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

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Abstract

The regulation of benthic nitrogen (N) cycling by multiple interactions among bacteria, macrofauna, and primary producers is poorly understood. 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 N₂ 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. To this purpose, the effects of two bioturbators (the detritivorous *Sparganophilus tamesis* and the filter-feeding *Corbicula* spp.) and two primary producer growth forms (the rooted macrophyte *Vallisneria spiralis* and microphytobenthos) on benthic N cycling were studied. An array of N-processes were measured along a complexity gradient (from bare sediments to all combinations of the above mentioned organisms), and experimental outcomes were analyzed via ecological network analysis (ENA). This suite of algorithms, applied to the microscale, revealed differential partitioning of N fluxes among bare sediments (highest denitrification rates), sediments with macrofauna (highest recycling), and sediments with rooted plants (highest N-fixation). N₂ losses and inputs were significantly reduced when all components were represented, and N requirements by primary producers were to a large extent supported by the activity of macrofauna. Ecological interactions in biodiverse benthic systems promoted an efficient exploitation of sedimentary N pools, increased the coupling between recycling and uptake, and maximized N use efficiency at the expenses of losses and imports.

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 (NH₄⁺), altering concentration gradients across the sediment–water interface (Soana et al. 2012). They, also, release oxygen (O₂) 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 while 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

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

further influenced by macrofauna (Bartoli et al. 2003b; Ferguson et al. 2004; Mermillod-Blondin et al. 2008).

Macrofauna affect 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 (Pelegri 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 mobilize 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 modeling 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.

Materials and methods

Experimental design

Our experimental approach involves the incubation of microcosms under controlled conditions after an acclimatization

period (Dalsgaard 2000; Ribaudo et al. 2011; Soana et al. 2015; Racchetti et al. 2017). Water, sediments, plants, and macrofauna were collected from a perifluvial area of the Mincio River (Northern Italy) in November 2013. Sediments of the sampled areas consisted of fluffy, organic material colonized by patchy meadows of the submersed macrophyte *V. spiralis*. Sediment porosity (0–10 cm depth, average \pm SD) was 0.77 ± 0.11 , while organic C, organic N and total P averaged $9.93\% \pm 0.93\%$, $0.56\% \pm 0.1\%$, and $0.06\% \pm 0.02\%$ of sediment dry weight, respectively. The concentrations of inorganic N in the water at the beginning of the experiment were 4.3 ± 0.3 , 1.6 ± 0.4 , and $114.2 \pm 10.5 \mu\text{M}$ for NH_4^+ , NO_2^- , and NO_3^- , respectively. Macrofauna were dominated by the oligochaete *S. tamesis* and by the bivalve *Corbicula* spp. (Gherardi et al. 2008; Rota et al. 2014). Both taxa were found in bare as well as in vegetated sediments.

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). Nearly 20 L of surface sediment (0–10 cm depth) were sieved in situ to remove macrofauna and large debris. It was then homogenized and transferred into cylindrical Plexiglass microcosms (i.d. 7.5 cm, height 10 cm, wall thickness 0.5 cm, $n = 64$). Additional sediment was collected and sieved to recover a sufficient number of oligochaetes and bivalves. The fluffy, organic sediment at the sampling site allowed gentle collection of *V. spiralis* by hand, preserving intact the rhizosphere. Two individuals of each species were then added or transplanted into each microcosm, reflecting in situ densities. All microcosms were then transferred into a large aquarium containing in situ, well-stirred and aerated water maintained at 20°C. Nearly 30% of the aquarium water was replaced every 3 d, and this pre-incubation phase lasted 5 weeks. A light-dark 12-h period was reproduced; halogen lamps provided an irradiance of $300 \mu\text{E m}^{-2} \text{s}^{-1}$ at the sediment–water interface. Based on previous experiments, we considered the pre-incubation period as sufficient for the development of microalgal mats; for the plants to overcome the transplant stress and grow; for the roots and the macrofauna to modify the sedimentary environment; for the bacterial communities associated to roots, burrows and biodeposits to develop; and for solute microgradients to establish (Racchetti et al. 2010, 2017; Ribaudo et al. 2011; Soana et al. 2012, 2015). In the conditions S, SC, SO, and SOC microphytobenthos developed during the pre-incubation period at the sediment–water interface, while algal growth was attenuated by the canopy of *V. spiralis* in the remaining microcosms.

The day before the incubation each microcosm was placed underwater in a Plexiglass liner with an inner diameter perfectly fitting the microcosm outer diameter and a

height of 40 cm. The liner was then bottom-capped and provided with a stirring system (more details about an analogous experimental set up can be found in Racchetti et al. 2017). Total dissolved inorganic carbon (TCO_2), ammonium (NH_4^+), nitrite (NO_2^-), nitrate (NO_3^-), and molecular nitrogen (N_2) net flux rates were then measured in the light and in the dark in four replicate microcosms per condition. Details of the incubation procedures are reported in Soana et al. (2015). Briefly, a short-term (1–2 h) batch incubation started when a gas-tight transparent lid was positioned on the top of each core. Initial and final water samples were collected from each replicate core just before the starting and just after the end of the incubation, when top lids were removed. A stirring magnetic bar, driven by an external motor at 40 rpm, ensured homogeneous chemical conditions within each core's water phase. Incubation time was kept at a minimum intentionally, to keep O_2 concentrations in the overlying water within 20% of the initial values. Dissolved N_2 was measured via membrane inlet mass spectrometry as N_2/Ar ratios (Kana et al. 1994). TCO_2 was measured via 0.1 M HCl titration, and dissolved inorganic nitrogen forms (NH_4^+ , NO_2^- , and NO_3^-), were measured by standard spectrophotometric techniques (Soana et al. 2015). 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, $^{15}\text{NO}_3^-$, from a stock 15 mM $\text{Na}^{15}\text{NO}_3$ solution was added to the water phase of each of the replicate cores. Labeled 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 h, the sediment and water were gently mixed together. An aliquot of the slurry was transferred to a 12.5 mL gas-tight vial; $^{14}\text{N}^{15}\text{N}$ and $^{15}\text{N}^{15}\text{N}$ 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.

As the IPT is destructive, chlorophyll *a* (Chl *a*) concentration, porosity, density, and water content were determined just before sediment and water mixing on sediment subsamples collected with a cut-off syringe, as described in Benelli et al. (2017). Exchangeable and pore water NH_4^+ and pore water NO_3^- concentrations were also determined. Exchangeable NH_4^+ was extracted from fresh sediments with 1 M KCl. Pore water NH_4^+ and NO_3^- concentrations were determined

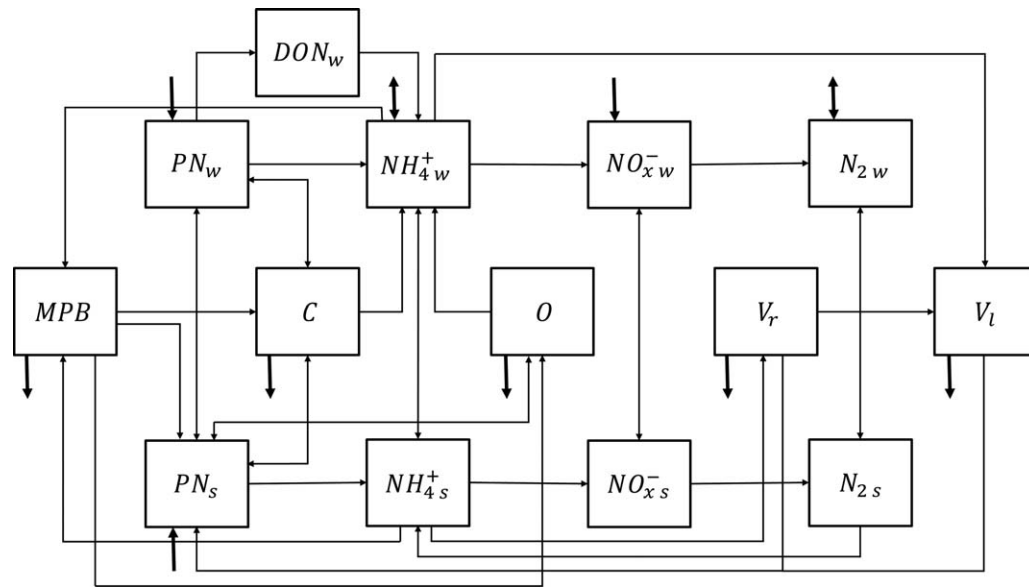


Fig. 1. Graph showing all compartments and connecting flows in the most complex experimental condition (SOVC) including: *Corbicula* spp. (C); *S. tamesis* (O); *V. spiralis*, divided in two different compartments (leaves (V_l) and roots (V_r)); and benthic microalgae (MPB). The other compartments represent organic and inorganic N compounds in the water column and in the sediment. Their designations are found in the text.

after sediment squeezing in the upper 1 cm sediment horizon and gradients with bottom water were calculated. Diffusive fluxes of NH_4^+ and NO_3^- across the sediment–water interface were then calculated by applying the Fick’s First Law (Berner 1980). Macrophytes and macrofauna were collected from each core by sediment sieving. Survival rates were verified, and then the macrofauna and the plants were desiccated at 70°C to constant weight for dry weight determination. All experimentally measured or calculated nitrogen fluxes among biotic and abiotic compartments that were used to construct the various networks are reported in Supporting Information (Supporting Information Table S1), together with a short description or reference on how calculations were made.

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 nine compartments which represented inorganic and organic N compounds in the water column (“ $\text{NH}_4^+_w$,” “ $\text{NO}_3^-_w$,” “ N_2_w ,” “ DON_w ,” “ PN_w ”) and in the sediment (“ $\text{NH}_4^+_s$,” “ $\text{NO}_3^-_s$,” “ N_2_s ,” “ PN_s ”). Some of these compartments (“ $\text{NH}_4^+_w$,” “ $\text{NO}_3^-_w$,” “ N_2_w ,” “ $\text{NH}_4^+_s$,” “ $\text{NO}_3^-_s$,” “ N_2_s ”) were measured

whereas the remaining were estimated, as detailed below or in Supporting Information Table S1.

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. 1). All standing stock values were obtained from the experiment. For N compounds molar concentration values of starting condition were standardized to $\mu\text{mol N m}^{-2}$. 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 $\mu\text{mol N m}^{-2}$ (Fink et al. 2006; Pinardi et al. 2009; Atkinson et al. 2010). N in microphytobenthos was calculated from Chl *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).

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 $0.001 \mu\text{mol N m}^{-2} \text{h}^{-1}$ (Christian et al. 2010). A comprehensive list of methods is presented in the Supporting Information (Supporting Information Table S1). All flow rates were expressed as $\mu\text{mol N m}^{-2} \text{h}^{-1}$.

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_{2w}) 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. 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. 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. Details about the Input Analysis algorithm are presented in the Supporting Information (Supporting Information Appendix S2). 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 eight 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. 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. The algorithms for TDM calculation are presented in the Supporting Information (Supporting Information Appendix S1). 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

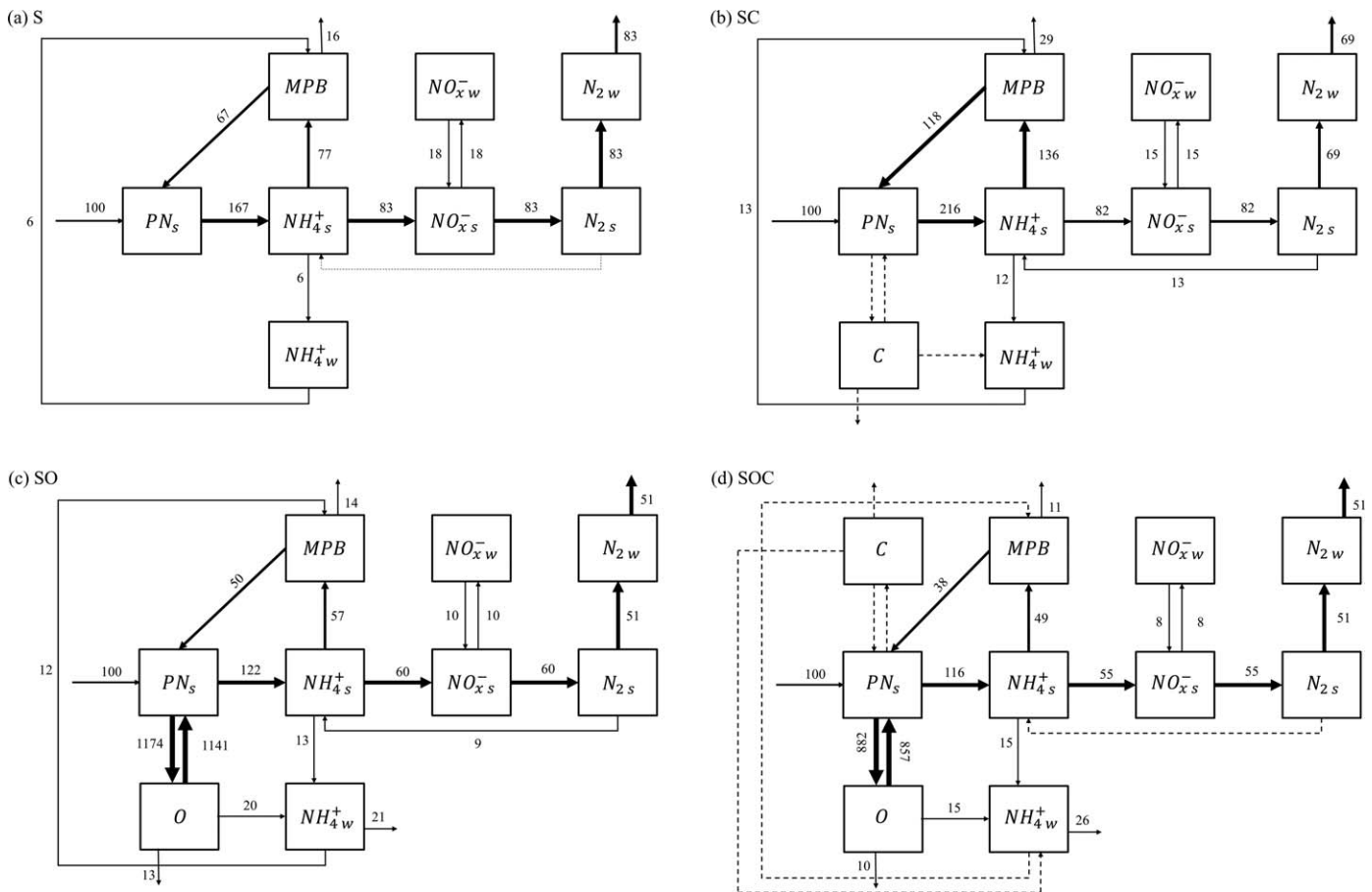


Fig. 2. Input analysis relative to the compartment PN_s for the condition (a) S (bare sediment), (b) SC (sediment with *Corbicula* spp.), (c) SO (sediment with *S. tamesis*), (d) SOC (sediment with *Corbicula* spp. and *S. tamesis*). Results are presented as graphs showing the benthic compartments and 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%.

flows (Figs. 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 eight experimental conditions averaged across incubations in the light and dark. Thus, each network reflects the integrated daily conditions. Figure 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. 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

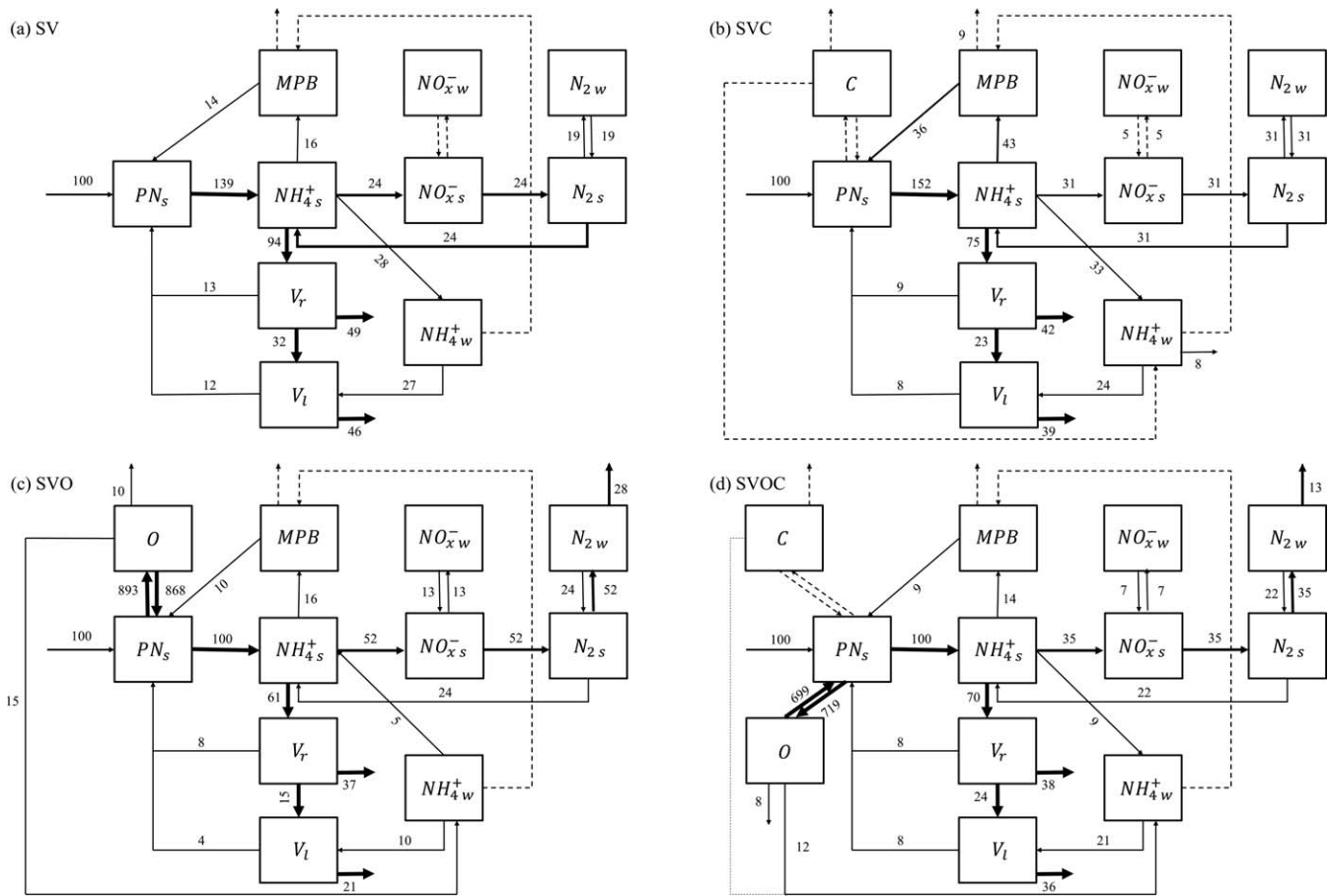


Fig. 3. Input analysis relative to the compartment PN_s for the conditions (a) SV (sediment with *V. spiralis*), (b) SVC (sediment with *V. spiralis* and *Corbicula* spp.), (c) SVO (sediment with *V. spiralis* and *S. tamesis*), (d) SVOC (sediment with *V. spiralis*, *Corbicula* spp. and *S. tamesis*). 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%.

(related to MPB and N-fixation) even if this component was not directly involved in large N flows.

S. tamesis (SO, Fig. 2c) reworked sediment, causing large fluxes and associated frequencies (ingestion, 1174%, and defecation, 1141%) 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. 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 $NH_4^+_w$ modeled 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. 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. 2d) it can be inferred that this organism played an indirect role within the system.

In the condition SV (Fig. 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 $\text{NH}_4^+_{\text{w}}$ was higher than for other conditions (28%), no increase (i.e., export) in water-column NH_4^+ occurred. NH_4^+ 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 - $\text{NH}_4^+_{\text{s}}$ - MPB - PN_s) decreased in SV as compared to all previous conditions.

Macrophyte presence strongly controlled how PN_s was processed when *Corbicula* spp. was added to vegetated sediments (SVC, Fig. 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 - $\text{NH}_4^+_{\text{s}}$ - MPB - PN_s (from 14% to 36% for MPB - PN_s) and the loop $\text{NH}_4^+_{\text{s}}$ - $\text{NO}_x^-_{\text{s}}$ - N_2_{s} - $\text{NH}_4^+_{\text{s}}$ (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 $\text{NH}_4^+_{\text{w}}$ export (8%); neither *V. spiralis* leaves nor MPB fully consumed this $\text{NH}_4^+_{\text{w}}$ surplus. No N_2 exited the system as in the condition SV.

Condition SVO (Fig. 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 ($\text{NH}_4^+_{\text{s}}$ - $\text{NO}_x^-_{\text{s}}$ - N_2_{s} - $\text{NH}_4^+_{\text{s}}$, 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 ($\text{NH}_4^+_{\text{s}}$ - $\text{NH}_4^+_{\text{w}}$) 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. 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 fueled 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 $\text{NH}_4^+_{\text{w}}$ pool. However, no accumulation of NH_4^+ was found as primary producer's uptake equaled or exceeded these inputs. In all conditions with *V. spiralis*, recycling along the loop $\text{NH}_4^+_{\text{s}}$ - $\text{NO}_x^-_{\text{s}}$ - N_2_{s} - $\text{NH}_4^+_{\text{s}}$ 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. 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 1000 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 1000 input analysis configurations from simulations of the eight conditions, such as those presented in Figs. 2, 3. This allowed us to derive distributions for all the fractional flows from PN_s . We then selected four 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 $\text{MPB} \rightarrow \text{PN}_s$, $\text{NH}_4^+_{\text{s}} \rightarrow \text{NO}_x^-_{\text{s}}$, $\text{N}_2_{\text{s}} \rightarrow \text{NH}_4^+_{\text{s}}$, $\text{NH}_4^+_{\text{s}} \rightarrow \text{NH}_4^+_{\text{w}}$. Also, exports, representing increases in standing stock, from MPB, N_2_{w} , V_1 and V_r were assessed. We compared the values of these flows across the eight 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 details and outcomes of the GLM Model as well as those of the Tukey contrasts are detailed in the Supporting Information (Supporting Information Appendix S3). 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.

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

Table 1. Dependency values (%) of benthic microalgae (MPB) in the eight 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

(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 TCO₂ data.

Table 1 reports the dependency (i.e., both direct and indirect) values of microphytobenthos in the eight experimental conditions. Microphytobenthos uptake in all conditions was supported largely by the NH₄⁺_s pool (dependency values on NH₄⁺_s ranging from 79% to 96%) and only for a small extent by NH₄⁺_w (11–35%). In all conditions without the macrophyte, dependency on NH₄⁺_s (79–93%) 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 NH₄⁺_s was particulate N in the sediment. Smaller fractions were provided by N₂_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 NH₄⁺ mainly from sediments (95%). N₂_w became an important source for NH₄⁺_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 N₂_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.

Tables 2, 3 report the dependency values of *V. spiralis* leaves and roots, respectively. The only direct N input to

Table 2. Dependency values (%) of *V. spiralis*' leaves (V_l) in the eight 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 3. Dependency values (%) of *V. spiralis*' roots (V_r) in the eight 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

roots was the NH₄⁺ from the sediment (dependency value of 100%), while NH₄⁺_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 NH₄⁺_w ranged from 44% to 56%). Other dependency values showed similar trends for both roots and leaves. When macrofauna, especially *S. tamesis*, were 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 N₂_s and on N₂_w were lower with macrofauna, especially with the oligochaete (Tables 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* spp. (on average 8% for the leaves and 3% for the roots).

To evaluate the support of the different compartments on denitrification, dependency values of N₂_w in dark conditions were analyzed in detail (Table 4). N₂_w was assessed as it reflected both denitrification in the sediment with subsequent diffusion of N₂_s as well as the potential for denitrification in the water column. In all dark incubations N₂_w showed complete dependence (i.e., 100%) on N₂ produced in the sediment by denitrification of NO_x⁻. Nitrate in pore water derives from two processes: the diffusion of NO_x⁻ from the water column

Table 4. Dependency values (%) of N_{2w} in the eight 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_{2s}	100	100	100	100	100	100	100	100
N_{2w}	0	0	0	0	0	0	0	0

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%, while 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.

Statistical significance through Monte Carlo simulation was applied also to the outcome of this analysis. We calculated the TDM for each of the random 1000 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. The results of this simulation are detailed in the Supporting Information (Supporting Information Appendix S4).

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 among 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* spp. in N cycling remains less clear and largely indirect. The rooted plants tended toward 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 modeling 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 h. 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 labeled and unlabeled nitrate in the denitrification zone (Risgaard-Petersen and Jensen 1997). The addition of labeled 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 labeled and unlabeled 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 $^{15}\text{NH}_4^+$ labeled 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_{2w} on NO_x^- . 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 $\text{PN}_s\text{-NH}_4^+\text{-MPB-PN}_s$. 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 Figs. 2b,d, 3b,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_{2w} 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* spp.) and organisms ventilating their burrows (*S. tamesis*). Excreted NH_4^+ may be transported within burrows and oxidized along nitrifier-rich burrow walls (Pelegri and Blackburn 1995). Such outcome was not evident from our results as the fraction of PN_s input leaving the system as N_{2w} 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 tubedwelling 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_1 and V_r compartments. N was stored in plant biomass (in our model represented as exports from V_r and V_1), 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; Volkman 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 toward 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* spp. results in a higher recycling activity along the path $\text{PN}_s\text{-NH}_4^+\text{-MPB-PN}_s$ 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^+ . 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 facilitate 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^- . This situation was inferred from the different N_2 dependency values, which increased for PN_s and decreased for NO_x^- 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 1892; 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^- 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 increases NH_4^+ 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 salmon 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.

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Conflict of Interest

None declared.

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