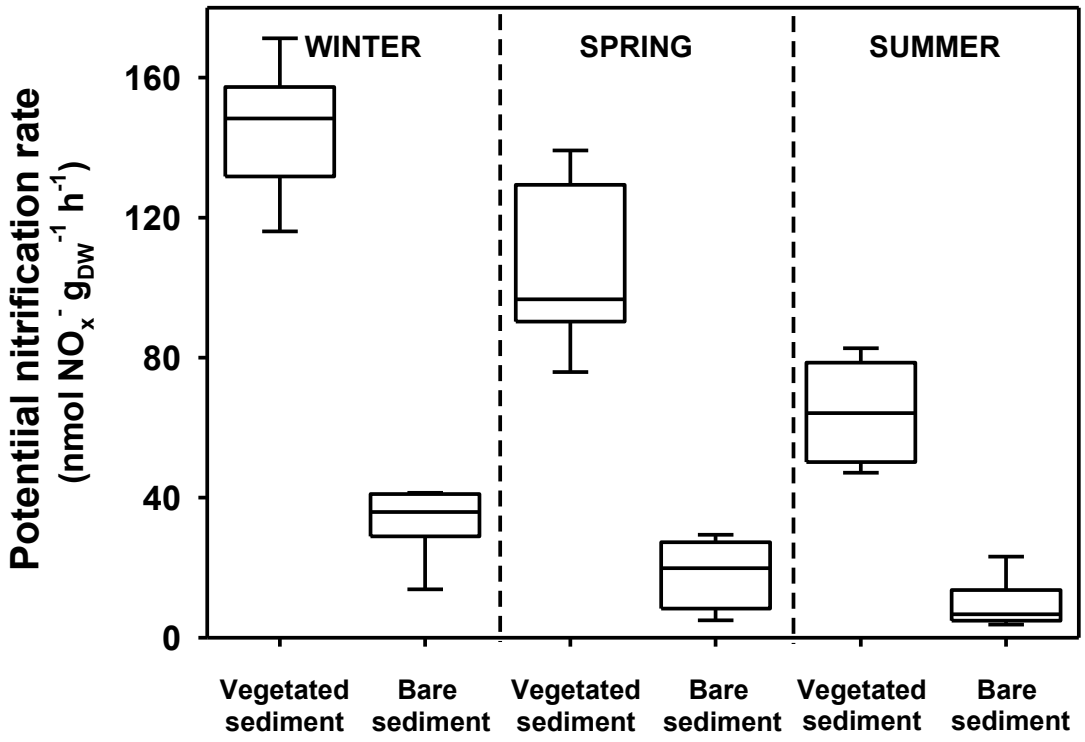

Fig.5.3. Potential nitrification in vegetated and bare sediments in the three sampled seasons (n=9).

Due to methodological problems, potential denitrification rates are not available for bare sediment in summer. Presence of plant didn't significantly affect potential denitrification activity ($p>0.05$). Average values (pooled data) were 44±11, 22±11 and 42±5 nmol N g_{DW}^{-1} h^{-1}, in winter, spring and summer, respectively.

5.4. Discussion

5.4.1. *V. spiralis* effect on pore water chemistry in eutrophic sediments

Pore water characterization was performed after extraction by centrifugation from the first 10 cm depth sediment horizon. This technique produces spatially averaged data and no indications about the vertical or lateral successions of solute gradient and microbial pathways can be inferred. However, such data are useful for assessing the net influence on pore water features of the macrophyte activity along the annual cycle. The aim of my experiment was to test whether, even in an organic-rich sediment, *V. spiralis* could modify the interstitial chemical environment and buffer the chemical changes occurring in such situations. The processes trough which the roots interact with sediment (ROL and uptake) are not constant in time and space (for example, ROL could not occur all along the entire root system). However, if interstitial chemistry differences between vegetated and plant-free sediment are evident also from the analyses of integrated pore water, it is a clear indication that the macrophyte exerts a relevant influence on pore water conditions.

Along its annual cycle *V. spiralis* maintains an oxygenated rhizosphere in the colonized sediments. Pore water redox potential in vegetated sediments was constantly higher than in adjacent bare ones, presumably as a consequence of the oxygen released from roots. These results support previous direct or indirect evidences that this aquatic plant transports a relevant amount of the photosynthetically produced oxygen towards the below-ground tissues (Chapter 4; Racchetti et al., 2010; Ribaudo et al., 2011). This ability is an essential ecological adaptation providing protection to roots against hostile reduced substrates. Indeed vegetated sediments were characterized by lower concentrations of potentially toxic species. Several studies of adjacent vegetated and unvegetated patches have suggested that sediments are generally more oxidized when vegetation is present but this evidence is mainly restricted to oligotrophic sites (Carpenter et al., 1983; Jaynes and Carpenter, 1986; Wigand et al., 1997). My results question such conclusions, as the effects of *V. spiralis* on pore water features seemed not to be impeded by the organic character of the sediment. *V. spiralis* roots demonstrated to be able to increase redox potential even in eutrophic, potentially strongly reduced sediments. The sampling station is in fact downstream the sewage of a huge wastewater treatment plant (>300,000 equivalent inhabitants) and the sediment is muddy and quite enriched with labile organic matter. Moreover, from my data on integrated pore water characterization, I can speculate that the oxidized zones are not only limited to small areas around

the root tips as previous reveled for other submerged plants (Flessa, 1994), but the effect is at larger scale, probably at the level of the whole meadow. A positive redox potential even when temperature is high implies that the oxygen flux leaked from the root system is enough to overcome the local oxygen consumption from anaerobic degradation pathways and chemical re-oxidations. The relevant effect of *V. spiralis* in organic-rich sediments can be a direct consequence of both high ROL rates or elevated root density in the meadows. Results from laboratory incubations via oxygen-depleted solutions showed that *V. spiralis* ROL rates were in the upper range reported in the literature for submerged plants and persisted even in darkness (see Chapter 4). Moreover, in dense plant populations, most of the sediment in the root zone is in close contact with root systems. The sediment can be strongly influenced by gas and solute exchanges with the roots and the oxygen supply by plants may overcome the uptake by the sediment between the roots themselves. On the countrary, macrophytes with a limited ability to transport oxygen or that have evolved root barriers to ROL to survive the oxygen shortage (Colmer, 2003) may have a negligible influence on sediment dynamics in eutrophic systems. When labile carbon deposition increases, the sediment oxygen consumption can exceed the plant ability to maintain an oxidized environment.

The balance between the sediment oxygen consumption and the magnitude of the root oxygen release controls nutrient and redox-sensitive species availability in pore water. Extraction and analyses of pore water in bare and vegetated sediments revealed relevant seasonal differences in the chemical composition. Redox sensitive species showed similar patterns with a progressively increase from winter to summer. The accumulation of anaerobic metabolism end-products (reducing power) in interstitial water along with increasing temperature resulted in a steady Eh decrease. The build-up of DIC, CH_4 and ammonium was consistent with relevant anaerobic organic matter degradation. However, oxygen release by the roots maintained a more oxidized interstitial environment and lower concentrations of reduced solutes as a consequence, compared to the corresponding unvegetated substrates all along the annual cycle. Methane and reduced forms of iron and manganese are target compounds providing indications of the plant activity with respect to oxygen release by roots (ROL presence and rate). In the presence of *V. spiralis*, pore water methane concentrations were significantly attenuated, likely due to the presence of oxidized micro-niches that inhibit methanogenesis or promote methanotrophy (Jespersen et al., 1998; Van Der Nat and Middelburg, 1998; Sorrel et al., 2002). Decrease in pore water SRP concentrations in vegetated sediments could not only be explained by plant uptake (Carignan and Kalff, 1980), but

also by precipitation of ferric and manganic oxy-hydroxides and the subsequent phosphorus adsorption. Only very few studies have dealt with the effect of plants on pore water silica (Mi et al., 2008). Plant presence decreased DRSi concentrations likely due to a combination of root uptake and influence on redox-dependent silica mobilization from particles (Michalopoulos and Aller, 1995; Struyf and Conley, 2009; Querné et al., 2012).

5.4.2. *V. spiralis* effect on sediment microbial activity

Redox potential is a key variable that not only controls the availably of a wide range of nutrients and metals but also affects bacterial activity. Since nitrification cannot proceed in the absence of oxygen, oxygen-releasing plants can have a major impact on N cycling in otherwise anoxic sediment layers. The root zone of aerenchymatous plants may form a niche for the aerobic bacteria, which oxidize ammonia to nitrite and nitrate. A number of studies has proven the stimulation of nitrification by oxygen-releasing plants in freshwater sites by both activity measurements and estimations of ammonia- and nitrite-oxidizing bacteria abundance (Caffrey and Kemp, 1990; Bodelier et al., 1996; Ruiz-Rueda et al., 2009; Forshay and Dodson, 2011). However, rooted macrophytes can directly affect nitrification not only by creating a more suitable environment for aerobic processes but also by ammonium assimilation. In sediments where NH_4^+ plant demand is high compared to production, competition between nitrifiers and plants may occur, especially in N-limited systems (Sousa et al., 2012). I have measured potential nitrification rates in *V. spiralis* vegetated sediments from 3 to 8 times higher than those in bare sediments, confirming that ROL enhanced nitrification in the rhizosphere and that competition for ammonium was attenuated. As a consequence, *V. spiralis* increased NO_x^- concentrations compared to the corresponding bare substrates. Due to the eutrophic conditions, nitrogen isn't probably a limiting factor in the investigated site. Nitrifiers saturate at NH_4^+ concentrations which are low (50 to 100 µM) (Henriksen and Kemp, 1988) compared to pore water ammonium pools measured in my site. Moreover, nitrate availability in water column could have enhanced plant N uptake from the leaves.

Potential nitrification rates obtained in the present study were greater than those previously reported for *Littorella uniflora* rhizosphere (Herrmann et al., 2008) and in the upper range of activities measured in freshwater sediments colonized by a variety of other submerged species (Caffrey and Kemp, 1990 and references therein; Coci et al., 2008). Seasonal patterns of potential nitrification are usually closely dependent upon temperature and pore water ammonium

availability, resulting in maximum summer rates (Caffrey and Kemp, 1990; Forshay and Dodson, 2011). Differently, I measured winter rates significantly higher than spring and summer ones, as already reported by some authors (Bodelier et al., 1996; Sousa et al., 2012). Potential nitrification rates obtained in optimal conditions of oxygen and ammonium availability can be regarded as a proxy for the abundance of active nitrifiers (Risgaard-Petersen et al., 2004). Higher activity in winter can reflect a greater proportion of active or non-resting cells, due to more favorable conditions. The decline in activity is very probably the result of oxygen limitation with progressively increasing temperature.

The highest rates of potential nitrification were observed during winter when oxygen penetrates deep into the sediment and when competition for ammonium with plants is usually weak. However, in the studied site nitrification seemed not to be limited by ammonium availability: smaller rates were in fact detected in summer in bare sediment despite pore water ammonium reached its seasonal peak. Oxygen shortage is most likely due to a decrease in water oxygen solubility in combination with elevated heterotrophic microbial oxygen consumption due to the high summer temperatures, resulting in a progressive inhibition of the nitrifiers in the shift winter-summer. Due to their low oxygen affinity, nitrifying bacteria can be out-competed by heterotrophic and chemolithotrophic bacteria when oxygen is limiting (Henriksen and Kemp 1988). Moreover, a variety of reduced compounds, such as sulfide, could adversely affect the growth and activity of the nitrifying community (Sears et al., 2004).

Similar to nitrification, also denitrification potential can be positively affected by the occurrence of rooted plants. Several studies have measured a significant increase in nitrate reduction activity in the rhizosphere in comparison with adjacent non-vegetated sediments (Caffrey and Kemp, 1990; Ruiz-Rueda et al., 2009; Forshay and Dodson, 2011). The enhancement in potential rates is mainly connected to the excretion of organic compounds by roots (Sherr and Payne, 1978; Karjalainen et al., 2001), even if the simultaneous release of oxygen and nitrate uptake can result in a more complex plant effect on denitrifier community. Differently, in my study area, potential denitrification rates were quite homogeneous in adjacent vegetated and bare sediment patches. Carbon limitation most likely didn’t occur since the sediment is quite enriched with labile matter (Pinardi et al., 2009; Racchetti et al., 2010). Moreover, unlike nitrifiers, which have a specialized metabolism, bacteria populations using nitrate as a terminal electron acceptor are facultative anaerobes also capable of other various metabolic pathways (Hattori, 1983).

An underestimation of potential denitrification rates can't be excluded, due to some methodological problems. However, as already shown in previous studies (Caffrey and Kemp, 1990; Ruiz-Rueda et al., 2009), denitrification potentials were constantly lower than the corresponding nitrification potentials, resulting in NO_x^- accumulation in pore water. Pore water DIN content were usually lower in vegetated sediments compared to the bare ones. The nitrogen cycle might have been stimulated mainly by promotion of nitrification through root oxygen release. These interactions have a high ecological relevance since they promote net N removal from the systems contributing to counteract eutrophication events and retard the plant community succession.

Results from the present study demonstrated that *V. spiralis* plays an important role in regulating the dynamics of nutrients and gasses in pore water of an organic-rich sediment (> 10% as loss on ignition). Adjacent sediment patches bare and with dense growth of the rooted macrophytes have displayed great differences in pore water chemistry and nitrification potential. Less accumulation of reduced species suggested that degradation processes occur mainly via aerobic pathways or that reduced compounds are quickly re-oxidized. This is consistent with intense oxygen pumping into the rhizosphere, especially in summer, via high density root systems.

6. Effects of increasing organic matter loads on pore water features of vegetated and plant-free sediments

6.1. Aim

Tolerant submerged plants have the potential to colonize even substrates theoretically hostile for roots. However, whether and to what extent the macrophyte presence may buffer chemical changes occurring in pore waters as a consequence of organic enrichment has been scarcely investigated. The object of this experiment was to evaluate how the organic enrichment affects the redox related processes in bare and *V. spiralis* vegetated sediments. I hypothesize significant differences of pore water features (less reduced conditions) in *V. spiralis* colonized sediments compared to the bare ones and I assume that such differences persist over a wide range of sedimentary organic matter content. To verify this assumption, I monitored pore water features in plant-covered and plant-free sediment microcosms containing increasing OM amounts, simulating an eutrophication gradient. The comparison of pore water chemical environment in vegetated and unvegetated sediment, along steep OM gradients, can give multiple indications about the effect of plant presence on biogeochemical dynamics. Chemical pore water composition provides evidences about the main microbial processes and the degree of coupling between reductions and oxidations. A significant difference in the presence or absence of plants suggest an active role of primary producers in the detoxification.

6.2. Material and Methods

6.2.1. Sampling procedure

The experiment was carried out during the exponential growth phase of *V. spiralis* and lasted 17 days. Plants, sediment and water were collected in March 2011 from the shallow water eutrophic site of the Mincio River (Massimbona location, Northern Italy, ~1.5 m depth). About 100 shoots were carefully collected by scuba divers, minimizing root damage for the subsequent transplant (average plant biomass ~8 g fresh weight). Over 10 l of unvegetated sediment was collected from the top horizon (0-10 cm) by means of Plexiglass liners. Shoots and sediment were brought to the laboratory fully submerged by river water.

6.2.2. Experimental setup

Within 2 h from the collection, the sediment was sieved through a 2 mm mesh to remove coarse plant debris, macrofauna and stones, and homogenized. The sediment was then divided into three equally sized portions (~2 l): one was not manipulated and served as control (C) and the others were enriched with two different amounts of labile organic matter in the form of powdered fish feed. Average organic C, N and P content of the sediment were 1.72, 0.56 and 0.06% respectively (Pinardi et al., 2009; Racchetti et al., 2010). Pellets of commercially available fish feed (49% organic C, 8% organic N and 1% organic P) were dried at 50°C and ground to a powder: 5 and 10 g of fish feed per liter of sediment ware added to the two aliquots of sieved sediment respectively (+5 and +10), then carefully homogenized by hand. The addition of fish feed pellets aimed at reproducing the enrichment with labile organic matter, an event which normally occurs in eutrophic environments, as those where phytoplankton blooms occur or those receiving effluents from sewage plants or fish farms. Previous studies have proven the stimulation of microbial metabolism with the organic enrichments adopted (Mascarò et al., 2009; Valdemarsen et al., 2009) and the labile carbon inputs are comparable to those measured annually close to fish farms (Holmer et al., 2007; Holmer and Frederiksen, 2007).

Sediments of the three organic levels (C, +5 and +10) were transferred into cylindrical Plexiglass tubes (i.d. 3 cm, height 10 cm) and 24 microcosms were set up for each OM level, for a total of 72 microcosms (Fig. 6.1 and Fig. 6.2). Healthy *V. spiralis* shoots were carefully washed several times with river water to remove the original sediment from roots and epiphytes from leaves. In 12 tubes for each OM level, one shoot randomly selected was transplanted. Microcosms were then maintained in tanks with river water at the temperature of 18-20°C and subjected to a 12/12h light/dark cycle at an irradiance of about 250 µmol photons m^{-2} s^{-1} (Photosynthetically Active Radiation, PAR) by means of fluorescent tubes. Water temperature was measured with a YSI Multiple Probe (mod 556) and PAR intensity with a LI-192 Underwater Quantum Sensor and a LI-250A Light Meter (Li-Cor, Lincoln, NE, U.S.A.). Irradiance intensity and light/dark cycle length during the experiment reflected the *in situ* average values for the sampling period. Water in the incubation tanks was stirred continuously with aquarium pumps and regularly replaced with fresh river water to prevent algal growth. Microcosms were exposed to the same river water to eliminate possible effects of nutrient availability resulting from the release from enriched sediments.

During the 17-day incubation, 3 replicates for each experimental condition (C_V, control vegetated sediment; C_B, control bare sediment; $+5_V$, 5g enriched vegetated sediment; $+5_B$, 5g enriched bare sediment; $+10_V$, 10g enriched vegetated sediment; $+10_B$, 10g enriched bare sediment) were sequentially sacrificed with the purpose of monitoring pore water feature changes. The first microcosm sacrifice was performed after 6 days, in order to allow the re-establishment of solute microgradients in interstitial water and to give plants enough time to recover from the transplant stress. The time interval of 3-4 days between samplings was chosen to allow detection of iron and manganese pool changes. The total experimental time of 17 days was set in order to avoid the system degeneration given the limited sediment volume of each microcosm.

Pore water was extracted by mixing and transferring microcosm sediment to 50-ml tubes followed by centrifugation for 10 min at 3000 rpm. Sediment extrusion was performed in a glove-bag under N_2 atmosphere. The supernatant was analyzed for Eh, CH_4, Fe^{2+}, Mn^{2+}, PO_4^{3-} and NH_4^+. In the vegetated sediments, leaves were cut at the base prior to centrifugation in order to avoid mechanical damage. Leaves and roots recovered from microcosms were washed with distilled water and desiccated separately at 70°C until constant weight.

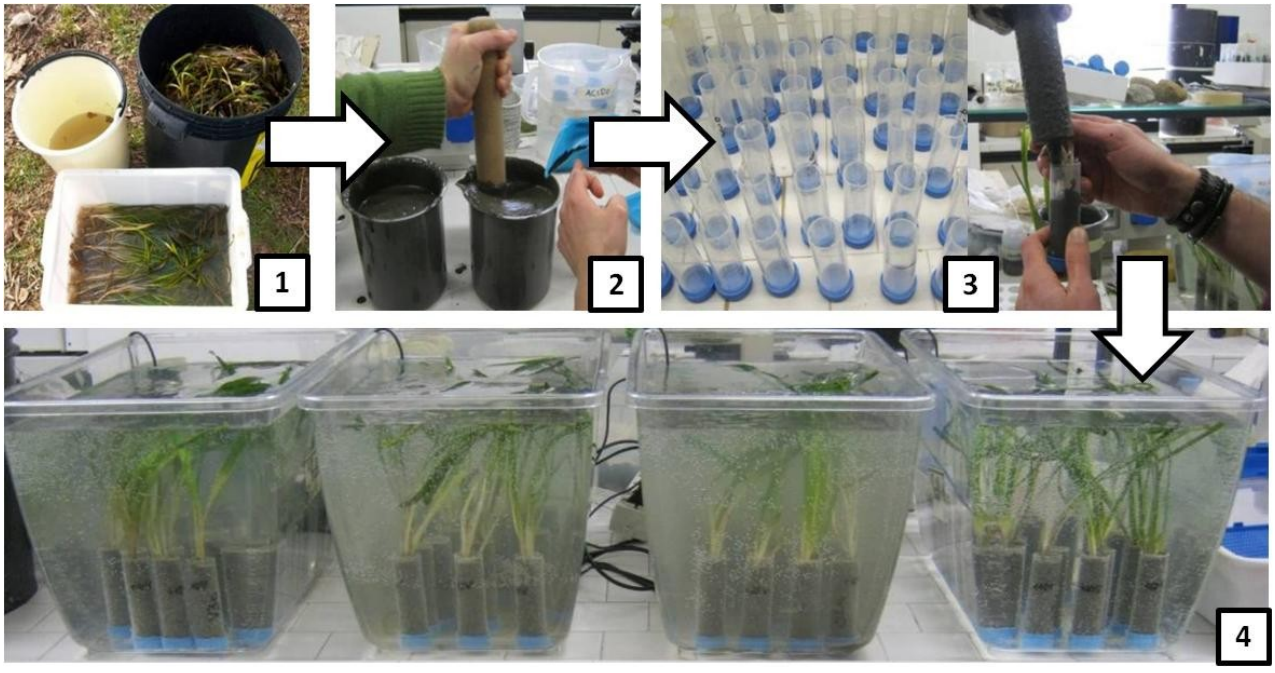

Fig. 6.1. Experimental procedure: 1) sampling of sediment and plants; 2) sediment enrichment; 3) microcosm set-up and transplant; 4) microcosm incubation.

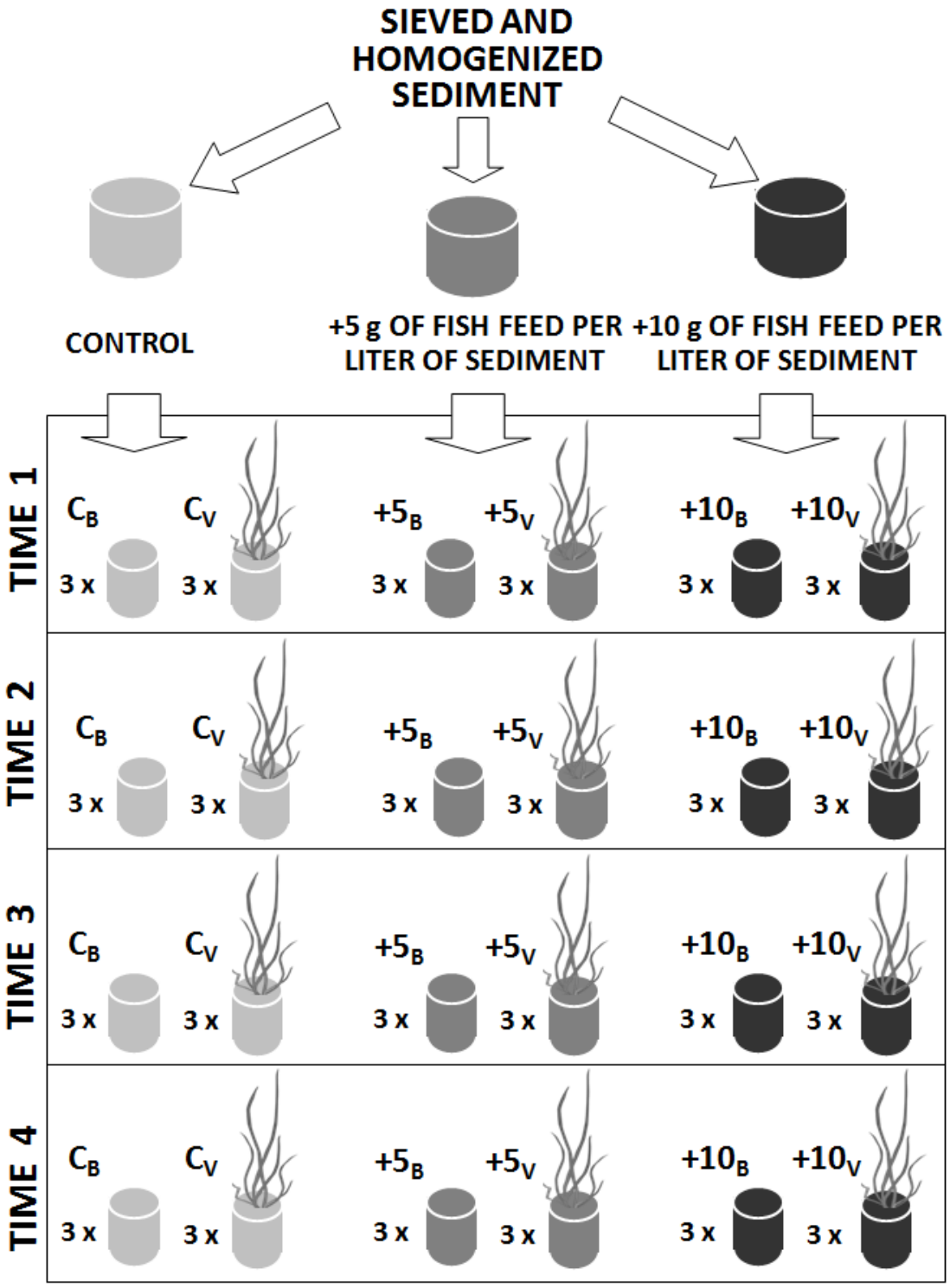

Fig. 6.2. Experimental design. An organic matter gradient (C, +5 and +10) was arranged adding two different amounts of fish feed to sieved and homogenized sediment collected from the Mincio River (northern Italy). 72 microcosms were set up, 24 for each organic matter level, half of which transplanted with a single individual of *V. spiralis*. Pore water features were analyzed at the beginning of the experiment, just after fish feed addition, and after 6 (time 1), 10 (time 2), 13 (time 3) and 17 days (time 4).

6.2.3. Pore water analyses

Redox potential was measured with a potentiometric electrode (Radiometer, DK) connected to a high impedance mV-meter (Crison micro pH 2002, ES). Pore water was then returned to the glove bag and sub-sampled for dissolved CH_4 determination. CH_4 analyses were performed with the headspace equilibration method (McAuliffe, 1971; 2 ml headspace in a 6 ml gas-tight vial) by means of a Fisons 9000 series gaschromatograph equipped with a flame ionization detector (FID). The remaining pore water was filtered through glass fibre filters (Whatman GF/F) for metal and nutrient analyses. Subsamples for Fe^{2+}, Mn^{2+} and PO_4^{3-} were immediately acidified (20 µL of 0.5 M HCl per mL of sample) to avoid precipitation and stored at 5°C until analysis. Fe^{2+} and Mn^{2+} were determined by flame atomic absorption spectroscopy on a Varian model AA240 Atomic Absorption Spectrometer. Nutrients were analyzed with standard spectrophotometric techniques. NH_4^+ was measured on pore water samples immediately after extraction, using salicylate and hypochlorite in the presence of sodium nitroprussiate (Bower and Holm-Hansen, 1980). PO_4^{3-} was measured after reaction with ammonium molybdate and potassium antimonyl tartrate and reduction with ascorbic acid (Valderrama, 1977).

6.2.4. Sediment analyses

Aliquots of control sediment were analyzed for bulk density (measured as the weight of a volume of 5ml fresh material) and water content after drying at 70°C until constant weight. OM content (%) was quantified as loss on ignition (LOI) at 350°C for 3 h on about 0.2 g of dry powdered sediment. Total iron and manganese contents (TFe and TMn) were obtained via acid digestion (Andersen, 1976); metal concentrations were analyzed in the supernatant by means of flame atomic absorption spectroscopy. The most easily reducible Mn fraction was extracted with a buffered ascorbate solution on an aliquot of homogenized fresh sediment, according to the procedure described by Magen et al. (2011). Manganese concentrations were measured with flame atomic absorption spectroscopy. Reduced (Fe^{2+}) and oxidized (Fe^{3+}) labile iron pools were measured via HCl (0.5 M) extraction (Kostka and Luther, 1994). Fe^{2+} was measured after reaction with ferrozine (Stookey, 1970). All results are given in terms of sediment dry weight (DW).

Potential nitrification rates were measured in dark aerobic incubations at room temperature on a shaker table, as already described in Chapter 5 (Hansen et al., 1981; Caffrey et al., 2007). Briefly, a known volume of sediment (1ml) was added to 20 ml of filtered water from the site, enriched with NH_4Cl to give a final concentration of 100 µM. After 20 h, an aliquot of the slurry was centrifuged

at 3000 rpm for 10 min, filtered through a GF/F filter and analyzed for nitrate plus nitrite. NO_3^- was measured after reduction to NO_2^- with activated cadmium. NO_2^- was determined spectrophotometrically using sulphanilamide and N-(1-naphtyl)ethylendiamine (Golterman et al., 1978). Potential nitrification rates were calculated from accumulation of NO_2^-+NO_3^- over time and expressed in terms of sediment dry weight (DW).

6.2.5. Statistical analyses

The effect of OM enrichment, presence of *V. spiralis* and time on pore water features (Eh, CH_4, Fe^{2+}, Mn^{2+}, PO_4^{3-}, NH_4^+) was tested by means of a three-way ANOVA. Potential nitrification rates were tested via two-way ANOVA, with OM enrichment and presence of *V. spiralis* as factors. Correlative relationships among pore water variables were identified using the Pearson's Correlation Coefficient (r). Differences were considered not significant if $p>0.05$. Normality and homoscedasticity of data were examined and Box-Cox transformation was used when necessary. Statistical analyses were performed with R statistical package (R-Development Core Team, 2011). Average values are reported with associated standard error (SE).

6.3. Results

6.3.1. Pore water analyses

Temporal changes in pore water features during the 17 days are reported in Fig. 6.3. OM level, presence of plant and time all had a highly significant effect on interstitial water chemistry evolution, as evidenced by the results of the three-way ANOVA (Tab. 6.1).

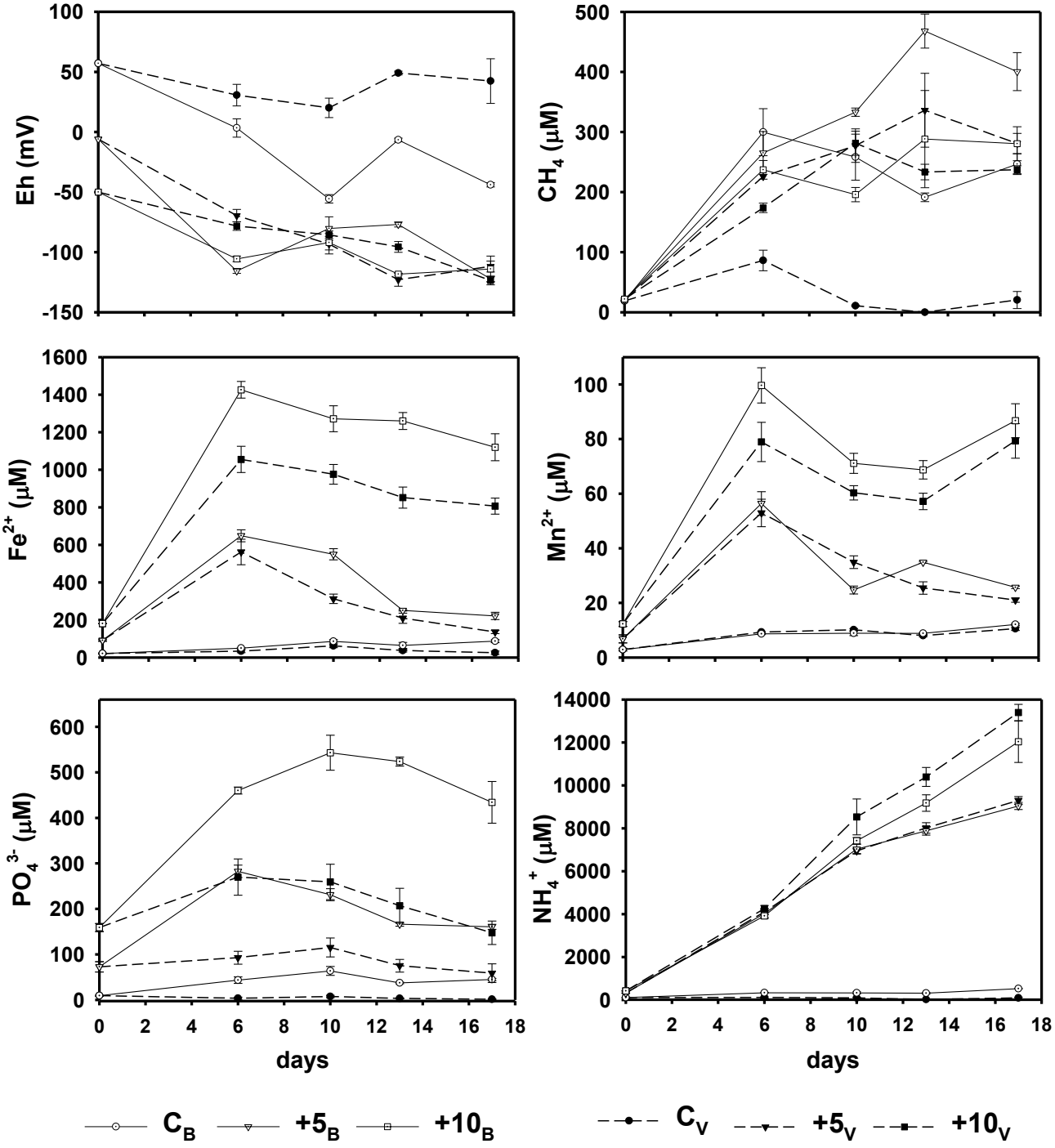

Fig. 6.3. Trends of pore water Eh, CH_4, Fe^{2+}, Mn^{2+}, PO_4^{3-} and NH_4^+ measured in each experimental condition during the 17-day incubation period (C_V, control vegetated sediment; C_B, control bare sediment; $+5_V$, 5g enriched vegetated sediment; $+5_B$, 5g enriched bare sediment; $+10_V$, 10g enriched vegetated sediment; $+10_B$, 10g enriched bare sediment; average $\pm$ standard error, n=3).

Table 6.1. Results of the three-way ANOVA performed to test the effects of OM level, presence of plants and time on pore water features (Eh, CH_4, Fe^{2+}, Mn^{2+}, PO_4^{3-} and NH_4^+). *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$; NS=not significant

Variable	Factor	df	MS	F	*p*
Eh	OM level	2	75499	1336.18	***
	Presence of plant	1	9841	174.16	***
	Time	3	1550	27.44	***
	OM level x Presence of plant	2	5805	102.74	***
	OM level x Time	6	1294	22.90	***
	Presence of plant x Time	3	533	9.43	***
	OM level x Presence of plant x Time	6	1870	33.10	***
	Error	43	57		
CH_4	OM level	2	17348	108.29	***
	Presence of plant	1	16520	103.12	***
	Time	3	143	0.89	NS
	OM level x Presence of plant	2	6901	43.08	***
	OM level x Time	6	1245	7.77	***
	Presence of plant x Time	3	193	1.20	NS
	OM level x Presence of plant x Time	6	248	1.55	NS
	Error	46	160		
Fe^{2+}	OM level	2	377.90	1915.22	***
	Presence of plant	1	19.89	100.82	***
	Time	3	7.18	36.38	***
	OM level x Presence of plant	2	0.07	0.35	NS
	OM level x Time	6	5.01	25.38	***
	Presence of plant x Time	3	0.25	1.28	NS
	OM level x Presence of plant x Time	6	0.32	1.61	NS
	Error	47	0.2		
Mn^{2+}	OM level	2	63.34	2079.29	***
	Presence of plant	1	0.34	11.22	**
	Time	3	1.16	38.16	***
	OM level x Presence of plant	2	0.14	4.53	*
	OM level x Time	6	0.86	28.10	***
	Presence of plant x Time	3	0.19	6.12	***
	OM level x Presence of plant x Time	6	0.10	3.30	**
	Error	48	0.03		
PO_4^{3-}	OM level	2	987.64	479.63	***
	Presence of plant	1	588.33	285.71	***
	Time	3	25.04	12.16	***
	OM level x Presence of plant	2	5.69	2.76	NS
	OM level x Time	6	2.68	1.30	NS
	Presence of plant x Time	3	4.30	2.09	NS
	OM level x Presence of plant x Time	6	2.64	1.28	NS
	Error	48	2.06		
NH_4^+	OM level	2	40253	4932.80	***
	Presence of plant	1	139	17.04	***
	Time	3	1727	211.66	***
	OM level x Presence of plant	2	509	62.41	***
	OM level x Time	6	491	60.18	***
	Presence of plant x Time	3	3	0.33	NS
	OM level x Presence of plant x Time	6	10	1.22	NS
	Error	48	8		

The presence of roots, in particular in the early stage of the incubation, significantly affected Eh values, which were comparatively less reduced than in bare sediments. During the course of the experiment, redox potential of C_V microcosms remained always above 0 mV, while a decline from +57 to about -56 mV was observed in C_B microcosms. In enriched sediments, Eh values underwent similar trends, with a significant distinction between vegetated and bare microcosms only up to day 6. The accumulation of anaerobic metabolism end-products in interstitial water of organic-rich sediment resulted in a steady Eh decrease (down to -133 mV) during the rest of the incubation time. At the end of the experiment the presence of *V. spiralis* did not appreciably affect redox status in enriched sediment.

Dissolved CH_4 concentrations exhibited a distinct pattern in vegetated and bare sediments of C treatment. *V. spiralis* presence exerted a significant effect on pore water CH_4 concentrations that decreased significantly from day 10 and remained always below 40 µM during the rest of the incubation. On the contrary, in C_B sediments concentrations were always higher than 180 µM. Enriched microcosms showed large gas bubble formation during the course of the experiment and CH_4 escaped with sediment resuspension. Concentrations in enriched sediments were constantly greater (up to 470 µM) than in C ones, but the patterns were more erratic and the effect of plant presence not always clear. Pore water CH_4 was negatively correlated with redox potential ($r=-0.709$, $p<0.01$; pooled data, n=81).

Interstitial Fe^{2+} and Mn^{2+} concentrations exhibited distinct patterns in vegetated and bare sediments at each OM level. Soluble iron and manganese species in pore water ranged from 6 to 1370 µM and from 4 to 107 µM respectively. Dissolved iron was generally one order of magnitude greater than dissolved manganese, reflecting the different magnitude of sedimentary metal pools (see later in the text). Differences between vegetated and bare sediments were less evident in C microcosms, where Fe^{2+} and Mn^{2+} concentrations were always less than 115 and 12 µM respectively. Fe^{2+} accumulated quickly in pore water of enriched sediments from day 1 to day 6, both in vegetated and bare microcosm. In +10 sediments Fe^{2+} concentrations remained essentially unchanged from day 6 till the end of the experiment, while in +5 sediments they decreased significantly. Mn^{2+} concentrations followed the same pattern up to day 6, while during the rest of the experimental time the trends were more erratic. Both pore water Fe^{2+} and Mn^{2+} were negatively correlated with redox potential (for Fe^{2+} $r=-0.629$, for Mn^{2+} $r=-0.679$, $p<0.01$; pooled data, n=81).

PO_4^{3-} concentrations showed a distinctive pattern in vegetated and bare microcosms of all OM levels. PO_4^{3-} values were undetectable or up to 12 µM in C_v microcosms while always greater than 30 µM in C_B microcosms. In enriched sediments PO_4^{3-} concentrations ranged between 50 and 300 µM and between 100 and 620 µM in +5 and +10 sediments, respectively. PO_4^{3-} values were significantly higher in bare than in vegetated sediments of each OM level and they showed similar trends, with an increase from day 1 to day 6 followed by stable or slightly decreased concentrations towards the end of the experiment. Pore water phosphorus and metal concentrations were highly correlated (for Fe^{2+}, r=0.920; for Mn^{2+} r=0.822; p<0.01, pooled data, n=81) (Fig. 6.4).

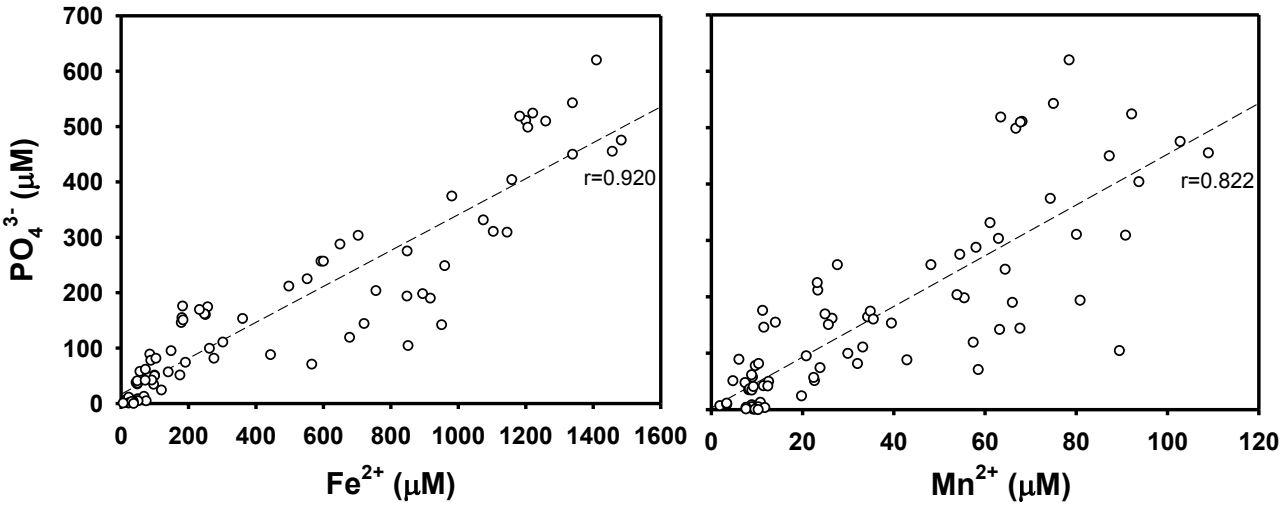

Fig. 6.4. Correlations between dissolved metals (Fe^{2+} and Mn^{2+}) and PO_4^{3-} concentrations in microcosm pore water (pooled data, n=81). Pearson's Correlation Coefficient (r) is reported.

NH_4^+ was produced immediately by hydrolysis of macromolecules upon OM addition and pore water concentrations exhibited distinct trends in control and enriched sediments. NH_4^+ values were always lower than 160 and 540 µM in C_v and C_B microcosms, respectively. In enriched sediments NH_4^+ concentrations increased quickly and constantly during the whole experimental time: at the end of the incubation values were about 9000 and 12000 µM in +5 and +10 sediments, respectively (pooled data). From day 6, in +10 treatment, NH_4^+ concentrations were generally higher in vegetated microcosms than in bare ones.

6.3.2. Sediment analyses

Control sediment used for microcosm set up was muddy with a bulk density of 1.3 g cm^{-3}, a water content of 66% and an OM content (as LOI) of 9.9%. Average TFe and TMn were 223.2±5.0 and 2.5±0.1 µmol g $_{DW}^{-1}$, respectively. Ascorbate extractable Mn was 0.14±0.02 µmol g $_{DW}^{-1}$,

representing about 6% of total sedimentary content. Total acid extractable iron (Fe(II) + Fe(III)) was about 58.0±1.4 µmol g_{DW}^{-1} of which on average 4.1±0.7 µmol g_{DW}^{-1} in the oxidized form.

At the end of the experiment, sediment nitrification activity significantly differed among the treatments ($p<0.001$) (Fig. 6.5). Nitrification rates in control sediments were generally higher than 150 nmol NO_x^- g_{DW}^{-1} h^{-1} and not statistically different between vegetated and bare microcosms ($p>0.05$). Rates measured in enriched vegetated substrate were 5 and 25-fold lower for +5 and +10 treatments, respectively, than those measured in control ones. Nitrification activity was not detectable in enriched bare sediments.

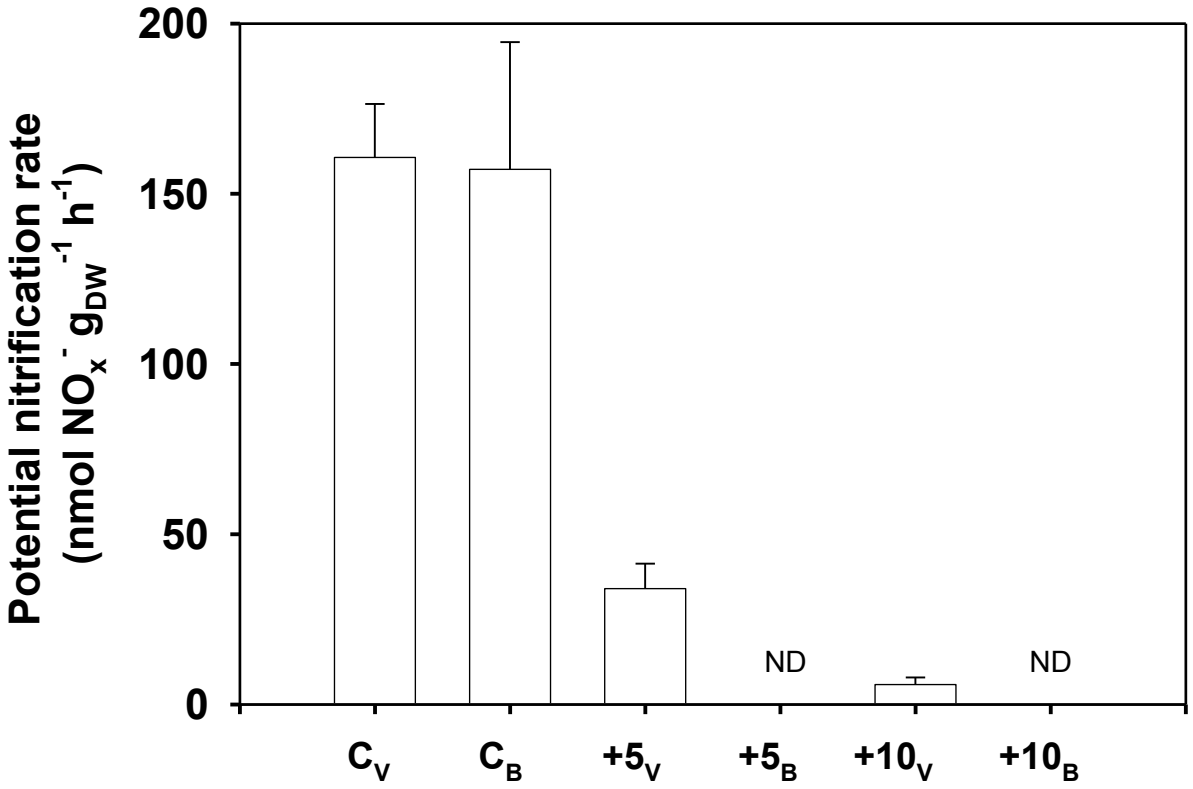

Fig. 6.5. Potential nitrification rates measured in sediments of each experimental condition on day 17 (C_V, control vegetated sediment; C_B, control bare sediment; $+5_V$, 5g enriched vegetated sediment; $+5_B$, 5g enriched bare sediment; $+10_V$, 10g enriched vegetated sediment; $+10_B$, 10g enriched bare sediment; average ± standard error, n=3). Rates in $+5_B$ and $+10_B$ sediments were not calculated as the accumulation of NO_x^- during the assay was not detectable.

6.4. Discussion

6.4.1. OM enrichment, shift to anaerobic metabolism and accumulation of potentially toxic end-products in pore water

Aquatic environments impacted by human activities as urban canals and ponds are generally enriched with high amounts of labile organic matter, that turns sediment reduced and harmful to roots (Cotano and Villate, 2006; Poté et al., 2008; Pulido et al., 2010). To mimic the chemical conditions that are established in such situations I added an easily degradable source of OM with a low C/N ratio (∼7) and a high protein content to a sediment with an elevated OM background. To these features are usually associated high microbial activity, oxygen exhaustion, shift to strictly anaerobic metabolism and accumulation of reduced solutes and nutrients in pore water (Mascaró et al., 2009; Valdemarsen et al., 2009). The chosen levels of organic enrichment simulated those of highly impacted sediments as those below fish farms, which represent an extreme of eutrophication gradient (Holmer et al., 2007). The macromolecular quality of the added OM plays a key role in turning the sediment into a hostile environment for roots. Refractory OM, undergoing extremely slow decomposition, may not affect significantly the pore water redox status, while on the contrary, under even a slight increase of extremely labile OM sediment may become very reducing and harmful to roots (Lenssen et al., 1999; Pulido et al., 2010).

Organic enrichment was performed in a sediment with an elevated OM background (∼10% as LOI). From the fish feed composition I calculated that the sedimentary organic carbon pool increased by about 16% and 31% in +5 and +10 enrichment, respectively. The addition of fish feed augmented sedimentary nitrogen pool by 7.8% and 15.5% in +5 and +10 treatments, respectively; similarly, the phosphorus pool increased by 9.0% and 18.1%, respectively. However, the relatively low fish feed inputs consisted of fresh, extremely reactive OM compared to that stocked within control sediments, leading to an immediate stimulation of mineralization processes. High chemical and microbial oxygen consumption probably minimized the thickness of oxic layers around roots. Enriched microcosms had dark brown sediments and no oxidized halos were evident in the rhizosphere (Fig. 6.6). Sediment chemistry was very susceptible to OM addition and resulted in an immediate accumulation of anaerobic metabolism end-products. Pore water Eh values fell within the range in which nitrate, manganese and iron are the more energetically favorable electron acceptors (Schüring et al., 1999; Neubauer et. al, 2008), even if some limitations were probably connected with this measurement. The Eh measurable by a platinum electrode is in fact

determined mainly by reactive species such as oxygen, Fe^{2+} and Mn^{2+}. Other soluble phase redox couples such as SO_4^{2-}/H_2S, CO_2/CH_4, NO_3^-/N_2 and N_2/NH_4^+ do not directly influence the measurement of the redox potential which is in conclusion only partially informative of the intensity of oxidation–reduction processes (Schüring et al., 1999).

Release of gas bubbles from the +5 and +10 treatments during handling of microcosms was an evident sign of the effects of organic enrichment on sedimentary processes. High CH_4 concentrations in pore water suggested that methanogenesis became a significant degradation pathway when other electron acceptor pools were depleted (Jespersen et al., 1998; Neubauer et. al, 2008). Pore water methane concentrations measured in +10 microcosms, especially the bare ones, could be partially underestimated because of frequent gas escape from sediment. Coupled nitrification-denitrification was probably limited by low pore water nitrate concentrations due to nitrification inhibition by reducing conditions. The sedimentary oxidized iron pool was only about 7% of the total Fe HCl extractable, and so quickly consumed by microbial reduction or abiotic reoxidation of reduced compounds. Manganese and sulfate reduction were probably constrained by low concentrations of these electron acceptors: reactive manganese pool was about two order of magnitude smaller than the iron one and sulphate content is usually relatively small in freshwater sediments (Holmer and Storkholm, 2001). Although not measured in this study, free S^{2-} may have been present as a consequence of OM degradation via sulfate reduction: sulfide smell and very dark sediments could be in fact detected during microcosm handling. The depletion of pore water Fe^{2+} and Mn^{2+} after day 6 in both enriched treatments suggested the precipitation of iron monosulfides or pyrite and manganese sulfide (Murray, 1995; Webb et al., 1998).

Degradation of the added OM may have led to the formation and accumulation in pore water of reduced inorganic (Fe^{2+}, Mn^{2+}, S^{2-}, NH_4^+) and organic (ethylene, acids, alcohols) species at levels toxic to freshwater macrophytes (van Wijck et al., 1992; Britto and Kronzucker, 2002; Gibbs and Greenway, 2003; Wu et al., 2009). For example, in the reduced forms, iron becomes soluble thus more readily bioavailable for uptake by root. The accumulation in tissue can produce significant physiological and biochemical responses, as inhibiting synthesis of chlorophyll and proteins and thus altering photosynthesis and respiration (Xing et al., 2010). Ammonium and iron stress could occur, since interstitial concentrations in enriched substrates were much greater than those found in previous toxicity studies (Talbot and Etherington, 1987; van Wijck et al., 1992; Britto and Kronzucker, 2002).

All *V. spiralis* specimens survived the transplant and they were alive during the course of the 17-day experiment (visual observations). However, while plants in control sediment appeared healthy, those in enriched substrates showed evident signs of stress, especially in +10 treatment, probably as a consequence of oxygen shortage and high levels of phytotoxins in pore water. Decrease in root development occurred in more reducing sediments: root decay (blackening and increasing flaccidity) was clearly visible in all the specimens exposed to organic rich substrates (Fig. 6.6). Plants were anchored with just the primary root and shedding of all the lateral fine roots was evident. This could be considered as an index of adaptive plasticity of *V. spiralis* to minimize oxygen loss in high demand sediment and maintain the sufficient supply to primary root tissue. Longitudinal oxygen diffusion in roots is in fact enhanced by morphological features such as a small number of lateral roots (Colmer, 2003). Moreover plants in enriched substrates produced plagiotropic stolons that spread horizontally above ground and formed new ramets out of the microcosms. Previous studies have proven that *V. spiralis*, if established in adverse conditions, can escape hostile sediment patches by clonal growth (Xiao et al., 2006). In addition to stressful chemical conditions, organic enrichment may also affect the physical properties of the substrate, leading to soft and less consolidated sediment and increasing the risk of uprooting (Pulido et al., 2010; Raun et al., 2010).

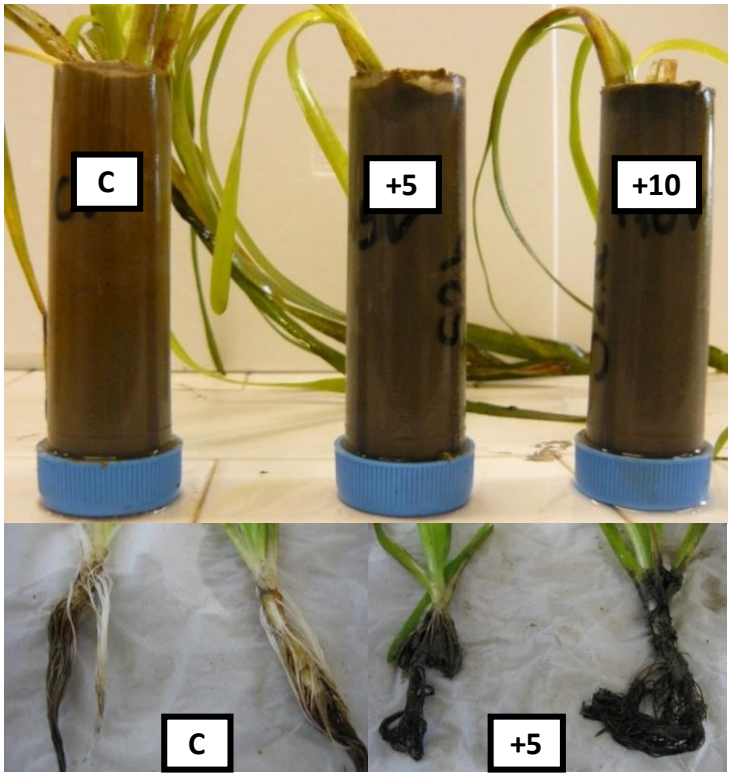

Fig. 6.6. Some particulars of microcosms and root systems of the three organic matter levels.

6.4.2. Effect of *V. spiralis* on pore water chemistry

V. spiralis can affect the rhizosphere chemistry as a consequence of direct (solute uptake) and indirect effects (ROL). In order to evaluate the direct effect of plant presence on pore water features, I estimated N and P theoretical uptake during the first six days of the experiment. I assumed that *V. spiralis* assimilated preferentially NH_4^+ and PO_4^{3-} from the roots, as previously reported (Carignan and Kalff, 1980; Racchetti et al., 2010). I considered for this macrophyte a mean net growth rate in spring of 0.055 d^{-1} (for the total biomass), a conservative nitrogen content of 30.7 mg N g_{DW}^{-1} and a conservative phosphorus content of 7.4 mg P g_{DW}^{-1} (Pinardi et al., 2009). In the first 6 days I calculated that each plant assimilated on average ~260 and ~29 µmol of N and P, respectively. Nitrogen uptake was appreciable only in control sediments, where difference in NH_4^+ trends and pools between bare and vegetated sediment suggested a tight coupling between ammmonification and assimilation by roots. In fact pore water NH_4^+ concentrations remained almost constant in the presence of *V. spiralis* while they increased in bare sediments. In +5 and +10 treatments ammonification largely exceeded the plant uptake and NH_4^+ accumulated at high rates without clear differences between vegetated and bare microcosms. On the other hand, towards the end of the experiment NH_4^+ concentrations were generally higher in vegetated microcosms than in bare ones, especially for the +10 treatment. Oxygen released by root may have stimulated the aerobic degradation of OM resulting in greater pore water NH_4^+. Moreover, organic compounds exuded by the roots, representing an additional source of easily degradable OM, could have fuelled microbial metabolism (Karjalainen et al., 2001; Dennis et al., 2010).

Different results were obtained for phosphorus where in all organic levels the presence of *V. spiralis* affected SRP concentrations. In control microcosms this was likely a combination of direct (uptake) and indirect (precipitation with oxidized iron) effects due to the presence of roots. I speculate that, as for nitrogen, the regeneration of mineral phosphorus was coupled with the assimilation by the plant. On the contrary, in +5 and +10 treatments organic phosphorus mineralization largely exceeded plant uptake, meaning that the indirect effect (creation of an oxic rhizosphere by ROL) was probably responsible for the observed differences in the presence and absence of roots. Accumulation of SRP in enriched bare sediments compared to vegetated ones resulted from the reduction of iron oxides/hydroxides and the consequent mobilization of the adsorbed phosphorus, as shown by the highly significant correlation between pore water PO_4^{3-} and Fe^{2+} concentrations.

Oxygen release by the roots of *V. spiralis* resulted in a generally more oxidizing environment (higher Eh) and lower concentrations of reduced solutes (CH_4, Fe^{2+} and Mn^{2+}). In control sediment, the low methane content of vegetated microcosms could be ascribed to a combination of methanogenesis inhibition and methane oxidization stimulation (Jespersen et al., 1998). Moreover control microcosms presented reddish-brown sediment mainly due to oxidized iron species and visible *V. spiralis* roots were surrounded by a <2mm thick layer of light brown substrate as a consequence of oxidized conditions (visual observations). Oxygen pumping by roots leads to iron oxidation within the rhizosphere and the consequent formation of insoluble iron plaques coating the roots (St-Cyr and Campbell, 1996; Sand-Jensen et al., 2008). Red-colored deposition on roots were clearly detectable only on root surface in control substrates (visual observations) and could be considered as a proxy of ROL.

Even in enriched microcosms, lower levels of reduced iron and manganese species were found in pore water of vegetated sediment, while higher concentrations in bare sediments suggested a greater mobilization of those elements. Moreover differences in Fe^{2+} and Mn^{2+} concentrations between bare and *V. spiralis* rooted sediments were maintained from day 6 till the end of the experiment. I calculated that, in order to explain the difference in metal concentrations (Fe^{2+}+Mn^{2+}) between vegetated and bare sediment during the first six days of the incubation, 2.8±1.8 and 8.4±3.0 nmol of oxygen equivalents per g of fresh sediment per day were needed in +5 and +10 treatments, respectively. Considering the very limited penetration depth in enriched substrates, such oxygen amount was likely provided by ROL. Macrophyte capacity of releasing oxygen in the rhizosphere is an essential ecological adaptation to protect root systems from phytotoxic concentrations of reduced compounds, even if this strategy does not provide unlimited tolerance in increasingly reducing sediments (Colmer, 2003). Direct measurements of ROL for *V. spiralis* are not available, but Li and coauthors (2011) reported for *V. natans* an oxygen loss of about 1000 µmol O_2 g $_{DW}$ $root^{-1}d^{-1}$, determined by means of Ti^{3+}-citrate method. Supposing a similar rate of release for *V. spiralis*, the oxygen amount supplied by plants in the enriched microcosms (expressed on unit of root biomass, 0.89±0.69 and 7.03±2.02 µmol O_2 g $_{DW}$ $root^{-1}d^{-1}$ in +5 and +10 treatments, respectively) represented only <1% of ROL. However, red-colored iron plaques were not detectable on root surfaces in enriched substrates, probably as a consequence of the rapid depletion of oxygen released by roots, due to high sediment oxygen demand.

Finally, other evidences suggested that roots were still active even in highly stressful conditions and, as a consequence, ROL played a significant role in regulating biogeochemical dynamics even

in OM-rich substrates. The nitrification potential test can be considered a tool for screening toxicity in sediment and the oxidizing activity is presumably proportional to nitrifying bacteria abundance (Caffrey et al., 2007). In organic enriched sediments, *V. spiralis* promoted the maintaining of the nitrifying community, even if its activity decreased from +5 to +10 treatment. Indeed potential nitrification rates were not detectable in enriched bare sediments, most likely due to the instauration of a reduced environment, harmful to the strictly aerobic nitrifiers. The decline in nitrification activity along the OM gradient was probably a result of oxygen shortage due to high sedimentary uptake not completely balanced by ROL. Moreover a variety of compounds, such as sulfide and ammonia, could have adversely affected the growth and activity of the nitrifying community (Anthonisen et al., 1976; Strauss and Lamberti, 2000; Sears et al., 2004).

Results from the present study demonstrated that the addition of fish feed represents an effective way to simulate sedimentary enrichment by very labile organic matter, as that occurring in many eutrophic aquatic environments undergoing primary producers bloom and collapse phases. Fish feed immediately stimulated sediment metabolism and deeply altered its redox status, with a series of cascade effects on pore water solutes.

In conclusion, I provide multiple, indirect evidences based on the analysis of pore water that *V. spiralis* is a tolerant macrophyte that can withstand large perturbations of sedimentary features and it can partly buffer the negative effects of such organic enrichment, which was on purpose rather extreme. Despite damaged, the macrophyte had a significant effect on SRP, Fe^{2+} and Mn^{2+} pore water concentrations. *V. spiralis* probably reduced its root biomass and maintained a minimum oxygen release in the rizhoshere, as suggested by potential nitrification rates.

7. Benthic nitrogen cycling under increasing sedimentary organic matter loads

7.1. Aim

Under N-limiting conditions, the activity of benthic vegetation generally inhibits denitrification rates because of the competition for N with bacteria involved in nitrogen cycling (nitrifiers and denitrifiers). In eutrophic riverine environments nitrogen is not limiting neither in water column (especially in the form of nitrate, due to runoff from the human-impacted watershed) nor in pore water (due to great availability from mineralization processes of accumulating organic matter). This condition would stimulate the assimilation of nitrate from the canopy of tolerant macrophytes, making the contribution of root uptake less important. An interesting question is therefore to evaluate whether the shift in the preferential N uptake compartment can slow down the competition in the rhizosphere between plant and bacteria. I speculate that, along a gradient of increasing N availability (i.e. organic gradient in sediment), N loss from sediment via nitrification-coupled denitrification can be stimulated. With an augment in sedimentary organic content, dentrification rates may increase as N limitation is attenuated, and then would likely decline as sediments become highly reduced and potentially toxic to denitrifiers.

The aim of the present work was to compare assimilatory (plant uptake) and dissimilatory (denitrification) N processes in non-N limiting conditions. Light and dark gas (O_2, CH_4) and nitrogen (NO_3^-, NO_2^-, NH_4^+) fluxes at the sediment-water interface and denitrification rates were measured in *V. spiralis* vegetated and bare microcosms along an organic matter gradient. Denitrification coupled to nitrification in the rizosphere of *V. spiralis* in light and dark conditions was also assessed.

7.2. Materials and Methods

7.2.1. Sampling procedure and microcosm setup

In summer 2011 sediment, water and *V. spiralis* specimens were collected from a shallow water eutrophic site (Mincio River, Massimbona location) (Fig. 7.1). The experiment was carried out during the biomass peak phase of the macrophyte (Pinardi et al., 2009). The hybrid approach

introduced by Ribaudo et al. (2011) based on incubations of microcosms under controlled conditions after an *in situ* acclimatization period was adopted.

Over 70 l of sediment from the upper 10 cm depth horizon were collected via Plexiglass cores and sieved with a 2 mm mesh in order to remove coarse plant debris, macrofauna and stones, and then homogenized. Thereafter sediment was divided and transferred into five 12 l tanks. One tank was left untreated and served as control (C) while the others were added with increasing amounts of organic matter in the form of commercially available fish feed pellets (49% organic C, 8% organic N and 1% organic P), previously dried at 50°C and ground to a powder on a mortar. 1, 2.5, 5 and 10 g of ground fish feed pellets per liter of sediment ware added to the four sieved sediment samples, then carefully homogenized by hand (Fig. 7.1 and Fig. 7.2). The previous laboratory microcosm experiment has confirmed the stimulation of microbial metabolism with the used OM type and the adopted levels of enrichment (see Chapter 6). The characterization (bulk density, porosity and OM content) of control sediment is reported in Chapter 6. Simultaneously, over 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 chosen for the subsequent transplant. Sediments of each OM level were transferred into cyclindrical Plexiglass microcosms of three different dimensions for the measurements of benthic metabolism (see later in the text). For each OM level, 6 microcosms with the outer diameter of 4 cm were left unvegetated. Randomly selected individuals of *V. spiralis* similar in size were transplanted in microcosms of two different dimensions: 6 cyclindrical microcosms with the outer diameter of 8 cm (3 shoots in each) and 2 cyclindrical microcosms with the outer diameter of 20 cm (20 shoots in each). This procedure was repeated for each OM level (Fig. 7.2). Plant density in microcosms reflected that previously measured *in situ* in summer months and already reproduced in other microcosm experiments (Racchetti, 2010; Ribaudo et al., 2011). All the microcosms were located on the river bottom, within vegetated and non-vegetated patches, and left *in situ* for about 10 days under natural conditions of temperature, irradiance, water chemistry and flow. After the acclimatization period, microcosms were transferred underwater into Plexiglass liners with compatible diameter, minimizing the disturbance to plant canopy and carefully retrieved from the river. Simultaneously over 200 l of river water were collected for pre-incubation and incubation procedures. Within two hours from the recovery, all liners were brought fully submerged to the laboratory for further processing.

In the laboratory, vegetated and unvegetated microcosms were kept in pre-incubation tanks submerged by river water continuously aerated with aquarium pumps and maintained at field temperature (about 24°C). Microcosms were subject to a 16/8h light/dark cycle at an irradiance of about 400 µmol photons m^{-2} s^{-1} (Photosynthetically Active Radiation, PAR) by means of 1000-W halogen lamps prior to the start of the experiment and between one incubation and the other. Water temperature was measured with a 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 maintained in the same tank to eliminate possible effects of nutrient availability resulting from the release from enriched sediments. However, water was regularly replenished to avoid extensive nutrient accumulation and to minimize algal growth. The preincubation period lasted a 24h light-dark cycle.

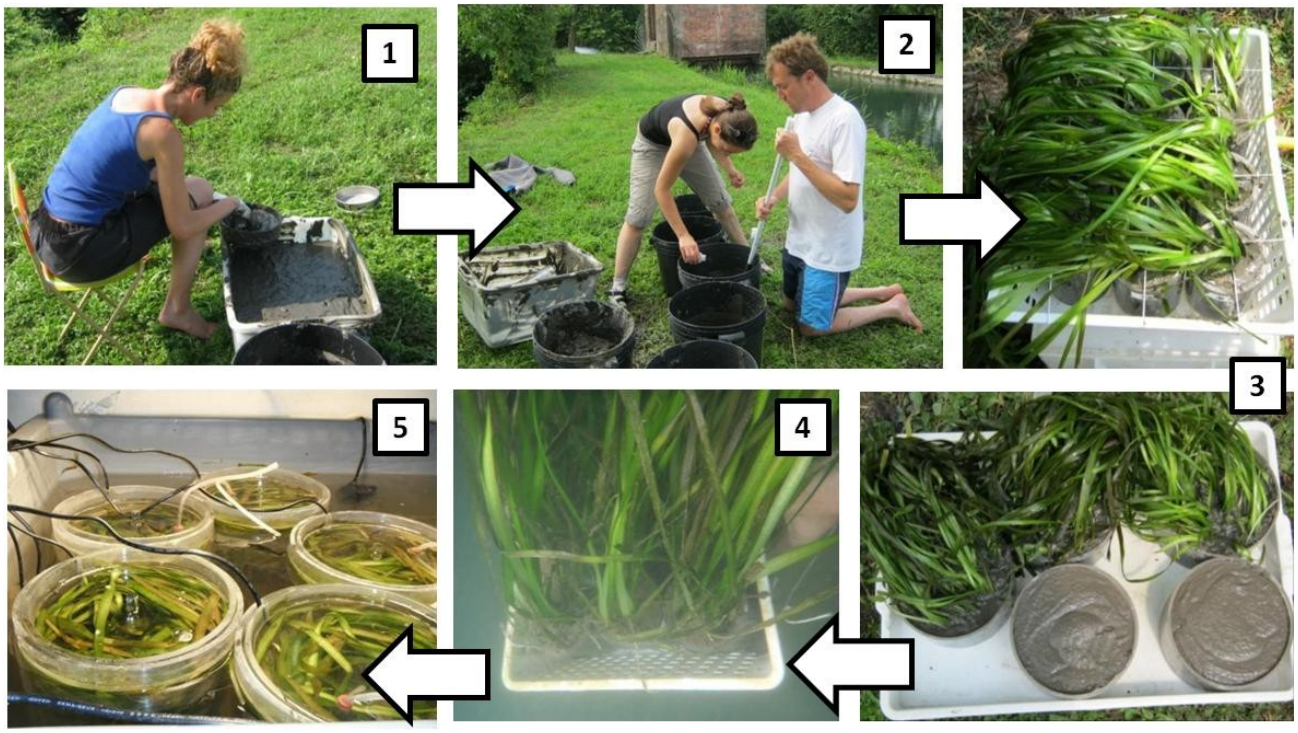

Fig. 7.1. Experimental procedure: 1) sediment sieving; 2) sediment enrichment; 3) microcosm set-up; 4) microcosm recovery after the *in situ* acclimatization period; 5) laboratory incubations.

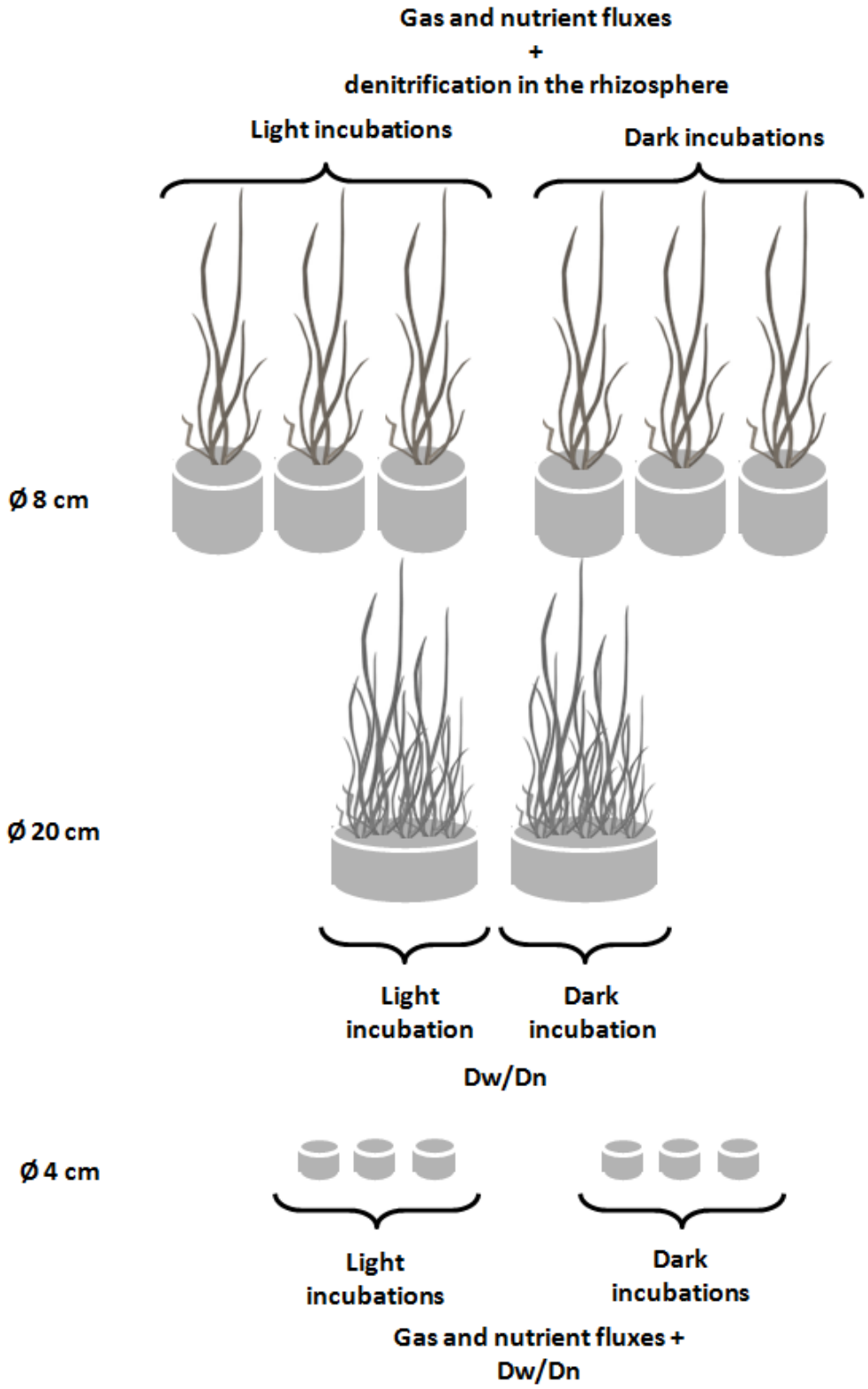

Fig. 7.2. Experimental design. Different types of microcosms set up for each organic matter level for incubations purpose (fluxes of gases and nutrients, denitrification in the rhizosphere, denitrification of water column nitrate – Dw – and surface denitrification coupled to nitrification – Dn).

7.2.2. Measurements of gas and nutrient fluxes

Microcosms were incubated at *in situ* temperature according to standard procedures (Dalsgaard et al., 2000) for flux measurements of O_2 (sediment oxygen demand, SOD), methane (CH_4), and dissolved inorganic nitrogen forms (NO_3^-, NO_2^-, NH_4^+). For each OM level, three 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. For incubation procedure, microcosms were transferred into transparent Plexiglass liners with a compatible diameter (height 30 cm). 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 and driven by an external motor (40 rpm). Light incubations were performed at an irradiance of about 400 µmol photons m^{-2} s^{-1} by means of halogen lamps. Incubation time (<3h) was set in order to keep the variation in oxygen concentration within 20% of the initial value. When incubations started, the water in the tank was lowered just below the top of the liners and each core was sealed with a Plexiglass transparent lid with a water sampling port. Water samples (60 ml) for gas and nutrient determinations were collected at regular time intervals using plastic syringes. Samples for gas determinations were transferred to glass-tigh vials (12 ml Exetainer®, Labco, High Wycombe, UK). For oxygen analyses Winkler reagents were immediately added, while for CH_4 analyses saturated mercuric chloride solution was added to prevent biological activity. Samples for nutrient determinations were filtered through Whatman GF/F glass fiber filters, transferred to polyethylene vials (NO_3^-, NO_2^-, NH_4^+) and frozen for later analyses. O_2 was measured with Winkler titration (APHA, 1981). 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 (FID). Ammonium was determined on a double bean Jasco V-550 spectrophotometer (Bower and Holm-Halsen, 1980). Nitrite and nitrate were measured on a Technicon AutoAnalyser II (Armstrong et al., 1967). Gas and nutrient hourly fluxes were calculated with a linear regression of concentrations versus time and expressed as rate per square meter.

7.2.3. Denitrification associated with the rhizosphere

Following measurements of dissolved gas and nutrient fluxes, vegetated microcosms (Ø 8 cm) were incubated to estimate the coupled nitrification-denitrification activity in the rhizosphere of *V. spiralis* by means of a modified version of the Isotope Pairing Technique (Caffrey and Kemp, 1992;

Nielsen, 1992). The methodology assumes that several centimetres below the water-sediment interface nitrate for denitrification is produced only by nitrification within the sediment and the source of the required oxygen can only be the oxygen released by the macrophyte. The assay is based on the direct addition of $^{15}NH_4^+$ to the rhizosphere and the quantification of $^{15}N_2$ produced from denitrification coupled to nitrification. Each microcosm was provided with 36 side ports (4 series of 9 ports each) filled with silicon glue, allowing the injection of solutions in pore water. In each port 250 µl of an anoxic 10mM $^{15}NH_4Cl$ solution (98 atom % ^{15}N enrichment) was injected by means of glass syringes (Hamilton 725RN 250 µl), for a total volume of 9 ml per microcosm. The syringe was pulled gently outwards while injecting the labelled solution, to ensured an homogeneous distribution throughout the rhizosphere. Interstitial ammonium concentrations were measured on sediment samples of the five OM levels after the *in situ* acclimatation period. The added volume of labelled solution was set to increase sedimentary ammonium concentrations of at least 30%.

For incubation procedure, microcosms were transferred into transparent Plexiglass liners with a compatible diameter (height 30 cm). For each OM level, three vegetated (Ø 8 cm) microcosms were used for the light treatment and the same number for the dark treatment. Light incubations were performed at an irradiance of about 400 µmol photons m^{-2} s^{-1} by means of halogen lamps. 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 and driven by an external motor (40 rpm). When incubations started (at *in situ* temperature, 24°C), the water in the tank was lowered just below the top of the liners and each core was sealed with a Plexiglass transparent lid with a water sampling port. After ~4h, sediment and water phases 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). A volume of 200 µl of zinc chloride solution (7 M) was added to each sample to inhibit microbial activity. Samples were stored refrigerated until labelled N_2 analysis. $^{14}N^{15}N$ and $^{15}N^{15}N$ abundance were analyzed by mass spectrometry (Delta V Advantage, Thermo Scientific) at the Department of Geological Sciences, Stockholm University, Sweden. Nitrification-coupled denitrification rates in the rizhosphere were calculated according to Rysgaard et al. (1998).

7.2.4. Denitrification of water column nitrate and surface denitrification coupled to nitrification

Following measurements of dissolved gas and nutrient fluxes, denitrification rates were estimated with the Isotope Pairing Technique (Nielsen, 1992). 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 the other one in dark condition.

For incubation procedure, bare and vegetated microcosms were transferred into transparent Plexiglass liners with a compatible diameter (height 30 cm). The IPT allows to quantify total denitrification (Dtot), denitrification of nitrate diffusing to the anoxic sediment from the water column (Dw) and denitrification of nitrate produced by nitrification within the sediment (Dn). At the beginning of the incubation, labelled nitrate (15 mM $Na^{15}NO_3$ solution, 98 atom% enrichment) was added to the water column to have a final ^{15}N atom% of at least 30% (Dalsgaard et al., 2000). The nitrate concentration was measured prior to the addition of $^{15}NO_3^-$ and immediately before the cores were closed with floating lids (about 15 minutes after the addition, to allow the $^{15}NO_3^-$ porewater profile to reach a steady state), in order to calculate the $^{14}N/^{15}N$ ratio in the initial nitrate pool. Incubations were performed at *in situ* temperature (~24°C) and lasted about 3 h to avoid change in oxygen concentration larger than 20% of the initial value. For light treatment, an irradiance of about 400 µmol photons m^{-2} s^{-1} was provided by means of halogen lamps. At the end of incubations, 3 sub-cores were sampled in each vegetated microcosm and slurry samples were collected and analyzed as previously described. For bare microcosms the procedure was the same adopted for measurement of denitrification associated with the rhizosphere. Denitrification rates were calculated according to the equations and assumptions of Nielsen (1992).

7.2.5. Estimation of *V. spiralis* theoretical N uptake

At the end of all incubations, plants were collected from each microcosms by sediment sieving with a 2 mm mesh. *V. spiralis* specimens were rinsed to remove epiphytes and sediment residues. Plants of each microcosm (separated in above and below ground tissues) were desiccated separately at 70 °C until constant weight. Theoretical N assimilation was calculated from plant net production rates and assuming average values of C to O (see Chapter 4) and C to N ratios (Racchetti et al., 2010) in *V. spiralis* photosynthetic tissues. In order to assign oxygen fluxes to *V. spiralis* activity alone and estimate net production rates, ecosystemic fluxes in vegetated microcosms were corrected for fluxes measured in the corresponding bare sediments.

7.2.6. Statistical analyses

The effects of OM level and light condition (light/dark) on dependent variables (gas and nutrient fluxes and denitrification rates) were tested by means of analysis of variance (two-way ANOVA). For each dependent variable, data from bare and vegetated microcosms were analysed separately to simplify the model and exclude any predictable significance connected to plant activity. Previous studies have already demonstrated that benthic metabolism is significantly affected by *V. spiralis* presence (Pinardi et al., 2009; Racchetti et al., 2010; Ribaudo et al, 2011). Normality (Shapiro–Wilk test) and homoscedasticity (Levene's test) of datasets were previously examined and Box-Cox transformation was used when necessary. Differences were considered not significant if $p>0.05$. Analyses were performed with R statistical package (R-Development Core Team, 2011). In the graphs average values are reported with associated standard deviation (sd).

7.3. Results

7.3.1. Gas fluxes

Results of the two-way ANOVA (Table 7.1) highlighted that the organic enrichment in sediment affected all benthic gas and nutrient fluxes, both in bare and vegetated microcosms. Oxygen fluxes were about one order of magnitude higher in microcosms with *V. spiralis* compared to bare sediments along the whole organic matter gradient (Fig. 7.3 a, b). In bare sediments SOD ranged between 2.8±0.3 (level A) and 6.6±0.7 mmol m^{-2} h^{-1} (level E). A sedimentary oxygen consumption was detected both in light and dark conditions in all the five organic matter levels. Within each organic level, dark and light oxygen fluxes were not significantly different ($p>0.05$), underlining the absence of microphytobenthos activity (Fig. 7.3 a). Oxygen uptake increased along the OM gradient from level A to level E, with the last one having an SOD on average 2.5 times higher than control substrate. A slight decrease in respiration rates of level D with respect to level C was probably a consequence of a not completely homogeneous mixing of the added organic matter (fish feed pellet) to the background sediment. Plant presence tended to increase the global respiration of the system. In vegetated sediments, ecosystemic oxygen fluxes (due to a combination of plant and sediment activity) ranged between -20.6±6.5 and -31.7±5.1 mmol O_2 m^{-2} h^{-1} and between 18.5±8.2 and 65.9±15.9 mmol O_2 m^{-2} h^{-1}, in dark and light condition, respectively (Fig. 7.3 b). Dark oxygen uptake was not different among organic matter levels. Light oxygen

release increased markedly from level A to level B and then it decreased in the following levels (p=0.001).

Methane fluxes ranged between 9.1±1.1 and 1127.8±131.8 µmol CH_4 m^{-2} h^{-1} and between -141.5±89.4 and 2350.3±1008.0 µmol CH_4 m^{-2} h^{-1}, in bare and vegetated microcosms, respectively, and they were dependent upon the organic matter level (p<0.001) (Fig. 7.3 c,d). Plant presence significantly affected both the direction and the magnitude of benthic methane exchanges. In bare microcosms, CH_4 fluxes were close to the detection limit in control sediments and increased progressively along the organic matter gradient (Fig. 7.3 c). For vegetated microcosms, both in the control sediment (A) and in the substrate with the lower organic addition (B), methane exchanges were directed from the water column to the sediment with a greater gas consumption in light conditions (Fig. 7.3 d). In level C CH_4 fluxes were close to zero, while from level D the vegetated sediment turned from a sink to a net CH_4 source both in light and dark conditions, with emission rates always greater than 1000 µmol CH_4 m^{-2} h^{-1} and a general increase from D to E level.

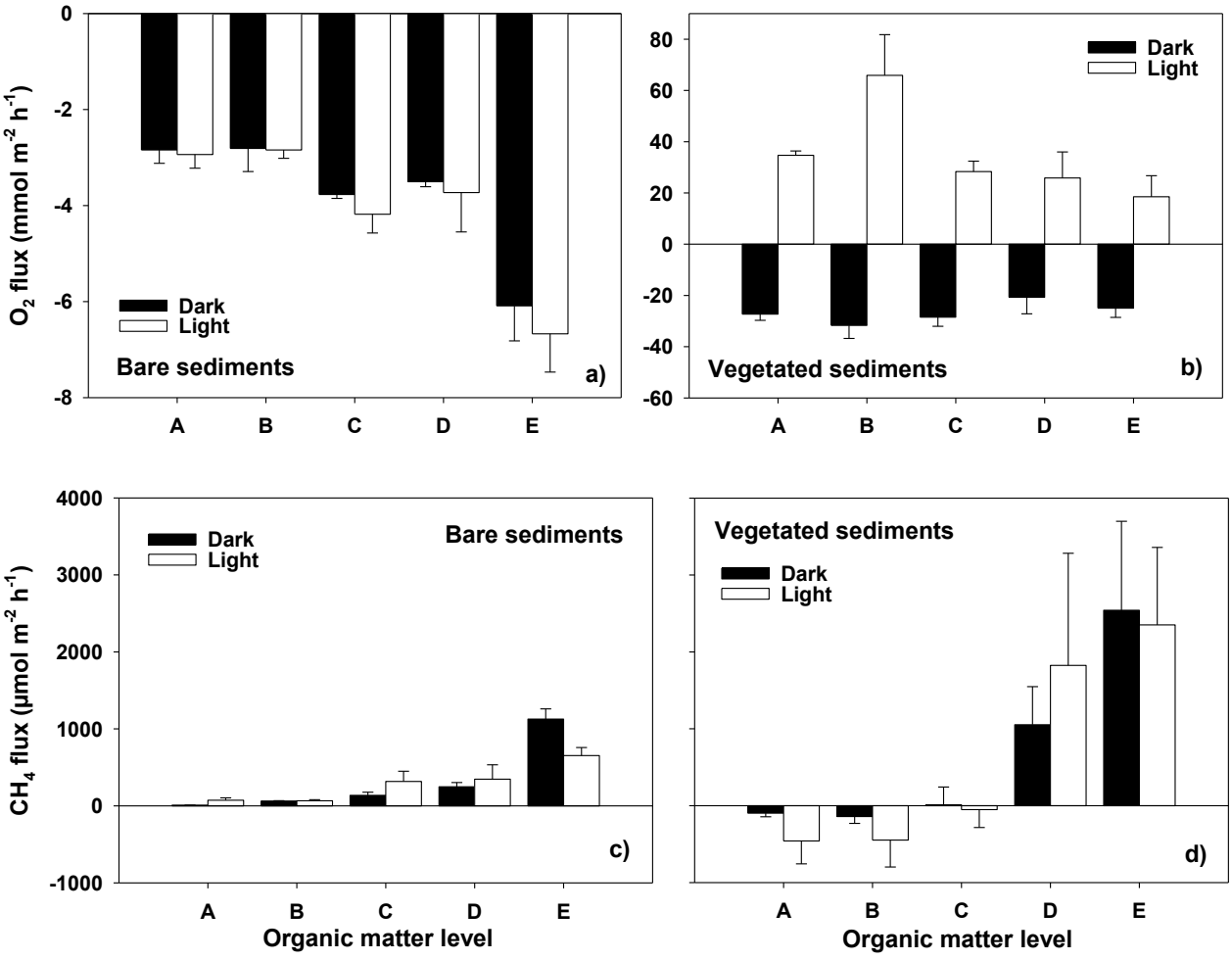

Figure 7.3. Light and dark O_2 and CH_4 fluxes measured in bare and *V. spiralis* vegetated microcosms of the five organic matter levels. Average values ± std. dev. are reported (n=3).

Table 7.1. Results of the two-way ANOVA performed to test the effect of light/dark condition and organic level on gas and nutrient fluxes measured in bare and vegetated microcosms. *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$; NS=not significant

			Bare microcosms			Vegetated microcosms		
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		

7.3.2. Inorganic nitrogen fluxes

Ammonium fluxes ranged between 167.0±133.2 and 1622.5±205.3 µmol N m^{-2} h^{-1} and between -2564.0±1196.5 and 4575.6±592.5 µmol N m^{-2} h^{-1}, in bare and vegetated microcosms, respectively (Fig. 7.4 a,b), and they were dependent upon the organic matter level ($p<0.001$). Significant differences were also detected between dark and light conditions ($p<0.001$). Bare sediments were always a source of ammonium along the whole organic matter gradient, with emission rates generally higher in light condition (Fig. 7.4 a). Differently, *V. spiralis* vegetated sediments were a net ammonium source in all the OM enrichments only in dark conditions and with a general augment with increasing organic matter additions (Fig. 7.4 b). Plant presence increased ammonium release from the sediment compared to the corresponding plant-free treatment. In light condition vegetated sediments were a net ammonium sink till level C with maximum rates measured in level B (∼-2560 µmol N m^{-2} h^{-1}). In the last two levels, sediments regenerate

ammonium also in the light but with emission rates generally lower than the corresponding dark ones.

Nitrate fluxes ranged between -1089.1±126.3 and -186.4±68.8 µmol N m^{-2} h^{-1} and between -5717.8±1445.0 and 1205.7±296.3 µmol N m^{-2} h^{-1}, in bare and vegetated microcosms (Fig. 7.4 c,d), respectively, and they were dependent upon the organic matter level ($p<0.001$). Significant differences were also detected between dark and light conditions ($p=0.019$). Plant-free sediments were always a nitrate sink even if a regular pattern between light and dark conditions and along the OM gradient was not detected (Fig. 7.4 c). An evident increase in nitrate uptake was recorded only in the last level where rates more than doubled those measured in control substrates. Contrary to what observed with ammonium, *V. spiralis* vegetated sediments were a nitrate trap in dark conditions along the whole organic gradient (Fig. 7.4 d) and the plant presence increased the nitrate consumption rate magnitude compared to bare sediments. In light condition fluxes from the sediment to the water column were detected, with a peak measured in level B. In the last two levels sediments became a net sink of nitrate also in the light and with consumption rates generally higher than the corresponding dark ones.

Nitrite fluxes ranged between -15.0±4.0 and 58.3±15.7 µmol N m^{-2} h^{-1} and between 97.0±57.5 and 482.1±153.6 µmol N m^{-2} h^{-1}, in bare and vegetated microcosms (Fig. 7.4 e,f), respectively, and they were dependent upon the organic matter level ($p<0.001$). Contrary to nitrate, nitrite fluxes in bare microcosms were always directed toward the water column, with the only exception of level E in dark condition, where a consumption of nitrite was recorded even if of small magnitude (Fig. 7.4 e). A nitrite release was also detected from *V. spiralis* colonised sediments in all organic levels and both in light and dark conditions (Fig. 7.4 f). For each organic level, nitrite fluxes in bare sediments were constantly smaller (up to one order of magnitude) than the corresponding ones measured when plants were present.

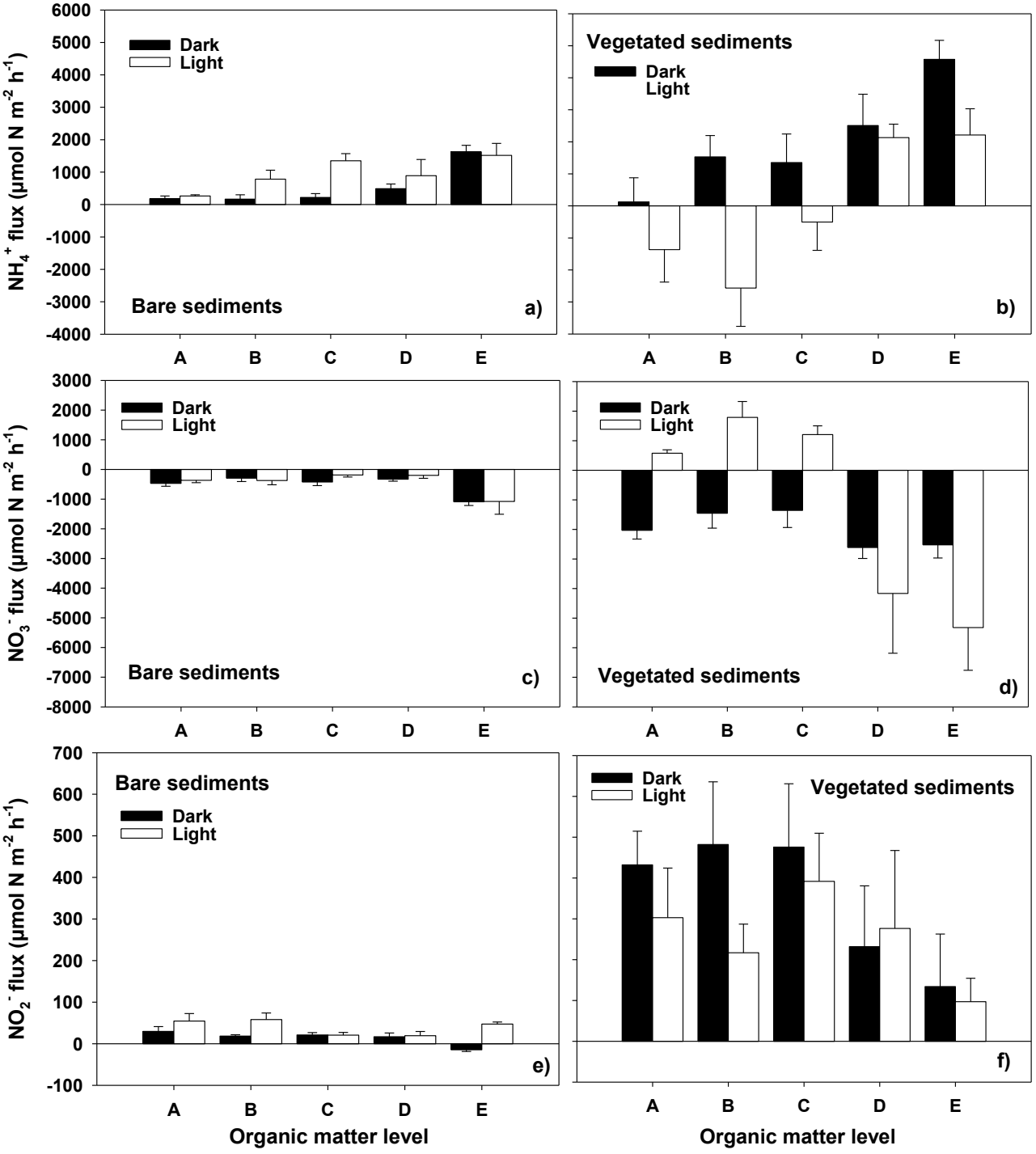

Figure 7.4. Light and dark nitrogen fluxes (NH_4^+, NO_3^-, NO_2^-) measured in bare and *V. spiralis* vegetated microcosms of the five organic matter levels. Average values ± std. dev. are reported (n=3).

7.3.3. Denitrification associated with the rhizosphere

As $^{15}NH_4^+$ was the only form of labelled nitrogen within incubated microcosms, labelled N_2 ($^{29}N_2$ and $^{30}N_2$) evolved during the experiment was produced only as a consequence of ammonium oxidation coupled to reduction of nitrate (nitrification-coupled denitrification). Detectable and reliable signals allowed the calculation of denitrification rates in all experimental conditions, both in light and dark, and along the whole organic gradient. Rates of denitrification coupled to nitrification in the rizosphere ranged between 5.0±0.3 and 27.8±9.80 µmol N $m^{-2}h^{-1}$ and between 4.0±0.8 and 62.3±10.4 µmol N $m^{-2}h^{-1}$, in dark and light conditions, respectively (Fig. 7.5). Denitrification rates followed two different patterns along the organic matter gradient in light and dark conditions. In the dark, denitrification coupled to nitrification decreased progressively with increasing organic addition to the sediment. Rates of level E were about one fifth of those measured in control sediment (level A). Differently, light rates peaked in level B and then progressively decreased along the organic gradient. Only from level B to D a significant difference in light and dark rates was detected, with light denitrification being from 1.2 to 4-fold higher than dark one. The two-way ANOVA showed that rates were dependent also on the interaction between the two factors (light/dark condition and organic level, $p<0.001$).

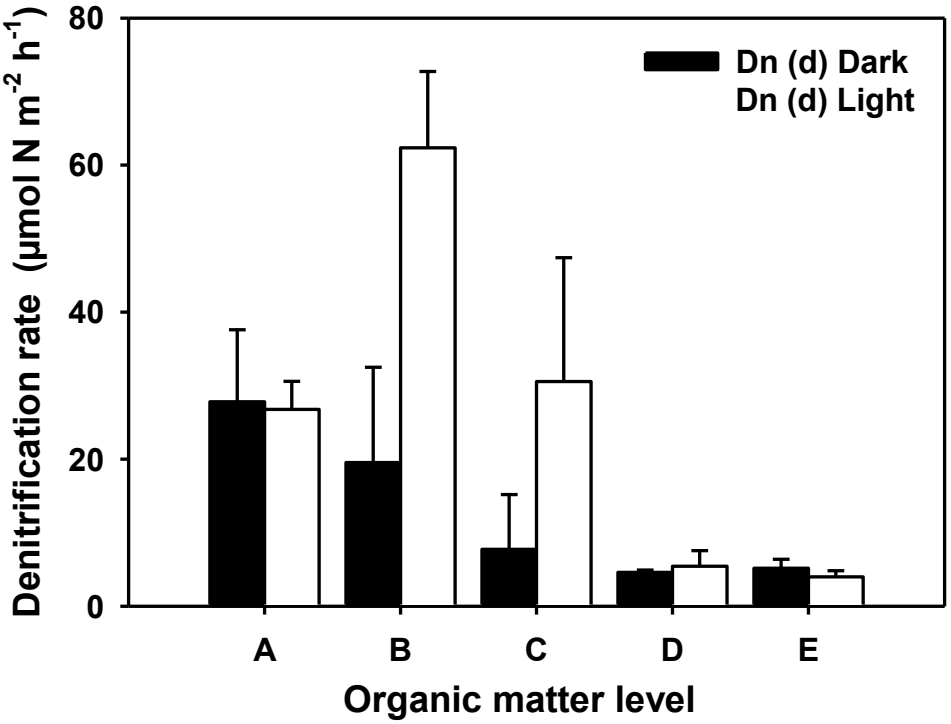

Figure 7.5. Light and dark denitrification rates associated with the rizosphere in *V. spiralis* vegetated microcosms of the five organic matter levels (Dn (d)- Deep denitrification coupled to nitrification). Average values ± std. dev. are reported (n=3).

7.3.4. Denitrification of water column nitrate and surface denitrification coupled to nitrification

Both in bare and vegetated sediments, water column nitrate was the dominant source of substrate fuelling denitrification (Fig. 7.6. a,b). In light condition, Dw showed an evident increasing trend along the organic gradient in vegetated sediment. In dark condition, both in presence and absence of the plant, rates were not significantly different from level A to level D. Only denitrification rates measured in the most enriched substrate (level E) differed, being on average 4 times higher.

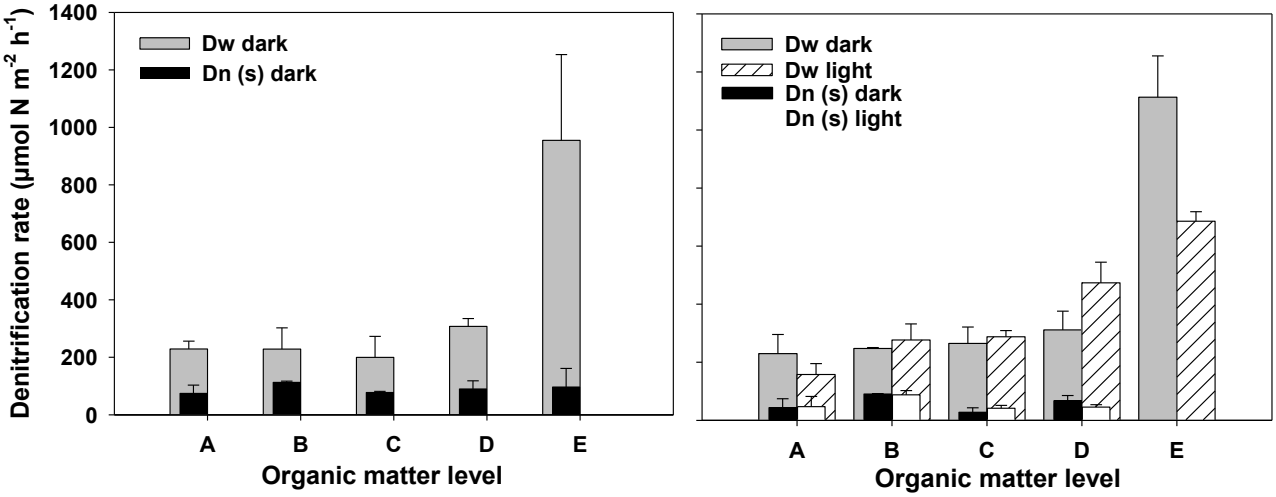

Figure 7.6. Denitrification rates, splitted in the contribution of Dw (denitrification of nitrate from the water column) and Dn (s) (denitrification of nitrate produced by nitrification in the surficial sediment) in bare (a) and *V. spiralis* vegetated (b) microcosms of the five organic matter levels. In vegetated sediments, Dn rates were not detectable in level E. Average values ± std. dev. are reported (n=3).

7.3.5. Estimation of *V. spiralis* theoretical N uptake

Average biomass (above+belowground) of transplanted plants ranged between ~250 g_{DW} m^{-2} in level E and ~620 g_{DW} m^{-2} in level B. *V. spiralis* N demand calculated from net production rate and assuming a conservative PQ (Photosynthetic Quotient) value of 0.69 and a C:N ratio of 12 for photosynthetic tissues ranged between ~3000 and 8300 µmol N $m^{-2}h^{-1}$, with a maximum in level B.

7.4. Discussion

7.4.1. Changes in benthic metabolism along the organic gradient

A number of studies have investigated the effect of nutrient and OM inputs on seagrass meadows in coastal and marine environments and the changes in benthic dynamics. Loadings of labile OM have been suggested to result in marine plant decline, fuelling bacterial metabolims and shift to anoxic pathways, promoting sediment anoxia and accumulation of toxic reduced compounds (Terrados et al., 1999; Perez et al., 2007; Díaz-Almela et al., 2008 and references therein). In contrast, much less is known about the effect of organic enrichment on benthic processes of freshwater environments colonised by macrophytes more tolerant to eutrophication. Relatively few researches have been addressed to measure and compare N fluxes and processing in vegetated and plant-free sediments in organic-rich substrate conditions. An interesting question is to evaluate the ability of tolerant macrophytes to actually act as a buffer to organic enrichment and to maintain, even in eutrophic conditions, the ecosystem services connected to their presence, such as N removal throught a combination of uptake and stimulation of coupled nitrification-denitrification.

The aim of this experiment was to mimic a natural or human-induced eutrophication process exacerbating the sediment metabolism, in order to investigate the macrophyte tolerance towards increasing stressful conditions. Addition of fish feed pellets (high protein content and low C/N) represented an effective way to simulate sedimentary enrichment by very labile organic matter (in terms of oxygen availability and redox status), as that occurring in many eutrophic aquatic environments impacted by urban sewages or sedimentation of phytoplanktonic or fast-growing plant detritus. This form of organic matter has been already used in other studies dealing with the physiological effects induced by interstitial reduced conditions to very sensitive aquatic plants (Møller and Sand-Jensen, 2011; Raun et al., 2010).

Immediately after sediment homogenization and OM addition, microcosms from the different levels were quite similar in sediment colour. However, once recovered from the river botton after the acclimatization period, a visual check across the transparent liner walls revealed that microcosms developed differently according to their OM enrichment. Control microcosms (level A) presented light brown-reddish sediment indicative of oxidized iron species. Otherwise all the artificially enriched microcosms had dark brown-blackish sediments with the exception of a surface layer (<5mm) that appeared light brown, but only in sediments of levels B and C. In the last

two levels, the oxidized portion was restricted to the uppermost ~1 mm layer. When *V. spiralis* roots were observed near the microcosm walls of levels A and B, they were surrounded by an oxidized 1-2 mm thick layer of light brown sediment, suggesting the presence of oxidized conditions (Fig. 7.7). However, no oxidized halos were evident around roots in levels C, D and E. High chemical and microbial oxygen consumption probably minimized the thickness of oxic layers in very OM enriched substrates. Red-colored iron plaques were detectable on root surfaces of plants recovered from microcosms till level C (Fig. 7.8). The rapid and complete depletion of oxygen released by roots due to high sediment oxygen demand probably explained the absence of oxidized metal coating belowground tissues in the most enriched substrates.

Fig. 7.7. Some particulars of the enriched microcosms after the acclimatization period.

As already demonstrated by the previous laboratory experiment (see Chapter 6), sediment chemistry was very susceptible even to small additions of labile OM in the form of fish feed pellets. Stimulation of degradation processes resulted in an increasing stressful condition to macrophyte roots, due to accumulation of anaerobic metabolism end-products and deeply alteration of pore water redox status (van Wijck et al., 1992; Britto and Kronzucker, 2002; Gibbs and Greenway, 2003; Wu et al., 2009). All *V. spiralis* specimens were alive after the *in situ* acclimatization period and during the incubation procedures. An oxygen release in the water column due to photosynthetic processes was detected in all the vegetated microcosms during light incubations. Production rates were not significantly affected by the sedimentary organic

content and comparable to those previously measured in summer in the same studied site (Pinardi et al., 2009; Ribaudo et al., 2011).

However, plants in enriched substrates, especially from levels D and E, showed evident signs of stress in the above-ground tissues. High labile OM contents caused a dramatic reduction in root biomass. Once recovered from microcosm after incubations, *V. spiralis* specimens were anchored with just the primary root and shedding of all the lateral fine roots was evident. There are evidences that *V. spiralis* do not form diffusive barriers (layers of suberin or lignin just below the root surface) that prevent oxygen release into the sediment (Lemoine et al., 2012) as described for other aquatic plants inhabiting reducing sediments (Colmer, 2003). This macrophyte can probably overcome the risk of root damage in anoxic sediments by reducing the biomass, minimizing root surface exposure to the hostile pore water environment and maintaining a sufficient oxygen supply to the root apex. Even if still active, root systems were probably strongly damaged by the most extreme organic enrichments, as a consequence of oxygen shortage and high concentrations of phyto-toxins in pore water. Indeed, all below-ground tissues from levels D and E sediments appeared blackish and seemed to be rotting (Fig. 7.8).

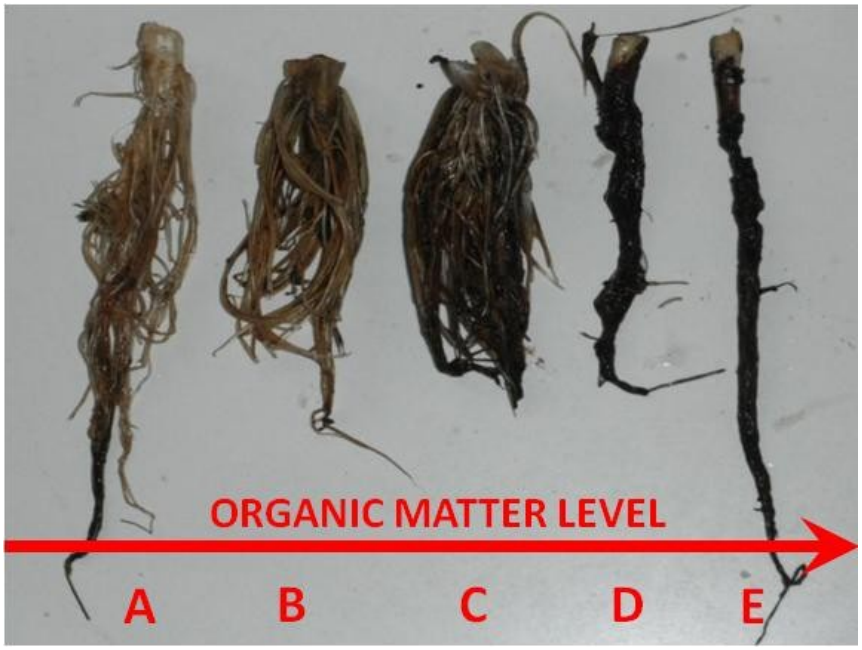

Fig. 7.8. Root systems of plants recovered from the five organic matter levels.

Easily degradable OM stimulated sediment metabolism with a series of cascade effects on benthic solute exchanges. However, plant presence play a crucial role in driving the changes in biogeochemical dynamics. In plant-free condition, OM addition stimulated oxygen consumption to

sustain degradation processes and triggered its uptake from the water column. Rates of carbon mineralization increased with the enrichment degree of the sediments as shown by the progressively higher consumption of oxygen and nitrate and concurrent release of methane and ammonium. Sedimentary oxygen demand increased by a factor of three along the organic gradient and rates were similar in dark and light conditions suggesting that benthic metabolism was driven mainly by heterotrophic activity. The organic content and the oxygen consumption of the substrates aimed at reproducing the eutrophication gradient were within ecologically relevant scales. Oxygen fluxes detected in the most enriched sediment were comparable to those measured during summer in naturally organic-rich sediments of temperate aquatic bodies (Longhi et al., 2008; Racchetti et al., 2011).

Among anaerobic reactions denitrification and methanogenesis likely dominated the organic matter degradation. Elevated nitrate fluxes toward the sediment suggest that denitrification was an important process due to high nitrate availability in the water column. Iron and manganese reductions probably didn't play a significant role in anaerobic decomposition, as the magnitude of oxidized metal pools in the sediment was low (see Chapter 6). Release of gas bubbles during microcosm handling and incubation procedures, especially from the sediment of the last two levels, was a clear sign of highly reduced conditions stimulating methanogenesis.

Microcosms with and without plants behaved differently with respect to sediment-water gas exchanges and nitrogen cycling. As expected, sedimentary processes were significantly influenced by *V. spiralis*, due both to its rhizosphere oxygenation capacity and great N requirements. However, the plant effect on benthic dynamics changed along the OM gradient. Plant presence tended generally to increase the magnitude of solute fluxes and switched the benthic metabolism from eterotrophic to autotrophic. This capacity has been previously demonstrated in less OM impacted sediments (Ribaudo et al., 2011), but it was maintained also in the condition of a rather extreme artificial OM enrichment. Till level C, *V.* spiralis presence was not only able to buffer methane release from the sediment but also to reverse the 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, methane fluxes directed toward the benthic compartment could also be related to oxic conditions in the sediment promoting both its biotic or abiotic oxidation. ROL can stimulate deep aerobic respiration, far from the uppermost oxic sediment layer, and the oxidation of anaerobic metabolism end-products. Indeed higher rates of methane consumption were detected in light conditions when ROL rates

are usullay higher as a consequence of photosynthetic activity (see Chapter 4). Differently, vegetated sediments of the last two enriched levels became a methane source greater than the corresponding bare ones, probably as a consequence 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 soil layers (Beckett et al., 2001; Colmer, 2003). This pathway can result in greater CH_4 emissions from areas inhabited by aerenchymatous plants with respect to bare sediments. Moreover, rooted macrophytes can also provide litter and root exudates as a carbon source for methanogenic bacteria (Joabsson et al., 1999).

Bare sediments acted always as an ammonium source with progressively greater release along the OM gradient, and in light condition compared to dark, likely due to higher oxygen penetration in the sediment stimulating ammonification. Plant presence was able to turn the sediment into a net ammonium sink till level C in light conditions. Ammonium production by microbial ammonification was more than compensated by plant uptake and nitrification in the sediment stimulated by the oxic conditions of the rhizosphere promoted by ROL. In the last two levels, ammonium production by mineralization of high OM loads exceeded plant N requirements and oxidation capacity of the sediment.

The large availability of nitrate in the water column (> 70 μM) was associated with elevated uptake rates in bare sediment to sustain mainly denitrification. However, along the whole OM gradient, nitrate consumption rates were always higher than the corresponding denitrification rates fed by nitrate diffusing into the anoxic sediment from the water column (from ~50% to ~90% of the total nitrate demand). Other processes apart from carbon-driven (heterotrophic) denitrification can contribute to nitrate conversion to N_2, such as dissimilative nitrate reduction coupled to chemolithoautotrophic oxidations of reduced compounds of sulfur and iron (Burgin et al., 2012; Melton et al., 2012). Since the IPT method allows the detection of the total N_2 produced during the incubations, the discrepancy between nitrate consumption rates and Dw could be probably due to incomplete nitrate conversion to N_2 (production of NO_2^- or N_2O) or to underestimation of real denitrification rates because of an insufficient $^{15}NO_3^-$ labeling or a not homogeneus mixing with pore water nitrate. However, even if not measured in the present experiment, the contribution of DNRA (Dissimilative Nitrate Reduction to Ammonium) to sediment nitrate uptake could not be excluded. DNRA is favored in organic rich and sulfidic sediments with low nitrate availability (Tiedje, 1988; McGlathery et al., 2007; Gardner and McCarthy, 2009). Only a few studies have simultaneously measured denitrification and DNRA in freshwater sediments, but

rates of nitrate conversion to ammonium are usually small if compared to denitrification (Nizzoli et al, 2010 and references therein).

Plant presence modified the magnitude and, up to level C, also the direction of nitrate fluxes. In the dark, vegetated sediments acted as a greater nitrate sink compared to plant-free condition, creating in the rhizosphere a more developed mosaic of oxic and anoxic microenvironments where denitrification can be enhanced. Differently in light condition, nitrate release from the sediment up to level C was likely a consequence of nitrification promoted by ROL, as also proven by ammonium fluxes directed towards the sediment. Nitrite production, detected in vegetatd sediment both in light and dark conditions, may be a consequence of ammonium oxidation to nitrite by epiphytic organisms growing on the dense canopy. Recent experiments have reported that ammonia-oxidizing bacteria can 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).

7.4.2. Does the absence of N limitation promote the N removal via coupled nitrification/denitrification in vegetated sediments?

Benthic vegetation activity can either stimulate or depress nitrification and denitrification, according to N availability in the system. In the rhizosphere, rates of coupled nitrification/denitrification may be dependent upon the relative influences of oxygen release by the plant roots and competition between the roots and nitrifying and denitrifying bacteria for ammonium, nitrite and nitrate. Nitrification-coupled denitrification in the rhizosphere of rooted aquatic plants has been investigated by both direct measurements (perfusion techniques, injection of labelled ammonium in the pore water; Reddy et al., 1989; Risgaard-Petersen and Jensen, 1997; Ottosen et al., 1999) or indirect estimations (N mass balance in vegetated sediments; Flindt, 1994). The results of previous studies are highly contradictory. High rates have been measured in the rhizosphere of plants with a great capacity to release oxygen in sediment that promotes nitrification (Reddy et al., 1989; Risgaard-Petersen and Jensen, 1997), whereas other studies performed in marine or coastal systems have reported that in N limitation condition, rates of nitrification-coupled denitrification are generally low compared to seagrass uptake (Risgaard-Petersen et al., 1998; Ottosen et al., 1999; Welsh et al., 2000). Indeed, plant roots regulate pore water ammonium and nitrate concentrations and thus, indirectly, rates of nitrification and denitrification in the rhizosphere. Competition for N resources between plant and bacterial

communities can starve nitrifiers and denitrifiers, thereby limiting denitrification as a N sink. Higher rates detected in the dark indicate that the competition between roots and bacteria for inorganic N has a greater influence on rates of coupled nitrification/denitrification than the potential stimulation of nitrification by ROL during the photosynthetic period. However, a few studies have addressed coupled nitrification denitrification in the rhizosphere of aquatic plants colonizing eutrophic sites. An increase of nitrate reductase enzymatic activity has been demonstrated for freshwater plants exposed to increasing levels of nitrate (Cedergreen and Madsen, 2003). In such circumstances, nitrate availability in the water column should stimulate N leaf uptake and roots could act mostly as anchors and probably have a minor relevance as regulators of pore water chemistry, at least regarding nutrients. The greater availability of water column nitrate could reduce the competition for nitrogen within the sediment and may contribute to higher N removal via denitrification. In my experiment, inorganic nitrogen availability in the water column and in pore water reversed the general outcome of most analogous studies. Dn rates in control sediment were not significantly different in light and dark conditions and in the first two enriched level (B and C) higher rates were detected in light, as a probable consequence of greater oxygen availability from ROL. The higher Dn measured in level B compared to control sediment was probably caused by a combination of higher ammonium availability (greater ammonium regeneration from OM decomposition) and higher ROL by the plant to counteract the more hostile condition in the sediment. An enhancing in anaerobiosis intensity (low Eh) could have promoted aerenchyma formation to facilitate gas transport mechanisms (Colmer, 2003). Lemoine et al. (2012) have measured an increase in root porosity and oxygen release potential in *V. spiralis* specimens grown in anoxic sediments compared to more oxygenated ones. During the acclimatization period *V. spiralis* specimens from level B could have increased their root porosity to allow the colonization of more OM impacted substrates. However, the time needed to develop such morphological adaptation is still to be investigated. From level C, Dn decreased progressively along the organic gradient. A further organic enrichment stimulates benthic respiration and ammonium production via ammonification, but simultaneously limits nitrification, resulting in higher NH_4^+ efflux and lower denitrification coupled to nitrification. Even oxygen directly injected by root in the deep sediment is not enough to promote the existence of oxic sites for nitrification and sustain ammonium oxidation. In sediments with high oxygen demands, nitrification is usually limited, due to aerobic heterotrophs and other chemoautotrophic bacteria, which have higher affinity for oxygen, outcompeting ammonia oxidizing bacteria (Henriksen and Kemp, 1988).

Moreover, acculmulation of reduced species, such as sulphide, can have an inhibitory effect, especially to nitrifiers (Strauss and Lamberti, 2000; Sears et al., 2004). Plants of the most enriched sediments could have been impacted negatively by the hostile pore water conditions, resulting in a progressively loss of oxygen release capacity. As already explained, signs of stress were evident in below-ground tissues of plants from the most OM-impacted substrates (Fig. 7.8).

In conclusion, the vegetated sediments were a significantly higher N trap with respect to unvegetated sediments along the whole organic matter gradient (Table 7.2). In the presence of *V. spiralis*, N retention capacity was mainly supported by plant uptake due to elevated N requirements.

Table 7.2. Nitrogen retention in vegetated and bare sediments along the organic matter gradient, espressed as daily summer rates (16/8 h light/dark). Different contributions to N retention are reported: Dw (denitrification of nitrate from the water column), Dn (s) (denitrification of nitrate produced by nitrification in the surficial sediment), Dn (d) (deep denitrification coupled to nitrification) and macrophyte uptake.

	Organic level	Dw	Dn (s)	Dn (d)	Uptake	Total retention (mmol N m^{-2} d^{-1})
Vegetated sediment	A	4.37	1.11	0.65	72.49	78.61
	B	6.41	2.13	1.15	132.75	142.44
	C	6.72	0.89	0.55	62.00	70.17
	D	10.07	1.27	0.12	56.68	68.15
	E	19.88	0.00	0.11	47.53	67.52
Bare sediment	A	5.50	1.79			7.28
	B	5.49	2.71			8.20
	C	4.79	1.87			6.66
	D	7.38	2.16			9.54
	E	22.92	2.32			25.24

Dw rates were significantly stimulated by the sediment organic enrichment, likely as a consequence of high availability of nitrate and easy degradable organic matter, substrates for heterotrophic denitrifier bacteria. Even in the most enriched condition, the sediment didn't loose its capacity of nitrogen removal via denitrification. Macrophyte presence caused a deeper oxygen penetration by ROL and an increase in the development of oxic-anoxic interfaces, stimulating benthic N removal via coupled nitrification-denitrification. However, with a progressively greater OM content in sediment, Dn contribution decreased. Oxygen shortage in the sediment resulted in a lower denitrification efficiency, with more nitrogen recycled to the water column as ammonium and less nitrogen nitrified and then lost to the atmosphere via denitrification. The inhibition of nitrification within the sediment disconnected the link between N-mineralisation and N-removal via coupled nitrification-denitrification.

8. General considerations and conclusions

I provided multiple direct and indirect evidences that *V. spiralis* is a tolerant macrophyte that can colonize organic rich substrates and withstand large perturbations of sedimentary features. Even substrates potentially hostile to root development (high organic content and low redox potential) do not seem to affect its function of *ecosystem engineer* as benthic metabolism regulator. This macrophyte plays a crucial role in driving benthic exchanges of gases and nutrients and in controlling pore water chemistry and microbial activity in organic matter impacted environments.

In temperate shallow aquatic bodies large seasonal variations of water temperature result in a wide range of benthic respiration rates which are coupled to changes of pore water redox. To cope with such sediment modifications, *V. spiralis* transfers progressively higher amounts of oxygen to roots in the transition winter-summer. Maximum radial oxygen loss occurs in early autumn and probably overlaps with the lowest sediment redox. At the end of the summer, the exhaustion of energy yielding electron acceptor pools is in fact coupled to input of labile organic matter from senescent primary producers, further exacerbating the demand of oxidized compounds to support degradation processes. Tolerant rooted plants respond to more reduced sediment conditions by increasing tissue porosity, or by forming a diffusive barrier to oxygen leakage from root surfaces. Evidences suggest that *V. spiralis* seems to adopt the first strategy and increase the oxygen transport to the rhizosphere when the conditions become potentially hostile to its survival. Even if the amount of oxygen release by roots represents only a relatively small fraction in the plant oxygen economy, it is interesting to discuss the relevance of this root-mediated leakage in the context of the oxygen balance of eutrophic benthic compartments. Here, heterotrophic activity consumes elevated oxygen quota from the water column. The oxygen injected in the pore water by a *V. spiralis* meadow in the summer period represents a significant portion of the daily benthic demand and can deeply affect the biogeochemical dynamics, with relevant implications for the quality of both sediment and water column compartments in term of oxidation status.

Seasonal characterization of pore water features in adjacent bare and vegetated sediment patches has displayed great differences in interstitial chemistry and microbial activity. *V. spiralis* influences pore water environment both directly (by nutrient uptake) and indirectly (by root oxygen release). Low accumulation of reduced species suggests that degradation processes occur mainly via aerobic pathways or that reduced compounds are quickly re-oxidized. Plant presence promotes the maintenance of a larger nitrifying community in the rhizosphere due to more oxic conditions. This is consistent with intense oxygen pumping, especially in summer, via high density root

systems. These outcomes question the conclusions of previous studies that rooted vegetation can control sediment biogeochemistry only in oligotrophic conditions.

The addition of labile organic matter to sediments causes profound and long-lasting effects on biogeochemistry and can deeply affect rooted plant growth and survival. *V. spiralis* is able to cope with organic additions to sediments as those occurring in water bodies undergoing eutrophication processes. Its relevant oxygen leakage in the rhizosphere can partially buffer the negative effects of organic enrichment, maintaining lower contents of potentially toxic compounds and reducing internal nutrient loads. Local oxidation of the rhizosphere has a strong impact not only on sediment dynamics but can also affect the water column chemistry. Benthic compartment plays a major role in the transformation and recycling of organic matter loads, but the presence of submerged meadows significantly affects water-sediment fluxes due to a combination of uptake and oxygenation capacity. Indeed, an oxidised condition in the sediment attenuates the release of nutrient to the water column and their availability to other primary producers. In particular, *V. spiralis* stands play a crucial role in controlling N dynamics and fate, with negative feedbacks for eutrophication processes. In moderate organic enriched substrates, radial oxygen loss promotes deep sediment nitrification and the coupling with denitrification implies a loss of remineralized N, thus enhancing the ecosystem capacity to control N contamination. Furthermore, the high nitrogen availability both in water column and pore water attenuates the competition between plants and nitrifying and denitrifying bacteria, favoring nitrogen removal through a combination of vegetation uptake and dissimilative microbial processes. The role of tolerant rooted macrophytes, such as *V. spiralis*, to act as regulators of benthic-pelagic nutrient fluxes varies along the organic gradient. Vegetated sediments are usually greater N traps compared to bare ones but, with an increasing organic content, their N removal capacity is progressively lost due to a combination of nitrification inhibition and plant stress induced by very reduced conditions. This fact disconnects the link between N mineralisation and N removal via coupled nitrification-denitrification, thus compromising the N removal capacity, a paramount ecosystem service provided by benthic vegetation.

The organic gradient artificially reproduced in the present study was on porpuse wide and rather extreme in order to investigate *V. spiralis* tolerance. Since organic enrichment occurring in most impacted aquatic environments is generally moderate and progressive compared to that adopted in my experiments, this macrophyte, due to its elevated physiological plasticity, could be considered an interesting low cost option in programs aimed at improving sediment conditions

and favoring ecosystem recovery. The experiments performed have proven that this plant can modify benthic dynamics with positive feedbacks for restoration of aquatic bodies (i.e. regeneration of ferric iron buffer and phosphorus retention in sediment, stimulation of coupled nitrification-denitrification with net nitrogen loss) and maintain the provided ecosystem services even in OM rich sediments. It can be preferred as a pioneer species for an initial “purification” of organic impacted substrates, favouring the subsequent reestablishment of other less tolerant rooted plants. High internal nutrient loadings in sediments are frequently reported as an important mechanism delaying aquatic ecosystem recovery after a reduction of the external loadings. Plant presence can contribute to partially lighten this load by injecting oxygen in deep sediments.

From a methodological point of view, the comparative analysis of pore water chemistry and benthic exchanges in vegetated and bare sediments was proposed as an indirect evaluation of the plant ability to cope with organic rich substrates and of its physiological status. Anyway additional experiments are certainly necessary to confirm if this macrophyte can survive in very organic impacted substrates over long periods and investigate what strategies (physiological and morphological adaptations) are adopted to overcome the oxygen shortage stress.

9. *Side Project*

Origin, transformations and export of nitrogen loads in sub-basins of the Po River Plain

During the three years as a PhD student I was involved in several projects aimed at investigating nitrogen dynamics at the watershed scale in the Po River Plain. Nitrogen sources, sinks, and major transformations were assessed for different sub-basins of the Po River catchment, such as Oglio and Mincio (in the framework of regional projects aimed at defining fluvial restoration strategies and the minimum vital flow), Parma (PRIN 2008 project "Nitrogen loads in the Po river basin: biogeochemical processes, transformations and effects in lowland reaches, transitional and coastal waters", funded by the Italian Ministry of University and Research) and Po di Volano (EU-Water Project "Transnational integrated management of water resources in agriculture for the EUropean WATER emergency control").

Here I report a list of the manuscripts written in the context of this side project and a summary of the main outcomes obtained for the Oglio River basin. This watershed has been investigated by the Department of Life Sciences (University of Parma) since 2007 in the framework of several projects. The whole detailed analysis performed in the Oglio River basin was the object of two papers recently published in international journals (Soana et al., 2011 - CLEAN; Bartoli et al., 2012 - Biogeosciences).

- Bartoli M., Racchetti E., Delconte C.A., Sacchi E., **Soana E.**, Laini A., Longhi D., Viaroli P. 2012. Nitrogen balance and fate in a heavily impacted watershed (Oglio River, Northern Italy): in quest of the missing sources and sinks. Biogeosciences 9, 361–373.

- **Soana E.**, Racchetti E., Laini A., Bartoli M., Viaroli P. 2011. Soil budget, net export and potential sinks of nitrogen in the lower Oglio River watershed (northern Italy). CLEAN – Soil, Air, Water 39, 956–965.

- **Soana E.**, Racchetti E., Pinardi M., Bartoli M., Viaroli P. 2010. Nitrogen mass balances and aquatic denitrification relevance: a watershed scale study for lower Oglio and Mincio Rivers. EURAC-Book 57, 103–112, Proceedings of the XIX SItE (Italian Society of Ecology) Congress (in Italian).

- **Soana E.**, Racchetti E., Romani F., Longhi D., Gardi C., Bartoli M. 2010. Nitrogen loading and associated environmental risk in the lower Oglio River basin (Northern Italy). Biologia Ambientale 24, 87–96, Proceedings of the XVIII SItE (Italian Society of Ecology) Congress (in Italian).

- Castaldelli G., **Soana E.,** Pierobon E., Mastrocicco M., Racchetti E., Bignami A., Bartoli M. Nitrogen budget in a lowland coastal area within the Po River Basin (Northern Italy): multiple evidences of equilibrium between sources and internal sinks. Environmental Management, accepted.

9.1. Topic context

Increased reactive nitrogen (N) input to the biosphere by human activities and widespread simplification of land use mosaic in watersheds have resulted in the loss of important ecosystemic functions, gradual saturation of N buffering capacity by terrestrial areas and augmented N loads towards surface waters (Valiela and Bowen, 2002; Galloway et al., 2003; Schlesinger, 2009). Increased manure production and spreading, use of industrially fixed nitrogen fertilizers, fixation by crops and atmospheric deposition have resulted in a release of reactive nitrogen into the environment greatly exceeding N-removal processes (Puckett, 1995; Cassman et al., 2002; Galloway et al., 2008). Rivers are particularly vulnerable because they link terrestrial and coastal ecosystems, aggregating stressors occurring at the landscape scale (Yates et al., 2007). Human-driven alterations such as channelization, impoundment, water withdrawals, reduction of riparian vegetation and wetlands have enhanced soil erosion and runoff processes, affected the biogeochemistry of riparian and in-stream zones and reduced the efficiency of the river network in mitigating N fluxes (Pinay et al., 2002; Bernot and Dodds, 2005).

Farming activity is the major responsible for the current high N loads, because of the availability of a N pool generally exceeding soil metabolic capability and crop requirements (Rotz, 2004). Since N losses from agro-ecosystems represent not only an ecological but also an economic damage (Oenema, 2006), law regulation on fertilizers use, livestock diets and agricultural best management practices implementation have improved N use efficiency (Di and Cameron, 2002; Rotz, 2004; Yates et al., 2007). However, the expected water quality enhancement has not been fulfilled yet, due to a combination of unbalance between livestock manure production and availability of agricultural lands for spreading, mismatch between timing of fertilizer application and nutrient crop demand (Crews and Peoples, 2005) and ecosystems inertia in controlling N export (Grimvall et al., 2000).

The control of N inputs to aquatic systems is of general public interest, due to their known ecosystemic consequences, namely eutrophication (Hilton et al., 2006; Howarth and Marino, 2006) and the potential toxicity risks posed by high nitrate (NO_3^-) concentration in water resources (Camargo and Alonso, 2006).

In this framework, the mass balance approach represents a useful tool to describe qualitatively and quantitatively the N fluxes to and from agro-ecosystems and provides a readily communicable guidance to improve N management (Oenema, 2006). Since hydrologic processes largely determine N surplus fate, watershed as spatial unit represents a study scale more appropriate than statistical regions (Campling et al., 2005). River planning, management and requalification programs should in fact embrace watershed rather than administrative boundaries (Schlesinger et al., 2006).

Worldwide N budgets estimate that, on a long-term basis, up to ~75% of the N load generated within catchments is generally retained and not exported via river discharge (Seitzinger et al., 2006; Boyer et al., 2006; Schlesinger, 2009). The retained nitrogen is the result of several biogeochemical processes among which some are well studied (i.e. crop uptake) while others are scarcely investigated (i.e. storage in soils, percolation to groundwater, denitrification in lotic ecosystems) and represent large unknown terms in N budgets (Kulkarni et al., 2008). Because of their spatial and temporal heterogeneity, and due to the lack of detailed information on N pathways and fluxes, the relative importance of those mechanisms buffering N surplus is difficult to quantify at watershed scale (van Breemen et al., 2002). Even if denitrification is accepted as the major sink of N excess in the landscape, it is usually not explicitly accounted in N budgets at the watershed level but estimated indirectly by difference. Some papers have recently pointed out the necessity to increase the level of detail in N balance studies by means of high-resolution local databases and to compare model application outcomes with field measurements (Boyer et al., 2006; Van Drecht et al., 2005).

Open questions about the fate of the nitrogen surplus in impacted watersheds concern where and for how long does the excess nitrogen accumulate, and what processes and transformations does it undergo.

The aim of this work was to assess the nitrogen metabolic capacity of the lower Oglio River watershed (3,840 km^2), a basin that lays within one of the most heavily exploited areas of Northern Italy for agriculture and animal farming. Data and information from multiple sources were used with the goal of quantifying the N sources, sinks, and major transformations within this watershed.

GIS techniques application provided a spatial distribution of N sources and sinks. Repeated monitoring of Oglio River for total nitrogen concentrations and water flow allowed the calculation

of net N export while measured and modelled denitrification rates allowed to speculate N loss in aquatic compartments (wetlands and channel networks) within the basin.

9.2. The Oglio River basin

The Oglio River is a left-side tributary of the Po River catchment, the largest hydrographic system in Italy (71,000 km^2, about one quarter of whole national territory) and a strategic area for the Italian economy (intensive agriculture and farming activities, industry and human settlement). The fluvial reach called "lower Oglio" is 156 km long and its watershed embraces an area of approximately 3,840 km^2 across four provinces of the Lombardy Region (Bergamo, Brescia, Cremona and Mantova). The lower Oglio River is fed by the Iseo Lake, the fourth largest Italian lake, and it is regulated upstream by the Sarnico dam. Water management strategy aims to minimize outflows from the lake during winter months and release water during summer months to feed artificial channels for agriculture requirements and hydroelectric plants. Mella, Chiese, Strone and Cherio Rivers are the four major tributaries, together with an extended network of drainage channels scattered in the agricultural lands. Agriculture is the dominant land use in the basin, comprising over 58% of total land cover, characterized by maize-based intensive farming and livestock practices. The lower Oglio River basin is representative of the Po River Plain in terms of climatic condition, land use and agronomic management.

9.3. N-balance calculation: the contribution of diffuse and point sources

A detailed nitrogen mass balance was conducted according to the *soil system budget* approach (Oenema et al., 2003) integrating statistics and water quality surveys data. The budget was calculated on an annual basis as the net difference between N inputs (livestock manure, synthetic fertilizers, atmospheric deposition, biological fixation and wastewater sludge) and N outputs (crop uptake, ammonia volatilization and denitrification in soils) within the productive agricultural lands system of the catchment. In order to quantify and compare the contribution by different sources - point and diffuse - in generating the N loads entering the river network, N loads from urban areas were also estimated.

Calculations were performed at a spatial resolution of individual municipalities (over 200) then aggregated to the watershed scale by means of GIS techniques. The overall analyses gained

robustness from the comprehensive and high-resolution datasets available for this area and the use of site-specific agronomic coefficients (for more details see Soana et al., 2011).

The calculations indicated that total N input in the Oglio River basin was over 100,000 t yr^{-1} and that most of such input was due to manure (50%) and to synthetic fertilizers (36%) (Table 9.1). Output terms accounted for about 60,000 t N yr^{-1} and were mostly sustained by crop uptake (65%). The difference between inputs and outputs indicates an excess of about 40,000 t N y^{-1}. All the municipalities showed a N surplus, but the N input and output patterns varied widely spatially within the catchment (Fig. 9.1).

Table 9.1. Nitrogen balance in the lower Oglio River basin computed for the year 2008. Data are expressed as tons of nitrogen produced or consumed per year in the whole basin or as kilograms of nitrogen produced or consumed per year per hectare of arable land (AL).

N balance terms	t N yr^{-1}	kg N ha^{-1} AL yr^{-1}
INPUT		
Livestock manure	51,512	232
Synthetic fertilizers	33,564	151
Biological fixation	12,182	35
Atmospheric deposition	1,800	8
Wastewater sludge	1,057	5
$\sum$ input	100,115	450
OUTPUT		
Crop uptake	38,915	175
NH_3 volatilization	12,704	57
Denitrification in soils	8,440	38
$\sum$ output	60,060	270
Balance	40,056	180

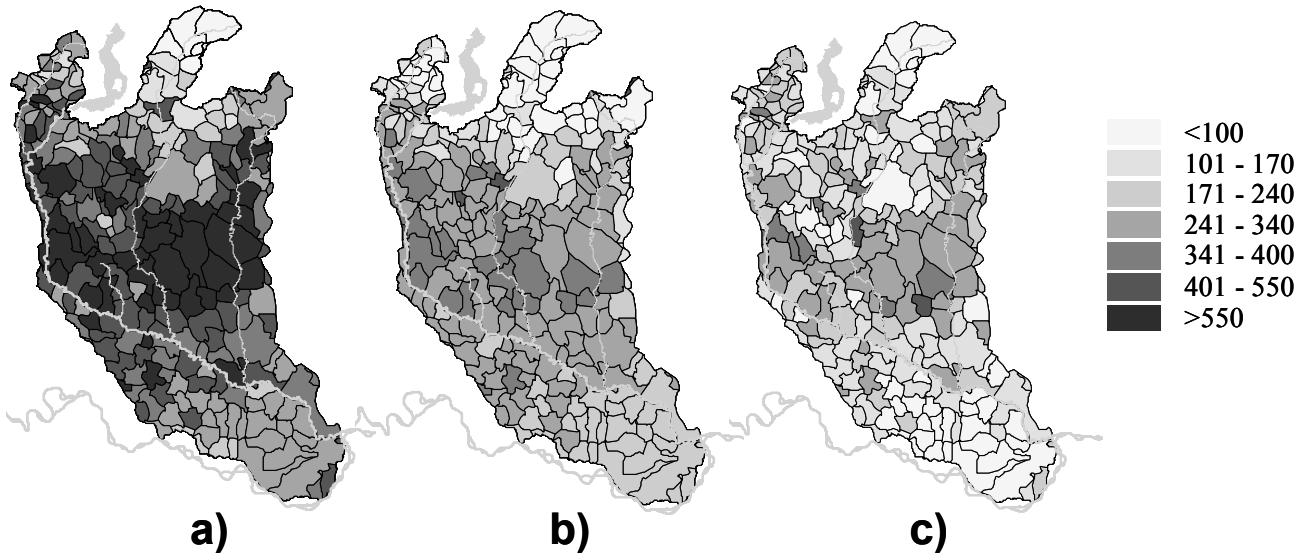

Fig. 9.1. Spatial distribution of N inputs (a), outputs (b) and surplus (c). Units are kg N ha AL^{-1} yr^{-1} (from Soana et al., 2011).

The comparison between N input and output suggested an elevated N surplus in this watershed, averaging 180 kg N ha^{-1} arable land (AL) yr^{-1}. The N surplus varied greatly across the basin (Fig. 9.1) and the most critical zone is the middle plain of the Oglio River, where some municipalities have a N surplus exceeding 400 kg N ha^{-1} AL yr^{-1}. To put this surplus in context, the total amount of manure recommended by the European Community (Nitrates Directive, 91/976/EEC) to be spread on arable lands (not N surplus) varies between 170 and 340 kg N ha^{-1} AL yr^{-1}, for vulnerable and non-vulnerable areas, respectively.

N loads from urban areas were quantified in over 5,800 t N yr^{-1}, representing only about 6% of the total N input from diffuse sources. Moreover, this calculation was likely a great overestimate of the true N load discharged into surface waters since over 85% of the total population is connected to the sewage system and most of the wastewater treatment plants operate denitrification.

9.4. N-export from the basin and diffuse contamination in the aquatic compartments

Although N surplus itself may not be used to directly estimate the actual environmental impact of the farming system, it's a proxy of N pollution risk for aquatic ecosystems (Schröder et al., 2004). In the Oglio River basin, the excessive manure spreading has led to a large anthropogenic excess of bioavailable nitrogen to the watershed and to broad-scale diffuse contamination by nitrate.

Elevated concentrations of $N\text{-}NO_3^-$ were detected in the Oglio River, in most of its tributaries, in all wetlands hydraulically connected with the river and in groundwater (Racchetti et al., 2011; Soana et al., 2011; Bartoli et al., 2012) (Fig. 9.2). This element was an evident sign of N-saturation in the terrestrial but probably also in the aquatic portions of the watershed (Mullholand et al., 2008).

The annual N load exported from the Oglio River basin was estimated by means of the available dataset of flow and water chemistry data collected at the closing section and approached 13,000 t N yr^{-1}, with over 90% as nitrate. The outputs from agricultural lands and export via river discharge were estimated in about 60% and 13% of the total inputs, respectively. About 27% of the anthropogenic inputs added to the landscape (about 26,800 t N yr^{-1}) were not exported via river discharge and retained within the basin by processes still to be identified. This suggests that there are efficient mechanisms causing an internal net N loss or retention. These mechanisms could permanently remove N by dissimilative processes (denitrification) or store and/or transport N in other environmental reservoirs (soil, groundwater).

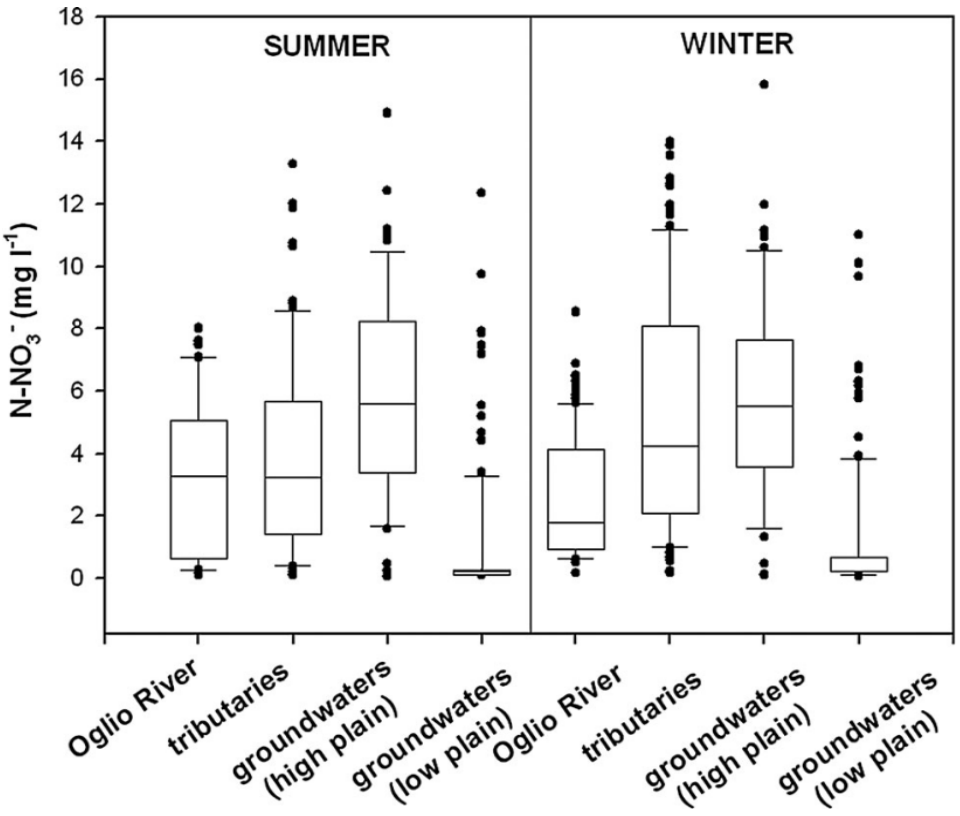

Fig. 9.2. Boxplot reporting nitrate concentrations measured in the Oglio River (n=20 sampling stations), in its main tributaries (n=60) and in the surface aquifer (n>100, data splitted into those collected in the high and low plain portions of the watershed) (from Soana et al., 2011).

9.5. What's the fate of the "missing N"?

The role of aquatic compartments (wetlands, secondary drainage channel network and associated buffer strips) in N load abatement via denitrification was assessed. Racchetti et al. (2011) reported sediment denitrification rates in several riverine wetlands of the Oglio River basin, measured seasonally by means of the isotope pairing technique. Denitrification rates were up to two orders of magnitude higher in perifluvial wetlands hydraulically connected to the NO_3^- rich Oglio River (150-1,260 kg N ha^{-1} yr^{-1}, average value 400 kg N ha^{-1} yr^{-1}) than those measured in hydraulically isolated sites. Supposing all wetlands within the watershed to be hydraulically connected to the Oglio River, the potential N removal in lentic ecosystems was calculated in ~250 t N yr^{-1}, considering their total area and the maximum measured denitrification rate. N loss via benthic denitrification was a very small amount compared to that generated within the basin or exported to the Po River, representing only <1% of the basin N surplus due to the limited surfaces of these ecosystems (<200 ha) compared to agricultural lands (>200,000 ha).

By means of a GIS analysis, the total stream length within the lower Oglio River basin was evaluated in over 12,500 km, 95% of which constituted by low-order ditches. Due to a lack of direct measurements of denitrification rates in the secondary drainage channels, the theoretical nitrate removal capacity was estimated by means of hydrochemical data collected during field surveys over several tributaries and according to the equation proposed by Christensen et al. (1990) (for more details see Soana et al., 2011). The maximum theoretical denitrification rate was extended all over the surface actually occupied by the ditch network (about 6,250 ha) in this geographical area. The calculated theoretical N removal was equivalent to 5,500 t N, denitrified during the 5-month period when the system is active for irrigation practices. In addition, assuming the highest denitrification rates reported in the literature (Mander et al., 1997), an additional amount of ~3,000 t N yr^{-1} of the surplus in the catchment was estimated to be removed in vegetated buffer strips adjacent to the secondary drainage network (linear extension of about 9,500 km).

The contribution of aquatic environments to N removal was not enough to explain the discrepancy between N surplus and N export out of the basin. The final fate of this "missing N amount" is at present not known, even if some evidences support the hypothesis that groundwater can represent a significant N sink in this watershed. In the Oglio River basin, nitrate distribution in groundwater from the shallow unconfined aquifer is not uniform (Fig. 9.3). Concentrations near or above the threshold for drinking water standards (11.68 mg N-NO_3^- l^{-1}) are commonly observed in

the higher plain portion of the basin. This northern part is particularly vulnerable to diffuse contamination, due to the combined effects of coarse-grained soils, flood-based irrigation practices, and widespread corn cultivation, a crop that requires large N amendments. This area also receives a large excess input of animal manure, leading to the greatest N surplus in the basin (Fig. 9.1).

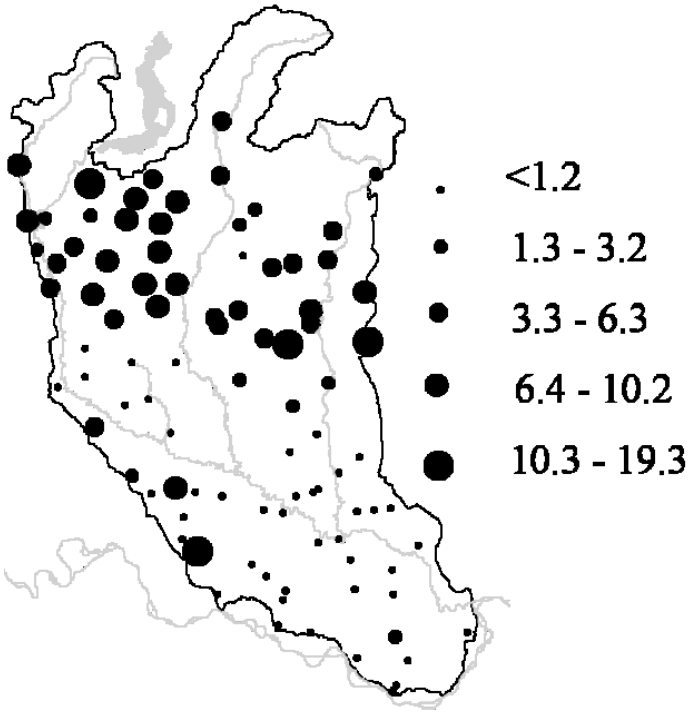

Fig. 9.3. Mean nitrate concentrations in the surficial aquifer within the Oglio River basin (mg N-NO_3^- l^{-1}, data from 2002-2008) (from Soana et al., 2011).

However, there are evidences of nitrogen transport to the surface water network, particularly in the transition zone between the high and the low plain where the river is fed by the surficial aquifer ("springs belt" zone). Water samples collected in lowland springs adjacent to the northern section of the Oglio River, and reflecting the water quality of the surface aquifer in the study area, had NO_3^- concentrations up to 20 mg N l^{-1} and were supersaturated with nitrous oxide (Laini et al., 2011). Therefore, groundwater is likely an N sink in the short-term, especially if compared to the rapid turnover of surface waters, but acts as a N source in longer periods of time (>20 years). Relevant N loads can be conveyed by springs and recycled to the surface water, acting as an internal source of pollution in the basin. Detailed investigation by hydrogeologists is needed, in order to clarify the path of deep and surface groundwater and to date the nitrogen that is recycled by springs. This will allow for an estimation of the time required by groundwater to recover from nitrate pollution if N loads are significantly reduced in the future.

10. References

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

Andersen, J.M., 1976. An ignition method for the determination of total phosphorus in lake sediments. Water Res. 10, 329-331.

Anderson, L.G., Hall, P.O.J., Iverfeldt, A., van der Loeff, M.M.R., Sundby, B., Westerlund, S.F.G., 1986. Benthic respiration measured by total carbonate production. Limnol. Oceanogr. 31, 319–329.

Anthonisen, A.C., Loehr, R.C., Prakasam, T.B.S., Srinath, E.G., 1976. Inhibition of nitrification by ammonia and nitrous acid. J. Water Pollut. Contr. Fed. 48, 835–852.

Armstrong, F.A.J., Sterus, C.R., Strickland, J.D.H., 1967. The measurement of upwelling and subsequent biological processes by means of the Technicon AutoAnalyzer and associated equipment. Deep-Sea Res. 14, 381–389.

Bal, K., Brion, N., Jupsin, H., Dehairs, F., Vasel, J-L, Meire P., 2009. Macrophytes and nutrient dynamics: process and field studies in the upper reaches of lowland rivers. "Manudyn II". Final Report Phase 1. http://www.belspo.be/belspo/SSD/science/Reports/MANUDYN_FinalRep%20Ph1_DEF.pdf.

Barko, J.W., Gunnison, G., Carpenter, S. R., 1991. Sediment interactions with submerged macrophyte growth and community dynamics. Aquat. Bot. 41, 41–65.

Barko, J.W., Smart, R.M., 1986. Sediment-related mechanisms of growth limitation in submersed macrophytes. Ecology 67, 1328–1340.

Bartoli M., Racchetti E., Delconte C.A., Sacchi E., Soana E., Laini A., Longhi D., Viaroli P., 2012. Nitrogen balance and fate in a heavily impacted watershed (Oglio River, Northern Italy): in quest of the missing sources and sinks. Biogeosciences 9, 361–373.

Beckett, P.M., Armstrong, W., Armstrong, J., 2001. Mathematical modelling of methane transport by *Phragmites*: the potential for diffusion within the roots and rhizosphere. Aquat. Bot. 69, 293–312.

Bernot, M.J., Dodds, W.K., 2005. Nitrogen retention, removal, and saturation in lotic ecosystems. Ecosystems 8, 442–453.

Blanch, S.J., Ganf, G.G., Walker, K.F., 1998. Growth and recruitment in *Vallisneria americana* as related to average irradiance in the water column. Aquat. Bot. 61, 181–205.

Bodelier, P.L.E., Libochant, J.A., Blom, C.W.P.M., Laanbroek, H.J., 1996. Dynamics of nitrification and denitrification in root- oxygenated sediments and adaptation of ammonia-oxidizing bacteria to low-oxygen or anoxic habitats. Appl. Environ. Microbiol. 66, 4100–4107.

Bornette, G., Puijalon, S., 2011. Response of aquatic plants to abiotic factors: a review. Aquat. Sci. 73, 1–14.

Bower, C.F., Holm-Hansen, T.T., 1980. A salicylate-hypoclorite method for determing ammonia in seawater. Can. J. Fish. Aquat. Sci. 37, 794–798.

Boyer, E.W., Howarth, R.W., Galloway, J.N., Dentener, F.J., Green, P.A., Vörösmarty, C.J., 2006. Riverine nitrogen export from the continents to the coasts. Global Biogeochem. Cycles. 20, GB1S91.

Britto, D.T., Kronzucker, H.J., 2002. NH_4^+ toxicity in higher plants: a critical review. J. Plant Physiol. 159, 567–584.

Brouwer, E., Bobbink, R., Roelofs, J.G.M., 2002. Restoration of aquatic macrophyte vegetation in acidified and eutrophied softwater lakes: an overview. Aquat. Bot. 73, 405–431.

Brune, A., Frenzel, P., Cypionka, H., 2000. Life at the oxic-anoxic interface: microbial activities and adaptations. FEMS Microbiol. Rev. 24, 691–710.

Burgin, A.J., Hamilton, S.K., Jones, S.E., Lennon, J.T., 2012. Denitrification by sulfur-oxidizing bacteria in a eutrophic lake. Aquat. Microb. Ecol. 66, 283–293.

Caffrey, J.M., Kemp, W.M., 1990. Nitrogen cycling in sediments with estuarine populations of *Potamogeton perfoliatus* and *Zostera marina*. Mar. Ecol. Prog. Ser. 66, 147–160.

Caffrey, J.M., Kemp, W.M., 1991. Seasonal and spatial patterns of oxygen production, respiration and root-rhizome release in *Potamogeton perfoliatus* L. and *Zostera marina* L. Aquat. Bot. 40, 109–128.

Caffrey, J.M., Kemp W.M., 1992. Influence of the submersed plant, *Potamogeton perfoliatus* L., on nitrogen cycling in estuarine sediments. Limnol. Oceanogr. 37, 1483–1495.

Caffrey, J.M., Bano, N., Kalanetra, K., Hollibaugh, J.T., 2007. Ammonia oxidation and ammonia-oxidizing bacteria and archaea from estuaries with differing histories of hypoxia. ISME Journal 1, 660–662.

Camargo, J.A., Alonso, A., 2006. Ecological and toxicological effects of inorganic nitrogen pollution in aquatic ecosystems: A global assessment. Environ. Int. 32, 831–849.

Campling, P., Terres, J.M., Vande Walle, S., Van Orshoven, J., Crouzet, P., 2005. Estimation of nitrogen balances from agriculture for EU-15: spatialisation of estimates to river basins using the CORINE Land Cover. Phys. Chem. Earth 30, 25–34.

Caraco, N., Cole J., Findlay, S., Wigand, C., 2006. Vascular plants as engineers of oxygen in aquatic systems. BioScience 56, 219–225.

Carignan, R., Kalff, J., 1980. Phosphorus sources for aquatic weeds: water or sediments. Science 207, 987–989.

Carpenter, S.R., Elser, J.J., Olsen, K.M., 1983. Effects of roots of *Myriophyllum verticillatum* on sediment redox conditions. Aquat. Bot. 17, 243 – 249.

Carr, G.M., Chambers, P.A., 1998. Macrophyte growth and sediment phosphorus and nitrogen in a Canadian prairie river. Fresh. Biol. 39, 525–536.

Cassman, K.G., Dobermann, A., Walters, D.T., 2002. Agroecosystems, nitrogen-use efficiency, and nitrogen management. Ambio 31, 132–140.

Cedergreen, N., Madsen, T.V., 2003. Nitrate reductase activity in roots and shoots of aquatic macrophytes. Aquat. Bot. 76, 203–212.

Christensen, P.B., Nielsen, L.P., Sørensen, J., Revsbech, N.P., 1990. Denitrification in nitrate-rich streams: diurnal and seasonal variations related to benthic oxygen metabolism. Limnol. Oceanogr. 35, 640–651.

Christensen, P.B., Sørensen, J., 1986. Temporal variation of denitrification activity in plant-covered, littoral sediment from lake Hampen, Denmark. Appl. Environ. Microbiol. 51, 1174– 1179.

Coci, M., Bodelier, P.L.E., Laanbroek, H.J., 2008. Epiphyton as a niche for ammonia-oxidizing bacteria: detailed comparison with benthic and pelagic compartments in shallow freshwater lakes. Appl. Environ. Microbiol. 74, 1963–1971.

Coci, M., Nicol, G.W., Pilloni, G.N., Schmid, M., Kamst-van Agterveld, M.P., Bodelier, P.L.E., Laanbroek, H. J., 2010. Quantitative assessment of ammonia-oxidizing bacterial communities in the epiphyton of submerged macrophytes in shallow lakes. Appl. Environ. Microbiol. 76, 1813–1821.

Colmer T. D., 2003. Long-distance transport of gases in plants: a perspective on internal aeration and radial oxygen loss from roots. Plant Cell Environ. 26, 17–36.

Cotano, U., Villate, F. 2006. Anthropogenic influence on the organic fraction of sediments in two contrasting estuaries: A biochemical approach. Mar. Pollut. Bull. 52, 404–414.

Crews, T.E., Peoples, M.B., 2005. Can the synchrony of nitrogen supply and crop demand be improved in legume and fertilizer-based agroecosystems? A review. Nutr. Cycl. Agroecosys. 72, 101–120.

Cronk, J.K., Fennessy, M.S., 2001. Wetland plants: biology and ecology. CRC Press/Lewis Publishers, Boca Raton, FL.

Dalsgaard, T., Nielsen, L.P., Brotas, V., Viaroli, P., Underwood, G.J.C., Nedwell, D.B., Sundbäck, K., Rysgaard, S., Miles, A., Bartoli, M., Dong, L., Thornton, D.C.O., Ottosen, L.D.M., Castaldelli, G., Risgaard-Petersen, N., 2000. Protocol Handbook for NICE-Nitrogen Cycling in Estuaries: A Project Under the EU Research Programme. Marine Science and Technology (MAST III). National Environmental Research Institute, Silkeborg, Denmark, 62 pp.

Dennis, P.G., Miller, A.J., Hirsch, P.R., 2010. Are root exudates more important than other sources of rhizodeposits in structuring rhizosphere bacterial communities? FEMS Microbiol. Ecol. 72, 313–327.

Di, H.J., Cameron, K.C., 2002. Nitrate leaching in temperate agroecosystems: sources, factors and mitigating strategies. Nutr. Cycl. Agroecosys. 46, 237–256.

Díaz-Almela, E., Marbà, N., Álvarez, E., Santiago, R., Holmer, M., Grau, A., Mirto, S., Danovaro, R., Petrou, A., Argyrou, M., Karakassis, I., Duarte, C.M., 2008. Benthic input rates predict seagrass (*Posidonia oceanica*) fish farm-induced decline. Mar. Pollut. Bull. 56, 1332–1342.

Dodds W.K., 2006. Eutrophication and trophic state in rivers and streams. Limnol. Oceanogr. 51, 671–680.

Eriksson, P.G., Weisner, S.E.B., 1999. An experimental study on effects of submersed macrophytes on nitrification and denitrification in ammonium rich aquatic systems. Limnol. Oceanogr. 44, 1993–1999.

Fang, C., Moncrieff, J.B., 2001. The dependence of soil CO_2 efflux on temperature. Soil Biol. Biochem. 33, 155–165.

Fenchel, T., Finlay, B., 2008. Oxygen and the spatial structure of microbial communities. Biol. Rev. 83, 553–569.

Fenchel, T., King, G.M., Blackburn, T.H., 1998. Bacterial biogeochemistry: the ecophysiology of mineral cycling. Academic press, 305 pp.

Flessa, H., 1994. Plant-induced changes in the redox potential of the rhizospheres of the submerged vascular macrophytes *Myriophyllum verticillatum* L. and *Ranunculus circinatus* L. Aquat. Bot. 47, 119–129.

Flindt, M.R., 1994. Measurements of nutrient fluxes and mass balances by on-line in situ dialysis in a *Zostera marina* bed culture. Verhandlungen Internationale Vereinigung fur theoretische und angewandte Limnologie 25, 2259–2264.

Forshay, K.J., Dodson, S.I., 2011. Macrophyte presence is an indicator of enhanced denitrification and nitrification in sediments of a temperate restored agricultural stream. Hydrobiologia 668, 21–34.

Galloway, J.N., Aber, J.D., Erisman, J.W., Seitzinger, S.P., Howarth, R.W., Cowling, E.B., Cosby, B.J., 2003. The Nitrogen Cascade. BioScience 53, 341–356.

Galloway, J.N., Townsend, A.R., Erisman, J.W., Bekunda, M., Cai, Z., Freney, J.R., Martinelli, L.A., Seitzinger, S.P., Sutton, M.A., 2008. Transformation of the nitrogen cycle: Recent trends, questions, and potential solutions. Science 320, 889–892.

Gardner, W.S., McCarthy, M.J., 2009. Nitrogen dynamics at the sediment–water interface in shallow, sub-tropical Florida Bay: why denitrification efficiency may decrease with increased eutrophication. Biogeochemistry 95,185–198.

Gibbs, J., Greenway, H., 2003. Mechanisms of anoxia tolerance in plants. I. Growth, survival and anaerobic catabolism. Funct. Plant Biol. 30, 1–47.

Golterman, H.L., Clymo, R.S., Ohnstand, M.A.M., 1978. Methods for physical and chemical analysis of fresh waters, I.B.P. Handbook Nr. 8, Blackwell, Oxford.

Grimvall, A., Stalnacke, P., Tonderski, A., 2000. Time scales of nutrient losses from land to sea - a European perspective. Ecol. Eng. 14, 363–371.

Hansen, J.I., Henriksen, K., and Blackburn, T.H., 1981. Seasonal distribution of nitrifying bacteria and rates of nitrification in coastal marine sediments. Microb. Ecol. 7, 297–304.

Harley, M.T., Findlay, S., 1994. Photosynthesis-irradiance relationships for three species of submersed macrophytes in the tidal freshwater Hudson River. Estuaries 17, 200–205.

Hasegawa, N., Hori, M., Mukai, H., 2008. Seasonal changes in eelgrass functions: current velocity reduction, prevention of sediment resuspension, and control of sediment water column nutrient flux in relation to eelgrass dynamics. Hydrobiologia 596, 387–399.

Hattori, A., 1983. Denitrification and dissimilatory nitrate reduction. In: Carpenter, E.J , Capone, D.G. (eds.) Nitrogen in the marine environment. Academic Press, New York, p. 191-232.

Hauxwell, J., Frazer, T.K., Osenberg, C.W., 2007. An annual cycle of biomass and productivity of *Vallisneria americana* in a subtropical spring-fed estuary. Aquat. Bot. 87, 61–68.

Hebert, A.B., Morse, J.W., Eldridge, P.M., 2007. Small-scale heterogeneity in the geochemistry of seagrass vegetated and non-vegetated estuarine sediments: causes and consequences. Aquat. Geochem. 13, 19–39.

Heilman, M.A., Carlton, R.G., 2001. Methane oxidation associated with submersed vascular macrophytes and its impact on plant diffusive methane flux. Biogeochemistry 52, 207–224.

Henriksen, K., Hansen, J.I., Blackburn, T.H., 1981. Rates of nitrification, distribution of nitrifying bacteria, and nitrate fluxes in different types of sediment from Danish waters. Mar. Biol. 61, 299–304.

Henriksen, K., Kemp, W.M., 1988. Nitrification in estuarine and coastal marine sediments: methods, patterns and regulating factors. In: Blackburn, T. H., Sorensen, J. (eds.) Nitrogen cycling in coastal marine environments. John Wiley and Sons, New York. p. 207–250.

Herrmann, M., Saunders, A.M., Schramm, A., 2008. Archaea dominate the ammonia-oxidizing community in the rhizosphere of the freshwater macrophyte *Littorella uniflora*. Appl. Environ. Microbiol. 74, 3279–3283

Herrmann, M., Saunders, A.M., Schramm, A., 2009. Effect of lake trophic status and rooted macrophytes on community composition and abundance of ammonia-oxidizing prokaryotes in freshwater sediments. Appl. Environ. Microbiol. 75, 3127–3136.

Hilton, J., O'Hare, M., Bowes, M.J., Jones, J.I., 2006. How green is my river? A new paradigm of eutrophication in rivers. Sci. Total Environ. 365, 66–83.

Hines, M.E., 2006. Microbially mediated redox cycling at the oxic–anoxic boundary in sediments: comparison of animal and plants habitats. Water Air Soil Poll. 6, 523–536.

Holmer, M., Frederiksen, M.S., 2007. Stimulation of sulfate reduction rates in Mediterranean fish farm sediments inhabited by the seagrass *Posidonia oceanica*. Biogeochemistry 85, 169–184.

Holmer, M., Marba, N., Diaz-Almela, E., Duarte, C.M., Tsapakis, M., Danovaro, R., 2007. Sedimentation of organic matter from fish farms in oligotrophic Mediterranean assessed through bulk and stable isotope ($\delta^{13}C$ and $\delta^{15}N$) analyses. Aquaculture 262, 268–280.

Holmer, M., Storkholm, P., 2001. Sulphate reduction and sulphur cycling in lake sediments: a review. Fresh. Biol. 46, 431–451.

Howarth, R.W., Marino, R., 2006. Nitrogen as the limiting nutrient for eutrophication in coastal marine ecosystems: Evolving views over three decades. Limnol. Oceanogr. 51, 364–376.

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.

Jaynes, M.L., Carpenter, S.R., 1986. Effects of vascular and nonvascular macrophytes on sediment redox and solute dynamics. Ecology 67, 875–882.

Jeppesen, E., Søndergaard, M., Jensen, J.P., Havens, K.E., Anneville, O., Carvalho, L. and others, 2005. Lake response to reduced nutrient loading – an analysis of contemporary long-term data from 35 case studies. Fresh. Biol. 50, 1747–1771.

Jespersen, D.N., Sorrell, B.K., Brix, H., 1998. Growth and root oxygen release by *Typha latifolia* and its effects on sediment methanogenesis. Aquat. Bot. 61, 165-180.

Joabsson, A., Christensen, T.R., 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.

Kemp, W.M, Murray, L., 1986. Oxygen release from roots of the submersed macrophyte *Potamogeton perfoliatus* L.: regulating factors and ecological implications. Aquat. Bot. 26, 271–283.

Kemp, W.M., Lewis, M.R., Jones, T.W., 1986. Comparison of methods for measuring production by the submersed macrophyte, *Potamogeton perfoliatus* L. Limnol. Oceanogr. 31, 1322–1334.

Knowles, R., 1982. Denitrification. Microbiol. Rev. 46, 43–70.

Koretsky, C.M., Haas, J.R., Miller, D., Ndenga, N.T., 2006. Seasonal variations in pore water and sediment geochemistry of littoral lake sediments (Asylum Lake, MI, USA). Geochem. Trans. 7, 11.

Kostka, J, Luther, G., 1994. Partitioning and speciation of solid phase iron in salt-marsh sediments. Geochim. Cosmochim. Acta 58, 1701–1710.

Kreiling, R.M., Richardson, W.B., Cavanaugh, J.C., Bartsch, L.A., 2011. Summer nitrate uptake and denitrification in an upper Mississippi River backwater lake: the role of rooted aquatic vegetation. Biogeochemistry 104, 309–324.

Kulkarni, M.V., Groffman, P.M., Yavitt, J.B., 2008. Solving the global nitrogen problem: it's a gas! Front. Ecol. Environ. 6, 199–206.

Lai, W., Zhang, Y., Chen, Z., 2012. Radial oxygen loss, photosynthesis, and nutrient removal of 35 wetland plants. Ecol. Eng. 39, 24–30.

Laini, A., Bartoli, M., Castaldi, S., Viaroli, P., Capri, E., Trevisan, M., 2011. Greenhouse gas (CO_2, CH_4 and N_2O) in lowland springs within an agricultural impacted watershed (Po River Plain, Northern Italy). Chem. Ecol. 27, 177–187.

Lamers, L.P., van Diggelen, J.M., Op den Camp, H.J., Visser, E.J., Lucassen, E.C., Vile, M.A., Jetten, M.S., Smolders, A.J., Roelofs, J.G., 2012. Microbial transformations of nitrogen, sulfur, and iron dictate vegetation composition in wetlands: a review. Front. Microbiol. 3, 156.

Laskov, C., Horn, O., Hupfer, M., 2006. Environmental factors regulating the radial oxygen loss from roots of *Myriophyllum spicatum* and *Potamogeton crispus.* Aquat. Bot. 84, 333–340.

Lemoine, D.G., 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.

Lenssen, J.P.M., Menting, F.B.J., van der Putten, W.H., Blom C. W.P.M., 1999. Effects of sediment type and water level on biomass production of wetland plant species. Aquat. Bot. 64, 151–165.

Les, D.H., Jacobs, S.W.L., Tippery, N.P., Chen, L., Moody, M.L., Wilstermann-Hildebrand, M., 2008. Systematics of *Vallisneria* (Hydrocharitaceae). Syst. Bot. 33, 49–65.

Li, H., Ye, Z.H., Wei, Z.J., Wong, M.H., 2011. Root porosity and radial oxygen loss related to arsenic tolerance and uptake in wetland plants. Environ. Pollut. 159, 30–37.

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.

Magen, C., Mucci, A., Sundby, B., 2011. Reduction rates of sedimentary Mn and Fe oxides: an incubation experiment with Arctic Ocean sediments. Aquat. Geochem. 17, 629–643.

Mander, U., Kuusemets, V., Lohmus, K., Mauring, T., 1997. Efficiency and dimensioning of riparian buffer zones in agricultural catchments. Ecol. Eng. 8, 299–324.

Mann, K.J., Wetzel, R.G., 2000. Effects of the emergent macrophyte *Juncus effusus* L. on the chemical composition of interstitial water and bacterial productivity. Biogeochemistry 48, 307–322.

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. Chem. Technol. 1, 46–51.

McClain, M.E., Boyer, E.W., Dent, C.L., Gergel, S.E., Grimm, N.B, Groffman, P.M., Hart, S.C., Harvey, J.W., Johnston, C.A., Mayorga, E., McDowell, W.H., Pinay, G., 2003. Biogeochemical hot spots and hot moments at the interface of terrestrial and aquatic ecosystems. Ecosystems 6, 301–312.

McGlathery, K.J., Sundbäck, K., Anderson, I.C., 2007. Eutrophication in shallow coastal bays and lagoons: the role of plants in the coastal filter. Mar. Ecol. Prog. Ser. 348, 1–18.

Melton, E.D., Schmidt, C., Kappler, A., 2012. Microbial iron(ii) oxidation in littoral freshwater lake sediment: the potential for competition between phototrophic vs. nitrate-reducing iron(ii)-oxidizers. Front Microbiol. 3, 197.

Mi, W.J., Zhu, D.W., Zhou, Y.Y., Zhou, H.D., Yang, T.W., Hamilton, D.P., 2008. Influence of *Potamogeton crispus* growth on nutrients in the sediment and water of Lake Tangxunhu. Hydrobiologia 603, 139–146.

Michalopoulos, P., Aller, R.C., 1995. Rapid clay mineral formation in Amazon Delta sediments: reverse weathering and oceanic elemental cycles. Science 270, 614–617.

Møller, C.L., Sand-Jensen, K., 2011. High sensitivity of *Lobelia dortmanna* to sediment oxygen depletion following organic enrichment. New Phytol. 190, 320–331.

Mulholland, P.J., Helton, A.M., Poole, G.C., Hall, R.O. Jr., Hamilton, S.K., Peterson, B.J., Tank, J.L., Ashkenas, L.R., Cooper, L.W., Dahm, C.N., Dodds, W.K., Findlay, S.E.G., Gregory, S.V., Grimm, N.B., Johnson, S.L., Mcdowell, W.H., Meyer, J.L., Valett, H.M., Webster, J.R., Arango, C.P., Beaulieu, J.J., Bernot, M.J., Burgin, A.J., Crenshaw, C.L., Johnson, L.T., Niederlehner, B.R., O'Brien, J.M., Potter, J.D., Sheibley, R.W., Sobota, D.J., Thomas, S.M., 2008. Stream denitrification across biomes and its response to anthropogenic nitrate loading. Nature 452, 202–205.

Murray, T.E., 1995. The correlation between iron sulfide precipitation and hypolimnetic phosphorus accumulation during one summer in a softwater lake. Can. J. Fish. Aquat. Sci. 52, 1190–1194.

Neubauer, S.C., Emerson, D., Megonigal, J.P., 2008. Microbial oxidation and reduction of iron in the root zone and influences on metal mobility. In: Violante, A., Huang, P.M., Gadd, G.M. (Eds), Biophysico-chemical processes of heavy metals and metalloids in soil environments. John Wiley & Sons Inc., Hoboken, New Jersey.

Nielsen L. P., 1992. Denitrification in sediment determined from nitrogen isotope pairing. FEMS Microbiol. Ecol. 86, 357–362.

Nikolausz, M., Kappelmeyer, U., Székely, A., Rusznyák, A., Márialigeti, K., Kästner, M. 2008. Diurnal redox fluctuation and microbial activity in the rhizosphere of wetland plants. Eur. J. Soil Biol. 44, 324–333.

Nixon, S.W., 1995. Coastal marine eutrophication: a definition, social causes, and future concerns. Ophelia. 41, 199–219.

Nixon, S.W., 2009. Eutrophication and the macroscope. Hydrobiologia 629, 5–19.

Nizzoli, D., Carraro, E., Nigro, V., Viaroli, P., 2010. Effect of organic enrichment and thermal regime on denitrification and dissimilatory nitrate reduction to ammonium (DNRA) in hypolimnetic sediments of two lowland lakes. Water Res. 44, 2715 – 2724.

Oenema, O., 2006. Nitrogen budgets and losses in livestock systems. Int. Congr. Ser. 1293, 262 – 271.

Oenema, O., Kros, H., De Vries, W., 2003. Approaches and uncertainties in nutrient budgets: implications for nutrient management and environmental policies. Eur. J. Agron. 20, 3–16.

Ottosen, L.D.M., Risgaard-Petersen, N., Neilsen, L.P., 1999. Direct and indirect measurements of nitrification and denitrification in the rhizosphere of aquatic macrophytes. Aquat. Microb. Ecol. 19, 81–91.

Pérez, M., Invers, O., Ruiz, J.M., Frederiksen, M.S., Holmer, M., 2007. Physiological responses of the seagrass *Posidonia oceanica* to elevated organic matter content in sediments: An experimental assessment. J. Exp. Mar. Biol. Ecol. 344, 149–160.

Pezeshki, S.R., 2001. Wetland plant responses to soil flooding. Environ. Exp. Bot. 46, 299–312.

Philippot, L., Hallin, S., Börjesson, G., Baggs, E. M. 2009. Biochemical cycling in the rhizosphere having an impact on global change. Plant Soil 321, 61–81.

Pinardi, M., Bartoli, M., Longhi, D., Marzocchi, U., Laini, A., Ribaudo, C., Viaroli, P., 2009. Benthic metabolism and denitrification in a river reach: a comparison between vegetated and bare sediments. J. Limnol. 68, 133–145.

Pinardi, M., Bartoli, M., Longhi, D., Viaroli, P., 2011. Net autotrophy in a fluvial lake: The relative role of phytoplankton and floating-leaved macrophytes. Aquat. Sci. 73, 389–403.

Pinay, G., Clément, J.C., Naiman, R.J., 2002. Basic principles and ecological consequences of changing water regimes on nitrogen cycling in fluvial systems. Environ. Manage. 30, 481–491.

Pokorný, J., Orr, P.T., Ondok, J.P., Denny, P.,1989. Photosynthetic quotients of some aquatic macrophyte species. Photosynthetica 23, 494–506.

Poté, J., Haller, L., Loizeau, J., Garcia Bravo, A., Sastre, V., Wildi, W. 2008. Effects of a sewage treatment plant outlet pipe extension on the distribution of contaminants in the sediments of the Bay of Vidy, Lake Geneva, Switzerland. Bioresource Technol. 99, 7122–7131.

Povidisa, K., Delefosse, M., Holmer, M., 2009. The formation of iron plaques on roots and rhizomes of the seagrass *Cymodocea serrulata* (R. Brown) Ascherson with implications for sulphide intrusion. Aquat. Bot. 90, 303–308.

Puckett, L.J., 1995. Identifying the major sources of nutrient water pollution. Envir. Sci. Tech. 29, 408–414.

Pulido, C., Lucassen, E.C.H.E.T., Pedersen, O., Roelofs, J.G.M., 2010. Influence of quantity and lability of sediment organic matter on the biomass of two isoetids, *Littorella uniflora* and *Echinodorus repens*. Fresh. Biol. 56, 939–951.

Qiu, D., Wu, Z., Liu, B., Deng, J., Fu, G., He, F., 2001. The restoration of aquatic macrophytes for improving water quality in a hypertrophic shallow lake in Hubei Province, China. Ecol. Eng. 18, 147–156.

Querné, J., Ragueneau, O., Poupart, N., 2012. In situ biogenic silica variations in the invasive salt marsh plant, *Spartina alterniflora*: A possible link with environmental stress. Plant Soil 352, 157–171.

R Development Core Team, 2011. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria, ISBN 3-900051-07-0, URL http://www.R-project.org.

Racchetti, E., 2010. Nitrogen loads, mass balance, net export and regulation of denitrification: a watershed scale analyis. Doctoral thesis, PhD in Ecology. University of Parma (in Italian).

Racchetti, E., Bartoli, M., Ribaudo, C., Longhi, D., Brito, E.Q.L., Naldi, M., Iacumin, P., Viaroli, P., 2010. Short term changes in pore water chemistry in river sediments during the early colonization by *Vallisneria spiralis*. Hydrobiologia 652, 127–137.

Racchetti, E., Bartoli, M., Soana, E., Longhi, D., Christian, R.R., Pinardi, M., Viaroli P., 2011. Influence of hydrological connectivity of riverine wetlands on nitrogen removal via denitrification. Biogeochemistry 103, 335–354.

Raun, A.L., Borum, J., Sand-Jensen, K., 2010. Influence of sediment organic enrichment and water alkalinity on growth of aquatic isoetid and elodeid plants. Fresh. Biol, 55, 1891-1904.

Reddy, K.R., Patrick, W.H. Jr, Lindau, C.W., 1989. Nitrification-denitrification at the plant root–sediment interface in wetlands. Limnol. Oceanogr. 34, 1004–1013.

Ribaudo, C., Bartoli, M., Racchetti, E., Longhi, D., Viaroli, P., 2011. Seasonal fluxes of O_2, DIC and CH_4 in sediments with *Vallisneria spiralis*: indications for radial oxygen loss. Aquat. Bot. 94, 134–142.

Risgaard-Petersen, N., Dalsgaard, T., Rysgaard, S., Christensen, P.B., Borum, J., McGlathery, K., Nielsen, L.P., 1998. Nitrogen balance of a temperate eelgrass *Zostera marina* bed. Mar. Ecol. Prog. Ser. 174, 281–291.

Risgaard-Petersen, N., Jensen, K., 1997. Nitrification and denitrification in the rhizosphere of the aquatic macrophyte *Lobelia dortmanna* L. Limnol. Oceanogr. 42, 529–537.

Risgaard-Petersen, N., Meyer, R.L, Revsbech, N.P., 2005. Denitrification and anaerobic ammonium oxidation in sediments: effects of microphytobenthos and NO_3^- . Aquat. Microb. Ecol. 40, 67–76.

Risgaard-Petersen, N., Nicolaisen, M.H., Revsbech, N.P., Lomstein, B.A., 2004. Competition between ammonia oxidizing bacteria and benthic microalgae. Appl. Environ. Microbiol. 70, 5528−5537.

Rotz, C.A., 2004. Management to reduce nitrogen losses in animal production. J. Anim. Sci. 82, 119–137.

Ruiz-Rueda, O., Hallin, S., Baneras, L., 2009. Structure and function of denitrifying and nitrifying bacterial communities in relation to the plant species in a constructed wetland. FEMS Microbiol. Ecol. 67, 308–319.

Sand-Jensen, K., 1997. Macrophytes as biological engineers in the ecology of Danish streams, in: Sand-Jensen, K., Pedersen, O. (Eds.), Freshwater biology: priorities and development in Danish research. The Freshwater Biological Laboratory. Copenhagen: University of Copenhagen and G.E.C. Gad Publishers.

Sand-Jensen, K., Prahl, C., Stokholm, H., 1982. Oxygen release from roots of submerged aquatic macrophytes. Oikos 38, 349–354.

Sand-Jensen, K., Borum, J., 1991. Interactions among phytoplankton, periphyton, and macrophytes in temperate freshwaters and estuaries. Aquat. Bot. 41, 137–175.

Sand-Jensen, K., 1998. Influence of submerged macrophytes on sediment composition and near-bed flow in lowland streams. Fresh. Biol. 39, 663–679.

Sand-Jensen, K., Riis, T., Vestergaard, O., Larsen, S., 2000. Macrophyte decline in Danish lakes and streams over the past 100 years. J. Ecol. 88, 1030–1040.

Sand-Jensen, K., Borum, J., Binzer, T., 2005. Oxygen stress and reduced growth of *Lobelia dortmanna* in sandy lake sediments subject to organic enrichment. Fresh. Biol. 50, 1034–1048.

Sand-Jensen, K., Møller, K.L., Raun, A.L., 2008. Outstanding *Lobelia dortmanna* in iron armor. Plant Signal. Behav. 3, 882–884.

Scheffer, M., Carpenter, S., Foley, J., Folke, C., Walker, B., 2001. Catastrophic shifts in ecosystems. Nature 413, 591–596.

Scheffer, M., Szabó, S., Gragnani, A., van Nes, E.H., Rinaldi, S., Kautsky, N., Norberg, J., Roijackers, R.M.M., Franken, R.J.M., 2003. Floating plant dominance as a stable state. PNAS 100, 4040–4045.

Schlesinger, W.H., 2009. On the fate of anthropogenic nitrogen. PNAS 106, 203–208.

Schlesinger, W.H., Reckhow, K.H., Bernhardt, E.S., 2006. Global change: The nitrogen cycle and rivers. Water Resour. Res. DOI, 10.1029/2005WR004300.

Schröder, J.J., Scholefield, D., Cabral, F., Hofman, G., 2004. The effects of nutrient losses from agriculture on ground and surface water quality: the position of science in developing indicators for regulation. Environ. Sci. Policy 7, 15–23.

Schüring, J., Schulz, H.D., Fischer, W.R., Böttcher, J., Duijnisveld, W.H.M., 1999. Redox: fundamentals, processes and applications, Springer-Verlag, New York.

Sculthorpe, C.D., 1967. The biology of aquatic vascular plants. London: Edward Arnold.

Sears, K., Alleman, J.E., Barnard, J.L., Oleszkiewicz, J.A., 2004. Impacts of reduced sulfur components on active and resting ammonia oxidizers. J. Ind. Microbiol. Biotechnol. 31, 369–378.

Seitzinger, S., Harrison, J.A., Bohlke, J.K., Bouwman, A.F., Lowrance, R., Peterson, B., Tobias, C., van Drecht, G., 2006. Denitrification across landscapes and waterscapes: a synthesis. Ecol. Appl. 16, 2064–2090.

Sherr, B.F., Payne, W.J., 1978. Effect of the *Spartina alterniflora* root-rhizome system on salt marsh soil denitrifying bacteria. Appl. Environ. Microbiol. 35, 724–729.

Sirivedhin, T., Gray, K.A., 2006. Factors affecting denitrification rates in experimental wetlands: field and laboratory studies. Ecol. Eng. 26, 167–181.

Smith, R.D., Dennison, W.C., Alberte, R.S., 1984. Role of seagrass photosynthesis in root aerobic processes. Plant Physiol. 74, 1055–1058.

Smolders, A.J.P., Lucassen, E.C.T.E.T., Roelofs, J.G.M., 2002. The isoetid environment biogeochemistry and threats. Aquat. Bot. 73, 325–350.

Soana E., Racchetti E., Laini A., Bartoli M., Viaroli P. 2011. Soil budget, net export and potential sinks of nitrogen in the lower Oglio River watershed (Northern Italy). CLEAN – Soil, Air, Water 39, 956–965.

Sorrell, B.K., Armstrong, W. 1994. On the difficulties of measuring oxygen release by root systems of wetland plants. J. Ecol. 82, 177–183.

Sorrell, B.K., Downes, M.T., Stanger, C.L. 2002. Methanotrophic bacteria and their activity on submerged aquatic macrophytes. Aquat. Bot. 72, 107–119.

Sousa, A.I., Lillebø A.I, Risgaard-Petersen, N., Pardal, M.A., Caçador, I., 2012. Denitrification: an ecosystem service provided by salt marshes. Mar. Ecol. Prog. Ser. 448, 79–92.

St-Cyr, L., Campbell, P.G.C., 1996. Metals (Fe, Mn, Zn) in the root plaque of submerged aquatic plants collected in situ: Relations with metal concentrations in the adjacent sediments and in the root tissue. Biogeochemistry 33, 45-76.

Stookey, L.L., 1970. Ferrozine: a new spectrophotometric reagent for iron. Anal. Chem. 42, 779–781.

Strauss, E.A., Lamberti, G.A., 2000. Regulation of nitrification in aquatic sediments by organic carbon. Limnol. Oceanogr. 45, 1854–1859.

Struyf, E., Conley, D.J., 2009. Silica: an essential nutrient in wetland biogeochemistry. Front. Ecol. Environ. 7, 88–94.

Talbot, R.J., Etherington, J.R., 1987. Comparative studies of plant growth and distribution in relation to waterlogging. XIII. The effect of Fe^{2+} on photosynthesis and respiration of *Salix caprea* and *S. cinerea* spp. *oleifolia*. New Phytol. 105, 575–583.

Terrados, J., Duarte, C.M., Kamp-Nielsen, L., Agawin, C.M., Gacia, E., Lacap, D., Fortes, M.D., 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.

Thamdrup, B., Dalsgaard, T., 2002. Production of N_2 through anaerobic ammonium oxidation coupled to nitrate reduction in marine sediments. Appl. Environ. Microbiol. 68, 1312–1318.

Tiedje, J.M., 1988. Ecology of denitrification and dissimilatory nitrate reduction to ammonium. In: Zehnder, A.J.B. (ed) Biology of anaerobic microorganisms. Wiley, New York.

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.

Valderrama, J.C., 1977. Methods used by the Hydrographic Department of the National Board of Fisheries. Goteborg, Sweden.

Valiela, I., Bowen, J.L., 2002. Nitrogen sources to watersheds and estuaries: role of land cover mosaics and losses within watersheds. Environ. Poll. 118, 239–248.

van Bodegom, P.M., de Kanter, M., Bakker, C., Aerts, R., 2005. Radial oxygen loss, a plastic property of dune slack plant species. Plant Soil. 271, 351–364.

van Breemen, N., Boyer, E.W., Goodale, C.L., Jaworski, N.A., Paustian, K., Seitzinger, S.P., Lajtha, K., Mayer, B., Van Dam, D., Howarth, R.W., Nadelhoffer, K.J., Eve, M., Billen, G., 2002. Where did all the nitrogen go? Fate of nitrogen inputs to large watersheds in the northeastern U.S.A. Biogeochemistry 57, 267–293.

Van Der Nat, F.W.A., Middelburg, J.J. 1998. Effects of two common macrophytes on methane dynamics in freshwater sediments. Biogeochemistry 43, 79–104.

Van Drecht, G., Bouwman, A.F., Boyer, E.W., Green, P., Siebert, S., 2005. A comparison of global spatial distributions of nitrogen inputs for nonpoint sources and effects on river nitrogen export. Global Biogeochem. Cycles DOI, 10.1029/2005GB002454.

van Wijck, C., de Groot, C.J., 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.

Viaroli, P., Bartoli, M., Giordani, G., Naldi, M., Orfanidis, S., Zaldívar, J.M., 2008. Community shifts, alternative stable states, biogeochemical controls and feedbacks in eutrophic coastal lagoons: a brief overview. Aquat. Conserv. 18, S105–S117.

Visser, E.J.W., Colmer, T.D., Blom, C.W.P.M., Voesenek, L.A.C.J., 2000. Changes in growth, porosity, and radial oxygen loss from adventitious roots of selected mono- and dicotyledonous wetland species with contrasting types of aerenchyma. Plant Cell Environ. 23, 1237–1245.

Wang, J., Yu, D., 2007. Influence of sediment fertility on morphological variability of *Vallisneria spiralis* L. Aquat. Bot. 87, 127–133.

Webb, J.S., McGinness, S., Lappin-Scott, H.M., 1998. Metal removal by sulphate-reducing bacteria from natural and constructed wetlands. J. Appl. Microbiol. 84, 240–248.

Welsh, D.T., Bartoli, M., Nizzoli, D., Castaldelli, G., Riou, S.A., 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.

Wigand, C., Stevenson, J.C., Cornwell, J.C. 1997. Effects of different submersed macrophytes on sediment biogeochemistry. Aquat. Bot. 56, 233–244.

Wigand, C., Wehr, J., Limburg, K., Gorham, B., Longergan, S., Findlay, S., 2000. Effect of *Vallisneria americana* (L.) on community structure and ecosystem function in lake mesocosms. Hydrobiologia 418, 137–146.

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

Xiao, K., Yu, D., Wang, J., 2006. Habitat selection in spatially heterogeneous environments: a test of foraging behaviour in the clonal submerged macrophyte *Vallisneria spiralis*. Fresh. Biol. 51, 1552–1559.

Xie, Y., An, S., Yao, X., Xiao, K., Zhang, C., 2005. Short-time response in root morphology of *Vallisneria natans* to sediment type and water-column nutrient. Aquat. Bot. 81, 85–96.

Xing, W., Li, D., Liu, G., 2010. Antioxidative responses of *Elodea nuttallii* (Planch.) H. St. John to short-term iron exposure. Plant Physiol. Biochem. 48, 873–878.

Yates, A.G., Bailey, R.C., Schwindt, J.A., 2007. Effectiveness of best management practices in improving stream ecosystem quality. Hydrobiologia 583, 331–344.