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Contrasting Effects of Bioturbation Studied in Intact and Reconstructed Estuarine Sediments

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Abstract: Macrofauna can produce contrasting biogeochemical effects in intact and reconstructed sediments. We measured benthic fluxes of oxygen, inorganic carbon, and nitrogen and denitrification rates in intact sediments dominated by a filter and a deposit feeder and in reconstructed sediments added with increasing densities of the same organisms. Measurements in reconstructed sediments were carried out 5 days after macrofauna addition. The degree of stimulation of the measured fluxes in the intact and reconstructed sediments was then compared. Results confirmed that high densities of bioturbating macrofauna produce profound effects on sediment biogeochemistry, enhancing benthic respiration and ammonium recycling by up to a factor of ~3 and ~9, respectively, as compared to control sediments. The deposit feeder also increased total denitrification by a factor of ~2, whereas the filter feeder activity did not stimulate nitrogen removal. Moreover, the effects of deposit feeders on benthic fluxes were significantly higher (e.g., on respiration and ammonium recycling) or different (e.g., on denitrification) when measured in intact and reconstructed sediments. In intact sediments, deposit feeders enhanced the denitrification coupled to nitrification and had no effects on the denitrification of water column nitrate, whereas in reconstructed sediments, the opposite was true. This may reflect active burrowing in reconstructed sediments and the long time needed for slow growing nitrifiers to develop within burrows. Results suggest that, in bioturbation studies, oversimplified experimental approaches and insufficient preincubation time might lead to wrong interpretation of the role of macrofauna in sediment biogeochemistry, far from that occurring in nature.

Keywords: benthic fluxes; denitrification; bioturbation; *Ruditapes philippinarum*; *Alitta succinea*

1. Introduction

Bioturbation by macrofauna is a fascinating topic that has fueled a diversified body of scientific research [1–6]. Macrofauna couple the pelagic and benthic environments in various ways. For instance, feces and pseudofeces deposition by filter feeders, containing viable and active phytoplankton, displaces pelagic primary production at the sediment level [7,8]. Filter feeders activity, such as that of clams, may process the entire water column of shallow water bodies many times per day, assuring water transparency, increased light penetration, and benthic primary production [9–11]. Filter feeders also excrete large amounts of the mineralized phytoplankton biomass back into the water column [12–14]. Biodeposits can increase the organic content of sediments and stimulate both aerobic and anaerobic

metabolic pathways [15,16]. Filtration and excretion thus have the potential of maintaining pelagic and benthic primary production elevated. Deposit feeders, such as polychaete worms, explore surficial or deep sediment horizons to extract food and avoid predation. As oxygen (O_2) penetration in sediments is extremely limited, burrowing macrofauna have evolved different ways to cope with anoxia and potentially toxic concentrations of compounds such as ammonia or sulfide. Burrow architectures may vary among species, but most deposit feeders ventilate their burrows by injecting and circulating oxic bottom water or by extracting pore water from sediments [17–19]. Such a need to get rid of metabolic end-products and to import O_2 for their own respiration produces different biogeochemical side-effects on sediments [20]. For example, the volume of oxidized sediments in bioturbated areas is much higher than that in their not bioturbated counterparts, which means that the oxidation status of different elements is different depending on the presence of macrofauna. Ecosystem-level implications are multiple as oxidized sediments may retain elements avoiding their release to the water column or favor their permanent loss via coupled oxic–anoxic processes [21,22].

Macrofauna, through bioturbation but also feeding strategy and ecological interactions, affect both the physical and biological environments in sediments and in the water column. During their life span, macrofauna contribute to the creation and become part of a complex network of micro- and macro-organisms and of chemical and biological processes [23–25]. With this respect, little is known about the complex and intimate mutual interactions within holobionts, which are associations between microbes and macrofauna, and that affect benthic functioning [6,26].

Research activities in the field of bioturbation, with the aim of understanding density-dependent effects of macrofauna on selected processes, have largely adopted experimental approaches based on the addition of a single species to reconstructed sediments (e.g., sieved or freeze-thaw, in order to remove other macrofauna species, then homogenized and packed back into microcosms) [27–30]. Results from this kind of studies have allowed us to understand how important macrofauna is for a number of biogeochemical processes, including aerobic and anaerobic microbial respiration rates, nutrient recycling, reworking, and mixing of new and old organic matter and oxidation of chemically reduced metal pools [31–33]. The level of enhancement or inhibition of specific processes operated by macrofauna and measured with such approaches has been likely used to calibrate models, to upscale processes under varying macrofauna density or to propagate the effects to the water column [34]. However, such an approach may lead to a partial understanding of the true effect of macrofauna due to multiple factors. One of them is the absence of other macrofauna groups that may compete with, facilitate, or inhibit the targeted species. Another one is the time taken for an organism living in sediment, which is necessary to engineer the environment and, therefore, to change the chemical conditions and the biological communities (e.g., meiofauna and microbes). Due to these factors, we hypothesized that processes measured under in situ conditions may deeply differ from those measured shortly after sediment manipulation and macrofauna addition. We also hypothesized that such differences may vary along different macrofauna functional groups.

To test these hypotheses, we measured aerobic, anaerobic (denitrification), total respiration, and nutrient fluxes in intact and reconstructed sediments collected from two estuarine areas dominated by a filter feeder and a deposit feeder. Specific objectives were i) to analyze the effects of bioturbation on different benthic processes in intact and reconstructed sediments and ii) to evaluate whether differences between the two experimental conditions (i.e., intact versus reconstructed) depend upon macrofauna functional groups.

2. Materials and Methods

2.1. Study Sites

Samples were collected from the Sacca di Goro Lagoon (Figure 1), a shallow microtidal lagoon within the Po River Delta, Northern Italy (44.78–44.83° N, 2.25–12.33° E) [35]. The lagoon is approximately triangular in shape, has a surface area of 27 km^2, an average depth of 1.5 m, and it

is connected to the sea by a large mouth. The lagoon presents a western portion influenced by the nutrient-rich freshwater inlet of the Po di Volano, a central portion with marine influence and an eastern, confined area cultivated with clams (*Ruditapes philippinarum*). The average annual temperature and precipitation in this area are 17 ± 1 °C and 631 ± 117 mm, respectively. For the aims of this study, two stations were selected as representative of a pristine and of a heavily impacted lagoon area and located in the western and in the eastern portion of the lagoon, respectively. Station 1 has turbid water due to the Po di Volano freshwater inputs and has muddy, highly bioturbated sediments. During summer, the macrofauna community is dominated by the polychaete *Alitta succinea*. Station 2 has transparent water and sandy sediments and is located within a licensed area for clam farming; the dominant macrofauna is represented by the clam *R. philippinarum*.

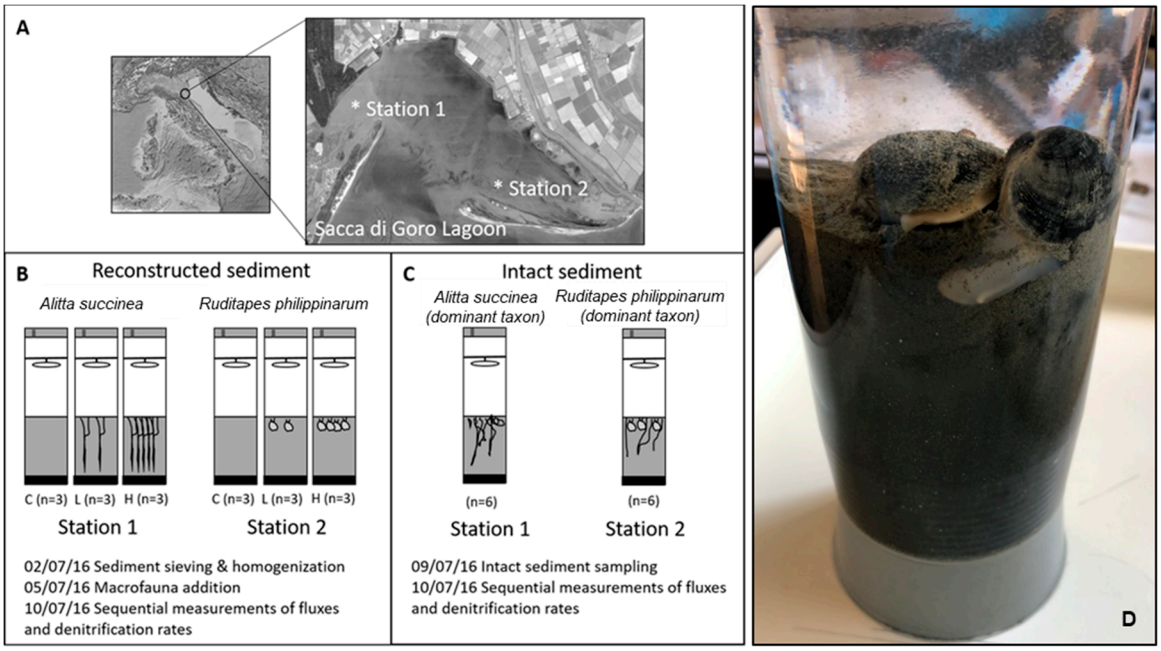

Figure 1. (**A**) shows the location of the two sampling stations in Sacca di Goro Lagoon (Po River Delta, Northern Italy). (**B**) shows reconstructed sediments from the two stations, added with no (C, control) or increasing densities of *Alitta succinea* (L, low, 3 ind core^{-1}, and H, high, 6 ind core^{-1}) or *Ruditapes philippinarum* (L, low, 2 ind core^{-1}, and H, high, 4 ind core^{-1}). (**C**) shows randomly collected intact sediments from the two stations, with natural macrofauna community, dominated by the species indicated. (**D**) shows a reconstructed sediment core with burrowing individuals of *Ruditapes philippinarum*.

2.2. Sampling Activities

2.2.1. Reconstructed Sediment Cores

Sediments and water were collected in July 2016. On 2 July 2016, nearly 50 L of sediment and 100 L of water were collected from the two stations. As both stations were shallow (<1 m depth), the water was collected by hand in 25 L tanks, whereas the sediment was collected with a shovel and transferred into 20 L buckets. Water and sediments were brought to the laboratories at Ferrara University within 2 h. Here, sediments from the two stations were (i) sieved (0.5 mm) in two tanks containing in situ water to remove large macrofauna, (ii) homogenized, and (iii) left to settle for 3 days. The tanks with sieved sediments and in situ water were maintained in the dark at in situ temperature, and the overlying water was kept oxic by air bubbling. Individuals of *A. succinea* and of *R. philippinarum* retrieved during the sieving were maintained alive in separate small aquaria with a few cm of sediments and 10 cm of well aerated water phase. On 5 July 2016, 9 cores (inner

diameter = 8 cm, height = 30 cm) were subsampled from each tank. Water and sediment phases were subsampled by pushing vertically transparent Plexiglas cores in the tanks. Once retrieved, all cores were leveled in order to have a sediment height of 10 cm and a water column of 17 cm. Cores with muddy sediments from Station 1 were treated as follows: 3 cores were used as control, 3 cores were added with 3 individuals of *A. succinea*, and 3 cores were added with 6 individuals of *A. succinea*, to reproduce densities (600 and 1200 ind m^{-2}, respectively) comparable to those measured in situ. In a few minutes, all added polychaetes burrowed into the sediments. Cores with sandy sediments from Station 2 were treated as follows: 3 cores were used as control, 3 cores were added with 2 clams (*R. philippinarum*), and 3 cores were added with 4 clams, to simulate densities at farmed areas (400 and 800 ind m^{-2}, respectively). Within 30 min from clams addition all organisms burrowed within the sand, and only siphons were visible. All 9 + 9 cores were provided with stirring units (a rotating magnet suspended 5 cm above the sediment–water interface driven by an external motor at 40 rpm) and submersed in separate incubation tanks containing aerated and well mixed water from the two stations inside a temperature-controlled room maintained in the dark at the in situ temperature (25 °C).

2.2.2. Intact Sediments

On 9 July 2016, a second sediment and water sampling was carried out during which at each station 6 Plexiglas cores (inner diameter = 8 cm, height = 30 cm) were randomly collected by hand for flux and denitrification measurements and 5 Plexiglas cores (inner diameter = 4 cm, height = 20 cm) were collected for sediment characterization (for the methods see [14,36,37]). Cores with disturbed vertical profiles or with visually broken clam shells along walls were immediately discarded. Besides intact sediments, nearly 100 L of water from each site was collected for preincubation and incubation periods. Water column temperature, pH, and salinity were measured at the two sites by means of a multiple probe (mod 556, YSI Instrument, Yellow Spring, OH, USA). All the material was brought to the laboratories at Ferrara University within 2 h. Here, intact sediment cores from the two sites were submersed into two 50 L tanks containing in situ aerated and well mixed water inside a temperature-controlled room maintained in the dark at the in situ temperature. Intact cores for flux measurements were provided with a rotating magnet, suspended 5 cm above the sediment–water interface driven by an external motor at 40 rpm.

2.3. Dark Incubations for Flux and Denitrification Measurements

On 10 July 2016, 5 days after macrofauna addition in reconstructed cores and after an overnight preincubation of the intact sediment cores, two sequential dark incubations were performed for the measurements of net solute fluxes across the sediment–water interface and of denitrification rates. During the first incubation, measurements of benthic fluxes of dissolved O_2, dissolved inorganic carbon (DIC), molecular nitrogen (N_2), and dissolved inorganic nitrogen (DIN = NH_4^+ + NO_2^- + NO_3^-) were performed with a start–end approach [38]. The incubation lasted between 2 and 3 h in order to keep O_2 drops from the initial values between 25 and 40%. Incubation time was set according to regular check (0.5 h) of dissolved O_2 in the water phase via a microelectrode (OX-50, Unisense A/S, Aarhus, Denmark) through the cores lid. At time, zero water samples (n = 4) were collected from each incubation tank. At time, final water samples were collected from each core water phase through a valve in the cores lid. Incubation started when gas-tight lids were positioned on the top of each core. Water samples were collected via 60 mL glass syringes; 20 mL was disposed in 12 mL exetainers (Exetainer®, Labco Limited, High Wycombe, UK) and added with Winkler reagents for O_2 measurements, 20 mL was disposed in 12 mL exetainers for DIC determination, which was measured with 0.1 N HCl titration with an automatic titration unit (ABU91, Radiometer, Copenhagen, Denmark) and a combined pH sensor [39]. An aliquot of 20 mL was filtered (Whatman GF/C, Cytiva, Marlborough, MA, USA) and transferred to scintillation vials for nutrient analysis. Dissolved inorganic nitrogen was determined spectrophotometrically. Ammonium (NH_4^+) was determined using salicylate and hypochlorite in the presence of sodium nitroprussiate [40]. Nitrate (NO_3^-) was determined

after reduction to nitrite (NO_2^-) in the presence of cadmium, and NO_2^- was determined using sulphanilamide and N-(1-naphthyl)ethylenediamine [41,42]. Fluxes were defined as the difference between final and initial concentrations of solutes in the water column multiplied by the volume of the core water phase and divided by the area of the sediment and the incubation time (as reported in [38]). The respiratory quotient RQ, which is the ratio of dissolved inorganic carbon to oxygen fluxes (absolute values), was calculated.

After the incubation for flux measurements, lids were removed, the water in each tank was renewed, and the cores were submersed. Cores were maintained open and stirred in in situ oxygenated and well mixed water in the dark and at the in situ temperature. Approximately 5 h later, denitrification measurements started. The water level in each tank was lowered just below the cores opening, in order to separate the cores from the tank water. Labelled NO_3^- from a stock solution (20 mM, sodium nitrate-^{15}N, ≥98 atom%, Sigma-Aldrich, Merk KGaA, Darmstadt, Germany) was added to the water column of each core in order to have a final $^{15}NO_3^-$ concentration of 25 µM (Isotope Pairing Technique -IPT- [43]). Before and after 5 min from the addition of the labelled NO_3^- a subsample (5 mL) of the water phase was collected, filtered, and dispensed into 10 mL plastic vials for NO_3^- analysis. Labelled NO_3^- was allowed to diffuse into the anoxic horizon and then a gas-tight lid was positioned on the top of each core and the incubation started. The incubation length of the denitrification experiment overlapped that of the flux measurements. At the end of the incubation, the cores were opened, slurred, and samples were collected for labelled N_2 analyses via membrane inlet mass spectrometry (MIMS, Bay instrument, Easton, MD, USA) [44]. Water samples for labelled N_2 analyses were poisoned with $ZnCl_2$ to stop microbial activity. Rates of total denitrification, denitrification coupled to nitrification (Dn), and water column nitrate-driven denitrification (Dw) were calculated, as described in [43]. Denitrification efficiency (DE) was calculated as the ratio between denitrification and cumulative flux of N from sediment (N_2 + DIN) and expressed as a percentage [45]. The slurry in each core was then sieved (0.5 mm) in order to collect all macrofauna; macrofauna were recognized, and dry weight was quantified after drying the soft tissue at 70 °C to a constant weight.

2.4. Statistical Analysis

Differences among net solute fluxes or denitrification rates measured in the intact cores of the two stations and in the reconstructed cores of C, L, and H treatments were tested via one-way analysis of variance (ANOVA) followed by the post hoc Holm Sidak test. The dependency of solute fluxes or denitrification rates on macrofauna biomass were tested using linear regression analysis. Normality of residuals was confirmed numerically with a Shapiro–Wilks test.

The comparison of regressions between the biomass and measured process rates or calculated respiratory quotients and denitrification efficiency in intact and reconstructed cores were performed via analysis of covariance (ANCOVA). The level for statistical significance was set at 0.05. Statistical analyses were performed with R software v. 3.5.1 (The R Foundation for Statistical Computing, Vienna, Austria) [46].

3. Results and Discussion

3.1. General Features of the Study Sites

Station 1 had soft, organic sediments, with lower density and higher porosity, organic matter, C and N contents, and chlorophyll *a* as compared to Station 2 (Table 1). The biomass of macrofauna was one order of magnitude higher at Station 2 mostly due to high size of the cultivated *R. philippinarum* (Table 1). At Station 1, the dominant organism retrieved from sediments was the deep burrower *A. succinea*, with a density of 870 ± 530 ind m^{-2}. Besides *A. succinea*, the macrofauna community at Station 1 included other small polychaetes and occasional individuals of *Monocorophium insidiosum*, *Chironomus salinarius*, *Hydrobia* spp., and *Capitella capitata*, whose pooled biomass was <10% of the total. At Station 2, *R. philippinarum* dominated the macrofauna community, with a density of 630 ± 490 ind m^{-2}. Besides

clams, sediments also hosted a few individuals of *M. insidiosum* and *Echinogammarus* spp., whose pooled biomass was <1% of the total. Station 1 was influenced by the freshwater inputs of the Po di Volano and had lower salinity and higher concentrations of DIN as compared to Station 2, with NO_3^- as dominant chemical species (Table 1).

Table 1. Sediment descriptors, including the biomass of the dominant macrofauna (*Alitta succinea* and *Ruditapes philippinarum* at Station 1 and 2, respectively) and water physico-chemical characteristics.

Sediment Features							
	Density	**Porosity**	**Organic Matter**	**C**	**N**	**Chl *a***	**Macrofauna**
	($g\ mL^{-1}$)		(%)	(%)	(%)	($mg\ m^{-2}$)	($g_{dw}\ m^{-2}$)
Station 1	1.14 ± 0.11	0.74 ± 0.06	6.03 ± 0.07	1.79 ± 0.04	0.20 ± 0.01	37.3 ± 9.8	16.0 ± 9.1
Station 2	1.74 ± 0.02	0.47 ± 0.01	1.61 ± 0.08	0.35 ± 0.02	0.04 ± 0.00	4.7 ± 0.8	220.8 ± 292.1
Granulometry							
	<0.063 mm (%)	0.063 < x < 0.125 mm (%)	0.125 < x < 0.5 mm (%)		0.5 < x < 2 mm (%)		>2 mm (%)
Station 1	24.4	22.0	50.4		3.1		0
Station 2	10.7	35.2	51.3		2.7		0
Water features							
	Temperature (°C)	Salinity	pH		NH_4^+ (μM)	NO_2^- (μM)	NO_3^- (μM)
Station 1	25	5	7.77		1.8	6.6	31.6
Station 2	25	17	7.96		14.6	1.3	3.1

3.2. Benthic Respiration, DIN Fluxes, and Rates of Denitrification in Intact Sediment Cores

Respiration rates tended to be higher at Station 2, dominated by *R. philippinarum*, however differences were not significant (Figure 2). Similar net O_2 uptake and DIC release at the two stations, despite large difference in organic matter content, were probably the result of much higher contribution of macrofauna metabolic activity in the sandy Station 2, sustained by clams. Both stations recycled large and not statistically different amounts of NH_4^+. At the muddy, organic-rich Station 1, such amounts are likely sustained by high microbial ammonification, whereas at Station 2, such amounts are likely excreted by clams [47]. Net inorganic carbon to NH_4^+ efflux ratios averaged 6.9 ± 0.7 and 13.9 ± 4.7 at Station 1 and 2, respectively, suggesting in general the labile nature of the available organic matter. The low ratio measured at Station 1, very close to that of phytoplankton, may be due to much higher rates of NH_4^+ oxidation within sediments via nitrification [22]. This is confirmed by higher net NO_2^- and NO_3^- fluxes, averaging 119.35 ± 5.35 and 122.27 ± 15.11 μmol N $m^{-2}h^{-1}$, respectively, and by denitrification rates of NO_3^- produced within sediments, all significantly higher at Station 1. Elevated nitrification rates at Station 1 are probably supported by the activity of burrowers, increasing O_2 availability within NH_4^+-rich pore waters [27,48,49].

Rates of total denitrification were higher at Station 1 due to higher denitrification coupled to nitrification and to higher denitrification of water column NO_3^- (Table 1). Denitrification efficiency was higher by a factor of 3 at Station 1 (15%) than at Station 2, due to similar NH_4^+ regeneration but much higher denitrification. Despite such difference, at both sites the major dissolved inorganic N flux was towards the water column as recycled NH_4^+, whereas the fraction lost to the atmosphere was minor.

3.3. Benthic Respiration, DIN Fluxes and Rates of Denitrification in Reconstructed Cores

Increasing densities of *A. succinea* and *R. philippinarum* in reconstructed sediments significantly stimulated benthic respiration, including the consumption of O_2 and the production of DIC (Figure 3). The degree of stimulation of O_2 and DIC fluxes was different along with the density gradient, resulting in an increase in the respiratory quotient, approaching the unit in the high density treatments. Both macrofauna functional groups largely enhanced NH_4^+ regeneration, whereas their net effects on NO_2^- and NO_3^- fluxes were less marked likely due to simultaneous stimulation of multiple microbial

processes (e.g., nitrification and denitrification, Figure 3) [50]. The isotope pairing technique revealed a significant effect of *A. succinea* on the removal of water column NO_3^- via denitrification (Dw), whereas the same was not apparent with *R. philippinarum* [28]. Both macrofauna groups did not stimulate denitrification coupled to nitrification (Dn) (Figure 3). In reconstructed sediments, denitrification efficiency tended to decrease along with increasing macrofauna densities ($p = 0.088$ and $p = 0.152$ for *A. succinea* and *R. phlippinarum*, respectively) confirming a major effect of both deposit-feeding and filter-feeding macrofauna biomass on N recycling than on N losses [5]. Denitrification efficiencies overlapped values determined in intact sediments and averaged 15% (range 11–18%) and 5% (range 2–8%) in the *A. succinea* and *R. philippinarum* treatments, respectively.

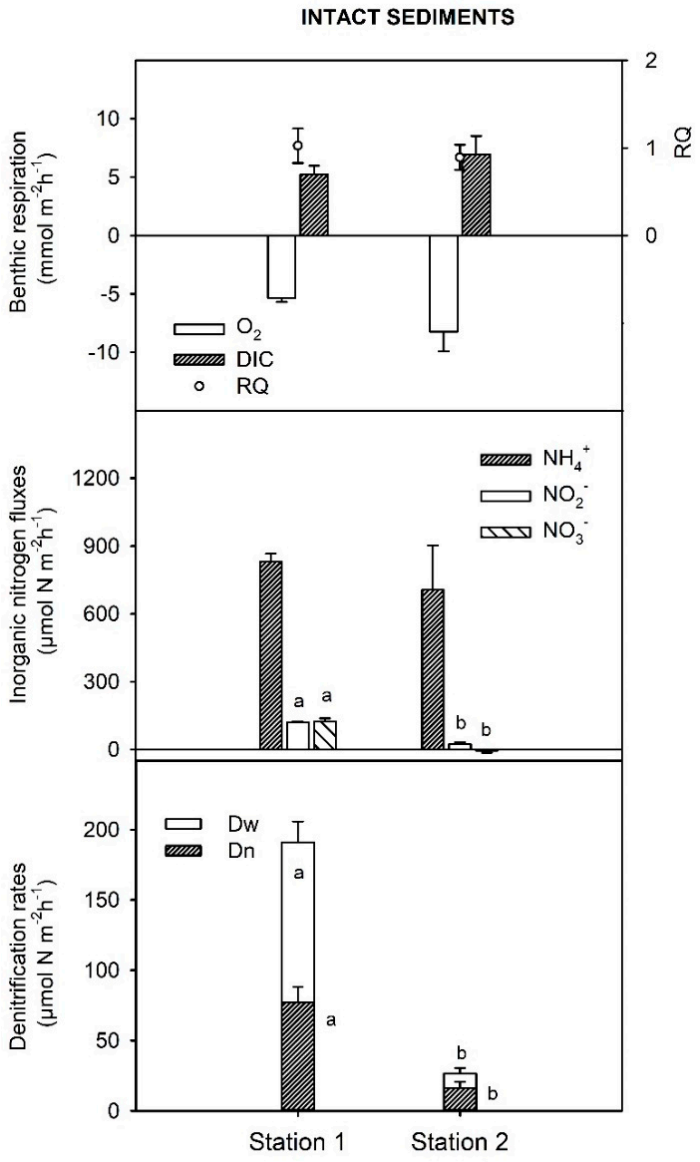

Figure 2. Dark fluxes of dissolved O_2 and dissolved inorganic carbon (DIC), dissolved inorganic nitrogen (DIN), and rates of denitrification measured in intact sediment cores at the two stations. In the upper panel, the *y*-axes on the right reports the respiratory quotient (RQ), calculated as the ratio between DIC and O_2 fluxes (absolute value). Where present, letters above bars indicate statistically significant differences. Averages ± standard errors ($n = 6$) are reported.

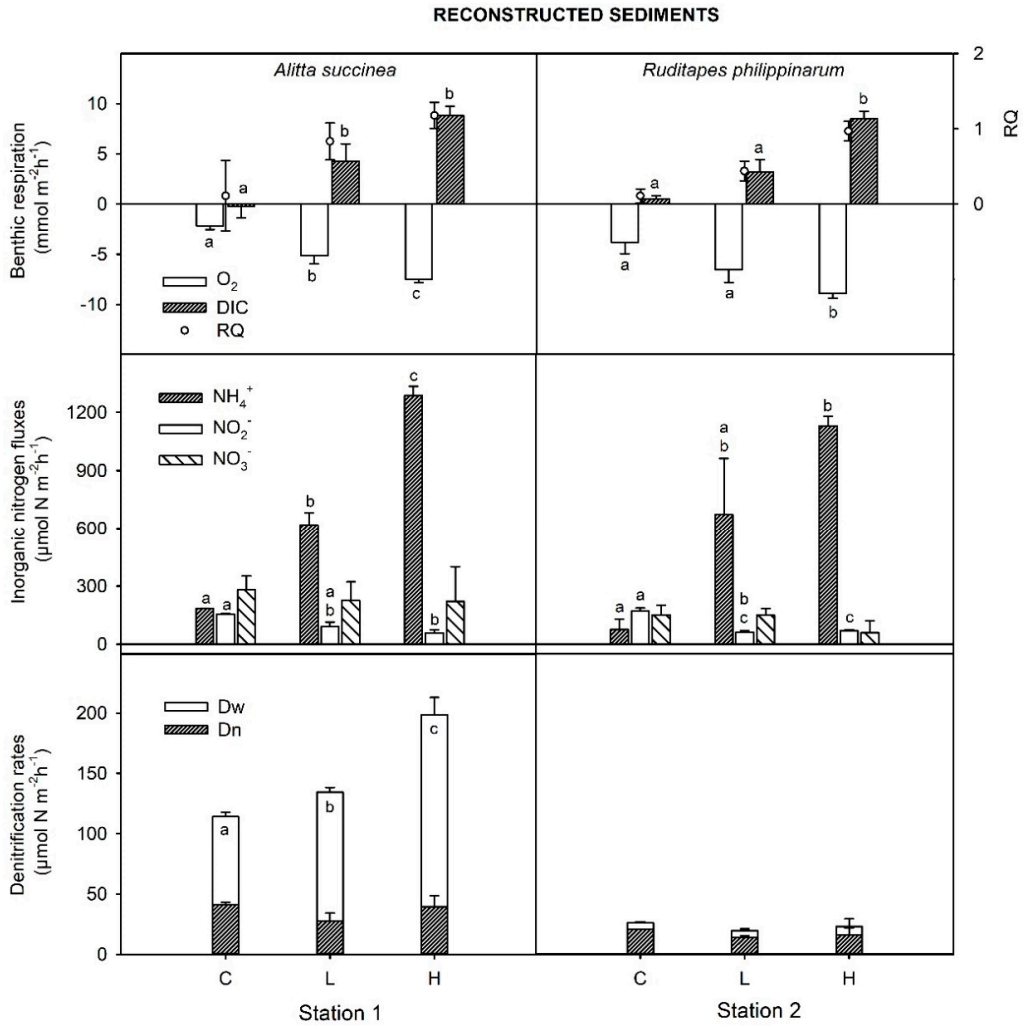

Figure 3. Dark fluxes of dissolved O_2 and DIC, DIN, and rates of denitrification measured in reconstructed sediments added with *Alitta succinea* (Station 1) and *Ruditapes philippinarum* (Station 2). C indicates the control treatment, L the low density treatment (600 and 400 ind m^{-2} for polychaetes and clams, respectively), and H the high density treatment (1200 and 800 ind m^{-2} for polychaetes and clams, respectively). In the upper panels, the *y*-axes on the right reports the respiratory quotient (RQ), calculated as the ratio between DIC and O_2 fluxes (absolute value). Where present, letters above bars indicate statistically significant differences. Averages ± standard errors (n = 3) are reported.

These results allow to perform simple linear regressions between measured process rates and macrofauna biomass to calculate the degree of enhancement of a certain process (Table 2). The biomass of added *A. succinea* (10.3 ± 3.7 and 21.4 ± 4.8 g_{dw} m^{-2} for L and H, respectively) was an order of magnitude lower than that of added *R. philippinarum* (222.2 ± 37.5 and 410.7 ± 68.8 g_{dw} m^{-2} for L and H, respectively), however the effects produced on benthic biogeochemistry was comparable, except for denitrification rates. Burrowing *A. succinea* contributed less to respiration and excretion than *R. philippinarum*, but produced a much larger effect on microbial activity, compensating the different biomass. This interpretation is supported by the higher degree of stimulation of Dw by *A. succinea*. Denitrification in fact is not performed by deposit-feeding macrofauna but by the microbes growing along burrows that take advantage from the macrofauna-mediated bioirrigation, transporting NO_3^--rich water within sediments [8,19,31]. Burrowing and ventilation activities moreover mobilize pore water solutes as NH_4^+, which is released in large amounts in *A. succinea* L and H treatments. We speculate that fluxes measured in sediments with *R. philippinarum* are instead mostly sustained by clams excretion [51,52].

Table 2. Slope, intercept, level of significance, and r^2 values of linear regressions between solutes fluxes (μmol $m^{-2}h^{-1}$), respiratory quotient, denitrification rates (μmol $m^{-2}h^{-1}$), and efficiency and macrofauna biomass of *Alitta succinea* and *Ruditapes philippinarum* (g_{dw} m^{-2}) retrieved in intact and reconstructed cores from the two stations. For slopes and intercepts, standard errors are reported in brackets.

	Alitta succinea								***Ruditapes philippinarum***							
	Intact Sediments				**Reconstructed Sediments**				**Intact Sediments**				**Reconstructed Sediments**			
	Slope	**Intercept**	***p***	**r^2**	**Slope**	**Intercept**	***p***	**r^2**	**Slope**	**Intercept**	***p***	**r^2**	**Slope**	**Intercept**	***p***	**r^2**
O_2	−81.75 (25.67)	−4031.78 (461.74)	0.033	0.72	−158.53 (13.25)	−2283.70 (291.41)	<0.001	0.95	−12.80 (2.94)	−5417.04 (1016.76)	0.012	0.83	−6.98 (2.27)	−4149.12 (969.48)	0.018	0.57
DIC	−137.11 (75.55)	7417.94 (1359.12)	0.144	0.45	264.35 (45.38)	−144.03 (997.82)	<0.001	0.83	12.57 (2.26)	4150.56 (781.78)	0.005	0.89	12.09 (1.75)	112.07 (748.16)	<0.001	0.87
RQ	−0.04 (0.02)	1.71 (0.29)	0.055	0.64	0.04 (0.01)	−0.09 (0.28)	0.015	0.59	−0.0001 (0.0006)	0.918 (0.207)	0.850	0.01	0.001 (0.0002)	0.067 (0.082)	<0.001	0.88
NH_4^+	5.90 (3.86)	736.22 (69.37)	0.201	0.37	31.43 (2.71)	169.36 (59.60)	<0.001	0.95	0.87 (0.69)	514.09 (239.52)	0.276	0.28	1.47 (0.40)	145.15 (169.82)	0.008	0.66
NO_2^-	1.13 (0.45)	−101.37 (8.12)	0.067	0.61	−2.66 (0.71)	145.65 (15.53)	0.007	0.67	−0.03 (0.02)	30.42 (7.14)	0.286	0.27	−0.15 (0.05)	149.04 (19.52)	0.014	0.60
NO_3^-	−2.46 (1.63)	161.53 (29.26)	0.205	0.36	−2.19 (4.68)	279.11 (102.81)	0.654	0.03	−0.05 (0.02)	5.76(8.53)	0.103	0.53	−0.09 (0.11)	148.70 (46.32)	0.458	0.08
DIN	4.57 (4.49)	999.12 (80.79)	0.367	0.21	26.59 (4.77)	594.12 (104.93)	<0.001	0.82	0.79 (0.68)	550.26 (234.96)	0.307	0.26	1.24 (0.40)	442.88 (168.65)	0.017	0.58
Dw	1.28 (0.93)	93.19 (16.65)	0.239	0.32	2.48 (0.29)	71.43 (6.30)	<0.001	0.91	0.004 (0.005)	9.33(1.75)	0.432	0.16	0.001 (0.001)	5.66 (0.59)	0.434	0.09
Dn	2.61 (0.75)	35.31 (13.51)	0.025	0.75	0.06 (0.30)	34.99 (6.53)	0.852	0.01	−0.007 (0.017)	17.90 (5.91)	0.705	0.04	−0.009 (0.007)	19.98 (2.86)	0.213	0.21
Dtot	3.89 (0.61)	128.50 (10.88)	0.003	0.91	2.53 (0.33)	106.42 (7.29)	<0.001	0.89	−0.003 (0.016)	27.22 (5.67)	0.885	0.01	−0.008 (0.008)	25.64 (3.27)	0.329	0.14
DE	0.21 (0.08)	11.73 (1.52)	0.068	0.61	−0.11 (0.05)	15.20 (1.09)	0.054	0.43	−0.005 (0.006)	6.21(2.08)	0.436	0.16	−0.007 (0.003)	6.29 (1.20)	0.042	0.47

3.4. Macrofauna Stimulate Differentially Biogeochemical Processes in Intact and in Reconstructed Sediments

Table 2 reports the slopes of linear regressions between measured fluxes or calculated rates and biomass of *A. succinea* and *R. philippinarum* in intact and reconstructed sediment cores, whereas Table 3 reports the results of the ANCOVA where such regressions were compared. As a general outcome, the enhancement of specific processes by the two functional groups of macrofauna (e.g., oxygen uptake, NH_4^+ efflux and denitrification of water column NO_3^- –Dw–) suggested much larger effects of the deposit feeder as compared to the filter feeder (Table 2). For example, the increase in benthic oxygen consumption per gram of dry weight of the burrower varied between 81 and 156 μmol $g_{dw}^{-1}h^{-1}$. This is likely due to the construction and irrigation of subsurface burrow structures, to the increased sediment–water exchange surface and to the stimulation of microbial activity within sediments [28].

Table 3. Results of the ANCOVA between the slopes of fluxes or calculated respiratory quotients and denitrification efficiency and macrofauna biomass in intact and reconstructed sediments from the two stations. While for *Ruditapes philippinarum*, most comparisons are not significant, for *Alitta succinea* the two conditions lead to rather different slopes.

	Alitta succinea		***Ruditapes philippinarum***	
	F	***p***	**F**	***p***
O_2	6.559	0.027	2.486	0.143
DIC	16.723	0.002	0.029	0.869
RQ	10.439	0.008	7.809	0.017
NH_4^+	20.424	0.001	0.662	0.433
NO_2^-	7.909	0.017	4.115	0.067
NO_3^-	0.001	0.976	0.057	0.816
DIN	5.550	0.038	0.371	0.555
Dw	2.149	0.171	0.586	0.459
Dn	11.740	0.006	0.121	0.735
Dtot	3.409	0.092	0.118	0.737
DE	9.041	0.012	0.089	0.771

Interestingly, the stimulatory effects of *A. succinea* on most measured benthic fluxes, with NO_3^- as the only exception, were generally different in intact and reconstructed sediments, whereas this was not true for *R. philippinarum* (Tables 2 and 3). In reconstructed sediment cores *A. succinea* produced much higher enhancement of O_2 respiration and of water column NO_3^- consumption via denitrification (by a factor of 2) and of NH_4^+ recycling (by a factor 5) as compared to intact sediments. In unmanipulated sediments, *A. succinea* stimulated denitrification of NO_3^- produced via nitrification (Dn), which is expected, whereas this was not apparent in reconstructed sediments. In the latter, *A. succinea* stimulated the denitrification of water column NO_3–, likely due to recently constructed burrows with sharp redox gradients and limited numbers of nitrifying bacteria on their walls.

Besides differences in the degree of stimulation of certain processes, measurements in intact and reconstructed bioturbated sediments led sometimes to contrasting effects of macrofauna. For example, *A. succinea* in intact cores enhanced DIC retention in sediments, whereas the same organism in reconstructed cores stimulated DIC release to the water (Tables 2 and 3). Opposite effects by burrowing fauna were apparent also for NO_2^- fluxes.

Interestingly, the effects of *R. philippinarum* on solute fluxes in intact and reconstructed cores were not statistically different (Tables 2 and 3). We speculate that clams drive fluxes mostly through their metabolic activities but produce a limited effect, at least in the short-term, on sedimentary variables and on microbial communities within sediments.

4. Conclusions

Results of this study confirm the key role of bioturbating macrofauna in benthic biogeochemistry, with deposit feeders simulating the removal of nitrogen via denitrification and increasing the

denitrification efficiency of the benthic system. Deposit feeders also increase the rates of oxygen consumption, partly due to chemical or biological oxidation of anaerobic metabolism end products. As such, they may buffer the toxicity of sulphides and act upon redox-sensitive processes such as P retention, ultimately producing positive feedbacks on benthic biodiversity and negative feedbacks on eutrophication. In the Sacca di Goro lagoon, clams are cultivated, and their density is orders of magnitude higher than under natural conditions. Clams are demonstrated to favor benthic-pelagic coupling, but with such densities, they may determine a too rapid (re)cycling of excreted nutrients associated to ingested phytoplankton. As clams produce a scarce stimulation of denitrification, they maintain low the denitrification efficiency, and they may promote (or sustain) eutrophic conditions.

The present study suggests that results of experiments with sediments and macrofauna manipulations need to be carefully considered. We demonstrated here that the degree of stimulation of commonly measured biogeochemical processes as aerobic, anaerobic (e.g., denitrification), and total respiration or nutrient fluxes by macrofauna can be substantially different when calculated in intact, not manipulated natural sediments compared to reconstructed microcosms. Reasons are probably multiple and include, first of all, the consequences of sediment sieving and packing, which deeply alter vertical gradients, organic matter distribution, the sediment redox, the pore water chemistry and likely affect important fractions of reactive components of the organic matter pools. Sieving also removes all the macrofauna in order to highlight the biogeochemical effects of a single species. This helps to have very clear, often linear, and highly significant results; on the other hand, the simplification of the community removes an unaccountable number of ecological interactions that shape the true functioning of the benthic ecosystems. Results obtained from manipulated sediments are real, as they are measured, but are likely never found in the field, due to oversimplification of the experimental approach, including the removal of chemical gradients and multiple ecological interactions. Aquatic ecologists often use manipulative approaches, where they remove species to analyze the effect of a few targeted organisms. Results from this work, and in particular, fluxes measured in manipulated sediments, suggest that clear and easy-to-interpret results are obtained from these manipulations. The intensity of process stimulation calculated in manipulative studies can then be coupled to data on macrofauna abundances in order to upscale the effects of bioturbation at the whole system level. In doing so, one can get results that are quite far from the reality. Something similar likely occurs when incubating a deep burrower or a clam in a small vial to calculate their respiration and excretion rates; they will try to dig through the glass walls during the incubation and their metabolic activity will be much higher as compared to when they are laying in their burrows, surrounded and protected by sediments. A major fraction of the effects of macrofauna on sediments are indirect, and associated to the way they affect microbial communities and metabolic activities and, with them, chemical microgradients within sediment, which are flux drivers. Measurements in manipulative experiments should also consider these aspects and consider or quantify how long it takes for macrofauna to produce a steady state in terms of microbial communities, activities, and chemical gradients in manipulated sediments. The latter require a long conditioning time before measurements, as the appropriate preincubation period is probably closer to weeks than to days.

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