Università degli Studi di Ferrara

Ph.D. Course
in
Evolutionary Biology and Ecology

In cooperation with:
Università degli Studi di Parma
Università degli Studi di Firenze

CYCLE XXXI

COORDINATOR Prof. Guido Barbujani

Hot moments and hot spots of benthic nitrogen cycling along environmental gradients

Scientific/Disciplinary Sector (SDS) BIO/07

Candidate
Dr. Benelli Sara

Supervisor
Prof. Fano Elisa Anna

Years 2015/2018

Contents

Thesis abstract

In shallow water ecosystems, the functioning of benthic compartment depends on the interactions between the physico-chemical environment and the community of micro and macroorganisms. This environment displays steep redox gradients and multiple interfaces, where fully oxic layers may be adjacent to sulfidic sediments. The biological communities have evolved adaptations, creating a network of coupled processes and multiple feedbacks. The sedimentary environment is complex and difficult to colonize but is full of opportunities for organisms, as it may be more stable than the water column, and it may receive a large flux of energy. It is also challenging for scientists, as sediment can be manipulated in laboratory in order to validate key ecological questions generally addressed in terrestrial environments, among which those related to diversity and functioning relationships.

This PhD thesis investigates some aspects of the benthic system functioning (*bsf*) along gradients including light, nutrient availability and those of species or functional diversity. The ecological interactions between trophic levels and physical environment create the complexity, whereas the functioning here refers to organic matter mineralization, nutrient cycling and transport across sediment-water interface. Benthic animals create horizontal and vertical heterogeneities in sediments and affect the benthic-pelagic coupling through their bioturbation activities. Bioturbation affects the heterotrophic component of benthic system, but also primary producers growth. The main aim of the thesis is to understand the effects of microbes, macrofauna and primary producers on benthic system processes along multiple environmental gradients. The general hypothesis is that a complex benthic system exploits better the available resources by increasing nutrient recycling and decreasing nutrient import or loss.

The thesis is divided in five chapters that revise the effects of complexity on ecosystem functioning. Traditionally, processes were studied along gradients of decreasing species and functional diversity. Results from these experiments were usually characterized by unidirectional large fluxes of energy and matter. Recently, researches have begun to apply more complex approaches that usually include both the experimental and modelling tools.

After a brief overview of the ecological research over the last few decades (Chapter 1), I introduce three experimental works in which the effects of the heterotrophic component on *bsf* was investigated (Chapter 2). These studies demonstrate that benthic invertebrates are drivers of biogeochemical processes, by controlling the fate of organic matter and nutrients as well as fluxes of nutrients between sediment and water. However, in natural environments their large effects could be smoothed in presence of primary producers that assimilate nutrients from both the water column and the sediment. The co-occurrence of macrofauna and benthic primary

producers was therefore investigated by means of two experiments. They demonstrated that process stimulation is species-specific and benthic microalgae can act as a buffer for regenerated nutrients (Chapter 3). Finally, I investigated interactions among multiple trophic levels, along a gradient of inorganic N availability and organic matter content (Chapter 4). In a eutrophic site, the co-occurrence of two different primary producers and macrofauna groups increased the recycling of nutrients within the system, suggesting a better exploitation of N sources. Results highlight the important role of macrofauna as facilitator of N availability in the benthic and pelagic compartments. On the contrary, microbial N fixation was the most important process that sustained primary production under oligotrophy. Primary producers N requirements suppressed N loss via denitrification. In the last chapter, I draw the main conclusions of my work and some aspects that could be developed in the future (Chapter 5).

Chapter 1

Introduction

In the past decade, an increasing number of ecological studies addressed the effects of biodiversity loss on ecosystem functioning (Schulze and Mooney 1993; Loreau et al. 2002; Thébault and Loreau 2003). In particular, the main concerns regarded the loss of species to be associated with the loss of ecological functions and services which could influence the structure of an ecosystem by affecting its productivity, biogeochemical cycles and the physical formation of habitats (Vaughn 2010; Cardinale et al. 2012). Most studies analyzed only few aspects of biodiversity and ecosystem functions, investigating how diversity within species may affect the processes that play important roles in the ecosystem, as for example primary production (Gamfeldt and Hillebrand 2008). For what concern benthic environments, and in particular shallow water bodies, a wide range of studies have been conducted regarding different topics as microbial ecology, population dynamics of primary producers, meio and macrofauna ecology and biogeochemical cycles (Gray and Elliott 2009). The first attempts to understand the direct effects of macrofauna on benthic biogeochemical processes involved laboratory studies that allowed to control and vary a broad range of experimental conditions (Banta et al. 1999; Ferguson and Eyre 2013). Typically, these investigations were carried out removing environmental variability by sediment sieving and with the construction of micro and mesocosms containing an increasing number of organisms of a single trophic level. These studies considered only the heterotrophic component of the benthic system (Pelegrí and Blackburn 1995; Nizzoli et al. 2006; Bonaglia et al. 2013). Results from these works were extremely predictable as the measured biogeochemical processes were directly or indirectly related to the biomass of the targeted species. However, these works have been important in the understanding of species-specific features about physiology, in situ density, respiration, excretion, etc. Although studies on a single trophic level represented the first attempts to join community and ecosystem (material and energy flow) ecology, they were insufficient to understand the role of the species in the ecosystem functioning in natural environments, since the latter are more complex and comprise multiple trophic levels (Hooper et al. 2005; Duffy 2009).

Recently, the research in this field has shifted from the traditional experimental approaches towards more complex designs that would help in understanding how the species interact among multiple trophic levels and how this can affect the ecosystem functioning. For instance, experiments performed using larger spatial and temporal scales and taking in consideration

different trophic levels may lead to a more comprehensive picture of the direct and indirect effects of species diversity on ecosystem processes. In multi-trophic systems, these effects are difficult to predict, since the targeted process rates could increase, decrease or stay the same (Thébault and Loreau 2003; Vaughn 2010). A couple of articles showed the analysis on multiple interactions between organisms and the abiotic components in freshwater environments (Chase and Knight 2006; Mermillod-Blondin and Lemoine 2010). These studies described the activity of different functional groups of macrofauna related to native and non-native macrophytes growth. They demonstrated that macrofauna enhanced the growth of native macrophytes, either by removing epiphytes from macrophyte leaves or mobilizing dissolved nutrients that were readily taken up by macrophytes. These examples show the importance of using holistic and inclusive approaches to have deeper knowledge about a particular ecosystem and its function in a particular period of time. Taking into account species belonging to different trophic levels seems to be the key factor for this approach (Ieno et al. 2006).

Investigations targeting multiple organisms and multiple trophic levels require large experimental and analytical efforts and can take advantage by qualitative and quantitative modelling. In fact, combining experimental with modelling approaches, the indirect and hidden interactions among the species, not measurable with experiments alone, could be better understood and more realistic scenarios could be provided (Gamfeldt and Hillebrand 2008). The application of models and the use of statistical tools would be useful to describe at larger scale the ecosystem functioning and to predict further changes (Loreau et al. 2001; Thébault and Loreau 2003; Cardinale et al. 2012).

Starting from a simplified experimental approach that focused on single trophic levels, this PhD thesis aims at analyzing how three interplaying factors, such as light, background nutrient levels and biodiversity, regulate process rates and the coupling of benthic nitrogen (N) transformations along a gradient of increasing complexity. The processes involved in N cycling are reported in Fig. 1.1 (modified from Canfield et al. 2005). Molecular N can be fixed by N-fixing bacteria, which turn it into a form that is more usable by complex organisms. Hence, N fixation can counteract N loss by denitrification. Organic N forms can be transformed to inorganic N in the water column and in the sediment. Both organic and inorganic N forms may diffuse from the water column to the sediment and vice versa. Nitrogen can either be buried in the deep sediment as organic N or adsorbed and mineral-bound ammonium (NH_4^+) as inorganic in sediment. Then, organic N at the sediment surface and along the water column may be ammonified through ammonification or N mineralization and transformed into NH_4^+ in the water column and in the sediment. In both compartments NH_4^+ may be oxidized by nitrifying microorganisms under oxic conditions. NH_4^+ and nitrite (NO_2^-) oxidations form NO_3^- via nitrification; in the sediment

NO_3^- could be transported downward by diffusion to the anoxic sediment. Here, NO_3^- could be reduced to N_2 by denitrification or to NH_4^+ by dissimilatory nitrate reduction to ammonium (DNRA). Denitrification, together with the reduction to N_2 coupled to the oxidation of NH_4^+ (anammox), contribute to the loss of N from the system to the atmosphere.

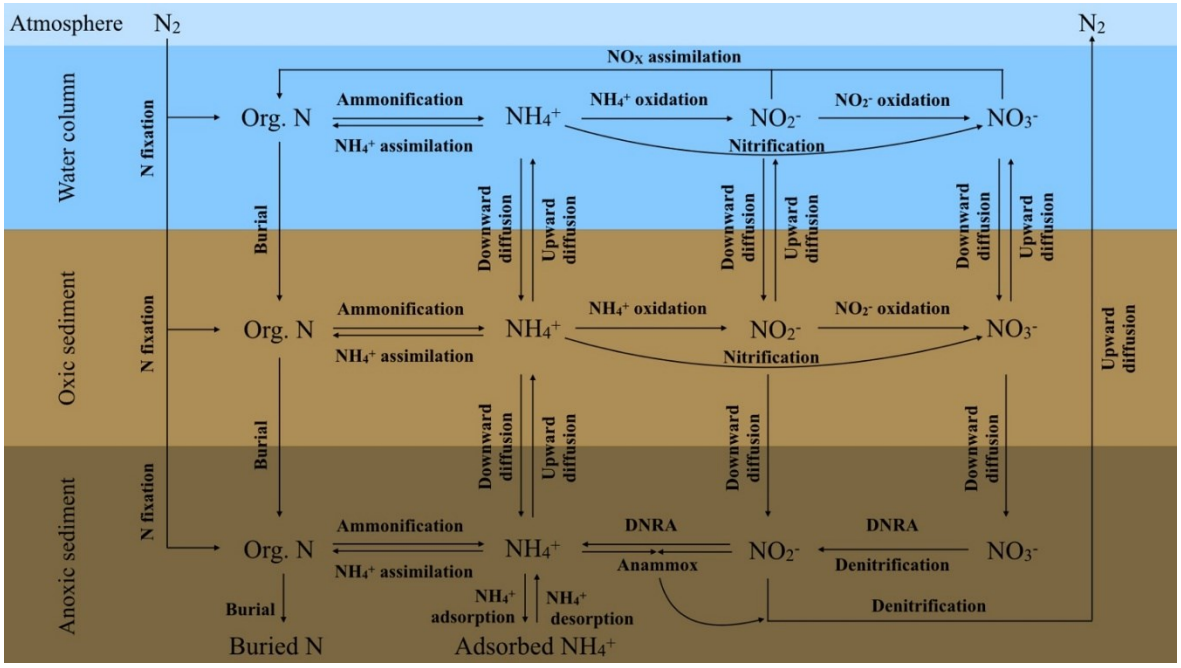


Fig. 1.1 Nitrogen cycling in freshwater systems (modified from Canfield et al. 2005).

The idea is to take into account the emerging properties associated to the interactions among multiple trophic levels as well as the hot moments and the hot spots where key N processes occur (Groffman et al. 2009). In the benthic systems, there could be small areas (hot spots) and brief periods (hot moments) where specific N processes may be enhanced or inhibited with the presence of organisms. For instance, in a shallow water environment where macrophyte meadows are present and receive light during the day, O_2 may be lost through the roots (ROL) and hence a process like the coupling between nitrification and denitrification could be stimulated. The rhizosphere in the light period represents the hot spot for N cycling compared to a sediment without macrophytes, whereas light represents the hot moment when ROL occurs with multiple implications on N cycling (Marzocchi et al. 2019). Another example of the hot moments and the hot spots of benthic N cycling in a shallow water system is represented by the burrowing animals. They dig into the sediment and flush their burrows with overlying water by creating the hot spots for the coupled nitrification-denitrification process along the burrow walls. The ventilation and bioirrigation activities may represent the hot moments for nitrification deep in the sediments (Stief 2013). These concepts will be detailed again by means of the single studies reported in this PhD thesis.

The main hypothesis is that a large nutrient regeneration in simplified heterotrophic systems is replaced by nutrient retention and recycling in illuminated, biodiverse sediments. In the latter, different functional groups of macroinvertebrates shape multiple processes of benthic N cycling through the interaction with primary producers and microbial communities. The focus on N cycling and macrofauna seems particularly promising with respect to the evaluation of complexity-functioning hypotheses for a few main reasons. First, N cycling is based on a large number of different reactions and processes, mediated by specialized heterotrophs as well as by autotrophs. Then, macroinvertebrates include a large number of functional groups that interact with the components of aquatic environments, including water, sediments, phytoplankton, microphytobenthos, macrophytes and microbial communities. On the basis of the type of feeding, macrofauna can be divided into five categories: suspension feeders, surface deposit feeders, sub-surface deposit feeders, herbivores and carnivores (Rosenberg 2001). Among them, burrowing animals constitute the major bioturbators of the sediment. With bioturbation I refer to all transport processes carried out by animals that directly or indirectly affect sediment matrices. These processes include particle 'reworking' (i.e. movement of particles) and burrow 'ventilation' (i.e. movement of water)(Kristensen et al. 2012). Bioturbation may have implications on habitat formation or alteration, on other species or on microbial communities that drive biogeochemical transformations. Furthermore, macrofauna can change sediment distribution and particle erosion through burrowing, feeding and movement, and thus it may enhance both the direct regeneration of nutrients to the water column and, indirectly, the surface area available for microbial activity (Henriksen et al. 1980; Aller 1988). Finally, the availability of methods to analyze in detail N reactions in laboratory experiments represents a unique possibility to test feedbacks and interactions. The experimental approach used in this thesis reproduces the steps of the evolution of the ecological research and it is based on a widely recognized protocol for biogeochemical studies (Dalsgaard et al. 2000). This method consists of short-term laboratory experiments that involve incubations of benthic chambers or cores containing either intact or reconstructed sediments, with or without macrofauna and/or primary producers.

Besides the introduction (Chapter 1) and the general conclusions (Chapter 5), the thesis consists of three main chapters. Chapters 2 to 4 mirror the temporal evolution of the ecological methods investigating the relationship between complexity and functioning, shifting from traditional towards complex and holistic experiments. Each experiment has specific hypotheses and objectives and focused on the ecosystem-level implication of bioturbation by benthic animals, in dark and illuminated sediments.

In chapter 2, three experiments analyze the effects of the heterotrophic component of the benthic system on its functioning. “Ecosystem functioning”, in a benthic perspective, is here analyzed in terms of mineralization efficiency and nutrient recycling. Sediments that display low mineralization capacity tend to accumulate organic carbon and are prone to become chemically reduced and therefore macrofauna poor. Sediments that display large effluxes of mineral nutrients generally maintain eutrophic conditions in the pelagic environment. Sediments that re-use (recycle) nutrients minimizing import and export processes are those working better in this perspective. Process rates measured in the dark may vary on the basis of different functional groups of macrofauna: filter feeders are known to regenerate large amount of dissolved inorganic nutrients to the water column, whereas burrowing deposit feeders may favor processes that dissipate or retain nutrients. The traditional approach used in these studies refers to dark incubation of one single species, to sediment sieving and to the addition of organisms to reconstructed sediments. However, attempts were made to simulate as much as possible the realistic conditions, by adding for instance a number of organisms that reflected in situ density. Long pre-incubation of the reconstructed sediments was done in order to allow the establishment of bacterial communities, of diagenetic processes and of steady-state conditions (Stocum and Plante 2006).

In chapter 3, the combined effect of macrofauna and primary producers on benthic biogeochemistry is evaluated by means of sediment core incubations in the dark and in the light. The autotrophic component is now introduced in these experiments as it may compensate through uptake and oxygen production the increased heterotrophy by macrofauna. It may also act sinergically with macrofauna, increasing sediment resilience towards perturbations. This chapter contains two studies related to the role of chironomid larvae and mussels in enhancing or inhibiting the growth of pelagic algae in a hypertrophic environment. Chironomid larvae dig deep burrows in the sediment and their bioturbation may inhibit the growth of pelagic algae by nutrient retention within the sediment. Similarly, microphytobenthos may enhance nutrient retention by assimilating nutrients from both the sediment and the water column and may oxidize surface sediments promoting processes as phosphorus retention or N loss via nitrification and denitrification.

In chapter 4, the effects of the interactions among macrofauna, primary producers and bacteria on N cycling are studied along a trophic gradient, which is reflected by the inorganic nitrogen availability and the sedimentary content of organic matter. The hypotheses here were multiple and addressed how the presence of different macrofauna functional groups might smooth the competition among microbes and primary producers and how losses and imports vary along biodiversity gradients. This chapter includes the most complex experiments realized in my

thesis, which were planned together with the help of experts in the field of the Ecological Network Analysis. The application of such qualitative and quantitative modelling tool allowed to estimate hidden loops within the benthic community and, in particular, the dependency of primary producers from the activity of bacteria and macrofauna.

References

Aller R.C. (1988) Benthic fauna and biogeochemical processes in marine sediments: the role of burrow structures. In: Blackburn T.H., & Sørensen J. (eds) Nitrogen cycling in coastal marine environments. John Wiley & Sons, Chichester, 301 – 338.

Banta G.T., Holmer M., Jensen M.H., & Kristensen E. (1999) Effects of two polychaete worms, *Nereis diversicolor* and *Arenicola marina*, on aerobic and anaerobic decomposition in a sandy marine sediment. Aquatic Microbial Ecology, 19: 189 – 204.

Bonaglia S., Bartoli M., Gunnarsson J.S., Rahm L., Raymond C., Svensson O., ... & Brüchert V. (2013) Effect of reoxygenation and *Marenzelleria* spp. bioturbation on Baltic Sea sediment metabolism. Marine Ecology Progress Series, 482: 43 – 55.

Canfield D.E., Kristensen E., & Thamdrup B. (2005) Aquatic geomicrobiology. Gulf Professional Publishing.

Cardinale B.J., Duffy J.E., Gonzalez A., Hooper D.U., Perrings C., Venail P., ... & Kinzig A.P. (2012) Biodiversity loss and its impact on humanity. Nature, 486: 59 – 67.

Chase J.M., & Knight T.M. (2006) Effects of eutrophication and snails on Eurasian watermilfoil (*Myriophyllum spicatum*) invasion. Biological Invasions, 8: 1643 – 1649.

Dalsgaard T., Nielsen L.P., Brotas V., Viaroli P., Underwood G., Nedwell D., ... & Dong L. (2000) Protocol handbook for NICE-Nitrogen Cycling in Estuaries: a project under the EU research programme: Marine Science and Technology (MAST III), 1 – 62.

Duffy J.E. (2009) Why biodiversity is important to the functioning of real-world ecosystems. Frontiers in Ecology and the Environment, 7: 437 – 444.

Ferguson A., & Eyre B. (2013) Interaction of benthic microalgae and macrofauna in the control of benthic metabolism, nutrient fluxes and denitrification in a shallow sub-tropical coastal embayment (western Moreton Bay, Australia). Biogeochemistry, 112: 423 – 440.

Gamfeldt L., & Hillebrand H. (2008) Biodiversity effects on aquatic ecosystem functioning–maturation of a new paradigm. International Review of Hydrobiology, 93: 550 – 564.

Gray J.S., & Elliott M. (2009) Ecology of marine sediments: from science to management. Oxford University Press, London.

Groffman P.M., Butterbach-Bahl K., Fulweiler R.W., Gold A.J., Morse J.L., Stander E.K., … & Vidon P. (2009) Challenges to incorporating spatially and temporally explicit phenomena (hotspots and hot moments) in denitrification models. Biogeochemistry, 93: 49 – 77.

Henriksen K., Hansen J.I., & Blackburn T.H. (1980) The influence of benthic infauna on exchange rates of inorganic nitrogen between sediment and water. Ophelia International Journal of Marine Biology, Supplement, 1: 249 – 256.

Hooper D.U., Chapin F.S., Ewel J.J., Hector A., Inchausti P., Lavorel S., ... & Schmid B. (2005) Effects of biodiversity on ecosystem functioning: a consensus of current knowledge. Ecological Monographs, 75: 3 – 35.

Ieno E.N., Solan M., Batty P., & Pierce G.J. (2006) How biodiversity affects ecosystem functioning: roles of infaunal species richness, identity and density in the marine benthos. Marine Ecology Progress Series, 311: 263 – 271.

Kristensen E., Penha-Lopes G., Delefosse M., Valdemarsen, T., Quintana, C.O., & Banta G.T. (2012) What is bioturbation? The need for a precise definition for fauna in aquatic sciences. Marine Ecology Progress Series, 446: 285 – 302.

Loreau M., Naeem S., Inchaus P., Bengtsson J., Grime J.P., Hector A., ... & Tilman D. (2001) Biodiversity and ecosystem functioning: current knowledge and future challenges. Science, 294: 804 – 808.

Loreau M., Naeem S., & Inchausti P. (2002) Biodiversity and Ecosystem Functioning: Synthesis and Perspectives. Oxford University Press, London.

Marzocchi U., Benelli S., Larsen M., Bartoli M., & Glud R.N. (2019) Spatial heterogeneity and short-term oxygen dynamics in the rhizosphere of *Vallisneria spiralis*: Implications for nutrient cycling. Freshwater Biology, 64: 532 – 543.

Mermillod-Blondin F., & Lemoine D.G. (2010) Ecosystem engineering by tubificid worms stimulates macrophyte growth in poorly oxygenated wetland sediments. Functional Ecology, 24: 444 – 453.

Nizzoli D., Welsh D.T., Fano E.A., & Viaroli P. (2006) Impact of clam and mussel farming on benthic metabolism and nitrogen cycling, with emphasis on nitrate reduction pathways. Marine Ecology Progress Series, 315: 151 – 165.

Pelegrí S.P., & Blackburn T.H. (1995) Effects of *Tubifex tubifex* (Oligochaeta: Tubificidae) on N-mineralization in freshwater sediments, measured with ^{15}isotopes. Aquatic Microbial Ecology, 9: 289 – 294.

Rosenberg R. (2001) Marine benthic faunal successional stages and related sedimentary activity. Scientia Marina, 65: 107 – 119.

Schulze E.D., & Mooney H.A. (1993) Biodiversity and ecosystem function. Berlin, Germany: Springer-Verlag.

Stief P. (2013) Stimulation of microbial nitrogen cycling in aquatic ecosystems by benthic macrofauna: Mechanisms and environmental implications. Biogeosciences, 10: 7829 – 7846.

Stocum E.T., & Plante C.J. (2006) The effect of artificial defaunation on bacterial assemblages of intertidal sediments. Journal of Experimental Marine Biology and Ecology, 337: 147 – 158.

Thébault E., & Loreau M. (2003) Food-web constraints on biodiversity–ecosystem functioning relationships. Proceedings of the National Academy of Sciences, 100: 14949 – 14954.

Vaughn C.C. (2010) Biodiversity losses and ecosystem function in freshwaters: emerging conclusions and research directions. BioScience, 60: 25 – 35.

Chapter 2

The dark side of the interactions between macrofauna and microbially-mediated sedimentary fluxes

Preface

Early bioturbation studies have been generally focused on the role of macrofauna on nutrient cycling (Kristensen 1984; Pelegrí et al. 1994; Svensson et al. 1997; Bartoli et al. 2000). In particular, they have been focusing on macrofauna direct (e.g., excretion) and indirect (e.g., stimulation of microbial activity) effects on nutrient regeneration and respiration rates (Kristensen et al. 2012; Baranov et al. 2016; Vanni and McIntyre 2017). The latter effect is particularly relevant in freshwater and brackish ecosystems as it includes the dissimilatory reduction of nitrate (NO_3^-) to molecular nitrogen (N_2) by denitrification, which is the only process that permanently removes reactive N from eutrophic ecosystems (Cornwell et al. 1999; Nizzoli et al. 2007; Stief 2013). Bioturbation studies have (over)emphasized the role of the heterotrophic component on the functioning of the benthic system as they have mainly addressed "dark" rates, even in illuminated sediments (Pelegrí et al. 1994; Bartoli et al. 2000; Nizzoli et al. 2006). However, such initial approach was necessary to remove some potentially confounding actors (e.g., primary producers) from the stage and to simplify the large network of connections between ecosystem biotic and abiotic compartments. Whereas most of these initial studies focused on density-dependent effects of macrofauna, where increasing, sometimes unrealistic, densities were intentionally manipulated, the effects of large and rare organisms were seldom considered. To explore this topic and to demonstrate that even rare organisms play a role in benthic biogeochemistry, the effect of the bivalve *Sinonodonta woodiana* on river metabolism was analyzed. I hypothesized that large bivalves are biogeochemical hot spots, contributing to benthic metabolism (Section 2.1). Short-term incubations of intact bare sediments and intact sediments with bivalves were performed in the dark under controlled laboratory conditions. Other two biogeochemical studies are described in this chapter: one dealing with two abundant tube-dwelling organisms present in two lagoons (Section 2.2) and another one dealing with a widespread oligochaete found in eutrophic and oligotrophic environments (Section 2.3). Tube-dwelling animals like chironomid larvae are generally very abundant in eutrophic environments as they are very tolerant to low oxygen and high sulfide concentrations. Chironomid larvae may oxidize deep sediments by injecting water through ventilation and bioirrigation in their burrows. Sediment cores containing an increasing

number of organisms were reconstructed and, after 3 weeks of acclimatization, the benthic N processes were measured. The effect of chironomid larvae on nitrification and denitrification was compared to the effect of another tube-dwelling organism, the amphipod *Corophium insidiosum*. Intact sediment cores with variable biomass of the surface burrower *C. insidiosum* were randomly collected from a shallow water lagoon. It was hypothesized a different stimulation of nitrification and denitrification processes due to different ventilation activities. The continuous ventilation by *C. insidiosum* would increase the nitrification rates and therefore the coupled nitrification and denitrification. The intermittent ventilation of chironomid larvae would increase the injection of water enriched in O_2 and NO_3^- deep in the sediments and therefore the denitrification of water column nitrate would be higher. The last work reported in this chapter highlights the role of a deep burrower oligochaete in shaping microbial N processes in two environments with different trophic conditions. Intact sediment cores were collected from a branch of the Mincio River and from a littoral zone of the Cazaux-Sanguinet Lake and an increasing number of organisms was added to each core. Oligochaetes can deeply vary the sediment redox potential by the injection of oxic water through burrow ventilation and bioirrigation. The studied oligochaete, *Sparganophilus tamesis*, is allochthonous in both study sites and is characterized by potential large invasiveness: one of the aims of this study was to understand whether such organism may produce changes in the chemistry of the water column, by increasing nutrient fluxes from the benthic to the pelagic compartment. This may produce adverse effects for local communities of isoetid plants in the Cazaux-Sanguinet Lake.

Section 2.1 is modified from Benelli S., Bartoli M., Racchetti E., Moraes P.C., Zilius M., Lubiene I., & Fano E.A. (2017) Rare but large bivalves alter benthic respiration and nutrient recycling in riverine sediments. Aquatic Ecology, 51: 1 – 16.

Section 2.2 is modified from Moraes P.C., Zilius M., Benelli S., & Bartoli M. (2018) Nitrification and denitrification in estuarine sediments with tube-dwelling benthic animals. Hydrobiologia, 819: 217 – 230.

Section 2.3 is a manuscript (Benelli S., Bartoli M., Ribaudo C., & Fano E.A.) that have been recently submitted to Water Special Issue “The Role of Macrobiota in Aquatic Nutrient Cycling”.

Section 2.1

Rare but large bivalves alter benthic respiration and nutrient recycling in riverine sediments

Introduction

In soft sediments, bioturbation has profound effects on the physical, geochemical and biological properties of the substratum, as well as on benthic-pelagic coupling (Aller 1982; Palmer et al. 1997). Such effects depend upon feeding, defecation, irrigation, excretion and reworking activities (Kristensen et al. 2012). If and how macrofauna affects sedimentary processes was investigated under different perspectives and with a wide range of experimental approaches (Pelegrí and Blackburn 1995; Hansen et al. 1996; Prins et al. 1998; Vaughn and Hakenkamp 2001; Stief 2013). Earlier studies have focused on small, active and abundant organisms, with densities sometimes over 10,000 ind m^{-2}, and generally burrowing in the sediment (Gallepp 1979; Pelegrí et al. 1994; Svensson 1997). To this purpose, incubations of sieved sediments added with increasing numbers of small organisms were performed, to test for process rates versus macrofauna density (Pelegrí and Blackburn 1995; Svensson 1997; Nizzoli et al. 2007). Burrowing macrofauna was demonstrated to stimulate the activity of bacteria, and to alter the ratio between advective and diffusive processes and the volume of oxic and anoxic sediments (Na et al. 2008; Bartoli et al. 2009). Burrowers may simultaneously increase nitrogen (N) loss and enhance its recycling from pore water, particularly during early colonization phases (Bartoli et al. 2000; Nizzoli et al. 2007). Stief (2013) analyzed the net effects of macrofauna on N, a deeply studied *bioturbated* nutrient, and concluded that in most studies macrofauna stimulated NH_4^+ recycling to a much larger extent than N loss via denitrification. Burrowers may also enhance the retention capacity of sediments toward phosphorus (P), increasing the pool of reactive iron (Norkko et al. 2012).

More recently, farmed or invasive filter feeding bivalves as *Tapes philippinarum* or *Dreissena polymorpha* received large attention due to their ability to control or stimulate phytoplankton, oxygen (O_2) and nutrient dynamics, sometimes at the whole ecosystem level (Caraco et al. 2000; Bartoli et al. 2001; Higgins and Zanden 2010; Stadmark and Conley 2011; Ruginis et al. 2014). Due to their high densities, filter-feeding bivalves may remove large amounts of particulate matter from the water column and produce high amounts of biodeposits as feces and pseudofeces, which enhance microbial activity and alter sediment properties (Nizzoli et al. 2014; Ruginis et al. 2014). They also excrete large amounts of nutrients, promoting the so-

called benthic-pelagic coupling, potentially favoring pelagic or benthic primary producers other than phytoplankton (Bartoli et al. 2001 and 2003; Murphy et al. 2015).

The effect of large bivalves, averaging few individuals per square meter, has been less studied (Nalepa et al. 1991; Strayer et al. 1994; Vaughn et al. 2004; Strayer 2014). This is likely due to their metabolism, which is supposed to be slow, to their low density and to their limited movements within sediments. These three combined factors are considered to produce little effects on the benthic system. Furthermore, large, occasional bivalves are difficult to include in random sediment sampling, unless many replicate cores with large diameters are collected (Glud and Blackburn 2002). As they are generally not included in benthic flux measurements, their contribution to the metabolism of sediment is underestimated or simply not considered.

In this work, we analyzed the effects of a large mollusk (*Sinanodonta woodiana,* Lea 1834) on benthic respiration and nutrient regeneration, using intact sediment cores collected in a canal with transparent water and soft sediments. The mollusk under investigation can reach several hundreds of grams as wet weight and moves producing clearly visible paths on the sediment surface, probably due to feeding, reproductive needs or water level variations (Balfour and Smock 1995; Schwalb and Pusch 2007). Burrowing and lateral movements of *S. woodiana*, due to the dimension of the mollusk, may produce a large sediment reworking. *S. woodiana* may eat sediments around siphons (i.e. benthic microalgae), resuspend and filter recently deposited matter or may feed pedally, as suggested by different works on freshwater mollusks (Hakenkamp and Palmer 1999; Raikow and Hamilton 2001; Nichols et al. 2005). These possibilities provide a competitive advantage in clear water areas with limited phytoplankton availability. Vertical and lateral movements by large unionids or incomplete closing of the valves may introduce O_2 and nitrate (NO_3^-) rich water within sediments, with a net reoxidation of chemically reduced compounds (i.e. iron and manganese pools) and stimulation of O_2 and NO_3^- respiration. Simultaneously, sediment reworking by this large organism may introduce in subsurface sediments labile organic matter as benthic algae, feces and pseudofeces that may stimulate anaerobic processes as metal reduction or methanogenesis. Different studies analysed the effect of bivalves on benthic biogeochemistry in phytoplankton-rich ecosystems, and quantified the balance between suspended matter removal via filtration and benthic regeneration (Nizzoli et al. 2006; Murphy et al. 2015). Comparatively, the effect of large mollusks on benthic-pelagic coupling and microbial respiration in oligotrophic sites, where sediments may be an important food source, was less studied (Strayer 2014). We hypothesized that these organisms may represent biogeochemical hot spots exerting both direct (i.e. due to their metabolism) and indirect (due to their feeding strategy and sediment reworking) effects, relevant at the whole benthic system scale.

Material and methods

Sampling procedure

Water, sediments and bivalves were collected in November 2015 from a canal tributary of the Mincio River (Northern Italy). The sampling site is characterized by shallow (z=30 cm), transparent (planktonic chl *a* < 1 µg L^{-1}), NO_3^--rich (30 < NO_3^- < 50 µM) and slow flowing (< 10 cm s^{-1}) water originating from a nearby spring and overlying a thick layer (~40 cm) of fine organic sediments. In the whole area, the siphons of *S. woodiana* were clearly visible, identifying the position of single or small groups (2-4) of organisms (Fig. 2.1.1). In Italy, *S. woodiana* is a non-native and invasive species, documented since 1996 (Manganelli et al. 1998). The density of the bivalve was estimated by random positioning of 1 m^2 quadrats on the sediment surface (n=10) and by counting the individuals.

Intact sediment cores with (n=13) and without (n=11) mollusks were collected by means of Plexiglass liners (inner diameter=20 cm, height=40 cm) in order to measure benthic respiration and nutrient fluxes. Liners were pushed by hand in the fluffy sediment; nearly 25 cm of sediment and 15 cm of water were included. In the liners with mollusks one individual per core was collected. PVC stoppers provided with 2 O-rings were inserted in the liner bottom and undisturbed intact cores with clear water phase were retrieved. Additional sediment cores devoid of mollusks (n=3, i.d.=5 cm, height=30 cm) were collected for sediment characterization: density, porosity, organic matter, concentrations of ammonium (NH_4^+), soluble reactive phosphorus (SRP) and reactive silica (SiO_2) in pore water and for potential microbial activities. Besides the individuals within the incubation cores, bivalves of different sizes were also collected by hand for metabolic measurements and were stored in a tank with in situ aerated water. Nearly 200 L of water was collected from the canal for preincubation and incubation procedures.

Sedimentary features and potential microbial activities

The upper 10 cm vertical sediment horizon was extruded with a piston from each of the 5 cm i.d. cores and homogenized. Bulk density was determined as the ratio between wet weight and volume (5 ml) of sediment (n=3); the sediment was collected by means of a cut-off 5 ml syringe filled with the sediment homogenate. The water content was determined after desiccation of the same fresh sediment volume at 70 °C until constant weight; porosity was calculated as the ratio between the volume of water and that of fresh sediment. Organic matter content (OM) was measured as percentage of weight loss on ignition (450 °C, 2 h) from dried, powdered sediment. In addition, nearly 20 ml of fresh sediment (n=3) was centrifuged to extract pore water that was analyzed spectrophotometrically for NH_4^+, SRP and SiO_2 interstitial concentration as detailed

below. These concentrations reflect the average along the upper 10 cm horizon. Chlorophyll (chl *a*) was extracted from about 1 g of fresh sediment taken in situ in the proximity of siphons (n=6) and > 1 m far from them (n=6). The upper sediment layer (0.5 cm) was collected via cut-off syringes inserted vertically by hand in the sediment (Fig. 2.1.1). Fresh sediments were immediately transferred in vials containing 10 ml of 90 % acetone and stored 24 h in the dark. The extracted chlorophyll was thereafter determined after centrifugation and filtration according to Lorenzen (1967).

Two ml of surface sediments from the sub-oxic zone (from the 0.5-1.5 cm horizon, n=18) were collected and transferred to 12-ml glass Exetainers (Labco, UK), containing a glass bead. Six treatments, each with 3 replicates, were then applied. The Exetainers were amended with: 1) NO_3^--free water; 2) NO_3^--free water and *S. woodiana* feces and pseudofeces; 3) 20 µM $^{15}NO_3^-$ water; 4) 20 µM $^{15}NO_3^-$ water and *S. woodiana* feces and pseudofeces; 5) NO_3^--free and 10 µM $^{15}NH_4^+$ water and 6) 20 µM $^{15}NH_4^+$ and 20 µM $^{14}NO_3^-$ water. The 6 treatments targeted potential CH_4 production in absence (1) and presence (2) of bivalve fecal material, potential denitrification in absence (3) and presence (4) of bivalve fecal material, and the occurrence of anammox (5 and 6), respectively. The bivalve fecal material was collected with a 50-ml syringe from the bottom of a large beaker containing in situ water and bivalves, without sediment. The collected material was concentrated via centrifugation and supernatant removal; thereafter 1 ml of the fecal material, corresponding to 0.2 g_{DW}, was added to each Exetainer. All added water was previously bubbled with N_2 to remove any O_2 and CH_4 traces. Once filled, all vials were capped leaving no air bubbles, transferred into a rotating shaker and incubated for 24 hours in the dark at 20 °C; afterwards 200 µl 7 M $ZnCl_2$ was added to the samples to inhibit microbial activity at the end of incubation. $^{14}N^{15}N$ and $^{15}N^{15}N$ abundance in N_2 and dissolved CH_4 were analyzed by membrane inlet mass spectrometer (MIMS, Bay instruments, USA). More details on these assays are reported in Thamdrup and Dalsgaard (2002).

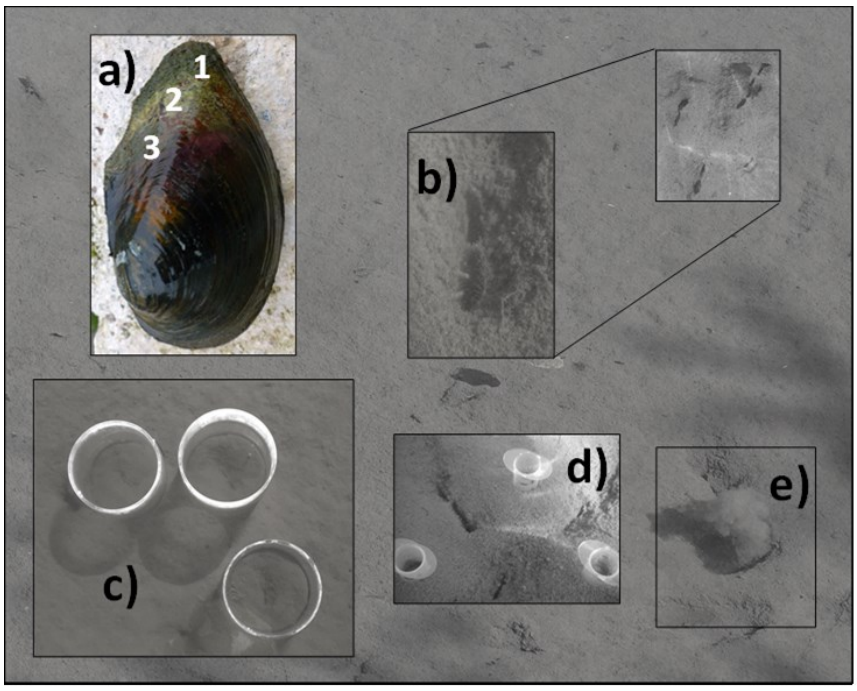


Fig. 2.1.1 The background picture shows the surface sediment of the canal under investigation, where *S. woodiana* individuals (a) were clearly visible through their siphons (b), allowing sampling undisturbed sediment cores with and without bivalves (c). The upper sediment layer was collected via cut-off syringes inserted vertically by hand in the sediment to extract chl *a* in the proximity of siphons and > 1 m far from them (d). Recovered organisms had epiphytic algae (1), growing in the upper extreme of the shell, as well as biofilms of whitish (2) and purple bacteria (3). Sediments around *S. woodiana* were frequently resuspended by the bivalve (e).

Measurement of benthic fluxes and S. woodiana *metabolism*

Once collected, water, sediment samples and bivalves were transferred to the laboratory within one hour. Cores were submersed with the top open in a large incubation tank containing well-mixed and aerated in situ water maintained at the ambient temperature (20 °C). The day after the sampling all cores were incubated in the dark for dissolved gas and nutrient flux measurements (Dalsgaard et al. 2000). Incubations started when a gas-tight lid with a sampling port and a compensation valve was positioned on the liners top. The water inside each liner was gently mixed by a small aquarium pump, which provided a minimum flow (1 L min^{-1}) to avoid stagnation and to guarantee homogeneous conditions within the cores, but excluding sediment resuspension. Incubations lasted 2 hours; water samples (nearly 80 ml) were collected at the beginning and at the end of the incubation and analyzed for dissolved gas (O_2, CH_4, N_2, and TCO_2) and nutrients (NH_4^+, NO_2^-, NO_3^-, SiO_2 and SRP). Samples for O_2, CH_4, N_2 and TCO_2 were transferred to 12-ml exetainers (Labco, UK); with the exception of TCO_2 they were poisoned with 100 µl of 7 M $ZnCl_2$. Dissolved O_2 was measured by means of polarography with a microelectrode (Unisense, Denmark). Dissolved CH_4 and N_2 were measured via MIMS.

TCO_2 was measured via 6 end points 0.1 N HCl titration (Anderson et al. 1986). Samples for the determination of inorganic forms of N and Si were filtered through Whatman GF/F glass fiber filters, transferred into 20-ml plastic vials and analyzed with standard spectrophotometric techniques (Golterman et al. 1978; Bower and Holm-Hansen 1980). Samples for SRP determination were filtered and transferred to glass vials and analyzed spectrophotometrically (Valderrama 1977). Fluxes were calculated according to the equation below:

$$Flux\ x = \frac{([x]_f - [x]_i) \times V}{A \times t}$$

where $[x]_f$ and $[x]_i$, expressed in µM or mM, are the concentrations of the solute x at the end and at the start of the incubation, respectively, V (L) is the volume of the core water phase, A (m^2) is the area of the sediment and t (h) is the incubation time.

The top lids were thereafter removed and the water in the tank replaced with fresh in situ water. In the afternoon, a sequential incubation was performed aiming at the measurement of denitrification rates with the isotope pairing technique (IPT, Nielsen 1992). Briefly, 10 ml of a 15 mM $^{15}NO_3^-$ stock solution was added to the water phase of each liner to have a final concentration of labelled nitrate of 30 µM. The top lids were then positioned and the cores were incubated in the dark for 2 hours. At the end of the incubation, the lids were removed and the whole sediment and water phase gently slurried, subsampled, transferred in Exetainers and poisoned with 200 µl of 7 M $ZnCl_2$ for labelled N_2 analysis. The latter was performed within one week by means of MIMS. At the end of the procedure, the cores were sieved through a 0.5 mm mesh in order to visual-check for the occurrence of other macrofauna and to retrive the bivalves. The revised version of the IPT was not used at the sampling site as sediment slurries demonstrated the absence of anammox. The rates of denitrification were calculated according to the equations and assumptions of Nielsen (1992): $D_{15} = p(^{15}N^{14}N) + 2p(^{15}N^{15}N)$ and $D_{14} = p(^{15}N^{14}N) + 2p(^{14}N^{14}N)$, where D_{15} and D_{14} = rates of denitrification based on $^{15}NO_3^-$ and $^{14}NO_3^-$, respectively; and $p(^{14}N^{14}N)$, $p(^{15}N^{14}N)$ and $p(^{15}N^{15}N)$= rates of production of labelled and unlabelled N_2 species. Because the $p(^{14}N^{14}N)$ cannot be readily measured, estimation of D_{14} was obtained from: $D_{14} = D_{15} \times p(^{15}N^{14}N) / 2p(^{15}N^{15}N)$. The proportion of D_{14} supported by unlabelled NO_3^- from the water column (D_W) was calculated from: $D_W = D_{15} \times f / (1-f)$, where f = mole fraction of $^{14}NO_3^-$ in the water column. The coupled nitrification-denitrification (D_N) was calculated by difference as: $D_N = D_{14}-D_W$.

Individuals of *S. woodiana* retrived from the cores were then gently brushed to remove biofilm growing on the shell and individually incubated in the dark to analyze gas and nutrient exchange between the mollusks and the water phase. Organisms were incubated in the same set of cores,

but in the absence of sediment. The procedure was exactly the same described above. All incubated individuals were characterized for wet weight (g_{WW}) and for shell-free dry weight (g_{SFDW}), after drying the soft tissue at 70 °C to a constant weight.

Statistical analyses

The non-parametric Mann-Whitney U-Test was used to test differences between the concentration of chl *a* close to and far from the siphons of *S. woodiana* and between potential denitrification and CH_4 production activities in sediments with and without *S. woodiana* feces. The same test was used to investigate differences between diffusive benthic fluxes measured in the three conditions: a) sediment alone (S) VS sediment with *S. woodiana* (S+A), b) S+A VS *S. woodiana* alone (A) and c) S VS A. Fluxes measured in S+A and A were related to the shell-free dry biomass of the incubated organisms with a simple linear regression analysis. Differences between slopes were tested by Student *t*-test. Statistical significance was set at $p<0.05$. Statistical analyses were performed with Sigma Plot 12.0.

Results

Sediment features

Chlorophyll *a* concentration in the upper sediment horizon revealed a large availability of benthic labile carbon associated with algal material. Sedimentary chl *a* tended to be higher close to *S. woodiana* siphons than far from mollusks (217 ± 13 VS 197 ± 7 mg chl *a* m^{-2}, average ± SE) but differences were not statistically significant (Mann-Whitney U-Test, $p>0.05$, Table 2.1.1). The 0-10 cm sediment profile was extremely fluffy, displaying low density, high porosity and elevated content of OM (Table 2.1.1). Pore water analysis of inorganic nutrients suggested high availability of NH_4^+ and SiO_2, with comparable concentrations, and scarce mobility of SRP, likely trapped in the organic matrix (Table 2.1.1).

Table 2.1.1 Sedimentary features at the sampling site. Chlorophyll *a* (chl *a*) was extracted from the upper 1 cm horizon, close (n=6) and far (n=6) from the bivalve siphons, but as differences were not significant data were pooled. All the remaining variables were measured on integrated sediment samples (n=3), along the upper 0-10 cm vertical horizon. Average values ± standard errors are reported.

Features	**Average ± SE**
Chl *a* (mg m^{-2})	207 ± 7
Density (g cm^{-3})	1.04 ± 0.02
Porosity	0.94 ± 0.02
OM (%)	24.1 ± 0.2
Pore water	
NH_4^+ (µM)	211 ± 14
SiO_2 (µM)	163 ± 15
SRP (µM)	0.5 ± 0.1

Sediments displayed elevated rates of denitrification potential, with the process not saturated even at 200 µM $^{15}NO_3^-$ concentration (data not shown). Results from slurry measurements (Table 2.1.2) suggested that the addition of *S. woodiana* feces did not stimulate the production of N_2 via denitrification and that of CH_4 compared to unamended sediment. Measured rates were in fact not statistically different (Mann-Whitney U-Test, $p>0.05$, Table 2.1.2). $^{29}N_2$ was not detected in any of the treatments, including those with $^{15}NH_4^+$, suggesting anammox was unimportant in N_2 production at the study site and supporting the application of the IPT to determine denitrification rates.

Table 2.1.2 Results from anoxic incubation of sediment slurries added with ^{14}N nitrate-free water (Control), ^{14}N nitrate-free water and *S. woodiana* feces and various combinations of labelled and unlabelled NO_3^- and NH_4^+ and biodeposits. See the text for more details on the 6 treatments. Average values ± standard errors are reported (n=3).

Treatment	**Denitrification** (nM N $h^{-1}ml^{-1}$)	**Anammox** (nM N $h^{-1}ml^{-1}$)	**Methanogenesis** (nM C $h^{-1}ml^{-1}$)
Control	-	-	53.6 ± 8.2
H_2O + feces	-	-	68.7 ± 6.3
20 µM $^{15}NO_3^-$	8.5 ± 4.5	0.0 ± 0.0	65.8 ± 5.9
20 µM $^{15}NO_3^-$ + feces	7.6 ± 3.8	0.0 ± 0.0	66.8 ± 3.7
20 µM $^{15}NH_4^+$ + 20 µM $^{14}NO_3^-$	-	0.0 ± 0.0	64.5 ± 2.8
10 µM $^{15}NH_4^+$	-	0.0 ± 0.0	88.5 ± 7.3

S. woodiana *features*

The density of *S. woodiana* at the study area was 6.0 (± 1.8; SE) ind m^{-2}; among bivalves we found a few individuals of *Corbicula* spp. (< 1 m^{-2}). Recovered macrofauna included also oligochaetes, among which the non-native *Sparganophilus tamesis* and chironomid larvae. However, their occurrence was sporadic likely due to the sampling season or to the absence of macrophytes (Rota et al. 2014). We cannot exclude the occurrence of other taxa as our macrofauna analysis was not exhaustive and mainly targeting potentially relevant bioturbating organisms besides *S. woodiana*. The population of *S. woodiana* was composed mostly of large individuals, with an average wet weight of 283 (± 33; SE) g_{WW} ind^{-1}, corresponding to a shell-free dry weight of 12.9 (± 1.5; SE) g_{SFDW} ind^{-1}. The upper portion of the bivalve outer shell was colonized by a thick layer of microalgae whereas the rest was colonized by bacterial biofilms (Fig. 2.1.1).

Benthic respiration and bivalve metabolism

The presence of the bivalve affected both aerobic and anaerobic benthic respirations. All the cores containing sediments with bivalves had one single large individual and displayed significantly higher O_2 consumption, TCO_2, N_2 and CH_4 production as compared to sediments without *S. woodiana* (Mann-Whitney U-Test, $p<0.01$, Fig. 2.1.2). Benthic O_2 demand measured in S+A overlapped the rate measured in A and it was nearly 3.6-fold higher than the rate measured in S (Fig. 2.1.2). Similar results were obtained for TCO_2 production that was significantly higher in S+A compared to S and not significantly different in S+A and A (Mann-Whitney U-Test, $p<0.01$ and $p=0.3$, respectively, Fig. 2.1.2). These results suggest that most of the increase of O_2 consumption and TCO_2 production was due to the mollusk metabolism.

The effect of *S. woodiana* on N_2 and CH_4 fluxes was also significant, but not related to the mollusk metabolism and therefore not direct (Fig. 2.1.2). Rates of total denitrification (D_N+D_W) measured via the IPT were significantly higher, by a factor of 2, in S+A than in S (Mann-Whitney U-Test, $p<0.001$, Fig. 2.1.2). The presence of the bivalve stimulated both the reduction of NO_3^- diffusing to the anoxic sediment from the water column (D_W) and of the NO_3^- produced via nitrification (D_N), but only differences between D_N in S+A and in S were significant (Mann-Whitney U-Test, $p<0.05$, Fig. 2.1.2). Net N_2 fluxes were positive, suggesting the dominance of denitrification over N fixation at the study site (Fig. 2.1.2). However, rates were lower as compared to those measured with the IPT, due to methodological issues or to the occurrence of some N fixation. Net N_2 efflux was enhanced by a factor of 3.1 in S+A versus S cores (Mann-Whitney U-Test, $p<0.01$). Diffusive CH_4 efflux increased by a factor of 94, from 0.03 (± 0.90, SE) to 2.60 (± 0.01, SE) mmol $m^{-2}h^{-1}$ measured in S and S+A, respectively (Mann-Whitney U-

Test, $p<0.001$, Fig. 2.1.2). Rates of N_2 production in incubations with *S. woodiana* alone revealed that the contribution of the mollusk to the flux measured in S+A was small, as rates in S and A were similar (Mann-Whitney U-Test, $p>0.05$)(Fig. 2.1.2). Fluxes of CH_4 in incubations with *S. woodiana* alone were statistically different from fluxes measured in S and S+A (Mann-Whitney U-Test, $p<0.01$ and $p<0.001$, respectively).

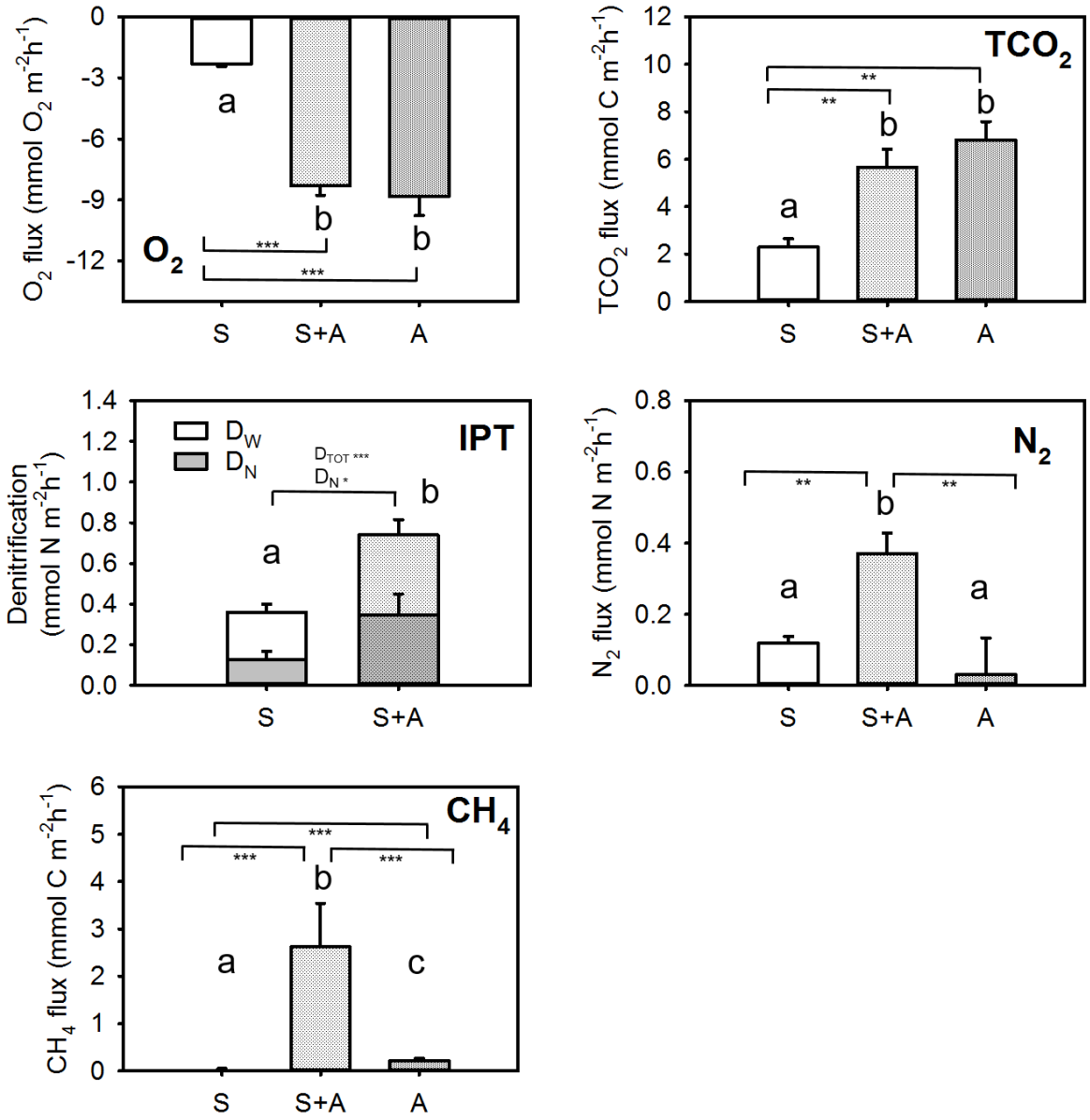


Fig. 2.1.2 Dark benthic fluxes of dissolved O_2, TCO_2, N_2 and CH_4 measured in incubations of bare sediments (S, n=11), sediments with *S. woodiana* (S+A, n=13) and single individuals of the bivalve (A, n=13). Rates of denitrification of water column NO_3^- (D_W) and of coupled nitrification-denitrification (D_N), were measured with the isotope pairing technique (IPT) only in S and S+A. Averages ± standard errors are reported. Different letters above bars indicate statistical differences between fluxes (* $p<0.05$, ** $p<0.01$ and *** $p<0.001$).

Oxygen demand and TCO_2, N_2 and CH_4 production measured in S+A increased linearly with the shell-free dry weight of *S. woodiana* retrived from each core (Table 2.1.3). Also O_2 demand measured in A increased linearly with the shell-free dry biomass of the incubated organisms, with a slope not statistically different from that calculated in sediments with *S. woodiana.* On the contrary, for CH_4 and N_2 the comparison between the slopes were close to significant (Table 2.1.3).

To calculate the average benthic respiration rates in the study area we gave appropriate weights to measured fluxes (nearly 80 and 20 % to S and S+A rates, respectively). Such weights were obtained downscaling fluxes measured in the S+A cores, where bivalve density was nearly 32 ind m^{-2} (1 ind per core with a core surface of 314 cm^2) to the in situ *S. woodiana* density which was nearly 5 times lower (6 ind m^{-2}). The weighted average O_2, TCO_2, N_2 and CH_4 fluxes in the study area were 3.51 ± 0.14, 2.97 ± 0.27, 0.17 ± 0.02 and 0.55 ± 0.18 (all averages ± SE) mmol $m^{-2}h^{-1}$, respectively, with a bivalve biomass of 95 (± 10; SE) g_{SFDW} m^{-2}. Such values are 1.5, 1.3, 1.4 and 19.7-fold higher than those measured in sediments without mollusks and suggest a similar stimulation of aerobic respiration and denitrification by the bivalve and a much higher stimulation of methanogenesis.

Benthic nutrient fluxes and bivalve excretion

Bare sediments were sinks for dissolved nutrients whilst sediments with *S. woodiana* recycled large amounts of NH_4^+, SiO_2 and SRP to the water column (Fig. 2.1.3). The fluxes of NH_4^+ and SiO_2 measured in S+A and A were significantly different from those measured in S (Mann-Whitney U-Test, $p<0.01$). In the presence of *S. woodiana* the fluxes of SRP were also reversed, but due to small scale high variability the difference between the conditions S+A and S was not significant. In the incubations of *S. woodiana* alone, excretion rates of NH_4^+, SiO_2 and SRP overlapped fluxes measured in S+A cores (Fig. 2.1.3).

The fluxes of nitrite were low if compared to those of NH_4^+ and NO_3^- in both S and S+A, but rates measured in the two conditions were statistically different (Mann-Whitney U-Test, $p<0.01$). The flux of NO_3^- had a different trend if compared with the other dissolved nutrients, being net consumed in both S and S+A, without a significant difference. A net release of NO_3^- and NO_2^- was measured in the incubation of *S. woodiana* alone.

The weighted average flux of NH_4^+, NO_3^-, NO_2^-, DIN, SRP and SiO_2 in the study area was 102 ± 27, -159 ± 124, -23 ± 7, -78 ± 95, -0.16 ± 2.51 and -54 ± 52 (all averages ± SE) µmol $m^{-2}h^{-1}$, respectively. The regressions between NH_4^+, NO_2^-, DIN and SRP fluxes measured in S+A and the shell-free dry weight of *S. woodiana* retrived from incubated sediments were all significant whilst that of SiO_2 was close to significant (Table 2.1.3). Similar outcomes were obtained with

linear regressions between excreted nutrients (A, mollusk alone incubation) and the shell free dry biomass of the incubated individuals (Table 2.1.3). As the slopes of the two regressions were for most parameters not statistically different ($p>0.05$) we address the metabolism of *S. woodiana* as driving factor for benthic nutrient recycling.

On a molar basis, the stoichiometry of the dissolved mineral forms of C, N, Si and P regenerated to the water column in presence of *S. woodiana* was 509:51:28:1. The C:N ratio (~ 10) suggests that the food source of *S. woodiana* has an elevated N content and it is probably labile organic matter. The N:Si ratio suggests that such pool is richer in N compared to Si and the N:P ratio suggests P limitation, even if most ingested P is generally released through feces and pseudofeces.

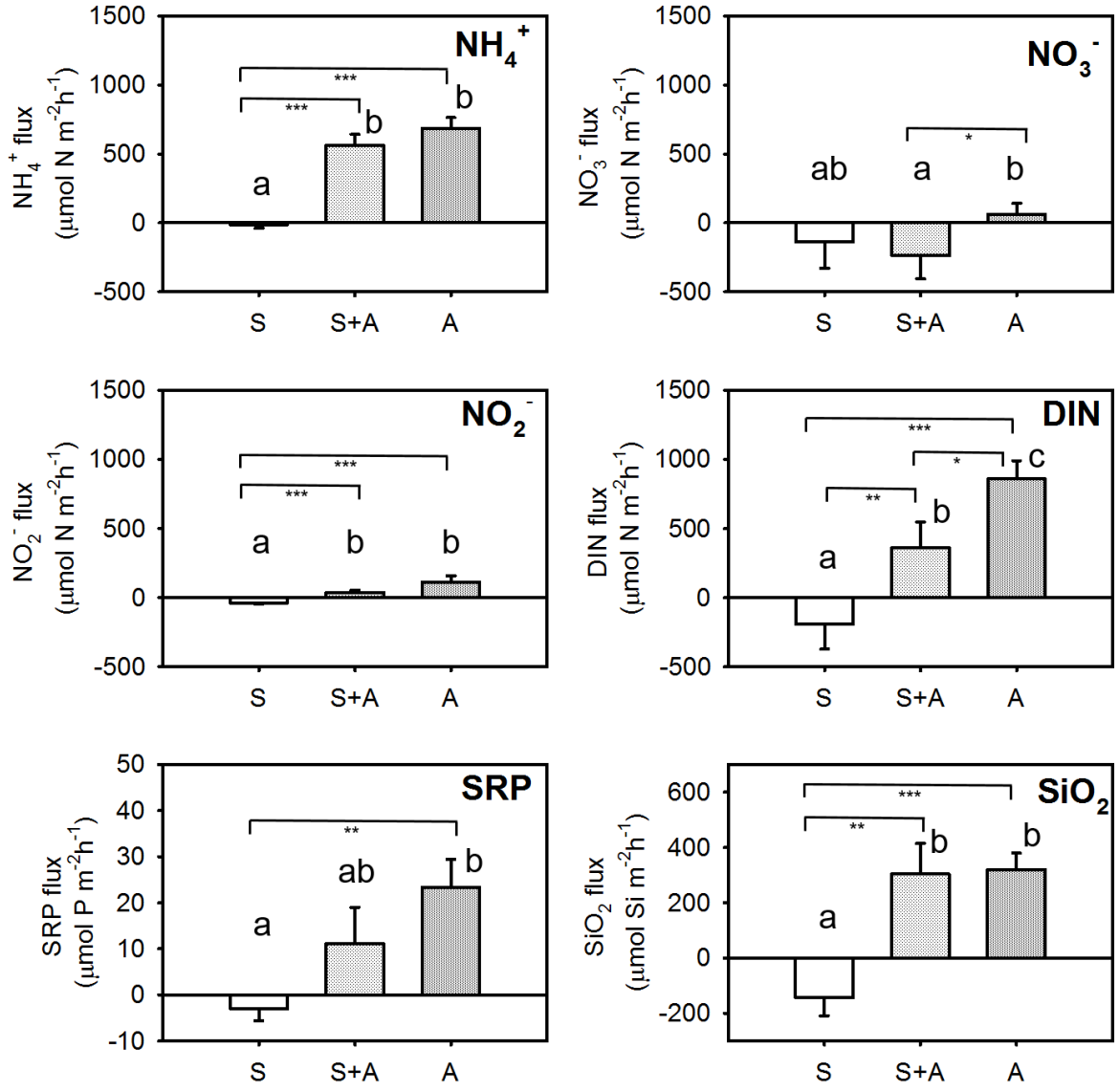


Fig. 2.1.3 Dark benthic fluxes of NH_4^+, NO_3^-, NO_2^-, DIN, SRP and SiO_2 measured in incubations of bare sediments (S, n=11), sediments with *S. woodiana* (S+A, n=13) and single individuals of the bivalve (A, n=13). Averages ± standard errors are reported. Different letters above bars indicate statistical differences between fluxes (* $p<0.05$, ** $p<0.01$ and *** $p<0.001$).

Table 2.1.3 Linear regression between benthic fluxes (S and S+A) and the shell-free dry biomass of *S. woodiana* recovered from incubated cores (S and S+A VS dry biomass)(n=24), linear regression between the mollusk alone fluxes (A) and the shell-free dry biomass of *S. woodiana* (A VS dry biomass)(n=21) and results from a t test (p-value) comparing the two slopes. When one of the two slopes was not significantly different from zero we did not perform the test (n.d.). Units for O_2, TCO_2 slope and intercept are mmol $g_{SFDW}^{-1}h^{-1}$ and mmol $m^{-2}h^{-1}$, respectively. For all remaining parameters slope and intercept units are µmol $g_{SFDW}^{-1}h^{-1}$ and µmol $m^{-2}h^{-1}$, respectively.

Measure	Rates measured in S and S+A VS dry biomass			Rates measured in A VS dry biomass			Slope comparison (two-tailed p-value)
O_2	slope	-0.28±0.03	p≤0.001	slope	-0.40±0.09	p≤0.001	p=0.201
	intercept	-2.82±0.44	p≤0.001	intercept	0		
TCO_2	slope	0.16±0.04	p≤0.01	slope	0.41±0.56	p=0.437	n.d.
	intercept	2.56±0.67	p≤0.001	intercept	0		
N_2	slope	9.89±3.48	p≤0.01	slope	3.07±1.13	p≤0.05	p=0.069
	intercept	155.62±50.77	p≤0.01	intercept	0		
CH_4	slope	101.89±48.24	p≤0.05	slope	12.91±5.67	p≤0.05	p=0.080
	intercept	396.27±689.23	p=0.571	intercept	0		
NH_4^+	slope	28.86±3.77	p≤0.001	slope	32.37±9.58	p≤0.01	p=0.730
	intercept	12.21±54.77	p=0.826	intercept	0		
NO_3^-	slope	-9.15±11.02	p=0.417	slope	-4.80±3.75	p=0.220	n.d.
	intercept	-104.33±166.75	p≤0.05	intercept	0		
NO_2^-	slope	2.79±1.09	p≤0.05	slope	0.95±1.35	p=0.487	n.d.
	intercept	-25.75±15.33	p=0.112	intercept	0		
DIN	slope	19.43±9.08	p≤0.05	slope	30.65±13.55	p≤0.05	p=0.481
	intercept	-95.59±134.75	p=0.478	intercept	0		
SRP	slope	0.87±0.42	p≤0.05	slope	1.32±0.48	p≤0.01	p=0.487
	intercept	-3.94±5.97	p=0.517	intercept	0		
SiO_2	slope	13.51±7.22	p=0.075	slope	7.16±6.44	p=0.281	n.d.
	intercept	-29.48±105.25	p=0.782	intercept	0		

Discussion

Large, low-density bivalves affect benthic metabolism

Our results demonstrate that a few individuals of a large bivalve affect significantly benthic respiration rates (both aerobic and anaerobic paths, via their metabolism, biodeposition and bioturbation) and nutrient recycling (NH_4^+, SiO_2 and SRP, via direct excretion). The outcomes of this work conform to other studies targeting the effects of farmed or reef-forming bivalves on sediment biogeochemistry, where similar results are reported (Bartoli et al. 2001; Nizzoli et al. 2006; Ruginis et al. 2014). However, only a few studies focused simultaneously on key benthic respiration paths and on the three nutrients N, Si and P (Table 2.1.4). At the in situ density of 6 ind m^{-2} the bivalve stimulated the respiration of O_2 and NO_3^- and the production of CH_4, reversed the fluxes of NH_4^+ and attenuated the sedimentary uptake of SRP and SiO_2. To our knowledge, the stimulation of methanogenesis by a bivalve was never reported before. As it was hypothesized, *S. woodiana* is a biogeochemical hot spot affecting benthic processes, but its role is probably neglected in routine measurements due to its low density, which makes its inclusion in random core sampling difficult.

S. woodiana *as bioturbator: lateral and vertical movements and ecosystem implications*

S. woodiana is reported as suspension feeder, displacing organic particles from the pelagic to the benthic compartment (Pusch et al. 2001). At the study site, which is fed by groundwater, chl *a* was very low, suggesting low phytoplankton biomass and food limitation. This may explain the low mollusk density or suggests alternative food sources to the bivalve as sediments, via coupled resuspension and filtration or pedal-feeding (Vaughn et al. 2008). Freshwater bivalves redirect and concentrate nutrients and organic matter from the pelagic to the benthic food web through biodeposition of feces and pseudofeces, but if they feed on sediments, choosing high quality food particles, they rework sediments and favor the regeneration of mineral nutrients to the water column. The upper shell portion of *S. woodiana* hosted a diversified community of algae whereas that within sediments provided habitat for microbial communities, including purple bacteria. As large amounts of nutrients are regenerated through the exhalant siphon, the occurrence of algae at the upper extreme of the mollusk is not surprising and may result in coupled uptake of regenerated N, P and Si.

The bivalve burrowing activity causes sediment bioturbation, which increases the O_2 and NO_3^- consumption by the sediment and influences the release of sediment-borne nutrients to the water column. Different studies have demonstrated large, apparently erratic lateral movements of unionid mussels, up to 226 cm per week (Schwalb and Push 2007). Horizontal migrations might be related to reproductive needs, to bring animals with opposite sex closer during spawning,

which may explain the groups of 2-4 individuals found at the study site (Amyot and Downing 1998). Such explanation fits with what was demonstrated by Burla (1971), with limited lateral movements in the winter and higher in the summer. Alternative hypotheses consider the relevance of water flow and flood, the presence of predators and that of pests like *D. polymorpha*. Unionid mussels move to deeper waters to avoid exposure to the atmosphere and back to shallower areas when water level rises (McMahon 1991). Furthermore, benthic microalgae may develop in different patches depending on turbidity and nutrient availability and microphytobenthos may represent a food source to unionid mussels. Horizontal movements may be related to feeding, to access to areas not depleted in food or with higher food quality and the movement itself may be part of the feeding mechanisms (McMahon 1991). Food could be ingested through a focused water current into the anterior portion of unionids (Nichols et al. 2005). If pedal-feeding occurs in unionids this may explain erratic movements and how unionids are able to meet their nutritional needs even at very low seston concentrations (Pusch et al. 2001). Reid et al. (1992) and Yeager et al. (1994) demonstrated that if juvenile bivalves actively pedal feed their growth is faster than if they only filter water, but this behavior is still not clear for adult forms of unionids. However, Raikow and Hamilton (2001) showed that unionids were consuming 80 % deposited and 20 % suspended material studying a stream labelled with ^{15}N. We speculate that *S. woodiana* adults may feed both pedally and filtering water when there is a low phytoplankton biomass, as observed for *Corbicula* spp. (Hakenkamp and Palmer 1999).

Water movements created during horizontal or vertical movements may alter the ratio between diffusive and advective solute exchange, favor the injection of O_2 and NO_3^--rich water in anoxic sediments and the mobilization of pore water nutrients or dissolved gas as CH_4. All these speculations agree with our results. The gardening effect demonstrated for ants and invoked for burrowing macrofauna may also be carried out by unionids, via sediment fertilization, nutrient mobilization and improvement of light penetration. Resuspension of sediment with benthic microalgae, due to vertical or lateral movement, would provide food to these large organisms and a competitive advantage in clear waters.

Detailed information on sediment-related processes, explaining observed results, are lacking and impede a deep understanding of the functional role of unionid mussels in aquatic ecosystems (Vaughn and Hakenkamp 2001; Strayer 2014). A better understanding of the roles of burrowing bivalves in nutrient cycling and storage in lotic and lentic habitats is needed. In particular, it is central to focus on the origin of food material and on if and how bivalves obtain food from sediments. Furthermore, it is important to investigate the differences in feeding between juvenile and adult bivalves and what is being consumed (sediment bacteria, detritus,

microphytobenthos). Further investigations are necessary to know what biotic and abiotic factors regulate bivalve activity. For instance, temperature may stimulate O_2 uptake and nutrient fluxes in sediment with and without mollusks (Zhang et al. 2011). The present study is limited as it addresses one season and it focuses on heterotrophic processes, whereas future studies should investigate a range of temperatures and the processes occurring in the light, including feedbacks between *S. woodiana* and the community of autotrophs.

Direct and indirect contribution of the bivalve to sediment metabolism

Oxygen consumption and TCO_2 production in sediments with *S. woodiana* were affected by the mollusk's metabolic activity, significantly enhancing rates measured in bare sediment. Similar results are reported by Bartoli et al. (2001), Nizzoli et al. (2006) and Ruginis et al. (2014) for cultivated *T. philippinarum* and *M. galloprovincialis* and for the reef-forming *D. polymorpha*, respectively. The slopes of the regressions of S+A or A fluxes VS the dry flesh biomass were not statistically different (Table 2.1.3), suggesting a prevalent direct role of the mollusk's metabolism and, if present, a minor role of biodeposits in enhancing O_2 and TCO_2 fluxes. Similar respiration rates in S+A and A suggest higher metabolic activity of *S. woodiana* when incubated outside the sediment.

The effect of *S. woodiana* on anaerobic respiration and on methanogenesis in particular, is a novel and interesting outcome, deserving future studies as it is scarcely explored in the literature (Ruginis et al. 2014). Enhanced production of CH_4 and N_2 suggests an indirect effect of *S. woodiana* on anaerobic metabolism, which may be due to different factors. Biodeposition may enhance anaerobic activities as already shown in different works, even if potential denitrification and methanogenesis were not stimulated by the addition of feces and pseudofeces to sediment slurries. Large CH_4 efflux in the presence of *S. woodiana* (up to 2.6 mmol C $m^{-2}h^{-1}$) may be a consequence of displacement of labile particles in subsurface anoxic sediments where methane production is the dominant path. Another explanation could be related to the feeding behavior of the mollusk. Ingested bacteria may produce CH_4 in the mollusk gut, as demonstrated by Traganza et al. (1979) in anoxic micro niches within herbivorous zooplankton digestive track. However, when *S. woodiana* individuals were incubated alone, there was a little production of CH_4. This may be a consequence of the interruption of sediment-based feeding and cleaning of the gut. To our knowledge there are no papers describing the effects of freshwater mollusks on methane production. Different works describe symbiosis between methanotrophic bacteria and deep-sea mussels, with the metabolism of mussels supported by the bacteria (Childress et al. 1986). Symbioses between bivalves and methanotrophs provide the bacteria with access to CH_4 and O_2 and the bivalve

host with a source of organic carbon; rates of CH_4 consumption are comparable to those of O_2 respiration. Analogously, CH_4 oxidizing bacteria were demonstrated to actively grow along the macrofauna burrow walls, resulting in decreased CH_4 effluxes in bioturbated versus not bioturbated sediments. In our case CH_4 fluxes are enhanced in the presence of unionids, suggesting also advective pore water movements stimulated by the activity of the large bivalve in such organic and methane-rich sediments.

The presence of the mollusk stimulated also denitrification, as reported for bivalves in fresh and brackish waters (Pelegrí and Blackburn 1995; Nizzoli et al. 2006; Ruginis et al. 2014). In this study, the high rate of total denitrification is a consequence of two factors: one is the high concentration of NO_3^- in the water, that stimulates D_W, and the second is the bioturbation by *S. woodiana* that stimulates both components, D_W and D_N. The nitrate production measured during the incubation of bivalves alone suggests the presence of nitrifying bacteria associated with the mollusk (Welsh and Castaldelli 2004). The movement of the bivalve increases NO_3^- and O_2 penetration in the sediment and the combination of O_2 and the high amount of excreted NH_4^+ could enhance the process of nitrification and the coupled process of denitrification. D_N increased by 63 % in presence of *S. woodiana* whilst D_W increased by 40 %. Furthermore, net N_2 efflux confirms the importance of denitrification in presence of *S. woodiana* and highlights the indirect effect of the mollusk on this process. These net fluxes were lower than the rates measured with the IPT likely due to the occurrence of some N fixation or because the IPT measures also the labelled N_2 accumulated within sediments. Previous studies targeting the effects of mollusks on denitrification report for brackish waters a stimulation of the process. However, rates were one or two orders of magnitude lower than those reported in the present study. Pelegrí and Blackburn (1995) measured denitrification rates between 11 and 13 µmol N $m^{-2}h^{-1}$ in sediments with *Cerastoderma* sp. and *M. arenaria*, whereas Nizzoli et al. (2006) measured rates between 70 and 180 µmol N $m^{-2}h^{-1}$ in sediments with *T. philippinarum*. In a freshwater lake, Ruginis et al. (2014) found that *D. polymorpha* stimulated N loss by a factor of 1.5, but reported denitrification rates were two orders of magnitude lower than those from this study. Denitrification efficiency calculated from the ratio between denitrification rates and total inorganic N effluxes (DIN+N_2) across the sediment–water interface was 100 % in S and decreased to 66 % in S+A, due to enhanced NH_4^+ recycling by *S. woodiana*. This is in agreement with the general conclusions of Stief (2013) on the net effects of bioturbation on benthic N cycling, with the stimulation of ammonification and recycling prevailing over N loss. However, in the canal under study, the presence of *S. woodiana* locally alters benthic N dynamics whereas, on average, weighed fluxes of N_2, NO_x and NH_4^+ suggest that denitrification efficiency remains unaltered at 100 %. The local net regeneration of NH_4^+ by *S. woodiana* in

fact is compensated for the large consumption of NO_3^- and by N_2 production, resulting in negative weighed DIN fluxes.

Nutrient fluxes in the presence of S. woodiana

Our results conform to previous studies reporting enhancement of nutrient regeneration in the presence of bivalves (Nalepa et al. 1991; Baker and Hornbach 2001; Ruginis et al. 2014). Sediments with *S. woodiana* net released the mineral forms of the three macronutrients NH_4^+, SRP and SiO_2, and incubations of single *S. woodiana* individuals suggest that the bivalve excretion was mainly responsible for such release, as reported in Bartoli et al. (2001) and Ruginis et al. (2014).

The nutrient excretion rates in relation to the density and biomass of mollusks were investigated in several aquatic ecosystems (Table 2.1.4). To our knowledge, only a few studies dealt with large mollusks present in low densities such as unionids. Furthermore, few investigations analyzed the mollusk-related alteration of N, Si and P ecological stoichiometry, which may affect primary producer communities. Small bivalves with high densities have a comparable effect on nutrient excretion as low-density but large individuals as *S. woodiana* (Table 2.1.4).

The effect of filter feeders on benthic nutrient cycling was studied more in detail compared to the effect of bivalves exploiting food sources from the sediment, a medium that may contain low quality organic matter as compared to the water column. At the study site, the sediment had an elevated organic matter content (24 %), largely composed of macrophyte fragments from the riparian area (*P. australis*, *Carex* spp. and *A. donax*). We may speculate that this organic matter is recalcitrant, with elevated C:N ratios, as nutrient fluxes in bare sediments were all negative, suggesting the need for external nutrient source for mineralization activities. It appears therefore interesting that the ratio between TCO_2 and NH_4^+ fluxes in sediments with *S. woodiana* (nearly 10) are similar to those measured in sediments with bivalves feeding on phytoplankton. In other words, this suggests that *S. woodiana* is able to select high quality, labile organic matter within the sediment matrix, such as microphytobenthos or bacteria. Filter feeders, in particular in riverine environments, may concentrate organic matter by removal of phytoplankton from running waters and biodeposition, whilst organisms feeding on sediments rework the bottom pools of organic matter and improve benthic-pelagic coupling in nutrient-limited environment. We calculated at the study site a molar DIN:SRP ratio of 1100 in the water column, suggesting a strong P limitation, which is exacerbated by negative SRP fluxes measured in bare sediment. The activity of *S. woodiana* resulted in reversed SRP fluxes, but the ratio of excreted NH_4^+ and SRP remained elevated (51), and did not alleviate P limitation. Mobilization of SRP by *S. woodiana* may be related to the pristine environments in which such

organism has evolved (i.e. nutrient limited, with low phytoplankton biomass). Such considerations align with what suggested by Strayer (2014), asking for more studies targeting the effects of freshwater mollusks on sedimentary dynamics, the fate of biodeposits and the mollusks nutrient sources. An interesting review (Hölker et al. 2015) analyzes how small chironomid larvae, via their burrowing, bioturbation and filtration activity, may potentially regulate the regime shift between clear and turbid state of shallow lakes but that such a relevant issue is always neglected in limnological studies. Hölker and coauthors list relevant aspects that are missing for a detailed comprehension of the effect of tiny ecosystem engineers in the whole biogeochemical cycles and regulation of planktonic organisms. Similarly, the activity of large, low-density bivalves such as *S. woodiana* should be included in gas and nutrient budgets and, more in general, should be analyzed with respect to the autotrophic and heterotrophic communities, in the context of the whole system functioning.

Table 2.1.4 Oxygen respiration and nutrient excretion rates for freshwater and marine bivalves are combined with individual biomass and in situ density in order to calculate the contribution of their activities to benthic fluxes. Such upscaling of SFDW (shell free dry weight) is based on bivalve metabolism, which allows comparison of net effects of species characterized by a wide range of dimensions and in situ densities. Averages ± standard errors are reported; n.d. – no data were available.

References	Temp. °C	Family	Species	Respiration and excretion ($\mu mol\ g_{SFDW}^{-1} h^{-1}$)				SFDW ($g\ ind^{-1}$)	Density ($ind\ m^{-2}$)	Contribution of bivalves to fluxes ($\mu mol\ m^{-2} h^{-1}$)			
				O_2	NH_4^+	PO_4^{3-}	SiO_2			O_2	NH_4^+	PO_4^{3-}	SiO_2
This study	20	Unionidae	*Sinanodonta woodiana*	-14±8	1.9±1.4	0.03±0.05	0.02±1.00	12.9±7.32	6±2	-1,111	145	2	1
Murphy et al. 2015	21	Veneridae	*Mercenaria mercenaria*	-25	2	n.d.	n.d.	0.31±0.40	821±83	-6,363	509	n.d.	n.d.
Ruginis et al. 2014	16	Dreissenidae	*Dreissena polymorpha*	-185±88	17.0±7.5	0.66±0.30	5.00±2.70	0.005±0.004	11,822±6,745	-10,935	1,005	39	296
Zhang et al. 2011	15	Cyrenidae	*Corbicula fluminea*	-29±1	5.0±1.3	0.11±0.05	n.d.	0.065	1,072	-1,991	348	8	n.d.
Conroy et al. 2005	22	Dreissenidae	*Dreissena polymorpha*	n.d.	1.8±0.2	0.21±0.01	n.d.	0.005	1,800±500	n.d.	16	2	n.d.
	22	Dreissenidae	*Dreissena bugensis*	n.d.	1.9±0.2	0.17±0.01	n.d.	0.007	2,200±500	n.d.	30	3	n.d.
Magni and Montagni 2005	20	Dreissenidae	*Ruditapes philippinarum*	n.d.	4.8±2.1	1.90±1.74	5.10±5.10	0.036±0.026	1,000	n.d.	173	68	184
	20	Mytilidae	*Musculista senhousia*	n.d.	10.5±1.2	1.40±0.14	5.15±0.49	0.04±0.01	1,500	n.d.	633	84	309
Baker and Hornbach 2001	20	Unionidae	*Actinonaias ligamentina*	-12±1	2.2±0.3	n.d.	n.d.	5	15	-900	165	n.d.	n.d.
	20	Unionidae	*Amblema plicata*	-10±1	1.9±0.3	n.d.	n.d.	8	15	-1,200	228	n.d.	n.d.
Bartoli et al. 2001; Nizzoli et al. 2006	22	Veneridae	*Tapes philippinarum*	-30	6.2	0.22	0.84	0.32±0.02	741	-7,114	1,470	52	199
Kiibus and Kautsky 1996	25	Cyrenidae	*Corbicula africana*	-59±5	12.5±1.1	n.d.	n.d.	0.21±0.06	0.06	-1	n.d.	n.d.	n.d.
	25	Mutelidae	*Aspatharia wahlbergi*	-10±5	2.8±1.8	0.02±0.02	n.d.	4.29±2.14	20.43	-915	245	2	n.d.
	25	Unionidae	*Caelatura mossambicensis*	-18±3	4.7±0.9	0.05±0.01	n.d.	1.68±0.50	0.34	-10	3	n.d.	n.d.
Yamamuro and Koike 1993	26	Cyrenidae	*Corbicula japonica*	n.d.	1.4±0.4	0.04±0.01	n.d.	2.63	13	n.d.	49	1	n.d.
Nalepa et al. 1991	21	Unionidae	*Lampsillis radiata siliquoidea*	n.d.	9.7±3.5	1.90±0.10	n.d.	1.6	2	n.d.	31	6	n.d.

References

Aller R.C. (1982) The effects of macrobenthos on chemical properties of marine sediment and overlying water. In: McCall P.L., & Tevest M.J.T. (eds), Animal-sediment relations: the biogenic alteration of sediment, 2nd edn. Plenum Press, New York, 53 – 102.

Amyot J.P., & Downing J. (1998) Locomotion in *Elliptio complanata* (Mollusca: Unionidae): a reproductive function?. Freshwater Biology, 39: 351 – 358.

Anderson L.G., Hall P.O.J., Iverfeldt Å., Van Der Loeff M.M.R., Sundby B., & Westerlund S.F.G. (1986) Benthic respiration measured by total carbonate production. Limnology and Oceanography, 31: 319 – 329.

Baker S.M., & Hornbach D.J. (2001) Seasonal metabolism and biochemical composition of two unionid mussels, *Actinonaias ligamentina* and *Amblema plicata*. Journal of Molluscan Studies, 67: 407 – 416.

Balfour D.L., & Smock L.A. (1995) Distribution, age structure, and movements of the freshwater mussel *Elliptio complanata* (Mollusca: Unionidae) in a headwater stream. Journal of Freshwater Ecology, 10: 255 – 268.

Bartoli M., Nizzoli D., Welsh D.T., & Viaroli P. (2000) Short-term influence of recolonisation by the polycheate worm *Nereis succinea* on oxygen and nitrogen fluxes and denitrification: A microcosm simulation. Hydrobiologia 431: 165 – 174.

Bartoli M., Nizzoli D., Viaroli P., Turolla E., Castaldelli G., Fano E.A., & Rossi R. (2001) Impact of *Tapes philippinarum* farming on nutrient dynamics and benthic respiration in the Sacca di Goro. Hydrobiologia, 455: 203 – 212.

Bartoli M., Naldi M., Nizzoli D., Roubaix V., & Viaroli P. (2003) Influence of clam farming on macroalgal growth: A microcosm experiment. Chemistry and Ecology, 19: 147 – 160.

Bartoli M., Longhi D., Nizzoli D., Como S., Magni P., & Viaroli P. (2009) Short term effects of hypoxia and bioturbation on solute fluxes, denitrification and buffering capacity in a shallow dystrophic pond. Journal of Experimental Marine Biology and Ecology, 381: 105 – 113.

Bower C.E., & Holm-Hansen T. (1980) A salicylate-hypochlorite method for determining ammonia in seawater. Canadian Journal of Fisheries and Aquatic Sciences, 37: 794 – 798.

Burla H. (1971) Gerichtete Ortsveränderung bei Muscheln der Gattung *Anodonta* im Zürichsee. Vierteljahrsschrift Der Naturforschenden Gesellschaft in Zürich, 116: 181 – 194.

Caraco N.F., Cole J.J., Findlay S.E.G., Lampman G.G., Pace M.L., & Strayer D.L. (2000) Dissolved oxygen declines in the Hudson River associated with the invasion of the zebra mussel (*Dreissena polymorpha*). Environmental Science & Technology, 34: 1204 – 1210.

Childress J.J., Fisher C.R., Brooks J.M., Kennicutt II M.C., Bidigare R., & Anderson A.E. (1986) A methanotrophic marine molluscan (Bivalvia, Mytilidae) symbiosis: mussels fueled by gas. Science, 233: 1306 – 1308.

Conroy J.D., Edwards W.J., Pontius R.A., Kane D.D., Zhang H., Shea J.F., & Culver A. (2005) Soluble nitrogen and phosphorus excretion of exotic freshwater mussels (*Dreissena* spp.): potential impacts for nutrient remineralization in western Lake Erie. Freshwater Biology, 50: 1146 – 1162.

Dalsgaard T., Nielsen L.P., Brotas V., Viaroli P., Underwood G., Nedwell D., ... & Dong L. (2000) Protocol handbook for NICE-Nitrogen Cycling in Estuaries: a project under the EU research programme: Marine Science and Technology (MAST III), 1 – 62.

Gallepp G.W. (1979) Chironomid influence on phosphorus release in sediment-water microcosms. Ecology, 60: 547 – 556.

Glud R.N., & Blackburn N. (2002) The effects of chamber size on benthic oxygen uptake measurements: A simulation study. Ophelia, 56: 23 – 31.

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.

Hakenkamp C.C., & Palmer M.A. (1999) Introduced bivalves in freshwater ecosystems: the impact of *Corbicula* on organic matter dynamics in a sandy stream. Oecologia, 119: 445 – 451.

Hansen K., King G.M., & Kristensen E. (1996) Impact of the soft-shell clam *Mya arenaria* on sulfate reduction in an intertidal sediment. Aquatic Microbial Ecology, 10: 181 – 194.

Higgins S.N., & Zanden M.J.V. (2010) What a difference a species makes: a meta–analysis of dreissenid mussel impacts on freshwater ecosystems. Ecological Monographs, 80: 179 – 196.

Hölker F., Vanni M.J., Kuiper J.J., Meile C., Grossart H.P., Stief P., ... & Hupfer M. (2015) Tube-dwelling invertebrates: Tiny ecosystem engineers have large effects in lake ecosystems. Ecological Monographs, 85: 333 – 351.

Kiibus M., & Kautsky N. (1996) Respiration, nutrient excretion and filtration rate of tropical freshwater mussels and their contribution to production and energy flow in Lake Kariba, Zimbabwe. Hydrobiologia, 331: 25 – 32.

Kristensen E., Penha-Lopes G., Delefosse M., Valdemarsen T., Quintana C.O., & Banta G.T. (2012) What is bioturbation? The need for a precise definition for fauna in aquatic sciences. Marine Ecology Progress Series, 446: 285 – 302.

Lorenzen C.J. (1967) Determination of chlorophyll and phaeopigments: spectrophotometric equations. Limnology and Oceanography, 12: 343 – 346.

Magni P., & Montani S. (2005) Laboratory experiments on bivalve excretion rates of nutrients. Water Encyclopedia, 4: 6 – 11.

Manganelli G., Bodon M., Favilli L., Castagnolo L., & Giusti F. (1998) Checklist of the species of the Italian fauna, terrestrial and freshwater mollusks. Bollettino Malacologico Italiano, 33: 151 – 156.

McMahon R.F. (1991) Mollusca: Bivalvia. In: Thorp J.H., & Covich A.P. (eds) Ecology and classification of North American freshwater invertebrates. Academic Press, San Diego, California, 315 – 399.

Murphy A.E., Anderson I.C., & Luckenbach M.W. (2015) Enhanced nutrient regeneration at commercial hard clam (*Mercenaria mercenaria*) beds and the role of macroalgae. Marine Ecology Progress Series, 530: 135 – 151.

Na T., Gribsholt B., Galaktionov O.S., Lee T., & Meysman F.J.R. (2008) Influence of advective bio-irrigation on carbon and nitrogen cycling in sandy sediments. Journal of Marine Research, 66: 691 – 722.

Nalepa T.F., Gardner W.S., & Malczyk J.M. (1991) Phosphorus cycling by mussels (Unionidae: Bivalvia) in Lake St. Clair. Hydrobiologia, 219: 239 – 250.

Nichols S.J., Silverman H., Dietz T.H., Lynn J.W., & Garling D.L. (2005) Pathways of food uptake in native (Unionidae) and introduced (Corbiculidae and Dreissenidae) freshwater bivalves. Journal of Great Lakes Research, 31: 87 – 96.

Nielsen L.P. (1992) Denitrification in sediment determined from nitrogen isotope paring. FEMS Microbiology Ecology, 86: 357 – 362.

Nizzoli D., Welsh D.T., Fano E.A., & Viaroli P. (2006) Impact of clam and mussel farming on benthic metabolism and nitrogen cycling, with emphasis on nitrate reduction pathways. Marine Ecology Progress Series, 315: 151 – 165.

Nizzoli D., Bartoli M., Cooper M., Welsh D.T., Underwood G.J.C., & Viaroli P. (2007) Implications for oxygen, nutrient fluxes and denitrification rates during the early stage of sediment colonisation by the polychaete *Nereis* spp. in four estuaries. Estuarine, Coastal and Shelf Science, 75: 125 – 134.

Nizzoli D., Welsh D.T., Longhi D., & Viaroli P. (2014) Influence of *Potamogeton pectinatus* and microphytobenthos on benthic metabolism, nutrient fluxes and denitrification in a freshwater littoral sediment in an agricultural landscape: N assimilation versus N removal. Hydrobiologia, 737: 183 – 200.

Norkko J., Reed D.C., Timmermann K., Norkko A., Gustafsson B.G., Bonsdorff E., … & Conley D.J. (2012) A welcome can of worms? Hypoxia mitigation by an invasive species. Global Change Biology, 18: 422 – 434.

Palmer M., Covich A.P., Finlay B.J., Gibert J., Hyde K.D., Johnson R.K., … & Strayer D.L. (1997) Biodiversity and ecosystem processes in freshwater sediments. Ambio, 26: 571 – 577.

Pelegrí S.P., Nielsen L.P., & Blackburn T.H. (1994) Denitrification in estuarine sediment stimulated by the irrigation activity of the amphipod *Corophium volutator*. Marine Ecology Progress Series, 105: 285 – 290.

Pelegrí S.P., & Blackburn T.H. (1995) Effects of *Tubifex tubifex* (Oligochaeta: Tubificidae) on N-mineralization in freshwater sediments, measured with ^{15}isotopes. Aquatic Microbial Ecology, 9: 289 – 294.

Prins T.C., Smaal A.C., & Dame R.F. (1998) A review of the feedbacks between bivalve grazing and ecosystem processes. Aquatic Ecology, 31: 349 – 359.

Pusch M., Siefert J., & Walz N. (2001) Filtration and respiration rates of two unionid species and their impact on the water quality of a lowland river. In: Bauer G., Wächtler K. (eds)

Ecology and evolution of the freshwater mussels Unionidae. Springer, Berlin, Germany, 317 – 326.

Raikow D.F., & Hamilton S.K. (2001) Bivalve diets in a midwestern U.S. stream: A stable isotope enrichment study. Limnology and Oceanography, 46: 514 – 522.

Reid R.G.B., McMahon R.F., Foighil D.O., & Finnigan R. (1992) Anterior inhalant currents and pedal feeding in bivalves. The Veliger, 35: 93 – 104.

Rota E, Bartoli M., & Laini A. (2014) First time in Italy. Is the elusive aquatic megadrile *Sparganophilus* Benham, 1892 (Annelida, Clitellata) accelerating its dispersal in Europe?. Journal of Limnology, 73: 482 – 489.

Ruginis T., Bartoli M., Petkuviene J., Zilius M., Lubiene I., Laini A., & Razinkovas-Baziukas A. (2014) Benthic respiration and stoichiometry of regenerated nutrients in lake sediments with *Dreissena polymorpha*. Aquatic Sciences, 76: 405 – 417.

Schwalb A.N., & Pusch M.T. (2007) Horizontal and vertical movements of unionid mussels in a lowland river. Journal of the North American Benthological Society, 26: 261 – 272.

Stadmark J., & Conley D.J. (2011) Mussel farming as a nutrient reduction measure in the Baltic Sea: consideration of nutrient biogeochemical cycles. Marine Pollution Bulletin, 62: 1385 – 1388.

Stief P. (2013) Stimulation of microbial nitrogen cycling in aquatic ecosystems by benthic macrofauna: Mechanisms and environmental implications. Biogeosciences, 10: 7829 – 7846.

Strayer D.L., Hunter D.C., Smith L.C., & Borg C. (1994) Distribution, abundance, and role of freshwater clams (Bivalvia: Unionidae) in the freshwater tidal Hudson River. Freshwater Biology, 31: 239 – 248.

Strayer D.L. (2014) Understanding how nutrient cycles and freshwater mussels (Unionoida) affect one another. Hydrobiologia, 735: 277 – 292.

Svensson J.M. (1997) Influence of *Chironomus plumosus* larvae on ammonium flux and denitrification (measured by the acetylene blockage- and the isotope pairing-technique) in eutrophic lake sediment. Hydrobiologia, 346: 157 – 168.

Thamdrup B., & Dalsgaard T. (2002) Production of N_2 through anaerobic ammonium oxidation coupled to nitrate reduction in marine sediments. Applied and Environmental Microbiology, 68: 1312 – 1318.

Traganza E.D., Swinnerton J.W., & Cheek C.H. (1979) Methane supersaturation and ATP-zooplankton blooms in near-surface waters of the Western Mediterranean and the subtropical North Atlantic Ocean. Deep-Sea Research Part I: Oceanographic Research Papers, 26: 1237 – 1245.

Valderrama J.C. (1977) Methods used by the hydrographica department of the national board of fisheries. Goteborg, Sweden.

Vaughn C.C., & Hakenkamp C. (2001) The functional role of burrowing bivalves in freshwater ecosystems. Freshwater Biology, 46: 1431 – 1446.

Vaughn C.C., Gido K.B., & Spooner D.E. (2004) Ecosystem processes performed by unionid mussels in stream mesocosms: species roles and effects of abundance. Hydrobiologia, 527: 35 – 47.

Vaughn C.C., Nichols S.J., & Spooner D.E. (2008) Community and foodweb ecology of freshwater mussels. Journal of the North American Benthological Society, 27: 409 – 423.

Welsh D.T., & Castaldelli G. (2004) Bacterial nitrification activity directly associated with isolated benthic marine animals. Marine Biology, 144: 1029 – 1037.

Yamamuro M., & Koike I. (1993) Nitrogen metabolism of the filter-feeding bivalve *Corbicula japonica* and its significance in primary production of a brackish lake in Japan. Limnology and Oceanography, 38: 997 – 1007.

Yeager M.M., Cherry D.S., & Neves R.J. (1994) Feeding and burrowing behaviors of juvenile rainbow mussels *Villosa iris* (Bivalvia: Unionidae). Journal of the North American Benthological Society, 13: 217 – 222.

Zhang L., Shen Q., Hu H., Shao S., & Fan C. (2011) Impacts of *Corbicula fluminea* on oxygen uptake and nutrient fluxes across the sediment-water interface. Water, Air, and Soil Pollution. 220: 399 – 411.

Section 2.2

Nitrification and denitrification in estuarine sediments with tube-dwelling benthic animals

Introduction

Nitrification is a critical step of benthic nitrogen (N) cycling, directly regulated by the availability of oxygen (O_2) and ammonium (NH_4^+) within sediments but also by pore water pH, the activity of benthic algae, the occurrence of free sulfides and the quality and quantity of organic matter (Rysgaard et al. 1994 and 1995; Strauss et al. 2002; Dollhopf et al. 2005). In the absence of nitrification, the NH_4^+ produced within sediments is transported to the water column, whereas in the presence of nitrification it is converted to nitrate (NO_3^-) and may be further denitrified (Rysgaard et al. 1995). The coupling of ammonification, nitrification and denitrification depends upon the organic matter (OM) input to sediments and the presence of benthic animals and may consistently reduce the internal recycling of N (Eyre and Ferguson 2009; Stief 2013). Under moderate OM load the efficiency of denitrification may be elevated, resulting in limited efflux of inorganic N from sediments, whereas under elevated OM load denitrification decreases substantially (Burgin and Hamilton 2007; Eyre and Ferguson 2009). Large organic matter inputs in fact increase total sediment respiration, resulting in lower O_2 penetration within sediments, lower nitrification and coupled nitrification-denitrification and higher ammonium efflux (Henriksen et al. 1981; Eyre and Ferguson 2009).

Burrowing benthic animals have a well demonstrated, stimulatory effect on different pathways of benthic N cycling, including nitrification and denitrification (Mayer et al. 1995; Rysgaard et al. 1995; Pelegrí and Blackburn 1995a; Stief 2013). This is due to sediment reworking, production of labile OM in the form of pellets and injection of O_2 and NO_3^--rich water through burrows by ventilation and bioirrigation (Kristensen et al. 2012). Benthic animals increase the spatial and biogeochemical heterogeneity of sediments (Lewandowski et al. 2007), creating mosaics of oxic and anoxic micro- or macroniches where the abundance and activity of microbial communities may largely exceed those of surrounding, non-bioturbated sediments (Kristensen et al. 1991; Gutiérrez and Jones 2006; Kristensen et al. 2012). By ventilating their burrows, besides the introduction of oxidized compounds within sediments, benthic animals increase the mobility of solutes, which in uninhabited sediments is regulated by molecular diffusion (Waldbusser and Marinelli 2006). Within functional groups, the effects of benthic animals on sediment biogeochemistry may be species-specific due to different size, metabolic rates and behavioural aspects (Pelegrí and Blackburn 1995b; Møller and Riisgård 2006;

Murphy et al. 2018). Therefore, depending on combinations of benthic animals' specific traits and sediment features, biogeochemical reactions and solute exchange across the sediment–water interface differ widely between bioturbated and non-bioturbated sites and within sediments bioturbated by organisms with different ecological traits (Zhang et al. 2010).

The bioturbation by benthic tube-dwelling animals (e.g., polychaetes, corophiid amphipods and chironomid larvae) plays an important role in regulating nitrification and denitrification in estuarine and freshwater environments (e.g., Nizzoli et al. 2007; Stief et al. 2009; Hölker et al. 2015). By constructing burrows, these animals increase the surface of the sediment-water interface and pump bottom water through the burrows, increasing both O_2 and NO_3^- penetration in sediment horizons where these electron acceptors are depleted (Kristensen and Hansen 1999; Kristensen 2000). They may therefore stimulate both microbial nitrification and denitrification (Svensson et al. 2001; Kristensen et al. 2012). Chironomid larvae and corophiid amphipods may attain high densities in estuarine and freshwater systems, and through their burrowing activities may significantly stimulate N removal (Pelegrí and Blackburn 1996; Svensson 1997; Stief et al. 2009). Different authors observed abundance-dependent stimulation of coupled nitrification-denitrification and of denitrification of water column NO_3^- in sediments with chironomid larvae and corophiid amphipods (Pelegrí and Blackburn 1994). These effects of benthic animals on N removal were mainly studied with the isotope pairing technique (IPT, Nielsen 1992), even if methodological concerns were raised (Pelegrí and Blackburn 1994; Rysgaard et al. 1995; Steingruber et al. 2001). The IPT method is based on the addition of ^{15}N-NO_3^- to the water phase of micro- or mesocosms and the quantification of the produced $^{29}N_2$ and $^{30}N_2$. The calculations of the IPT assume a homogeneous ratio of $^{14}NO_3^-$ and $^{15}NO_3^-$ in the denitrification zone, which in bioturbated sediments is unlikely (Steingruber et al. 2001). A few studies reported direct measurements of nitrification or of denitrification with methods alternative to the IPT. Some measurements were based on the use of specific inhibitors (Belser and Mays 1980; Sloth et al. 1992), assuming that their effects on target measurement were negligible, which is not always true (e.g., Cornwell et al. 1999; Fulweiler et al. 2015).

Aims of this work were to reconstruct benthic N cycling and in particular microbial nitrification and denitrification processes in two distinct eutrophic estuaries, colonized by benthic animals with different ecological traits. Sediments with chironomid larvae (Diptera: *Chironomus plumosus*) were collected from the Curonian Lagoon (SE Baltic Sea), whereas sediments with corophiid amphipods (*Corophium insidiosum)* were collected from the Sacca di Goro Lagoon (NW Adriatic Sea). These burrowers have different tolerance (higher in *C. plumosus*) to high OM loads and low O_2 levels and colonize different sediment horizons. Burrows of *C. plumosus*

extend down to 10–15 cm depth whereas those of *C. insidiosum* are confined in surface (2–5 cm) sediment layers (Gamble 1970).

We hypothesized that the presence of chironomid larvae in organic-loaded sediment produces a minor effect on nitrification and a major effect on water column NO_3^- denitrification. This is due to low bioirrigation and ventilation activities by chironomid larvae and dominant O_2 and NO_3^- consumption for microbial respiration. The latter is favored by short path length to reach the denitrification zone as a consequence of intermittent ventilation and limited O_2 penetration along deep burrow lining (Rysgaard et al. 1994; Roskosch et al. 2010). We hypothesized on the contrary high rates of nitrification and low rates of water column NO_3^- denitrification in the presence of *C. insidiosum*, due to elevated rates of continuous ventilation, active oxidation of burrows and longer paths of NO_3^- to reach the denitrification zone (Rysgaard et al. 1994; Møller and Riisgård 2006). Contrarily to most published experiments, where denitrification is measured by the IPT, our approach is based on measurements of net N_2 fluxes by the N_2/Ar and of net NO_3^- effluxes via the $^{15}NO_3^-$ dilution techniques, in sediment cores (Rysgaard et al. 1993; Kana et al. 1994). Combining net inorganic N fluxes with the above-mentioned measurements, we were able to calculate nitrification and denitrification rates in bioturbated sediments collected from different estuarine areas, avoiding the methodological problems of the IPT.

Material and methods

Sample collection and experimental setup

Two distinct experiments based on batch dark incubation of microcosms, were carried out using estuarine sediments and benthic animals collected in the Curonian and Sacca di Goro Lagoons. Both lagoons are shallow, eutrophic and undergo algal blooms, due to large riverine input of nutrients (Viaroli et al. 2006; Gasiūnaitė et al. 2008). Site-specific experimental approaches were used in the selected lagoons due to different spatial distributions of the taxa of benthic animals. In the Curonian Lagoon, due to low abundance and high spatial variability of chironomid larvae, sediments were collected and sieved to recover the target organism. Homogenized and defaunated sediment was used to reconstruct identical cores where chironomid larvae were added or not. In the Sacca di Goro Lagoon, where *C. insidiosum* is very abundant and quite homogeneously distributed, intact sediment cores were collected randomly, maintained in the dark for a short period and incubated.

Experiment 1

In the Curonian Lagoon, surface sediments (the upper 10 cm) were collected in April 2016 using a hand corer at a muddy site (median grain size 0.034 mm) located in the central portion

of the lagoon (55°17.2' N, 21°01.3' E; depth 3.5 m). Collected sediments were immediately sieved (0.5 mm mesh size) to recover fourth instar stage individuals of *C. plumosus*, which represent in the sampled area the dominant taxon with densities of 1,700 ± 1,300 ind m^{-2} (average ± std; Zettler and Daunys 2007). Nearly 200 L of water was also collected for experiment maintenance. In the laboratory, the homogenized sieved sediments were transferred into transparent cylindrical bottom-capped liners (i. d. 8 cm, 30 cm length) in order to reconstruct 12 identical microcosms with sediment height 15 cm. All microcosms were then carefully filled with lagoon water (salinity < 1) to create a water column overlying sediments of nearly 10 cm, and provided with a magnetic bar placed 5 cm above the sediment. Three treatments were created: 4 microcosms served as control without organisms, 4 microcosms were added with 3 individuals of *C. plumosus* each (low abundance, 600 ind m^{-2}) and 4 microcosms were added with 9 individuals of *C. plumosus* each (high abundance, 1,800 ind m^{-2}). After visual inspection that all chironomid larvae burrowed, which occurred after a few minutes from their addition, the 12 microcosms were fully submerged with the top open in a 75 L tank, containing aerated and well-stirred lagoon water, maintained at in situ temperature (14 °C). An external magnet rotating at 40 rpm ensured water stirring within each microcosm and its exchange with the tank water. The microcosms were preincubated for 3 weeks in the dark in order 1) to allow chironomid larvae to burrow and consolidate burrow structures, 2) to allow bacterial growth along burrows, 3) to establish chemical gradients across interfaces and 4) to avoid development of benthic algae and N uptake. Twice per week, nearly half of the water in the incubation tank was renewed with fresh in situ water.

Experiment 2

In the Sacca di Goro Lagoon, intact sediment cores (i.d. 8 cm, length 30 cm, n=11) were randomly collected in May 2016 by hand in the plume area of the Po di Volano River (salinity ~ 4), which is dominated by alluvial sediments (median grain size 0.024 mm) with high clay content (44° 49′ N, 12° 17′ E, depth 1.2 m)(Zilius et al. 2015). In this area, *C. insidiosum* dominates benthic animal assemblages, with abundance attaining up to 20,000 ind m^{-2} (Bartoli et al. 2012). In addition, nearly 100 L of water was collected for core maintenance and incubation. Once collected, all cores were levelled to have 15 cm of sediment and 10 cm of water column, bottom capped, provided with a magnetic bar suspended 5 cm above the sediment and transferred with the top open in a tank filled with aerated and well-stirred in situ water. An external magnet rotating at 40 rpm ensured water exchange between the core and the tank. Cores were preincubated in the dark for 3 days at the in situ temperature (19 °C), to exclude N uptake from benthic microalgae (Rysgaard et al. 1995).

Measurement of dissolved oxygen and inorganic nitrogen net fluxes

After the preincubation period, the microcosms from the two experiments underwent a short-term batch incubation in the dark, according to Dalsgaard et al. (2000). After collection of initial water samples from the incubation tank (t_i, 60 ml, n=4), a gas-tight top lid was placed on each microcosm and the incubation started. Incubations lasted 5 and 2 hours for the Curonian and Sacca di Goro Lagoon sediments, respectively, due to different water temperatures. Incubation time was set in order to keep the O_2 concentration within 20 – 30 % of the initial value. At the end of the incubation, the lids were removed and another water aliquot (t_f, 60 ml) was collected from each microcosm. Initial and final water samples were disposed in 12-ml exetainers (Labco®) for dissolved O_2 and dinitrogen (N_2) analyses and were fixed with 100 µl of 7 M $ZnCl_2$. In addition, a second aliquot of 20 ml was filtered (Whatman GF/F filters) and transferred into 20-ml plastic vials for dissolved inorganic nitrogen (NH_4^+ and NO_x=NO_2^-+NO_3^-) determination. Details on the analytical techniques are reported below. Net fluxes across the sediment-water interface were calculated according to the equation:

$$F_x = \frac{(C_f - C_i) \times V}{A \times t}$$

where F_x (mmol or µmol $m^{-2}h^{-1}$) is the flux of O_2 or the inorganic N forms, C_f and C_i are final and initial concentrations (mM or µM), V (L) is the volume of the microcosms water phase, A (m^2) is the area of the sediment and t (h) is the incubation time.

Measurements of nitrate efflux via $^{15}NO_3^-$ dilution

After flux measurements, the microcosms were submerged in the incubation tank to renew the water and shortly after (2 h) a second dark incubation was performed in order to determine the efflux of $^{14}NO_3^-$ from sediments due to nitrification (Rysgaard et al. 1994). At the beginning of the experiment, the water level in the incubation tank was lowered just below the core top opening and each core was amended with labelled potassium nitrate ($K^{15}NO_3$ 98 % atom, Cambridge Isotope Laboratories, MA, USA) to a final concentration of 50 ± 5 µM $^{15}NO_3^-$ (corresponding at both estuaries in nearly 50 % enrichment of in situ $^{14}NO_3^-$). An aliquot of water (15 ml) was collected from each microcosm 5 minutes after the addition of $^{15}NO_3^-$ (t_i). An additional water sample was collected after 8 h (t_f). All water samples were filtered (Whatman GF/F filters) and transferred into 20 ml HDPE vials for further treatment. During the incubation the microcosms were left open and with the stirring system on, in order to maintain O_2 levels close to saturation and to not limit nitrification. Dissolved O_2 concentration in the water overlying sediments was frequently monitored via microelectrodes (OX-50,

Unisense). The collected water aliquots were analyzed via spectrophotometry for the concentration of total dissolved nitrate ($^{14}NO_3^-$+$^{15}NO_3^-$) and via membrane inlet mass spectrometry for the concentration of $^{15}NO_3^-$ (see below for analytical methods). The net efflux of $^{14}NO_3^-$ from sediment (µmol $m^{-2}h^{-1}$) was calculated from:

$$NO_3^- \; efflux = \left[\frac{C(f-i)}{0.366-f}\right] \times \frac{V}{A \times t}$$

where *C* (µM) is the initial concentration of $^{14}NO_3^-$+$^{15}NO_3^-$, *i* and *f* are the ^{15}N atom % of NO_3^- at the beginning (t_i) and at the end (t_f) of the incubation, 0.366 is the natural abundance of ^{15}N, *V* (L) is the volume of the water in the microcosms, *A* (m^2) is the surface of the incubated sediment and t (h) is the incubation time (t_f-t_i).

Sediment characterization

After the second incubation, three cores from each experiment (randomly selected from the high-abundance treatment in the Curonian Lagoon experiment and from the Sacca di Goro Lagoon experiment), were processed for the determination of organic matter, pore water NH_4^+ and potential nitrification rates. Sediments were extruded and sliced in the following layers: 0–1, 1–2, 2–3, 3–5 and 5–10 cm. From each slice, after homogenization, 12 ml of sediments were subsampled with a cut-off syringe for different treatments: a) 5 ml for organic carbon (C_{org}) and total nitrogen (TN) content analysis, b) 5 ml for pore water extraction and c) 2 ml was collected for potential nitrification rates. All the remaining sediment, including sliced (70 – 90 % of initial volume) and not sliced cores, was sieved (0.5 mm mesh size) in order to analyze benthic animals abundance and biomass.

Sediment C_{org} and TN content were analyzed with elemental analyzer (FlashEA 1112, Thermo Scientific). Samples were acidified with 1 N HCl in order to remove carbonates and then dried. The pore water was extracted by squeezing sediment under N_2 at 1.5 bar (KC-Denmark). After filtration (Whatman GF/F filters), the pore water was transferred into plastic vials and frozen for later NH_4^+ analysis. Rates of potential nitrification were obtained by oxic slurries containing 2 ml of fresh sediment suspended in 30 ml of in situ water, enriched with NH_4^+ to a final concentration of nearly 200 µM. Water samples (5 ml) were collected at the beginning and at the end of the slurry incubation (8 hours) at room temperature (21 °C), then they were centrifuged, filtered (Whatman GF/F filters) and analyzed for combined nitrite and nitrate (NO_x^- =NO_2^-+NO_3^-). After pore water extraction and measurements of potential nitrification sediments were also carefully inspected in order to recover all benthic animals. The rates of potential nitrification (*PN*, nmol N $cm^{-3}h^{-1}$) were calculated from:

$$PN = \frac{[NO_x^-]_f - [NO_x^-]_i}{t \times [S]}$$

where $[NO_x^-]_i$ and $[NO_x^-]_f$ (nM) are the initial and final concentrations of NO_x^- in the slurry, t is the incubation time and $[S]$ is the concentration of the slurry (cm^3 of fresh sediment L^{-1}).

Analytical methods

The concentrations of dissolved O_2 and N_2 were measured as O_2/Ar and N_2/Ar ratios with a membrane inlet mass spectrometer (MIMS; Bay Instruments, sensitivity 0.2 µM), multiplied by the theoretical Ar concentration (Weiss 1970; Kana et al. 1994). Dissolved nitrite (NO_2^-) and NO_x^- were measured with a continuous flow analyzer (San^{++}, Skalar, sensitivity 0.3 µM) using standard colorimetric methods (Grasshoff et al. 1983). NO_3^- was calculated as the difference between NO_x^- and NO_2^-. Dissolved NH_4^+ was analyzed manually by means of the salicylate-hypochlorite method, using nitroprussiate as catalyst (Bower and Holm-Hansen 1980). $^{15}NO_3^-$ was quantitatively converted into $^{29}N_2$ and $^{30}N_2$ via anoxic slurries where water samples were added with denitrifying bacteria. The produced $^{29}N_2$ and $^{30}N_2$ were analyzed with a MIMS.

Statistical analyses

In the Experiment 1, differences between benthic fluxes along treatments were tested via one-way ANOVA. The ANOVA assumptions, data normality and homogeneity of variance, were checked using Shapiro-Wilks test. In the case of heteroscedasticity, data were transformed. For significant factors, post-hoc pairwise comparisons were performed using the Holm-Sidak test. In both experiments, the relation between biomass of benthic animals and net flux of different N forms and $^{14}NO_3^-$ efflux were analyzed by linear regression. Statistical significance was set at $p < 0.05$. All the analyses were performed using Sigma Plot 11.0 program.

Results

Experiment 1

After three weeks of preincubation, reconstructed control sediments appeared dark grey with the exception of the upper ~ 6 mm horizon that was light brown. Sediments with low and high abundance of *C. plumosus* displayed a network of burrows surrounded by brownish halos along a dark grey sediment column (10 cm). The sedimentary content of C_{org} and TN averaged 3.9 ± 0.3 % and 0.50 ± 0.04 %, respectively. The pore water NH_4^+ in sediments with *C. plumosus* was rather constant along the 0 – 10 cm vertical profile with a mean concentration of 7 µM (Fig. 2.2.1). The rates of PN measured in the upper 4 cm sediment layer, which was the most

bioturbated sediment horizon, peaked at the sediment-water interface with 95 ± 23 nmol N $cm^{-3}h^{-1}$; thereafter PN rates gradually decreased along the vertical profile (Fig. 2.2.1).

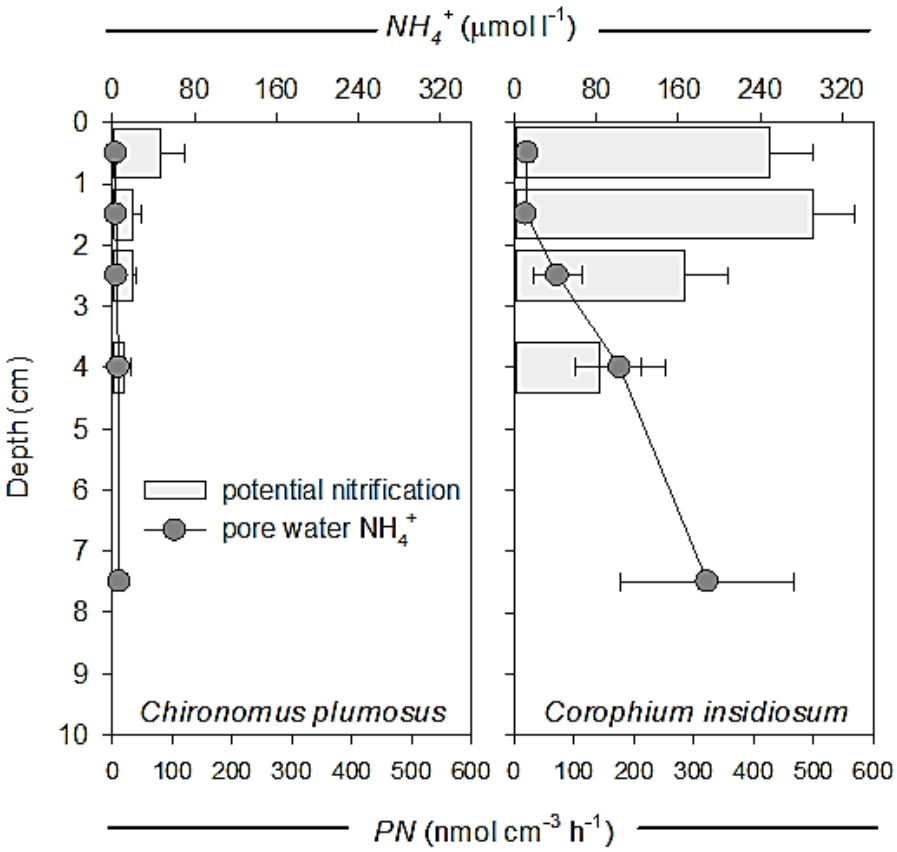


Fig. 2.2.1 Pore water profiles of ammonium (grey circles) and rates of potential nitrification (grey bars) in sediments bioturbated by *Chironomus plumosus* (left panel) and *Corophium insidiosum* (right panel). Mean rates and standard errors are reported (n=3).

Sediment O_2 demand was significantly different among treatments (Table 2.2.1, ANOVA, F=22.8, p=0.001) and rates measured in high abundance treatment were significantly higher than in control and low abundance treatments (Holm-Sidak test, $p<0.05$). The net fluxes of NH_4^+, NO_2^- and NO_3^- were not significantly different among treatments (Table 2.2.1, ANOVA, F=0.4, p=0.665 for NH_4^+; F=0.8, p=0.474 for NO_2^-; F=0.1, p=0.875 for NO_3^-). NO_3^- flux to sediment tended to increase with chironomid larvae abundance, however the trend was not significant as it was masked by a large variability. The net efflux of N_2 was significantly greater in the presence of chironomid larvae (ANOVA, F=395.6, $p<0.001$), with the highest production measured in high abundance treatment (Holm-Sidak test, $p<0.05$). The regressions between net fluxes of inorganic N and the biomass of benthic animals suggested that *C. plumosus*

significantly enhanced only the N_2 production (Fig. 2.2.2, r^2=0.65, p=0.002). The slope of the regression was 50.96 ± 11.79 µmol $g_{DW}^{-1}h^{-1}$.

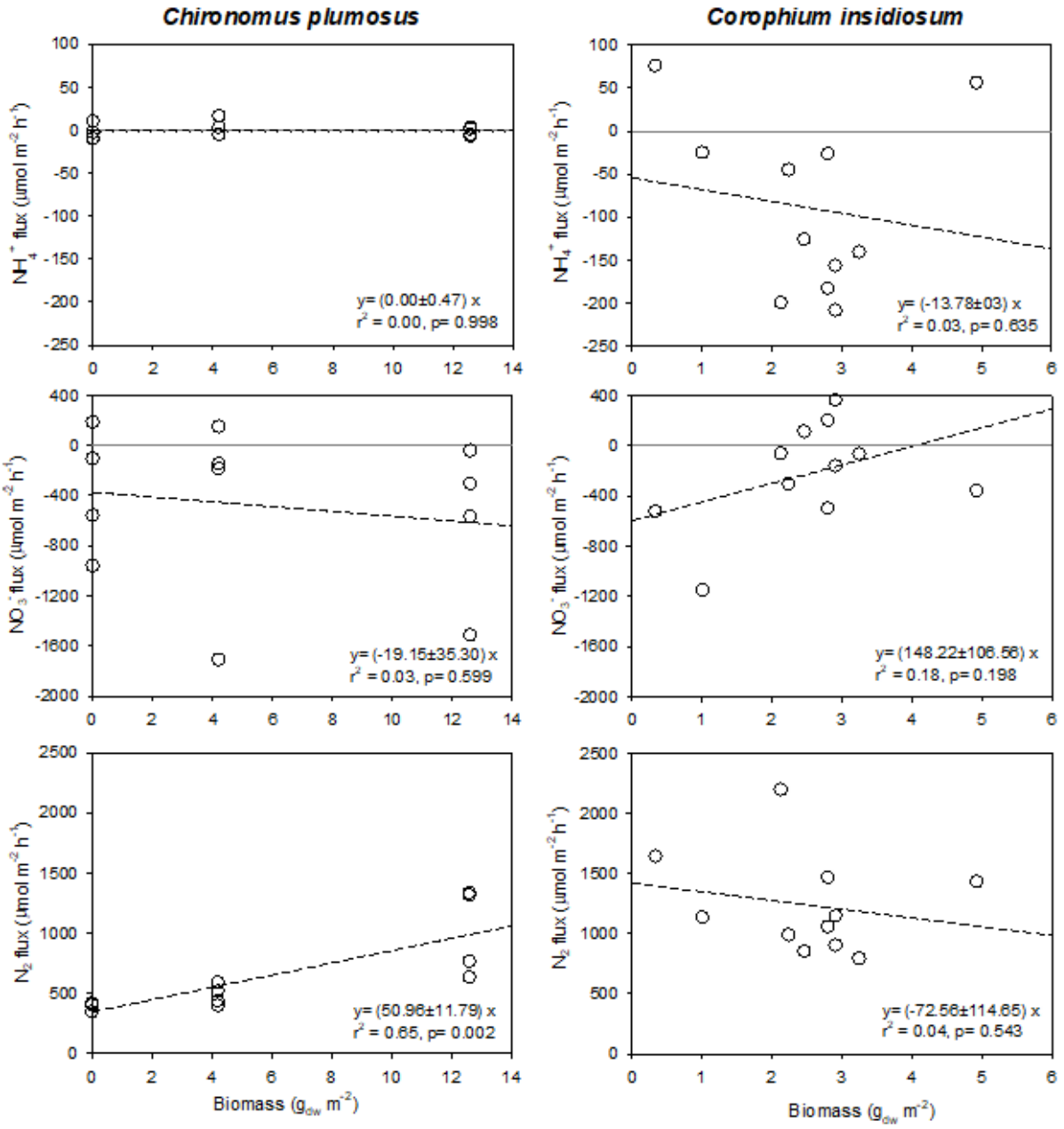


Fig. 2.2.2 Linear regressions between net inorganic nitrogen fluxes (NH_4^+, NO_3^- and N_2) and dry biomass of *Chironomus plumosus* (left panel) and *Corophium insidiosum* (right panel) recovered from incubated cores.

The $^{14}NO_3^-$ efflux from sediment to bottom water due to nitrification (from 19.1 to 55.9 µmol $m^{-2}h^{-1}$) was significantly stimulated by *C. plumosus.* There was a positive and significant

regression between the chironomid biomass and $^{14}NO_3^-$ effluxes, with a calculated slope of 2.45 ± 0.97 µmol $g_{DW}^{-1}h^{-1}$ (Fig. 2.2.3).

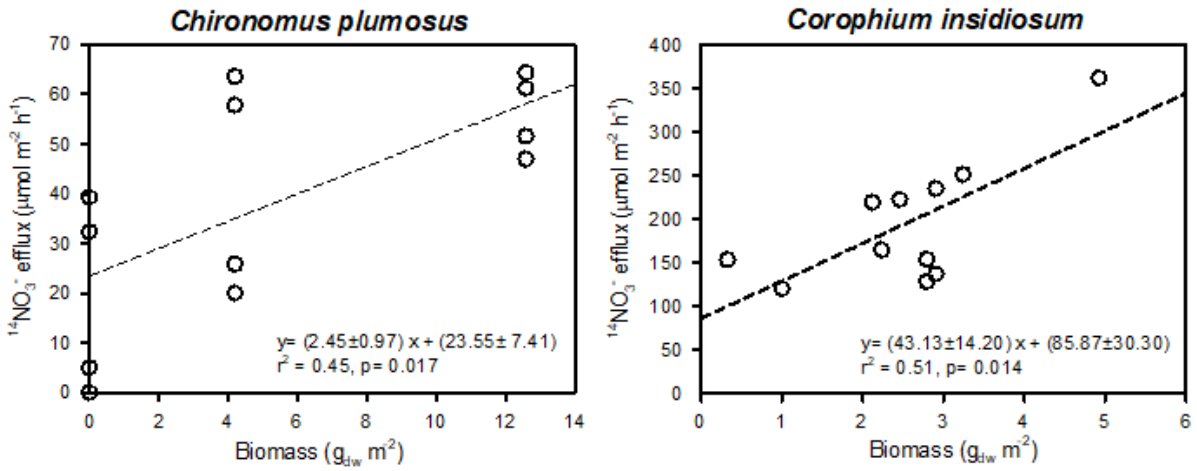


Figure 2.2.3 Linear regressions between the $^{14}NO_3^-$ efflux from sediment and dry biomass of *Chironomus plumosus* (left panel) and *Corophium insidiosum* (right panel) recovered from incubated cores.

At the end of the experiment, sediments were sieved to check chironomid larvae survival and measure their size. All the added chironomid larvae were recovered alive; the body length varied between 22 and 28 mm and the biomass averaged 4.2 ± 0.15 and 12.5 ± 0.27 g_{DW} m^{-2} in low (600 ind m^{-2}) and high (1,800 ind m^{-2}) abundance treatments, respectively.

Experiment 2

Intact sediments appeared homogeneously bioturbated by *C. insidiosum,* with an upper 3 – 5 cm thick light brown layer overlying reduced sediments. The C_{org} and TN sedimentary content averaged 4.0 ± 0.5 % and 0.34 ± 0.01 %, respectively. The NH_4^+ concentrations in pore water were low only in the upper 2 cm sediment layer (12.1 ± 5.8 µM), whereas they gradually increased with depth up to 188.3 ± 85.1 µM. The rates of PN were elevated all along the sediment profile with a peak of 498 ± 80 nmol N $cm^{-3}h^{-1}$ observed in the 1 – 2 cm layer.

Oxygen consumption in sediments with *C. insidiosum* varied from -13,243 to -3,669 µmol $m^{-2}h^{-1}$ (Table 2.2.1). Whereas, the fluxes of NH_4^+, NO_2^- and NO_3^- were in the range of -25.2 – -208.8, -123.5 – 15.1 and -1,153 – 365 µmol $m^{-2}h^{-1}$, respectively. The net flux of N_2 ranged between 791 and 2,195 µmol $m^{-2}h^{-1}$. The regressions between *C. insidiosum* biomass and inorganic N fluxes were not significant (Fig. 2.2.2, $p>0.05$).

The $^{14}NO_3^-$ efflux from sediment to bottom water due to nitrification varied from 119.6 to 361.6 µmol $m^{-2}h^{-1}$. Also *C. insidiosum* significantly stimulated the production of NO_3^- via nitrification

($p<0.05$); the slope of the regression between corophiid amphipods biomass and $^{14}NO_3^-$ efflux was 43.13 ± 14.20 µmol $g_{DW}^{-1}h^{-1}$ (Fig. 2.2.3). *C. insidiosum* stimulated the process of nitrification by a much larger extent, approximately 15-fold more, compared to the chironomid larvae (t-test, t=-2.8, p=0.011). The randomly collected cores had individuals of *C. insidiosum* (body length 3 to 4 mm), averaging 4,509 ± 2,119 ind m^{-2} (min – max, 600 – 8,800 ind m^{-2} corresponding to 3 – 44 ind $core^{-1}$), and representing the dominant species. The biomass of the corophiid amphipods averaged 2.5 ± 1.2 g_{DW} m^{-2}. The dense U-shaped burrow network extended over the upper ~3 – 6 cm and overlapped the light brown surficial sediment layer. Three cores contained also 1 or 2 small individuals of the polychaete *Neanthes succinea* with size between 3 and 4 cm, burrowing deeper within sediments.

Table 2.2.1 Net fluxes of oxygen (O_2) and inorganic nitrogen (NH_4^+, NO_2^-, NO_3^-, N_2) and nitrate efflux ($^{14}NO_3^-$) due to nitrification measured in dark incubations of sediments from the Curonian and Sacca di Goro Lagoons. Mean fluxes and standard errors are reported.

Site	Species	Abundance	O_2	NH_4^+	NO_2^-	NO_3^-	N_2	$^{14}NO_3^-$ efflux
		ind m^{-2}	µmol $m^{-2}h^{-1}$	µmol $m^{-2}h^{-1}$	µmol $m^{-2}h^{-1}$	µmol $m^{-2}h^{-1}$	µmol $m^{-2}h^{-1}$	µmol $m^{-2}h^{-1}$
Curonian Lagoon	*Chironomus plumosus*	0	-1,035 ± 70*	-3.0 ± 4.7	-6.8 ± 1.5	-487.9 ± 232.3	395.1 ± 16.7*	19.1 ± 9.8
		600	-1,203 ± 76*	2.3 ± 5.2	-6.4 ± 2.3	-594.7 ± 451.2	486.5 ± 42.4*	41.8 ± 11.0
Sacca di Goro Lagoon	*Corophium insidiosum*	1,800	-1,957 ± 146**	-2.0 ± 2.5	-9.6 ± 1.8	-731.3 ± 346.8	1,013 ± 182**	55.9 ± 4.1
		4,500	-7,611 ± 962	-89.2 ± 30.5	-53.2 ± 12.3	-222.5 ± 126.2	1,236 ± 126	194.8 ± 21.7

Note: different number of asterics indicates significant differences ($p < 0.05$); negative values = sediment uptake.

Discussion

The need of site-specific experimental approaches

Two distinctive experimental approaches were used due to different spatial distributions of the targeted benthic animals. In the experiment 1, due to low abundance and high spatial variability of chironomid larvae, we used reconstructed sediments with different abundance of *C. plumosus*. A 3 weeks preincubation was considered as sufficient to consolidate burrows, and for microbial communities, chemical gradients, and diagenetic processes to establish (Stocum and Plante 2006). Such relatively long preincubation period avoids artefacts associated to measurements performed the day after benthic animals addition. They include large stimulation of pore water solutes efflux during active burrowing, insignificant enhancement of processes as nitrification due to slow growth of nitrifiers in new oxic niches and absence of steady state. We acknowledge that sieving introduces other, potentially important artefacts in redox-dependent cycles because sediments are homogenized, vertical gradients are removed, dissolved organic matter is redistributed or partially lost and chemically-reduced pools are partly re-oxidized due to manipulation. Sieving may also simplify the benthic community, but not at the considered site where chironomid larvae represent the dominant organism (Zettler and Daunys 2007). On the other hand, the creation of homogeneous microcosms is a widely used approach in bioturbation studies because it allows addressing experimentally species-specific effects (Bartoli et al. 2000; Svensson et al. 2001). The chosen approach may represent an acceptable compromise at the Curonian Lagoon site, to avoid the risk of collecting unmanipulated cores without chironomid larvae.

Benthic nitrogen cycling in sediment bioturbated by C. plumosus *and* C. insidiosum

We combined the results from net flux measurements of inorganic N and $^{14}NO_3^-$ efflux due to nitrification to produce a conceptual scheme of benthic N cycling at the two sites (Table 2.2.2 and Fig. 2.2.4). We analyzed benthic N cycling in the Sacca di Goro Lagoon, with high abundance of *C. insidiosum*, and in the Curonian Lagoon, using results from the high abundance treatment of *C. plumosus*, as densities overlap those reported in the literature for this area (Zettler and Daunys 2007). Our main assumption was that net N_2 fluxes, at these eutrophic estuarine sites, were entirely driven by denitrification of water column NO_3^- and of NO_3^- produced within sediments by nitrifiers, excluding therefore the occurrence of anammox or N fixation. We remark that our purpose is not to compare rates measured at the two bioturbated sites, as the two experiments differ in terms of incubation temperature, sediment type, quality of organic matter, sampling approach, abundance and size of benthic animals, that preclude

direct comparison. Rather, our main aim is to analyze how different tube-dwelling animals may shape key processes of benthic N cycling. At the two sites, NO_3^- concentration in the water column was elevated and similar (102 and 110 µM in the Sacca di Goro and Curonian Lagoons, respectively), excluding the need to import N via an energy costly process as N fixation. Anammox was not detected at the two sites with specific assays (data not shown), as reported for other organic rich and eutrophic estuaries (e.g., Brin et al. 2014).

Table 2.2.2 Equations used to calculate the nitrogen flows reported in Fig. 2.2.4. Ammonium pool (comprising both ions weakly attached to solid particles and freely dissolved in pore water) is assumed to be in steady state.

No.	Pathway of nitrogen	Determination	Equation
1	NH_4^+ uptake	measured	–
2	Ammonification and excretion	calculated	(3) - (1)
3	Nitrification	calculated	(4) + (7)
4	$^{14}NO_3^-$ efflux	measured	–
5	NO_3^- uptake	measured	–
6	$*NO_x^-$ uptake	calculated	(5) - (4)
7	Coupled nitrification-denitrification	calculated	(8) - (6)
8	N_2 efflux	measured	–

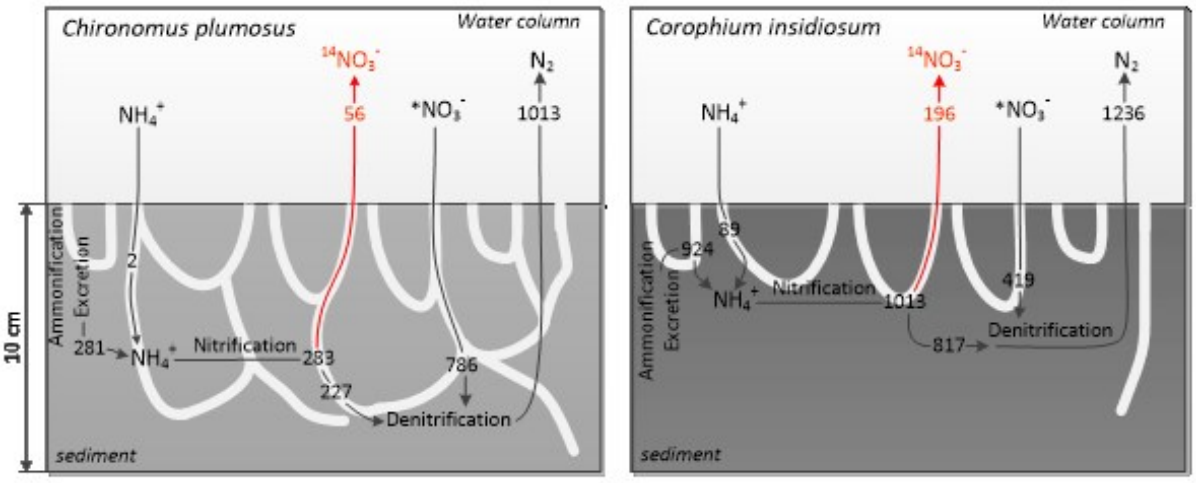


Fig. 2.2.4 Flow schemes for the benthic pathways of nitrogen in *Chironomus plumosus* (left panel) and *Corophium insidiosum* (right panel) bioturbated sediments, calculated combining measured fluxes and process rates (Table 2.2.1). The net rates are expressed on a hourly basis per unit of sediment surface (µmol $m^{-2}h^{-1}$). Anammox, dissimilative NO_3^- reduction to NH_4^+ (DNRA) and N fixation rates are not shown as rates are assumed to be negligible.

Although the production of N_2 was similar in both sites with high abundance of benthic animals, in the presence of *C. plumosus*, N_2 production was primarily fuelled by NO_3^- diffusing to the denitrification zone from the overlying water column (76 %), whereas in sediment inhabited by *C. insidiosum* coupled nitrification-denitrification was the main source of NO_3^- to denitrifiers (66 %). This result is supported by the fact that N_2 efflux was significantly correlated with *C. plumosus* abundance, whereas the NO_3^- efflux from nitrification was significantly correlated with the *C. insidiosum* abundance. *C. plumosus* had a scarce effect on nitrification (and consequently on denitrification coupled to nitrification) whereas corophiid amphipods had no effects on net N_2 fluxes. The latter may be explained in terms of contrasting effects of *C. insidiosum* on denitrification coupled to nitrification (e.g., enhancement) and on water column NO_3^- reduction (e.g., inhibition). Our calculations reveal that sediments inhabited by *C. plumosus* had nearly 2-fold higher NO_3^- uptake as compared to sediments with *C. insidiosum*. The flow scheme estimates that the amount of NH_4^+ required for NO_3^- production via nitrification was by 3-fold higher in sediments inhabited by *C. insidiosum.* Ammonification is expected to be the main pathway fuelling nitrification at both sites as excretion of NH_4^+ by tube-dwelling animals never exceeds of 20 % of immobilized NH_4^+ pool (e.g., Tuominen et al. 1999). However, additional NH_4^+ was taken from overlying water, suggesting limited ammonium availability in sediments.

C. plumosus *and* C. insidiosum *effect on nitrification and denitrification*

Despite comparable values of organic carbon and total N content, pore water NH_4^+ was lower in the Curonian Lagoon sediments (and decreasing along increasing abundance of chironomid larvae) as compared to concentrations measured in the Sacca di Goro Lagoon. This may suggest a different quality and reactivity of the organic matter at the two sites. Our conceptual schemes of benthic N cycling (Fig. 2.2.4) suggest that ammonification (+excretion) rates were nearly 3-fold lower in sediments with chironomid larvae, likely due to lower temperature, recalcitrant organic matter or low O_2 levels within sediments and inefficient anaerobic mineralization. The latter would explain different rates of potential nitrification measured at the two sites, which express the number of active bacterial cells able to oxidize NH_4^+. The vertical profile of PN in the high chironomid larvae abundance treatment was similar to that reported for not bioturbated sediments. In defaunated sediments from the Curonian Lagoon PN is limited by O_2 penetration, peaks close to the sediment-water interface and decreases exponentially with depth. On the contrary, sediments with corophiid amphipods had simultaneously higher NH_4^+ concentrations and higher PN rates, along a horizon of 4 cm, suggesting elevated O_2 transport within sediments. These results suggest different bioirrigation and ventilation activities by the two burrowers

(generally continuous for corophiid amphipods and intermittent for chironomids) and different degree of tolerance for chemically reduced conditions in the pore water (Pelegrí and Blackburn 1996; Kajan and Frenzel 1999; Møller and Riisgård 2006). It is also important to stress that the size of the two organisms was different and that smaller organisms are more active than larger ones in terms of metabolism (higher excretion per unit biomass) and burrow ventilation. Results also suggest very different within sites stimulation of nitrification by the two organisms, with much larger stimulation operated by the corophiid amphipods. The stimulation of N cycling by tube-dwelling animals is demonstrated by different studies (Pelegrí and Blackburn 1995a, 1996; Svensson 1997; Stief and de Beer 2006). The degree of stimulation depends on burrowing mode such as burrow shape and depth, and ventilation activity (Mermillod-Blondin et al. 2004). The tube-dwellers *C. plumosus* and *C. insidiosum* continuously irrigate their burrows and have a large effect on N cycling, particularly on nitrification, denitrification and NH_4^+ regeneration (Stief and de Beer 2006; Lewandowski et al. 2007). The most important mechanism, by which these organisms may stimulate nitrification, is the continuous renewal of O_2 in their burrows, which stimulates ammonification and the oxidation of produced NH_4^+ (Stief and de Beer 2002; Mermillod-Blondin et al. 2004).

The slope of the linear regression between $^{14}NO_3^-$ efflux and biomass of benthic animals showed much stronger effect on nitrification by *C. insidiosum* than by *C. plumosus*. This might be attributed to different burrowing depths. Due to scarce tolerance to low O_2 content, *C. insidiosum* lives in U-shaped burrows close to the surface sediment, whereas *C. plumosus* is more tolerant to O_2 shortage and typically digs deeper. Furthermore, *C. insidiosum* is more abundant, smaller and more active than *C. plumosus*. Unlike chironomid larvae, which display intermittent ventilation (Pelegrí and Blackburn 1996), corophiid amphipods display continuous ventilation of burrows via pleopods (Møller and Riisgård 2006). Such interspecific difference can determine different water velocity, giving different O_2 levels within burrows (Mermillod-Blondin et al. 2004; Riisgård 2007; Brand et al. 2013). Our results indicate that NH_4^+ concentration in pore water varied differently with depth in presence of the two species. In the presence of *C. plumosus*, NH_4^+ was depleted in the pore water along the vertical profile, suggesting limited ammonification and removal of pore water NH_4^+ during the early stages of sediment colonization, when high effluxes overlap digging activities. Also, the upper sediment layers with *C. insidiosum* where depleted in NH_4^+, but our data suggest alternative explanations (high and coupled ammonification and nitrification, as evidences by potential rates and measured $^{14}NO_3^-$ effluxes). We do not know the grazing pressure of the two analysed organisms on bacteria growing along the burrows wall, and if this could be relevant for the obtained results (Stief and de Beer 2002).

Site-specific characteristics like the presence of deep-burrowers and/or different sediment grain size or organic matter reactivity might regulate NH_4^+ content in surface sediment. In the intact cores from the Sacca di Goro Lagoon, we found in some cores few individuals of the polychaete *N. succinea,* which is typically a deeper burrower as compared to *C. insidiosum*. *N. succinea* creates dense burrow networks which can enhance NH_4^+ mobilization from deep to surface sediments (Nizzoli et al. 2007). Interestingly, our results showed negligible NH_4^+ efflux in all cores, suggesting a limited effect of this deep burrower experiment 2 and that all produced ammonium was nitrified, resulting in NO_3^- release from sediment. Grain size was very different at the two study sites, and may explain different benthic N cycling at Sacca di Goro and Curonian Lagoons. However, Henriksen et al. (1981) analyzed nitrification and its regulation along a wide gradient of sediment grain size and other descriptors along the Danish coasts and demonstrated that grain size was not an explanatory variable for nitrification, which was more regulated by the availability of ammonium and oxygen. Sediment O_2 consumption was highly different at the two sites that were studied. This may be due to different incubation temperature but may also suggests that muddy sediments in the Curonian Lagoon contain mainly organic matter which is refractory to microbial degradation whereas sediments in the Sacca di Goro Lagoon contain more labile organic matter that fuels intense heterotrophic activity. Consequently, this can maintain the elevated NH_4^+ pool in sediments that may be nitrified in the presence of burrowing benthic animals.

Conclusions

In the Sacca di Goro Lagoon, the amphipod *C. insidiosum* intensively bioirrigates its surface burrows and produces a large effect on nitrification, which sustains denitrification and most of the measured N_2 production. In the Curonian Lagoon, *C. plumosus* digs deeper within strictly anoxic sediments and produces a small effect on nitrification, likely due to a combination of lower NH_4^+ and O_2 availability to nitrifiers. However, it stimulates denitrification of water column NO_3^-. In dry periods, when NO_3^- concentrations in estuarine waters generally drop, benthic N loss in sediments with chironomid larvae might be strongly attenuated whereas in sediments with corophiid amphipods N removal should remain elevated due to the different underlying mechanisms. Our work aligns with the studies suggesting how the ecological traits of benthic animals, together with site-specific sediment features, affect differently the N-related microbial communities and the mechanisms regulating benthic N cycling.

References

Bartoli M., Nizzoli D., Welsh D.T., & Viaroli P. (2000) Short-term influence of recolonisation by the polychaete worm *Nereis succinea* on oxygen and nitrogen fluxes and denitrification: a microcosm simulation. Hydrobiologia, 431: 165 – 174.

Bartoli M., Castaldelli G., Nizzoli D, & Viaroli P. (2012) Benthic primary production and bacterial denitrification in a Mediterranean eutrophic coastal lagoon. Journal of Experimental Marine Biology and Ecology, 438: 41 – 51.

Belser L.W., & Mays E.L. (1980) Specific inhibition of nitrite oxidation by chlorate and its use in assessing nitrification in soils and sediments. Applied Environmental Microbiology, 39: 505 – 510.

Bower C.E., & Holm-Hansen T. (1980) A salicylate-hypochlorite method for determining ammonia in seawater. Canadian Journal of Fish and Aquatic Science, 37: 794 – 798.

Brand A., Lewandowski J., Hamann E., & Nützmann G. (2013) Advection around ventilated U-shaped burrows: A model study. Water Resources Research, 49: 2907 – 2917.

Brin L.D, Giblin A.E., & Rich J.J. (2014) Environmental controls of anammox and denitrification in southern New England estuarine and shelf sediments. Limnology and Oceanography, 59: 851 – 860.

Burgin A.J., & Hamilton S.K. (2007) Have we overemphasized the role of denitrification in aquatic ecosystems? A review of nitrate removal pathways. Frontiers in Ecology and the Environment, 5: 89 – 96.

Cornwell J.C., Kemp W.M., & Kana T.M. (1999) Denitrification in coastal ecosystems: methods, environmental controls, and ecosystem level controls, a review. Aquatic Ecology, 33: 41 – 55.

Dalsgaard T., Nielsen L.P., Brotas V., Viaroli P., Underwood G., Nedwell D., ... & Dong L. (2000) Protocol handbook for NICE-Nitrogen Cycling in Estuaries: a project under the EU research programme: Marine Science and Technology (MAST III), 1 – 62.

Dollhopf S.L., Hyun J.H., Smith A.C., Adams H.J., O'Brien S., & Kostka J.E. (2005) Quantification of ammonium-oxidizing bacteria and factors controlling nitrification in salt marsh sediments. Applied Environmental Microbiology, 71: 240 – 246.

Eyre B.D., & Ferguson A.J.P. (2009) Denitrification efficiency for defining critical loads of carbon in shallow coastal ecosystems. Hydrobiologia, 629: 137 – 146.

Fulweiler R.W., Heiss E.M., Rogener M.K., Rogener M.K., Newell S.E., LeCleir G.R., … & Wilhelm S.W. (2015) Examining the impact of acetylene on N-fixation and the active sediment microbial community. Frontiers in Microbiology, 6: 418.

Gamble J.C. (1970) Anaerobic survival of the crustaceans *Corophium volutator*, *C. arenarium* and *Tanais chevreuxi*. Journal of the Marine Biological Association of the United Kingdom, 50: 657 – 671.

Gasiūnaitė Z.R., Daunys D., Olenin S., & Razinkovas A. (2008) The Curonian lagoon. In Schiewer, U. (eds), Ecology of Baltic coastal waters, Springer Berlin Heidelberg, Berlin, 197 – 215.

Grasshoff K., Ehrhardt M., & Kremling K. (1983) Methods of Seawater analysis. 2nd eds, Verlag Berlin Chemie, Berlin.

Gutiérrez J.L., & Jones C.G. (2006) Physical ecosystem engineers as agents of biogeochemical heterogeneity. Bioscience, 56: 227 – 236.

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. Marine Biology, 61: 299 – 304.

Hölker F., Vanni M.J., Kuiper J.J., Meile C., Grossart H.P., Stief P., ... & Hupfer M. (2015) Tube-dwelling invertebrates: Tiny ecosystem engineers have large effects in lake ecosystems. Ecological Monographs, 85: 333 – 351.

Kajan R., & Frenzel P. (1999) The effect of chironomid larvae on production, oxidation and fluxes of methane in a flooded rice soil. FEMS Microbiology Ecology, 28: 121 – 129.

Kana T.M., Darkangelo C., Hunt D., Oldham J.B., Bennett G.E., & Cornwell J.C. (1994) A membrane inlet mass spectrometer for rapid high-precision determination of N_2, O_2, and Ar in environmental water samples. Analytical Chemistry, 66: 4166 – 4170.

Kristensen E. (2000) Organic matter diagenesis at the oxic/anoxic interface in coastal marine sediments, with emphasis on the role of burrowing animals. Hydrobiologia, 426: 1 – 24.

Kristensen E., Jensen M.H., & Aller R.C. (1991) Direct measurement of dissolved inorganic nitrogen exchange and denitrification in individual polychaete (*Nereis virens*) burrows. Journal of Marine Research, 49: 355 – 377.

Kristensen E., & Hansen K. (1999) Transport of carbon dioxide and ammonium in bioturbated (*Nereis diversicolor*) coastal, marine sediments. Biogeochemistry, 45: 147 – 168.

Kristensen E., Penha-Lopes G., Delefosse M., Valdemarsen T., Quintana C.O., & Banta G.T. (2012) What is bioturbation? The need for a precise definition for fauna in aquatic sciences. Marine Ecology Progress Series, 446: 285 – 302.

Lewandowski J., Laskov C., & Hupfer M. (2007) The relationship between *Chironomus plumosus* burrows and the spatial distribution of pore-water phosphate, iron and ammonium in lake sediments. Freshwater Biology, 52: 331 – 343.

Mayer M.S., Schaffner L., & Kemp W.M. (1995) Nitrification potentials of benthic macrofaunal tubes and burrow walls: effects of sediment NH_4^+ and animal irrigation behaviour. Marine Ecology Progress Series, 121: 157 – 169.

Mermillod-Blondin F., Rosenberg R., François-Carcaillet F., Norling K., & Mauclaire L. (2004) Influence of bioturbation by three benthic infaunal species on microbial communities and biogeochemical processes in marine sediment. Aquatic Microbial Ecology, 36: 271 – 284.

Møller L.F., & Riisgård H.U. (2006) Filter feeding in the burrowing amphipod *Corophium volutator*. Marine Ecology Progress Series, 322: 213 – 224.

Murphy A.E., Nizzoli D., Bartoli M., Smyth A.R., Castaldelli G., & Anderson I.C. (2018) Variation in benthic metabolism and nitrogen cycling across clam aquaculture sites. Marine Pollution Bulletin, 127: 524 – 535.

Nielsen L.P. (1992) Denitrification in sediment determined from nitrogen isotope paring. FEMS Microbiology Ecology, 86: 357 – 362.

Nizzoli D., Bartoli M., Cooper M., Welsh D.T., Underwood G.J.C., & Viaroli P. (2007) Implications for oxygen, nutrient fluxes and denitrification rates during the early stage of sediment colonisation by the polychaete *Nereis* spp. in four estuaries. Estuarine, Coastal and Shelf Science, 75: 125 – 134.

Pelegrí S.P., & Blackburn T.H. (1994) Bioturbation effects of the amphipod *Corophium volutator* on microbial nitrogen transformations in marine sediments. Marine Biology, 121: 253 – 258.

Pelegrí S.P., & Blackburn T.H. (1995a) Effects of *Tubifex tubifex* (Oligochaeta: Tubificidae) on N-mineralization in freshwater sediments, measured with ^{15}N isotopes. Aquatic Microbial Ecology, 9: 289 – 294.

Pelegrí S.P., & Blackburn T.H. (1995b) Effect of bioturbation by *Nereis* sp*., Mya arenaria* and *Cerastoderma* sp. on nitrification and denitrification in estuarine sediments. Ophelia, 42: 289 – 299.

Pelegrí S.P., & Blackburn T.H. (1996) Nitrogen cycling in lake sediments bioturbated by *Chironomus plumosus* larvae, under different degrees of oxygenation. Hydrobiologia, 325: 231 – 238.

Riisgård H.U. (2007) Biomechanics and energy cost of the amphipod *Corophium volutator* filter-pump. Biological Bulletin, 212: 104 – 114.

Roskosch A., Morad M.R., Khalili A., & Lewandowski J. (2010) Bioirrigation by *Chironomus plumosus*: advective flow investigated by particle image velocimetry. Journal of North American Benthological Society, 29: 789 – 802.

Rysgaard S., Risgaard-Petersen N., Nielsen L.P., & Revsbech N.P. (1993) Nitrification and denitrification in lake and estuarine sediments measured by the ^{15}N dilution technique and isotope pairing. Applied Environmental Microbiology, 59: 2093 – 2098.

Rysgaard S., Risgaard-Petersen N., Sloth N.P., Jensen K., & Nielsen L.P. (1994) Oxygen regulation of nitrification and denitrification in freshwater sediments. Limnology and Oceanography, 39: 1643 – 1652.

Rysgaard S., Christensen P.B., & Nielsen L.P. (1995) Seasonal variation in nitrification and denitrification in estuarine sediment colonized by benthic microalgae and bioturbating infauna. Marine Ecology Progress Series, 126: 111 – 121.

Sloth N.P., Nielsen L.P., & Blackburn T.H. (1992) Nitrification in sediment cores measured with acetylene inhibition. Limnology and Oceanography, 37: 1108 – 1112.

Steingruber S.M., Friedrich J., Gächter R., & Wehrli B. (2001) Measurement of denitrification in sediments with the ^{15}N isotope pairing technique. Applied Environmental Microbiology, 67: 3771 – 3778.

Stief P. (2013) Stimulation of microbial nitrogen cycling in aquatic ecosystems by benthic macrofauna: mechanisms and environmental implications. Biogeosciences, 10: 7829 – 7846.

Stief P., & de Beer D. (2002) Bioturbation effects of *Chironomus riparius* on the benthic N-cycle as measured using microsensors and microbiological assays. Aquatic Microbial Ecology, 27: 175 – 185.

Stief P., & de Beer D. (2006) Probing the microenvironment of freshwater sediment macrofauna: Implications of deposit-feeding and bioirrigation for nitrogen cycling. Limnology and Oceanography, 51: 2538 – 2548.

Stief P., Poulsen M., Nielsen L.P., Brix H., & Schramm A. (2009) Nitrous oxide emission by aquatic macrofauna. Proceedings of the National Academy of Sciences, 106: 4296 – 4300.

Stocum E.T., & Plante C.J. (2006) The effect of artificial defaunation on bacterial assemblages of intertidal sediments. Journal of Experimental Marine Biology and Ecology, 337: 147 – 158.

Strauss E.A., Mitchel N.L, & Lamberti G.A. (2002) Factors regulating nitrification in aquatic sediments: effects of organic carbon, nitrogen availability, and pH. Canadian Journal of Fish and Aquatic Science, 59: 554 – 563.

Svensson J.M. (1997) Influence of *Chironomus plumosus* L. on ammonium flux and denitrification (measured by the acetylene blockage- and the isotope pairing-technique) eutrophic lake sediment. Hydrobiologia, 346: 157 – 16.

Svensson J.M., Enrich-Prast A., & Leonardson L. (2001) Nitrification and denitrification in a eutrophic lake sediment bioturbated by oligochaetes. Aquatic Microbial Ecology, 23: 177 – 186.

Tuominen L., Mäkelä K., Lehtonen K.K., Haahti H., Hietanen S., & Kuparinen J. (1999) Nutrient fluxes, porewater profiles and denitrification in sediment influenced by algal sedimentation and bioturbation by *Monoporeia*. Estuarine, Coastal and Shelf Science, 49: 83 – 97.

Viaroli P., Giordani G., Bartoli M., Naldi M., Azzoni R., Nizzoli D., … & Fano E.A. (2006) The Sacca di Goro lagoon and an arm of the Po River. In Wangersky P.J. (eds) The Handbook of Environmental Chemistry, Estuaries, vol. 5. H. Springer-Verlag, Berlin, 197 – 232.

Waldbusser G.G., & Marinelli R.L. (2006) Macrofaunal modification of porewater advection: role of species function, species interaction, and kinetics. Marine Ecology Progress Series, 311: 217 – 231.

Weiss R.F. (1970) The solubility of nitrogen, oxygen and argon in water and seawater. Deep Sea Research and Oceanographic Abstracts, 17: 721 – 735.

Zettler M.L., & Daunys D. (2007) Long-term macrozoobenthos changes in a shallow boreal lagoon: comparison of a recent biodiversity inventory with historical data. Limnologica, 37: 107 – 185.

Zhang L., Gu X., Fan C., Shang J., Shen Q., Wang Z., & Shen J. (2010) Impact of different benthic animals on phosphorus dynamics across the sediment-water interface. Journal of Environmental Science, 22: 1674 – 1682.

Zilius M., Giordani G., Petkuviene J., Lubiene I., Ruginis T., & Bartoli M. (2015) Phosphorus mobility under short-term anoxic conditions in two shallow eutrophic coastal systems (Curonian and Sacca di Goro lagoons). Estuarine, Coastal and Shelf Science, 164: 134 – 146.

Section 2.3

Contrasting effects of an alien worm on benthic N cycling in muddy and sandy sediments

Introduction

In aquatic ecosystems invasive species have by default a negative connotation, with very limited exceptions in the literature (Norkko et al. 2012). Invasion by alien species may affect the interactions of species within communities and the cycling of energy and matter within ecosystems and produce cascade consequences and sequential shifts from pristine conditions (Strayer 2010). This might be the case of bioturbating fauna, which may support both bacteria and primary producers by mobilizing refractory or scarcely bioavailable (e.g., deep and buried) organic matter, adding new nutrient input to the system (Kristensen 1988). If this can be true in well preserved ecosystems, the invasion of heavily impacted ecosystems by alien species can paradoxically produce unexpected trajectories (Norkko et al. 2012).

Invasive macrofauna, able to colonize and spread even under oligotrophic conditions, may determine a large nutrient mobilization within the sediments and from the sediments to the water column (Hietanen et al. 2007). It is difficult to predict long-term net effects of alien species on biogeochemical functioning and on communities (Strayer 1999). Generally, the highest impact takes place within the early invasion phase, when alien species spread and reach peak density thanks to a strong competition capacity or to the abundance of resources (Crooks 2002). Later on, pristine communities tend to reorganize and contrast the invasion, resulting in a decrease of density of the invader and to a trajectory back to the original condition. This may require different time spans, depending on a large number of factors. In aquatic ecosystems, one of the most detailed story of invasion relates to *Dreissena polymorpha*, due to the impressive densities of this reef forming filter feeder and its capacity to invade a wide range of environments, from plankton-rich eutrophic to oligotrophic aquatic ecosystems (Strayer et al. 1999; Strayer 2009; Ruginis et al. 2014; Sousa et al. 2014). *D. polymorpha* was studied due to its supposed capacity to control and reverse eutrophication, an apparent paradox for an alien species (Fahnenstiel et al. 1995). Specific hypotheses postulated the reduction in plankton biomass due to high filtration rates, increase of water transparency, increase of benthic production and regime shift reversing turbid status into transparent one (Cha et al. 2012). Such trajectory was only partially verified as multiple analyzed systems revealed that such results might be short-term or not valid in system with limited access to phytoplankton or where excreted nutrients stimulate new algal growth (Gardner et al. 1995; Caraco et al. 2006). Another

interesting example of biogeochemical study of the effect of an alien species in marine environment is that of *Marenzelleria* spp. in the Baltic Sea. This invasion was also carefully studied due to the critical conditions of the Baltic area and to the contrasting effects produced by this species. It was demonstrated in fact that *Marenzelleria* spp. inhibited denitrification and stimulated the dissimilative nitrate reduction to ammonium (DNRA). The latter process is negative for an eutrophic ecosystem. However, *Marenzelleria* spp. enhanced the oxidation of sediments and the long-term retention of phosphorus (P), which both may favor the recolonization of pristine macrofauna. This aspect is good for an impacted and anoxic sea bottom (Hietanen et al. 2007; Kristensen et al. 2011; Bonaglia et al. 2013; Quintana et al. 2013; Kauppi et al. 2015). Macrofauna may produce contrasting effects on nutrient cycling, as it may stimulate nutrients permanent or temporal retention or loss or favor their mobilization (Norkko et al. 2012). In eutrophic environments, retention and losses are preferred over recycling, to contrast excess carbon production, infilling and so on (Benelli et al. 2018). In oligotrophic settings, macrofauna communities contribute to the slow cycling of elements and help the activity of primary producers (Vanni 2002).

In this study, we analyzed the effect of an alien worm in two freshwater environments: the eutrophic Mincio River (Northern Italy) and the oligotrophic Cazaux-Sanguinet Lake (Atlantic Coast of Southern France). *Sparganophilus tamesis* comes from North America and is now spreading all over Europe in a wide range of shallow environments generally colonized by macrophytes but with variable trophic levels and sedimentary features (Rota et al. 2018). We analyzed the effect of *S. tamesis* on rates of benthic respiration and nutrient dynamics hypothesizing a net stimulation of N_2 production in the eutrophic site and N recycling in the oligotrophic site. The latter hypothesis is supported by the fact that this invasive worm may exploit as nutrient source refractory fragments of macrophyte, scattered within the upper sediment horizon. As such, it may mobilize N otherwise associated and buried as particulate nitrogen (PN) and favor through burrow ventilation the displacement of this N-PN from the sediment to the water column, in the ammonium (NH_4^+) form. Ultimately, we hypothesized that there would be a limited stimulation of denitrification and anammox due to low organic pools in sediment and low nitrate (NO_3^-) in the water column but high excretion and release of NH_4^+. In the eutrophic settings, the contribution of *S. tamesis* on N loss via denitrification could be positive for a system with high nutrient background level. Therefore, the main aim was to compare the role of the *S. tamesis* in two different freshwater environments in order to understand if a system could benefit from the invasion by an alien species.

Material and methods

Sampling procedure and sediment characterization

Water, sediments and oligochaetes were collected from two sites: a branch of the Mincio River in proximity of Goito (MN, Northern Italy) and a littoral zone of the Cazaux-Sanguinet Lake (Atlantic Coast of Southern France). Both the sampling sites were shallow (~50 cm) with transparent water. The Italian site is characterized by high nutrients concentration in the water column (DIN~200 µM) and muddy sediment, whereas Cazaux-Sanguinet Lake is a nutrient-poor (DIN~5 µM) and a soft water (DIC~0.5 mM) environment with sediments predominantly sandy (Ribaudo et al. 2017).

Intact sediment cores (n=12 for Mincio and n=16 for Cazaux-Sanguinet) were collected by means of Plexiglass liners (inner diameter=4 cm, height=20 cm) vertically inserted into the sediment in order to have nearly 12 cm of sediment and nearly 8 cm of water phase. Individuals of *S. tamesis* collected from the same sites were then added to the cores in order to have variable biomass: 0 g_{dw} m^{-2} (n=3), 35 ± 7 g_{dw} m^{-2} (n=3), 55 ± 11 g_{dw} m^{-2} (n=3), 65 ± 3 g_{dw} m^{-2} (n=3) for the Mincio River and 0 g_{dw} m^{-2} (n=4), 45 ± 13 g_{dw} m^{-2} (n=4), 65 ± 8 g_{dw} m^{-2} (n=4), 85 ± 4 g_{dw} m^{-2} (n=4) for the French site (Rota et al. 2018). Nearly 50 L of in situ water was collected for preincubation and incubation procedures.

Additional 4 Plexiglass liners (inner diameter=4 cm, height=20 cm) were collected from the sites for sediment characterization. The upper sediment layer (0–10 cm) vertical sediment horizon was extruded with a piston and homogenized with a spatula and a sub-sample of 5 ml of fresh sediment was dried at 70 °C for 48 h for density, porosity and organic matter analyses. The homogenized sediment was collected by means of a cut-off 5 ml syringe. Bulk density was measured as the weight of a volume of 5 ml fresh material and porosity was calculated after drying at 70 °C until constant weight. Organic matter content (OM) was measured as percentage of weight loss on ignition (450 °C, 2 h) from dried, powdered sediment.

Incubation setup and measurement of benthic fluxes

Once collected, water and intact sediment cores added with oligochaetes were transferred to the laboratory within two hours. Cores were submersed with the top open in a large incubation tank containing well-mixed and aerated in situ water maintained at ambient temperature (24 ± 0.5 °C). A week after the sampling, all cores were closed and dissolved gas and nutrient fluxes were measured in the darkness. Incubation procedure was standard, with initial and final samplings from each core water phase, as detailed in Dalsgaard et al. (2000). Incubations lasted 2 h for Mincio sediment cores and 3 h for the French sediment cores and started when a gas-tight lid

with a sampling port and a compensation valve was positioned on the liners top. A Teflon-coated stirring bar gently mixed the water inside each liner to avoid stagnation and to guarantee homogeneous conditions within the cores. At the beginning and at the end of the incubations an aliquot of water was transferred to a 12 ml glass vial (Exetainer®, Labco Limited, High Wycombe, UK) and fixed with 100 µl of 7 M $ZnCl_2$ for O_2 analysis by means of Membrane Inlet Mass Spectrometer (MIMS, Bay Instrument, sensitivity 0.2 µM). In addition, an aliquot of 20 ml was filtered (Whatman GF/F glass fiber filters) and transferred to a plastic vial for NH_4^+, NO_3^- and nitrite (NO_2^-) analyses performed with standard spectrophotometric techniques (Golterman et al. 1978; Bower and Holm-Hansen 1980). Fluxes were calculated according to the equation below:

$$Flux\ x = \frac{\left([x]_f - [x]_i\right) \times V}{A \times t}$$

where *xf* and *xi*, expressed in µM or mM, are the concentrations of the solute x at the end and at the start of the incubation, respectively, V (L) is the volume of the core water phase, A (m^2) is the area of the sediment and t (h) is the incubation time.

The top lids were thereafter removed and the water in the tank replaced with fresh in situ water. In the afternoon, a sequential incubation was performed aiming at the measurement of denitrification rates with the isotope pairing technique (IPT, Nielsen 1992). Briefly, 0.1 ml (Cazaux-Sanguinet) and 0.5 ml (Mincio) of a 20 mM $^{15}NO_3^-$ stock solution was added to the water phase of each liner to have a final concentration of labelled nitrate of 10 and 50 µM at the oligotrophic and eutrophic sites, respectively. The top lids were then positioned and the cores were incubated in the dark for 3 hours. At the end of the incubation, the lids were removed, the whole sediment and water phase gently mixed to create a slurry, which was subsampled and transferred to the Exetainers, then poisoned with 200 µl of 7 M $ZnCl_2$ for labelled N_2 analysis by means of MIMS. At the end of the procedure, the cores were sieved in order to check for the occurrence of other macrofauna and to retrieve the oligochaetes. The revised version of the IPT was not used at the sampling sites as sediment slurries demonstrated the absence of anammox (Benelli unpublished). The rates of denitrification were calculated according to the equations and assumptions of Nielsen (1992): $D_{15} = p(^{15}N^{14}N) + 2p(^{15}N^{15}N)$ and $D_{14} = p(^{15}N^{14}N) + 2p(^{14}N^{14}N)$, where D_{15} and D_{14} = rates of denitrification based on $^{15}NO_3^-$ and $^{14}NO_3^-$, respectively; and $p(^{14}N^{14}N)$, $p(^{15}N^{14}N)$ and $p(^{15}N^{15}N)$ = rates of production of labelled and unlabeled N_2 species. Because the $p(^{14}N^{14}N)$ cannot be readily measured, estimation of D_{14} was obtained from: $D_{14} = D_{15} \times p(^{15}N^{14}N) / 2p(^{15}N^{15}N)$. The proportion of D_{14} supported by unlabeled NO_3^- from the water column (D_W) was calculated from: $D_W = D_{15} \times f / (1-f)$, where f

is a mole fraction of $^{14}NO_3^-$ in the water column. The coupled nitrification-denitrification (D_N) was calculated by difference as: $D_N = D_{14}-D_W$.

Individuals of *S. tamesis* retrieved from the cores were analyzed for the wet (g_{ww}) and dry weights (g_{dw}, after drying the soft tissue at 70 °C to a constant weight).

The sum of the inorganic N forms (DIN= $NH_4^+ + NO_3^- + NO_2^-$) was calculated in order to estimate the denitrification efficiency (DE) that is calculated as:

$$DE = \frac{D_{TOT}}{DIN + D_{TOT}} \times 100$$

where Dtot is the sum of D_W and D_N and DIN is the sum of NH_4^+, NO_3^- and NO_2^- (only positive values). Denitrification efficiency represents the fraction of mineralized N that is released to the water column as N_2. When DE is 100% it suggests tightly coupled ammonification, nitrification and denitrification and no inorganic N efflux.

Statistical analyses

Differences between sedimentary features at the two sites were tested by one-way ANOVA. The effect of variable *S. tamesis* biomass on process rates was analyzed via linear regression; differences between slopes were tested by one-way ANOVA.

Results

Sedimentary features

At the two sites, sediment density, porosity and organic matter content were significantly different (one-way ANOVA, $p<0.001$). In the Mincio River sediments were soft, muddy and very organic, whereas sandy sediment of Cazaux-Sanguinet Lake displayed high density, low porosity and low organic matter content (Table 2.3.1). The Mincio River sediments appeared dark and chemically reduced, with a sharp redox discontinuity a few mm below the interface whereas those of the Cazaux-Sanguinet lake were light brown and homogeneously oxidized along the upper 10-15 cm depth profile.

Table 2.3.1 Sediment features measured at the two sampling sites obtained by pooling the upper 10 cm sediment horizon. The reported values correspond to the average ± standard error.

	Mincio	**Cazaux-Sanguinet**
Sediment typology	muddy	sandy
Density (g cm^{-3})	1.34 ± 0.08	1.83 ± 0.09
Porosity	0.77 ± 0.04	0.36 ± 0.05
Organic matter content (%)	9.2 ± 0.70	0.11 ± 0.03

Benthic fluxes along increasing S. tamesis *biomass*

Microbial respiration rates measured in sediments without macrofauna was significantly higher in the Mincio River, likely due to higher organic content (one-way ANOVA, $p<0.001$). At both sites, the addition of increasing *S. tamesis* biomass resulted in increased sediment O_2 demand (SOD)(Fig. 2.3.1).

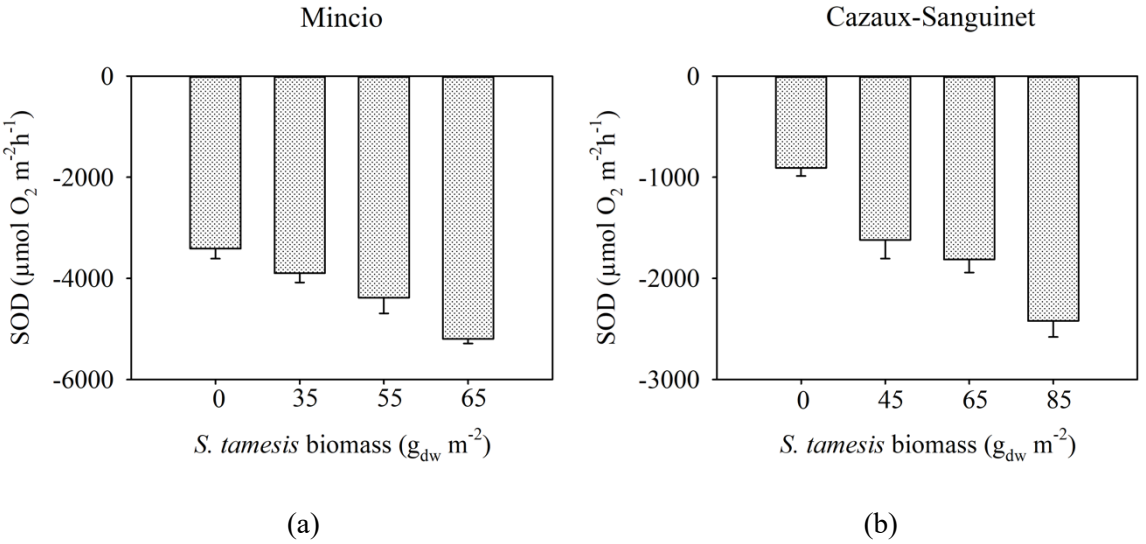


Fig. 2.3.1 Sediment Oxygen Demand in the four treatments in the Mincio River (a) and in the Cazaux-Sanguinet Lake (b). Averages ± standard errors are reported. Note different scales on y-axis.

Denitrification rates measured with the IPT are reported in figure 2.3.2. N_2 production increased in the 4 treatments along with increasing *S. tamesis* biomass at both sites. Compared to the aerobic respiration, denitrification rates were high in the Mincio sediments (Fig. 2.3.2a), whereas they were very low even with the high biomass of oligochaetes in the sandy sediments of Cazaux-Sanguinet Lake (Fig. 2.3.2b). In the 4 treatments denitrification supported by nitrification averaged 82 ± 22 and 0.38 ± 0.15 µmol $m^{-2}h^{-1}$, in Mincio sediments and Cazaux-Sanguinet sediments, respectively, without significant differences between control and bioturbated sediments. In Cazaux-Sanguinet Lake, D_N represented nearly 50 % of total denitrification in control sediments, whereas this share was reduced to 6 % in the highest biomass treatment.

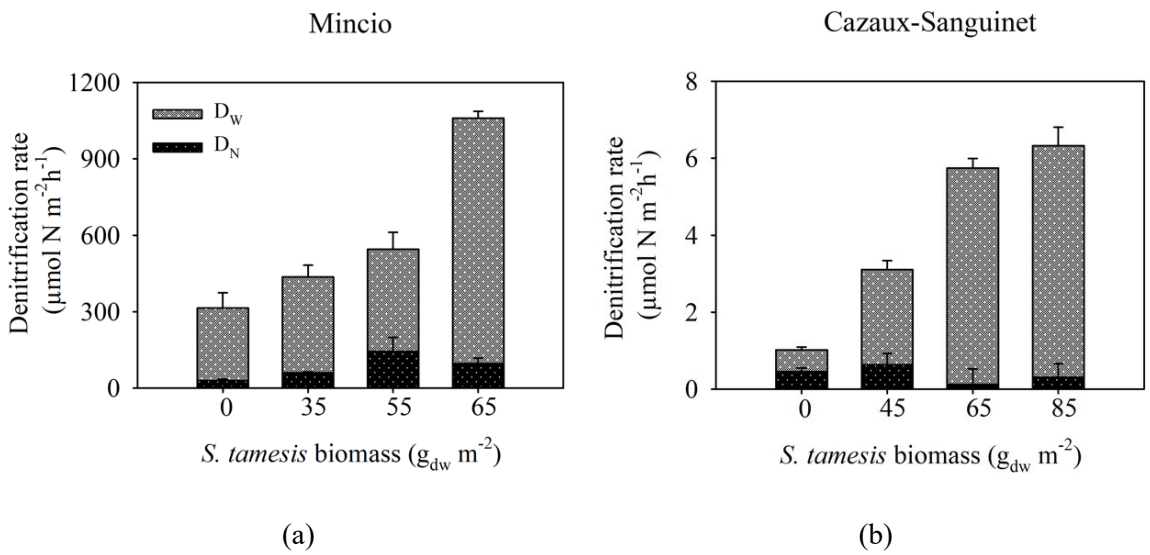


Fig. 2.3.2 Denitrification rates measured via IPT in the four treatments in the Mincio River (a) and in the Cazaux-Sanguinet Lake (b). D_W represents the denitrification of NO_3^- diffusing to anoxic sediments from the water column, whereas D_N is the denitrification of NO_3^- produced by nitrification in the sediment. Averages ± standard errors are reported. Note different scales on y-axis.

Comparing the effect produced by the worm on the processes measured at two sites, a different enhancement factor was calculated for sediment oxygen demand (SOD) and total denitrification (D_W+D_N)(Fig. 2.3.3). In particular, at both sites increasing worm biomass stimulated more the nitrate-based than the aerobic respiration. Furthermore, the stimulatory effect of *S. tamesis* on both processes was relatively higher in the Cazaux-Sanguinet Lake than in the Mincio River (one-way ANOVA, SOD $p<0.001$, Dtot $p<0.05$).

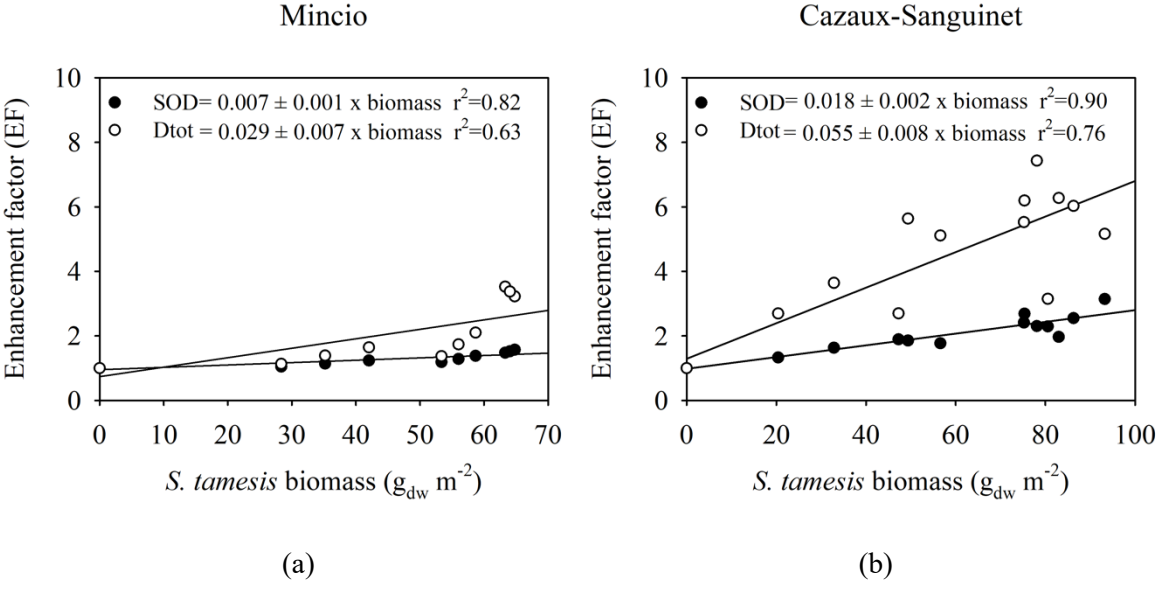
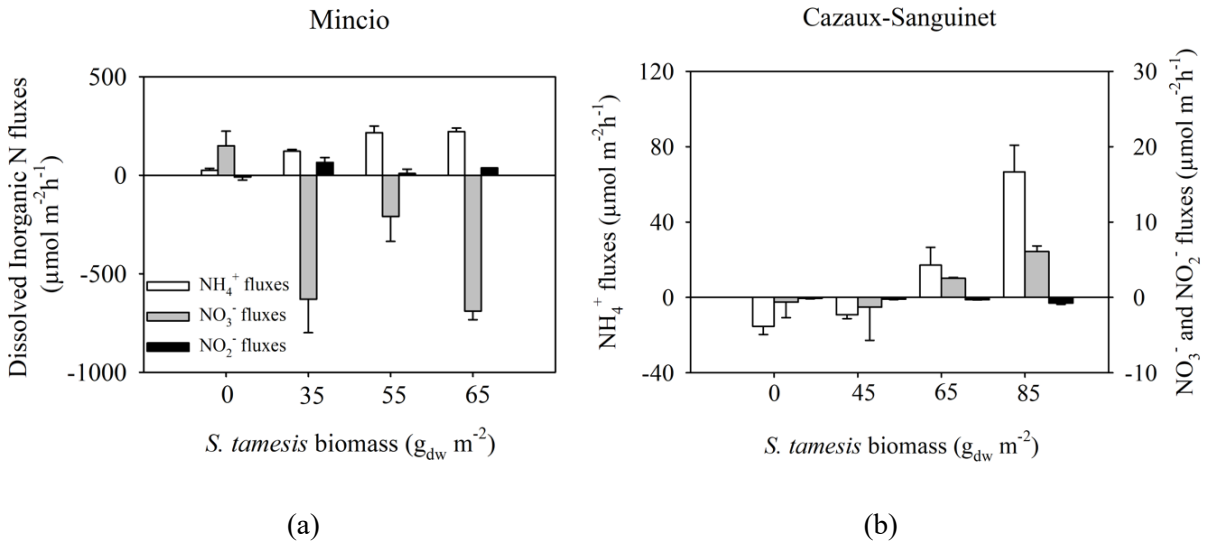


Fig. 2.3.3 Enhancement factors of *S. tamesis* biomass on SOD and denitrification rates. Results of linear regressions are reported in the graphs. The relative stimulations were calculated dividing the rates measured in the bioturbated sediments by the rates measured in control sediments, in the Mincio River (a) and in the Cazaux-Sanguinet Lake (b). Averages ± standard errors are reported.

Measured fluxes of the dissolved inorganic N forms had different trends, depending on *S. tamesis* biomass (Fig. 2.3.4). In the Mincio River, bare sediments acted as source of NH_4^+ and NO_3^-. Along increasing oligochaete biomass ammonium fluxes augmented whereas the presence of the worms reversed NO_3^- fluxes, which turned highly negative (Fig. 2.3.4a). In Cazaux-Sanguinet Lake, in absence and in presence of the low oligochaete biomass, sediments acted as sink for all N forms, whereas in sediments with the worm biomass equal or higher than 65 g_{dw} m^{-2} the fluxes were reversed and NH_4^+ and NO_3^- were regenerated to the water column ($NH_4^+ > NO_3^-$)(Fig. 2.3.4b). Nitrite fluxes were low and negative in all treatments.



(a) (b)

Fig. 2.3.4 Benthic fluxes of N forms (NH_4^+, NO_3^- and NO_2^-) expressed in µmol $m^{-2}h^{-1}$ measured during dark incubation in the sediment from Mincio River (a) and from the Cazaux-Sanguinet Lake (b). Averages ± standard errors are reported. Note different scales on y-axes.

The mean value of DE in the 4 treatments increased along with the increase of oligochaete biomass in Mincio sediments (Fig. 2.3.5a), whereas it decreased along with the increase of worm biomass in the French site, as a result of increasing and positive NH_4^+ fluxes (Fig. 2.3.5b).

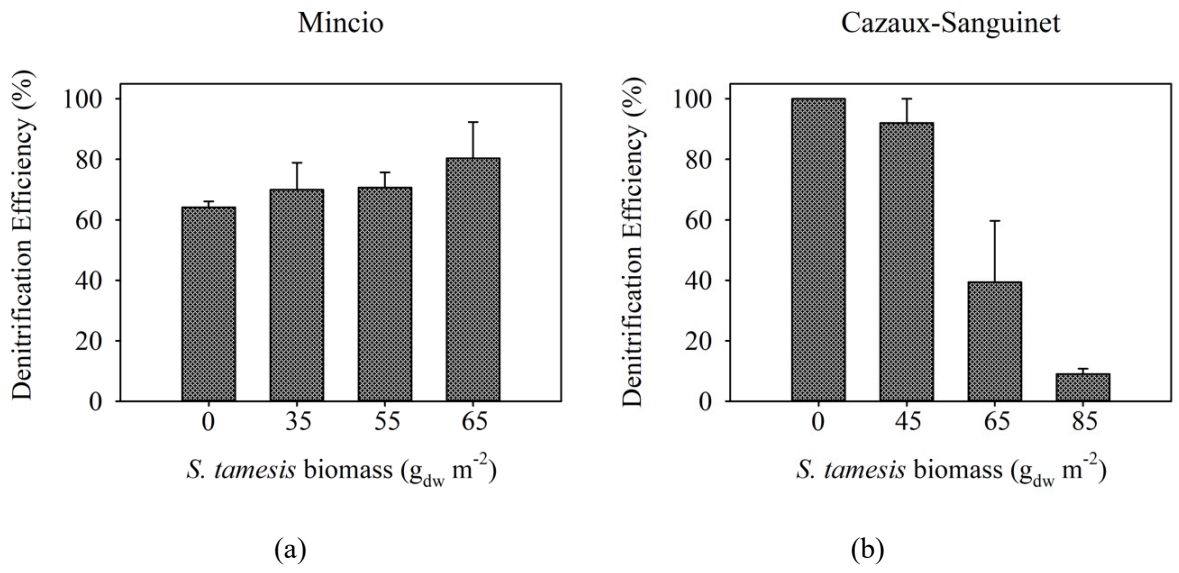


Fig. 2.3.5 Denitrification efficiency (%) calculated in the 4 treatments of (a) the Mincio River and of (b) the Cazaux-Sanguinet Lake. Averages ± standard errors are reported.

Discussion

We have investigated biogeochemical issues related to an alien, plastic species that we have found as abundant in contrasting environments, the eutrophic Mincio River and the oligotrophic Cazaux-Sanguinet Lake (Rota et al. 2018). Our results suggest that *S. tamesis* produced opposite effects at the two sites on benthic N cycling. Such effects might be considered as positive (e.g., increased N removal from nutrient-enriched Mincio) or negative (e.g., increased N recycling in the oligotrophic Cazaux-Sanguinet) for the invaded ecosystem. Analogous results are reported after the invasion of the bivalve *D. polymorpha*, whose ecosystem implications can be opposite and site-specific (e.g., spanning from the control of algal blooms to their enhancement) (Caraco et al. 2006, Benelli et al. 2019). Also for the invasive worm *Marenzelleria* spp. different authors report contrasting ecosystem level effects, depending upon the invaded site and the temporal scale considered (Kristensen et al. 2011; Norkko et al. 2012; Bonaglia et al. 2013).

S. tamesis was found at both sites in proximity of native macrophytes: *Vallisneria spiralis* in Italy (Magri et al. 2018) and *Lobelia dortmanna* in the Cazaux-Sanguinet Lake (Rota et al. 2018). The close association with macrophytes might depend on the favorable chemical conditions promoted by roots via radial oxygen loss (Marzocchi et al. 2019), that may increase the survival of worm cocoons. It is suggested for this species a large plasticity (Rota et al. 2018), that allows successful invasion in dramatically different sedimentary environments as those from the study sites. Different food availability or quality locally affect worms growth, as adults retrieved from the two areas differ for size: larger in muddy sediments and smaller in sandy

sediments. The two sites displayed very different sediment oxygen demand, higher in Mincio, as muddy sediments were organic-rich and chemically reduced, whereas sandy sediments at Cazaux-Sanguinet were organic-poor and more oxidized. The presence of oligochaetes increased O_2 consumption at both sites, due to the worm direct metabolic contribution and to indirect stimulation of microbial or chemical processes (Nizzoli et al. 2007; Bartoli et al. 2009). However, the degree of stimulation was different and larger at the sandy site. We calculated that a worm biomass of 100 g_{dw} m^{-2} might stimulate sediment oxygen demand by a factor of 1.8 in sandy sediments compared to an enhancement factor of 0.7 calculated for muddy sediments. Such difference could be due to different porosity and solute transport in sandy *vs* muddy sediments.

Similarly, rates of total denitrification were different and higher by 2 orders of magnitude at the Mincio site. Such difference is likely a combination of higher organic carbon and higher NO_3^- concentration in the water column of the Mincio site, resulting in elevated production of N_2 mainly from D_W (Pinardi et al. 2009). The presence of *S. tamesis* increased the rates of denitrification at both sites, but as for oxygen demand the degree of stimulation was different and higher in Cazaux-Sanguinet Lake. With a worm biomass of 100 g_{dw} m^{-2}, the enhancement factor of N_2 production in the sandy sediments was 5.5, whereas that calculated for the Mincio sediments was 2.9. Both denitrification enhancement factors were higher than those calculated for oxygen respiration. A higher relative stimulation of denitrification as compared to O_2 consumption was reported also by other authors in similar bioturbation experiments (Pelegrí et al. 1994). In the Mincio River denitrification rates support a substantial fraction of CO_2 production (see next sections), whereas in the Cazaux-Sanguinet Lake denitrification is quantitatively irrelevant as compared to the aerobic respiration.

In the Mincio sediments, the presence of the oligochaete reversed the role of the sediment from a source to a sink for NO_3^-, whereas the sediments acted always as NH_4^+ source. The presence of *S. tamesis* augmented NH_4^+ production, suggesting an important contribution to N regeneration by the reworking activities and the excretion by the oligochaetes (Nizzoli et al. 2007; Bartoli et al. 2009).

However, in the Mincio sediments the presence of worms stimulated NO_3^- consumption to an extent that always exceeded NH_4^+ production, resulting in negative DIN fluxes. In Cazaux-Sanguinet Lake, a worm biomass equal or higher than 65 g_{dw} m^{-2} reversed the role of sediment from N sink to N source, with NH_4^+ efflux as main driver of the net DIN regeneration. The comparison between the two sites suggests that the net effect of *S. tamesis* on net DIN fluxes depends on the environmental conditions, specifically the background nitrate level and the organic content.

Burrow ventilation and bioirrigation, with a few exceptions, always result in a higher consumption of O_2 and NO_3^- (Aller 1988; Pelegrí and Blackburn 1995). This is due to increased sediment-water interfaces and to thinner oxic layers within burrows along the sediment vertical profile (Nielsen et al. 2004; Kristensen et al. 2012; Kristensen et al. 2014). A few species of burrowers (e.g., *Marenzelleria* spp.) do not stimulate denitrification but rather NO_3^- ammonification due to specific pore water movement (Kristensen et al. 2011). Some studies on N cycling in faunated sediments support the major contribution of NO_3^- from the overlying water to the total N_2 production (Pelegrí et al. 1994; Jensen et al. 1996; Svensson 1997; Karlson et al. 2005). This is likely true at the eutrophic sites with elevated NO_3^- concentration in the water column (Kristensen et al. 2014). Other studies demonstrated that nitrification and hence the coupled nitrification-denitrification had the highest contribution to N_2 production (Na et al. 2008). The latter result likely depends on factors as the sediment redox or the NH_4^+ availability in the upper sediment layer. In sulfidic sediments, nitrification can be suppressed and the same can be true in oligotrophic sandy sites with elevated benthic microalgal activity and little exchangeable pools of NH_4^+. In the case of Cazaux-Sanguinet Lake, we believe that the low activity of the population of nitrifiers and the limited stimulation of nitrification by the oligochaete can be explained by NH_4^+ limitation, high competition with plants and benthic algae and by general inhibition of these bacteria by primary producers (Risgaard-Petersen 2003).

On the other hand, the limited relevance of denitrification of water column NO_3^- in Cazaux-Sanguinet Lake strongly depended on NO_3^- concentration in the overlying water, which was very low (<5 μM). In the Mincio sediments the high NH_4^+ availability enhanced the process of nitrification and therefore its share in the total N_2 production.

From NO_3^- reduction, it was also estimated that the denitrification in bare sediments contributed differently in the two sites: 11.5 % and 0.1 % of the total mineralization rate in Mincio and Cazaux-Sanguinet sites, respectively. O_2 fluxes were converted into CO_2 production assuming an O_2 : CO_2 = 1:1 stoichiometry, whereas denitrification was converted into CO_2 production assuming a NO_3^- : CO_2 stoichiometry of 1.25 (Richards 1965). In the presence of the highest oligochaetes biomass denitrification contributed 25.5 % and 0.3 % of total CO_2 production. Hence, in both sediment a highest biomass of *S. tamesis* enhanced the contribution of the anaerobic denitrification to total mineralization rates, but such contribution was completely different at the two sites. The contrasting effects of *S. tamesis* on benthic N cycling at the two sites is confirmed by the calculation of denitrification efficiency, which increased in the Mincio River along with increasing worms biomass. *S. tamesis* stimulated the process of denitrification and reduced the recycling of inorganic N forms to the water column. The opposite result was found in the Cazaux-Sanguinet Lake. Here, the denitrification efficiency measured in bare

sediment was 100 %, which suggests a tightly coupled ammonification, nitrification and denitrification and no inorganic N efflux. Along with increasing oligochaetes biomass denitrification efficiency decreased in the Cazaux-Sanguinet Lake due to increased NH_4^+ recycling.

Oxygen and DIN fluxes were related to the dry biomass of the incubated organisms with a linear regression. Assuming a 1:1 ratio of O_2 and CO_2 fluxes, we calculated a C:N molar ratios of excreted nutrients (8 for the Mincio River and 19 for Cazaux-Sanguinet Lake) from the slopes of the regression. The C:N ratios calculated for Cazaux-Sanguinet fall within the range reported by Sand-Jensen et al. (2005) for the detritus of *L. dortmanna* (C:N = 19-26). This result suggests that *S. tamesis* in Cazaux-Sanguinet Lake likely feeds on *L. dortmanna* detritus, which is characterized by a low N content. The C:N calculated for Mincio reflected the C:N molar ratio of more labile organic matter.

Overall results from the present study demonstrated that the alien worm *S. tamesis* could have both positive and negative effects on the invaded ecosystem, depending on the trophic conditions of the environment. At the eutrophic site, the high density of burrowers that dig and rework the sediment, flush oxic and nitrate-enriched water into a reduced sediment, stimulate the process of denitrification, which could alleviate the high nutrient load of the system. At the oligotrophic site, bare sediment and sediment with a low biomass of oligochaetes per m^2 act as buffers for inorganic N, whereas the increase of *S. tamesis* biomass enhanced the stimulation of benthic organic matter mineralization and nutrient cycling. Consequently, if the density of *S. tamesis* would dramatically increase, the benthic-pelagic coupling of this shallow-water system would change, favoring the growth of pelagic primary producers and, hence, modifying the overall lake functioning. The effects of alien species may vary with time and a rapid expansion of *S. tamesis* combined with changes in environmental conditions (e.g., higher temperature regimes) may result in higher mobilization of nutrients buried within sediments. This may produce undesired effects such as the growth of epiphytic algae on the native isoetid *L. dortmanna* or of phytoplankton in the water column. In fact, previous studies demonstrated that > 80 % of the inorganic N requirements of *L. dortmanna* is absorbed by the roots, in the forms of NO_3^- rather than NH_4^+ (Schuurkes et al. 1986). It is therefore likely that higher ammonium availability would favor microalgal primary production. Higher phytoplanktonic or epiphytic productivity may in turn enrich sediments with labile organic matter and create other positive feedbacks on benthic respiration and nutrient recycling (Sand-Jensen et al. 2005). All these cascade effects may hamper protected isoetid vegetation and ultimately result in a regime shift of the lake.

References

Aller R.C. (1988) Benthic fauna and biogeochemical processes in marine sediments: the role of burrow structures. In: Blackburn T.H., & Sørensen J. (eds) Nitrogen cycling in coastal marine environments. John Wiley & Sons, Chichester, 301 – 338.

Bartoli M., Longhi D., Nizzoli D., Como S., Magni P., & Viaroli P. (2009) Short term effects of hypoxia and bioturbation on solute fluxes, denitrification and buffering capacity in a shallow dystrophic pond. Journal of Experimental Marine Biology and Ecology, 381: 105 – 113.

Benelli S., Bartoli M., Zilius M., Vybernaite-Lubiene I., Ruginis T., Petkuviene J., & Fano E.A. (2018) Microphytobenthos and chironomid larvae attenuate nutrient recycling in shallow-water sediments. Freshwater Biology, 63: 187 – 201.

Benelli S., Bartoli M., Zilius M., Vybernaite-Lubiene I., Ruginis T., Vaiciute D., … & Fano E.A. (2019) Stoichiometry of regenerated nutrients differs between native and invasive freshwater mussels with implications for algal growth. Freshwater Biology, 00: 1 – 13.

Bonaglia S., Bartoli M., Gunnarsson J. S., Rahm L., Raymond C., Svensson O., … & Brüchert V. (2013) Effect of reoxygenation and *Marenzelleria* spp. bioturbation on Baltic Sea sediment metabolism. Marine Ecology Progress Series, 482: 43 – 55.

Bower C.E., & Holm-Hansen T. (1980) A salicylate-hypochlorite method for determining ammonia in seawater. Canadian Journal of Fisheries and Aquatic Sciences, 37: 794 – 798.

Caraco N.F., Cole J.J., & Strayer D.L. (2006) Top down control from the bottom: regulation of eutrophication in a large river by benthic grazing. Limnology and Oceanography, 51: 664 – 670.

Cha Y., Stow C.A., & Bernhardt E.S. (2012) Impacts of dreissenid mussel invasions on chlorophyll and total phosphorus in 25 lakes in the USA. Freshwater Biology, 58: 192–206.

Crooks J.A. (2002) Characterizing ecosystem-level consequences of biological invasions: The role of ecosystem engineers. Oikos, 97: 153 – 166.

Dalsgaard T., Nielsen L. P., Brotas V., Viaroli P., Underwood G., Nedwell D., ... & Dong L. (2000) Protocol handbook for NICE-Nitrogen Cycling in Estuaries: a project under the EU research programme: Marine Science and Technology (MAST III), 1 – 62.

Fahnenstiel G.L., Lang G.A., Nalepa T.F., & Johengen T.H. (1995) Effects of zebra mussel (*Dreissena polymorpha*) colonization on water quality parameters in Saginaw Bay, Lake Huron. Journal of Great Lakes Research, 21: 435 – 448.

Gardner W.S., Cavaletto J.F., Johengen T.H., Johnson J.R., Heath R.T., & Cotner Jr J.B. (1995) Effects of the zebra mussel, *Dreissena polymorpha*, on community nitrogen dynamics in Saginaw Bay, Lake Huron. Journal of Great Lakes Research, 21: 529 – 544.

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.

Hietanen S., Laine A.O., & Lukkari K. (2007) The complex effects of the invasive polychaetes *Marenzelleria* spp. on benthic nutrient dynamics. Journal of Experimental Marine Biology and Ecology, 352: 89 – 102.

Jensen K.M., Jensen M.H., & Kristensen E. (1996) Nitrification and denitrification in Wadden Sea sediments (Konigshafen, Island of Sylt, Germany) as measured by nitrogen isotope pairing and isotope dilution. Aquatic Microbial Ecology, 11: 181 – 191.

Karlson K., Hulth S., Ringdahl K., & Rosenberg R. (2005) Experimental recolonisation of Baltic Sea reduced sediments: survival of benthic macrofauna and effects on nutrient cycling. Marine Ecology Progress Series, 294: 35 – 49.

Kauppi L., Norkko A., & Norkko J. (2015) Large-scale species invasion into a low-diversity system: spatial and temporal distribution of the invasive polychaetes *Marenzelleria* spp. in the Baltic Sea. Biological Invasions, 17: 2055 – 2074.

Kristensen E. (1988) Benthic fauna and biogeochemical processes in marine sediments: the role of burrow structures. In: Blackburn T.H., & Sørensen J. (eds) Nitrogen cycling in coastal marine environments. John Wiley & Sons, Chichester, 275 – 299.

Kristensen E., Hansen T., Delefosse M., Banta G.T., & Quintana C.O. (2011) Contrasting effects of the polychaetes *Marenzelleria viridis* and *Nereis diversicolor* on benthic metabolism and solute transport in sandy coastal sediment. Marine Ecology Progress Series, 425: 125-139.

Kristensen E., Penha-Lopes G., Delefosse M., Valdemarsen T., Quintana C.O., & Banta G.T. (2012) What is bioturbation? The need for a precise definition for fauna in aquatic sciences. Marine Ecology Progress Series, 446: 285 – 302.

Kristensen E., Delefosse M., Quintana C.O., Flindt M.R., & Valdemarsen T. (2014) Influence of benthic macrofauna community shifts on ecosystem functioning in shallow estuaries. Frontiers in Marine Science, 41: 1 – 14.

Magri M., Benelli S., Bondavalli C., Bartoli M., Christian R.R., & Bodini A. (2018) Benthic N pathways in illuminated and bioturbated sediments studied with network analysis: Network analysis of benthic N processes. Limnology and Oceanography, 63: S68–S84.

Marzocchi U., Benelli S., Larsen M., Bartoli M., & Glud R.N. (2019) Spatial heterogeneity and short-term oxygen dynamics in the rhizosphere of *Vallisneria spiralis*: Implications for nutrient cycling. Freshwater Biology, 64: 532 – 543.

Na T., Gribsholt B., Galaktionov O.S., Lee T., & Meysman F.J.R. (2008) Influence of advective bio-irrigation on carbon and nitrogen cycling in sandy sediments. Journal of Marine Research, 66: 691 – 722.

Nielsen L.P. (1992) Denitrification in sediment determined from nitrogen isotope paring. FEMS Microbial Ecology, 86: 357 – 362.

Nielsen O.I., Gribsholt B., Kristensen E., & Revsbech N.P. (2004) Microscale distribution of oxygen and nitrate in sediment inhabited by *Nereis diversicolor*: Spatial patterns and estimated reaction rates. Aquatic Microbial Ecology, 34: 23 – 32.

Nizzoli D., Bartoli M., Cooper M., Welsh D.T., Underwood G.J.C., & Viaroli P. (2007) Implications for oxygen, nutrient fluxes and denitrification rates during the early stage of sediment colonisation by the polychaete *Nereis* spp. in four estuaries. Estuarine, Coastal and Shelf Science, 75: 125 – 134.

Norkko J., Reed D.C., Timmermann K., Norkko A., Gustafsson B.G., Bonsdorff E., … & Conley D.J. (2012) A welcome can of worms? Hypoxia mitigation by an invasive species. Global Change Biology, 18: 422 – 434.

Pelegrí S.P., Nielsen L.P., Blackburn T.H. (1994) Denitrification in estuarine sediment stimulated by the irrigation activity of the amphipod *Corophium volutator*. Marine Ecology Progress Series, 105: 285 – 290.

Pelegrí S.P., & Blackburn T.H. (1995) Effects of *Tubifex tubifex* (Oligochaeta: Tubificidae) on N-mineralization in freshwater sediments, measured with ^{15}isotopes. Aquatic Microbial Ecology, 9: 289 – 294.

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. Journal of Limnolology, 68: 133 – 145.

Quintana C.O., Kristensen E., & Valdemarsen T. (2013) Impact of the invasive polychaete *Marenzelleria viridis* on the biogeochemistry of sandy marine sediments. Biogeochemistry, 115: 95 – 109.

Ribaudo C., Bertrin V., Jan G., Anschutz P., & Abril G. (2017) Benthic production, respiration and methane oxidation in *Lobelia dortmanna* lawns. Hydrobiologia, 784: 21 – 34.

Richards F.A. (1965) Anoxic basins and fjords. In: Ryley J.P., Skirrow G. (eds) Chemical oceanography. Academic Press, London, 611 – 645.

Risgaard-Petersen N. (2003) Coupled nitrification-denitrification in autotrophic and heterotrophic estuarine sediments: On the influence of benthic microalgae. Limnology and Oceanography, 48: 93 – 105.

Rota E., Benelli S., Erséus C., Soors J., & Bartoli M. (2018) New data and hypotheses on the invasiveness, habitat selection, and ecological role of the limicolous earthworm *Sparganophilus tamesis* Benham, 1892. Fundamental and Applied Limnology, 192: 129 – 136.

Ruginis T., Bartoli M., Petkuviene J., Zilius M., Lubiene I., Laini A., & Razinkovas-Baziukas A. (2014) Benthic respiration and stoichiometry of regenerated nutrients in lake sediments with *Dreissena polymorpha*. Aquatic Sciences, 76: 405 – 417.

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

Schuurkes J.A.A.R., Kok C.J., & den Hartog C. (1986) Ammonium and nitrate uptake by aquatic plants from poorly buffered and acidified waters. Aquatic Botany, 24: 131 – 146.

Sousa R., Novais A., Costa R., & Strayer D.L. (2014) Invasive bivalves in fresh waters: Impacts from individuals to ecosystems and possible control strategies. Hydrobiologia, 735: 233 – 251.

Strayer D.L. (1999) Effects of alien species on freshwater mollusks in North America. Journal of the North American Benthological Society, 18: 74 – 98.

Strayer D.L., Caraco N.F., Cole J.J., Findlay S., & Pace M.L. (1999) Transformation of freshwater ecosystems by bivalves: a case study of zebra mussels in the Hudson River. BioScience, 49: 19 – 27.

Strayer D.L. (2009) Twenty years of zebra mussels: lessons from the mollusk that made headlines. Frontiers in Ecology and the Environment, 7: 135 – 141.

Strayer D. L. (2010) Alien species in fresh waters: Ecological effects, interactions with other stressors, and prospects for the future. Freshwater Biology, 55: 152 – 174.

Svensson J.M. (1997) Influence of *Chironomus plumosus* larvae on ammonium flux and denitrification (measured by the acetylene blockage- and the isotope pairing-technique) in eutrophic lake sediment. Hydrobiologia, 346: 157 – 168.

Vanni M.J. (2002) Nutrient cycling by animals in freshwater ecosystems. Annual Review of Ecology and Systematics, 33: 341 – 370.

Main findings and general remarks

These bioturbation studies demonstrate that the ecological traits of benthic animals affect differently the N-related microbial communities and the mechanisms regulating benthic N cycling and that results are context-dependent (e.g., they depend on sediment features and nutrient background). They demonstrate that large and rare bivalves create biogeochemical hot spots by enhancing the aerobic and anaerobic metabolisms in and nutrient mobilization from the benthic system. Therefore, it appears important to include such large but rare organisms in the benthic budgets as their effects was demonstrated to be relevant. Smaller but more abundant surface and deep burrowers enhanced N dissipation by similar extent, but through two different underlying mechanisms. Molecular N originates from the NO_3^- produced by coupled nitrification and denitrification in the presence of amphipods and from water column NO_3^- in the presence of chironomid larvae. Invasive macrofauna may rework sediments, mobilize refractory N pools and increase NH_4^+ regeneration and availability. Such effect could be different on the basis of the environment the alien species invade. In fact, the allochthonous worms that live in the eutrophic site enhance N loss by denitrification, whereas in the oligotrophic ecosystem characterized by rare and highly specialized macrophytes, as isoetids, they stimulate the regeneration and mobilization of dissolved nutrients, which may cause the growth of epiphytes and then negatively impact pristine macrophyte communities.

Although the results presented in this chapter are important to understand the role of some widespread organisms, I acknowledge that they reflect only the dark part of daily rates and hence allow to have only a partial comprehension of the net daily metabolism in illuminated sediments. The latter include the activity of benthic primary producers and the multiple interactions among plants, macrofauna and microbes. Moreover, sediment sieving and animal addition to intact sediments change the natural conditions even though they allow to perform laboratory experiments. To partially overcome these problems, all reconstructed sediments were pre-incubated for 3 weeks to let the establishment of bacterial communities and of diagenetic processes, as the experiment in section 2.2 showed (Stocum and Plante 2006).

In the next chapter, the autotrophic component is included in the analysis of benthic functioning (Sundbäck and Granéli 1988; MacIntyre et al. 1996; Underwood and Kromkamp 1999). In particular, bottom-up and top-down interactions between heterotrophic and autotrophic benthic components are considered. This is mostly important in shallow, illuminated sediments where bacteria, macrofauna and benthic primary producers coexist (Herren et al. 2017). Early bioturbation studies have excessively drawn the attention on the heterotrophic pathways of the benthic system, but macrofauna, and filter feeders or grazers in particular, may as well be

considered as the “fertilizer” or the “gardener” of the benthic primary producers (Tang and Kristensen 2007; Lohrer et al. 2010). As such, macrofauna may promote autotrophic processes to an extent that is even higher than those of microbes. If this is true, macrofauna would help the temporary or permanent “sink” ecosystem service of sediments for nutrients.

Preface and general remarks references

Baranov V., Lewandowski J., Romeijn P., Singer G., & Krause S. (2016) Effects of bioirrigation of non-biting midges (Diptera: Chironomidae) on lake sediment respiration. Scientific Reports, 6: 27329.

Bartoli M., Nizzoli D., Welsh D.T., & Viaroli P. (2000) Short-term influence of recolonisation by the polychaete worm *Nereis succinea* on oxygen and nitrogen fluxes and denitrification: A microcosm simulation. Hydrobiologia, 431: 165 – 174.

Cornwell J.C., Kemp W.M., & Kana T.M. (1999) Denitrification in coastal ecosystems: methods, environmental controls, and ecosystem level controls, a review. Aquatic Ecology, 33: 41 – 55.

Herren C.M., Webert K.C., Drake M.D., Vander Zanden M.J., Einarsson Á., Ives A.R., & Gratton C. (2017) Positive feedback between chironomids and algae creates net mutualism between benthic primary consumers and producers. Ecology, 98: 447 – 455.

Kristensen E. (1984) Effect of natural concentrations on nutrient exchange between a polychaete burrow in estuarine sediment and the overlying water. Journal of Experimental Marine Biology and Ecology, 75: 171 – 190.

Kristensen E., Penha-Lopes G., Delefosse M., Valdemarsen T., Quintana C.O., & Banta G.T. (2012) What is bioturbation? The need for a precise definition for fauna in aquatic sciences. Marine Ecology Progress Series, 446: 285 – 302.

Lohrer A.M., Halliday N.J., Thrush S.F., Hewitt J.E., & Rodil I.F. (2010) Ecosystem functioning in a disturbance-recovery context: Contribution of macrofauna to primary production and nutrient release on intertidal sandflats. Journal of Experimental Marine Biology and Ecology, 390: 6 – 13.

MacIntyre H.L., Geider R.J., & Miller D.C. (1996) Microphytobenthos: The ecological role of the “Secret Garden” of unvegetated, shallow-water marine habitats. I. Distribution, abundance and primary production. Estuaries, 19: 186 – 201.

Nizzoli D., Welsh D.T., Fano E.A., & Viaroli P. (2006) Impact of clam and mussel farming on benthic metabolism and nitrogen cycling, with emphasis on nitrate reduction pathways. Marine Ecology Progress Series, 315: 151 – 165.

Nizzoli D., Bartoli M., Cooper M., Welsh D.T., Underwood G.J.C., & Viaroli P. (2007) Implications for oxygen, nutrient fluxes and denitrification rates during the early stage of sediment colonisation by the polychaete *Nereis* spp. in four estuaries. Estuarine, Coastal and Shelf Science, 75: 125 – 134.

Pelegrí S.P., Nielsen L.P., & Blackburn T.H (1994) Denitrification in estuarine sediment stimulated by the irrigation activity of the amphipod *Corophium volutator*. Marine Ecology Progress Series, 105: 285 – 290.

Stief P. (2013) Stimulation of microbial nitrogen cycling in aquatic ecosystems by benthic macrofauna: mechanisms and environmental implications. Biogeosciences, 10: 7829 – 7846.

Stocum E.T., & Plante C.J. (2006) The effect of artificial defaunation on bacterial assemblages of intertidal sediments. Journal of Experimental Marine Biology and Ecology, 337: 147 – 158.

Sundbäck K., & Granéli W. (1988) Influence of microphytobenthos on the nutrient flux between sediment and water: A laboratory study. Marine Ecology Progress Series, 43: 63 – 69.

Svensson J.M. (1997) Influence of *Chironomus plumosus* larvae on ammonium flux and denitrification (measured by the acetylene blockage- and the isotope pairing-technique) in eutrophic lake sediment. Hydrobiologia, 346: 157 – 168.

Tang M., & Kristensen E. (2007) Impact of microphytobenthos and macroinfauna on temporal variation of benthic metabolism in shallow coastal sediments. Journal of Experimental Marine Biology and Ecology, 349: 99 – 112.

Underwood G.J.C., & Kromkamp J. (1999) Primary production by phytoplankton and microphytobenthos in estuaries. In: Nedwell D.B., & Raffaelli D.G. (eds) Advances in Ecological Research, Academic Press, 29: 93 – 153.

Vanni M.J., & McIntyre P.B. (2016) Predicting nutrient excretion of aquatic animals with metabolic ecology and ecological stoichiometry: a global synthesis. Ecology, 97: 3460 – 3471.

Chapter 3

Macrofauna activity and its direct and indirect effects on microbes and primary producers

Preface

The ecological interactions among trophic levels have been poorly studied in the past from a biogeochemical perspective. Littoral, shallow water environments are zones where multi-trophic interactions between producers and consumers often occur. Benthic algae may form dense mats covering the surface sediment layer (Revsbech et al. 1981; MacIntyre et al. 1996). During the light period, benthic algae create a thicker oxic layer on sediment surface that may produce a series of cascade effects on biogeochemical processes as the increase of nitrification, P and N uptake and retention and regeneration of benthic geochemical buffers (Rysgaard et al. 1995; Risgaard-Petersen 2003; Ferguson et al. 2007). Benthic macrofauna may graze on microphytobenthos and may affect sediment particle distribution by reworking activities, change sediment redox conditions, enhance mineralization process and excrete large amount of dissolved inorganic nutrients that could be readly taken up and reallocated in the upper sediment horizon (Kristensen 1984; Aller 1994; Caraco et al. 1997; Stief 2013). Hence, macrofauna may feed directly on benthic and pelagic algae maintaining elevated or even enhancing their growth potential (Stadmark and Conley 2011).

In this chapter, the effects of primary producers and benthic macrofauna on N cycling have been analyzed by means of light and dark incubations of sediment cores. I hypothesized an increase of nutrient retention within the sediment when chironomid larvae and microphytobenthos are co-present, due to reworking and ventilation activities and uptake, respectively (Section 3.1). Other groups of macrofauna with different ecological traits may have completely opposite effects on nutrient retention and algal growth. Filter-feeding mussels may live on hard substrates or burrowed in the sediment, in clumps or singly. The well-known effects of mussels on the pelagic and benthic compartments are the regeneration of dissolved inorganic nutrients via excretion and the biodeposition of feces and pseudofeces on sediment surface. I hypothesized a different stoichiometry of regenerated nutrients on the basis of mussel species. I also hypothesized that such different stoichiometry may promote algal succession and in particular the dominance of cyanobacteria. The effects of native and allochthonous mussels on algal growth were analyzed by means of intact sediment cores incubations in the light and in the dark (Section 3.2).

Section 3.1 is modified from Benelli S., Bartoli M., Zilius M., Vybernaite-Lubiene I., Ruginis T., Petkuviene J., & Fano E.A. (2018) Microphytobenthos and chironomid larvae attenuate nutrient recycling in shallow-water sediments. Freshwater Biology, 63: 187 – 201.

Section 3.2 is modified from Benelli S., Bartoli M., Zilius M., Vybernaite-Lubiene I., Ruginis T., Vaiciute D., Petkuviene J., & Fano E.A. (2019) Stoichiometry of regenerated nutrients differs between native and invasive freshwater mussels with implications for algal growth. Freshwater Biology, 00: 1 – 13.

Section 3.1

Microphytobenthos and chironomid larvae attenuate nutrient recycling in shallow-water sediments

Introduction

Shallow, bioturbated sediments are sites of intense biogeochemical processes, including carbon fixation, nutrient uptake and organic matter mineralization (MacIntyre et al. 1996; Miller et al. 1996). They are also sites of multiple interactions among trophic levels: macrofauna may keep elevated the growth potential of microphytobenthos through feeding, excretion and bioturbation activities. This results in a positive feedback between the biomass and growth of benthic algae and the biomass and growth of primary consumers (Herren et al. 2017). Macrofauna may also stimulate the activity of bacteria as well as different chemical processes (Kristensen 1984; Aller 1994). Mutualistic interactions in benthic communities are poorly studied but they may produce important effects on nutrient dynamics (Herren et al. 2017). Among these, the minimization of losses (i.e. large effluxes to the water column) or imports (i.e. N fixation) are expected (Magri et al. 2018).

The transition between transparent and turbid status in shallow aquatic ecosystems and its reversal has been traditionally interpreted in terms of nutrient excess or deficiency and of pelagic or benthic grazing pressure (Scheffer et al. 1993; Caraco et al. 1997; Scheffer 1998; Carpenter et al. 2003). Turbid waters characterize systems with high nutrient levels and low or inefficient grazing pressure, sustaining high phytoplankton biomass (Malone 1992; Scheffer 1998). However, the benthic compartment may promote water clarity in the presence of large densities of filter-feeding macrofauna (Caraco et al. 1997) or when sediments retain nutrients and minimize internal recycling (Sundbäck et al. 2000; Hölker et al. 2015). Whereas the role of zooplankton grazing on algal abundance is well studied, the role of benthos on pelagic production in shallow ecosystems is poorly investigated (Officer et al. 1982; Stadmark and Conley 2011; Hölker et al. 2015).

Photosynthesis by benthic microalgae may represent an important fraction of total primary production in shallow water ecosystems (MacIntyre et al. 1996; Underwood 2008). Microphytobenthos may form thick mats on the sediment surface, where the availability of light and nutrients may result in high rates of primary production, biomass turnover and nutrient uptake, translocation and retention (Revsbech et al. 1981; Underwood and Kromkamp 1999). Active mats of benthic algae, deeply affecting surface sediment biogeochemistry, are described in lakes (Herren et al. 2017), shallow bays (Bartoli et al. 2003), coastal areas (Sundbäck et al.

2006) and estuaries (Cabrita and Brotas 2000). The benthic carbon flux driven by microphytobenthos may support the activities of bacteria, meiofauna and macrofauna (Miller et al. 1996). In the light period, benthic algae may significantly enhance sediment oxygen penetration and assimilate dissolved nutrients from both the water column and the pore water (Revsbech et al. 1981; Rysgaard et al. 1995). A thicker oxic layer produces cascade effects on a number of processes in the proximity of the water-sediment interface, which is a critical zone due to its steep redox gradients and intense microbial and geochemical activity (MacIntyre et al. 1996; Sundbäck et al. 2000; Risgaard-Petersen 2003). Some of these effects have been analyzed in detail, for example the stimulation or competitive inhibition of nitrification and denitrification, which ultimately depend on the background nitrogen level and on the net autotrophy or heterotrophy of the benthic system (Risgaard-Petersen 2003). Different works report how active mats of benthic algae act as a trap for nutrient regenerated from the sediment, and prevent their diffusion to the water column (Bartoli et al. 2003; Sundbäck et al. 2004). Net autotrophic sediments may exert a bottom-up control on pelagic primary production via nutrient uptake and retention and promote clear water status (Cerco and Seitzinger 1997; Sundbäck et al. 2000). Heterotrophic sediments may instead regenerate large nutrient amounts to the water column and stimulate or maintain elevated rates of pelagic primary production (Risgaard-Petersen 2003; Bartoli et al. 2009; Pinardi et al. 2009).

Benthic fauna may exert a top-down control of pelagic primary production but it may simultaneously excrete large amounts of nutrients and increase the organic matter content of sediment via biodeposition, stimulating microbial activity and mineralization at the interface (Caraco et al. 1997; Stief 2013; Ruginis et al. 2014; Benelli et al. 2017). Bivalves may remove phytoplankton (Newell et al. 2007; Higgins and Vander Zanden 2010) but they also may stimulate its growth in transparent water enriched with excreted nutrients, resulting in an unclear net effect on pelagic production (Stadmark and Conley 2011). As filter-feeding burrowers, some chironomid larvae may, on the contrary, displace particles from the water column to their burrows and, simultaneously, favor N loss and P retention via burrows ventilation and bioirrigation (Svensson and Leonardson 1996; Lewandowski and Hupfer 2005; Shang et al. 2013; Hölker et al. 2015). High densities of filter-feeding sediment dwelling invertebrates may therefore exert a top-down and bottom-up control of phytoplankton growth and promote a clear-water status (Hölker et al. 2015).

Such control may be further increased by the mutualistic interactions between microphytobenthos and tube-dwelling invertebrates, resulting in even larger nutrient uptake and retention (Herren et al. 2017). In addition, bioturbating fauna can affect the physical and chemical processes within sediments. Bioturbation includes particles reworking, ventilation of

burrows and bioirrigation by animals living on or in the substratum (Kristensen et al. 2012). Such kind of activity may alter sediment-water fluxes and change vertical and horizontal gradients of pore water chemical compounds (Shull et al. 2009). Burrow ventilation, that is the pumping of oxygen (O_2), nitrate (NO_3^-) and particles-rich waters within the sediment for respiration and feeding purposes (Kristensen et al. 2012; Hölker et al. 2015), has many consequences on the biogeochemistry of sediments including the increases of the oxidized sediment volume (Kristensen 1984). The structure and the metabolic activity of the microbial communities that are living along the burrow walls could also change and biogeochemical microniches may form (Stief and de Beer 2002; Bertics and Ziebis 2009; Laverock et al. 2010). Whereas the role of benthic algae and macrofauna on sediment fluxes was analyzed separately in multiple studies, little is known about their combined effects (Miller et al. 1996; Tang and Kristensen 2007; Lohrer et al. 2010; Herren et al. 2017). As both microphytobenthos and tube-dwelling organisms may exert a strong control of regenerated nutrients, their synergistic effect may be quantitatively important in shallow eutrophic ecosystems, limiting internal recycling.
In this study, we incubated sediments with and without chironomid larvae in the dark and in the light to evaluate the effects on benthic metabolism of macrofauna and microphytobenthos, when they are co-occurring. We performed this study using sediments collected from a shallow eutrophic lagoon, where chironomid larvae represent the dominant macrofauna (Zettler and Daunys 2007). Chironomids improve P retention, stimulate denitrification and remove phytoplankton via filtration (Svensson 1997; Lewandowski et al. 2007; Hölker et al. 2015). We hypothesized that the mutualism between chironomid larvae and microphytobenthos (sensu Herren et al. 2017) may stimulate nutrient retention within sediments, minimize recycling to the pelagic environment and may favor water clarity.

Material and methods

Sampling procedure and microcosms establishment

In March 2016, chironomid larvae (*Chironomus plumosus*), sediment and water were collected from a muddy site (55°17.2388' N, 21°01.2898' E, depth 2.5 m) within a confined area in the Lithuanian part of the Curonian Lagoon. The sediment sampled for the experiment had a density of 1.28 g cm^{-3}, a porosity of 0.94 and a water content of 93.6 %; the C/N molar ratio of the upper 0-10 cm horizon was 7.6. Muddy sediments from the sampling site host large numbers of chironomid larvae (Markiyanova 2016*)*, with reported densities averaging 1,700 ± 1,300 ind m^{-2} (Zettler and Daunys 2007). In the laboratory, nearly 15 L of the collected sediment was sieved (0.5 mm mesh size) in order to remove large debris, chironomid larvae and other

occasional macrofauna, and gently mixed to a slurry. Then, the sediment homogenate was transferred into 12 bottom capped Plexiglass liners (height=30 cm, inner diameter=8 cm), to reconstruct a 15 cm thick sediment column with an overlying 14 cm thick water column. Thereafter in situ water was gently added, after positioning on the sediment surface a floating polystyrene disc (height=1 cm, inner diameter=7.5 cm) to avoid sediment resuspension. At the end of this procedure, 12 identical reconstructed sediment cores were obtained. Three treatments, each with 4 replicates, were realized: control sediment without macrofauna (C), sediment with low (L) and sediment with high (H) density of chironomid larvae. Treatments with low and high densities were prepared by adding 3 and 9 larvae per core, respectively, corresponding to 600 and 1,800 ind m^{-2} and within in situ densities (Zilius et al. 2014; Markiyanova 2016). All added chironomids burrowed deep inside sediments within 15 min, as suggested by ramified light halos along the vertical sediment profile (Fig. 3.1.1).

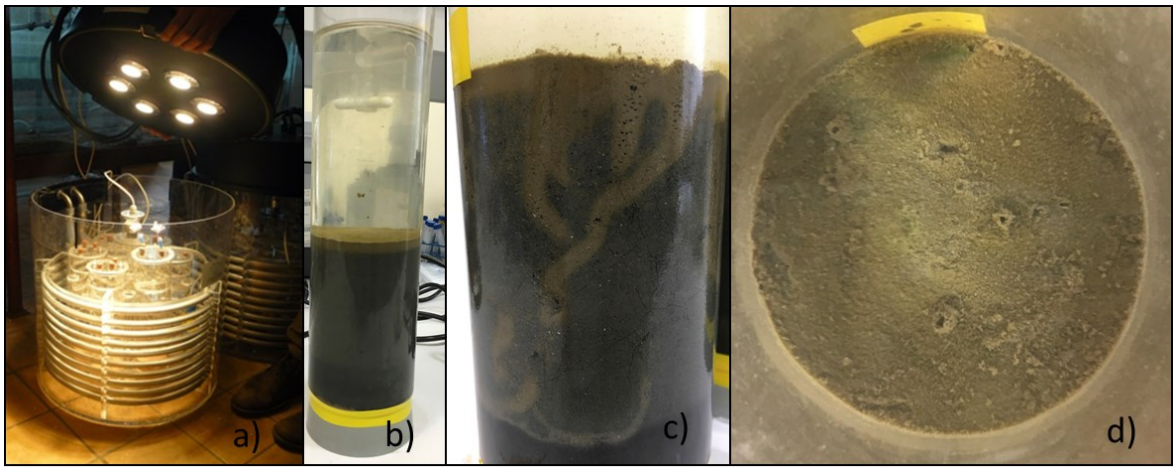


Fig. 3.1.1 The images display the incubation tanks we used and in particular the water temperature control and the core illumination systems (a), a control core (b), a core with chironomid larvae (c) and a patchy mat of benthic algae developed at the sediment-water interface during the preincubation period (d).

In each core, a magnetic bar was fixed 10 cm above the sediment interface to stir water avoiding sediment resuspension. Then, all the cores were submerged with the top open in a temperature-controlled (14 ± 0.2 °C) tank (100 L), containing aerated and well-stirred lagoon water. The tank was provided with a central magnet rotating at 40 rpm and driving all magnets inside the cores in order to ensure water exchange with the tank and to supply phytoplankton to chironomid larvae. The tank cover was provided with 6 halogen lamps (Osram Decostar, 35W), positioned above each core and producing an irradiance of 96 ± 12 µE $m^{-2}s^{-1}$ measured at the sediment-water interface with an underwater quantum sensor (LI-COR 192s) and over a 16 hours light and 8 hours dark period (Fig. 3.1.1). The cores were preincubated for a period of three weeks, in order to have a) stable vertical and horizontal chemical gradients after sediment

sieving and homogenization and chironomid larvae addition, b) established bacterial communities and microphytobenthos along burrows and on the sediment surface, respectively. During the preincubation period all microcosms were regularly checked for the development of light brown halos along chironomid larvae burrows and of benthic algal mats on the sediment surface, which occurred within 10 days (Fig. 3.1.1). A methodological work reports that bacterial communities recover after the stress of sediment sieving within 25 days and that defaunation with this technique is less impacting than sediment freezing or anoxia induction (Stocum and Plante 2006). Nearly 30 % of the water volume in the tank was renewed every 2 days in order to maintain in situ conditions in term of suspended matter, nutrient concentrations and chemical gradients across the sediment-water interface.

Benthic flux measurements

After the preincubation period, light and dark fluxes of dissolved gas and nutrients were measured via short-term batch incubations, as detailed in Ruginis et al. (2014). Incubations lasted 5 hours, in order to keep initial O_2 concentration (330 µM) within 20 % of the initial value. Lowest O_2 concentration at the end of the incubation was 258 µM, measured in a microcosm with high chironomid density. At the beginning and at the end of the incubation, dissolved O_2 concentration was measured with a microelectrode (Unisense A/S, DK) and an initial and a final water sample (100 ml) were collected with plastic syringes from each core from a one-way valve located in transparent, gas-tight top lids fixed in each liner at the beginning of the incubation. The collected volume was replaced with an equivalent amount of tank water entering the core from another valve. Each water sample underwent the same processing: an aliquot of 40 ml was transferred to 12-ml Exetainers (Labco, UK) for total inorganic carbon (TCO_2), dissolved O_2 and N_2 analyses. The last two were added with 100 µl of 7 M $ZnCl_2$ to stop microbial activity whereas TCO_2 was immediately titrated (see analytical methods below). An aliquot of 20 ml was filtered (GF/F glass-fiber filters) and transferred into 20-ml plastic vials in order to analyze dissolved inorganic N compounds: ammonium (NH_4^+), nitrite (NO_2^-) and nitrate (NO_3^-) and dissolved inorganic silica (SiO_2). DIN was calculated as the sum of ammonium, nitrite and nitrate (DIN = NH_4^++NO_2^-+NO_3^-). Another aliquot was filtered and transferred into 5-ml glass vials to measure soluble reactive phosphorus (PO_4^{3-}). Details on the analytical techniques are reported below. Dissolved gas and nutrient fluxes were calculated according to the equation below:

$$Flux_x = \frac{([x]_f - [x]_i) \times V}{A \times t}$$

where $[x]_f$ and $[x]_i$ are the concentrations (µM or mM) of the solute x at the end and at the start of the incubation, respectively, V (L) is the volume of the core water phase, A (m^2) is the area of the sediment and t (h) is the incubation time.

Daily fluxes (µmol $m^{-2}d^{-1}$ or mmol $m^{-2}d^{-1}$) were calculated according to the following equation:

$$Daily\ flux_x = (hourly\ dark\ flux \times h_D) + (hourly\ light\ flux \times h_L)$$

where h_D and h_L are the numbers of dark and light hours during incubation, respectively.

Net and gross O_2 fluxes were converted into theoretical net and gross nutrient uptake by benthic algae. Rates were multiplied by a photosynthetic quotient of 1.2 to convert O_2 production into C uptake (Sundbäck et al. 2004). We used oxygen data instead of measured TCO_2 fluxes as microbial processes (i.e. nitrification) may results in overestimation of C fixation rates by benthic algae. Calculated net and gross C fixation were divided by the Redfield ratio 106:16:15:1 and converted into inorganic N, Si and P uptake (Redfield 1958; Sigmon and Cahoon 1997).

Pore water extraction and diffusive fluxes calculation

At the end of light/dark incubations, the cores (n = 12) were sliced in 5 layers at 0–1, 1–2, 2–3, 3–5 and 5–10 cm intervals in order to analyze the vertical distribution of dissolved inorganic nutrients (NH_4^+, PO_4^{3-} and SiO_2) and metals (Fe^{2+} and Mn^{2+}). Briefly, the sediment was extruded and sliced in a N_2 filled glow bag. Pore water were obtained by gently squeezing with N_2 (1.5–3 bar) of discrete sediment slices through GF/F filters with a squeezer bench (KC-Denmark, DK). An aliquot for metal analysis was immediately transferred into 6-ml Exetainers, containing 100 µl of concentrated ultrapure HCl. Another aliquot (5 ml) was frozen for later NH_4^+, PO_4^{3-} and SiO_2 analyses.

Diffusive fluxes of all solutes across sediment layers were calculated from nutrient profiles in the sediment by applying the Fick's First Law (Berner 1980):

$$J = \frac{\delta C}{\delta z} \cdot Ds$$

where J is the diffusive flux of the solute (µmol $m^{-2}h^{-1}$), $\frac{\delta C}{\delta z}$ is the concentration gradient of the solute between adjacent sediment layers (µmol m^{-4}) and Ds is the diffusion coefficient of the solute in the sediment (m^2 h^{-1}), which was calculated from the following equation (Lerman 1979):

$$Ds = D_w^0 \cdot \theta^2$$

where D_w^0 is the diffusion coefficient ($m^2\ h^{-1}$) of the solute in the water (Wollast and Garrels 1971; Li and Gregory 1974) and θ^2 is the sediment tortuosity, calculated according to Boudreau (1997):

$$\theta^2 = 1 - 2ln\phi$$

where ϕ is the sediment porosity. Diffusion coefficients of nutrients and metals were corrected for in situ temperature (14 °C) according to the Stokes-Einstein relation (Li and Gregory 1974). Excess sediment from all slices was pooled and homogenized and a subsample of 5 ml was dried at 70 °C for 48 h in order to measure porosity.

Analytical methods

Dissolved O_2 was measured by means of polarography with a microelectrode (90 % response time in < 5 s, 50 µm tip; Unisense, Denmark). Dissolved N_2 was analyzed by membrane inlet mass spectrometer (MIMS, Bay instruments, USA). Dissolved N_2 concentrations were calculated from obtained N_2:Ar ratio and theoretical Ar concentration derived from Weiss (1970). TCO_2 was measured via 6 end points 0.1 N HCl microtitration (Anderson et al. 1986). Dissolved nutrients (NO_x^-=NO_3^-+NO_2^-, NO_2^-, PO_4^{3-} and SiO_2) from incubations and pore water samples were measured with a continuous flow analyzer (San^{++}, Skalar, sensitivity 0.3 µM) using standard colorimetric methods (Grasshoff et al. 1983). NO_3^- was calculated as the difference between NO_x^- and NO_2^-. NH_4^+ was analyzed spectrophotometrically using salicylate and hypochlorite with nitroprussiate as catalyst (Bower and Holm-Hansen 1980).

Statistical analysis

Two-way analysis of variance (ANOVA) was used to test the effects of the factors illumination (dark/light measurements) and chironomid larvae density (including their interactive effects) on benthic fluxes of dissolved gas and nutrients. The same analysis was used to test the effects of chironomid larvae density and sediment depth, including their interactions, on pore water solute concentrations. Homogeneity of variance was checked using the Levene Median test and data were transformed if significant heteroscedasticity was found. Only ammonium fluxes were log-transformed. For significant factors, a pairwise multiple comparison of means was carried out with the post-hoc Holm-Sidak test. Statistical significance was set at p level lower than 0.05. All the analyses were performed using Sigma Plot 11.0.

Results

Benthic metabolism

Both factors illumination and chironomid larvae density produced a significant effect on the fluxes of dissolved O_2, TCO_2 and N_2 (Fig. 3.1.2 and Table 3.1.1). In the light, net O_2 production and TCO_2 consumption along the three treatments C, L and H were measured, suggesting the development of an active algal mat and the occurrence of benthic photosynthesis. During the light incubation, sediments displayed negative N_2 fluxes (i.e. N fixation > denitrification) that became less negative in the H treatment, with the highest density of chironomid larvae. In the dark, increasing density of chironomid larvae stimulated aerobic (O_2, from -1.03 ± 0.07 to -1.96 ± 0.15 mmol $m^{-2}h^{-1}$), total (TCO_2, from 0.86 ± 0.56 to 2.33 ± 0.19 mmol $m^{-2}h^{-1}$) and anaerobic (NO_3^- reduction to N_2, from 0.40 ± 0.02 to 1.01 ± 0.18 mmol $m^{-2}h^{-1}$) sediment respirations, which nearly doubled in H as compared to C (Fig. 3.1.2). The respiratory quotient of sediments (data not shown) was close to unity and not significantly different among treatments. On a daily basis, the C and L treatments were net O_2 producing (i.e. net autotrophic) whereas H was net O_2 consuming (i.e. net heterotrophic). Daily N_2 fluxes were negative in the net autotrophic conditions C and L and positive in the heterotrophic treatment H. Daily TCO_2 fluxes were negative in all treatments.

Table 3.1.1 Results of two-way ANOVA testing the effects of the factors incubation condition (illumination) and treatment (control, low and high chironomid density) on gas (O_2, TCO_2 and N_2) and nutrient (NH_4^+, NO_3^-, NO_2^-, DIN, PO_4^{3-} and SiO_2) fluxes. Df, degree of freedom; SS, sum of squares; MS, mean of squares; F, F-statistics; P, P-value.

	Source of variation	Df	SS	MS	F	P
O_2 flux	Illumination	1	53.9	53.9	373.1	**<0.001**
	Treatment	2	8.0	4.0	27.7	**<0.001**
	Interaction	2	1.0	0.5	3.3	0.059
	Error	18	2.6	0.1		
	Total	23	65.5	2.8		
TCO_2 flux	Illumination	1	107.8	107.8	87.7	**<0.001**
	Treatment	2	11.5	5.8	4.7	**0.023**
	Interaction	2	4.1	2.1	1.7	0.215
	Error	18	22.1	1.2		
	Total	23	145.6	6.3		

	Source of variation	Df	SS	MS	F	P
	Illumination	1	6.7	6.7	234.3	**<0.001**
	Treatment	2	1.2	0.6	21.7	**<0.001**
N_2 flux	Interaction	2	0.1	0.0	1.0	0.378
	Error	18	0.5	0.0		
	Total	23	8.5	0.4		
	Illumination	1	0.3	0.3	0.7	0.422
	Treatment	2	2.1	1.1	2.4	0.118
NH_4^+ flux	Interaction	2	1.9	0.9	2.1	0.148
	Error	18	8.0	0.4		
	Total	23	12.3	0.5		
	Illumination	1	1.3	1.3	2.3	0.143
	Treatment	2	0.0	0.0	0.0	0.965
NO_3^- flux	Interaction	2	0.3	0.2	0.3	0.745
	Error	18	10.0	0.6		
	Total	23	11.6	0.5		
	Illumination	1	83.1	83.1	4.5	**0.049**
	Treatment	2	5.0	2.5	0.1	0.875
NO_2^- flux	Interaction	2	24.4	12.2	0.7	0.531
	Error	18	334.5	18.6		
	Total	23	447.0	19.4		
	Illumination	1	1.3	1.3	2.4	0.139
	Treatment	2	0.0	0.0	0.0	0.969
DIN flux	Interaction	2	0.3	0.2	0.3	0.742
	Error	18	10.1	0.6		
	Total	23	11.8	0.5		
	Illumination	1	7.4	7.4	22.3	**<0.001**
	Treatment	2	0.2	0.1	0.3	0.747
PO_4^{3-} flux	Interaction	2	0.1	0.0	0.1	0.910
	Error	18	5.9	0.3		
	Total	23	13.6	0.6		
	Illumination	1	340.2	340.2	3.5	0.078
	Treatment	2	2,052.3	1,026.1	10.5	**<0.001**
SiO_2 flux	Interaction	2	385.4	192.7	2.0	0.167
	Error	18	1,751.6	97.3		
	Total	23	4,529.4	196.9		

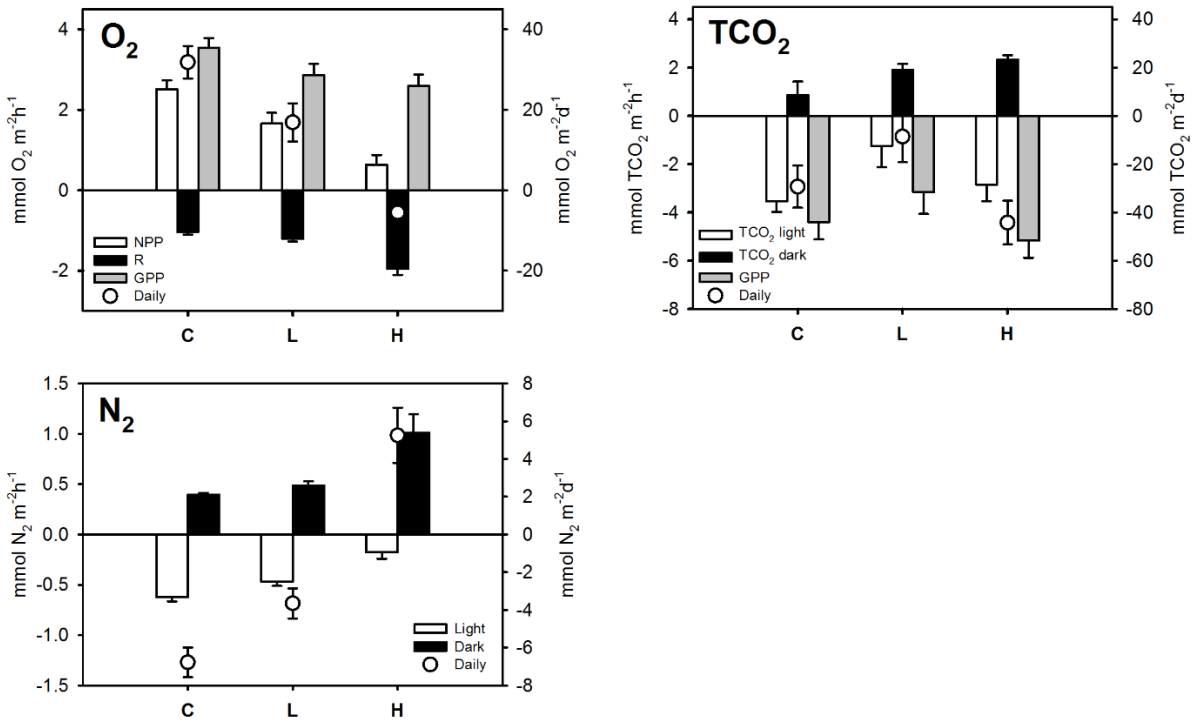


Fig. 3.1.2 Benthic fluxes of dissolved O_2, TCO_2 and N_2 measured in light (white bars) and dark (black bars) incubations of C, L and H treatments (n=4). Grey bars in O_2 and TCO_2 represent gross primary production. Averages ± standard errors are reported. All fluxes are expressed in mmol $m^{-2}h^{-1}$. Dots represent daily averages ± standard errors expressed in mmol $m^{-2}d^{-1}$.

Benthic nutrient fluxes

All forms of inorganic N were mostly net consumed by the sediment, regardless of the density of chironomid larvae (Fig. 3.1.3 and Table 3.1.1). Fluxes tended to be more negative in the light than in the dark, but differences were significant only for NO_2^- (Table 3.1.1). Fluxes of NO_3^- were nearly two orders of magnitude higher than those of NH_4^+ and NO_2^-, reflecting different concentrations of the three ions in the water column (0.9, 0.8 and 109 µM for NH_4^+, NO_2^- and NO_3^-, respectively). On a daily basis, DIN fluxes were all negative and mostly driven by NO_3^-, with values averaging -22.3 ± 3.0 mmol $m^{-2}d^{-1}$ (Fig. 3.1.3).

Fluxes of PO_4^{3-} were low and within ±1 µmol $m^{-2}h^{-1}$ (Fig. 3.1.3). Differences between light and dark incubations were significant, with small regeneration in the dark and uptake in the light; differences among the three treatments C, L and H were not significant (Table 3.1.1). As for DIN, also dissolved inorganic P daily fluxes were all negative and not statistically different among treatments, with values averaging -10.6 ± 3.5 µmol $m^{-2}d^{-1}$.

Reactive silica was the only macronutrient with chironomid larvae-dependent fluxes (Fig. 3.1.3 and Table 3.1.1). Increasing density of chironomids significantly stimulated the regeneration of SiO_2 in both light and dark conditions. SiO_2 fluxes tended to be more negative or less positive

in the light as compared to dark incubations, with differences close to significant level ($p=0.078$). SiO_2 daily fluxes were negative only in the C treatment (-98 ± 19 µmol $m^{-2}d^{-1}$) whereas they were increasingly positive in L (190 ± 108 µmol $m^{-2}d^{-1}$) and in H (378 ± 104 µmol $m^{-2}d^{-1}$).

Net and gross theoretical inorganic N, P and Si uptake, calculated from rates of O_2 production, were similar to NO_3^- uptake or N fixation rates but they were much higher than NH_4^+, NO_2^-, SiO_2 and PO_4^{3-} nutrient fluxes measured in the light and in the dark or calculated from pore water chemical gradients in the upper sediment layer (Table 3.1.2 and Figs. 3.1.3 and 3.1.4).

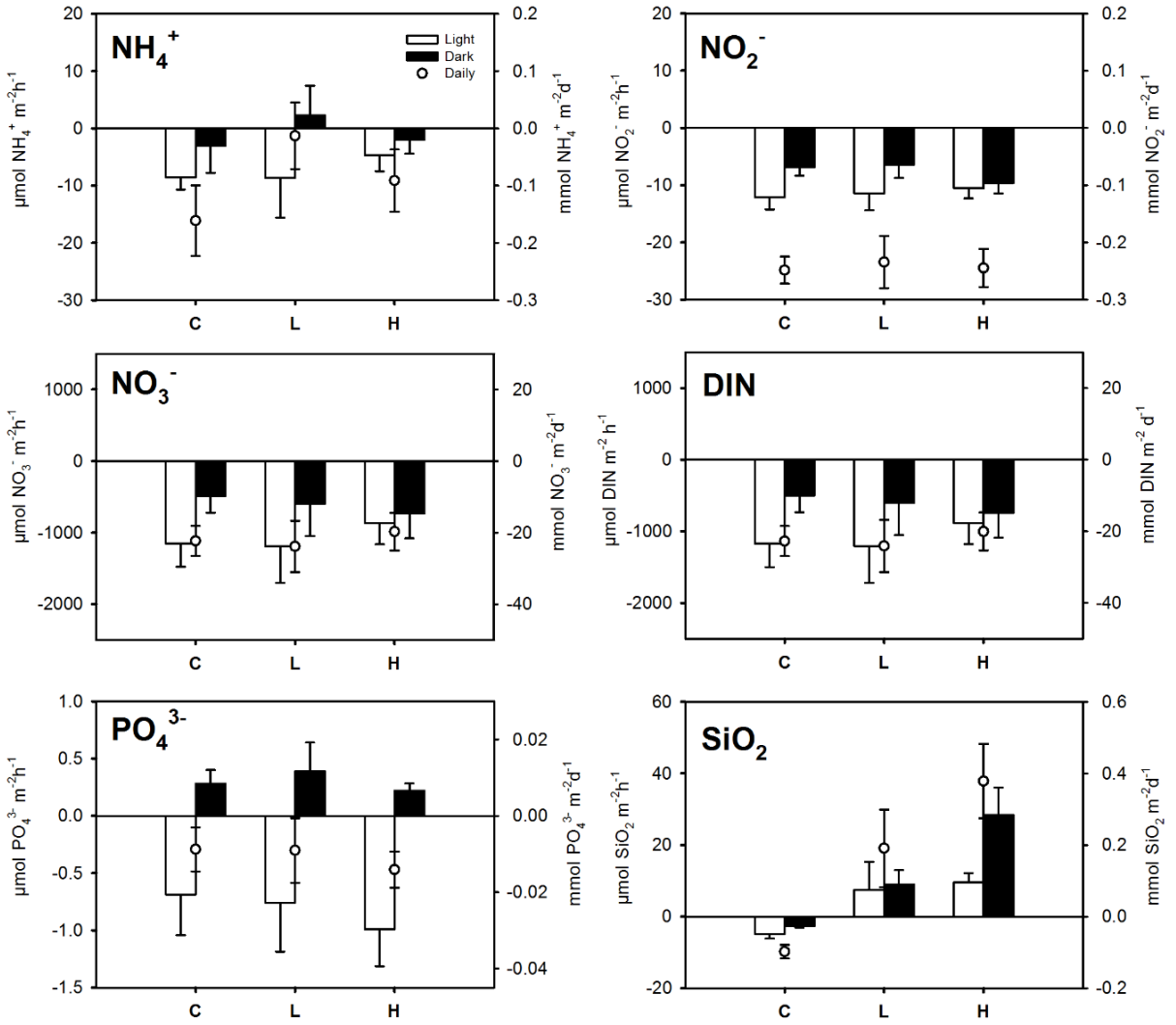


Fig. 3.1.3 Light and dark benthic fluxes of NH_4^+, NO_2^-, NO_3^-, DIN, PO_4^{3-} and SiO_2 measured in C, L and H treatments (n=4). Averages ± standard errors are reported. All fluxes are expressed in µmol $m^{-2}h^{-1}$. Dots represent daily averages ± standard errors of nutrient fluxes expressed in µmol or mmol $m^{-2}d^{-1}$.

Table 3.1.2 Theoretical net and gross nutrient uptake by benthic microalgae calculated from O_2 fluxes in light incubations of reconstructed sediment cores with no (C), low (L) and high (H) density of chironomid larvae.

Treatment	N-uptake		P-uptake		Si-uptake	
	Net	Gross	Net	Gross	Net	Gross
	(μmol N $m^{-2}h^{-1}$)		(μmol P $m^{-2}h^{-1}$)		(μmol Si $m^{-2}h^{-1}$)	
C	492 ± 45	695 ± 47	31 ± 4	43 ± 3	522 ± 48	738 ± 50
L	325 ± 53	561 ± 55	20 ± 3	35 ± 4	345 ± 57	596 ± 59
H	124 ± 48	508 ± 56	8 ± 2	32 ± 3	132 ± 51	540 ± 59

Pore water vertical profiles and diffusive fluxes

Pore water nutrient profiles and diffusive fluxes calculated across the interface and across adjacent sediment layers are plotted in figure 3.1.4. The two-way ANOVA suggests that differences among treatments C, L and H were highly significant (Table 3.1.3) but that they depended upon the sediment layer (treatment x depth significant interaction). Pooling depths, NH_4^+ concentrations were not statistically different in L and H treatments (Table 3.1.3), whereas they were higher in C (Holm-Sidak pairwise comparison, $p<0.001$). Regarding PO_4^{3-} and SiO_2, all treatments were different with concentrations in C > L > H (Holm-Sidak pairwise comparison, $p<0.001$). The comparison of layers suggested important nutrient-specific differences among treatments. In the control treatment, all the nutrients displayed increasing concentrations with depth, with values significantly higher from the top to the bottom layers. Values approached 206 ± 11, 17 ± 3 and 349 ± 32 µM at 7.5 cm depth for NH_4^+, PO_4^{3-} and SiO_2, respectively (Fig. 3.1.4). Such increase with depth was not occurring for the three nutrients in L and H treatments, where concentrations were not statistically different along the vertical profile (NH_4^+, PO_4^{3-}) or displayed an increase only in the upper layers (SiO_2)(Fig. 3.1.4).

Calculated diffusive fluxes across the sediment-water interface in C treatment were 8 ± 3, 0.4 ± 0.1 and 31 ± 3 µmol NH_4^+, PO_4^{3-} and SiO_2 $m^{-2}h^{-1}$, respectively, whereas they were much lower in L and H treatments (Fig. 3.1.4). In C, diffusive fluxes of the three nutrients across adjacent sediment layers peaked at 2-2.5 cm depth whereas they were strongly reduced, in particular for NH_4^+ and PO_4^{3-}, in L and H treatments.

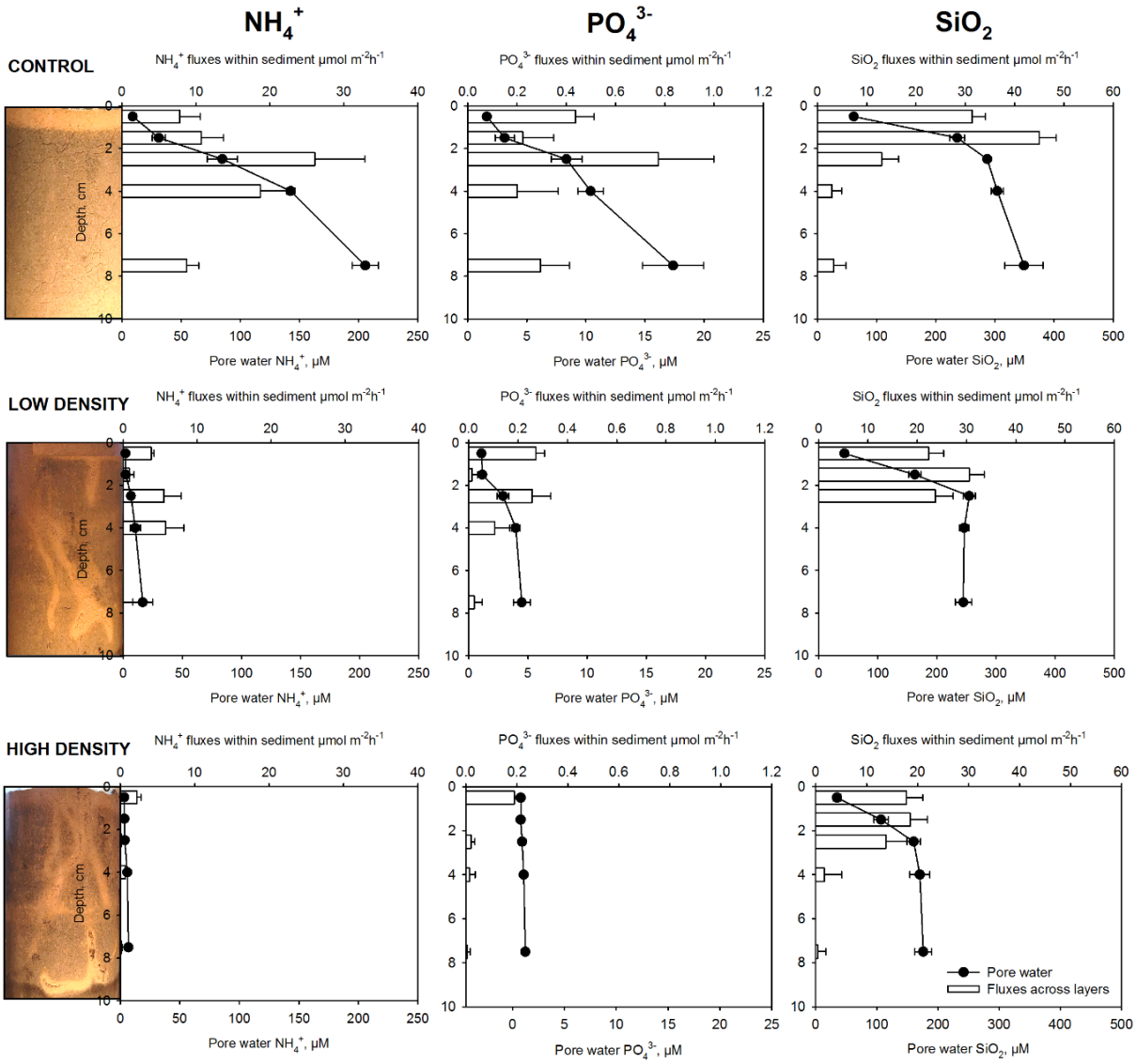


Fig. 3.1.4 Vertical profiles of pore water and diffusive fluxes of dissolved inorganic nutrients (NH_4^+, PO_4^{3-} and SiO_2) in C, L and H treatments. Dots represent nutrient concentrations (µM) in the different layers (averages ± standard errors) and the horizontal bars represent diffusive fluxes (µmol $m^{-2}h^{-1}$) across sediment layers (averages ± standard errors).

Vertical pore water profiles of Fe^{2+} and Mn^{2+} in the three treatments are shown in Fig. 5. There were significant differences among treatments along sediment horizons (two-way ANOVA, treatment x depth interaction, Table 3.1.3). Pooling all depths, the three treatments resulted statistically different with concentrations in C > L > H (Holm-Sidak pairwise comparison, $p<0.005$). The highest diffusive fluxes of Fe^{2+} and Mn^{2+} in control sediments were calculated at 2.5 and 2 cm depth, respectively (Fig. 3.1.5). In H treatment, there was virtually no accumulation of Fe^{2+} in pore water and diffusive fluxes were set to zero whereas a peak accumulation of Mn^{2+} was measured at 2.5 cm depth, coinciding with the layer of maximum diffusion upwards.

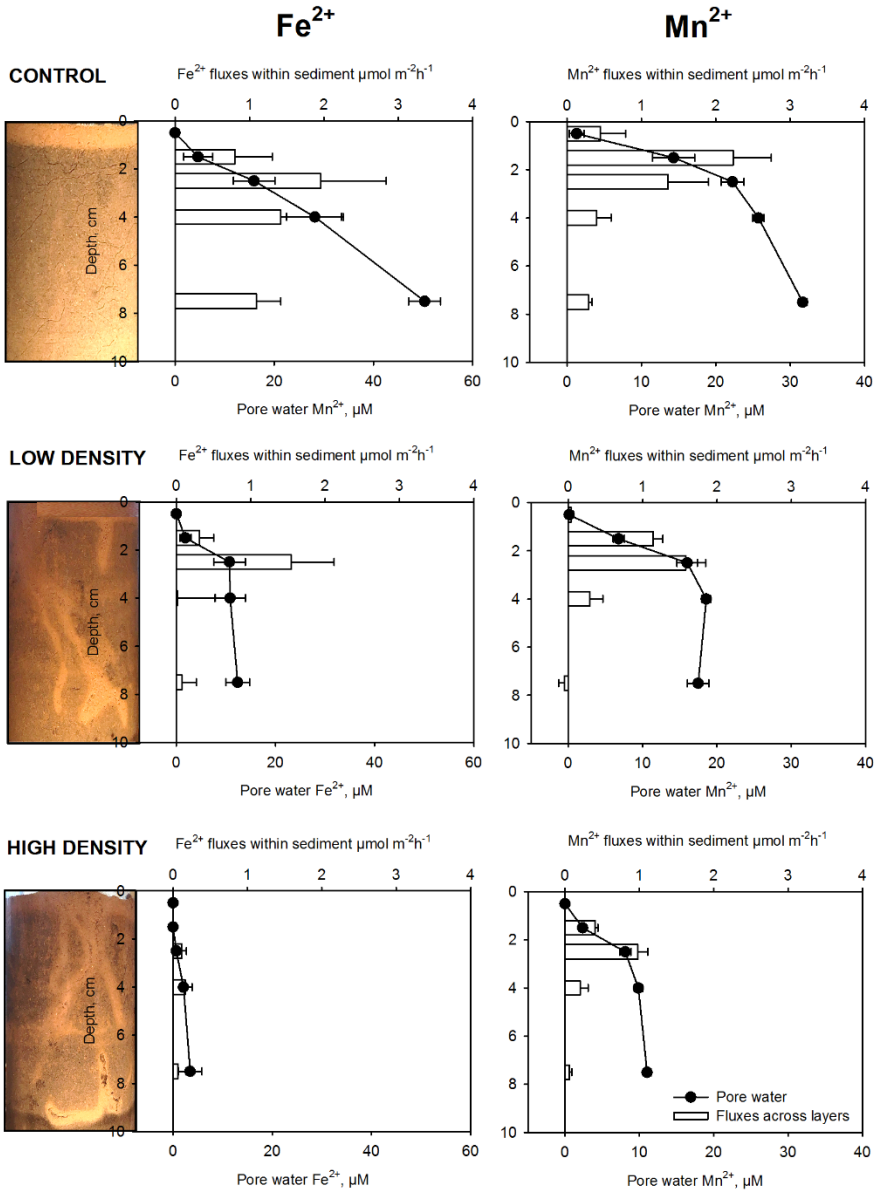


Fig. 3.1.5 Vertical profiles of pore water dissolved reduced metals (Fe^{2+} and Mn^{2+}) and their diffusive fluxes in C, L and H treatments. Dots represent metal concentrations (µM) in the different layers (averages ± standard errors) and the horizontal bars represent the diffusive fluxes (µmol $m^{-2}h^{-1}$) across sediment layers (averages ± standard errors).

Table 3.1.3 Results of two-way ANOVA testing the effects of the two factors treatment (control, low and high chironomid density) and depth on dissolved nutrient (NH_4^+, PO_4^{3-} and SiO_2) and metal (Fe^{2+} and Mn^{2+}) pore water concentrations.

	Source of variation	Df	SS	MS	F	P
NH_4^+	Treatment	2	104,737.1	52,368.5	451.2	**<0.001**
	Depth	4	41,635.9	10,409.0	89.7	**<0.001**
	Interaction	8	63,352.4	7,919.0	68.2	**<0.001**
	Residual	45	5,222.9	116.1		
	Total	59	214,948.3	3,643.2		
PO_4^{3-}	Treatment	2	566.4	283.2	95.0	**<0.001**
	Depth	4	340.1	85.0	28.5	**<0.001**
	Interaction	8	331.6	41.4	13.9	**<0.001**
	Residual	45	134.1	3.0		
	Total	59	1,372.2	23.3		
SiO_2	Treatment	2	139,176.7	69,588.4	100.2	**<0.001**
	Depth	4	358,587.9	89,647.0	129.0	**<0.001**
	Interaction	8	27,659.4	3,457.4	5.0	**<0.001**
	Residual	45	31,264.7	694.8		
	Total	59	556,688.8	9,435.4		
Fe^{2+}	Treatment	2	3,607.6	1,803.8	68.5	**<0.001**
	Depth	4	3,836.5	959.1	36.4	**<0.001**
	Interaction	8	3,294.6	411.8	15.6	**<0.001**
	Residual	45	1,185.2	26.3		
	Total	59	11,923.9	202.1		
Mn^{2+}	Treatment	2	1,648.0	824.0	170.7	**<0.001**
	Depth	4	3,158.2	789.6	163.5	**<0.001**
	Interaction	8	452.8	56.6	11.7	**<0.001**
	Residual	45	217.3	4.8		
	Total	59	5,476.3	92.8		

Df, degree of freedom; SS, sum of squares; MS, mean of squares; F, F-statistics; P, P-value.

Discussion

Macrofauna and microphytobenthos activities promote benthic N and P retention and loss

Results from this work suggest how the combined activity of benthic microalgae and chironomid larvae contributes not only to the net retention of inorganic N and P within the sediment, but also to their net uptake from the water column. Inorganic Si was net retained and assimilated by the benthic system only in control sediments without macrofauna, presumably due to uptake by a mat of benthic diatoms (Sigmon and Cahoon 1997), whereas a chironomid larvae density-dependent net regeneration was found. Macrofauna, including chironomid larvae, may excrete large amounts of inorganic N, P and Si or favor their mineralization, which may fuel large sediment efflux (Gallepp 1979; Devine and Vanni 2002). However, in the analyzed shallow sediments, processes retaining nutrients exceeded rates of N and P mineralization and excretion. These results are different from what reported on the net effect of macrofauna, in particular for benthic N cycling, where recycling generally exceeds denitrification (Stief 2013). They therefore suggest that changes induced by bioturbation on benthic biogeochemistry are species, element- and likely sediment-specific (Michaud et al. 2006).

Our results may be important for shallow eutrophic aquatic environments, where the combined activity of benthic algae and chironomid larvae may significantly attenuate the internal recycling, which is the flux of nutrients that sustain pelagic primary production. The dependency of phytoplankton on sediment regeneration may be quantitatively relevant in dry periods, when external loads decrease and the removal of nutrient at the sediment-water interface may promote water clarity. Such bottom-up control adds to the demonstrated active filtration of phytoplankton that chironomid larvae perform via burrow ventilation, by pumping large water volumes within sediments (Hölker et al. 2015).

Results of this study show also that pore water concentrations of inorganic N and P, and to a minor extent Si, decrease in bioturbated as compared to non-bioturbated sediments, likely due to coupled nitrification-denitrification (N) and co-precipitation (P). The latter is supported by significantly different accumulation of Fe^{2+} and Mn^{2+} in pore water of both low and high chironomid larvae density treatments, due to macrofauna-mediated sediment oxidation (Lewandowski et al. 2007). Results from flux measurements exclude a chironomid larvae-dependent efflux of NH_4^+ and PO_4^{3-} resulting from bioirrigation, that was evident only for SiO_2 efflux, significantly stimulated by chironomid larvae. Our results align with those of Lewandowski et al. (2007), suggesting that the burrowing, ventilation and bioirrigation

activities of chironomids change the chemistry of pore water, setting to zero the vertical gradients and the regeneration of N and P to the water phase.

The present study confirms also the importance of dark and light measurements when studying bioturbation in shallow sediments, as daily budgets allow to produce a more realistic picture of benthic functioning, which integrates the dark, heterotrophic period with the light, autotrophic one. We acknowledge that results from our study were obtained from manipulated and reconstructed sediments, but sediment sieving was demonstrated to produce a minor impact on bacterial communities and their recovery as compared to other defaunation techniques as the induction of anoxia and freezing (Stocum and Plante 2006). Furthermore, we preincubated our microcosms for 3 weeks before starting the measurements, which is close to the period of recovery of the bacterial community (25 days) reported in the Stocum and Plante (2006) experiment. Incubations performed shortly after the addition of burrowing macrofauna to reconstructed sediment may result in large efflux of pore water solutes, due to active burrowing, with overestimation of the role of macrofauna in natural sediments (Bartoli et al. 2000).

The light period and the role of benthic algae

Fluxes of dissolved oxygen and nutrients (Si in particular) measured in the light strongly support the development of benthic algae, including diatoms, on the sediment surface during the preincubation period. The occurrence of a homogeneous and a patchy light brown mat on control and bioturbated cores sediment surface, respectively, was also noticed by visual inspection.

Benthic primary production measured in C, L and H treatments (500-3,000 µmol C $m^{-2}h^{-1}$) falls within the range reported for shallow sediments (Nedwell and Raffaelli 1999). Such photosynthetic activity had a clear effect on nutrient dynamics, resulting in net daily uptake of inorganic N and P in all treatments, largely exceeding rates of mineralization and excretion. The presence of macrofauna reversed the sink role of sediments only for inorganic Si, which was net regenerated in L and H treatments. Our results align with previous investigations on the role of benthic algae as nutrient traps (Bartoli et al. 2003; Sundbäck et al. 2004 and 2006).

Estimated N requirements by benthic algae (Table 3.1.2) were much higher than the measured fluxes of NH_4^+ and NO_2^- in both light and dark incubations, whereas they were comparable with N_2 and NO_3^- fluxes measured in the light. This suggests that neither dark regeneration nor light uptake of NH_4^+ or NO_2^- satisfied microphytobenthos N requirements. Negative N_2 fluxes were always measured in the light in the three treatments; however, rates decreased from the most autotrophic condition C to the most heterotrophic condition H, in agreement with decreasing net O_2 production and calculated N demand. Such results conform to those reported by

Sundbäck et al. (2006), demonstrating that in autotrophic sediments assimilation prevails over denitrification. Comparable rates of N fixation are reported for estuarine sediments by Fulweiler et al. (2007), but in heterotrophic sediments incubated in the dark, and in oligotrophic lagoons in the light (Charpy et al. 2007). Also Scott et al. (2008), investigating microbial N cycling along a gradient of NO_3^- availability, measured negative N_2 fluxes and N fixation rates comparable to those reported in the present study. They concluded that cyanobacteria may be responsible for such rates.

Nitrate fluxes were always negative, with higher uptake during light as compared to dark incubations, suggesting NO_3^- as possible N source to microphytobenthos. In general, pelagic and benthic algae display a preference for NH_4^+ assimilation (Boyer et al. 1994; Nedwell and Raffaelli 1999). However, algae may switch to NO_3^- uptake when NH_4^+ is limiting or when the NO_3^- to NH_4^+ ratio is elevated, as in our experiment (50:1; McCarthy et al. 1977). Unfortunately, with our data we cannot quantify the fraction of NO_3^- consumption that goes to microphytobenthos, to denitrifiers or that may sustain bacterial growth. We also do not know the rates of N fixation and denitrification in the light, as our method measures the net N_2 flux, which is the sum of the two opposite processes (Kana et al. 1994). It remains therefore unknown the fraction of NO_3^- uptake and N fixation that is shared by bacteria and benthic algae. Such information would clarify why the sum of NO_3^- and N_2 fluxes in the light always exceeds calculated gross N uptake by microphytobenthos. In the Neuse River estuary, similar results were explained in terms of heterotrophic bacterial DIN uptake (Boyer et al. 1994).

Net and gross calculated Si uptake rates were much higher than fluxes measured at the sediment-water interface, suggesting that pore water represented the major source of SiO_2 to benthic algae. This speculation is based on the assumption that the mat of algae developed on the sediment surface was mainly composed of diatoms, requiring large amounts of inorganic Si in relation to other nutrients to synthesize their frustules (Sigmon and Cahoon 1997). This is plausible, as Si concentrations in pore water were comparatively higher than those of N and P. Alternatively, the comparison between theoretical and measured fluxes may suggest that benthic algae were not siliceous. However, there are differences between measured light and dark Si fluxes, supporting diatoms uptake at sediment-water interface. Similar arguments are true for inorganic P: calculated net and gross uptake largely exceeded fluxes measured in the light, suggesting that benthic algae extracted inorganic P from the pore water or may cope with limited P availability. Overall, N and P requirements by microphytobenthos resulted in fluxes to the sediments of both nutrients regardless of the presence of chironomid larvae. Concerning Si, only control sediments without chironomids were net Si sinks whereas benthic uptake only smoothed Si regeneration in L and H treatments.

Net autotrophic sediments were demonstrated to inhibit the activity of nitrifiers and denitrifiers, likely due to competition for N, alteration of O_2 and pH values in the upper sediment layer or production of specific inhibitory substances (Risgaard-Petersen 2003). In the surface sediments of the Curonian Lagoon photosynthesis at the interface resulted in negative N_2 fluxes, which were reversed in the dark, when denitrification prevailed. This is likely due to large availability of NO_3^- and active denitrification when benthic algae are less active, even if uptake by benthic algae was demonstrated also in the dark (Rysgaard et al. 1995).

The activity of benthic algae is limited by light penetration to the upper sediment microlayer and we may speculate that pore water solutes, diffusing from the sediment to the water column, are assimilated and retained in the algal biomass. This is always true in control sediments, where gradients from the sediment to the water are elevated for all the considered nutrients (Bartoli et al. 2003; Sundbäck et al. 2004). In the bioturbated sediments, on the contrary, chemical gradients are modified by the activity of larvae, with element-specific mechanisms. If microphytobenthos operates at the interface, the activity of chironomid larvae operates deeper into the sediments, within the upper 10-15 cm.

Benthic metabolism and pore water features in sediments bioturbated by chironomid larvae

A large number of studies analyzed the effect of chironomids on sediment biogeochemistry over a wide range of water temperatures (10-20 °C) and larvae densities (up to 12,000 ind m^{-2}); chironomids were generally demonstrated to stimulate aerobic and anaerobic benthic respiration, with a few exceptions (Stief and Hölker 2006). Our results align with previously published rates of oxygen uptake in chironomid larvae bioturbated ($-0.53<x<-2.5$ mmol $m^{-2}h^{-1}$) versus not bioturbated ($-0.40<x<-1.0$ mmol $m^{-2}h^{-1}$) sediments (Pelegrí and Blackburn 1996; Svensson 1997; Hansen et al. 1998; Shang et al. 2013; Soster et al. 2015). In the present work chironomids stimulated benthic oxygen consumption by a factor of 2, that falls within the range reported in the literature, between 1.2 and 3.6. This means that chironomid larvae have a deep impact on surface sediment metabolism. Such impact includes the larvae respiratory needs (6-8 µg O_2 $mg^{-1}{}_{AFDW}h^{-1}$ at 20 °C, reported in Baranov et al. 2016), together with the stimulation of microbial or chemical processes by larvae bioturbation.

Comparatively, the effects of chironomids bioturbation on benthic TCO_2 fluxes were less studied. Stief and Hölker (2006) demonstrated that predatory fish may induce changes in the behavior of chironomid larvae, that burrow deeper and reduce surface foraging, resulting in significantly lower rates of carbon mineralization and CO_2 fluxes. Hansen et al. (1998) determined a stimulation of TCO_2 production by a factor of ~2 in the presence of chironomids, comparable to that measured for O_2 and similar to the results of our study. Our TCO_2 and O_2

data suggest that chironomid larvae did not alter the respiratory quotient, meaning that they produced a similar stimulation of aerobic and anaerobic metabolism.

In our experiment, we did not measure appreciable CH_4 fluxes in any of the experimental conditions; in the dark rates varied between 0.7 ± 0.6 (C) and 1.9 ± 1.2 (H) μmol $m^{-2}h^{-1}$, whereas in the light rates varied between 0.4 ± 0.7 (C) and 1.1 ± 0.8 (H) μmol $m^{-2}h^{-1}$ (data not shown), without significant differences among light and dark rates and among treatments. On the contrary, high rates of N_2 production were measured in the dark. Denitrification, in excess of N fixation, varied from 395 ± 17 (C) to 1,012 ± 182 (H) μmol N $m^{-2}h^{-1}$, suggesting a stimulation of this process in the presence of chironomid larvae by a factor of 2.6. Shang et al. (2013) measured with the IPT much smaller rates and a stimulation of D_w (denitrification of water column nitrate) by a factor of ~12 and of D_n (coupled nitrification-denitrification) by a factor of ~ 3 with 2,264 ind m^{-2}. Svensson (1997) measured denitrification with the IPT under increasing NO_3^- concentrations in bioturbated vs not bioturbated sediments (2,000 ind m^{-2}) and found a large stimulation of D_w (by a factor of 3), with rates from ~120 to ~380 μmol $m^{-2}h^{-1}$ at NO_3^- concentration of 300 μM. Also Pelegrí and Blackburn (1996) demonstrated a slight increase of coupled nitrification-denitrification as compared to denitrification of water column nitrate, due to burrow ventilation, injection of nitrate-rich water into the sediments and to low-density of nitrifiers in the organic sediments inhabited by chironomids. Their reported rates (20-60 μmol N $m^{-2}h^{-1}$) are much lower than those reported in this study.

We can say that denitrification rates in the Curonian Lagoon sediments with chironomid larvae are among the highest reported in the literature and we can speculate that they are mainly sustained by high NO_3^- concentrations in the water column (100 μM in the study period). The nitrate consumption we have measured in fact has similar rates as those of N_2 efflux.

In eutrophic shallow aquatic systems, chironomid larvae are abundant and their sediment reworking, burrow ventilation and bioirrigation activities may alter the biogeochemistry of the whole benthic system (Lewandowski et al. 2007; Morad et al. 2010). Bioturbation may influence aerobic and anaerobic processes and the abundance of some microbial communities (Nogaro et al. 2008; Bertics and Ziebis 2009). Ventilation and bioirrigation activities introduce O_2 and NO_3^--rich water inside the sediments, and these solutes diffuse through the burrow walls augmenting the oxidized sediment volume (Lewandowski et al. 2007; Hölker et al. 2015). Pore water NH_4^+ decreased in presence of chironomids, likely due to its oxidation to NO_2^- and to NO_3^- by nitrifying bacteria, as hypothesized also by Lewandowski et al. (2007). As a result, in chironomid larvae bioturbated sediments the calculated NH_4^+ diffusive fluxes across the sediment-water interface were almost zero (Svensson 1997; Stief and de Beer 2002). Pore water concentration of PO_4^{3-} was also significantly reduced in sediments with chironomids, likely due

to co-precipitation with ferric iron and retention within sediments as insoluble Fe-hydroxides (Gunnars et al. 2002; Lewandowski et al. 2007). Contrarily to N, P, Fe and Mn, the concentrations of Si in pore water were affected by a much lower degree by chironomids, as this nutrient dynamics are much less redox-dependent, and Si mobility is more dependent upon physical and chemical processes (Schelske et al. 1986). This result is in agreement with the net fluxes we measured during incubations, where Si was the only nutrient regenerated to the water column in presence of chironomids, probably due to the flushing of Si-rich pore water.

Benthic processes in Curonian Lagoon shallow and bioturbated sediments: do they matter?

Results from the present study confirm the potential role of shallow and bioturbated sediments as bottom-up controllers of pelagic primary production, as evidenced by Hölker et al. (2015). Chironomid larvae are demonstrated to actively pump water within sediments and have the potential to filter the whole water column in shallow aquatic ecosystems (Morad et al. 2010). They may perform active control of phytoplankton, with a clearance rate comparable to that of filter-feeding pelagic zooplankton (Roskosch et al. 2010; Hölker et al. 2015). This means that in hypertrophic shallow system as the Curonian Lagoon they may contribute to control pelagic primary production. In a recent paper, Lesutiene et al. (2014) demonstrated that chironomid flesh displays a drop in ^{15}N signature after a cyanobacterial bloom, due to active incorporation of algal biomass with low ^{15}N and rapid turnover. In the same work, this effect was not evident in zebra mussel flesh, even of small individuals, suggesting much slower biomass turnover of these bivalves (Lesutiene et al. 2014). Furthermore, our study suggested that the combined action of chironomids and algae may retain regenerated nutrients within the sediments, with Si as only exception. This is also an interesting output for a hypertrophic freshwater lagoon that in summer is cyanobacteria-dominated, due to a number of different reasons. First of all, internal nutrient recycling may support blooms in the summer, when external loads from the basin are at their minimum level, due to sudden drop of discharge and nutrient concentration in the spring-summer transition (Lubiene et al. 2017). If sediments retain nutrients, and P in particular, whereas Si is regenerated, cyanobacteria lose the competitive advantage that they have in comparison with other, not harmful algal groups as diatoms (Pilkaityte and Razinkovas 2007). Other aspects acting in opposite direction should be taken into account. First, chironomid larvae density peaks in spring, when the experiment was performed, and when riverine nutrient concentrations and loads are still very high. This may result in large stimulation of water column denitrification, as shown in this study, but may not affect significantly the elevated N background. In the summer, chironomid larvae become flying insects that leave the sediments resulting in decreasing densities and associated ecosystem services. We may speculate that

burrows turn anoxic in a short whereas after flying out of insects and that trapped P may be suddenly regenerated to the water column, proportionally more than N, with a positive feedback for cyanobacteria (Zilius et al. 2015; Petkuviene et al. 2016). These aspects were hypothesized and discussed by Hölker et al. (2015) and require some laboratory experiments under controlled conditions to verify whether such redox-dependent P efflux occurs or not. Ultimately, such efflux and its intensity depend upon the reactivity of the sedimentary P pools associated to chironomid larvae burrows. In the Curonian Lagoon sediments, we measured a significant decrease of pore water Fe and Mn in the presence of chironomids, suggesting that the establishment of anoxia may reverse iron and manganese oxidation and favor P efflux. Another important aspect is that the Curonian Lagoon, despite shallow, is a turbid system with limited light penetration, and shallow sediments represent a minor fraction of the total surface. The Nemunas River, together with nutrients, transports to the Curonian Lagoon large amounts of phytoplankton and it is unlikely that pelagic or benthic filter feeders are able to control such load (Lubiene et al. 2017). Transparent water periods occur in the Lagoon, but they are short and confined into specific areas where for example densities of bivalves (or chironomids) are locally abundant. Excess nutrient delivery from the Nemunas watershed and unbalanced stoichiometry have resulted into extremely high pelagic primary production and cyanobacterial blooms, ultimately affecting the functioning of the benthic system through anoxia, organic enrichment and loss of macrofauna. If this trend will be reversed, recolonization of macrofauna and increased light penetration may favor the growth of microphytobenthos and the benthic system may contribute to maintain water clarity (Herren et al. 2017).

References

Aller R.C. (1994) Bioturbation and remineralization of sedimentary organic matter: Effects of redox oscillation. Chemical Geology, 114: 331 – 345.

Anderson L.G., Hall P.O.J., Iverfeldt Å., Van Der Loeff M.M.R., Sundby B., & Westerlund S.F.G. (1986) Benthic respiration measured by total carbonate production. Limnology and Oceanography, 31: 319 – 329.

Baranov V., Lewandowski J., Romeijn P., Singer G., & Krause S. (2016) Effects of bioirrigation of non-biting midges (Diptera: Chironomidae) on lake sediment respiration. Scientific Reports, 6: 27329.

Bartoli M., Nizzoli D., Welsh D.T., & Viaroli P. (2000) Short-term influence of recolonisation by the polycheate worm *Nereis succinea* on oxygen and nitrogen fluxes and denitrification: a microcosm simulation. Hydrobiologia, 431: 165 – 174.

Bartoli M., Nizzoli D., & Viaroli P. (2003) Microphytobenthos activity and fluxes at the sediment-water interface: Interactions and spatial variability. Aquatic Ecology, 37: 341 –

349.

Bartoli M., Longhi D., Nizzoli D., Como S., Magni P., & Viaroli P. (2009) Short term effects of hypoxia and bioturbation on solute fluxes, denitrification and buffering capacity in a shallow dystrophic pond. Journal of Experimental Marine Biology and Ecology, 381: 105 – 113.

Benelli S., Bartoli M., Racchetti E., Moraes P.C., Zilius M., Lubiene I., & Fano E.A. (2017) Rare but large bivalves alter benthic respiration and nutrient recycling in riverine sediments. Aquatic Ecology, 51: 1 – 16.

Berner R.A. (1980) Early Diagenesis - A Theoretical Approach. Princeton University Press, Princeton.

Bertics V.J., & Ziebis W. (2009) Biodiversity of benthic microbial communities in bioturbated coastal sediments is controlled by geochemical microniches. The ISME Journal, 3: 1269 – 1285.

Boudreau B.P. (1997) Diagenetic models and their implication: Modeling transport and reactions in aquatic sediments. Springer, Berlin.

Bower C.E., & Holm-Hansen T. (1980) A salicylate-hypochlorite method for determining ammonia in seawater. Canadian Journal of Fisheries and Aquatic Sciences, 37: 794 – 798.

Boyer J.N., Stanley D.W., & Christian R.R. (1994) Dynamics of NH_4^+ and NO_3^- uptake in the water column of the Neuse River Estuary, North Carolina. Estuaries, 17: 361 – 371.

Cabrita M.T., & Brotas V. (2000) Seasonal variation in denitrification and dissolved nitrogen fluxes in intertidal sediments of the Tagus estuary, Portugal. Marine Ecology Progress Series, 202: 51 – 65.

Caraco N.F., Cole J.J., Raymond P.A., Strayer D.L., Pace M.L., Findlay S.E., & Fischer D.T. (1997) Zebra mussel invasion in a large, turbid river: phytoplankton response to increased grazing. Ecology, 78: 588 – 602.

Carpenter S. R., Kinne O., & Wieser W. (2003) Regime shifts in lake ecosystems: pattern and variation. Outline of Excellence in Ecology, 15. Oldendorf/Luhe: International Ecology Institute.

Cerco C.F., & Seitzinger S.P. (1997) Measured and modeled effects of benthic algae on eutrophication in Indian River-Rehoboth Bay, Delaware. Estuaries, 20: 231 – 248.

Charpy L., Alliod R., Rodier M., & Golubic S. (2007) Benthic nitrogen fixation in the SW New Caledonia lagoon. Aquatic Microbial Ecology, 47: 73 – 81.

Devine J.A., & Vanni M.J. (2002) Spatial and seasonal variation in nutrient excretion by benthic invertebrates in a eutrophic reservoir. Freshwater Biology, 47: 1107 – 1121.

Fulweiler R.W., Nixon S.W., Buckley B.A., & Granger S.L. (2007) Reversal of the net dinitrogen gas flux in coastal marine sediments. Nature, 448: 180 – 182.

Gallepp G.W. (1979) Chironomid influence on phosphorus release in sediment-water microcosms. Ecology, 60: 547 – 556.

Grasshoff K., Ehrhardt M., & Kremling K. (1983) Methods of Seawater analysis. 2nd eds, Verlag Berlin Chemie.

Gunnars A., Blomqvist S., Johansson P., & Andersson C. (2002) Formation of Fe(III) oxyhydroxide colloids in freshwater and brackish seawater, with incorporation of phosphate and calcium. Geochimica et Cosmochimica Acta, 66: 745 – 758.

Hansen K., Mouridsen S., & Kristensen E. (1998) The impact of Chironomus plumosus larvae on organic matter decay and nutrient (N, P) exchange in a shallow eutrophic lake sediment following a phytoplankton sedimentation. Hydrobiologia, 364: 65 – 74.

Herren C.M., Webert K.C., Drake M.D., Vander Zanden M.J., Einarsson Á., Ives A.R., & Gratton C. (2017) Positive feedback between chironomids and algae creates net mutualism between benthic primary consumers and producers. Ecology, 98: 447 – 455.

Higgins S.N., & Vander Zanden M.J. (2010) What a difference a species makes: a meta–analysis of dreissenid mussel impacts on freshwater ecosystems. Ecological Monographs, 80: 179 – 196.

Hölker F., Vanni M.J., Kuiper J.J., Meile C., Grossart H.P., Stief P., ... & Hupfer M. (2015) Tube-dwelling invertebrates: Tiny ecosystem engineers have large effects in lake ecosystems. Ecological Monographs, 85: 333 – 351.

Kana T.M., Darkangelo C., Hunt M.D., Oldham J.B., Bennett G.E., & Cornwell J.C. (1994) Membrane inlet mass spectrometer for rapid high-precision determination of N_2, O_2, and Ar in environmental water samples. Analytical Chemistry, 66: 4166 – 4170.

Kristensen E. (1984) Effect of natural concentrations on nutrient exchange between a polychaete burrow in estuarine sediment and the overlying water. Journal of Experimental Marine Biology and Ecology, 75: 171 – 190.

Kristensen E., Penha-Lopes G., Delefosse M., Valdemarsen T., Quintana C.O., & Banta G.T. (2012) What is bioturbation? The need for a precise definition for fauna in aquatic sciences. Marine Ecology Progress Series, 446: 285 – 302.

Laverock B., Smith C.J., Tait K., Osborn A.M., Widdicombe S., & Gilbert J.A. (2010) Bioturbating shrimp alter the structure and diversity of bacterial communities in coastal marine sediments. The ISME Journal, 4: 1531 – 1544.

Lerman A. (1979) Geochemical processes: water and sediment environments. John Wiley & Sons, New York.

Lesutiene J., Bukaveckas P.A., Gasiunaite Z.R., Pilkaityte R., & Razinkovas-Baziukas A. (2014) Tracing the isotopic signal of a cyanobacteria bloom through the food web of a Baltic Sea coastal lagoon. Estuarine, Coastal and Shelf Science, 138: 47 – 56.

Lewandowski J., & Hupfer M. (2005) Effect of macrozoobenthos on two-dimensional small-scale heterogeneity of pore water phosphorus concentrations in lake sediments: A laboratory study. Limnology and Oceanography, 50: 1106 – 1118.

Lewandowski J., Laskov C., & Hupfer M. (2007) The relationship between *Chironomus plumosus* burrows and the spatial distribution of pore-water phosphate, iron and ammonium in lake sediments. Freshwater Biology, 52: 331 – 343.

Li Y.H., & Gregory S. (1974) Diffusion of ions in sea water and in deep-sea sediments. Geochimica et Cosmochimica Acta, 38: 703 – 714.

Lohrer A.M., Halliday N.J., Thrush S.F., Hewitt J.E., & Rodil I.F. (2010) Ecosystem functioning in a disturbance-recovery context: Contribution of macrofauna to primary production and nutrient release on intertidal sandflats. Journal of Experimental Marine Biology and Ecology, 390: 6 – 13.

Lubiene I., Zilius M., Giordani G., Petkuviene J., Vaiciute D., Bukaveckas P., & Bartoli M. (2017) Effect of algal blooms on retention of N, Si and P in Europe's largest coastal lagoon. Estuarine, Coastal and Shelf Science, 194: 217 – 228.

McCarthy J.J., Taylor R.W., & Taft J.L. (1977) Nitrogenous nutrition of the plankton of the Chesapeake Bay. 1. Nutrient availability and phytoplankton preferences. Limnology and Oceanography, 22: 996 – 1011.

MacIntyre H.L., Geider R.J., & Miller D.C. (1996) Microphytobenthos: The ecological role of the "Secret Garden" of unvegetated, shallow-water marine habitats. I. Distribution, abundance and primary production. Estuaries, 19: 186 – 201.

Magri M., Benelli S., Bondavalli C., Bartoli M., Christian R.R., & Bodini A. (2018) Benthic N pathways in illuminated and bioturbated sediments studied with network analysis. Limnology and Oceanography, 63: S68 – S84.

Malone T.C. (1992) Effects of water column processes on dissolved oxygen, nutrients, phytoplankton and zooplankton. In: Smith D., Leffler M., & Mackiernan G. (eds) Oxygen dynamics in the Chesapeake Bay. A synthesis of recent research. Maryland Sea Grant, College Park, 61 – 148.

Markiyanova M.F. (2016) Composition and distribution of sibling species of *Chironomus Meigen*, 1803 (Diptera, Chironomidae) in Curonian Lagoon of the Baltic Sea. Biology Bulletin, 43: 1422 – 1427.

Michaud E., Desrosiers G., Mermillod-Blondin F., Sundby B., & Stora G. (2006) The functional group approach to bioturbation: II. The effects of the *Macoma balthica* community on fluxes of nutrients and dissolved organic carbon across the sediment–water interface. Journal of Experimental Marine Biology and Ecology, 337: 178 – 189.

Miller D.C., Geider R.J., & MacIntyre H.L. (1996) Microphytobenthos: The ecological role of the "Secret Garden" of unvegetated, shallow-water marine habitats. II. Role in sediment stability and shallow-water food webs. Estuaries, 19: 202 – 212.

Morad M.R., Khalili A., Roskosch A., & Lewandowski J. (2010) Quantification of pumping rate of *Chironomus plumosus* larvae in natural burrows. Aquatic Ecology, 44: 143 – 153.

Nedwell D.B., & Raffaelli D.G. (1999) Advances in ecological research. Estuaries.

Newell R.I.E., Kemp W.M., Hagy III J.D., Cerco C.F., Testa J.M., & Boynton W.R. (2007) Top-down control of phytoplankton by oysters in Chesapeake Bay, USA: Comment on Pomeroy et al. (2006). Marine Ecology Progress Series, 341: 293 – 298.

Nogaro G., Mermillod-Blondin F., Montuelle B., Boisson J.C., & Gibert J. (2008) Chironomid larvae stimulate biogeochemical and microbial processes in a riverbed covered with fine sediment. Aquatic Sciences, 70: 156 – 168.

Officer C.B., Smayda T.J., & Mann R. (1982) Benthic filter feeding: A natural eutrophication control. Marine Ecology Progress Series, 9: 203 – 210.

Pelegrí S.P., & Blackburn T.H. (1996) Nitrogen cycling in lake sediments bioturbated by *Chironomus plumosus* larvae, under different degrees of oxygenation. Hydrobiologia, 325: 231 – 238.

Petkuviene J., Zilius M., Lubiene I., Ruginis T., Giordani G., Razinkovas-Baziukas A., & Bartoli M. (2016) Phosphorus cycling in a freshwater estuary impacted by cyanobacterial blooms. Estuaries and Coasts, 39: 1386 – 1402.

Pilkaityte R., & Razinkovas A. (2007) Seasonal changes in phytoplankton composition and nutrient limitation in a shallow Baltic lagoon. Boreal environment research, 12: 551 – 559.

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. Journal of Limnology, 68: 133 – 145.

Redfield A. (1958) The biological control of chemical factors in the environment. American Scientist, 46: 205 – 221.

Revsbech N.P., Jørgensen B.B., & Brix O. (1981) Primary production of microalgae in sediments measured by oxygen microprofile, $H^{14}CO_3^-$ fixation, and oxygen exchange methods. Limnology and Oceanography, 26: 717 – 730.

Risgaard-Petersen N. (2003) Coupled nitrification-denitrification in autotrophic and heterotrophic estuarine sediments: On the influence of benthic microalgae. Limnology and Oceanography, 48: 93 – 105.

Roskosch A., Morad M.R., Khalili A., & Lewandowski J. (2010) Bioirrigation by *Chironomus plumosus*: advective flow investigated by particle image velocimetry. Journal of the North American Benthological Society, 29: 789 – 802.

Ruginis T., Bartoli M., Petkuviene J., Zilius M., Lubiene I., Laini A., & Razinkovas-Baziukas A (2014) Benthic respiration and stoichiometry of regenerated nutrients in lake sediments with *Dreissena polymorpha*. Aquatic Sciences, 76: 405 – 417.

Rysgaard S., Christensen P.B., & Nielsen L.P. (1995) Seasonal variation in nitrification and denitrification in estuarine sediment colonized by benthic microalgae and bioturbating infauna. Marine Ecology Progress Series, 126: 111 – 121.

Scheffer M., Hosper S.H., Meijer M.L., Moss B., & Jeppesen E. (1993) Alternative equilibria in shallow lakes. Trends in Ecology and Evolution, 8: 275 – 279.

Scheffer M. (1998) Ecology of Shallow Lakes. Springer Science & Business Media.

Schelske C.L., Stoermer E.F., Fahnenstiel G.L., & Haibach M. (1986) Phosphorus enrichment, silica utilization, and biogeochemical silica depletion in the Great Lakes. Canadian Journal of Fisheries and Aquatic Sciences, 43: 407 – 415.

Scott J.T., McCarthy M.J., Gardner W.S., & Doyle R.D. (2008) Denitrification, dissimilatory nitrate reduction to ammonium, and nitrogen fixation along a nitrate concentration gradient in a created freshwater wetland. Biogeochemistry, 87: 99 – 111.

Shang J., Zhang L., Shi C. & Fan C. (2013) Influence of Chironomid Larvae on oxygen and nitrogen fluxes across the sediment-water interface (Lake Taihu, China). Journal of Environmental Sciences, 25: 978 – 985.

Shull D.H., Benoit J.M. Wojcik C., & Senning J.R. (2009) Infaunal burrow ventilation and pore-water transport in muddy sediments. Estuarine, Coastal and Shelf Science, 83: 277 – 286.

Sigmon D.E., & Cahoon L.B. (1997) Comparative effects of benthic microalgae and phytoplankton on dissolved silica fluxes. Aquatic Microbial Ecology, 13: 275 – 284.

Soster F.M., Matisoff G., Schloesser D.W., & Edwards W.J. (2015) Potential impact of *Chironomus plumosus* larvae on hypolimnetic oxygen in the central basin of Lake Erie. Journal of Great Lakes Research, 41: 348 – 357.

Stadmark J., & Conley D.J. (2011) Mussel farming as a nutrient reduction measure in the Baltic Sea: consideration of nutrient biogeochemical cycles. Marine Pollution Bulletin, 62: 1385 – 1388.

Stief P., & de Beer D. (2002) Bioturbation effects of *Chironomus riparius* on the benthic N-cycle as measured using microsensors and microbiological assays. Aquatic Microbial Ecology, 27: 175 – 185.

Stief P., & Hölker F. (2006) Trait-mediated indirect effects of predatory fish on microbial mineralization in aquatic sediments. Ecology, 87: 3152 – 3159.

Stief P. (2013) Stimulation of microbial nitrogen cycling in aquatic ecosystems by benthic macrofauna: Mechanisms and environmental implications. Biogeosciences, 10: 7829 – 7846.

Stocum E.T., & Plante C.J. (2006) The effect of artificial defaunation on bacterial assemblages of intertidal sediments. Journal of Experimental Marine Biology and Ecology, 337: 147 – 158.

Sundbäck K., Miles A., & Göransson E. (2000) Nitrogen fluxes, denitrification and the role of microphytobenthos in microtidal shallow-water sediments: An annual study. Marine Ecology Progress Series, 200: 59 – 76.

Sundbäck K., Linares F., Larson F., Wulff A., & Engelsen A. (2004) Benthic nitrogen fluxes along a depth gradient in a microtidal fjord: The role of denitrification and microphytobenthos. Limnology and Oceanography, 4: 1095 – 1107.

Sundbäck K., Miles A., & Linares F. (2006) Nitrogen dynamics in nontidal littoral sediments: Role of microphytobenthos and denitrification. Estuaries and Coasts, 29: 1196 – 1211.

Svensson J.M., & Leonardson L. (1996) Effects of bioturbation by tube-dwelling chironomid larvae on oxygen uptake and denitrification in eutrophic lake sediments. Freshwater Biology, 35: 289 – 300.

Svensson J.M. (1997) Influence of *Chironomus plumosus* larvae on ammonium flux and

denitrification (measured by the acetylene blockage- and the isotope pairing-technique) in eutrophic lake sediment. Hydrobiologia, 346: 157 – 168.

Tang M., & Kristensen E. (2007) Impact of microphytobenthos and macroinfauna on temporal variation of benthic metabolism in shallow coastal sediments. Journal of Experimental Marine Biology and Ecology, 349: 99 – 112.

Underwood G.J.C., & Kromkamp J. (1999) Primary Production by Phytoplankton and Microphytobenthos in Estuaries. In: Nedwell D.B., & Raffaelli D.G. (eds) Advances in Ecological Research, Academic Press, 29: 93 – 153.

Underwood G.J.C. (2008) Microphytobenthos. Encyclopedia of Ocean Sciences: Second Edition, 807 – 814.

Weiss R.F. (1970) The solubility of nitrogen, oxygen and argon in water and seawater. Deep-Sea Research and Oceanographic Abstracts, 17: 721 – 735.

Wollast R., & Garrels R.M. (1971) Diffusion coefficient of Silica in seawater. Nature-Physical Science.

Zettler M.L., & Daunys D. (2007) Long-term macrozoobenthos changes in a shallow boreal lagoon: Comparison of a recent biodiversity inventory with historical data. Limnologica, 37: 170 – 185.

Zilius M., Bartoli M., Bresciani M., Katarzyte M., Ruginis T., Petkuviene J., … & Razinkovas-Baziukas A. (2014) Feedback mechanisms between cyanobacterial blooms, transient hypoxia, and benthic phosphorus regeneration in shallow coastal environments. Estuaries and Coasts, 37: 680 – 694.

Zilius M., Giordani G., Petkuviene J., Lubiene I., Ruginis T., & Bartoli M. (2015) Phosphorus mobility under short-term anoxic conditions in two shallow eutrophic coastal systems (Curonian and Sacca di Goro lagoons). Estuarine, Coastal and Shelf Science, 164: 134 – 146.

Section 3.2

Stoichiometry of regenerated nutrients differs between native and invasive freshwater mussels with implications for algal growth

Introduction

Native mussel communities are considered at present as the most endangered group of organisms globally (Strayer and Dudgeon 2010). The decline of their diversity and abundance depends upon multiple factors, including water quality and habitat deterioration and the invasion of fast spreading alien species (Schloesser et al. 1996; Bowers and De Szalay 2004; Strayer 2010). The replacement of native with alien mussels may trigger changes in species diversity and ecosystem functioning of whole aquatic environments. Such consequences are poorly understood as they may vary from early to late invasion stages due to compensation responses, changes in community composition and food web structures (Schloesser et al. 1996; Caraco et al. 1997; Bowers and De Szalay 2004; Ozersky et al. 2012).

In pristine environments, freshwater mussels may comprise a major portion of the biomass of benthic macrofauna and are an important functional component of the macrofauna community (Vaughn and Hakenkamp 2001; Atkinson et al. 2013; Burlakova et al. 2014; Cyr et al. 2017; Ruginis et al. 2017). Mussels displace pelagic primary production at the benthic level, fertilize sediments via labile biodeposits and fasten nutrient turnover, by conversion of particulate nutrients into reactive inorganic forms (Strayer 1999; Gergs et al. 2009; Benelli et al. 2017). In nutrient-poor aquatic ecosystems native mussel beds may alleviate via excretion nitrogen (N) limitation and produce N, phosphorus (P) or silica (Si) co-limitation, depressing cyanobacteria and favoring diatom growth (Atkinson et al. 2013; Strayer 2014; Cyr et al. 2017).

Invasion by dreissenid mussels have stimulated many studies focusing on the functional role of invading organisms (Caraco et al. 1997; Higgins and Vander Zanden 2010; Strayer 2010; Ruginis et al. 2014). Dreissenids were shown to effectively decrease chlorophyll concentration in lakes and rivers due to their high densities and filtration rate (Strayer 2009; Cha et al. 2012). Some authors postulated the possibility to manage such invasion (e.g., with periodic removal of mussels biomass and associated N and P) in order to counteract eutrophication, but the amount of nutrient stocked in biomass is generally much lower than that circulated as particulate or dissolved forms (Stańczykowska and Lewandowski 1993; Heat et al. 1995; Arnott and Vanni 1996; Higgins and Vander Zanden 2010). Different studies suggested that top-down control of phytoplankton by zebra mussels (*Dreissena polymorpha*) is site-specific and depends upon a number of other factors such as water depth, stratification, turbidity and background nutrient

concentration (Conroy et al. 2005; Caraco et al. 2006; De Stasio et al. 2008). In Lake Erie, Zhang et al. (2008 and 2011) predicted small top-down control of phytoplankton by zebra mussels but large, mussels-mediated effects on the mobilization of inorganic N and P. Phytoplankton may peak even under elevated grazing pressure by dreissenids, suggesting complex mechanisms regulating blooms (Caraco et al. 1997). Dominance of cyanobacteria in dreissenid invaded lakes were interpreted on the basis of selective filtration or alteration of nutrient cycles and ecological stoichiometry, e.g., enhanced N removal via denitrification and/or enhanced reactive P mobilization via excretion or release from sediment (Bykova et al. 2006; De Stasio et al. 2008; Makhutova et al. 2013).

Zebra mussel biodeposits increase benthic heterotrophic activity via increased bacterial numbers and benthic secondary production (Gergs et al. 2009; Ozersky et al. 2012; Benelli et al. 2017). Increased heterotrophy, together with mussel metabolic requirements, resulted in decreased oxygen concentrations in river reaches (Caraco et al. 2000; Higgins and Vander Zanden 2010). Simultaneously, dreissenid activity may stimulate mechanisms that compensate for large benthic oxygen demand. Elevated filtration rates and nutrient mobilization in fact increase light penetration and stimulate the activity of benthic primary producers. There is no univocal agreement on the net effect of dreissenids on ecosystem autotrophy or heterotrophy (Caraco et al. 2006; Stadmark and Conley 2011). It likely depends upon different regulating factors such as the density of filter feeders (patchy colonies *versus* reefs), their metabolism and nutrient excretion rates, the morphometry of the invaded ecosystem (shallow *versus* deep), the background nutrient concentration, water turbidity and hydrodynamic factors (stratified or well-mixed)(Caraco et al. 1997; Yu and Culver 1999; Conroy et al. 2005; Caraco et al. 2006).

Native and invasive mussels may co-occur in aquatic ecosystems during the early stages of invasions or due to combinations of available trophic resources and niches, by minimizing competition and allowing coexistence (Russert Kraemer 1979). The co-occurrence allows investigating how benthic biogeochemistry varies depending on the dominance of native or invasive species. In this study, we investigated benthic biogeochemistry in a shallow, generally turbid and well-mixed area of the Nemunas River Delta (Curonian Lagoon, Baltic Sea) where native (the unionids *Unio tumidus* and *Anodonta anatina*) and invasive (*D. polymorpha*) mussels coexist. Unionids and dreissenids can be found as single individuals or in small clumps, respectively, or in association, with dreissenid colonies fouling unionid shells (Zaiko et al. 2009; Dzierzynska-Białonczyk et al. 2018). Dreissenids may take advantage of growing on unionids, and in particular in the proximity of their siphons, due to large water flows and higher oxygen and nutrient availability (Hörmann and Maier 2006); however, this can be different in sites with limited food availability and where competition can be high (Baker and Hornbach

2008). Previous studies reported that dreissenids may outcompete unionids a few years after the invasion, resulting in drastic decrease of their abundance and diversity (Bowers and De Szalay 2004). This is not the case in the Curonian Lagoon, were dreissenids invasion was documented decades ago and where dreissenids coexist with unionids (Zettler and Daunys 2007).

We analyzed the exchange of dissolved O_2, total inorganic carbon (DIC), N_2, CH_4 and nutrients (NH_4^+, SiO_2, PO_4^{3-}) in sediments with unionids and dreissenids when present alone and in association. All fluxes were measured in the light and in the dark in order to evaluate the effects of native and invasive mussels on benthic autotrophic and heterotrophic processes. We also determined the filtration and excretion rates of the mussels and tested their effect on phytoplankton growth.

We predicted higher heterotrophic activity in sediments colonized by the small, invasive *D. polymorpha* than in sediments with unionids due to higher filtration rates, biodeposition, microbial-mediated oxygen consumption and nutrient regeneration. This should result in higher potential stimulation of pelagic algal growth by *D. polymorpha*. It was also expected that benthic primary producers play an important role in attenuating nutrient recycling by mussels, due to benthic uptake and retention (Vaughn and Hakenkamp 2001; Atkinson et al. 2013).

Material and methods

In this study, we performed 3 sets of experiments each one targeting a specific objective. In experiment 1, light and dark incubations of intact sediments with native and invasive mussels addressed the effects of mussels on gas and nutrient exchange. In experiment 2, incubations of native and invasive mussels without sediments addressed their filtration, respiration and nutrient excretion rates. Finally, experiment 3 addressed the effects of native and invasive mussels on phytoplankton growth.

Sampling procedure and incubation setup

Intact sediment cores were collected by means of Plexiglass liners (height=30 cm, inner diameter=8 cm) from a shallow (1 m depth) sandy area within the Nemunas River Delta (55°20'25.9"N, 21°11'24.4"E) in September 2016. Low discharge and transparent water allowed collecting sediments with and without two families of mussels: Dreissenidae (*D. polymorpha*, an invasive species) and Unionidae (*U. tumidus* and *A. anatina*, two native species). Unionids were clearly visible, with half body burrowed within sediments; some were devoid of *D. polymorpha* whereas others were fouled by dreissenids. The sediment surface

hosted also patchy colonies of *D. polymorpha* attached on any debris. Encrusting algae were commonly found growing close to the upper portion of unionids and over dreissenid clumps.
We collected 16 intact sediment cores, 4 replicates for 4 treatments: control sediment without mussels (C), sediment with a clump of *D. polymorpha* (D), sediment with an individual of unionids (U) and sediment with a unionid fouled by dreissenids (D+U). After sampling, all liners were provided with a bottom lid and a magnetic bar fixed 10 cm above the sediment interface to stir the water and avoiding sediment resuspension. They were transferred in a cool box filled with in situ water and transported to the laboratory within 2 hours. Then, all the cores were submerged with the top open in a temperature-controlled (17 ± 0.1 °C) tank (100 L), containing aerated and well-stirred lagoon water. The tank was provided with a central magnet rotating at 40 rpm and driving all magnets inside the cores in order to ensure water exchange with the tank and to supply phytoplankton to mussels. The tank cover was provided with halogen lamps (Osram Decostar, 35 W), positioned above each core and producing an irradiance of 96 ± 12 μE $m^{-2}s^{-1}$ measured at the sediment-water interface with an underwater quantum sensor (Li-COR LI-192) and over a 12 hours light and 12 hours dark period. The cores underwent an overnight preincubation and were then processed to measure benthic fluxes (Experiment 1).
Besides intact sediment cores, nearly 200 L of in situ water and additional sets of mussels, including native and invasive species, were collected from the site to measure filtration, respiration and excretion rates (Experiment 2) and to measure phytoplankton growth with dialysis bags (Experiment 3).

Experiment 1: benthic respiration and nutrient fluxes

The day after the sampling, light and dark fluxes of dissolved gas and nutrients were measured via short-term batch incubations, as detailed in Benelli et al. (2018). Incubations lasted 2 hours, in order to keep O_2 concentration within ± 20 % of the initial value. Longer incubation times may result in large drops of O_2, creating limiting conditions for aerobic processes and resulting in wrong estimation of rates (Dalsgaard et al. 2000). Incubations started when transparent, gas-tight lids were positioned on the top of each core. At the beginning and at the end of the incubations, dissolved O_2 concentration was measured with a microelectrode and initial and final water samples (100 ml) were collected with plastic syringes from each core from an one-way valve located in the lids (Fig. 3.2.1a). The collected volume was replaced with an equivalent amount of tank water entering the cores from another valve. Each water sample underwent the same processing: an aliquot of 40 ml was transferred to two 12-ml Exetainers (Labco Scientific, UK), the first for DIC, which was immediately titrated and the second for

dissolved O_2, N_2 and CH_4 analyses. The latter was added with 100 µl of 7 M $ZnCl_2$ to stop microbial activity (analytical methods are reported below). An aliquot of 20 ml was filtered (GF/F glass-fiber filters, 0.7 µm) and transferred into 20 ml plastic vials in order to analyze ammonium (NH_4^+) and dissolved inorganic silica (SiO_2). Another aliquot of water was filtered and transferred into 5-ml glass vials to measure soluble reactive phosphorus (PO_4^{3-}). Details on the analytical techniques are reported below. Dissolved gas and nutrient fluxes were calculated according to the equation:

$$Flux_x = \frac{([x]_f - [x]_i) \times V}{A \times t}$$

where $[x]_f$ and $[x]_i$ are the concentrations (µM or mM) of the solute x at the end and at the start of the incubation, respectively, V (L) is the volume of the core water phase, A (m^2) is the area of the sediment and t (h) is the incubation time.

Daily fluxes (mmol $m^{-2}d^{-1}$) were calculated according to the following equation:

$$Daily\ flux_x = (hourly\ dark\ flux \times h_D) + (hourly\ light\ flux \times h_L)$$

where h_D and h_L are the number of dark and light hours during incubation, respectively.

At the end of the incubation, all the individuals were recovered and characterized for the flesh wet weight (g_{WW}) and for the flesh dry weight (g_{DW}), after drying the soft tissue at 70 °C to a constant weight.

Net and gross O_2 fluxes measured in the light were converted into theoretical net and gross nutrient uptake by benthic algae. Rates were multiplied by a photosynthetic quotient of 1.2 to convert O_2 production into C fixation (Sundbäck et al. 2004). We used oxygen data instead of measured DIC fluxes as microbial processes (i.e. nitrification) may results in overestimation of C fixation rates by benthic algae. Calculated net and gross C fixation were divided by the Redfield ratio 106:16:15:1 and converted into theoretical inorganic N, Si and P uptake (Redfield 1958).

Experiment 2: respiration, excretion and filtration rates by native and invasive mussels

Plexiglass liners identical to those described for experiment 1, but devoid of sediment, were used to measure rates of O_2 consumption and nutrients (NH_4^+, SiO_2 and PO_4^{3-}) excretion by mussels. Chlorophyll *a* (chl *a*) fluxes were also measured and considered as proxies of filtration rates. Three treatments, each with three replicates, were considered: in situ water with a dreissenid clump (D), in situ water with a single unionid (U) and in situ water with a single unionid fouled by dreissenids (D+U). The biomass of mussels in each replicate reproduced approximately the biomass in the intact cores of experiment 1. An additional set of three cores,

containing in situ water without mussels, was used as control. All treatments underwent a two hours dark incubation. At the beginning and at the end of the incubation, dissolved O_2 concentration was measured with a microelectrode, a water subsample was collected and filtered (GF/F) into plastic vials for dissolved inorganic nutrient analyses (NH_4^+ and SiO_2) and another aliquot was collected into glass vials to measure PO_4^{3-}. Additionally, 300 ml of water from the incubation tank (at the start of the incubation, n=3) and 300 ml of water from each core (at the end of the incubation) were collected and filtered (GF/F) to measure chl *a* fluxes. At the end of the incubation, all the individuals were recovered and characterized for the flesh wet weight (g_{WW}) and for the flesh dry weight (g_{DW}), after drying the soft tissue at 70 °C to a constant weight. Fluxes of chl *a*, normalized by the dry mussel flesh (µg $g_{dw}^{-1}h^{-1}$), were converted into µmol of particulate carbon (PC) removed from the water column by mussel filtration. To this purpose, we multiplied chl *a* fluxes by the factors of 2.5 and 3.3, that include most chl *a* : C conversion factors (µg : µmol) reported in the literature (Banse 1977). The PC flux (µmol C $g_{dw}^{-1}h^{-1}$) was then converted into particulate N (PN), particulate P (PP) and particulate Si (PSi) removed from the water column as phytoplankton. Due to large errors associated to such estimates, we used a range of 5-20 for the C:N stoichiometry, 25-400 for C:P stoichiometry and 0-10 for C:Si stoichiometry (all mol : mol). Oxygen fluxes were converted into DIC production assuming a DIC : O_2 = 1:1 stoichiometry. Rates measured in control cores were subtracted from treatments.

Experiment 3: phytoplankton growth assay

To measure phytoplankton growth in presence and absence of mussels, dialysis bags (n=16) were incubated for 3 days in a 10 L tank containing frequently renewed in situ water with and without mussels. The dialysis bags were filled each with ca. 600 ml of in situ water, filtered with a 50 µm-mesh to remove zooplankton but not phytoplankton. The water used to fill the dialysis bags was analyzed at the beginning of the incubation for the concentration of chl *a* and dissolved inorganic nutrients (NH_4^+, PO_4^{3-} and SiO_2). The dialysis bags were made from Spectra/por 1 dialysis membrane consisting of regenerated cellulose with a molecular weight cut off of 6-8 kDa (Mura et al. 1996). Each bag was cylindrical, with a diameter of 6 cm, a length of 15 cm and closed at the extremes with plastic ties. A single dialysis bag was put in each tank. Four treatments were tested, each with 4 replicate tanks: a dialysis bag submersed in water (C), in water with a dreissenid clump (D), in water with a single unionid (U) and in water with a unionid fouled with dreissenids (D+U)(Fig. 3.2.1b). At the end of the incubation, the dialysis bags were opened and, from each one, 300 ml of water were collected and filtered

(GF/F) to analyze chl *a* and dissolved inorganic nutrients. Phytoplankton growth rate (μ) was calculated via the equation:

$$B_f = B_0 * e^{\mu t}$$

$$\mu = \frac{(ln(B_f) - ln(B_0))}{t}$$

where B_f and B_0 are the concentrations of chl *a* at the end and at the beginning of the incubation (μg L^{-1}) and t is the incubation time (d).

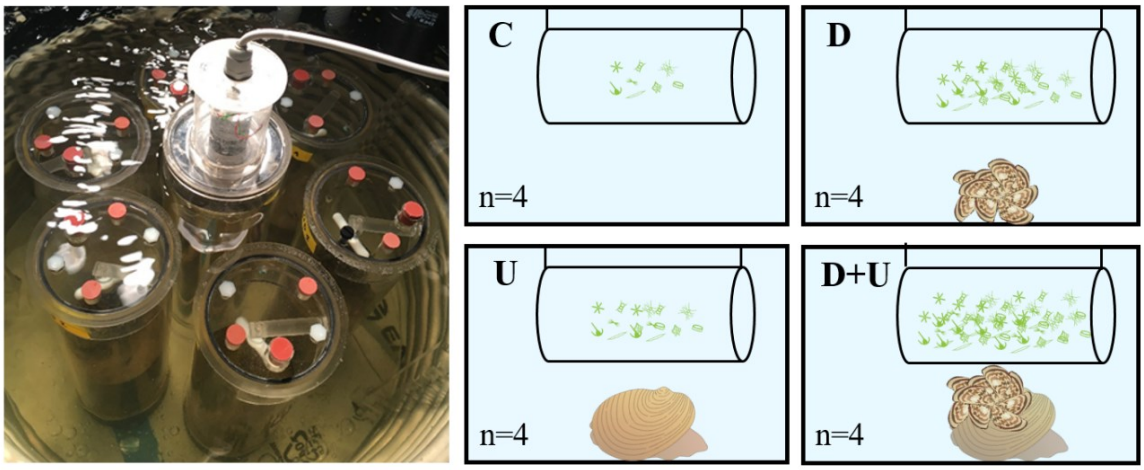


Fig. 3.2.1 Setup of the experiment targeting light and dark fluxes in intact sediments with and without mussels (a) and phytoplankton growth in dialysis bags submersed in tanks with different combination of mussels (b). See the text for more details.

Analytical methods

Dissolved O_2 was measured by means of polarography with a microelectrode (90 % response time in < 5 s, 50 μm tip; Unisense, Denmark). Dissolved N_2 and CH_4 were analyzed by membrane inlet mass spectrometer (MIMS, Bay instruments, USA)(Schlüter and Gentz 2008). Dissolved N_2 concentrations were calculated from obtained N_2:Ar ratio and theoretical Ar concentration derived from Weiss (1970). DIC was measured via six end points 0.1 N HCl microtitration (Anderson et al. 1986). Dissolved nutrients (PO_4^{3-} and SiO_2) from incubations were measured with a continuous flow analyzer (San^{++}, Skalar, sensitivity 0.3 μM) using standard colorimetric methods (Grasshoff et al. 1983). NH_4^+ was analyzed spectrophotometrically using salicylate and hypochlorite, with nitroprussiate as catalyst (Bower and Holm-Hansen 1980). Lipophilic pigments from water samples were extracted in 5 ml of 90 % acetone during 24 h at 4 °C. The extracts were centrifuged and chl *a* was measured by

spectrophotometry according to Lorenzen (1967). Absorption was measured before and after adding 1 M HCl to separate between chl *a* and phaeopigments.

Statistical analysis

Two-way analysis of variance (ANOVA) was used to test the effects of illumination (light/dark measurements), treatments (without or with different combination of mussels) and their interaction on benthic gas and nutrient fluxes. One-way ANOVA was used to test the effect of mussels on phytoplankton growth. The significance (p-value) was set at < 0.05 and pairwise multiple comparison was performed with the post-hoc Holm-Sidak test. All the statistical analyses were performed with Sigma Plot 11.0.

Results

Densities of native mussels at the study site averaged 4 ± 5 ind m^{-2} whereas density of invasive dreissenids averaged 92 ± 13 ind m^{-2}. Oxygen bubbles at the sediment surface indicated photosynthetic activity of benthic algae. During the sampling period, the discharge of the Nemunas River was nearly 300 m^3 s^{-1}, which corresponds to nearly 40 % of the annual average discharge and suggesting relatively low transport of nutrients and particulate matter at the study site and slow water turnover.

Experiment 1: benthic respiration and nutrient fluxes

Benthic O_2 fluxes were affected by the presence of mussels, which shifted, the benthic system from net autotrophic to net heterotrophic (Fig. 3.2.2a). Fluxes were more negative in sediments in presence of both mussels (D+U), with rates of -4.3 ± 0.7 mmol $m^{-2}h^{-1}$ and -12.8 ± 1.6 mmol $m^{-2}h^{-1}$ (mean ± standard error), measured in the light and in the dark, respectively (Fig. 3.2.2a), and significantly more negative than the other treatments (Holm-Sidak test, $p<0.05$). Both factors illumination and treatment produced a significant effect on O_2 fluxes (two-way ANOVA, Table 3.2.1). Gross primary production (GPP, obtained combining light and dark fluxes) was not different among treatments suggesting that there were active microalgae growing on the sediment and mussel surface in all treatments. The highest rate of DIC production was found in the dark in D+U treatment, with a rate of 6.3 ± 1.1 mmol $m^{-2}h^{-1}$ (Fig. 3.2.2b), which was significantly higher than in the other three treatments (Holm-Sidak test, $p<0.05$). Similar to O_2, both illumination and treatment factors produced a significant effect on the fluxes of dissolved DIC (two-way ANOVA, Table 3.2.1). The simultaneous presence of mussels (D+U) turned the benthic system from net DIC sink to a net DIC source (Fig. 3.2.2b). Bare sediments (C) displayed negative N_2 fluxes in the light and in the dark (Fig. 3.2.2c). In the

light, the presence of mussels set to zero the net N_2 uptake whereas in the dark it reversed the flux measured in C. The two-way ANOVA on N_2 fluxes revealed that both factors illumination and treatment, but not their interaction, were very close to the 5 % significant level (Table 3.2.1). Fluxes of CH_4 were generally low and variable among replicates and treatments. In the light, they were generally not measurable whereas in the dark CH_4 tended to be released from sediments, with higher rates measured in D and D+U (Fig. 3.2.2d). Differences between light and dark rates were significant (Holm-Sidak test, $p<0.05$), but not differences among treatments (two-way ANOVA, Table 3.2.1).

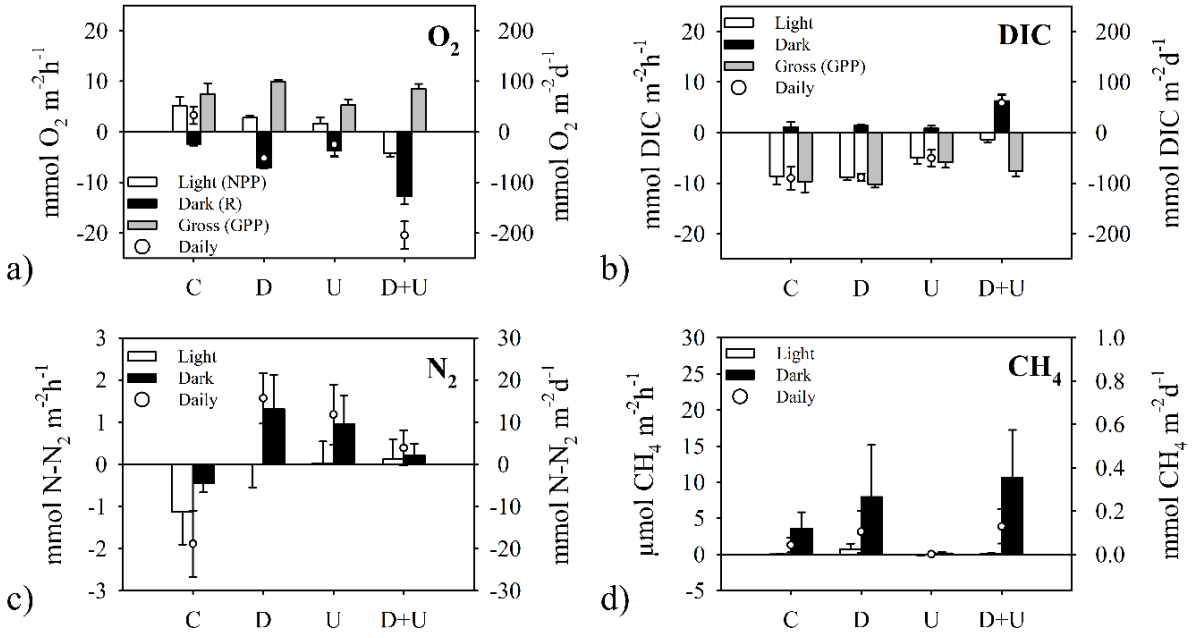


Fig. 3.2.2 Benthic fluxes of a) dissolved O_2, measured in light (NPP= Net Primary Production; white bars) and in dark (R= Respiration; black bars) incubations of C, D, U and D+U treatments (n=4). b) DIC; c) N_2 and d) CH_4 measured in light (white bars) and dark (black bars) incubations of C, D, U and D+U treatments (n = 4). Grey bars in O_2 and DIC graphs represent Gross Primary Production (GPP). Mean ± standard error are reported. All fluxes are expressed in µmol $m^{-2}h^{-1}$ or mmol $m^{-2}h^{-1}$. Dots represent daily mean ± standard error expressed in mmol $m^{-2}d^{-1}$.

Dissolved inorganic nutrients measured during light and dark incubations were reported in figure 3.2.3. There was a statistically significant interaction between illumination and treatment factors in NH_4^+ fluxes (two-way ANOVA, Table 3.2.1), suggesting that the effect of illumination depended on the treatment. The presence of mussels always resulted in a net NH_4^+ regeneration to the water column during the dark incubation (Fig. 3.2.3a), with rates that were significantly different from bare sediment (Holm-Sidak test, $p<0.05$). In the light, uptake processes measured in C were significantly reduced or reversed (D+U) by the presence of mussels. Only bare sediment (C) acted as a net NH_4^+ sink whereas in the presence of mussels

NH_4^+ was always regenerated. The factor illumination had no effect on PO_4^{3-} fluxes, that were strongly influenced by the presence of *D. polymorpha* clumps (Fig. 3.2.3b)(two-way ANOVA, Table 3.2.1). Bare sediments and sediments with unionids were PO_4^{3-} sinks, with negative fluxes measured in light and dark incubations. Sediments with *D. polymorpha* were on the contrary always PO_4^{3-} sources, regardless the illumination condition, with rates exceeding 30 µmol $m^{-2}h^{-1}$ measured in the dark in D+U (Fig. 3.2.3b). Fluxes of SiO_2 were negative in all treatments and illumination conditions (Fig. 3.2.3c); two-way ANOVA revealed significant differences only in the treatment factor, with the lowest SiO_2 uptake measured in D+U (Table 3.2.1). Mussels affected differentially the ecological stoichiometry of regenerated nutrients: both dreissenids and unionids, alone or in combination, alleviated N limitation by recycling large NH_4^+ amounts. Only dreissenids alleviated P limitation by recycling PO_4^{3-}, whereas both mussels had no appreciable effects on Si, which was never recycled to the water column.

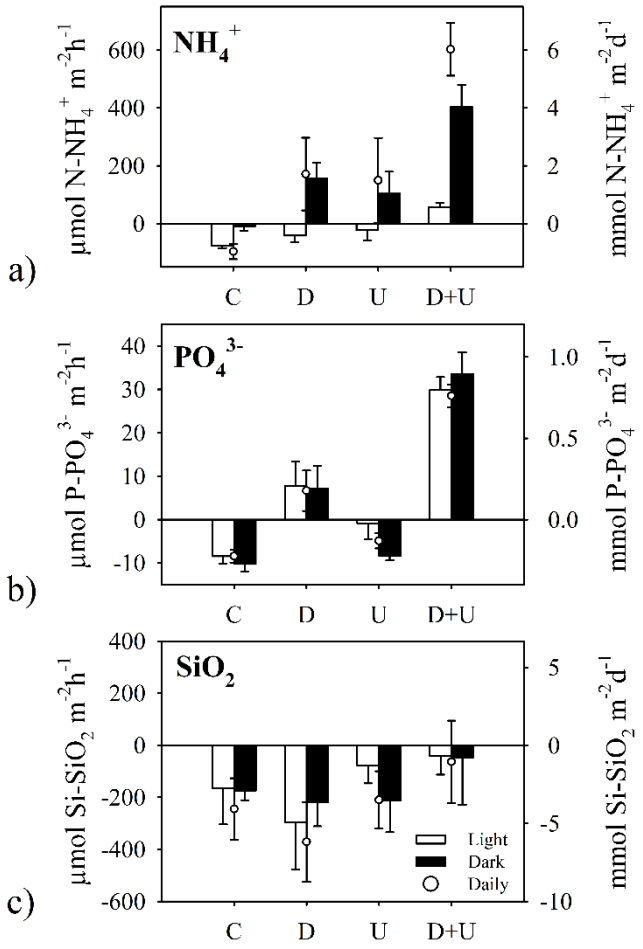


Fig. 3.2.3 Light and dark benthic fluxes of: a) NH_4^+, b) PO_4^{3-} and c) SiO_2 measured in C, D, U and D+U treatments (n=4). Mean ± standard error are reported. All fluxes are expressed in µmol $m^{-2}h^{-1}$. Dots represent daily mean ± standard error of nutrient fluxes expressed in mmol $m^{-2}d^{-1}$.

Table 3.2.1 Results of two-way ANOVA testing the effects of the factors incubation condition (illumination: light/dark) and treatment (control, D, U, D+U) on gas (O_2, DIC, N_2, CH_4) and nutrient (NH_4^+, PO_4^{3-} and SiO_2) fluxes. *df*, degree of freedom; SS, sum of squares; MS, mean of squares; F, F-statistics; *p*, *p*-value. Significant values are printed in bold.

	Source of variation	*df*	SS	MS	F	*p*
O_2						
	Illumination	1	472	472	84.4	**<0.001**
	Treatment	3	492	164	29.3	**<0.001**
	Interaction	3	20	7	1.2	0.341
	Residual	24	134	6		
	Total	31	1123	36		
DIC						
	Illumination	1	542	542	131.6	**<0.001**
	Treatment	3	228	76	18.3	**<0.001**
	Interaction	3	23	8	1.8	0.166
	Residual	24	100	4		
	Total	31	888	29		
N_2						
	Illumination	1	4	4	3.8	0.064
	Treatment	3	9	3	2.7	0.072
	Interaction	3	2	1	0.5	0.718
	Residual	24	28	1		
	Total	31	43	1		
CH_4						
	Illumination	1	225	225	4.3	**0.048**
	Treatment	3	149	50	1.0	0.429
	Interaction	3	132	44	0.8	0.482
	Residual	24	1243	52		
	Total	31	1775	57		
NH_4^+						
	Illumination	1	261818	261818	28.9	**<0.001**
	Treatment	3	361429	12476	13.3	**<0.001**
	Interaction	3	99582	33194	3.7	**0.026**
	Residual	24	217175	9049		
	Total	31	946477	31499		

	Source of variation	*df*	SS	MS	F	*p*
PO_4^{3-}						
	Illumination	1	19	19	0.3	0.561
	Treatment	3	9294	3098	58.0	**<0.001**
	Interaction	3	139	46	0.9	0.470
	Residual	24	1282	53		
	Total	31	10729	346		
SiO_2						
	Illumination	1	12058	12058	0.3	0.581
	Treatment	3	384698	128233	3.3	**0.037**
	Interaction	3	196426	65475	1.7	0.194
	Residual	24	924804	38533		
	Total	31	1510298	48719		

Experiment 2: respiration, excretion and filtration rates by native and invasive mussels

The dry flesh normalized respiration of mussels incubated in water (DIC fluxes, µmol C $g_{dw}^{-1}h^{-1}$) was double in dreissenids compared to unionids (Fig. 3.2.4; one-way ANOVA, p=0.05). Community respiration rates calculated for the D+U treatment displayed intermediate values between D and U. The mussels excretion rate of NH_4^+ did not differ among treatments, whereas PO_4^{3-} excretion measured in D was nearly seven time higher than that measured in the presence of unionids (U and D+U; one-way ANOVA, p=0.03). Silica was never excreted to the water column; fluxes were, on the contrary, small and negative (Fig. 3.2.4). On a molar basis, the DIC : NH_4^+ ratio of inorganic nutrients excreted by mussels were 9.8, 6.0 and 9.9 in the D, U and D+U treatments, respectively; whereas the NH_4^+ : PO_4^{3-} ratio were 3.5, 20.4 and 17.0 in the D, U and D+U treatments, respectively.

Figure 3.2.4 reports also conservative ranges of particulate nutrient fluxes, all derived from chl *a* filtration rates. Biomass-normalized rates of chl *a* removal and calculated particulate nutrients were not significantly different among treatments. The comparison of particulate fluxes (to the mussels) and dissolved nutrients excretion (from the mussels) reveals that: i) DIC production was always in excess to PC fluxes; ii) NH_4^+ excretion was always within the calculated range but close to the highest value of PN fluxes and iii) PO_4^{3-} excretion was within the range (in U and D+U) or higher (in D) than PP fluxes. Unmeasurable SiO_2 excretion supports the hypothesis that algal material filtered by mussels was not siliceous. Feces and pseudofeces were not collected at the end of the incubation.

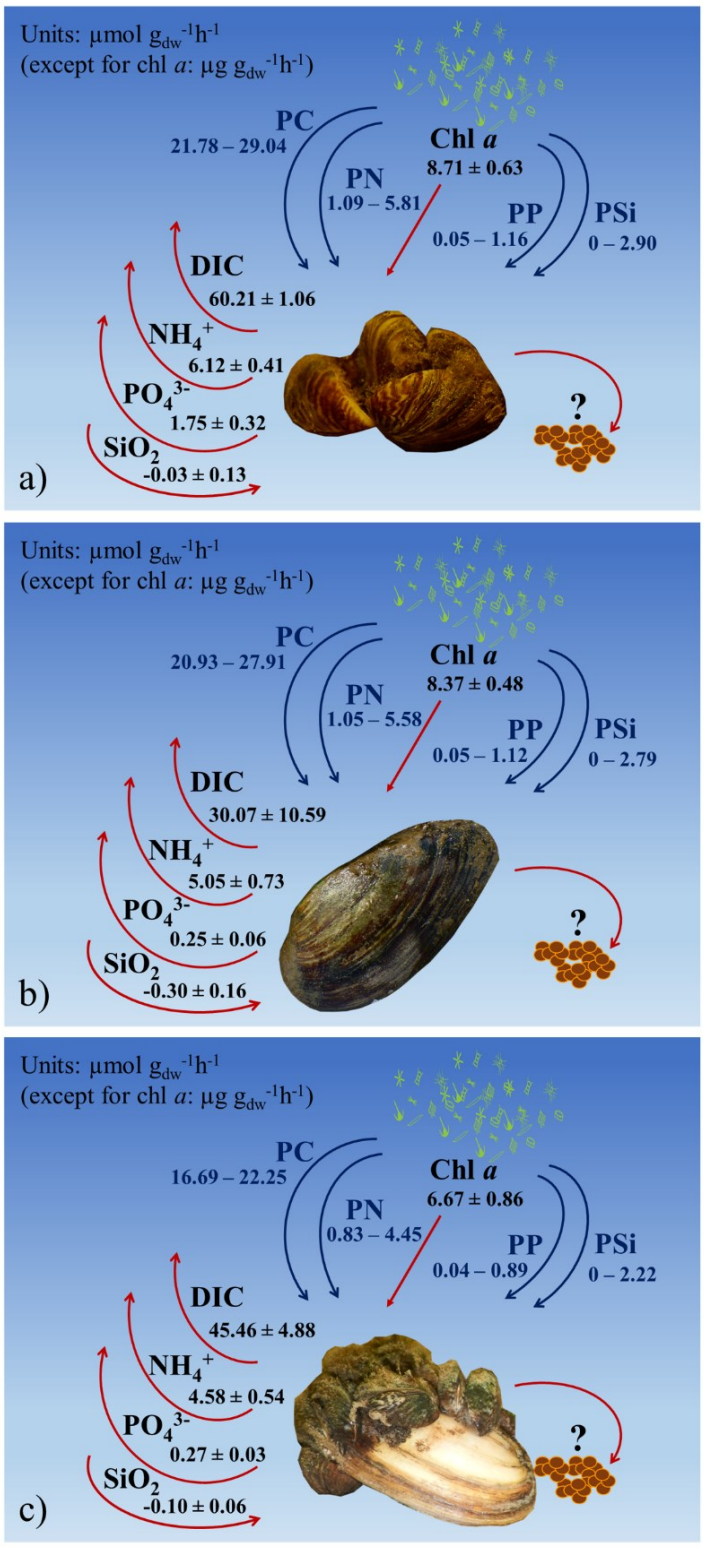


Fig. 3.2.4 Respiration and excretion rates measured in presence of *D. polymorpha* clumps (a), unionids (b) and both (c) are indicated by red arrows. Measured chl *a* fluxes filtrated by mussels are expressed in µg $g_{dw}^{-1}h^{-1}$ and indicated by red arrows. Ranges of particulate nutrients (carbon, PC; nitrogen, PN; phosphorus, PP and silica, PSi) fluxes derived from chl *a* are indicated by blue arrows in presence of *D. polymorpha* clumps (a), unionids (b) and both (c). Mean ± standard error are reported. All units are expressed in µmol $g_{dw}^{-1}h^{-1}$.

Experiment 3: phytoplankton growth assay

Phytoplankton growth rates measured in the four treatments are reported in figure 3.2.5a. Phytoplankton growth was significantly higher in presence of mussels and in particular in presence of *D. polymorpha* (Holm-Sidak test, $p<0.05$). Along the incubation period, NH_4^+ concentration decreased in all treatments as compared to initial value (t_0)(Fig. 3.2.5b). Reactive phosphorus concentration also decreased as compared to the initial value, except for D+U treatment (Fig. 3.2.5c). At the end of the experiment, SiO_2 displayed a marked decrease in presence of *D. polymorpha* by a factor of nearly three, whereas it was similar to initial value in U and C treatments (Fig. 3.2.5d).

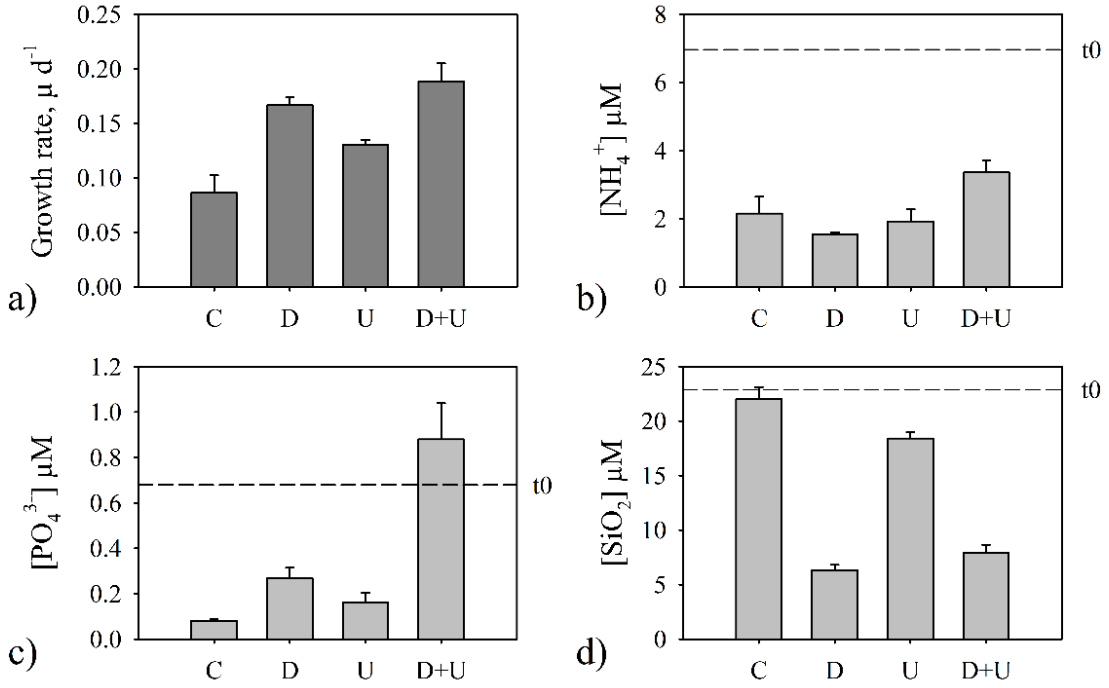


Fig. 3.2.5 Phytoplankton growth rates in the dialysis bags incubated for three days in control treatment (only water, C) and in presence of mussels (D, U and D+U) expressed in µ d^{-1} (a). Concentrations of dissolved inorganic nutrients b) NH_4^+; c) PO_4^{3-} and d) SiO_2), expressed in µM, in control treatment (only water, C) and in treatments with mussels at the end of experiment. The dash line is the reference value of nutrient concentrations at the beginning of the experiment (t0).

Discussion

Mussel beds as biogeochemical hot spots in sediments

This study contributes to our understanding of how invasions by non-native mussels may alter benthic biogeochemistry and nutrient availability in the pelagic environment, stimulating primary production (Heath et al. 1995; Strayer 1999; Conroy et al. 2005; Zhang et al. 2011; Ruginis et al. 2014). Results from this study confirm that filter-feeding mussels increase the net

heterotrophy of the benthic system, augmenting oxygen uptake, total dissolved inorganic carbon, nitrogen and phosphorus release and nitrogen loss via denitrification (Ruginis et al. 2014; Welsh et al. 2015; Benelli et al. 2017; Smyth et al. 2017). Light measurements highlight also the importance of benthic primary production for nutrient cycling in the absence and even more in the presence of filter feeders, with high and comparable rates of gross primary production in all the considered treatments. Mussels displace pelagic primary production at the sediment level and provide surface for algal growth (Atkinson and Vaughn 2015; Ozersky et al. 2018). Gross primary production and nutrient uptake in sediments with or without mussels largely attenuated the benthic regeneration or even reversed nutrient effluxes (Atkinson et al. 2018).

Dreissenids vary the ecological stoichiometry of regenerated nutrients

Through filtration, mussels remove particulate materials from the water column. A fraction of such algal material is assimilated by the mussels and stored for their growth, a fraction is excreted to the water column as dissolved inorganic nutrients, and a fraction is egested as biodeposits (feces and pseudofeces) to the sediment (Strayer 2014; Smyth et al. 2017; Vaughn 2018). In this study, we did not measure the quality and quantity of biodeposits. However, we found large interspecific metabolic differences between the two families of mussels, in terms of excreted nutrients, which afterwards may differentially stimulate pelagic primary production. These outcomes align with those reported by Vanni and McIntyre (2016), dealing with species-specific rates of nutrient excretion by aquatic animals and by Atkinson et al. (2013), dealing with the role of mussel aggregates as nutrient sources to the water column. They suggest also that whereas widely documented NH_4^+ excretion by some mussels (e.g., Unionidae) may balance N limitation, other mussels (e.g., Dreissenidae) may further unbalance the ecological stoichiometry of nutrients, due to large PO_4^{3-} production (Heath et al. 1995; Conroy et al. 2005; Ruginis et al. 2014). Results from this study show that mussels may reverse the sink role of sediments through the excretion of high rates of dissolved inorganic N and P to the water column, as reported for many other filter-feeding animals (Nalepa et al. 1991; Ruginis et al. 2017; Benelli et al. 2018). We demonstrated that this regeneration may support completely, or in part, the theoretical nutrient demand by benthic primary producers (Table 3.2.2). In particular, *D. polymorpha* regenerated proportionally more P than N, and such regeneration entirely supported inorganic P but not N requirements by benthic primary production. This may result in competition for N between algae and bacteria and may stimulate N uptake from the water column or from pore water or N fixation. Nitrogen and P excretion by unionid mussels was on the contrary in excess to nutrient requirements by algae (Table 3.2.2). The excretion

rates that we measured are among the values that Vanni et al. (2017) reported reviewing the literature in a range of temperature between 17-19 °C. The amount of nutrients excreted is generally proportional to the composition of particulate matter ingested (Vanni 2002). As *D. polymorpha* excreted high rates of inorganic PO_4^{3-} to the water column, we assume that its biodeposits are depleted in phosphorus (Gergs et al. 2009). These biodeposits may remain photosynthetically active and enhance benthic primary production (Roditi et al. 1996; Newell 2004) and benthic secondary production from pelagic resources (Gergs et al. 2009). The molar ratio of excreted inorganic nutrients (NH_4^+ : PO_4^{3-}) was 3.50 ± 0.87 for *D. polymorpha* and 20.43 ± 8.12 for unionids. These ratios are in line with the mean value found by Vanni et al. (2017) in the studies on dreissenids and unionids in the range of temperature between 17 and 19 °C. Changes in the ratios of available nutrients may alter the community of primary producers and may vary the background nutrient conditions of the system (Atkinson et al. 2013).

Benthic buffers of excreted nutrients

Mussels increase sediment heterotrophy and nutrient recycling but our results suggest that a large fraction of regenerated nutrients is assimilated and retained by primary producers within the benthic compartment. Flux measurements performed in the dark in sediment with mussels or the upscaling of mussel excretion rates provide a partial picture of the effects of an invasive species, overestimating its impact. A similar line of reasoning is reported by Caraco et al. (2000) analyzing the effects of dreissenids on dissolved oxygen decline in the Hudson River. The large discrepancy between in situ oxygen decline and that, much higher, predicted by mussel respiration rates was explained in terms of increased photosynthesis by macrophytes. The latter, due to dreissenid-mediated increase of light penetration, moderated the impact of the mussels. Combining biomass-specific excretion rates (Fig. 3.2.4) and the biomass of mussels recovered in the incubated sediment (Table 3.2.3) it was possible to calculate the theoretical nutrient release by mussels in the different experimental conditions (4^{th} column of Table 3.2.2). Heterotrophic measurements of nutrient fluxes reported in figure 3.2.3 are always much lower than those expected from mussel excretion, suggesting different retention mechanisms, operating also in the dark and including precipitation and co-precipitation, coupled nitrification and denitrification and uptake. Large differences between nutrient recycling via excretion measured with mussels alone and net nutrient regeneration in intact cores with mussels were generally found (Benelli et al. 2017; Ruginis et al. 2017; Murphy et al. 2018).

Table 3.2.2 Contribution of mussels N and P excretion to theoretical gross benthic primary production. Biomass-specific excretion rates measured in experiment 2 were multiplied by the biomass of mussels in experiment 1 and compared to the theoretical nutrient demand to sustain gross primary production. The latter was calculated form oxygen fluxes measured in the light and from algal nutrient stoichiometry. Mean ± standard error are reported. GPP=gross primary production, TNU=theoretical nitrogen uptake by benthic algae, TPU=theoretical phosphorus uptake by benthic algae.

	GPP	**TNU**	**TPU**	**Mussel excretion**		**Contribution to uptake**	
	($mmol\ m^{-2}h^{-1}$)	($\mu mol\ m^{-2}h^{-1}$)	($\mu mol\ m^{-2}h^{-1}$)	($\mu mol\ m^{-2}h^{-1}$)		(%)	
				NH_4^+	**PO_4^{3-}**	**N**	**P**
D	9.91 ± 0.63	1,794 ± 114	112 ± 7	499 ± 110	143 ± 60	28 ± 8	128 ± 62
U	5.38 ± 2.09	974 ± 379	61 ± 24	1,135 ± 392	56 ± 29	117 ± 340	92 ± 84
D+U	8.47 ± 2.20	1,534 ± 399	96 ± 25	2,393 ± 954	320 ± 167	156 ± 662	333 ± 261

Table 3.2.3 Biomass of mussels in the three treatments (D, U and D+U) incubated in experiment 1. Mean ± standard error are reported.

	Biomass of mussels ($g_{dw}\ m^{-2}$)		
	Dreissenids	**Unionids**	**Total**
D	82 ± 9	0	82 ± 9
U	0	225 ± 21	225 ± 21
D+U	140 ± 45	304 ± 97	444 ± 107

Such differences can be due to higher mussel metabolism when incubated without sediments and to a diversified community of microbes and algae, which are growing in sediments with mussels and may promote net uptake of the excreted solutes.

Dreissenids may favor cyanobacteria blooms

Our data allow direct comparison of the metabolism of a native and an invasive species in the delta area of the Nemunas River. The rates of phytoplankton removal by the two families of mussels were comparable on a mussel flesh dry weight basis, but nutrient regeneration was different, with significantly larger reactive P excretion by *D. polymorpha* (Heath et al. 1995; Ruginis et al. 2014; Vanderploeg et al. 2017). This suggests different metabolic pathways between mussels, as the nutrient source (i.e. the algal community and its nutrient stoichiometry) was the same during the incubations. The invasive species, singly or in association with the unionids, determined an unbalanced stoichiometry of regenerated nutrients, with a P excess that may favor the growth of N-fixing algae. Cyanobacteria blooms are regularly occurring in the Curonian Lagoon, which is a large freshwater estuary (Gasiūnaitė et al. 2008). The large nutrient inputs of the Nemunas River to the Curonian Lagoon (44,208 ± 12,677 tons total N yr^{-1}, 59,048 ± 10,770 tons total Si yr^{-1} and 1,547 ± 266 tons total P yr^{-1}) largely contribute to the hypertrophic status of this estuary (Vybernaite-Lubiene et al. 2018). Besides dissolved nutrients, the Nemunas River delivers large amounts of phytoplankton, with an estimated annual load of ~350 tons chl *a* y^{-1} (Vybernaite-Lubiene et al. 2017). Such loads undergo profound seasonal variations, with summer minima for inorganic nutrients and summer peaks for particulate forms. Interestingly, the ecological stoichiometry of dissolved inorganic N, Si and P displays a pronounced and steep drop in the critical period between the spring and the summer, when N and Si limitation establishes for 4-5 months (Vybernaite-Lubiene et al. 2018). Such limitation was generally considered to be the main reason favoring cyanobacteria blooms in the Curonian Lagoon. Our results suggest that other factors besides external loads, among which macrofauna activity and sediment recycling, may foster cyanobacteria blooms, by further unbalancing NH_4^+ : PO_4^{3-} ratios. We believe that nutrient regeneration by mussels is diluted within the huge loads delivered from September to May by the Nemunas River, but that internal recycling might represent an important fraction during summer, when river discharge and nutrient concentrations are at their minimum. In particular, large areas facing the Nemunas River Delta and invaded by dreissenids may represent benthic reactors turning fluvial chl *a* into reactive nutrients characterized by P excess. As such, mussels may be co-drivers of cyanobacteria blooms, as demonstrated in experimental or theoretical works by Conroy et al. (2005); Bykova et al. (2006); De Stasio et al. (2008); Zhang et al. (2008 and 2011). The peak

of dissolved inorganic P regeneration measured in D+U (30 µmol P $m^{-2}h^{-1}$) overlaps the highest P flux reported for the Curonian Lagoon benthic system. Such dramatic P regeneration was measured with the same batch incubation of intact sediments without mussels in a single occasion, during the occurrence of a large cyanobacteria bloom and was interpreted as a positive biogeochemical feedback to blooms (Zilius et al. 2014).

In the present study, the benthic system of the Nemunas River deltaic area did not release any dissolved reactive Si, regardless the presence of mussels. This suggests either a strong Si limitation of the benthic primary producers and a retention/translocation of Si at the sediment-water interface or the dominance in late summer of not siliceous algae feeding the mussels (Vybernaite-Lubiene et al. 2017 and 2018).

The shallow depth (< 1 m) of the study area, the minimum discharge of the Nemunas River and the absence of wind in the study period, combined with the relatively large population of mussels, resulted in transparent water with low chlorophyll concentrations. This might suggest top-down control of pelagic primary production by the filter-feeding community, at least under the specific hydrographic and meteorological conditions of the sampling period (Prins et al. 1998; Caraco et al. 2006). However, this is not the rule in the Curonian Lagoon, as for most of the year the water is turbid due to wind-wave action resuspending sediments, high river discharge, elevated phytoplankton biomass and limited capacity of benthic and pelagic grazers to control algal growth (Vybernaite-Lubiene et al. 2018). Elsewhere, dreissenid mussels were demonstrated to exert top-down control of phytoplankton, increase water transparency and promote benthic primary production (Caraco et al. 2000; Cha et al. 2012). Such outcome is likely true for well-mixed lakes with moderate nutrient loads, but it cannot be generalized to all the invaded aquatic ecosystems. Mussels have limited access to phytoplankton in stratified lakes, where the main effect of mussels is to fasten nutrient recycling (Yu and Culver 1999; Zhang et al. 2008). Caraco et al. (1997) suggested also that phytoplankton may compensate the grazing pressure of dreissenids in various ways, depending upon factors as turbidity or available nutrients, and resulting in sometimes opposite scenarios (e.g., much lower or higher phytoplankton concentrations). The dialysis bag experiment revealed strong enhancement of phytoplankton growth in presence of mussels, larger with *D. polymorpha* as compared to unionids. This result is robust in terms of comparison between the effects of native and invasive species on potential growth of phytoplankton but it should be carefully considered due to the specific and controlled laboratory conditions (e.g., removal of grazing, absence of sediments, etc.).

In conclusion, this study suggests that the replacement of native with invasive mussels may produce large changes in benthic nutrient cycling and phytoplankton growth. In particular, different ecophysiology of dreissenids results in different stoichiometry of benthic nutrient regeneration as compared to sediments with native mussels. Dreissenids, when incubated in intact sediment cores or in the water column without sediments, increased the recycling of reactive P; as such, they may favor or sustain the growth of cyanobacteria.

References

Anderson L.G., Hall P.O.J., Iverfeldt Å., Van Der Loeff M.M.R., Sundby B., & Westerlund S.F.G. (1986) Benthic respiration measured by total carbonate production. Limnology and Oceanography, 31: 319 – 329.

Arnott D.L., & Vanni M.J. (1996) Nitrogen and phosphorus recycling by the zebra mussel (*Dreissena polymorpha*) in the western basin of Lake Erie. Canadian Journal of Fisheries and Aquatic Sciences, 53: 646 – 659.

Atkinson C.L., Vaughn C.C., Forshay K.J., & Cooper J.T. (2013) Aggregated filter-feeding consumers alter nutrient limitation: Consequences for ecosystem and community dynamics. Ecology, 94: 1359 – 1369.

Atkinson C.L., & Vaughn C.C. (2015) Biogeochemical hotspots: Temporal and spatial scaling of the impact of freshwater mussels on ecosystem function. Freshwater Biology, 60: 563 – 574.

Atkinson C.L., Sansom B.J., Vaughn C.C., & Forshay K.J. (2018) Consumer aggregations drive nutrient dynamics and ecosystem metabolism in nutrient-limited systems. Ecosystems, 21: 521 – 535.

Baker S.M., & Hornbach D.J. (2008) Zebra mussels (*Dreissena polymorpha*) attached to native mussels (Unionidae) or inanimate substrates: Comparison of physiological rates and biochemical composition. The American Midland Naturalist, 160: 20 – 28.

Banse K. (1977) Determining the carbon-to-chlorophyll ratio of natural phytoplankton. Marine Biology, 41: 199 – 212.

Benelli S., Bartoli M., Racchetti E., Moraes P.C., Zilius M., Lubiene I., & Fano E.A. (2017) Rare but large bivalves alter benthic respiration and nutrient recycling in riverine sediments. Aquatic Ecology, 51: 1 – 16.

Benelli S., Bartoli M., Zilius M., Vyberanite-Lubiene I., Ruginis T., Petkuviene J., & Fano, E.A. (2018) Microphytobenthos and chironomid larvae attenuate nutrient recycling in shallow-water sediments. Freshwater Biology, 63: 187 – 201.

Bower C.E., & Holm-Hansen T. (1980) A salicylate-hypochlorite method for determining ammonia in seawater. Canadian Journal of Fisheries and Aquatic Sciences, 37: 794 – 798.

Bowers R., & De Szalay F.A. (2004) Effects of hydrology on unionids (Unionidae) and zebra mussels (Dreissenidae) in a Lake Erie coastal wetland. The American Midland Naturalist, 151: 286 – 300.

Burlakova L.E., Karatayev A.Y., Pennuto C., & Mayer C. (2014) Changes in Lake Erie benthos over the last 50 years: Historical perspectives, current status, and main drivers. Journal of Great Lakes Research, 40: 560 – 573.

Bykova O., Laursen A., Bostan V., Bautista J., & McCarthy L. (2006) Do zebra mussels (*Dreissena polymorpha*) alter lake water chemistry in a way that favours *Microcystis* growth?. Science of the Total Environment, 371: 362 – 372.

Caraco N.F., Cole J.J., Raymond P.A., Strayer D.L., Pace M.L., Findlay S.E., & Fischer D.T. (1997) Zebra mussel invasion in a large, turbid river: phytoplankton response to increased grazing. Ecology, 78: 588 – 602.

Caraco N.F., Cole J.J., Findlay S.E., Fischer D.T., Lampman G.G., Pace M.L., & Strayer D.L. (2000) Dissolved oxygen declines in the Hudson River associated with the invasion of the zebra mussel (*Dreissena polymorpha*). Environmental Science and Technology, 34: 1204 – 1210.

Caraco N.F., Cole J.J., & Strayer D.L. (2006) Top-down control from the bottom: regulation of eutrophication in a large river by benthic grazing. Limnology and Oceanography, 51: 664 – 670.

Cha Y., Stow C.A., & Bernhardt E.S. (2012) Impacts of dreissenid mussel invasions on chlorophyll and total phosphorus in 25 lakes in the USA. Freshwater Biology, 58: 192 – 206.

Conroy J.D., Edwards W.J., Pontius R.A., Kane D.D., Zhang H., Shea J.F., & Culver A. (2005) Soluble nitrogen and phosphorus excretion of exotic freshwater mussels (*Dreissena* spp.): Potential impacts for nutrient remineralization in western Lake Erie. Freshwater Biology, 50: 1146 – 1162.

Cyr H., Collier K.J., Clearwater S.J., Hicks B.J., & Stewart S.D. (2017) Feeding and nutrient excretion of the New Zealand freshwater mussel *Echyridella menziesii* (Hyriidae, Unionida): Implications for nearshore nutrient budgets in lakes and reservoirs. Aquatic Sciences, 79: 557 – 571.

Dalsgaard T., Nielsen L.P., Brotas V., Viaroli P., Underwood G., Nedwell D., ... & Dong L. (2000) Protocol handbook for NICE-Nitrogen Cycling in Estuaries: a project under the EU research programme: Marine Science and Technology (MAST III), 1 – 62.

De Stasio B.T., Schrimpf M.B., Beranek A.E., & Daniels W.C. (2008) Increased Chlorophyll a, phytoplankton abundance, and cyanobacteria occurrence following invasion of Green Bay, Lake Michigan by dreissenid mussels. Aquatic Invasions, 3: 21 – 27.

Dzierzynska-Białonczyk A., Jermacz Ł., Mackiewicz T., Gajewska J., & Kobak J. (2018) Mechanisms and impact of differential fouling of the zebra mussel *Dreissena polymorpha* on different unionid bivalves. Freshwater Biology, 63: 687 – 699.

Gasiūnaitė Z.R., Daunys D., Olenin S., & Razinkovas A. (2008) The Curonian Lagoon. Berlin; Heidelberg: Springer.

Gergs R., Rinke K., & Rothhaupt K.O. (2009) Zebra mussels mediate benthic-pelagic coupling by biodeposition and changing detrital stoichiometry. Freshwater Biology, 54: 1379 – 1391.

Grasshoff K., Ehrhardt M., & Kremling K. (1983) Methods of Seawater analysis. 2nd eds, Verlag Berlin Chemie.

Heath R.T., Fahnenstiel G.L., Gardner W.S., Cavaletto J.F., & Hwang S.J. (1995) Ecosystem-level effects of zebra mussels (*Dreissena polymorpha*): An enclosure experiment in Saginaw Bay, Lake Huron. Journal of Great Lakes Research, 21: 501 – 516.

Higgins S.N., & Vander Zanden M.J. (2010) What a difference a species makes: A meta–analysis of dreissenid mussel impacts on freshwater ecosystems. Concepts & Synthesis, 80: 179 – 196.

Hörmann L., & Maier G. (2006) Do zebra mussels grow faster on live unionids than on inanimate substrate? A study with field enclosures. International Review of Hydrobiology, 91: 113 – 121.

Lorenzen C.J. (1967) Determination of chlorophyll and pheo-pigments: Spectrophotometric equations. Limnology and Oceanography, 12: 343 – 346.

Makhutova O.N., Protasov A.A., Gladyshev M.I., Sylaieva A.A., Sushchik N.N., Morozovskaya I.A., & Kalachova G.S. (2013) Feeding spectra of bivalve mollusks Unio and Dreissena from Kanevskoe Reservoir, Ukraine: Are they food competitors or not?. Zoological Studies, 52: 56.

Mura M.P., Agustí S., del Giorgio P.A., Gasol J.M., Vaqué D., & Duarte C.M. (1996) Loss-controlled phytoplankton production in nutrient-poor littoral waters of the NW Mediterranean: In situ experimental evidence. Marine Ecology Progress Series, 130: 213 – 219.

Murphy A.E., Nizzoli D., Bartoli M., Smyth A.R., Castaldelli G., & Anderson I.C. (2018) Variation in benthic metabolism and nitrogen cycling across clam aquaculture sites. Marine Pollution Bulletin, 127: 524 – 535.

Nalepa T.F., Gardner W.S., & Malczyk J.M. (1991) Phosphorus cycling by mussels (Unionidae: Bivalvia) in Lake St. Clair. Hydrobiologia, 219: 239 – 250.

Newell R.I.E. (2004) Ecosystem influences of natural and cultivated populations of suspension-feeding bivalve molluscs: A review. Journal of Shellfish Research, 23: 51 – 61.

Ozersky T., Evans D.O., & Barton D.R. (2012) Invasive mussels alter the littoral food web of a large lake: stable isotopes reveal drastic shifts in sources and flow of energy. PLoS One, 7: e51249.

Ozersky T., Evans D.O., & Ginn B.K. (2015) Invasive mussels modify the cycling, storage and distribution of nutrients and carbon in a large lake. Freshwater Biology, 60: 827 – 843.

Prins T.C., Smaal A.C., & Dame R.F. (1998) A review of the feedbacks between bivalve grazing and ecosystem processes. Aquatic Ecology, 31: 349 – 359.

Redfield A. (1958) The biological control of chemical factors in the environment. American Scientist, 46: 205 – 221.

Roditi H.A., Caraco N.F., Cole J.J., & Strayer D.L. (1996) Filtration of Hudson River water by the zebra mussel (*Dreissena polymorpha*). Estuaries, 19: 824 – 832.

Ruginis T., Bartoli M., Petkuviene J., Zilius M., Vybernaite-Lubiene I., Laini A., &

Razinkovas-Baziukas A. (2014) Benthic respiration and stoichiometry of regenerated nutrients in lake sediments with *Dreissena polymorpha*. Aquatic Sciences, 76: 405 – 417.

Ruginis T., Zilius M., Vybernaite-Lubiene I., Petkuviene J., & Bartoli M. (2017) Seasonal effect of zebra mussel colonies on benthic processes in the temperate mesotrophic Plateliai Lake, Lithuania. Hydrobiologia, 802: 23 – 38.

Russert Kraemer L. (1979) *Corbicula* (Bivalvia: Sphaeriacea) vs. indigenous mussels (Bivalvia: Unionacea) in U.S. rivers: A hard case for interspecific competition?. American Zoologist, 19: 1085 – 1096.

Schloesser D.W., Nalepa T.F., & Mackie G. L. (1996) Zebra mussel infestation of unionid bivalves (Unionidae) in North America. American Zoologist, 36: 300 – 310.

Schlüter M., & Gentz T. (2008) Application of membrane inlet mass spectrometry for online and in situ analysis of methane in aquatic environments. Journal of the American Society for Mass Spectrometry, 19: 1395 – 1402.

Smyth A.R., Murphy A., Anderson I.C., & Song B. (2017) Differential effects of bivalves on sediment nitrogen cycling in a shallow coastal bay. Estuaries and Coasts, 41: 1147 – 1163.

Stadmark J., & Conley D.J. (2011) Mussel farming as a nutrient reduction measure in the Baltic Sea: Consideration of nutrient biogeochemical cycles. Marine Pollution Bulletin, 62: 1385 – 1388.

Stańczykowska A., & Lewandowski K. (1993) Effect of filtering activity of *Dreissena polymorpha* (Pall.) on the nutrient budget of the littoral of Lake Mikołajskie. Hydrobiologia, 251: 73 – 79.

Strayer D.L. (1999) Effects of alien species on freshwater mollusks in North America. Journal of the North American Benthological Society, 18: 74 – 98.

Strayer D.L. (2009) Twenty years of zebra mussels: Lessons from the mollusk that made headlines. Frontiers in Ecology and the Environment, 7: 135 – 141.

Strayer D.L. (2010) Alien species in fresh waters: Ecological effects, interactions with other stressors, and prospects for the future. Freshwater Biology, 55: 152 – 174.

Strayer D.L., & Dudgeon D. (2010) Freshwater biodiversity conservation: Recent progress and future challenges. Journal of the North American Benthological Society, 29: 344 – 358.

Strayer D.L. (2014) Understanding how nutrient cycles and freshwater mussels (Unionoida) affect one another. Hydrobiologia, 735: 277 – 292.

Sundbäck K., Linares F., Larson F., Wulff A., & Engelsen A. (2004) Benthic nitrogen fluxes along a depth gradient in a microtidal fjord: The role of denitrification and microphytobenthos. Limnology and Oceanography, 4: 1095 – 1107.

Vanderploeg H.A., Sarnelle O., Liebig J.R., Morehead N.R., Robinson S.D., Johengen T.H., & Horst G.P. (2017) Seston quality drives feeding, stoichiometry and excretion of zebra mussels. Freshwater Biology, 62: 664 – 680.

Vanni M.J. (2002) Nutrient cycling by animals in freshwater ecosystems. Annual Review of Ecology and Systematics, 33: 341 – 370.

Vanni M.J., & McIntyre P.B. (2016) Predicting nutrient excretion of aquatic animals with metabolic ecology and ecological stoichiometry: A global synthesis. Ecology, 97: 3460 – 3471.

Vanni M.J., McIntyre P.B., Allen D., Arnott D.L., Benstead J.P., Berg D.J., … & Zimmer K.D. (2017) A global database of nitrogen and phosphorus excretion rates of aquatic animals. Ecology, 98.

Vaughn C.C., & Hakenkamp C.C. (2001) The functional role of burrowing bivalves in freshwater ecosystems. Freshwater Biology, 46: 1431 – 1446.

Vaughn C.C. (2018) Ecosystem services provided by freshwater mussels. Hydrobiologia, 810: 15 – 27.

Vybernaite-Lubiene I., Zilius M., Giordani G., Petkuviene J., Vaiciute D., Bukaveckas P.A., & Bartoli M. (2017) Effect of algal blooms on retention of N, Si and P in Europe's largest coastal lagoon. Estuarine, Coastal and Shelf Science, 194: 217 – 228.

Vybernaite-Lubiene I., Zilius M., Saltyte-Vaisiauske L., & Bartoli M. (2018) Recent trends (2012–2016) of N, Si, and P export from the Nemunas River watershed: Loads, unbalanced stoichiometry, and threats for downstream aquatic ecosystems. Water, 10: 1178.

Weiss R.F. (1970) The solubility of nitrogen, oxygen and argon in water and seawater. Deep-Sea Research and Oceanographic Abstracts, 17: 721 – 735.

Welsh D.T., Nizzoli D., Fano E.A., & Viaroli P. (2015) Direct contribution of clams (*Ruditapes philippinarum*) to benthic fluxes, nitrification, denitrification and nitrous oxide emission in a farmed sediment. Estuarine, Coastal and Shelf Science, 154: 84 – 93.

Yu N., & Culver D.A. (1999) Estimating the effective clearance rate and refiltration by zebra mussels, *Dreissena polymorpha*, in a stratified reservoir. Freshwater Biology, 41: 481 – 492.

Zaiko A., Daunys D., & Olenin S. (2009) Habitat engineering by the invasive zebra mussel *Dreissena polymorpha* (Pallas) in a boreal coastal lagoon: Impact on biodiversity. Helgoland Marine Research, 63: 85 – 94.

Zettler M.L., & Daunys D. (2007) Long-term macrozoobenthos changes in a shallow boreal lagoon: comparison of a recent biodiversity inventory with historical data. Limnologica-Ecology and Management of Inland Waters, 37: 170 – 185.

Zhang H., Culver D.A., & Boegman L. (2008) A two-dimensional ecological model of Lake Erie: application to estimate dreissenid impacts on large lake plankton populations. Ecological Modelling, 214: 219 – 241.

Zhang H., Culver D.A., & Boegman L. (2011) Dreissenids in Lake Erie: an algal filter or a fertilizer?. Aquatic Invasions, 6: 175 – 194.

Zilius M., Bartoli M., Bresciani M., Katarzyte M., Ruginis T., Petkuviene J., ... & Razinkovas-Baziukas A. (2014) Feedback mechanisms between cyanobacterial blooms, transient hypoxia, and benthic phosphorus regeneration in shallow coastal environments. Estuaries and Coasts, 37: 680 – 694.

Main findings and general remarks

These studies demonstrate that the effects of the co-presence of primary producers and macrofauna are species-specific and context-dependent. The combined activity of chironomid larvae and benthic algae results in a top-down and bottom-up control of pelagic primary production and therefore in a retention of the regenerated nutrients. The invasive mussels *D. polymorpha* dramatically alter the stoichiometry of nutrient regeneration, enhancing reactive P release and stimulating algal growth. In shallow and well-mixed aquatic ecosystem pelagic algae may take advantage of such regeneration and filtration rates may not control the density of phytoplankton. The role of primary producers is extremely important in shallow water sediments where light penetrates until the bottom. As previously reported, bioturbation studies usually do not take into account the role of primary producers, resulting in an underestimation or overestimation of the effects of macrofauna on benthic process rates. The studies in this chapter are an example, still simplified, of the combined effect of macrofauna and benthic microalgae on a daily basis. They suggest that the tolerant chironomid larvae, which have the lowest rank when considered as a bioindicator, perform relevant ecosystem services in the eutrophic environments and act synergistically with benthic algae in order to remove pelagic algae, translocate nutrients within the benthic ecosystem and impede nutrient losses to the water column. This is extremely interesting from an ecological perspective: organisms able to colonize organic-rich and chemically reduced sediments act in a way to turn such sediments less hostile to other less tolerant organisms. Results of these studies suggest also that not all mussels net remove phytoplankton from the pelagic compartment and are therefore useful in order to control eutrophication via filter-feeding processes. The other side of the medal is that the same mussels may release via excretion large amounts of solutes in a transparent, algal-free medium, where pelagic primary producers may grow. Filter feeders, as such, keep pelagic algal growth at the maximum potential; furthermore, by selective filtration or by changing the stoichiometry of regenerated nutrients, they may enhance the growth of harmful algal groups. The two studies reported in this chapter add the autotrophic component to bioturbation studies and demonstrate how on a daily basis such component attenuates or even reverses the effects of macrofauna.

In the next chapter, the complexity-functioning relationship of the benthic compartment is further disentangled, as different functional groups of macroinvertebrates and different growth forms of primary producers are simultaneously studied in the light and in the dark. Such more complex experimental effort allows to better analyze what happens in natural environments and to produce a more realistic picture of benthic N processes.

Preface and general remarks references

Aller R.C. (1994) Bioturbation and remineralization of sedimentary organic matter: Effects of redox oscillation. Chemical Geology, 114: 331 – 345.

Caraco N.F., Cole J.J., Raymond P.A., Strayer D.L., Pace M.L., Findlay S.E., & Fischer D.T. (1997) Zebra mussel invasion in a large, turbid river: Phytoplankton response to increased grazing. Ecology, 78: 588 – 602.

Ferguson A., Eyre B., Gay J., Emtage N., & Brooks L. (2007) Benthic metabolism and nitrogen cycling in a sub-tropical coastal embayment: spatial and seasonal variation and controlling factors. Aquatic Microbial Ecology, 48: 175 – 195.

Kristensen E. (1984) Effect of natural concentrations on nutrient exchange between a polychaete burrow in estuarine sediment and the overlying water. Journal of Experimental Marine Biology and Ecology, 75: 171 – 190.

MacIntyre H.L., Geider R.J., & Miller D.C. (1996) Microphytobenthos: The ecological role of the "Secret Garden" of unvegetated, shallow-water marine habitats. I. Distribution, abundance and primary production. Estuaries, 19: 186 – 201.

Revsbech N.P., Jørgensen B.B., & Brix O. (1981) Primary production of microalgae in sediments measured by oxygen microprofile, $H^{14}CO_3^-$ fixation, and oxygen exchange methods. Limnology and Oceanography, 26: 717 – 730.

Risgaard-Petersen N. (2003) Coupled nitrification-denitrification in autotrophic and heterotrophic estuarine sediments: On the influence of benthic microalgae. Limnology and Oceanography, 48: 93 – 105.

Rysgaard S., Christensen P.B., & Nielsen L.P. (1995) Seasonal variation in nitrification and denitrification in estuarine sediment colonized by benthic microalgae and bioturbating infauna. Marine Ecology Progress Series, 126: 11 – 121.

Stadmark J., & Conley D.J. (2011) Mussel farming as a nutrient reduction measure in the Baltic Sea: Consideration of nutrient biogeochemical cycles. Marine Pollution Bulletin, 62: 1385 – 1388.

Stief P. (2013) Stimulation of microbial nitrogen cycling in aquatic ecosystems by benthic macrofauna: Mechanisms and environmental implications. Biogeosciences, 10: 7829 – 7846.

Chapter 4

Macrofauna, primary producers and microbes interaction: variations along gradients

Preface

Benthic macrofauna may act as a benthic facilitator, and smooth the negative interactions among organisms for space or a limiting resource (Ferguson and Eyre 2013; Stief 2013). An example is the negative interaction between bacteria, in particular denitrifiers, and primary producers that compete for N. Denitrifiers remove N from aquatic ecosystems whereas primary producers need this element and generally inhibit (e.g., via oxygen release or the production of allelopatic compounds) the activity of bacteria (Sundbäck et al. 2000). Macrofauna may have the role of facilitator by increasing the availability of an element by its extraction from a refractory or not bioavailable pool or by enhancing its transport within sediments (e.g., from diffusion to advection)(Mermillod-Blondin and Lemoine 2010; Ferguson and Eyre 2013). Facilitation is an understudied ecosystem interaction that generally characterizes stable and mature environments. I have considered the macrophyte rhizosphere as an interesting environment with alternating stable states (less oxygenated night and well oxygenated day) where multiple bacteria species and macrofauna interact. The rhizosphere is a three dimensionally complex environment, with steep and daily variable chemical gradients, that hosts a large biodiversity; here, multiple relationships between micro and macroorganisms may vary depending on factors like background nutrient levels (Krull 1970; Bogut et al. 2007).

This chapter shows the most holistic approach applied in this thesis in order to understand the relationship between complexity and ecosystem functioning in vegetated and bioturbated sediments. The multiple interactions occurring among different functional groups of benthic animals and different growth forms of primary producers were analyzed in a eutrophic environment by means of experimental (Section 4.1) and modelling (Section 4.2) approaches. At first, all N processes rates were measured by means of reconstructed sediment cores pre-incubated for 5 weeks and then incubated in the light and in the dark. Thereafter, the Ecological Network Analysis (thereafter ENA) was applied to experimental data to estimate the indirect, hidden effects and dependencies rates, otherwise not measurable. The same approach was applied to an oligotrophic site (Section 4.3) where intact sediment cores were collected from the littoral zone. Microphytobenthos and macrophytes were incubated in the light and in the dark with and without the presence of filter feeders. For the moment, only the measurements of

N processes are reported in the thesis, as the ENA is still in progress. The hypotheses underlying these two studies are multiple: I expected increasing competition between primary producers and bacteria in oligotrophic settings and therefore a more relevant role of macrofauna to facilitate N availability in nutrient-poor environments. I also expected variable denitrification to assimilation rates, higher in the nutrient-rich site. I finally expected large fluxes, both to and from the sediments (imports and exports) in the eutrophic condition and more recycling and better use of the available resources in the more oligotrophic settings.

Section 4.1 is a manuscript draft that will be submitted to Biogeochemistry.

Section 4.2 is modified from Magri M., Benelli S., Bondavalli C., Bartoli M., Christian R.R., & Bodini A. (2018) Benthic N pathways in illuminated and bioturbated sediments studied with network analysis. Limnology and Oceanography, 63: S68 – S84.
Additional Supporting Information may be found in the online version of this article (lno10724-sup-0001-suppinfo1).

Section 4.3 is a first manuscript draft. The experimental and modelling analyses are still in progress.

Section 4.1

Benthic N pathways in illuminated and bioturbated sediments studied with an experimental approach

Introduction

In illuminated sediments the benthic cycle of nitrogen (N) may undergo a complex regulation by interplaying communities of primary producers, macrofauna and microbes (Pelegrí and Blackburn 1994 and 1995; Rysgaard et al. 1995; Bartoli et al. 2003; Sundbäck et al. 2006; Pinardi et al. 2009). Benthic microalgae and rooted macrophytes assimilate inorganic N from bottom and pore water, mainly as ammonium (NH_4^+), and release oxygen (O_2) at the interface or in the sediment via roots, increasing the volume of oxic sediments (Risgaard-Petersen 2003; Soana and Bartoli 2014). Benthic primary producers may favor or inhibit N-related microbial communities through the production of O_2 and the release of exudates, the assimilation of inorganic N or the production of specific inhibitors (Risgaard-Petersen 2003). The presence of benthic primary producers in illuminated sediments adds new paths to the benthic N cycling as compared to dark sediments or alters the direction and intensity of benthic N fluxes. The assimilation and the creation of oxic niches within subsurface sediments by benthic primary producers may stimulate ammonification, nitrification and denitrification (Soana et al. 2015). The assimilation of NH_4^+ from the pore water alters concentration gradients as compared to bare sediment and may decrease or reverse diffusive fluxes (Pinardi et al. 2009). Similarly, subsurface nitrification and denitrification result in net N removal (Soana et al. 2015; Racchetti et al. 2017). Simultaneously, primary producers may strongly compete with bacteria under N-limiting conditions, inhibiting dissimilative microbial processes and N losses from the benthic system. They may stimulate microbial processes that import N as heterotrophic N fixation (McGlathery et al. 2007). Under N excess, primary producers-bacteria competition can be smoothed and other opposite interactions are expected, with primary producers' favoring microbial activity. The presence of macrofauna in the benthic system is also demonstrated to alter benthic N cycling via sediment reworking and conditioning, by mixing new and old organic matter, by burrowing, irrigation and ventilation activities and by benthic-pelagic coupling (Kristensen 2000; Nizzoli et al. 2007; Ruginis et al. 2014). Macrofauna may excrete large amounts of NH_4^+ or may favor its release from pore water, by stimulating ammonification via burrow ventilation (Stief 2013). Burrowers may stimulate subsurface nitrification and denitrification, resulting in increased N loss (Pelegrí et al. 1994; Svensson and Leonardson 1996). More recently, it was demonstrated that they may also stimulate N fixation, to

compensate losses (Bertics et al. 2010). The effects of other functional groups as scrapers is comparatively less studied, but they may favor rooted plants, avoiding excess growth of epiphytic organisms (Chase and Knight 2006). The effects of primary producers and macrofauna on benthic N cycling were generally studied with simplified experimental approaches targeting a single species. The risk is to get a partial representation of natural environments as multiple interactions among different organisms are not taken into account. For example, the inhibition of benthic primary producers on denitrifiers may be alleviated in presence of burrowing macrofauna, which is able to make more available refractory N pools in the sediments via ingestion, conditioning and production of labile feces. Similarly, the presence of filter feeders may promote benthic primary production by controlling the growth of pelagic algae and may enhance the regeneration of NH_4^+ that is readily taken up by plant leaves (Bartoli et al. 2003; Mermillod-Blondin and Lemoine 2010). It may also be expected that benthic primary production provides energy and high quality food in the close proximity of sediments, favoring the activity of macrofauna if compared to bare dark sediments, where the main source is the water column. Roots, via radial O_2 loss (ROL), may also turn the chemistry of pore water more favorable to burrowers and may alter the frequency of burrows ventilation, with a feedback on the behavior of macrofauna (Kristensen et al. 2000).

The benthic system, and the benthic N cycling in particular, may be assumed as a paradigmatic issue to test complexity-ecosystem functioning relationship (sensu Loreau 2010). The analysis of how ecosystems behave as a function of their species richness was traditionally carried out in terrestrial more than in aquatic environments (Tilman 1996). An interesting question, in the context of eutrophication phenomena and the loss of ecosystem functions, is to analyze whether gradients of complexity affect the capacity of the benthic system to retain N. The focus on N cycling seems particularly interesting with this respect as it is affected by bacterial consortiums (Racchetti et al. 2011), by macroinvertebrates (Pelegrí and Blackburn 1995), by benthic algae (Bartoli et al. 2003) and by rooted macrophytes (Soana and Bartoli 2014).

We hypothesized that the presence of macrofauna, by promoting benthic-pelagic coupling, may favor benthic primary production and that burrowers, more than filter feeders, may simultaneously promote N loss via denitrification. We also hypothesized that macrofauna may smooth the negative interactions between primary producers and microbes. To this purpose, we created microcosms with fluvial sediments combining two typologies of primary producers (benthic microalgae and rooted macrophytes) and bioturbating fauna (a burrower and a filter feeder) and, after a conditioning period, we measured in the light and in the dark benthic primary production, net inorganic N fluxes and sedimentary features, including pore water NH_4^+ and potential rates of N-related microbial processes.

Material and methods

Sampling procedure and microcosms setup

Nearly 20 L of sediment, water (~200 L) and many specimens of the rooted macrophyte *Vallisneria spiralis*, of the burrowing oligochaete *Sparganophilus tamesis* and of the filter-feeding bivalve *Corbicula* spp. (hereafter *Corbicula*, n=50 each) were collected from a branch of the Mincio River (Northern Italy). A detailed description of the sampling area is reported in Pinardi et al. (2009). The sampling site is characterized by cohesive, soft sediment (density 1.18 ± 0.03 g cm^{-3}, organic matter content 13.4 ± 0.6 %, mean ± st. err.) patchily colonized by dense meadows of *V. spiralis* (520 ± 40 shoots m^{-2}). Both bare and vegetated sediments present a simplified macrofauna community, mainly represented by *S. tamesis* and *Corbicula*, with densities of 582 ± 88 and 474 ± 67 individuals m^{-2}, respectively. Specimens of *V. spiralis* were gently extracted by hand from the sediment to minimize root damage. Healthy plants were selected and carefully washed to remove epiphytes from leaves and sediment from roots. Oligochaetes and bivalves were collected by sieving (500 µm mesh-size) surface sediments. Oligochaetes were frequently recovered from the rhizosphere of *V. spiralis* (Rota et al. 2014). Plants and macrofauna were then transferred into tanks containing aerated in situ water and brought to the laboratory within a few hours. In laboratory, the sediment was sieved (500 µm mesh-size) to remove large debris and macrofauna, homogenized and packed into cylindrical Plexiglass microcosms (inner diameter=7.2 cm, height=10 cm). Thirty-two microcosms were prepared to test a total of 8 treatments (4 replicates per treatment): bare sediment (S), sediment with *Corbicula* (SC), sediment with *S. tamesis* (SO), sediment with *S. tamesis* and *Corbicula* (SOC), sediment with *V. spiralis* (SV), sediment with *V. spiralis* + *Corbicula* (SVC), sediment with *V. spiralis* + *S. tamesis* (SVO), sediment with *V. spiralis* + *S. tamesis* + *Corbicula* (SVOC). In situ densities of plants, bivalves and oligochaetes were reproduced, adding two organisms of similar size to each microcosm. *V. spiralis* recovered quickly from the transplant (Racchetti et al. 2011), and bivalves and oligochaetes rapidly burrowed into the sediments. Microcosms were then transferred to a large tank containing aerated in situ water at 20 °C , and exposed to a 12/12 h light/dark cycle (light-phase irradiance=200 µE $m^{-2}s^{-1}$). Water was kept stirred by aquarium pumps and a third of the tank volume was replaced every two days with fresh in situ water over a period of 5 weeks. This acclimatization period was considered as sufficient for the establishment of bacterial communities and of diagenetic processes (Stocum and Plante 2006).

Measurements of gas and nutrient fluxes in reconstructed sediments

After the pre-incubation period, the microcosms were inserted underwater into transparent Plexiglass liners (height=40 cm, inner diameter=8 cm, fitting the microcosm outer diameter).

The bottom of each liner was sealed with a rubber stopper. A magnetic bar driven by an external motor (40 rpm) was suspended in the upper liner extreme and ensured homogenous stirring of the water column. All microcosms were then incubated with a start-end procedure (Dalsgaard et al. 2000; Soana et al. 2015) to measure gas fluxes of O_2, total dissolved inorganic carbon (DIC), molecular nitrogen (N_2), methane (CH_4), and nutrient fluxes of nitrate (NO_3^-), nitrite (NO_2^-) and NH_4^+ under dark and light conditions. The top of the liners was sealed with gas-tight transparent lid, without leaving a head-space. Water samples were collected with plastic syringes just before the lid positioning and after approximately 2 hours. Dissolved O_2 concentration was measured with an O_2 microsensor (Unisense, DK); the sensor was calibrated with 100 % and 0 % air-saturated water, maintained at the incubation temperature (20 °C). DIC was measured with 0.1 N HCl titration with an automatic titration unit (ABU91, Radiometer, DK) and a combined pH sensor (Anderson et al. 1986). Samples for N_2 and CH_4 determinations were transferred to gas-tight glass vials (12-ml Exetainer, Labco, High Wycombe, UK), fixed with 100 µl of $ZnCl_2$ 7 M, and analyzed via Membrane Inlet Mass Spectrometer (MIMS, Bay instrument, 0.2 µM sensitivity). Samples for dissolved inorganic nitrogen (DIN, i.e. NO_3^-, NO_2^-, and NH_4^+) determinations were filtered (Whatman GF/F glass fiber filters), transferred to polyethylene vials and analyzed spectrophotometrically. NO_3^- was measured after reduction to NO_2^- with activated cadmium, NO_2^- was measured using sulphanilamide and N-(1-naphtyl) ethylendiamine (Golterman et al. 1978). NH_4^+ was measured using salicylate and hypochlorite in the presence of sodium nitroprussiate (Bower and Holm-Hansen 1980). Gas and DIN fluxes were calculated with the following formula:

$$Flux\ x = \frac{\left([x]_f - [x]_i\right) \times V}{A \times t}$$

where $[x]_f$ and $[x]_i$, expressed in µM or mM, are the concentrations of the solute x at the end and at the start of the incubation, respectively, V (L) is the volume of the core water phase, A (m^2) is the area of the sediment and t (h) is the incubation time.

Sediment characterization and potential activities

At the end of the incubations, all liners were removed and microcosms were sampled for sediment characterization and to quantify the biomass of primary producers and macrofauna. To determine the dry weight of *V. spiralis*, the leaves were cut at the sediment-water interface, carefully rinsed with in situ water to remove epiphytes and dried at 70 °C until constant weight. The biomass of benthic microalgae was measured by collecting the upper 0.5 cm sediment horizon by cut-off plastic syringes (n=3 per microcosm). Chlorophyll a (chl *a*) was extracted from the sediment with 90 % acetone solution, for 24 hours in the dark. After the centrifugation

of the extract, the supernatant was filtered, transferred to quartz cuvettes and analyzed spectrophotometrically according to Lorenzen (1967).

The sediment column (nearly 10 cm) was sampled by means of a cut-off syringe and homogenized to obtain a depth-integrated sample. Sub-samples were then extracted to measure density, porosity, organic matter content, exchangeable and pore water NH_4^+ and to conduct assays for potential activities (i.e. denitrification, anammox and N fixation). Density was determined as the ratio between sediment wet weight and the volume; porosity was measured as the weight loss of a known sediment volume after desiccation at 70 °C. Organic matter content (OM) was quantified as loss on ignition at 450 °C for 4 h of ~ 0.3 g of dry, powdered sediment. Pore water NH_4^+ was analyzed after fresh sediment centrifugation at 3,000 rpm for 10 minutes; the supernatant was filtered and analyzed spectrophotometrically as described above. Exchangeable NH_4^+ was extracted from fresh sediment with 1 M KCl. After 1 hour extraction the slurry (sediment+KCl) was centrifuged and the supernatant filtered and analyzed as for pore water NH_4^+.

Potential denitrification and anammox rates were measured via anoxic slurry incubation where bacteria grew in their optimum condition: 2 ml of fresh sediment were transferred to 12-ml gas-tight glass Exetainers filled with in situ filtered water (two vials per each replicate). Samples were pre-incubated for 20 hours in the dark, in order to remove NO_3^- and O_2 traces. After this pre-incubation period, $^{15}NH_4^+$ was added to an Exetainer and $^{14}NH_4^+$+$^{15}NO_3^-$ were added to the second one. ^{15}N amendments were added from isotopic stock solutions with a 250 µl Hamilton syringe and an accessory needle inserted through the Exetainer lid septum to remove the excess of water during the injection; final inorganic N concentration was 200 µM. The samples were then placed on a rotating shaker and incubated for 6 hours at 20 °C; afterwards 100 µl 7 M $ZnCl_2$ was added to the samples with a syringe and an accessory needle to inhibit microbial activity at the end of incubation. $^{14}N^{15}N$ and $^{15}N^{15}N$ abundance in N_2 were analyzed by MIMS, as previously detailed. This assay allows to evaluate the potential rate of denitrification, the presence or absence of anammox and its relative contribution to total N_2 production (Thamdrup and Dalsgaard 2002). Potential N fixation rates were assessed using the acetylene reduction technique, via anoxic incubation of 2 ml of fresh sediment transferred to 12-ml gas-tight glass Exetainers filled with in situ water at 10 % acetylene saturation. The exetainers were placed on a rotating shaker and incubated for 6 hours. The acetylene and ethylene were then measured within one week by gas chromatography (Fisons 9000 series, equipped with a FID). Obtained acetylene reduction rates were corrected for the trace amounts of ethylene in the acetylene used and within the sediments at the beginning of the incubations. They were related to N fixation

rates using the stoichiometric relationship of 1 mol N_2 fixed for every 3 mol acetylene reduced (McGlathery et al. 1998).

At the end of flux incubations, sediment characterization and the measurement of potential activities, the sediment was gently washed and the roots of *V. spiralis*, the individuals of *S. tamesis* and *Corbicula* were retrieved. Dry weights of roots and of animals were determined as described above.

Statistical analyses

Differences between diffusive gas and nutrient fluxes measured in the light and in the dark were tested by means of two-way analysis of variance. One-way ANOVA was used to analyze differences among treatments for sediment features and potential activities. All pairwise multiple comparison was performed with Holm-Sidak or Tukey HSD tests. The significance (p-value) was set at level lower than 0.05.

Results

Benthic metabolism and nutrient fluxes

Benthic aerobic respiration increased along a gradient of complexity, with rates between -1.5 and -7 mmol O_2 $m^{-2}h^{-1}$ (Fig. 4.1.1a). Light O_2 fluxes were positive in all the treatments, suggesting photosynthetic activity of both microphytobenthos and macrophytes, but fluxes were significantly higher in presence of *V. spiralis*. The lowest rate was measured in treatment SOC that exhibited also the lowest concentration of chl *a* above the sediments. Gross primary production was significantly higher in presence of *V. spiralis* with values > 10 mmol O_2 $m^{-2}h^{-1}$. Daily O_2 budgets were null in SO, slightly positive in S and SC and negative in the other treatments. A net DIC production was observed in the dark in all treatments, DIC production mirrored O_2 consumption (Fig. 4.1.1b). The highest value was observed in SVOC. In the light DIC fluxes were higher in treatments with *V. spiralis*. SV and SVC treatments displayed significantly higher DIC fluxes than in the other treatments. The only exception from this observed trend was the treatment SOC, where net DIC fluxes were slightly positive. Gross primary production was positive in some treatments (SO, SOC and SVOC), null in SVO and negative in the other treatments. According to O_2 and DIC fluxes, respiratory quotient (RQ) was equal to 1 in the treatments S, SO and SC. These sediments were net heterotrophic. Contrarily, in the other treatments RQ was < 1, in particular in SOC. Photosynthetic quotient (PQ) was lower than 1 in two treatments (SV and SVC) and it was undeterminable in SOC. In most of the microcosms with *V. spiralis* PQ was higher than 1, as the O_2 produced was higher than the DIC consumed. The ability of transport O_2 to the sediment via the roots set to zero CH_4

emissions to the atmosphere (Fig. 4.1.1c). In bare sediment and sediment with macrofauna, CH_4 effluxes were high, with rates between 50 and 200 µmol C $m^{-2}h^{-1}$, highlighting the relevance of anaerobic processes. In the conditions with *V. spiralis* the daily CH_4 budgets were near the zero but positive in all the other treatments. Net N_2 fluxes measured at the water-sediment interface were positive in the dark conditions, with rates between 200 (SOC) and 1,000 (SVOC) µmol N $m^{-2}h^{-1}$ (Fig. 4.1.1d). Net N_2 fluxes in the light were positive in treatments without *V. spiralis*, whereas they were reversed in presence of macrophytes, suggesting the occurrence of N fixation and its dominance over denitrification. The most negative N_2 flux was measured in SV treatment, with a rate of -733.1 ± 193.3 µmol N $m^{-2}h^{-1}$. Daily N_2 budgets were all positive with the exception of SV and SVC.

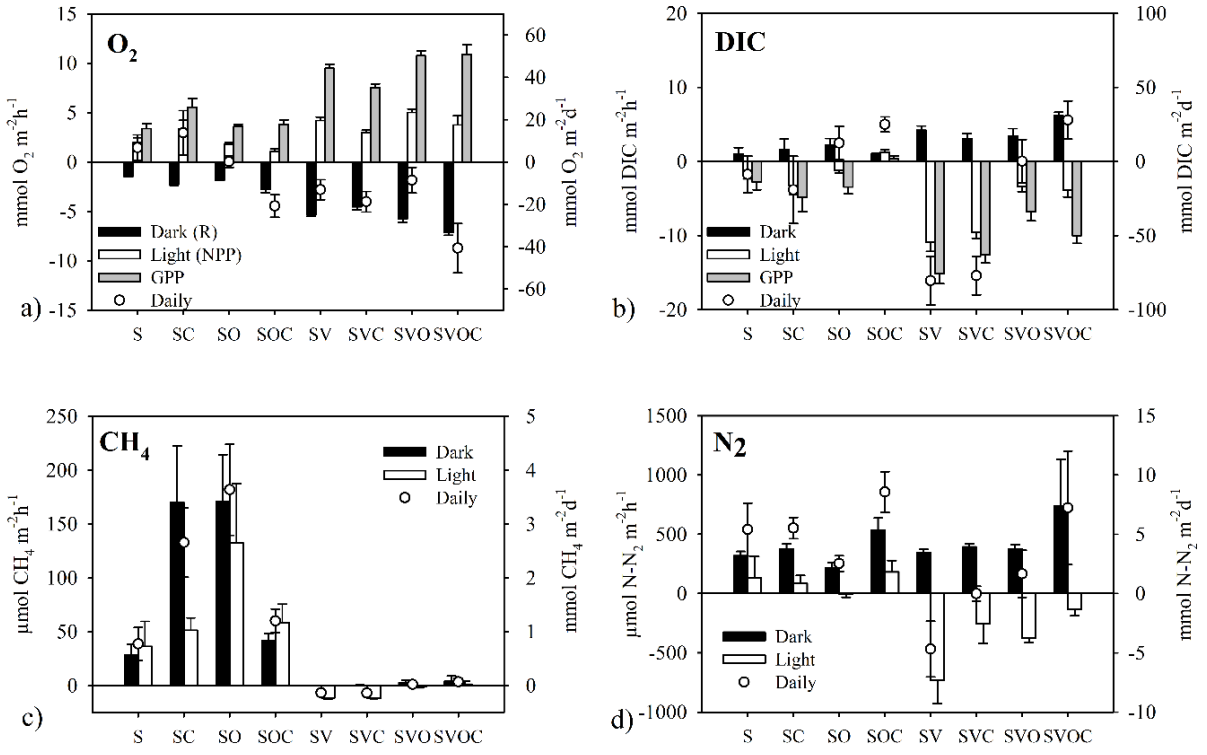


Fig. 4.1.1 Benthic fluxes of a) dissolved O_2, measured in dark (R= Respiration; black bars) and in light (NPP= Net Primary Production; white bars) incubations in the 8 treatments (n=4). b) Dissolved inorganic carbon (DIC); c) CH_4 and d) N_2 measured in light (white bars) and dark (black bars) incubations in the 8 treatments (n=4). Grey bars in O_2 and DIC graphs represent Gross Primary Production (GPP). Mean ± standard error are reported. All fluxes are expressed in µmol $m^{-2}h^{-1}$ or mmol $m^{-2}h^{-1}$. Dots represent daily mean ± standard error expressed in mmol $m^{-2}d^{-1}$.

In the dark NH_4^+ was regenerated to the water column, with the only exception of SVO treatment (Fig. 4.1.2a). Net NH_4^+ fluxes in the light were negative in presence of *V. spiralis*, suggesting the assimilation of this nutrient by the plant, and in SC treatment. In S, SO and SOC treatments, NH_4^+ fluxes were positive and significantly different from the other treatments. Daily NH_4^+ budgets were variable among treatments without any specific trend. Net NO_2^- fluxes were low compared with trends of other N forms (Fig. 4.1.2b). The highest value was found in

SO in the dark condition with a rate of 26.7 ± 5.3 µmol N $m^{-2}h^{-1}$. Nitrate fluxes in microcosms without *V. spiralis* were strongly influenced by the presence/absence of oligochaetes (Fig. 4.1.2c). In both light and dark conditions, in fact, *S. tamesis* caused an increase of NO_3^- consumption. The situation was completely different in presence of *V. spiralis*, where a low NO_3^- efflux was measured in light conditions, with the exception of SVOC. On the contrary, in dark incubations a consumption of NO_3^- was measured in all treatments, with the exception of bare sediment (S) and of the most complex treatment (SVOC). Dissolved inorganic nitrogen fluxes (DIN) were mostly driven by NO_3^- fluxes (Fig. 4.1.2d).

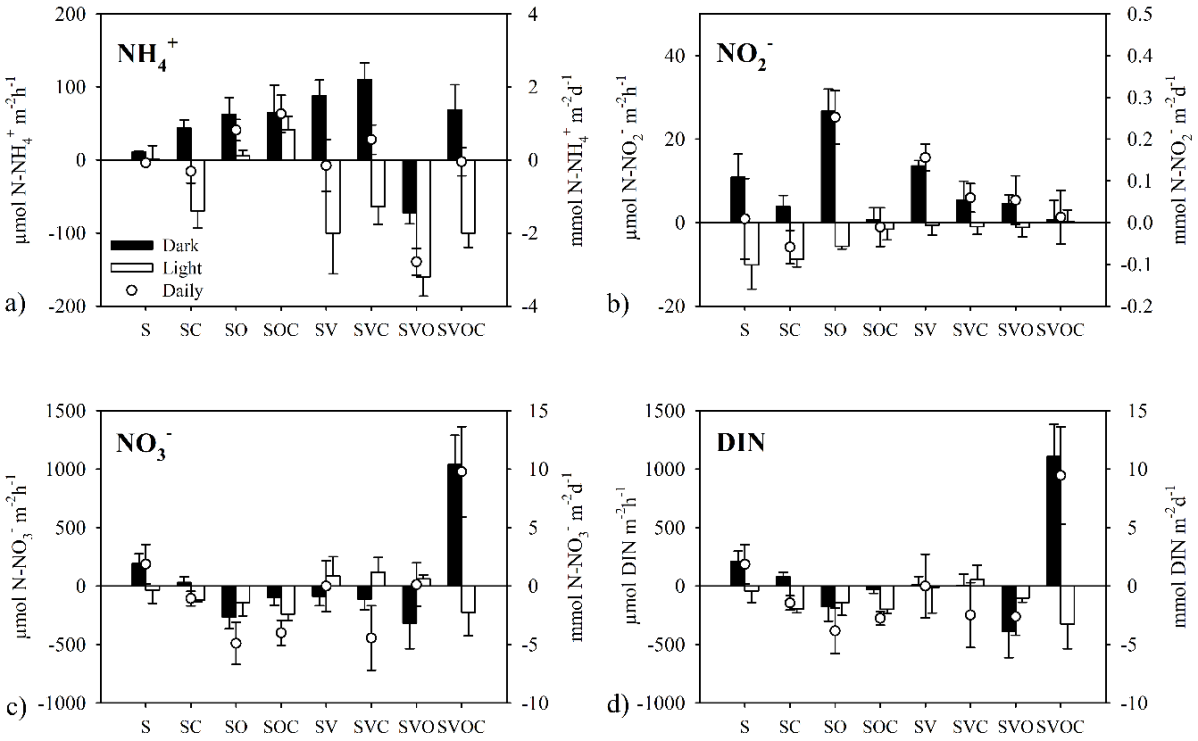


Fig. 4.1.2 Light and dark benthic fluxes of: a) NH_4^+, b) NO_2^-, c) NO_3^- and d) DIN measured in the 8 treatments (n=4). Mean ± standard error are reported. All fluxes are expressed in µmol $m^{-2}h^{-1}$. Dots represent daily mean ± standard error of nutrient fluxes expressed in mmol $m^{-2}d^{-1}$.

Sediment features and potential activities

The presence of oligochaetes, bivalves and macrophytes produced a significant effect on sediment features (Table 4.1.1). Sediment density was slightly lower in bioturbated sediments than in bare sediment, whereas sediment porosity increased in sediment with oligochaetes and decreased in sediments with bivalves but both parameters were not significantly different among treatments. Organic matter content did not differ among treatments and a depth-integrated value was around 13 %. Ammonium concentrations in pore water resulted lower in sediments with *V. spiralis* and higher in presence of *Corbicula*. The significant pairwise were: SC vs SVO, SC vs SV, S vs SVO (Tukey HSD, $p<0.05$). The same result was confirmed with

the measurement of exchangeable NH_4^+, as in presence of *V. spiralis* NH_4^+ concentrations were significantly lower compared to non-vegetated sediments (Tukey HSD, $p<0.05$). The presence of *Corbicula*, on the contrary, increased NH_4^+ concentration in sediments.

Table 4.1.1 Sedimentary features and NH_4^+ concentrations of the 8 experimental units. All reported numbers are averages ± standard error, n=4.

Treatment	Density	Porosity	OM content	Pore water NH_4^+	Exchangeable NH_4^+
	g cm^{-3}	ml ml^{-1}	%	µM	µmol cm^{-3}
S	1.20 ± 0.02	0.87 ± 0.01	13.0 ± 0.2	31.6 ± 3.6	0.31 ± 0.06
SC	1.17 ± 0.00	0.87 ± 0.00	13.1 ± 0.4	38.3 ± 4.5	0.62 ± 0.02
SO	1.19 ± 0.02	0.88 ± 0.01	13.3 ± 0.3	28.0 ± 3.8	0.40 ± 0.02
SOC	1.20 ± 0.00	0.87 ± 0.00	13.0 ± 0.3	25.7 ± 1.1	0.64 ± 0.12
SV	1.17 ± 0.01	0.86 ± 0.01	13.2 ± 0.7	6.0 ± 0.2	0.10 ± 0.00
SVC	1.19 ± 0.01	0.86 ± 0.00	12.8 ± 0.7	8.0 ± 0.3	0.09 ± 0.00
SVO	1.20 ± 0.01	0.88 ± 0.01	14.0 ± 2.4	5.4 ± 0.3	0.10 ± 0.01
SVOC	1.17 ± 0.01	0.85 ± 0.01	12.9 ± 0.4	10.1 ± 1.2	0.08 ± 0.02

Potential denitrification rates where significantly lower in treatments with *V. spiralis* compared to the other 4 treatments (one-way ANOVA, $p<0.001$; Fig. 4.1.3a). Nitrogen fixation rates resulted significantly higher in treatments with macrofauna and without *V. spiralis* (one-way ANOVA, $p<0.001$; Fig. 4.1.3b).

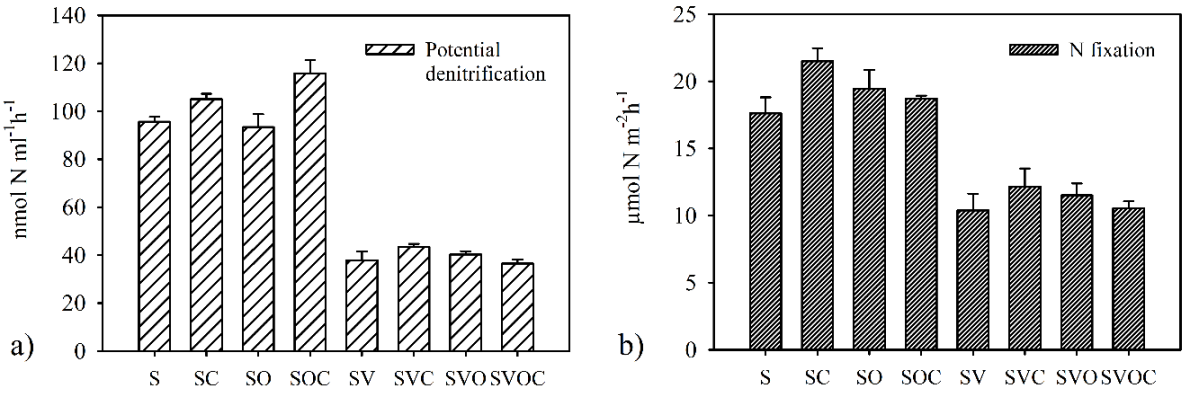


Fig. 4.1.3 Potential denitrification (a) and N fixation (b) measured in the 8 treatments by means of slurry incubations.

No anammox was measured in any treatment.

At the end of the experiment all macrophytes from the different treatments looked healthy, with new shoots and propagules and all introduced macrofauna was retrieved alive. The biomass of benthic microalgae (as chl *a* in the upper sediment layer), and that of macrophytes, oligochaetes and bivalves (as dry weight) measured at the end of the incubations are reported in table 4.1.2. Chl *a* concentrations differed among treatments (one-way ANOVA, $p<0.05$) and the lowest values were measured in treatments with oligochaetes.

Table 4.1.2 Biomass of benthic microalgae (MPB), macrophytes and macrofauna measured in the different microcosms at the end of the incubations. Chl *a* was used as a proxy of MPB abundance. Averages ± standard deviation (n=4) are reported.

	MPB	**Macrophytes**			**Macrofauna**	
	Chl *a*	**Leaves**	**Roots**	**Total**	**Oligochaetes**	**Bivalves**
	mg m^{-2}	g_{dw} m^{-2}	g_{dw} m^{-2}	g_{dw} m^{-2}	g_{dw} m^{-2}	g_{dw} m^{-2}
S	112.3 ± 20.0	-	-	-	-	-
SC	89.3 ± 13.0	-	-	-	-	57.6 ± 4.6
SO	67.7 ± 4.6	-	-	-	40.3 ± 5.2	-
SOC	55.2 ± 4.7	-	-	-	39.7 ± 6.8	66.1 ± 19.5
SV	85.2 ± 14.5	81.9 ± 12.6	72.9 ± 5.0	154.8 ± 15.4	-	-
SVC	100.0 ± 6.1	52.3 ± 12.2	52.6 ± 14.2	104.8 ± 23.7	-	44.6 ± 14.8
SVO	81.4 ± 11.0	59.6 ± 18.0	43.6 ± 12.9	103.2 ± 29.9	40.8 ± 9.2	-
SVOC	84.9 ± 4.6	70.9 ± 13.0	56.3 ± 13.6	127.8 ± 26.2	38.0 ± 5.1	64.1 ± 10.1

Discussion

At the end of the experiments, all the 32 microcosms were sieved to quantify the biomass of oligochaetes, bivalves, roots and leaves of macrophytes. After one month and half of maintenance in aquaria under controlled conditions, all the organisms were alive. Microcosms with oligochaetes were characterized by an extended system of burrows along all the 10 cm depth. Due to the presence of burrows, sediment was extremely fluffy, as shown in density and porosity measured values. A narrow sediment layer close to the burrows and to the roots was characterized by a light brown color, which represented the oxidized zones. Microcosms were maintained under controlled conditions for 5 weeks for the acclimatization period. Sediment surfaces were colonized by benthic microalgae with high level of biomass, as reported in data from the analysis of chl *a* and from O_2 net fluxes measured in the light in treatments without *V. spiralis*. Net and gross primary production resulted higher in presence of macrophytes both for O_2 and DIC fluxes, since the photosynthetic activity of microalgae could be affected by the

reworking activity of macrofauna, which covered with feces and pseudofeces the microalgal mats. Although we did not measure directly the ROL, CH_4 data suggest that part of the O_2 released was actively used by microbial communities for CH_4 oxidation (Jespersen et al. 1998; Racchetti et al. 2010). Furthermore, our results highlighted high NH_4^+ recycling in presence of macrofauna, which could favor the growth of benthic primary producers. In fact, as we found no significant differences in NO_3^- fluxes between day and night measurements, we excluded NO_3^- uptake by the primary producers. Despite high NO_3^- concentration in the water column, *V. spiralis* and microalgae preferred the assimilation of NH_4^+, since NO_3^- assimilation requires more energy (Miller and Cramer 2004; Volkmann et al. 2016).

Dissolved O_2, pH and conductivity measured during the acclimatization period in the eight tanks, highlighted differences among treatments since the second day after the realization of microcosms (data not shown). In particular, significant differences were found among treatments: the presence of macroinvertebrates determined O_2 under saturation and high conductivity. Vice versa, the presence of *V. spiralis* increased significantly O_2 level during light conditions whilst conductivity values decreased due to the photosynthetic activity and nutrients uptake. Ammonium concentration in pore water highlighted the macroscopic differences among the 8 experimental units related to the presence of bioturbators and macrophytes. Bioturbators through burrow activities, reworking and biodeposition determined an increase of inorganic N in pore water, whilst macrophytes determined an N decrease due to assimilative processes (uptake) or to the enhancement of nitrification and denitrification processes. Oxygen and DIC fluxes allowed estimating the theoretical N assimilation by primary producers. The calculation was based on the ratio between gross fluxes and the C:N ratio of benthic microalgae (~ 9; Sundbäck et al. 2004) and rooted macrophytes (~ 13; Pinardi et al. 2009; Racchetti et al. 2010). In all treatments, it was evident a strong discrepancy between measured N fluxes and the theoretical N demand. This difference could be explained in two different ways on the basis of primary producers' typology. In the case of benthic microalgae, the portion of missing N was assimilated from the pore water, where this nutrient is mostly concentrated, whereas data from pore water and microbial activities in microcosms with *V. spiralis* revealed a strong N limitation due to plant uptake. In fact, pore water and exchangeable NH_4^+ concentrations were low in sediments with *V. spiralis*. These results could explain the highly negative N_2 fluxes measured during light conditions. Overall, these results suggest that *V. spiralis* may favor the process of N fixation in the rhizosphere to support its N requirement. In particular, the treatment with only *V. spiralis* showed the highest N_2 flux, whereas the lowest flux (among the treatments with *V. spiralis*) was found in the presence of both macrofauna functional groups (SVOC). The discrepancy was likely due to the NH_4^+ mobilization by bioturbators, through direct excretion

and feces production, reworking and bioirrigation, as it was hypothesized. This result might suggest a positive feedback among macrofauna, mobilization of nutrients not bioavailable and primary production (Mermillod-Blondin and Lemoine 2010). To understand if macrofauna may facilitate *V. spiralis*, net and gross diffusive fluxes of O_2 and DIC were normalized by leaves biomass (Fig. 4.1.4). The normalized fluxes indicate a slightly higher O_2 production per gram of leaf in all the treatments with macrofauna compared to the control (SV), in particular it was higher in SVO treatment (one-way ANOVA, $p<0.05$). Normalized DIC fluxes showed a different trend. The most negative flux was measured in SVC, whilst the presence of the oligochaetes reduced DIC fluxes even more than SV. The latter result indicated the need for further studies in order to understand the role of this deep burrower in facilitating plant growth.

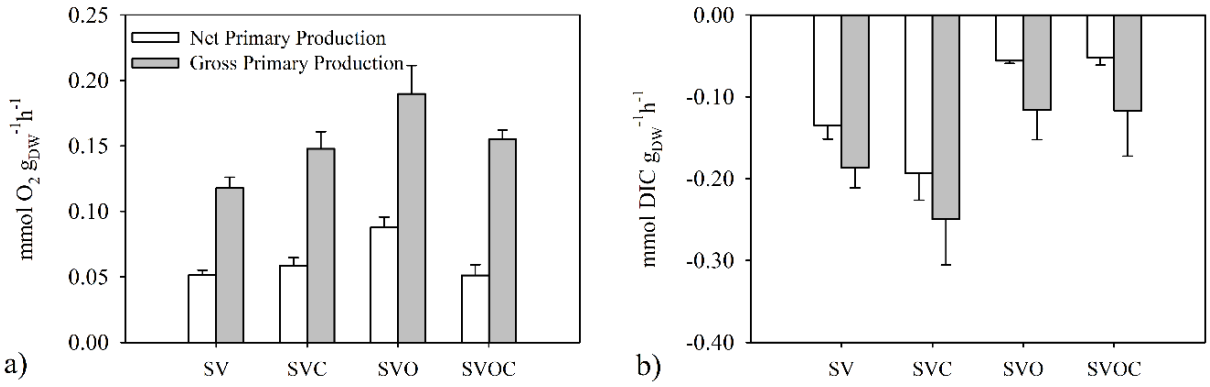


Fig. 4.1.4 Net (white bars) and gross (grey bars) fluxes of O_2 (a) and DIC (b) normalized by *V. spiralis* biomass.

The present study highlighted also the relationship between N and C fixation in presence of *V. spiralis*. By calculating the linear regression between N_2 and DIC fluxes measured during the light incubations in microcosms with *V. spiralis* we found a slope of 13 (Fig. 4.1.5). Despite standard errors associated to mean values, the result is extremely interesting. The C:N molar ratio that we found is very close to the C:N measured previously in other studies on *V. spiralis* (Pinardi et al. 2009; Racchetti et al. 2010) and it suggests that a relevant part of the N used by the plants comes from the atmosphere. The treatments which displayed the highest rates of N fixation had also the highest rates of primary production. This result represents a novelty in a system like Mincio River where there is high N availability in the water column. Nevertheless, the most abundant N form is NO_3^-, *V. spiralis* stimulated N fixation in order to import N that could be converted to NH_4^+ (Miller and Cramer 2004; Volkmann et al. 2016). Ammonium limitation in the water column could be the reason for the high N fixation in treatments with *V. spiralis*.

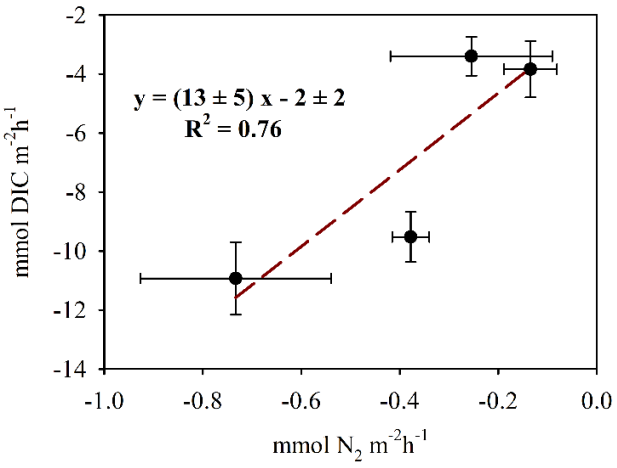


Fig. 4.1.5 Linear regression between net light flux of N_2 and of DIC measured in the 4 treatments with *V. spiralis*.

Further investigations are needed in order to understand the discrepancy between net N_2 fluxes measured during microcosm incubations and rates of N fixation measured with slurry incubation. We suppose that this difference could be due to the different bacterial activity when *V. spiralis* roots are present (microcosm incubation) or absent (slurry incubation).

References

Anderson L.G., Hall P.O.J., Iverfeldt A., Rutgers Van Der Loeff M.M., Sundby B., & Westerlund S.F.G. (1986) Benthic respiration measured by total carbonate production. Limnology and Oceanography, 31: 319 – 329.

Bartoli M., Nizzoli D., & Viaroli P. (2003) Microphytobenthos activity and fluxes at the sediment-water interface: Interactions and spatial variability. Aquatic Ecology, 37: 341 – 349.

Bertics V.J., Sohm J.A., Treude T., Chow C.E.T., Capone D.G., Fuhrman J.A., & Ziebis W. (2010) Burrowing deeper into benthic nitrogen cycling: the impact of bioturbation on nitrogen fixation coupled to sulfate reduction. Marine Ecology Progress Series, 409: 1 – 15.

Bower C.E., & Holm-Hansen T. (1980) A salicylate-hypochlorite method for determining ammonia in seawater. Canadian Journal of Fisheries and Aquatic Sciences, 37: 794 – 798.

Chase J.M., & Knight T.M. (2006) Effects of eutrophication and snails on Eurasian watermilfoil (*Myriophyllum spicatum*) invasion. Biological Invasions, 8: 1643 – 1649.

Dalsgaard T., Nielsen L.P., Brotas V., Viaroli P., Underwood G., Nedwell D., ... & Dong L. (2000) Protocol handbook for NICE-Nitrogen Cycling in Estuaries: a project under the EU research programme: Marine Science and Technology (MAST III), 1 – 62.

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.

Jespersen D.N., Sorrell B.K., & Brix H. (1998) Growth and root oxygen release by *Typha latifolia* and its effects on sediment methanogenesis. Aquatic Botany, 61: 165 – 180.

Kristensen E. (2000) Organic matter diagenesis at the oxic/anoxic interface in coastal marine sediments, with emphasis on the role of burrowing animals. Hydrobiologia, 426: 1 – 24.

Loreau M. (2010) Linking biodiversity and ecosystems: towards a unifying ecological theory. Philosophical Transactions of the Royal Society of London B: Biological Sciences, 365: 49 – 60.

Lorenzen C.J. (1967) Determination of chlorophyll and phaeopigments: Spectrophotometric equations. Limnology and Oceanography, 12: 343 – 346.

McGlathery K.J., Risgaard-Petersen N., & Christensen P.B. (1998) Temporal and spatial variation in nitrogen fixation activity in the eelgrass *Zostera marina* rhizosphere. Marine Ecology Progress Series, 168: 245 – 258.

McGlathery K.J., Sundbäck K., & Anderson I.C. (2007) Eutrophication in shallow coastal bays and lagoons: The role of plants in the coastal filter. Marine Ecology Progress Series, 348: 1 – 18.

Mermillod-Blondin F., & Lemoine D.G. (2010) Ecosystem engineering by tubificid worms stimulates macrophytes growth in poorly oxygenated wetland sediments. Functional Ecology, 24: 444 – 453.

Miller A.J., & Cramer M.D. (2004) Root nitrogen acquisition and assimilation. Plant and Soil, 274: 1 – 36.

Nizzoli D., Bartoli M., Cooper M., Welsh D.T., Underwood G.J.C., & Viaroli P. (2007) Implications for oxygen, nutrient fluxes and denitrification rates during the early stage of sediment colonisation by the polychaete *Nereis* spp. in four estuaries. Estuarine, Coastal and Shelf Science, 75: 125 – 134.

Pelegrì S.P., & Blackburn T.H. (1994) Bioturbation effects of the amphipod *Corophium volutator* on microbial nitrogen transformations in marine sediments. Marine Biology, 121: 253 – 258.

Pelegrì S.P., Nielsen L.P., & Blackburn T.H. (1994) Denitrification in estuarine sediment stimulated by the irrigation activity of the amphipod *Corophium volutator*. Marine Ecology Progress Series, 105: 285 – 290.

Pelegrì S.P., & Blackburn T.H. (1995) Effects of *Tubifex tubifex* (Oligochaeta:Tubificidae) on N-mineralization in freshwater sediments, measured with ^{15}N isotopes. Aquatic Microbial Ecology, 9: 289 – 294.

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. Journal of Limnology, 68: 133 – 145.

Racchetti E., Bartoli M., Ribaudo C., Longhi D., Brito L.E.Q., Naldi M., … & 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.

Racchetti E., Longhi D., Ribaudo C., Soana E., & Bartoli M. (2017) Nitrogen uptake and coupled nitrification–denitrification in riverine sediments with benthic microalgae and rooted macrophytes. Aquatic Sciences, 79: 487 – 505.

Rota E., Bartoli M., & Laini A. (2014) First time in Italy. Is the elusive aquatic megadrile *Sparganophilus* Benham, 1892 (Annelida, Clitellata) accelerating its dispersal in Europe?. Journal of Limnology, 73: 482 – 489.

Risgaard-Petersen N. (2003) Coupled nitrification-denitrification in autotrophic and heterotrophic estuarine sediments: On the influence of benthic microalgae. Limnology and Oceanography, 48: 93 – 105.

Ruginis T., Bartoli M., Petkuviene J., Zilius M., Lubiene I., Laini A., & Razinkovas-Baziukas A. (2014) Benthic respiration and stoichiometry of regenerated nutrients in lake sediments with *Dreissena polymorpha*. Aquatic Sciences, 76: 405 – 417.

Rysgaard S., Christensen P.B., & Nielsen L.P. (1995) Seasonal variation in nitrification and denitrification in estuarine sediment colonized by benthic microalgae and bioturbating infauna. Marine Ecology Progress Series, 126: 111 – 121.

Soana E., & Bartoli M. (2014) Seasonal regulation of nitrification in a rooted macrophyte (*Vallisneria spiralis* L.) meadow under eutrophic conditions. Aquatic Ecology, 16: 2064 – 2090.

Soana E., Naldi M., Bonaglia S., Racchetti E., Castaldelli G., Brüchert V., … & Bartoli M. (2015) Benthic nitrogen metabolism in a macrophyte meadow (*Vallisneria spiralis* L.) under increasing sedimentary organic matter loads. Biogeochemistry, 124: 387 – 404.

Stief P. (2013) Stimulation of microbial nitrogen cycling in aquatic ecosystems by benthic macrofauna: Mechanisms and environmental implications. Biogeosciences, 10: 7829 – 7846.

Stocum E.T., & Plante C.J. (2006) The effect of artificial defaunation on bacterial assemblages of intertidal sediments. Journal of Experimental Marine Biology and Ecology, 337: 147 – 158.

Sundbäck K., Linares F., Larson F., Wulff A., & Engelsen A. (2004) Benthic nitrogen fluxes along a depth gradient in a microtidal fjord: The role of denitrification and microphytobenthos. Limnology and Oceanography, 4: 1095 – 1107.

Sundbäck K., Miles A., & Linares F. (2006) Nitrogen dynamics in nontidal littoral sediments: Role of microphytobenthos and denitrification. Estuaries and Coasts, 29: 1196 – 1211.

Svensson J.M., & Leonardson L. (1996) Effects of bioturbation by tube-dwelling chironomid larvae on oxygen uptake and denitrification in eutrophic lake sediments. Freshwater Biology, 35: 289 – 300.

Thamdrup B., & Dalsgaard T. (2002) Production of N_2 through anaerobic ammonium oxidation coupled to nitrate reduction in marine sediments. Applied and Environmental Microbiology, 68: 1312 – 1318.

Tilman D. (1996) Biodiversity: Population versus ecosystem stability. Ecology, 77: 350 – 363.

Volkmann C., Halbedel S., Voss M., & Schubert H. (2016) The role of dissolved organic and inorganic nitrogen for growth of macrophytes in coastal waters of the Baltic Sea. Journal of Experimental Marine Biology and Ecology, 477: 23 – 30.

Section 4.2

Benthic N pathways in illuminated and bioturbated sediments studied with network analysis

Introduction

Benthic cycling of nitrogen (N) undergoes complex regulation by the interplay of primary producers, macrofauna and microbes (Mermillod-Blondin et al. 2008; Soana et al. 2015; Vila-Costa et al. 2016). Understanding how interactions at the community level affect N dynamics is relevant in the context of loss of both biodiversity and ecosystem services (Vadeboncoeur et al. 2003). This understanding comes from studies that combine community structure and biogeochemistry (Lohrer et al. 2010; Mermillod-Blondin and Lemoine 2010; Herren et al. 2017). But such studies often do not include measurements of multiple N processes. Detailed understanding of complex interactions is, thus, difficult to achieve with traditional observational and experimental approaches quantifying net rates (Loreau 2010).

Microphytobenthos and rooted macrophytes may have a variety of consequences to benthic ecosystems. They assimilate inorganic N from bottom and pore water, mainly as ammonium (NH_4^+), altering concentration gradients across the sediment-water interface (Soana et al. 2012). They, also, release oxygen (O_2) and exudates at the interface or within sediments via the roots, increasing the volume of oxic sediments and favoring microbial processes such as ammonification, nitrification and denitrification (Lemoine et al. 2012; Soana et al. 2015; Vila-Costa et al. 2016). Under N-limiting conditions, primary producers may inhibit both dissimilative microbial processes and N-losses from sediments whereas stimulating heterotrophic N fixation (Risgaard-Petersen and Jensen 1997; Bartoli et al. 2003a; McGlathery et al. 2007). Under N excess, primary producer-bacterial competition is ameliorated, and high rates of assimilation and denitrification may co-occur (Soana et al. 2015). The interactions between autotrophs and heterotrophic microbial communities depend, therefore, upon gradients of N availability, as further influenced by macrofauna (Bartoli et al. 2003b; Ferguson et al. 2004; Mermillod-Blondin et al. 2008).

Macrofauna affects benthic N cycling via sediment ingestion and reworking, burrowing, irrigation and ventilation activities, biodeposition and benthic-pelagic coupling (Kristensen 2000; Stief 2013). These animals can excrete large amounts of NH_4^+, favor its mobilization from pore water, and stimulate microbial ammonification (Stief 2013; Ruginis et al. 2014; Benelli et al. 2017). Activities by both surface and deep burrowers increase microbial N_2 production within sediments via coupled nitrification-denitrification (Pelegrí et al. 1994;

Nizzoli et al. 2007). This can, in turn, stimulate N fixation along burrows and compensate N losses (Henriksen et al. 1983; Bertics et al. 2010). Finally, macrofauna mobilizes deep or refractory sedimentary N pools, resulting in regeneration, which may favor primary production and lessen negative feedbacks between assimilative and dissimilative paths (Mermillod-Blondin et al. 2008).

The effects of primary producers and macrofauna on benthic N cycling are sometimes studied with simplified experimental approaches targeting single species or functional groups and focusing on single processes. This leads to a partial interpretation of the effects of multiple interactions among different organisms (Raffaelli et al. 2003; Loreau 2010). We have expanded the ability to understand benthic N cycling across gradients of community complexity. Specifically we combined three approaches: (1) microcosm experiments to reproduce the gradients in community structure, (2) measurements of an array of N-processes, and (3) construction of mass balance models with Ecological Network Analysis (ENA). Our strategy makes explicit the array of coupled paths determining net N fluxes. We investigated whether competitive interactions for N among primary producers and bacteria are ameliorated by different macrofaunal functional groups, via mobilization of sedimentary and pelagic N pools. We also analyzed how microbial processes, such as N fixation and denitrification driving net N_2 fluxes, are differentially stimulated or depressed by the combined activity of macrofauna and primary producers.

Mass balance network models and their analysis enable the observation and the quantification of direct and indirect relationships among network components as well as provide system-level attributes (Christian et al. 2016). Accordingly, Ecological Network Analysis (ENA, henceforth) can help disentangle the importance of co-occurring processes and the role of different taxa within a community. Ecological network analysis actually refers to a suite of algorithms that address numerous aspects of network flow structure (Borrett and Lau 2014). ENA has been used extensively to interpret food web interactions and compare web networks using system-level indices (Ulanowicz and Puccia 1990; Salas and Borrett 2011). It has also been applied to N cycles across a variety of ecosystems and scales (Christian et al. 2016). For example, Christian and Thomas (2003) and Small et al. (2014) traced the paths of different forms of imported N through the networks of two different aquatic ecosystems and determined the amount of recycling. Other studies focused on the role of a single compartment on N cycling, especially primary producers and their different growth forms (Christian et al. 1996). Hines et al. (2012) constructed and analyzed benthic N cycling with networks that highlighted microbial processes. They evaluated the strength of coupling between different microbial N processes and the importance of each compartment relative to others through both direct and indirect paths.

Hines et al. (2012) constructed and analyzed their networks at the cm^3 scale. But other analyses have spanned scales as large as the Baltic Sea (Wulff et al. 1989). In fact most ENA applications have been done at the large ecosystem scale (Christian et al. 2016). We uniquely applied ENA to the scale of the microcosm in this research. In microcosms the limited number of components does not necessarily simplify N cycling as the numbers of compartments and flows in our networks are comparable to those found in networks of larger ecosystems (Christian et al. 2016). Downscaling in fact is implemented to analyze processes at a finer resolution, and this reveals how net effects depend on multiple paths of exchange. ENA disentangles the nature of this dependence. In addition, measuring most of the pools and input data under controlled conditions overcomes the common limitation for larger systems that many input data are estimated or taken from the literature (Christian et al. 2016).

The purpose of this work was to analyze, via combined experimental and modelling approaches, the share of sedimentary N fluxes among bacteria, macrofauna and primary producers across a gradient of benthic complexity. We studied the role and the interactions of two bioturbators (*Sparganophilus tamesis* and *Corbicula* spp., a deposit and a filter feeder, respectively); two growth forms of primary producers (the rooted macrophyte *Vallisneria spiralis* and microphytobenthos); and N-related microbial communities on benthic N cycling in illuminated sediments. Specific questions were as follows: do different macrofaunal functional groups attenuate the competitive interactions for N between primary producers and bacteria, and if so how? Are microbial N fixation and denitrification differentially stimulated by the combined activity of macrofauna and primary producers, and if so how?

We hypothesized that a biodiverse benthic system should better exploit the benthic N availability and retain N than a simpler one. Retention occurs by avoiding losses both to the water column via increased recycling and to the atmosphere via decreased N_2 fluxes and by limiting energy-costly processes as N fixation. We also hypothesized that primary producer-bacterial competition is reduced in the presence of macrofauna due to mobilization of refractory N pools.

Material and methods

A detailed description of the experiment that generated the data for the network construction is reported in section 4.1. Briefly, a set of short-term batch incubations were performed in the dark and in the light (Dalsgaard et al. 2000). The experimental design consisted of eight conditions, each with n=8 replicated microcosms: bare sediment (S), sediment with *Corbicula* spp. (SC), sediment with *S. tamesis* (SO), sediment with *V. spiralis* (SV), sediment with *Corbicula* spp. and *S. tamesis* (SOC), sediment with *Corbicula* spp. and *V. spiralis* (SVC), sediment with *S.*

tamesis and *V. spiralis* (SVO), and sediment with *S. tamesis*, *Corbicula* spp. and *V. spiralis* (SVOC). Sequential to flux measurements, rates of denitrification were quantified in the light and in the dark via the isotope pairing technique (IPT, Nielsen 1992; Soana et al. 2015). Briefly, at the beginning of the experiment, $^{15}NO_3^-$, from a stock 15 mM $Na^{15}NO_3$ solution, was added to the water phase of each of the replicate cores. Labelled nitrate was added to provide a final ^{15}N atom% of at least 30 %. The cores were then closed with gas-tight lids and the incubation started. After 2 hours the sediment and water were gently mixed together. An aliquot of the slurry was transferred to a 12.5 ml gas-tight vial; $^{14}N^{15}N$ and $^{15}N^{15}N$ abundance in N_2 were analyzed by membrane inlet mass spectrometry. In the Mincio River sediments the contribution of anammox to N_2 production was demonstrated to be low (< 2 %, Soana et al. 2015), as generally reported for organic-rich freshwater sediments. We therefore assumed that the IPT is reliable in our system. Soana et al. (2015) demonstrated that in the Mincio River sediments the dissimilative nitrate reduction to ammonium (DNRA) was also a minor process. These authors demonstrated that denitrification of water column NO_3^- explained most of dark nitrate uptake by sediments and that denitrification was the dominant N sink. In our network analysis anammox and DNRA were included, but with minimal flows.

Network construction

Pools and fluxes associated with the various processes were measured and averaged for each experimental condition (n=8) under dark and light, and a total of 16 networks of benthic N cycling were constructed. The networks consisted of individual compartments representing standing stocks of N connected by flows of N. Flows with the outside described N imports and exports. Import of sediment PN was adopted as an accounting device to represent mineralization of this large, largely refractory pool. All networks shared 9 compartments which represented inorganic and organic N compounds in the water column ("$NH_4^+{}_w$", "$NO_x^-{}_w$", "$N_{2\,w}$", "DON_w", "PN_w") and in the sediment ("$NH_4^+{}_s$", "$NO_x^-{}_s$", "$N_{2\,s}$", "PN_s"). Some of these compartments ("$NH_4^+{}_w$", "$NO_x^-{}_w$", "$N_{2\,w}$","$NH_4^+{}_s$", "$NO_x^-{}_s$", "$N_{2\,s}$") were measured whereas the remaining were estimated.

Networks representing different conditions potentially included N pools for the macrofaunal taxa *S. tamesis* ("O") *Corbicula* spp. ("C") and the macrophyte *V. spiralis*. Appropriate networks included *V. spiralis* as two different compartments, one for N in leaves ("V_l") and another for N in roots ("V_r"). Every network also contained a compartment representing benthic microalgae (microphytobenthos = "MPB"), which naturally grew on sediment. The most complex condition included 14 active compartments (Fig. 4.2.1). All standing stock values were obtained from the experiment. For N compounds molar concentration values of starting

condition were standardized to µmol N m^{-2}. N pools in macrofauna, macrophytes and benthic algae were calculated from dry biomass at the end of experiment and elemental composition taken from the literature and, also, expressed as µmol N m^{-2} (Fink et al. 2006; Pinardi et al. 2009; Atkinson et al. 2010). N in microphytobenthos was calculated from chlorophyll *a* in the upper sediment layer, using a C : Chl *a* ratio of 40 (g:g, Winberg et al. 1973) and a 106:16 C:N molar ratio (Redfield 1958).

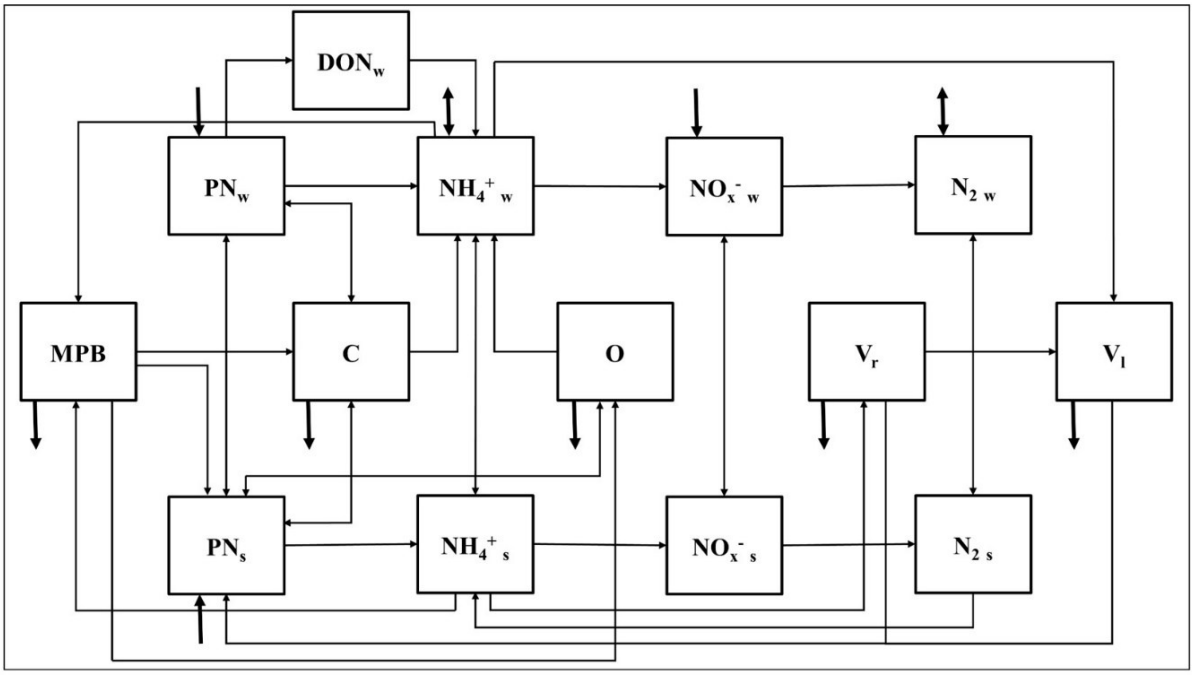


Fig. 4.2.1 Graph showing all compartments and connecting flows in the most complex experimental condition (SVOC) including: *Corbicula* spp. (C); *Sparganophilus tamesis* (O); *Vallisneria spiralis*, divided in two different compartments (leaves (V_l) and roots (V_r)); and benthic microalgae (MPB). The other compartments represent organic and inorganic N compounds in the water column and in the sediment. Their designations are found in the text.

Compartments were internally connected via feeding, biogeochemical, and detrital pathways. We quantified flows between compartments as well as flows connecting internal components to the outside system. We derived most flows from N and TCO_2 net fluxes at the sediment-water interface and denitrification rates obtained by the IPT. In some cases we used comparisons between dark and light incubations and comparisons between conditions of increasing complexity and in a few instances literature data. When a flow was considered negligible, it was given a minimal value of 0.001 µmol N $m^{-2}h^{-1}$ (Christian et al. 2010). All flow rates were expressed as µmol N $m^{-2}h^{-1}$.

A steady state assumption facilitates the interpretation of some analyses and is required for others; this means that the sum of matter entering any given compartment equals the sum leaving the compartment. Balancing networks to achieve steady state resulting from data uncertainty is a universal practice in network modeling (Allesina and Bondavalli 2003). As

stated previously, incubations were kept short to foster steady state, but some changes in standing stock occurred or were inferred. In these microcosms, therefore, imports and exports within the network models served as conveniences to facilitate analysis. They represented gain to and loss from the biologically active components of microcosms. Overall network steady state was achieved by balancing imports (most notably PN_s and $N_{2\ w}$) with exports as N_2 loss (i.e., through denitrification) and accumulation of biomass N. To balance each compartment, adjustments were made to the flows by acting mainly on those flows that were estimated; they were changed as needed to reach at least 15 % unbalanced in respect to each compartment throughput. This procedure was carried out on estimated flows first, leaving the values derived from direct measurements during the experimental phase unmodified. Finally the software "NetBalance" (Allesina and Bondavalli 2003) was applied for a final overall balancing of the networks.

Analyses

ENA is actually a suite of analyses and not one. We chose two widely used analyses based on matrix algebra (Borrett et al. 2013; Christian et al. 2016): input analysis and total dependency analysis. The structure of each network was represented by an exchange matrix of flows between compartments and vectors for import, export and biomass. This was depicted as a graph (Fig. 4.2.1). Analyses were conducted on the matrices and vectors using "WAND" (Allesina and Bondavalli 2004), a software developed for ENA applications.

Input analysis tracks the fate of specific forms of imported N as they pass through the system. As applied here and explained earlier, an atom of PN_s that is decomposed is represented as import (Fig. 4.2.1). It may pass through multiple compartments and transformations before being exported. Export is represented in our networks as N_2 loss (i.e., through denitrification) and accumulation of biomass N in our microcosm networks. Input analysis indicates the frequencies that imported N (sic, decomposed PN_s) pass through each pathway or process within and leaving the network. In general input analysis is calculated through a series of algorithms conducted on the initial exchange matrix and flow vectors. The analysis then calculates the frequencies of individual imports as apportioned to the various interactions within the system and exports. For example, one obtains an estimate of how much of the original decomposed PN_s is denitrified. These frequencies are often portrayed as percentages, although values greater than 100 can occur. These high values reflect rapid recycling within the system. Each import is considered separately and results were additive across imports; however, we only present the fate of PN_s decomposition - the major source of N for cycling within the microcosms.

We applied input analysis to our N networks representing the 8 experimental conditions. Data for these networks were obtained by averaging all dark and light estimated fluxes. We focused our analysis on the flow of PN "imported" to sediments to test the hypothesis of the relationship of biodiversity and N availability and because this input flow is the only one which is present in all the experimental schemes. This was not truly a flow of N physically from outside the microcosm; rather a process actually representing the portion of N depleted from the sediment by decomposition and assimilation. To guarantee a steady state condition this depletion of PN into the available N pools was offset as input into PN. Since this flow was estimated for every single network, direct comparison among experimental conditions as for input analysis was possible.

Total dependency analysis addresses the question: how dependent is the flow through one compartment on each other compartment? The dependence can be direct as that of microalgae on the uptake of NH_4^+ in sediments (Fig. 4.2.1). Or, more interestingly, it can be indirect as the dependence of microalgae on *V. spiralis* leaves where multiple transformations are needed prior to access by the microalgae. The algorithm for total dependency analysis produces a matrix (TDM) in which the coefficients express the fraction of each compartment's throughput that had previously resided at some point in another compartment (Hannon 1973). Compartmental throughput is the sum of either the inputs to or outputs from the compartment. In steady state the sum of inputs equals the sum of outputs. The TDM calculates the dependency of each compartment on each the other compartment relative to its throughput. The diagonal elements of the dependency matrix are interpreted as the fractions of throughput that compartments recycle back to themselves. We used this analysis to understand how the various components of the microcosm's N cycle support primary producers within the different conditions. We, also, assessed the dependency of the water column N_2 pool on all the biotic and abiotic compartments as an analysis of N loss from the systems.

Results

Input analysis

Results of input analysis are presented as graphs showing the main benthic compartments and the corresponding N flows (Figs. 4.2.2 and 4.2.3). We inferred that values indicate the percentage of decomposed ("imported") N-PN_s as it passes along each flow. Each graph represents one of the 8 experimental conditions averaged across incubations in the light and dark. Thus, each network reflects the integrated daily conditions.

Fig. 4.2.2a shows how 100 units of N entering as biologically available PN_s is distributed in the microcosm containing bare sediment (condition S). The flow from PN_s to $NH_4^+{}_s$, representing

gross N mineralization, was shared largely between MPB uptake (77 %) and coupled nitrification-denitrification (83 %). The former prevailed during the light phase and the latter during dark. Most of the N assimilated by primary producers (MPB) as $NH_4^+_s$ was recycled within the system returning to PN_s (67 %) and back to $NH_4^+_s$ and MPB. The transfer from $NH_4^+_s$ to $NH_4^+_w$, representing net N regeneration, was low (6 %) and it was found mainly in the dark. As a result, little to no accumulation of N-$NH_4^+_w$ occurred (i.e., what we calculate as "export" of $NH_4^+_w$). The largest portion of N was inferred to exit the system as N_2 (83 %), via denitrification.

In the presence of *Corbicula* spp. (SC, Fig. 4.2.2b) N recycling increased. Recycling may be traced as flows connecting sequential compartments returning to the original one. Such recycling patterns in ENA are called loops. A loop involving PN_s, $NH_4^+_s$ and MPB (216 % - 136 % - 118 %) showed more recycling in condition SC compared to condition S (167 % - 77 % - 67 %). The flows representing microalgal degradation, MPB uptake and PN_s mineralization, showing percentages higher than 100 %, indicate that most of the processes were substantially supported by recycling. MPB uptake of $NH_4^+_s$ during light period (136 %) competed with nitrification (flux from $NH_4^+_s$, to $NO_x^-_s$, 82 %). But coupled denitrification was considerable with 69 % of the initial input from PN_s leaving as N_2. This latter percentage was slightly lower than for condition S (83 %), due to a small fraction of N_2 (13 %) directed to $NH_4^+_s$ (N fixation). Thus, *Corbicula* enhanced N recycling (related to MPB and N fixation) even if this component was not directly involved in large N flows.

S. tamesis (SO, Fig. 4.2.2c) reworked sediment, causing large fluxes and associated frequencies (ingestion, 1,174 %, and defecation, 1,141 %) between the oligochaete and PN_s. Only a small fraction of the initial input to PN_s was assimilated by the oligochaete for growth, representing a biomass increase (13 %, depicted in Fig. 4.2.2c as an output flow for the system to ensure steady state). *S. tamesis* excretion (flux directed from O to $NH_4^+_w$, 20 %) together with the flux directed from $NH_4^+_s$ to $NH_4^+_w$ (somewhat higher than in the previous two conditions S and SC at 13 %) promoted a build-up of $NH_4^+_w$ modelled as a daily export (21 %). Compared to the effects in condition SC, PN_s in SO was transformed at lower frequencies through MPB uptake (57 %), recycling back to PN_s (50 %), nitrification (60 %, from $NH_4^+_s$ to $NO_x^-_s$), and loss as N_2 (51 %).

When both *Corbicula* and *S. tamesis* were present (SOC, Fig. 4.2.2d) recycling activities somewhat decreased relative to either species individually. The three major loops involving oligochaete (PN_s - O - PN_s), the microphytobenthos (PN_s - $NH_4^+_s$ - MPB - PN_s) and nitrogen fixation ($NH_4^+_s$ - $NO_x^-_s$ - $N_2{}_s$ - $NH_4^+_s$) resulted in smaller N flow frequencies compared to those observed in the previous conditions. Changes in the relative weight of recycling did not modify

the portion of N leaving the system as N_2, which was still 51 %. Excretion by macrofauna (15 %) with subsequent diffusion of NH_4^+ (flux from $NH_4^+{}_s$ to $NH_4^+{}_w$, 15 %) resulted in a net release of NH_4^+ to the water column. This corresponded to daily export of $NH_4^+{}_w$ of 26 % representing a virtual increase standing stock of $NH_4^+{}_w$. Also in the SOC condition the input analysis shows a reduced export from MPB and O relative to SO (from 13 to 10 % for compartment O and from 14 to 11 % for compartment MPB). Since no fraction of the PN import distributes to *Corbicula* (Fig. 4.2.2d) it can be inferred that this organism played an indirect role within the system.

In the condition SV (Fig. 4.2.3a) the benthic paths of PN_s input were strongly influenced by the assimilation of *V. spiralis* roots and leaves (94 %). This was tightly coupled to the ammonification of PN_s (139 %). Most of the PN input was then stored in *V. spiralis*' roots and leaves (i.e., accumulation in the two macrophyte compartments, 46 % for leaves, 49 % for roots; both portrayed as exports). Consequently, net N_2 loss was 0 %. Even though the portion of N entering $NH_4^+{}_w$ was higher than for other conditions (28 %), no increase (i.e., export) in water-column NH_4^+ occurred. $NH_4^+{}_w$ that had diffused from sediments was taken up to an equal degree by the high N requirement of macrophyte leaves. The fraction of the cycle associated to the coupled nitrification-denitrification (24 %), the fraction for MPB (16 %) and its related loop back to PN_s (PN_s - $NH_4^+{}_s$ - MPB - PN_s) decreased in SV as compared to all previous conditions.

Macrophyte presence strongly controlled how PN_s was processed when *Corbicula* was added to vegetated sediments (SVC, Fig. 4.2.3b). Note the role played by *Corbicula* in N dynamics was inferred to be largely indirect. Slightly smaller portions of N were stored in macrophyte biomass (comparing SV with SVC: from 49 to 42 % for roots and from 46 to 39 % for leaves). As in condition SC, the presence of the bivalve enhanced the fractions recycled along both the loop PN_s - $NH_4^+{}_s$ - MPB - PN_s (from 14 to 36 % for MPB - PN_s) and the loop $NH_4^+{}_s$ - $NO_x^-{}_s$ - $N_2{}_s$ - $NH_4^+{}_s$ (from 24 to 31 %). The diffusion rate of NH_4^+ from sediment to water column increased somewhat compared to SV (from 28 to 33 %). Once again a daily accumulation of NH_4^+ was inferred from $NH_4^+{}_w$ export (8 %); neither *V. spiralis* leaves nor MPB fully consumed this $NH_4^+{}_w$ surplus. No N_2 exited the system as in the condition SV.

Condition SVO (Fig. 4.2.3c) included macrophytes and *S. tamesis*. A large amount of recycling between the oligochaete and PN_s was inferred, as in SO and SOC. Compared to SV and SVC, PN input fractions that were assimilated and stored in macrophyte leaves (21 %) and roots (37 %) were low and N loss via denitrification (28 %) was relatively high. The observed high importance of nitrification-denitrification path (52 %) was related not only to an output of N_2 but also to a significant fraction of recycled N ($NH_4^+{}_s$ - $NO_x^-{}_s$ - $N_2{}_s$ - $NH_4^+{}_s$, 24 %). The fraction of N uptake by MPB (16 %) was less when compared to SVC but similar to SV. The exchange

of NH_4^+ between water and sediment ($NH_4^+_s$ - $NH_4^+_w$) was peculiar for this condition: the direction for this transfer was opposite to that measured in all other conditions (5 %), going from water to sediment.

The most complex condition (SVOC, Fig. 4.2.3d) demonstrated a flow pattern different from all others. SVOC had a lower percentage of PN_s flux exiting from the system as N_2 relative to SVO (from 28 % in SVO to 13 % in SVOC). And the condition had an increased fraction accumulated in macrophyte leaves (from 21 % in SVO to 36 % in SVOC). Benthic N fluxes distributed more evenly among multiple paths and fuelled coupled nitrification-denitrification. N fixation rate in SVOC as fraction of the input was similar to the SVO condition (22 %). SVOC had relatively low flow to microphytobenthos with corresponding low recycling (9 %). The flow of NH_4^+ diffusion to the water column conveyed 9 % of PN_s input and together with NH_4^+ excretion by macrofauna (12 %), thus contributed to less than half of enrichment of the $NH_4^+_w$ pool. However, no accumulation of NH_4^+ was found as primary producer's uptake equalled or exceeded these inputs. In all conditions with *V. spiralis,* recycling along the loop $NH_4^+_s$ - $NO_x^-_s$ - $N_{2\,s}$ - $NH_4^+_s$ was never lower than 22 % of the incoming PN input.

Statistical evaluation of ecological network analyses is not standardized. We assessed the conditions by which aforementioned flow apportionments (Figs. 4.2.2 and 4.2.3) could be inferred as statistically different. This was done by Monte Carlo simulation. For every experimental condition the original matrix of the flows was simulated 1,000 times by changing randomly each of its coefficients within a 30 % interval of variability. This percentage is large relative to most key empirical measurements in this study. Empirically measured rates had coefficients of variation typically within 10 and 20 %. We then obtained 1,000 input analysis configurations from simulations of the 8 conditions, such as those presented in Figures 4.2.2 and 4.2.3. This allowed us to derive distributions for all the fractional flows from PN_s. We then selected 4 major internal flows; i.e., those that were common to all conditions and some that were considered as key in the nitrogen budget. The flows were MPB → PN_s, $NH_4^+_s$ → $NO_x^-_s$, $N_{2\,s}$ → $NH_4^+_s$, $NH_4^+_s$ → $NH_4^+_w$. Also, exports, representing increases in standing stock, from MPB, $N_{2\,w}$, V_l and V_r were assessed. We compared the values of these flows across the 8 experimental conditions using a Generalized Linear Model coupled with the Tukey contrasts to assess which, among the different conditions, generated significantly different flow values. The Tukey contrasts shows that all the differences that we described above are statistically significant. We are confident that this reveals a true difference between the flows generated by the inputs in the different conditions.

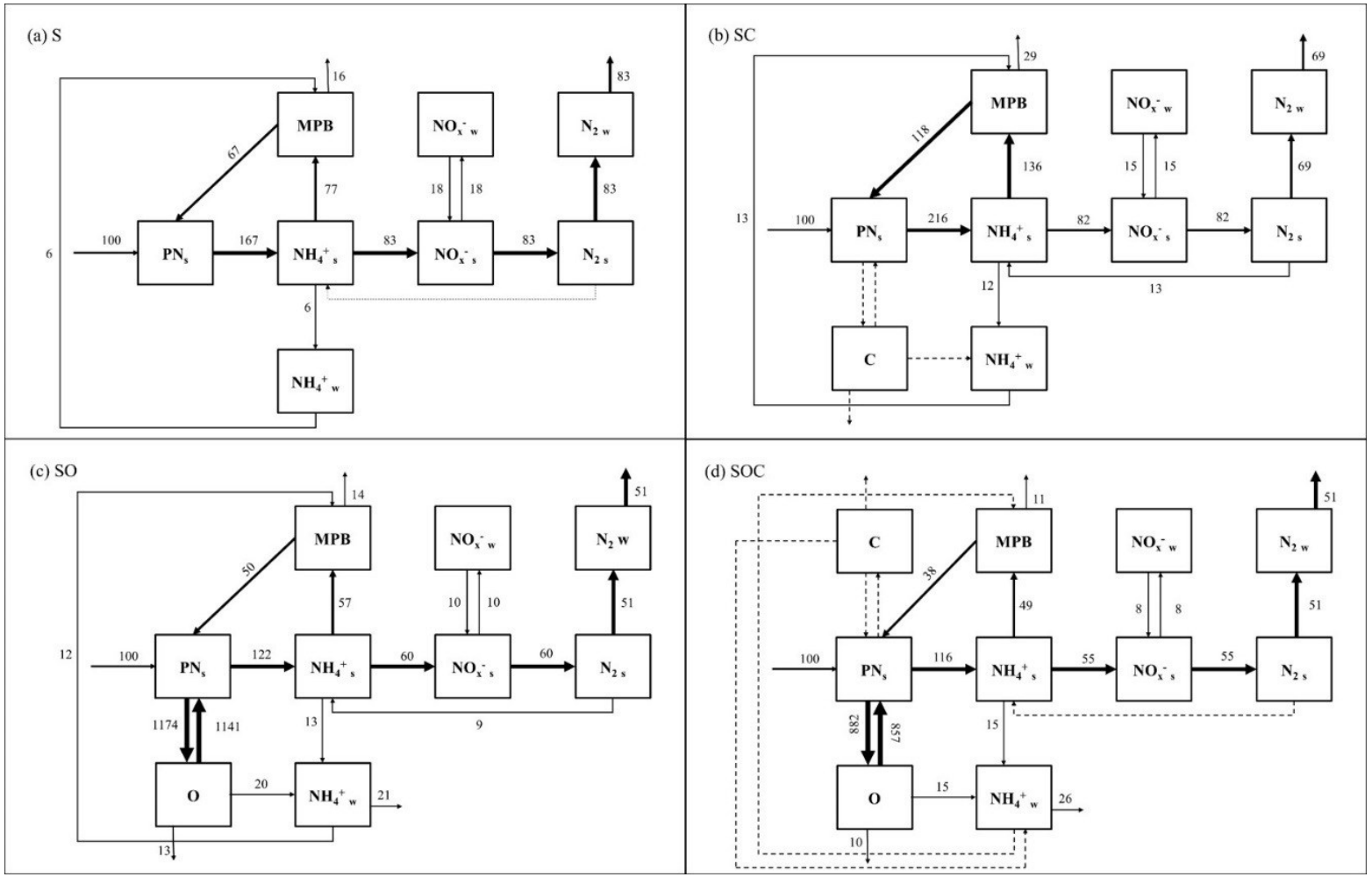


Fig. 4.2.2 Input analysis relative to the compartment PN_s for the condition (a) S (bare sediment), (b) SC (sediment with *Corbicula* spp.), (c) SO (sediment with *S. tamesis*), (d) SOC (sediment with *Corbicula* spp. and *S. tamesis*). Results are presented as graphs showing the benthic compartments and corresponding N flows. Values indicate the fractional flow (as a percentage) of the investigated N input to the system that passes along that flow. Data are relative to daily condition, obtained by averaging all dark and light estimated fluxes. Amounts higher than 100 % suggest more than one passage of an imported N atom along a path, in other words they indicate recycling. Dashed lines represent flows accounting for less than 5 %.

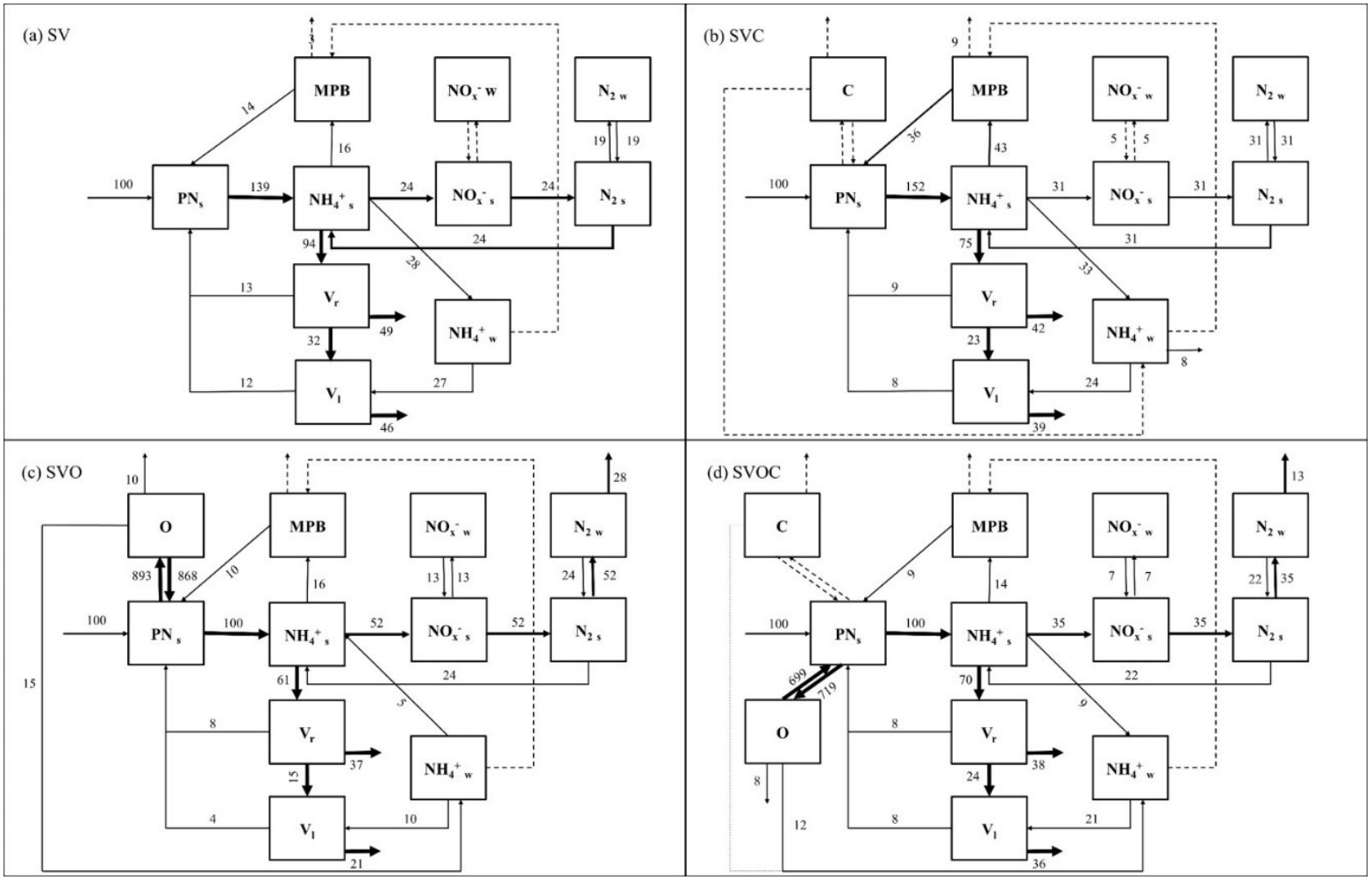


Fig. 4.2.3 Input analysis relative to the compartment PN_s for the conditions (a) SV (sediment with *V. spiralis*), (b) SVC (sediment with *V. spiralis* and *Corbicula* spp.), (c) SVO (sediment with *V. spiralis* and *S. tamesis*), (d) SVOC (sediment with *V. spiralis*, *Corbicula* spp. and *S. tamesis*). Results are presented as graphs showing the benthic compartments and corresponding N flows. Values indicate the fractional flow (as a percentage) of the investigated N input to the system that passes along that flow. Data are relative to daily condition, obtained by averaging all dark and light estimated fluxes. Amounts higher than 100 % suggest more than one passage of an imported N atom along a path, in other words they indicate recycle. Dashed lines represent flows accounting for less than 5 %.

Total Dependency Analysis

The total dependency analysis provides insight on how dependent each compartment is on all other compartments. Dependency is measured as the fraction of N entering a compartment that had previously passed through another. Both direct and indirect dependencies are represented, and the advantage of ENA in this case is in providing an estimate of indirect relationships and hence the extended pathways of supply. We focused on the dependence of primary producers (MPB and *V. spiralis*) upon other compartments; results of only light incubations are described to assess that uptake with the assumption that dark uptake was minimal. Primary production and N uptake were calculated from TCO_2 data.

Table 4.2.1 reports the dependency (i.e., both direct and indirect) values of microphytobenthos in the 8 experimental conditions. Microphytobenthos uptake in all conditions was supported largely by the $NH_4^+{}_s$ pool (dependency values on $NH_4^+{}_s$ ranging from 79 % to 96 %) and only for a small extent by $NH_4^+{}_w$ (11 % to 35 %). In all conditions without the macrophyte, dependency on $NH_4^+{}_s$ (79 % to 93 %) was only slightly more than the values relative to PN_s (from 66 % to 91 %). This suggests that most of the N reaching MPB through $NH_4^+{}_s$ was particulate N in the sediment. Smaller fractions were provided by $N_{2\ s}$ (dependency values between 2 % and 38 %) and recycling through macrofaunal activity. The coefficients relative to *S. tamesis* were also relevant, averaging 75 %, whereas those of *Corbicula* indicated little dependency (on average 12 %). Microphytobenthos did not receive direct N supply from macrofauna; but strong PN recycling activities, largely through the oligochaete, were responsible for this indirect contribution.

The presence of the macrophyte altered dependencies of MPB. In the presence of macrophytes without macrofauna (SV), MPB assimilated NH_4^+ mainly from sediments (95 %). $N_{2\ w}$ became an important source for $NH_4^+{}_s$ and subsequently for MPB (68 %). Dependency of MPB on PN_s decreased (27 %) for this condition (SV). Macrofaunal presence in vegetated sediment (SVC, SVO, SVOC), promoted more MPB dependence on PN_s (from 33 % to 45 %), particularly in the presence of the oligochaete. MPB dependence on $N_{2\ w}$ declined from conditions with only the macrophytes (68 % in SV) to conditions with macrofauna (60 % in SVC, 28 % in SVO and 30 % SVOC). Thus, both the presence of the macrophyte *V. spiralis* and the two macrofaunal species had impacts on the extended pathways by which microphytobenthos received N.

Table 4.2.1 Dependency values (%) of benthic microalgae (MPB) in the 8 different conditions, relative to light incubations. Only the compartments considered relevant for the analyzed process are shown.

	S	SC	SO	SOC	SV	SVC	SVO	SVOC
PN_s	91	66	81	85	27	33	41	45
$NH_4^+{}_s$	92	79	93	93	95	96	91	92
$NH_4^+{}_w$	15	35	23	15	13	11	21	12
$N_{2\,s}$	2	27	38	23	80	78	49	59
$N_{2\,w}$	0	0	0	0	68	60	28	30
C	-	13	-	11	-	4	-	4
O	-	-	75	75	-	-	41	39

Tables 4.2.2 and 4.2.3 report the dependency values of *V. spiralis* leaves and roots, respectively. The only direct N input to roots was the NH_4^+ from the sediment (dependency value of 100 %), whereas $NH_4^+{}_s$ only partly supplied leaves through roots. The remaining source of N to leaves was through direct uptake from the water column (dependency values of V_l on $NH_4^+{}_w$ ranged from 44 % to 56 %). Other dependency values showed similar trends for both roots and leaves. When macrofauna, especially *S. tamesis*, was present, dependency values of V_l and V_r on PN_s were higher compared to *V. spiralis* alone. Dependencies of V_l and V_r on $N_{2\,s}$ and on $N_{2\,w}$ were lower with macrofauna, especially with the oligochaete (Tables 4.2.2 and 4.2.3). The effect of macrofaunal reworking activities on macrophytes was evident with higher dependency values on *S. tamesis* (on average 29 % for the leaves and 42 % for the roots) than on *Corbicula* (on average 8 % for the leaves and 3 % for the roots).

Table 4.2.2 Dependency values (%) of *Vallisneria spiralis*' leaves (V_l) in the 8 different conditions, relative to light incubations. Only the compartments considered relevant for the analyzed process are shown.

	SV	SVC	SVO	SVOC
PN_s	21	26	36	36
$NH_4^+{}_s$	73	74	72	59
$NH_4^+{}_w$	50	56	44	51
$N_{2\,s}$	62	61	39	37
$N_{2\,w}$	53	47	20	21
C	-	9	-	8
O	-	-	26	32

Table 4.2.3 Dependency values (%) of *Vallisneria spiralis*' roots (V_r) in the 8 different conditions, relative to light incubations. Only the compartments considered relevant for the analyzed process are shown.

	SV	SVC	SVO	SVOC
PN_s	28	35	44	47
$NH_4^+{}_s$	100	100	100	100
$NH_4^+{}_w$	4	4	10	3
$N_{2\,s}$	85	82	54	64
$N_{2\,w}$	72	63	28	32
C	-	3	-	3
O	-	-	44	40

To evaluate the support of the different compartments on denitrification, dependency values of $N_{2\,w}$ in dark conditions were analyzed in detail (Table 4.2.4). $N_{2\,w}$ was assessed as it reflected both denitrification in the sediment with subsequent diffusion of $N_{2\,s}$ as well as the potential for denitrification in the water column. In all dark incubations $N_{2\,w}$ showed complete dependence (i.e., 100 %) on N_2 produced in the sediment by denitrification of $NO_x^-{}_s$. Nitrate in pore water derives from two processes: the diffusion of NO_x^- from the water column and the nitrification of ammonium in the oxic sediment. In all conditions dependency values relative to $NO_x^-{}_w$ (from 57 % to 77 %) were higher than values relative to $NH_4^+{}_s$ (from 27 % to 54 %). The largest difference was in condition SOC. The only exception occurred in condition SVO, where the difference was less pronounced compared to all the other conditions (dependency value on $NO_x^-{}_w$ was 57 %, whereas the value relative to $NH_4^+{}_s$ was 54 %). Further, denitrification dependency on $NH_4^+{}_w$ was 21 %, but such dependency was not found in any of the other conditions (data not shown). Effects on denitrification by the oligochaete, but not the bivalve, were evident when the macrophyte was also present. In SVO and SVOC denitrification dependency on $NH_4^+{}_s$ was highest and on $NO_x^-{}_w$ lowest among all treatments. As seen throughout these analyses, then, different community structure has significant effects on the quantitative pathways by which N flows through the benthic ecosystem.

Table 4.2.4 Dependency values (%) of $N_{2\,w}$ in the 8 different conditions, relative to dark incubations. Only the compartments considered relevant for the analyzed process are shown.

	S	SC	SO	SOC	SV	SVC	SVO	SVOC
PN_s	44	38	35	27	35	39	40	47
$NH_4^+{}_s$	44	38	35	27	35	39	54	47
$NO_x^-{}_s$	100	100	100	100	100	100	100	100
$NO_x^-{}_w$	64	68	70	77	70	67	57	62
$N_{2\,s}$	100	100	100	100	100	100	100	100
$N_{2\,w}$	0	0	0	0	0	0	0	0

Statistical significance through Monte Carlo simulation was applied also to the outcome of this analysis. We calculated the TDM for each of the random 1,000 networks that we described previously. For each single coefficient we counted how many simulations yielded a value higher than the actual coefficient and how many produced a lower value. In this way we reconstructed a distribution for each of the coefficients of the TDM. Finally, we observed whether the actual coefficient fell within the 95 % confidence interval of its simulated distribution. The actual values we obtained by performing TDM are not outside the 95 % confidence interval of the constructed distributions for the coefficients of the TDM. That is to say that the variability imposed to the flow values (30 %) does not change significantly the outcomes of the TDM analysis.

Discussion

Macrofauna mediates primary producers-bacteria particulate N pools fluxes

Results from the present study highlight higher sedimentary N recycling along a gradient of increasing benthic biodiversity. Modifying community structure to increase complexity supported strong mutualistic interactions between macrofauna and primary producers, attenuated N losses via denitrification and decreased the dependency of primary producers on N fixation. Such outcomes confirm the hypothesis of better exploitation of sedimentary N pool by primary producers when mediated by macrofauna. The latter, via functional-group specific mechanisms, discussed below, favor the utilization of decomposing particulate N by photosynthetic organisms. Our results confirm the expected decrease of N_2 fluxes from the sediment (via denitrification) and to the sediments (via N fixation). Ultimately, microbial-macrofaunal-primary producer interactions minimize the waste (net export, sensu ENA) of a precious nutrient and energy investment for the costly process of conversion of scarcely reactive

N_2 into bioavailable N. Our results provide a different perspective when compared to outcomes from traditional studies. In oligotrophic marine ecosystems, the competition between microbial communities and macrophytes is exacerbated by low N availability where large N fluxes go to plant biomass and little N is processed by bacteria (Risgaard-Petersen et al. 1998; McGlathery et al. 2007). However, recent studies analyzing biogeochemical processes in seagrass meadows where macrophytes and bivalves coexist revealed interesting and understudied symbioses among seagrasses, lucinid bivalves and their gill bacteria. Such symbioses result in sediment detoxification via pore water sulphide oxidation and radial oxygen loss, improved plant growth performances, and bivalve-mediated N transfer from N-fixers to seagrasses (Van Der Heide et al. 2012; Petersen et al. 2016).

The complexity gradient that we have created experimentally, analyzed through ENA, allowed the identification of large variations in the distribution of sedimentary N among processes, spanning from large fractions lost via denitrification in condition S to large import or reuse of N temporarily stored in plant biomass (SV). All networks with sediments alone or only with macrofauna tended to lose N from the benthic compartment, but the two macrofaunal taxa, and therefore functional groups, produced different effects on benthic N cycling. The effects of *S. tamesis* is more evident (large reworking, pellets production), whereas the role of *Corbicula* in N cycling remains less clear and largely indirect. The rooted plants tended towards dominance of assimilative processes, which drove main N paths in all treatments. The presence of macrofauna attenuated the dependency of plants on N fixation with a larger share of the N flux to the macrophytes among compartments and processes.

Novel methodological aspects of this work

A widespread, combined experimental and modelling approach to evaluate whole system benthic processes in lakes, lagoons and coastal areas consists of the upscaling of process rates measured in intact sediment cores or chambers. The novel aspect of this paper was to consider studied microcosms as benthic micro-environments, where we manipulated a natural, simplified community of primary producers and macrofauna. These manipulations produced multiple cascade effects on the diversified microbial consortium of N-related bacteria. By controlling conditions of sediment (by sieving and homogenization) and communities (same densities of individuals with similar size added in the different conditions), we removed a large fraction of environmental variability. Measured rates were characterized by much lower coefficients of variation (generally < 20 %) as compared to intact cores measurements (where 100 % variation or more is not unusual). Our microcosm results may differ from those under natural conditions, but they and their analysis highlight interesting perspectives on benthic N

cycling along a simplified biodiversity gradient. This suggests promising and intriguing results if a similar approach is applied to much more complex communities in freshwater or marine ecosystems, with other plants or macrofaunal functional groups.

The application of ENA to microcosm studies provides integration of separate N process measurements under controlled conditions. The high standard and low variability of these process measurements in turn foster accuracy of ENA. Most often ENA is based on networks whose data are inferred from the literature taken from conditions outside those of the system under investigation. This is what happens when ENA is applied to whole ecosystems, as it is impossible to measure everything, especially under controlled conditions. The microcosms described here were pre-incubated in fresh, in situ water for 1 month and then were incubated in the light and in the dark for 1-2 hours. The very short-term incubation allowed us to observe differences in concentrations of the most reactive solutes and therefore to calculate rates very close to-steady state conditions. At the end of the incubations concentration of target solutes changed by a small amount compared to initial values (within 10-30 %). This means that the pools of particulate matter (the sediment N-bulk, the N in fronds and roots, the N in macrofauna) virtually did not change but for a negligible amount. Short-term incubation ensured that during the process of measurements nutrients were not limiting or the degree of limitation did not change appreciably. This is true not only for inorganic N forms, but also for reactive P, not reported here.

We acknowledge that denitrification rates measured with the IPT may underestimate true denitrification due to two main reasons: 1) the occurrence of multiple oxic and anoxic niches within bioturbated or rooted sediments and 2) the occurrence of subsurface nitrification and denitrification within the *V. spiralis* rhizosphere. Multiple oxic and anoxic niches within sediments present a violation of an IPT assumption dealing with homogeneous mixing of labelled and unlabeled nitrate in the denitrification zone (Risgaard-Petersen and Jensen 1997). The addition of labelled nitrate to the water phase does not allow measurement of subsurface nitrification and denitrification, supported by radial oxygen loss (Soana et al. 2015; Racchetti et al. 2017). However, Soana et al. (2015) demonstrated that in the Mincio River a major fraction of N loss via denitrification occurs in the upper mm of the organic sediments, where labelled and unlabelled nitrate are homogeneously mixed, and that denitrification is mostly sustained by water column nitrate, as reported here. Soana et al. (2015) and Racchetti et al. (2017) report much lower rates of denitrification associated with *V. spiralis* roots, using $^{15}NH_4^+$ labelled pore water.

Benthic complexity results in better sharing of N fluxes among benthic actors

Results from the input analysis suggest that in the simplest experimental condition (S) a large fraction of the N remobilized from PN_s is lost from the benthic system. Denitrification accounts for a high fraction of mobilized PN_s through coupling of ammonification, nitrification and benthic denitrification and via denitrification of water column nitrate. This is in agreement with the outcomes of the total dependency matrix, which shows a strong dependency of $N_{2\,w}$ on $NO_x^-{}_w$. Further, a minor fraction of PN_s feeds microphytobenthos. High rates of denitrification have been demonstrated in a number of shallow eutrophic ponds of the Po River Plain (Racchetti et al. 2011) similar to conditions maintained in microcosms. Racchetti et al. (2011)'s results suggest that in the S condition available reactive N is in excess of N demand by microphytobenthos (Risgaard-Petersen and Jensen 1997). Elsewhere, under more oligotrophic settings, a strong inhibition of denitrification by microphytobenthos was demonstrated (Risgaard-Petersen 2003).

We progressively increased the biodiversity with two kinds of macrofauna (i.e., a filter-feeding bivalve and a deposit-feeding oligochaete) with different effects on the N cycle. The presence of the bivalve *Corbicula* promoted a higher fraction of PN_s into sustaining benthic production and circulating within the closed path PN_s - $NH_4^+{}_s$ - MPB - PN_s. As a consequence, a lower fraction is lost from the system. Filter-feeding bivalves have been demonstrated to affect different processes. They can relocate pelagic primary production to benthic primary production because most of the ingested algae remain viable when released as feces and pseudofeces onto the sediment surface. Bivalves can also excrete large amounts of ammonium, which can be readily taken up by primary producers (Bartoli et al. 2003b; Ruginis et al. 2014). What emerges from our results is that even if there are no large N flows from or to *Corbicula*, significant changes in benthic fluxes relative to bare sediment, support a relevant but indirect role played by the bivalve.

We assumed that almost 100 % of the food requirements by *Corbicula* is supplied from the water column in our networks and not from the sediment, consistent with the data at our disposal. However, *Corbicula* may potentially display pedal-feeding and therefore it may exploit the sediment as a nutrient source (Hakenkamp and Palmer 1999; Vaughn et al. 2008). Such feeding may be realistic at the study site where the concentration of phytoplankton is limited and may produce a different picture from that reported in Figures 4.2.2(b,d) and 4.2.3(b,d). Pedal-feeding would result in a higher fraction of PN_s going to direct *Corbicula* feeding and, consequently, in a higher sediment-derived NH_4^+ excretion sustaining MPB production.

Contrary to the need for pedal-feeding by *Corbicula*, the link of *S. tamesis* with PN_s is direct, with large flows related to ingestion and egestion, and indirect with reworking activities. Reworking activities were quite evident from visual inspection as the sediment surface was continuously covered with pellets. Oligochaete processing of N had extended effects. A significant fraction of PN_s input reached the water column through the $NH_4^+{}_w$ compartment as *S. tamesis* excretion, or from $NH_4^+{}_s$. Dependency of $NH_4^+{}_w$ from PN_s reached 94 %. Thus, the oligochaete favored the mobilization of N and sped $NH_4^+{}_s$ transfer to the water column. These results are consistent with others. The effect of burrowers on benthic N cycling has been extensively studied in the literature (Svensson et al. 2001; Nizzoli et al. 2007; Bonaglia et al. 2014), and most studies have demonstrated large macrofauna-mediated ammonium efflux from sediments during burrowing and ventilation activities. Such efflux is the result of direct excretion, mobilization of pore water solutes and increased ammonification due to sediment reworking (Stief 2013).

Larger ammonium availability due to macrofaunal excretion and bioturbation may have other effects. Benthic primary production may be stimulated and affect the N fraction recycled back to the sediments (Mermillod-Blondin and Lemoine 2010). Sediment reworking may inhibit the development of microalgal mats due to continuous pellet production and burial. Such inhibition may be compensated by higher growth due to rapid nutrient mobilization and vertical movements of diatoms to avoid light limitation. Thus, pellet production and N mobilization act in opposite directions upon MPB primary production. We tracked the fate of decomposing PN_s when oligochaetes were present and found NH_4^+ accumulation in the water phase (treated as an export flux in the network). This means that even if there is a wide availability of ammonium for MPB, other factors may limit primary producers preventing full use of available N. Further, over half of the N remobilized from sediment is lost by denitrification in condition SO. Here, confirmed in the results of TDM, the dependency coefficients of $N_{2\ w}$ were, compared to previous conditions, slightly higher on $NO_x^-{}_w$ and lower on PN_s. Burrowers have been demonstrated to stimulate N loss via denitrification, mainly through the increase of NO_x^- fluxes from the water column to the sediment (Nizzoli et al. 2007).

The *Corbicula* and *S. tamesis* condition (SOC) was most similar to that of *S. tamesis* alone. Relative mobilization of $NH_4^+{}_s$ was enhanced with proportional decreases of both microphytobenthos uptake and coupled nitrification-denitrification relative to conditions S or SC. Large N flows reached $NH_4^+{}_s$ with high dependency on PN_s (75 %), but a smaller fraction reached MPB.

When the two macrofauna functional groups are co-present, the reworking activity of the oligochaete prevails over the stimulating effect of *Corbicula* on microphytobenthos production.

We considered interactions between bivalves and burrowers within the benthic N cycling in our experimental design. Of particular interest was the potential for stimulation of coupled nitrification-denitrification from co-occurrence of organisms excreting large NH_4^+ amounts (*Corbicula*) and organisms ventilating their burrows (*S. tamesis*). Excreted NH_4^+ may be transported within burrows and oxidized along nitrifier-rich burrow walls (Pelegrí and Blackburn 1995). Such outcome was not evident from our results as the fraction of PN_s input leaving the system as $N_{2\,w}$ was equal for SO and SC conditions, suggesting the higher relevance on the coupled microbial processes of sediment reworking than excretion and ventilation. Bonaglia et al. (2014) demonstrated that the invasive polychaete *Marenzelleria viridis* stimulated proportionally more dissimilative reduction of nitrate to ammonium (DNRA) than denitrification. There was no effect of *M. viridis* on coupled nitrification-denitrification (D_n), contrary to what reported for most tube-dwelling organisms (Hölker et al. 2015). It is likely that very tolerant, opportunistic oligochaetes or polychaetes withstand chemically reduced conditions and do not need frequent ventilation of their burrows. The growth of nitrifiers is negatively affected, because of their sensitivity to low oxygen concentrations (Hansen et al. 1981). This would explain our results.

With the addition of *V. spiralis* (condition SV) the largest flows of N constituted macrophyte uptake, and the largest fraction of remobilized PN_s reached V_l and V_r compartments. N was stored in plant biomass (in our model represented as exports from V_r and V_l), reducing N mobility due to the long retention time within the plant organic pool. Uptake by plants depleted the sedimentary ammonium pool, attenuating its availability to MPB and to bacterial communities. The fractional flux from $NH_4^+{}_s$ to MPB is much lower in the presence of *V. spiralis*. This may have resulted from competition for N, but probably also for light, space and potentially inhibition from allelopathic effects (Gette-Bouvarot et al. 2015).

The presence of rooted macrophytes and the dominance of assimilative paths also depressed the dissimilative N pathways (Risgaard-Petersen and Jensen 1997) with no N_2 leaving the system. This result is in contrast to other studies carried out in shallow freshwater ecosystems characterized by high NO_x^- availability in water column (Pinardi et al. 2009; Nizzoli et al. 2014; Soana et al. 2015). They report a positive effect of different aquatic plants on both nitrification and denitrification, as high NO_x^- concentrations may attenuate the competition for N. As we found no significant difference in NO_x^- fluxes between day and night measurements, we excluded NO_x^- uptake by macrophytes. This is in accordance with other studies reporting that NH_4^+ represents the preferred N source of primary producers, because NO_x^- assimilation requires more energy (Miller and Cramer 2004; Volkmann et al. 2016). The sedimentary ammonium pool was not able to support all the nutrient requirements of the plants when only

supplied from PN_s. Based on TDM analysis, under these conditions that limit uptake or bacterial activities sedimentary ammonium may be enriched by new N, supplied via N fixation in the rhizosphere (Nielsen et al. 2001).

The presence of rooted macrophytes, such as *V. spiralis*, therefore may modify the benthic N dynamics considerably, conveying N towards assimilative pathways, reducing its mobility and increasing the fraction of N accumulated as organic matter (i.e., plant biomass). Sedimentary remobilized PN is stored temporarily or permanently in the system. Moreover, elevated N requirements to sustain the primary production result in a new N path from water to sediment (i.e., N fixation), which balances N loss from the benthic system via denitrification.

This picture changes with the addition of the bivalve (condition SVC). The presence of *Corbicula* results in a higher recycling activity along the path PN_s - $NH_4^+{}_s$ - MPB - PN_s due to the lower retention time compared to the macrophytes alone (Nizzoli et al. 2014). This increased recycling is strictly related to the relative increase of MPB productivity (the output from MPB is now 9 % of the input to PN_s, whereas in SV it was less than 3 %). It is interesting to note that the stimulatory effect of this bivalve on microphytobenthos primary production occurs regardless of whether rooted macrophytes are present or not, even though the magnitude of this effect is higher in the latter case. The greater availability of NH_4^+ is derived from the excretion of bivalves and the enhanced mineralization, probably from the presence of labile organic matter (feces and pseudofeces). Increased availability of NH_4^+ causes a decline in the dependency of both primary producers' on N fixation. This result, highlighted by the TDM, suggests that bivalves make sedimentary pools of N available to primary producers directly and indirectly.

The decreased dependency of primary producers on N fixation is more visible in the presence of *S. tamesis* (condition SVO) than of *Corbicula* (SVC) because of the higher mobilization of $NH_4^+{}_s$. Positive effects of bioturbators on vegetation growth have been reported in several studies and attributed to ability of the animals to reduce the anoxic stress in the sediment as well as to increase the rates of N mineralization. The increased dependency of *V. spiralis* on the PN_s pool in the presence of bioturbators highlights how macrofauna facilitates the transfer of N from the PN_s pool to the macrophytes through production of NH_4^+ (Mermillod-Blondin and Lemoine 2010; Schrama et al. 2015).

S. tamesis was influential also on the dissimilative pathways when co-present with *V. spiralis* (SVO). Coupled nitrification-denitrification increased and, as in conditions without the macrophytes, the loss of N_2 was an important export. The relative importance of coupled nitrification-denitrification increased compared to the denitrification of $NO_x^-{}_w$. This situation was inferred from the different $N_2{}_w$ dependency values, which increased for PN_s and decreased

for $NO_x^-{}_w$ in SVO and SVOC. This could be due to the effects of plants on oligochaete ventilation. Studies report that oligochaetes of the genus *Sparganophilus* live in closed association with macrophytes' roots (Benham 1982; Rota et al. 2014) and our observations confirmed this behavior. The roots of *V. spiralis* are characterized by an elevated radial oxygen loss (Soana and Bartoli 2013), and the oligochaete may take advantage of such O_2 leaking from roots, diffusing through its skin with a considerable savings of energy required for burrows ventilation. This could explain both the decreased dependency of denitrification rates on $NO_x^-{}_w$ and the increase of coupled nitrification-denitrification. The former is likely when associated with a decline in NO_x^- flux from the water column to the sediment, as advection from ventilation is reduced. The oligochaete may enhance ammonification by producing labile organic matter while processing and metabolizing sediments. This increase $NH_4^+{}_s$ availability as does direct excretion by the oligochaete. The sedimentary ammonium pool, combined with oxygen release from roots, could stimulate nitrification and denitrification (Soana and Bartoli 2014).

Conclusions

N network model construction and analysis is an underdeveloped application of ENA (Christian et al. 2011; 2016). Published N network models may be associated with one of two approaches: one focused on the biogeochemistry of N cycling (e.g., Christian and Thomas 2003, Hines et al. 2012) and another that focuses on N transfers within the food webs (Degan et al. 1994; Baird et al. 2011). Models constructed and analyzed in this work provide a perspective that includes both approaches. Detailed N dynamics are represented within experimental, controlled microcosms representing a gradient of biodiversity. The resultant network analyses have provided insights on indirect and system-level interactions of N processes with taxon and functional group occurrence. Specifically, the analyses tracked how the occurrence of different functional groups of macrofauna and growth forms of primary producers affected extended N sources for processes, process coupling, recycling, and N loss. The influence of different functional groups, however, was assessed through one taxon of each (i.e., a bivalve vs. an oligochaete, and a macrophyte vs a MPB community). The influence of species differences within each functional group remains to be done.

Some papers (Chase and Knight 2006; Mermillod-Blondin and Lemoine 2010; Van Der Heide et al. 2012; Hölker et al. 2015; Petersen et al. 2016; Herren et al. 2017) report interesting experimental studies inferring complex interactions among microbial communities, different macrofauna functional groups and micro and macro primary producers. Petersen et al. (2016) have demonstrated via molecular tools the symbiosis between lucinid bivalves and N-fixing bacteria and speculate on an N transfer mediated by the bivalve between bacteria and roots that

may favor seagrass growth in oligotrophic marine ecosystems. Hölker et al. (2015) suggest the relevance of chironomid larvae as benthic sinks for large fractions of pelagic primary production, largely exceeding the role of zooplankton in shallow lakes. Chase and Knight (2006) demonstrated better growth performance of macrophytes in the presence of snails that actively remove epiphytes from their leaves and theoretically promote water clarity under eutrophic settings. The often cited work of Levi et al. (2013) reports the importance to nitrifiers of N derived from dying salmons in extremely nutrient low creeks. Salmon carcasses represent imported N from the oceans and feed a large fraction of the trophic web. This generation of manuscripts, reveals unexplored and intriguing synergistic/mutualistic interactions that link community structure to nutrient cycling. These studies would greatly benefit by the application of ENA that can aid inferences of the dependency of myriad processes to apparently distant factors.

References

Allesina S., & Bondavalli C. (2003) Steady state of ecosystem flow networks: A comparison between balancing procedures. Ecological Modelling, 165: 221 – 229.

Allesina S., & Bondavalli C. (2004) WAND: An ecological network analysis user-friendly tool. Environmental Modelling & Software, 19: 337 – 340.

Atkinson C.L., Opsahl S.P., Covich A.P., Golladay S.W., & Conner L.M. (2010) Stable isotopic signatures, tissue stoichiometry, and nutrient cycling (C and N) of native and invasive freshwater bivalves. Journal of the North American Benthological Society, 29: 496 – 505.

Baird D., Asmusb H., & Asmusb R. (2011) Carbon, nitrogen and phosphorus dynamics in nine sub-systems of the Sylt-Rømø Bight ecosystem, German Wadden Sea. Estuarine, Coastal and Shelf Science, 91: 51 – 68.

Bartoli M., Nizzoli D., & Viaroli P. (2003a) Microphytobenthos activity and fluxes at the sediment-water interface: Interactions and spatial variability. Aquatic Ecology, 37: 341 – 349.

Bartoli M., Naldi M., Nizzoli D., Roubaix V., & Viaroli P. (2003b) Influence of clam farming on macroalgal growth: A microcosm experiment. Chemistry and Ecology, 19: 147 – 160.

Benelli S., Bartoli M., Racchetti E., Moraes P.C., Zilius M., Lubiene I., & Fano E.A. (2017) Rare but large bivalves alter benthic respiration and nutrient recycling in riverine sediments. Aquatic Ecology, 51: 1 – 16.

Benham W.B. (1892) A new English genus of aquatic Oligochaeta (*Sparganophilus*) belonging to the family Rhinodrilidae. Quarterly Journal of Microscopical Science archives, 34: 155 – 179.

Bertics V.J., Sohm J.A., Treude T., Chow C.E.T., Capone D.G., Fuhrman J.A., & Ziebis W. (2010) Burrowing deeper into benthic nitrogen cycling: The impact of bioturbation on

nitrogen fixation coupled to sulfate reduction. Marine Ecology Progress Series, 409: 1 – 15.

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

Borrett S.R., Christian R.R., & Ulanowicz R.E. (2013) Network Ecology, in Encyclopedia of Environmetrics. El-Shaarawi A.H., & Piegorsch W. (eds), John Wiley & Sons Ltd: Chichester, UK.

Borrett S.R., & Lau M.K. (2014) enaR: An R package for Ecosystem Network Analysis. Methods in Ecology and Evolution, 5: 1206 – 1213.

Chase J.M., & Knight T.M. (2006) Effects of eutrophication and snails on Eurasian watermilfoil (*Myriophyllum spicatum*) invasion. Biological Invasions, 8: 1643 – 1649.

Christian R.R., Forés E., Comin F., Viaroli P., Naldi M., & Ferrari I. (1996) Nitrogen cycling networks of coastal ecosystems: influence of trophic status and primary producer form. Ecological Modelling, 87: 111 – 129.

Christian R.R., & Thomas C.R. (2003) Network Analysis of Nitrogen Inputs and Cycling in the Neuse River Estuary, North Carolina, USA. Estuaries, 26: 815 – 828.

Christian R.R., Voss C.M., Bondavalli C., Viaroli P., Naldi M., Tyler A.C., … & Camacho-Ibar V. (2010) Chapter 4. Ecosystem Health Indexed through Networks of Nitrogen Cycling, 73 – 90. In: Coastal Lagoons Critical Habitats of Environmental Change. CRC Press.

Christian R.R., Camacho-Ibar V.F., Piehler M.F., & Smyth A.R. (2011) Understanding the Nitrogen Cycle through Network Models in Coastal Ecosystems, 383 – 396. In: Wolanski E. (eds), Treatise on Estuarine and Coastal Science, vol. 9: Estuarine and Coastal Ecosystem Modeling. Elsevier.

Christian R.R., Camacho-Ibar V.F., Piehler M.F., & Smyth A.R. (2016) Improved understanding of nitrogen cycle through network models in coastal ecosystems. In: Wolanski E., & McLusky D. (chief eds.), Treatise on Estuarine and Coastal Science. Elsevier, Amsterdam, Netherlands.

Dalsgaard T., Nielsen L.P., Brotas V., Viaroli P., Underwood G., Nedwell D., ... & Dong L. (2000) Protocol handbook for NICE-Nitrogen Cycling in Estuaries: a project under the EU research programme: Marine Science and Technology (MAST III), 1 – 62.

Degan L.A., Finn J.T., Hopkinson C.S., Giblin A.E., Peterson B.J., Fry B., & Hobbie J.E. (1994) Flow model analysis of the effects of organic matter-nutrient interactions on estuarine trophic dynamics. In: Dyer K.R., & Orth R.J. (eds), Changes in fluxes in estuaries: implications from science to management, Proceedings of ECSA22/ERF Symposium, 13–18 September 1992, Plymouth, Olsen and Olsen, 273–281.

Ferguson A.J.P., Bradley D.E., & Gay J.M. (2004) Benthic nutrient fluxes in euphotic sediments along shallow sub-tropical estuaries, northern New South Wales, Australia. Aquatic Microbial Ecology, 37: 219 – 235.

Fink P., Peters L., & Von Elert E. (2006) Stoichiometric mismatch between littoral invertebrates and their periphyton food. Archiv für Hydrobiologie, 165: 145 – 165.

Gette-Bouvarot M., Mermillod-Blondin F., Lemoine D.G., Delolme C., Danjean M., Etienne L., & Volatier L. (2015) The potential control of benthic biofilm growth by macrophytes: A mesocosm approach. Ecological Engineering, 75: 178 – 186.

Hakenkamp C.C., & Palmer M.A. (1999) Introduced bivalves in freshwater ecosystems: the impact of *Corbicula* on organic matter dynamics in a sandy stream. Oecologia, 119: 445 – 451.

Hannon B. (1973) The structure of ecosystems. Journal of Theoretical Biology, 41: 535 – 546.

Hansen J.I., Henriksen K., & Blackburn T.H. (1981) Seasonal distribution of nitrifying bacteria and rates of nitrification in coastal marine sediments. Microbial Ecology, 7: 297 – 304.

Henriksen K., Rasmussen M.B., & Jensen A. (1983) Effect of bioturbation on microbial nitrogen transformations in the sediment and fluxes of ammonium and nitrate to the overlaying water. Ecological Bulletins, 35: 193 – 205.

Herren C.M., Webert K.C., Drake M.D., Vander Zanden M.J., Einarsson Á., Ives A.R., & Gratton C. (2017) Positive feedback between chironomids and algae creates net mutualism between benthic primary consumers and producers. Ecology, 98: 447 – 455.

Hines D.E., Lisa J.A., Song B., Tobias C.R., & Borrett S.R. (2012) A network model shows the importance of coupled processes in the microbial N cycle in the Cape Fear River Estuary. Estuarine, Coastal and Shelf Science, 106: 45 – 57.

Hölker F., Vanni M.J., Kuiper J.J., Meile C., Grossart H.P., Stief P., ... & Hupfer M. (2015) Tube-dwelling invertebrates: Tiny ecosystem engineers have large effects in lake ecosystems. Ecological Monographs, 85: 333 – 351.

Kristensen E. (2000) Organic matter diagenesis at the oxic/anoxic interface in coastal marine sediments, with emphasis on the role of burrowing animals. Hydrobiologia, 426: 1 – 24.

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. Aquatic Ecology, 46: 191 – 200.

Levi P.S., Tank J.L., Tiegs S.D., Chaloner D.T., & Lamberti G.A. (2013) Biogeochemical transformation of a nutrient subsidy: Salmon, streams, and nitrification. Biogeochemistry, 113: 643 – 655.

Lohrer A.M., Halliday N.J., Thrush S.F., Hewitt J.E., & Rodil I.F. (2010) Ecosystem functioning in a disturbance-recovery context: Contribution of macrofauna to primary production and nutrient release on intertidal sandflats. Journal of Experimental Marine Biology and Ecology, 390: 6 – 13.

Loreau M. (2010) Linking biodiversity and ecosystems: towards a unifying ecological theory. Philosophical Transactions of the Royal Society of London B: Biological Sciences, 365: 49 – 60.

McGlathery K.J., Sundbäck K., & Anderson I.C. (2007) Eutrophication in shallow coastal bays and lagoons: The role of plants in the coastal filter. Marine Ecology Progress Series, 348: 1 – 18.

Mermillod-Blondin F., Lemoine D.G., Boisson J.C., Malet E., & Montuelle B. (2008) Relative influences of submersed macrophytes and bioturbating fauna on biogeochemical processes and microbial activities in freshwater sediments. Freshwater Biology, 53: 1969 – 1982.

Mermillod-Blondin F., & Lemoine D.G. (2010) Ecosystem engineering by tubificid worms stimulates macrophyte growth in poorly oxygenated wetland sediments. Functional Ecology, 24: 444 – 453.

Miller A.J., & Cramer M.D. (2004) Root nitrogen acquisition and assimilation. Plant and Soil, 274: 1 – 36.

Nielsen L.P. (1992) Denitrification in sediment determined from nitrogen isotope paring. FEMS Microbiology Ecology, 86: 357 – 362.

Nielsen L.B., Finster K., Welsh D.T., Donelly A., Herbert R.A., De Wit R., & Lomstein B.A.A. (2001) Sulphate reduction and nitrogen fixation rates associated with roots, rhizomes and sediments from *Zostera noltii* and *Spartina maritima* meadows. Environmental Microbiology, 3: 63 – 71.

Nizzoli D., Bartoli M., Cooper M., Welsh D.T., Underwood G.J.C., & Viaroli P. (2007) Implications for oxygen, nutrient fluxes and denitrification rates during the early stage of sediment colonisation by the polychaete *Nereis* spp. in four estuaries. Estuarine, Coastal and Shelf Science, 75: 125 – 134.

Nizzoli D., Welsh D.T., Longhi D., & Viaroli P. (2014) Influence of *Potamogeton pectinatus* and microphytobenthos on benthic metabolism, nutrient fluxes and denitrification in a freshwater littoral sediment in an agricultural landscape: N assimilation versus N removal. Hydrobiologia, 737: 183 – 200.

Pelegrí S.P., Nielsen L.P., & Blackburn T.H. (1994) Denitrification in estuarine sediment stimulated by the irrigation activity of the amphipod *Corophium volutator*. Marine Ecology Progress Series, 105: 285 – 290.

Pelegrí S.P., & Blackburn T.H. (1995) Effects of *Tubifex tubifex* (Oligochaeta: Tubificidae) on N-mineralization in freshwater sediments, measured with ^{15}isotopes. Aquatic Microbial Ecology, 9: 289 – 294.

Petersen J.M., Kemper A., Gruber-Vodicka H., Cardini U., van der Geest M., Kleiner M., ... & Antony C.P. (2017) Chemosynthetic symbionts of marine invertebrate animals are capable of nitrogen fixation. Nature Microbiology, 2: 16195.

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. Journal of Limnology, 68: 133 – 145.

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.

Racchetti E., Longhi D., Ribaudo C., Soana E., & Bartoli M. (2017) Nitrogen uptake and coupled nitrification–denitrification in riverine sediments with benthic microalgae and rooted macrophytes. Aquatic Sciences, 79: 487 – 505.

Raffaelli D., Emmerson M., Solan M., Biles C., & Paterson D. (2003) Biodiversity and ecosystem processes in shallow coastal waters: An experimental approach. Journal of Sea Research, 49: 133 – 141.

Redfield A. (1958) The biological control of chemical factors in the environment. American Scientist, 46: 205 – 221.

Risgaard-Petersen N., & Jensen K. (1997) Nitrification and denitrification in the rhizosphere of the aquatic macrophyte *Lobelia dortmanna* L. Limnology and Oceanography, 42: 529 – 537.

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. Marine Ecology Progress Series, 174: 281 – 291.

Risgaard-Petersen N. (2003) Coupled nitrification-denitrification in autotrophic and heterotrophic estuarine sediments: On the influence of benthic microalgae. Limnology and Oceanography, 48: 93 – 105.

Rota E., Bartoli M., & Laini A. (2014) First time in Italy. Is the elusive aquatic megadrile *Sparganophilus* Benham, 1892 (Annelida, Clitellata) accelerating its dispersal in Europe?. Journal of Limnology, 73: 482 – 489.

Ruginis T., Bartoli M., Petkuviene J., Zilius M., Vybernaite-Lubiene I., Laini A., & Razinkovas-Baziukas A. (2014) Benthic respiration and stoichiometry of regenerated nutrients in lake sediments with *Dreissena polymorpha*. Aquatic Sciences, 76: 405 – 417.

Salas A.K., & Borrett S.R. (2011) Evidence for the dominance of indirect effects in 50 trophic ecosystem networks. Ecological Modelling, 222: 1192 – 1204.

Schrama M., van Boheemen L.A., Olff H., & Berg M.P. (2015) How the litter-feeding bioturbators *Orchestia gammarellus* promotes late-successional saltmarsh vegetation. Journal of Ecology, 103: 915 – 924.

Small G. E., Sterner R.W, & Finlay J.C. (2014) An Ecological Network Analysis of nitrogen cycling in the Laurentian Great Lakes. Ecological Modelling, 293: 150 – 160.

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.

Soana E., & Bartoli M. (2013) Seasonal variation of radial oxygen loss in *Vallisneria spiralis* L.: An adaptive response to sediment redox?. Aquatic Botany, 104: 228 – 232.

Soana E., & Bartoli M. (2014) Seasonal regulation of nitrification in a rooted macrophyte (*Vallisneria spiralis* L.) meadow under eutrophic conditions. Aquatic Ecology, 16: 2064 – 2090.

Soana E., Naldi M., Bonaglia S., Racchetti E., Castaldelli G., Brüchert V., … & Bartoli M. (2015) Benthic nitrogen metabolism in a macrophyte meadow (*Vallisneria spiralis* L.) under increasing sedimentary organic matter loads. Biogeochemistry, 124: 387 – 404.

Stief P. (2013) Stimulation of microbial nitrogen cycling in aquatic ecosystems by benthic macrofauna: Mechanisms and environmental implications. Biogeosciences, 10: 7829 – 7846.

Svensson J.M., Enrich-Prast A., & Leonardson L. (2001) Nitrification and denitrification in a eutrophic lake sediment bioturbated by oligochaetes. Aquatic Microbial Ecology, 23: 177 – 186.

Ulanowicz R.E., & Puccia C.J. (1990) Mixed trophic impacts in ecosystems. Coenoses, 5: 7 – 16.

Vadeboncoeur Y., Jeppesen E., Vander Zanden M.J., Schierup H.H., Christoffersen K., & Lodge D.M. (2003) From Greenland to green lakes: Cultural eutrophication and the loss of benthic pathways in lakes. Limnology and Oceanography, 48: 1408 – 1418.

van der Heide T., Govers L.L., de Fouw J., Olff H., van der Geest M., van Katwijk M.M., ... & van Gils J.A. (2012) A three-stage symbiosis forms the foundation of seagrass ecosystems. Science, 336: 1432 – 1434.

Vaughn C.C., Nichols S.J., & Spooner D.E. (2008) Community and foodweb ecology of freshwater mussels. Journal of the North American Benthological Society, 27: 409 – 423.

Vila-Costa M., Pulido C., Chappuis E., Calviño A., Casamayor E.O., & Gacia E. (2016) Macrophyte landscape modulates lake ecosystem-level nitrogen losses through tightly coupled plant-microbe interactions. Limnology and Oceanography, 61: 78 – 88.

Volkmann C., Halbedel S., Voss M., & Schubert H. (2016) The role of dissolved organic and inorganic nitrogen for growth of macrophytes in coastal waters of the Baltic Sea. Journal of Experimental Marine Biology and Ecology, 477: 23 – 30.

Winberg G.G., Alimov A.F., Boullion V.V., Ivanova M.B., Korobtzova E.V., Kuzmitzkaya N.K., ... & Fursenko M.V. (1973) Biological productivity of two subarctic lakes. Freshwater Biology, 3: 177 – 197.

Wulff F., Field J.G., & Mann K.H. (1989) Network Analysis in Marine Ecology: Methods and Applications. Springer-Verlag, Berlin.

Section 4.3

Benthic uptake stimulates N fixation and suppresses denitrification and recycling in the oligotrophic Cazaux-Sanguinet Lake

Introduction

Shallow freshwater oligotrophic systems receive sufficient light to sustain high rates of primary production. Primary producers such as microalgae and rooted macrophytes have an important role in controlling nutrient dynamics and oxygen (O_2) production (Risgaard-Petersen 2003; Sand-Jensen et al. 1982). They assimilate inorganic nutrients from the water column and from the pore water and increase the volume of the oxic sediment by releasing O_2 and, hence, may favor processes, such as nitrification, deeper in the sediment (Risgaard-Petersen and Jensen 1997; Sundbäck et al. 2004; Lemoine et al. 2012; Ferguson and Eyre 2013; Vila-Costa et al. 2016).

Perennial plants like isoetids can grow in waters with low pH, low nutrient concentration and low organic matter content (Richardson et al. 1984). *Lobelia dortmanna* L. (hereafter *Lobelia*) is a common representative of the small isoetid plants dominating the vegetation in nutrient-poor lakes in the temperate zones of Europe and North America (Farmer 1989; Sand-Jensen et al. 2005). *Lobelia* is a slow-growing plant with a slow tissue turnover that grows in sandy sediments (Moeller 1978; Richardson et al. 1984; Pedersen and Sand-Jensen 1992). Isoetid species usually grow in low carbon dioxide (CO_2) and carbonate levels in waters, as their main carbon source comes from the sediment in which the concentrations are usually up to a 100-fold higher than in the water layer (Richardson et al. 1984; Smolders et al. 2002; Raun et al. 2010). *Lobelia* has a well-developed aerenchyma and a thick leaf cuticle that reduces the permeability of O_2 and CO_2, whereas the roots have no barriers to gas exchange with the sediment pore water (Sand-Jensen and Prahl 1982; Pedersen et al. 1995). As carbon dioxide is taken up from the sediment, the transfer of CO_2 from the roots to the leaves and the transfer of O_2 from the leaves to the roots, is possible due to the presence of air lacunae along the entire plant (Sand-Jensen and Prahl 1982). *Lobelia* can release the 85 % of the O_2 produced during photosynthesis by the roots (Pedersen et al. 1995). This process, called radial oxygen loss (ROL), has implications in the surrounding reduced sediments, such as the stimulation of the organic matter mineralization and the coupling between nitrification and denitrification. The latter results in a nitrogen (N) loss that plays an important role in maintaining the oligotrophic status of the environment (Olsen and Andersen 1994; Risgaard-Petersen and Jensen 1997;

Smolders et al. 2002). However, this N loss could be a disadvantage for primary producers that need N for their growth. Schuurkes et al. (1986) demonstrated that the 83 % of the inorganic N is absorbed by the roots of *Lobelia* and that it is able to assimilate nitrate (NO_3^-) rather than ammonium (NH_4^+).

The rhizosphere of isoetid plants could favor the presence of individuals of meio and macrofauna, by releasing O_2 through the roots. Furthermore, the plant can be favored by sediment reworking and inorganic nutrients mobilization in presence of benthic animals. Among macrofauna functional groups, filter-feeding animals can provide many ecosystem services such as particle filtration, nutrient recycling and storage, structural habitat and substrate modification (Vaughn 2018). In oligotrophic environments, nutrient limitation can be alleviated by bivalve excretion of soluble nutrients to the water column, which can be readily taken up by primary producers (Vaughn and Hakenkamp 2001; Atkinson et al. 2013). Furthermore, bivalves may process large amounts of particulate matter that is partly stored in the sediment as biodeposits that enrich the sediment with organic matter (Prins et al. 1998; Strayer 2014). It is well known that bivalves couple the water column and sediment compartments by removing particulate materials from the water column and depositing them to the sediment as feces and pseudofeces (Vaugnh 2018; Benelli et al. 2019). The widespread burrowing bivalve *Corbicula fluminea* (hereafter *Corbicula*) can directly impact nutrient dynamics in the water column, but also affect benthic processes as it can burrow into the sediments. Bivalve bioturbation can increase O_2 penetration deeper in the sediment and stimulate microbial metabolism (Vaughn and Hakenkamp 2001). *Corbicula* has known to be a filter-feeding bivalve; however, there are some evidences of pedal-feeding, where the cilia of the foot can collect the subsurface organic matter (Cleland 1988; Reid et al. 1992). Hakenkamp and Palmer (1999) demonstrated the pedal-feeding on burial organic sediment by *Corbicula* in streams characterized by sandy sediments and long period of low flow.

In the present work, the metabolism of a benthic oligotrophic system where *Lobelia* and *Corbicula* are co-present was studied. By means of intact sediments incubation, the effects of plant and bivalve interaction on biogeochemical processes were investigated. We hypothesized a higher assimilation/denitrification ratio (A/D) in the system with *Lobelia*, as the plants need N for their growth, and a lower ratio in presence of bivalves, as they can regenerate inorganic nutrients, through their excretion. Finally, we hypothesized that filter feeders may alleviate the competition for N between primary producers and bacteria.

Material and methods

Sampling and experimental design

The sampling was performed during the summer 2017 in the Cazaux-Sanguinet Lake (44.49683°N, 001.20005°W). This is an oligo-mesotrophic lake in the South-West of France (Ribaudo et al. 2017) hosting native and non-native macrophytes. Individuals of *Lobelia*, a submersed rooted native isoetid plant, and of *Corbicula*, a filter-feeding allochtonous bivalve, were collected by hand from a shallow zone along the littoral. During the sampling, 32 plexiglass cores (8 cm inner diameter and 30 cm height) containing a specimen of *L. dortmanna* were collected from the site. In half of this set of cores, two individuals of *Corbicula* were added to the sediment, as from a first survey of the site, we found 2 or more individuals of bivalve living within the roots of most of the sampled macrophytes. A second set of 32 cores (4 cm inner diameter and 20 cm height) was used to collect bare sediment from the same site. In 16 cores of this set, two individuals of *Corbicula* were added. At the end the treatments were four: bare sediment (S), sediment with *Lobelia* (SL), sediment with *Corbicula* (SC) and sediment with *Lobelia* and *Corbicula* (SLC).

Sediment features

Four additional cores (4 cm inner diameter and 20 cm height) were collected from the same site in order to characterize the substrate. The upper 10 cm vertical sediment horizon from each core was extruded with a piston and sliced in 5 layers: 0-1 cm, 1-2 cm, 2-3 cm, 3-5 cm and 5-10 cm. Bulk density was determined as the ratio between wet weight and volume (5 ml) of sediment; the sediment was collected by means of a cut-off 5-ml syringe filled with the sediment homogenate. Then it was dried at 70 °C until constant weight and porosity was calculated. Organic matter content (OM) was measured as the percentage of weight loss on ignition (450 °C, 4 h) from ~ 0.2 g of dried, powdered sediment. Chlorophyll a (chl *a*) was extracted from each layer and transferred into vials containing 10 ml of 90 % acetone and stored 24 h in the dark. The extracted chlorophyll was thereafter determined after centrifugation and filtration according to Lorenzen (1967).

Setup and procedure of intact core incubation

After collecting, all the cores were immediately brought to the laboratory and submerged overnight with the top open in two tanks containing aerated and well-stirred in situ water. Each core was equipped with a magnetic stirrer positioned 10 cm above the sediment surface, in order to keep the water mixed and not to resuspend the sediment. The tanks were provided with a central magnet rotating at 40 rpm and driving all magnets inside the cores. Two short-term

batch incubations were performed, both in the dark and in the light: in the first incubation net fluxes of gas and nutrients were measured and in the second one denitrification was measured using the revised version of the isotope pairing technique (IPT, Dalsgaard et al. 2000; Risgaard-Petersen et al. 2003), in order to exclude anammox. Both incubations lasted 2 hours for the small cores and 4 hours for the larger ones, in order to keep initial O_2 concentration within 20 % of the initial value, not to have limiting O_2 conditions in the cores that may underestimate aerobic processes. During the light phases the irradiance was 400 ± 50 µE $m^{-2}s^{-1}$ measured at the sediment–water interface with an underwater quantum sensor (LI-COR 192) and the temperature was 24 °C. The incubations started when the level of water in the tank was lowered and the top lids were added to each core. At the starting of the incubations, O_2 was measured by means of electrode and a sub-sample of water was taken taken from the tanks. One aliquot of 20 ml was collected by means of a plastic syringe to fill a 12-ml Exetainer (Labco®, High Wycombe, UK) and added with 100 µl of 7 M $ZnCl_2$ to stop bacterial activity for further analysis of N_2 and CH_4 by membrane inlet mass spectrometer (MIMS, Bay-Instrument, University of Ferrara). Another aliquot of water was filtered (Whatman GF/F filters) and transferred to a 100-ml glass bottle where alkalinity (Talk) was determined by Gran titration with 0.1 M HCl (Anderson et al. 1986) and dissolved inorganic carbon (DIC) calculated from pH and Talk measurements, the carbonic acid dissociation constant and the carbon dioxide solubility coefficient (Weiss 1970). An aliquot of 20 ml was filtered (GF/F glass fiber filters, 0.7 µm) and transferred to 20-ml plastic vials in order to analyze: ammonium (NH_4^+), nitrite (NO_2^-) and nitrate (NO_3^-). Dissolved inorganic nitrogen (DIN) was calculated as the sum of NH_4^+, NO_2^- and NO_3^-. At the end of the incubations, the same procedure was repeated. Dissolved NO_2^-, combined nitrites and nitrates (NO_x^-) were analyzed with a continuous flow analyzer (San^{++}, Skalar, sensitivity 0.3 µM) using standard colorimetric methods. Nitrate was calculated as the difference between NO_x^- and NO_2^-. Ammonium was analyzed spectrophotometrically using salicylate and hypochlorite, with nitroprussiate as catalyst (Bower and Holm-Hansen 1980).

Dissolved gas and nutrient fluxes were calculated according to the equation below:

$$Flux_x = \frac{\left([x]_f - [x]_i\right) \times V}{A \times t}$$

where $[x]_f$ and $[x]_i$ are the concentrations (µM or mM) of the solute x at the end and at the start of the incubation, respectively, V (L) is the volume of the core water phase, A (m^2) is the area of the sediment, and t (h) is the incubation time.

Daily fluxes (µmol $m^{-2}d^{-1}$ or mmol $m^{-2}d^{-1}$) were calculated according to the following equation:

$$Daily\ flux_x = (hourly\ dark\ flux \times h_D) + (hourly\ light\ flux \times h_L)$$

where h_D and h_L are the numbers of dark (10) and light (14) hours, respectively.

At the end of the first incubation, all the cores were submerged again with the top open in aerated and well-stirred in situ water. Then, two different volumes of a stock solution of 15 mM $^{15}NO_3^-$ were added to the water column of each core, in order to have two final concentrations of labelled nitrate of 10 and 30 µM, per each treatment. After few minutes from the addition, an aliquot of water was sampled to measure the actual concentration of NO_3^- at the initial time. The top lids were positioned in each core and the light and dark incubations started. They lasted two and four hours for 4 and 8 cm cores inner diameter, respectively. At the end of the incubations, the lids were removed and the whole sediment and water phase were gently slurried with a glass spatula to distribute labelled N_2, then an aliquot of slurry was subsampled and immediately transferred to Exetainers and poisoned with 200 µL of 7 M $ZnCl_2$ for labelled N_2 analysis. At the end of the procedure, the cores were sieved through a 0.5 mm mesh in order to retrive macrophytes and bivalves, which were then characterized for wet weight (g_{WW}), for the dry weight (g_{DW}) and for the shell-free dry weight (sfdw), after drying at 70 °C to a constant weight.

Statistical analysis

One-way analysis of variance was used to test for differences of sedimentary features along sediment depth. Two-way ANOVA was used to test for differences between condition (light and dark) and treatment (S, SL, SC and SLC), including their interaction. Data normality was tested using Shapiro-Wilk method and the homogeneity of variance with Levene test. In the case of heteroscedasticity, data were $\log(x^2)$ transformed. Post hoc pairwise comparisons were performed using Tukey HSD test to find out the significant differences among groups. The statistical significance was set at $p<0.05$. All the analyses were performed using the R program.

Results

Sediment features of the sampling site

The 10 cm sediment profile was sandy and displayed high density that increased with sediment depth but the differences in the layers were not statistically different (one-way ANOVA, $p=0.064$; Table 4.3.1). Porosity was low and not statistically different in the 5 sediment layers (one-way ANOVA, $p=0.604$) but it decreased along depth profile (Table 4.3.1). Organic matter content was very low and decreased with sediment depth (Table 4.3.1). The one-way ANOVA showed a statistical difference among the 5 layers ($p<0.01$). Chl *a* concentration was higher in

the first layers and lowered until 10 cm depth and the difference among the layers was significant (one-way ANOVA, $p<0.01$; Table 4.3.1). In this side of the lake, there was the highest density of *Lobelia* (~ 40 %).

Table 4.3.1 Sediment features of the sampling site in Cazaux-Sanguinet Lake. The values reported in the table correspond to the mean ± standard error of each sediment layer (n=4).

Sediment layers	**Density**	**Porosity**	**Organic matter**	**Chl *a***
cm	g cm^{-3}	ml ml^{-1}	%	mg m^{-2}
0-1	1.72 ± 0.03	0.40 ± 0.04	0.16 ± 0.01	113.34 ± 4.38
1-2	1.85 ± 0.06	0.34 ± 0.02	0.11 ± 0.01	48.33 ± 4.16
2-3	1.81 ± 0.02	0.34 ± 0.01	0.12 ± 0.01	36.82 ± 6.98
3-5	1.89 ± 0.05	0.35 ± 0.01	0.10 ± 0.01	28.38 ± 1.26
5-10	1.87 ± 0.04	0.35 ± 0.01	0.07 ± 0.02	13.09 ± 0.83

Benthic fluxes

Net O_2 fluxes measured during the light incubation were higher in bare sediment (S) compared to other treatments, with values around 2.00 ± 0.13 mmol $m^{-2}h^{-1}$ (mean ± standard error)(Fig. 4.3.1a). The presence of *Lobelia* did not increase O_2 production during the light phase in the benthic system. In the dark, the highest respiration rate was measured in SLC. Both factors condition and treatment produced a significant effect on O_2 fluxes, but not their interaction (two-way ANOVA, Table 4.3.2). Gross primary production (obtained combining light and dark fluxes) did not differ among the treatments, suggesting the presence of active benthic algae covering the sediments in the 4 treatments. Daily fluxes showed that the benthic system was net autotrophic in bare sediment and in sediment with *Corbicula*. In presence of *Lobelia*, the benthic system was net heterotrophic (Fig. 4.3.1a). Dissolved inorganic carbon net fluxes were generally higher than O_2 fluxes, suggesting an underestimation of the latter due to bubble formation not detectable with our standard methods. DIC fluxes in the light ranged from -12.07 ± 1.07 in S to -1.56 ± 2.75 mmol $m^{-2}h^{-1}$ in SL (Fig. 4.3.1b). In the dark, dissolved inorganic carbon was produced in treatments with *Lobelia*, whereas bare sediment and sediment with *Corbicula* displayed negative fluxes. The high dark fluxes contributed to a higher (more negative) GPP flux in SLC. Only in presence of *Corbicula*, GPP flux was close to zero. The two-way ANOVA on DIC fluxes revealed that condition, treatment and their interaction were significant. On a daily basis, the benthic system was a net DIC sink in all treatments with the

exception of SL treatment (Fig. 4.3.1b). Net N_2 fluxes were all negative during the light phase, with values up to -1.33 ± 0.25 and -1.66 ± 0.37 mmol N $m^{-2}h^{-1}$ in S and SC, respectively (Fig. 4.3.1c). In these treatments N_2 fluxes were significantly different from the ones measured in treatments with *Lobelia* (Tukey HSD test, Table 4.3.2). In the dark, all treatments displayed positive N effluxes that were not statistically different. Daily N_2 fluxes were generally driven by light fluxes, in particular in treatments without *Lobelia* (Fig. 4.3.1c). Denitrification rates measured with the IPT (data not shown) were higher in the dark in all treatments but with values close to 1 µmol $m^{-2}h^{-1}$. The main source of NO_3^- for the denitrification was coming from the coupled nitrification and denitrification that occurred in the sediment, with the only exception of sediments with *Lobelia* where the denitrification was zero.

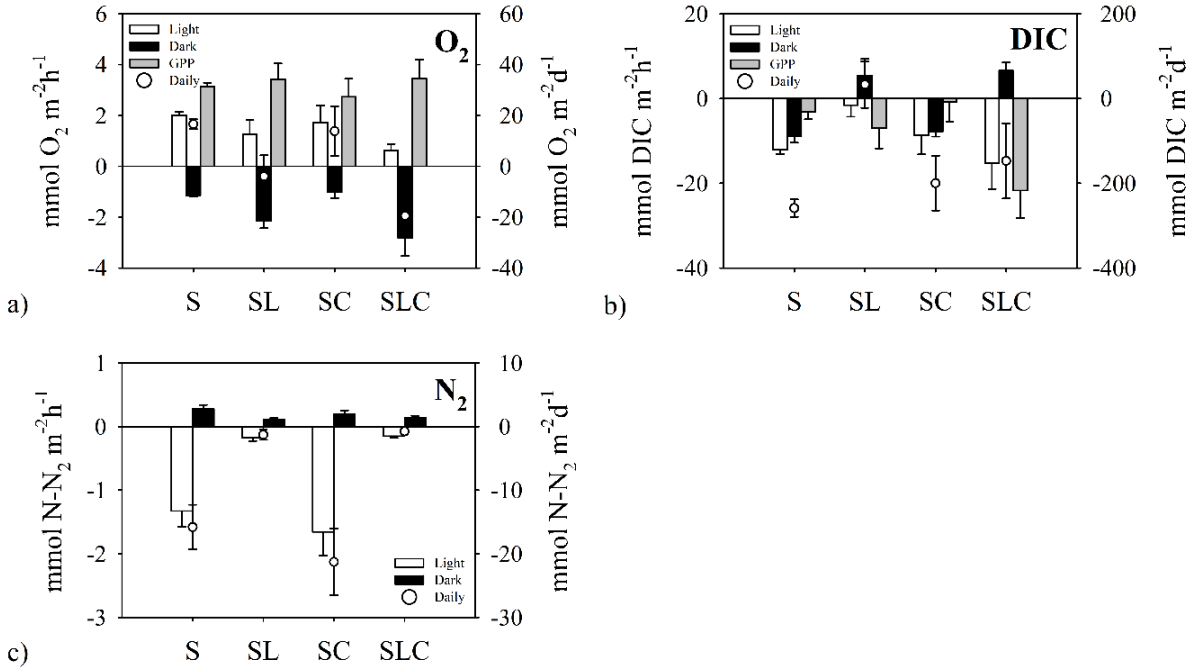


Fig. 4.3.1 Benthic fluxes of a) dissolved O_2 and b) DIC, measured in dark (black bars) and in light (white bars) incubations in the 4 treatments; c) N_2 measured in light (white bars) and dark (black bars) incubations in the 4 treatments. Grey bars in O_2 and DIC graphs represent Gross Primary Production (GPP). Mean ± standard error are reported. All fluxes are expressed in mmol $m^{-2}h^{-1}$. Dots represent daily mean ± standard error expressed in mmol $m^{-2}d^{-1}$.

Light ammonium fluxes were negative in all treatments, suggesting NH_4^+ assimilation by benthic primary producers. In the dark there was a net NH_4^+ release in SC treatment, whereas negative fluxes were displayed in the other treatments (Fig. 4.3.2a). Daily fluxes were negative in all treatments. The factor condition had no effect on NH_4^+ fluxes, whereas treatment and the interaction were significant (two-way ANOVA, Table 4.3.2). Nitrate fluxes were positive both in the light and in the dark in bare sediment and sediment with bivalves, whereas sediments with *Lobelia* were sink of NO_3^- both in the light and in the dark (Fig. 4.3.2b). Daily fluxes were negative with the plants and positive in the other treatments. Low negative nitrite fluxes with

values of a few µmol $m^{-2}h^{-1}$ were measured in all treatments and daily fluxes were close to zero (Fig. 4.3.2c). The sum of the three dissolved inorganic N forms gave the DIN fluxes in the 4 treatments: sediments with *Lobelia* acted as sink of DIN both in the light and in the dark, whereas S and SC were releasing DIN to the water column in the dark and consuming it in the light (Fig. 4.3.2d). Daily fluxes in these two latter treatments were close to zero, suggesting a balancing between light and dark fluxes. With the plants daily fluxes were always negative. Condition and treatment factors and their interaction were significant for the 3 inorganic forms of nitrogen (two-way ANOVA, Table 4.3.2).

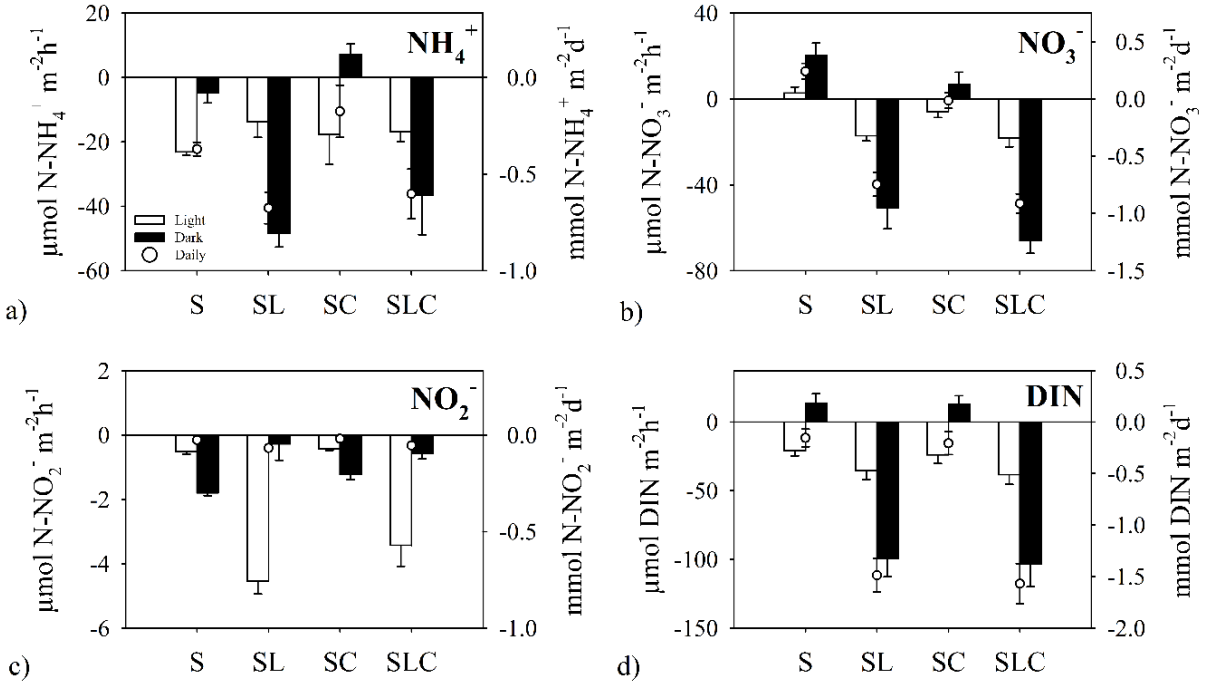


Fig. 4.3.2 Light and dark benthic fluxes of: a) NH_4^+, b) NO_3^-, c) NO_2^- and d) DIN measured in the 4 treatments. Mean ± standard error are reported. All fluxes are expressed in µmol $m^{-2}h^{-1}$. Dots represent daily mean ± standard error of nutrient fluxes expressed in mmol $m^{-2}d^{-1}$.

Table 4.3.2 Results of the two-way ANOVA testing the effects of the factors incubation condition (light and dark) and treatment (S, SL, SC, SLC) on benthic gas and nutrient fluxes. N_2 and NO_2^- fluxes were log(x^2) transformed. F, F- statistics; p, p-value. Significant values are printed in bold.

Dependent variable	Independent variable	F	p
O_2 fluxes	Condition	110.2	**<0.001**
	Treatment	5.7	**<0.01**
	Interaction	0.3	0.82
DIC fluxes	Condition	11.9	**<0.01**
	Treatment	5.3	**<0.01**
	Interaction	3.9	**<0.05**

Dependent variable	Independent variable	F	p
N_2 fluxes	Condition	22.3	**<0.001**
	Treatment	12.3	**<0.001**
	Interaction	5.8	**<0.01**
NH_4^+ fluxes	Condition	0.4	0.52
	Treatment	7.2	**<0.01**
	Interaction	10.9	**<0.001**
NO_3^- fluxes	Condition	11.0	**<0.01**
	Treatment	45.4	**<0.001**
	Interaction	18.1	**<0.001**
NO_2^- fluxes	Condition	5.5	**<0.05**
	Treatment	4.7	**<0.01**
	Interaction	26.5	**<0.001**
DIN fluxes	Condition	4.8	**<0.05**
	Treatment	32.2	**<0.001**
	Interaction	19.3	**<0.001**

Discussion

Intact sediments incubation is a useful method to understand the metabolism of the whole benthic system. Such approach sets boundaires of net processes in the light and in the dark but does not allow to disentangle the multiple, sometimes opposite flows mediated by microbes, meio and macrofauna and primary producers. To this purpose, measurements in the light and in the dark of net exchanges, performed in bare and vegetated sediments, with or without macrofauna, allow to infer the role of single components. Moreover, sequential incubations of fluxes under natural conditions and after the addition of tracers (e.g., $^{15}NO_3^-$) allow to quantify specific pathways (e.g., dentitrification, anammox or DNRA) and, by difference, to infer the relevance of additional processes (e.g., N fixation, or N uptake). We applied this combined approach to investigate the role of the isoetid *Lobelia*, the community of benthic and ephypitic algae, the bivalve *Corbicula*, and of bacteria as drivers of N cycling in the Cazaux-Sanguinet Lake. As hypothesized, in such oligotrophic environment *Lobelia* seems the main driver of N transformation, by means of direct uptake, via stimulation of large N import through fixation in the light period and via indirect inhibition of processes as denitrification. Our approach was not conclusive on the role of *Corbicula*, as the effect of this bivalve on benthic biogeochemistry

remains hidden. This is likely due to strong N limitation, elevated re-use of regenerated or excreted N, occurring in the light and in the dark.

The calculated photosynthetic quotient (PQ), which is the ratio between O_2 and DIC fluxes in the light, is lower than 1.2 (Strickland and Parsons 1972) in sediments with *Lobelia* indicating that the O_2 produced by photosynthesis was mainly transferred to the roots and not to the water column (Wium-Andersen 1971; Sand-Jensen et al. 1982). However, PQ was lower than 1.2 also in bare sediments, likely due to an underestimation of O_2 fluxes as in the cores we observed gas bubble formation during the incubation. Oxygen released through the roots may contribute to sediment oxidation and to the consequent increased microbial mineralization of organic matter. This process contributes to CO_2 formation that can be directly taken up by the plant (Pedersen and Sand-Jensen 1992; Pedersen et al. 1995; Smolders et al. 2002; Lenzewski et al. 2018). The respiratory quotient calculated from DIC : O_2 ratio in the dark was higher than the unit in sediments with *Lobelia*, suggesting an accumulation of reduction equivalents in the sediment. The rates of dissolved inorganic carbon and oxygen in sediments with *Lobelia* that we measured are in accordance with the results reported by Ribaudo et al. (2017) for vegetated sediments in the same lake. The only exception is for light DIC fluxes that were probably affected by carbonate dissolution or precipitation.

Nitrogen loss via denitrification was very low to undetectable in all treatments likely due to N limitation in this oligotrophic environment. We acknowledge that the IPT allows to measure only the removal of nitrate diffusing from the water column to the sediment and the nitrate produced in the upper sediment layer by nitrification. The first is low due to extremely low concentrations of nitrate (< 5 μM) and to root uptake of NO_3^-, as it was demonstrated by Schuurkes et al. (1986). The second is low likely due to ammonium limitation in the sandy sediments of the lake, in particular in the uppermost sediments where the high primary production rates of benthic algae represent the main inorganic N sink. Risgaard-Petersen (2003) also dimostrated in illuminated sediments a general inhibition of nitrification and denitrification operated by algae on bacteria, even under conditions of N availability. The same author (Risgaard-Petersen and Jensen 1997) reported measurable rates of subsurface denitrification coupled to nitrification in the rhizosphere of *Lobelia*, measured via $^{15}NH_4^+$ perfusion technique. Reported rates (20-40 μmol N $m^{-2}h^{-1}$) are higher than those reported in this work and represent a cost that the macrophyte withstands in an N limiting environment. Furthermore, as *Lobelia* drives much higher O_2 to the roots during the light phase, when it is photosynthetising, denitrification rates are higher in the light (when the plant needs N to assimilate) than in the dark. Risgaard-Petersen did not measure other processes besides denitrification, whereas results

from the present study support the occurrence of large N fixation rates, largely compensating such loss.

In the light, we measured negative N_2 fluxes in all conditions, suggesting a general dominance of N fixation over denitrification and likely supporting a large share of theoretical N demand by benthic primary producers. Such negative fluxes of N_2 rates peaked in the conditions without *Lobelia*, whereas they were considerably lower in the presence of the plant, likely due to some subsurface N_2 production and to the inhibition of this process by the O_2 released by plant roots. Fluxes of inorganic N supported the strong control operated by the macrophyte and by the benthic primary producers in general, with small rates of regeneration measured only in the dark, in the sediment alone and in the sediment with *Corbicula*.

A note from the author:

I need to perform a number of calculations on the present dataset, starting from the quantification of benthic primary production by algae and by Lobelia. *I need literature support to this purpose. Thereafter, I want to backcalculate the theoretical N requirements to support such primary production and put all measured fluxes (e.g., N fixation and denitrification) in such context. This, in order to quantify the relevance of N fixation in supporting primary production in this system and to analyze the assimilation/denitrification rates. Last, I would like to quantify the role of* Corbicula *in this oligotrophic system, as large excretion rates from the literature and net process measurement suggest a tight coupling between regeneration and uptake.*

References

Anderson L.G., Hall P.O.J., Iverfeldt Å., Van Der Loeff M.M.R., Sundby B., & Westerlund S.F.G. (1986) Benthic respiration measured by total carbonate production. Limnology and Oceanography, 31: 319 – 329.

Atkinson C.L., Vaughn C.C., Forshay K.J., & Cooper J.T. (2013) Aggregated filter-feeding consumers alter nutrient limitation: Consequences for ecosystem and community dynamics. Ecology, 94: 1359 – 1369.

Benelli S., Bartoli M., Zilius M., Vybernaite-Lubiene I., Ruginis T., Vaiciute D., ... & Fano E.A. (2019) Stoichiometry of regenerated nutrients differs between native and invasive freshwater mussels with implications for algal growth. Freshwater Biology, 00: 1 – 13.

Bower C.E., & Holm-Hansen T. (1980) A salicylate-hypochlorite method for determining ammonia in seawater. Canadian Journal of Fisheries and Aquatic Sciences, 37: 794 – 798.

Cleland J.D. (1988) Ecological and physiological considerations of deposit-feeding in freshwater bivalve, *Corbicula fluminea*. Masters Thesis, University of Texas, Arlington

Dalsgaard T., Nielsen L.P., Brotas V., Viaroli P., Underwood G., Nedwell D., ... & Dong L. (2000) Protocol handbook for NICE-Nitrogen Cycling in Estuaries: a project under the EU research programme: Marine Science and Technology (MAST III), 1 – 62.

Farmer A.M. (1989) *Lobelia Dortmanna* L. Journal of Ecology, 77: 1161 – 1173.

Ferguson A., & Eyre B. (2013) Interaction of benthic microalgae and macrofauna in the control of benthic metabolism, nutrient fluxes and denitrification in a shallow sub-tropical coastal embayment (western Moreton Bay, Australia). Biogeochemistry, 112: 423 – 440.

Hakenkamp C.C., & Palmer M.A. (1999) Introduced bivalves in freshwater ecosystems: The impact of *Corbicula* on organic matter dynamics in a sandy stream. Oecologia, 119: 445 – 451.

Moeller R.E. (1978) Seasonal changes in biomass, tissue chemistry, and net production of the evergreen hydrophyte, *Lobelia dortmanna*. Canadian Journal of Botany, 56: 1425–1433.

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. Aquatic Ecology, 46: 191 – 200.

Lenzewski N., Mueller P., Meier R.J., Liebsch G., Jensen K., & Koop-Jakobsen K. (2018) Dynamics of oxygen and carbon dioxide in rhizospheres of *Lobelia dortmanna* – A planar optode study of belowground gas exchange between plants and sediment. New Phytologist, 218: 131 – 141.

Lorenzen C.J. (1967) Determination of chlorophyll and phaeopigments: Spectrophotometric equations. Limnology and Oceanography, 12: 343 – 346.

Olsen K.R., & Andersen F.O. (1994) Nutrient cycling in shallow, oligotrophic Lake Kvie, Denmark. Part I. Effects of isoetids on the exchange of nitrogen between sediment and water. Hydrobiologia, 275: 255 – 265.

Pedersen O., & Sand-Jensen K. (1992) Adaptations of submerged *Lobelia dortmanna* to aerial life form: morphology, carbon sources and oxygen dynamics. Oikos, 65: 89 – 96.

Pedersen O., Sand-Jensen K., & Revsbech N.P. (1995) Diel pulses of O_2 and CO_2 in sandy lake sediments inhabited by *Lobelia dortmanna*. Ecology, 76: 1536 – 1545.

Prins T.C., Smaal A.C., & Dame R.F. (1998) A review of the feedbacks between bivalve grazing and ecosystem processes. Aquatic Ecology, 31: 349 – 359.

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. Freshwater Biology, 55: 1891 – 1904.

Reid R.G.B, McMahon R.F., Foighil D.O., & Finnigan R. (1992) Anterior inhalant currents and pedal feeding in bivalves. Veliger, 35: 93 – 104.

Ribaudo C., Bertrin V., Jan G., Anschutz P., & Abril G. (2017) Benthic production, respiration and methane oxidation in *Lobelia dortmanna* lawns. Hydrobiologia, 784: 21 – 34.

Richardson K., Griffiths H., Reed M.L., Raven J.A., & Griffiths N.M. (1984) Inorganic carbon assimilation in the Isoetids, *Isoetes lacustris* L. and *Lobelia dortmanna* L. Oecologia, 61: 115 – 121.

Risgaard-Petersen N., & Jensen K. (1997) Nitrification and denitrification in the rhizosphere of the aquatic macrophyte *Lobelia dortmanna* L. Limnology and Oceanography, 42: 529 – 537.

Risgaard-Petersen N. (2003) Coupled nitrification-denitrification in autotrophic and heterotrophic estuarine sediments: On the influence of benthic microalgae. Limnology and Oceanography, 48: 93 – 105.

Risgaard-Petersen N., Nielsen L.P., Rysgaard S., Dalsgaard T., & Meyer R.L. (2003) Application of the isotope pairing technique in sediments where anammox and denitrification coexist. Limnology and Oceanography: Methods, 1: 63 – 73.

Sand-Jensen K., & Prahl C. (1982) Oxygen exchange with the lacunae and across leaves and roots of the submerged vascular macrophyte, *Lobelia dortmanna* L. New Phytologist, 91: 103 – 120.

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., & Binzer T. (2005) Oxygen stress and reduced growth of *Lobelia dortmanna* in sandy lake sediments subject to organic enrichment. Freshwater Biology, 50: 1034 – 1048.

Schuurkes J.A.A.R., Kok C.J., & den Hartog C. (1986) Ammonium and nitrate uptake by aquatic plants from poorly buffered and acidified waters. Aquatic Botany, 24: 131 – 146.

Smolders A.J.P., Lucassen E.C.H.E.T., & Roelofs J.G.M. (2002) The isoetid environment: Biogeochemistry and threats. Aquatic Botany, 73: 325 – 350.

Strayer D.L. (2014) Understanding how nutrient cycles and freshwater mussels (Unionoida) affect one another. Hydrobiologia, 735: 277 – 292.

Strickland J.D., & Parsons T.R. (1972) A practical handbook of seawater analysis.

Vaughn C.C., & Hakenkamp C.C. (2001) The functional role of burrowing bivalves in freshwater ecosystems. Freshwater Biology, 46: 1431 – 1446.

Vaughn C.C. (2018) Ecosystem services provided by freshwater mussels. Hydrobiologia, 810: 15 – 27.

Vila-Costa M., Pulido C., Chappuis E., Calviño A., Casamayor E.O., & Gacia E. (2016) Macrophyte landscape modulates lake ecosystem-level nitrogen losses through tightly coupled plant-microbe interactions. Limnology and Oceanography, 61: 78 – 88.

Weiss R.F. (1970) The solubility of nitrogen, oxygen and argon in water and seawater. Deep Sea Research and Oceanographic Abstracts, 17: 721 – 735.

Wium-Andersen S. (1971) Photosynthetic uptake of free CO_2, by the roots of *Lobelia dortmanna*. Physiologia plantarum, 25: 245 – 248.

Main findings and general remarks

Experiments reported in this chapter tentatively respond to the stimulation of ecologists that stress the need of holistic, complex approaches. Such experiments are the only able to catch the effects of natural ecosystem complexity and therefore are the only able to predict the consequences of species loss or of species invasions on functioning.

Results from the experiment done under eutrophic conditions are somewhat surprising and partly different from what was hypothesized. In the most complex condition in fact, despite high N background and large N availability, fluxes from and to the benthic ecosystem were reduced whereas recycling increased within the benthic compartment. These results suggest that higher benthic biodiversity and functional diversity lead to better use of available resources and reduction of imports and losses. Furthermore, macrofauna acted as a facilitator and made available dissolved inorganic N that was taken up by primary producers. At the oligotrophic site, the results confirmed the hypotheses: most of N flows were directed to the primary producers and were supported by microbial N fixation; whereas the low denitrification rates suggest the need to keep N within the benthic compartment. As expected, the denitrification to N assimilation ratio varied dramatically in the two conditions, with much higher values at the N-rich site. The application of the Ecological Network Analysis at the microscale revealed interesting N dynamics between the compartments of the benthic system. Furthermore, it showed indirect interactions among multiple trophic levels that were not detectable with the experimental approach. For example, a major fraction of the N assimilated by the macrophyte was processed by macrofauna, even if there were no direct fluxes between the animals-plant compartments. In other words, ENA demonstrated the dependency of primary production from the particulate N pool, via the macrofauna facilitation. As the Ecological Network Analysis for the oligotrophic site is still in progress, it is not possible to find out if these N dynamics are true also for this system. However, from the experimental part, it appeared relevant the role of bacteria in driving N imports from outside the system. This suggests interactions between plants and N-fixers, as N fixation is an energetically costly process.

Next generation experimental studies on this topic may take advantage of multiple techniques now available, spanning from the combined use of stable ^{15}N isotopes ($^{15}NH_4^+$, $^{15}NO_3^-$, $^{30}N_2$), planar optodes techniques to analyze O_2 or other ions dynamics in sediments with roots and macrofauna, and molecular techniques to clarify if and how roots and macrofauna alter the microbial consortium in sediments. My personal idea is that illuminated sediments, with their autotrophic and heterotrophic communities, may represent a challenging opportunity to combine traditional community and ecosystem ecology into unified theoretical and

interpretative approaches. Relatively easy manipulation and much shorter time scales for aquatic plants growth as compared to terrestrial environments allow in fact to test large number of environmental gradients and human impacts, as biodiversity loss, invasion by alien species, nutrient enrichment or extreme phenomena as anoxia. Besides these aspects, energy and matter flows within aquatic environments can be measured with extreme accuracy, allowing to trace multiple paths and their variation along food webs.

Preface and general remarks references

Bogut I., Vidaković J., Palijan G., & Čerba D. (2007) Benthic macroinvertebrates associated with four species of macrophytes. Biologia, 62: 600 – 606.

Ferguson A., & Eyre B. (2013) Interaction of benthic microalgae and macrofauna in the control of benthic metabolism, nutrient fluxes and denitrification in a shallow sub-tropical coastal embayment (western Moreton Bay, Australia). Biogeochemistry, 112: 423 – 440.

Krull J.N. (1970) Aquatic plant-macroinvertebrate associations and waterfowl. The Journal of Wildlife Management, 34: 707 – 718.

Mermillod-Blondin F., & Lemoine D.G. (2010) Ecosystem engineering by tubificid worms stimulates macrophyte growth in poorly oxygenated wetland sediments. Functional Ecology, 24: 444 – 453.

Stief P. (2013) Stimulation of microbial nitrogen cycling in aquatic ecosystems by benthic macrofauna: Mechanisms and environmental implications. Biogeosciences, 10: 7829 – 7846.

Sundbäck K., Miles A., & Göransson E. (2000) Nitrogen fluxes, denitrification and the role of microphytobenthos in microtidal shallow-water sediments: An annual study. Marine Ecology Progress Series, 200: 59 – 76.

Chapter 5

Conclusions and future insights

Results from this thesis suggest that illuminated sediments, hosting diversified communities of bacteria, macrofauna and primary producers, represent ideal ecosystems to study key ecological questions. These include how the interactions between organisms and the physical environment change along environmental and biodiversity gradients and what are the effects produced by such changes on carbon and nutrient cycling or on the primary production. Native species loss or alien invasion, biodiversity and ecosystem functioning relationships are generally studied in terrestrial more than in aquatic ecosystems with large-scale experiments. However, these experiments are a few and limited, as they need huge efforts, large surfaces, sometimes long time span and large budgets. In this context, I believe that shallow water sediments provide a valid alternative in aquatic environments. The experiments reported in this thesis demonstrate that the complexity of the benthic system can be disentangled in relatively small scale laboratory experiments. Here, it is possible to remove or to add components under controlled conditions and analyze the response of the community. Within shallow benthic systems, N was elected in this thesis as an ideal nutrient for this kind of studies, for multiple reasons. First, its cycling is mediated by different reactions, including autotrophic (uptake) and heterotrophic (regeneration) processes, aerobic (e.g., nitrification) and anaerobic (e.g., nitrate ammonification) microbial transformations, and opposite pathways as net import (N fixation) or export (denitrification, anammox). Then, the physical and biological environment regulates such variety of reactions, by the competitive interactions among organisms or by facilitation processes within the community. This means that any variation in the physical or biological level is reflected by a change at some point of N cycling that can be detected with accuracy. Finally, an increasing number of techniques based on the use of the stable isotope in inorganic and organic molecules, planar optodes and pore water microplates, allow analyzing each N path and creating networks of mass transfer processes. Furthermore, N represents an extreme in aquatic environments as either it can be strongly limiting primary production and, consequently, the negative interactions between primary producers and bacteria are exacerbated, or it can be present in excess and therefore shared among organisms, smoothing the negative ecological interactions and resulting in its dissipation and inefficient use.

In my work, I investigated how light and the different background level of nutrients drive N dynamics along multiple trophic levels. Simplified and complex experimental approaches were applied in order to investigate the main N-related processes driven by benthic animals. Multiple

trophic levels leaded to complex ecological interactions, resulted in cascade effects and explained emerging properties at the whole system level, including the pelagic compartment. My results confirm the precious role of rare, but large organisms in benthic-pelagic coupling, the importance of *low ranked* macrofauna as chironomid larvae as pioneer species, regenerating geochemical buffers in organic loaded sediments and exerting a strong bottom-up and top-down control on pelagic primary production, in association with benthic algae. They also highlight how an invasive bivalve may alter the stoichiometry of nutrient regeneration and likely contribute to the dominance of harmful cyanobacteria, or favor a shift from low tolerant isoetids towards more tolerant macrophytes.

Results from this thesis are relevant in understanding the ecological role of the organisms under investigation. Regardless their origin, they could have positive or negative effects on the environment where they live. For instance, *Corbicula* spp. and *Sparganophilus tamesis* are allochthonous species in Europe and both were found in the oligotrophic Cazaux-Sanguinet Lake (France) and in the eutrophic Mincio River (Italy). Results from this thesis demonstrated the high ability of these invasive organisms to adapt to very different trophic conditions and sediment organic matter content. In the oligotrophic site, in fact, nutrient-poor water and refractory organic matter could make hostile the surrounding environment for macrofauna due to the limited food availability. To counteract the absence of planktonic food, *Corbicula* is able to change its way of feeding: from filtration of suspended particles to pedal-feeding. The latter included the feeding of organic matter from the sediment. The low organic matter content of these sites could also make difficult the survival of the oligochaete *S. tamesis*. In the Cazaux-Sanguinet Lake, *S. tamesis* biomass was lower than the individuals retrieved from the Mincio River. However, the invasion by the oligochaete suggests that it can survive also in these conditions. Therefore, the presence of macrofauna in these shallow water environments may have different implications on the survival of native rooted macrophytes: in the Mincio River, *Vallisneria spiralis* could take advantage from the presence of bioturbating organisms that mobilize nutrients and mineralize organic matter. Whereas in the Cazaux-Sanguinet Lake, the high nutrient availability could limit *Lobelia dortmanna* growth, as isoetids require very low nutrient levels in the water column and low organic matter content in the sediment. A hypothesized increase of worm density in the Cazaux-Sanguinet Lake could even endanger the persistence of the isoetids, due to a higher nutrient mobilization, which may stimulate the growth of pelagic algae or epiphytes, at the expenses of *Lobelia*. So, if the biomass of *S. tamesis* would grow excessively, nutrient stoichiometry of the lake may change and the isoetids would decline. The importance of these species is well known in the Atlantic Lakes of France and should be preserved with continuous monitoring plans of the other invasive species. The

objective of this thesis is not the analysis of the effects of macrofauna and macrophytes on the whole benthic system, but rather to investigate the effects of organisms and of the synergies between them on N processes at a finer scale. Further studies could take into consideration more organisms at the same time and could provide a global picture of the whole ecosystem.

The role of macrofauna in shallow, illuminated sediments is central in my work and it is fascinating for an ecologist. The hypothesis that macrofauna-plants interactions in organic-rich sediments may be beneficial for both via active exchange of oxygen (e.g., from roots via ROL to macrofauna through skin diffusion) and via increased sedimentary redox and nutrient mobilization (e.g., via sediment ingestion or conditioning and bioturbation) emerges from the *Vallisneria-Sparganophilus* intimate association but it is only partially demonstrated. Macrophytes-macrofauna interactions under oligotrophic conditions are likely more complex and may involve plants or macrofauna associations with microbes (e.g., N-fixing bacteria). Similar associations were usually studied in marine ecosystems, whereas they are poorly explored in freshwater environments and require molecular approaches.