



LETTER

Worms and submersed macrophytes reduce methane release and increase nutrient removal in organic sedimentsSara Benelli ^{1,2*} Marco Bartoli ^{2,3}¹Water Research Institute (CNR-IRSA) National Research Council, Verbania Pallanza, Italy; ²Department of Chemistry, Life Sciences and Environmental Sustainability, University of Parma, Parma, Italy; ³Marine Research Institute, University of Klaipeda, Klaipeda, Lithuania**Scientific Significance Statement**

Artificial drainage and irrigation canals have converted floodplains into productive agricultural areas, resulting in extensive loss of biodiversity and ecosystem services. The same canal networks represent an opportunity for restoration of lowland ecosystems due to the large area that canals cover nowadays. However, current canals management minimizes hydraulic resistance via mechanical removal of vegetation, which affects their potential to reduce greenhouse gas emissions and improve biological habitat and water quality. With manipulative laboratory experiments reproducing a gradient from bare sediments to sediments colonized by macrophytes and macrofauna, we provide evidence of important biogeochemical services offered by vegetation and associated invertebrates in organic sediments. Such experiments demonstrate a large decrease in methane emission and nutrient loss/retention in biodiverse (macrophytes and macrofauna colonized) vs. simplified (microbial-dominated) benthic communities.

Abstract

Organic sediments are greenhouse gas and nutrient hotspots. They may display lower methane (CH₄) emissions and increase nutrient retention when macrophytes and macrofauna are present, due to oxygen leakage from roots and bioirrigation. We tested this hypothesis via incubations of microcosms reproducing four treatments: bare sediment, sediment with oligochaetes, sediment with macrophytes, and sediment with both organisms. Along a 12-d experiment, CH₄ ebullition in bare sediment ($470 \pm 13 \text{ mmol m}^{-2}$) decreased by 67%, 88%, and 97% in the presence of plants, oligochaetes, and both organisms, respectively. Oligochaetes increased N₂ production by $\sim 200 \text{ mmol N m}^{-2}$ and nitrate consumption by a factor of 4, whereas macrophytes reduced nitrogen losses by $\sim 65 \text{ mmol N m}^{-2}$. All treatments acted as phosphate sink. Results suggest that the maintenance of vegetation and associated macrofauna in organic sediments promotes their combined ecosystem services, resulting in significant reduction of greenhouse gas emission and nutrient release to the water column.

*Correspondence: sara.benelli@unipr.it

Associate editor: Alberto Borges**Authors Contribution Statement:** SB conceptualization, laboratory analyses, data curation, writing–original draft. MB conceptualization, methodology, supervision, writing–original draft, funding acquisition.**Data Availability Statement:** Data are available from the Dryad Digital Repository: <https://doi.org/10.5061/dryad.ngf1vhht4>.

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

This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.

Eutrophication in inland waters has resulted in widespread loss of biodiversity, including dramatic reduction of rooted macrophytes and associated macrofauna (Egertson et al. 2004). The increase of pelagic primary production and sedimentation rates has resulted in large inputs of labile organic matter (OM) to surface sediments with a positive feedback for nutrient regeneration and a negative feedback for macrophytes recovery (Hilt et al. 2017). Macrophytes increase habitat complexity, have a structuring role, and support an array of biogeochemical services in aquatic ecosystems as O_2 production, nutrient uptake, and particulate matter retention (Sand-Jensen 1998). The latter increases the organic content of sediments, stimulating anaerobic metabolism (Soana et al. 2015). Simultaneously, macrophytes adaptations to anoxia as radial O_2 loss (ROL) from roots stimulate the growth of aerobic bacteria in sediments and the development of unique microbiome adjacent to roots, oxidize the rhizosphere, and favor phosphorus (P) retention and nitrogen (N) removal (Racchetti et al. 2010; Oliveira-Junior et al. 2018; Ávila et al. 2019; Marzocchi et al. 2019). The aerenchyma of emergent macrophytes connects the sediment to the atmosphere and may favor CH_4 evasion. Methane greenhouse net effect can be offset by plants CO_2 fixation rates (Van Der Nat and Middelburg 1998; Laanbroek 2010; Oliveira-Junior et al. 2020). ROL by submersed macrophytes might inhibit methanogenesis or favor aerobic CH_4 oxidation, reducing net emissions (Ribaudou et al. 2011; Kosten et al. 2016).

Similar biogeochemical services are provided by burrowing macrofauna through sediments reworking and improved net mineralization (Hölker et al. 2015). Bioirrigation and burrow ventilation with O_2 - and nitrate (NO_3^-)-rich water favor also N removal, P retention, CH_4 oxidation, or the inhibition of methanogenesis (Kajan and Frenzel 1999; Lewandowski et al. 2007; Kristensen et al. 2012).

In this study, we analyze the provision of benthic biogeochemical services by macrophytes and burrowing macrofauna reproducing a gradient from an oversimplified environment (microbial-dominated bare sediment) toward a system in which rooted macrophytes and macroinvertebrates are present. A few studies have previously addressed the synergic interactions among macrophytes and macrofauna on nutrient dynamics in freshwater ecosystems (Mermillod-Blondin and Lemoine 2010; Magri et al. 2018), whereas no studies have analyzed the combined biogeochemical services of plants and burrowing fauna on CH_4 dynamics. We hypothesize large reduction of CH_4 emission in the presence of roots and bioturbating macrofauna and that macrofauna facilitate aquatic macrophytes by mobilizing nutrients, reducing plant-bacteria competition, and resulting in high uptake and nutrients loss. We use model organisms commonly found in association with freshwater organic sediments: the submersed macrophyte *Vallisneria spiralis* and the oligochaete *Sparganophilus tamesis* (Benelli et al. 2019; Marzocchi et al. 2019).

Materials and methods

Water, sediment, shoots of *V. spiralis*, and individuals of *S. tamesis* were collected from a derivation of Mincio River (Northern Italy). These organisms are resistant to transplant and manipulation and can be maintained healthy in the laboratory (Magri et al. 2018). The collected sediment ($C = 9.44 \pm 0.88\%$, $N = 0.61 \pm 0.15\%$, $P = 0.07 \pm 0.01\%$) was sieved (500 μm -mesh), amended with labile OM and homogenized. The added OM (5 g L^{-1}) was in the form of powdered fish feed (42% C, 6% N, and 1% P) to simulate a C, N, and P input of ~ 100 , ~ 14 , and $\sim 2 \text{ g m}^{-2}$. Such input lays within the lower limit of particulate C, N, and P seasonal retention rates by macrophytes canopies (Sand-Jensen 1998) and produced a moderate increase ($< 5\%$) of the C, N, and P pools in the sediments. Sediments were transferred into opaque cylindrical plexiglass microcosms (i.d. 7 cm, height 10 cm, $n = 24$) to create four treatments, each with six replicates: bare sediment (S), sediment with oligochaetes (SO), sediment with macrophytes (SV), and sediment with oligochaetes and macrophytes (SOV; Fig. 1a). The density of oligochaetes and macrophytes (400 and 600 ind m^{-2} , respectively) reflected those in situ.

Microcosms were transferred to a temperature-controlled room maintained at $25 \pm 0.5^\circ C$ into a large aquarium (200 liters) filled with river water and provided with air bubbling to maintain O_2 saturation and homogeneous chemical conditions. Twelve microcosms (4 treatments \times 3 replicates) were equipped with transparent funnels having a diameter comparable to that of microcosms to convey gas bubbles to glass vials and to measure ebullitive CH_4 fluxes (Fig. 1b). The remaining microcosms underwent batch incubations to measure dissolved gas and nutrient fluxes (Fig. 1c). Both flux measurements started the day after microcosms setup. Microcosms were maintained under a 14.5 h light and 9.5 h dark light regime and with an irradiance set at $300 \mu E m^{-2} s^{-1}$, reflecting in situ average irradiance of the sampling period (July).

Ebullition rates of methane

After 0.4, 0.9, 1.9, 3.1, 4.2, 5.2, 6.2, 7.3, 8.2, 9.7, and 11.8 d from the starting of the experiment, the vials were removed from the funnels, closed underwater, weighed, and replaced. All vials were previously filled with water, closed, and weighed to allow calculation of the weight of the water displaced by bubbles and of gas volumes. The collected vials were added with 100 μL of water through the lid septum with a glass syringe and the same volume of gas was removed and transferred to 12-mL He flushed Exetainers (Labco) for CH_4 gas chromatographic analyses (FISONS 9000 GC, flame ionization detector, detection limit 10 ppb, precision $\pm 1\%$). The GC calibration was done via serial gas dilutions starting with 1-liter tedlar bag (Supelco) inflated with pure methane ($> 99.0\%$, Fluka Analytical) diluted into 250-mL of glass sampling bulbs (Supelco) pre-filled with He.

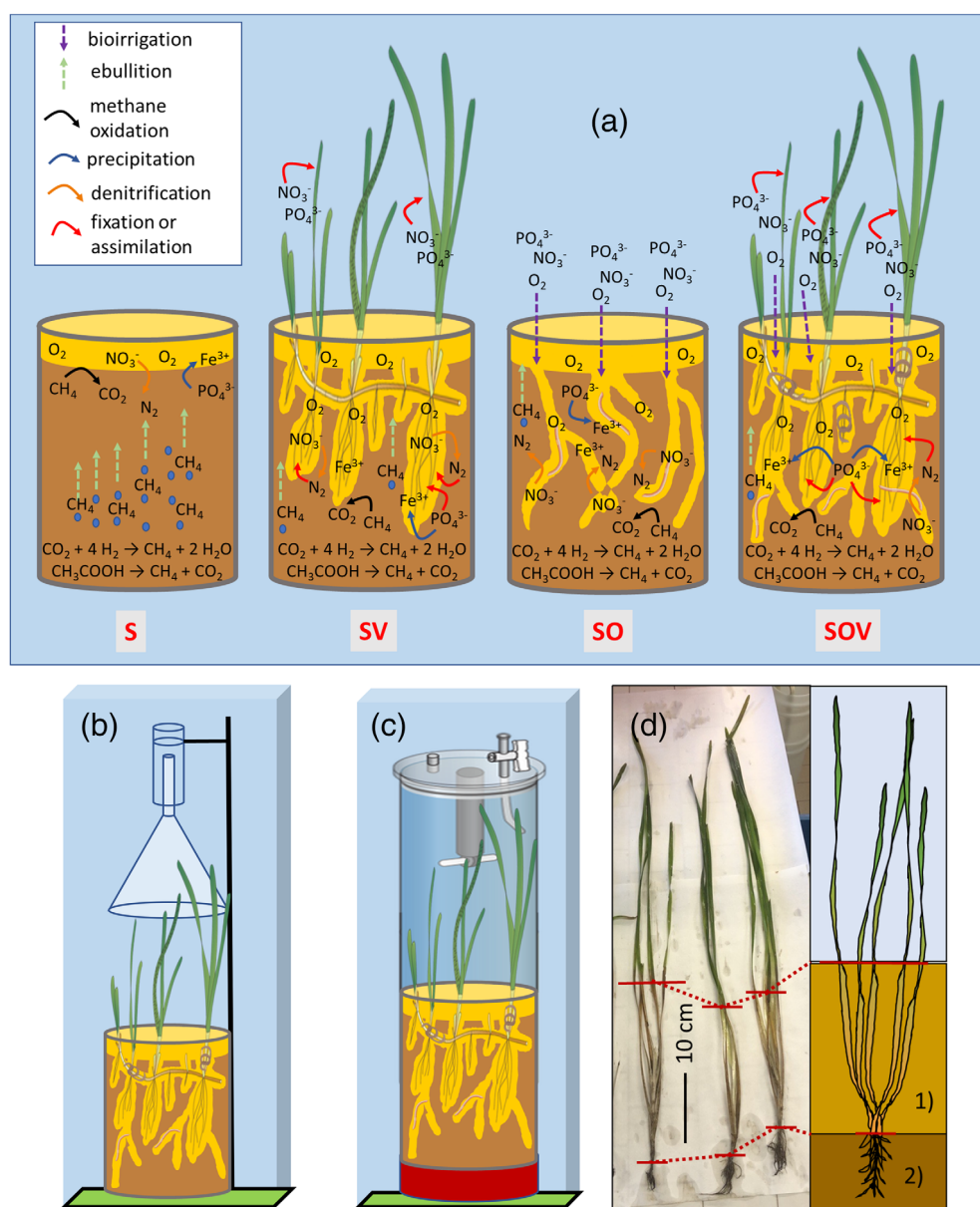


Fig. 1. Panel (a) depicts the biogeochemical services hypothesized in S (bare sediment), SV (sediment with macrophytes), SO (sediment with oligochaetes), and SOV (sediment with macrophytes and oligochaetes). They include the inhibition of methanogenesis or the oxidation of the produced methane, nutrient uptake by macrophytes, denitrification and precipitation of phosphate with ferric iron. Panels (b) and (c) show the setup for gas ebullition and dissolved gas and nutrient flux measurements, respectively. Panel (d) depicts the thickness of *V. spiralis* leaves laying within organic sediments (1) accumulated over pristine bottom sediments (2).

Methane ebullition rates ($\text{mmol CH}_4 \text{ m}^{-2} \text{ h}^{-1}$) were calculated dividing the amount of CH_4 produced by the surface area of microcosms and the time interval between successive vials collection. Rates were integrated along the incubation period to calculate the cumulative gas production ($\text{mmol m}^{-2} \text{ incubation period}^{-1}$).

Fluxes of dissolved gas and nutrients

The second set of microcosms underwent light and dark batch incubations after 1, 5, and 10 d from the beginning of

the experiment (Dalsgaard et al. 2000). Shortly before the incubation, microcosms were setup in plexiglass liners (i.d. 8 cm, height 30 cm) equipped with a stirring unit (Fig. 1c). The incubations started when liners were capped with gas-tight lids and lasted 2 h to keep O_2 concentration within $\pm 20\%$ of the initial value. Water samples were collected at the beginning and at the end of the incubation. Fluxes were calculated as the difference between final and initial concentrations of solutes in the water column multiplied by the volume of the water phase and divided by sediment

area and incubation time. Hourly fluxes were converted into daily fluxes by multiplying dark fluxes by 24 h in S and SO, and light fluxes by 14.5 h, and dark fluxes by 9.5 h in SV and SOV. Treatments S and SO underwent only dark incubations to mimic nonilluminated sediment without macrophytes. We acknowledge that microphytobenthos might have developed on sediment surface and that its contribution to benthic processes was neglected.

Dissolved O_2 was measured with a microelectrode (OX-50, Unisense A/S, DK) from each liner. Samples for dissolved CH_4 and molecular nitrogen (N_2) were collected via overflushing 12-mL Exetainers added with 100 μ L of 7 M $ZnCl_2$ to stop microbial activity. Dissolved CH_4 was measured by gas chromatography as described in Soana et al. (2015). Samples for N_2 were analyzed by a membrane inlet mass spectrometer (MIMS, Bay Instruments) and the concentrations were calculated from obtained $N_2 : Ar$ ratios and theoretical Ar concentrations. Additional water samples were filtered (GF/F glass-fiber filters) for spectrophotometric NO_3^- and phosphate (PO_4^{3-}) analyses (Valderrama 1977; Golterman et al. 1978). As the water in the incubation tank was not filtered or sterilized, dissolved CH_4 fluxes might be underestimated due to oxidation in the water column (Rudd et al. 1974). Such underestimation, occurring in all treatments, should not affect the relative differences in the measured rates.

Statistical analyses

The temporal trends of ebullitive CH_4 emissions were not linear and were modeled by generalized additive mixed model (gamm) using the R package mgcv (Wood 2017). Factor treatment was used as fixed variable, and smooths of factor time for each level of treatment were fitted, with replicates as random variables. Linear mixed models were applied to test differences in dissolved gas and nutrient fluxes due to the limited number of replicates of our experiment and robustness of models even if the distributional assumptions are not met (Schielzeth et al. 2020). Factors treatment and time were the fixed parameters, whereas replicates were set as random effects. All analyses were performed and graphs built with R statistical software (R Core Team 2018), and ggplot2 (Wickham et al. 2019), lme4 (Bates et al. 2019), and multcomp (Hothorn et al. 2008) packages.

Results

Methane ebullition rates

The production of CH_4 bubbles varied significantly among treatments along the course of the incubation (Fig. 2; Tables S1, S2). In S, SV, and SO treatments, ebullition rates increased, reached a maximum (S > SV > SO), and slowly declined (Fig. 2a). Methane ebullition peaked at $3.69 \pm 0.11 \text{ mmol m}^{-2} \text{ h}^{-1}$ in S after nearly 4 d from the beginning of the experiment. Smoothing curves and analysis revealed no significant temporal trend of ebullition only in

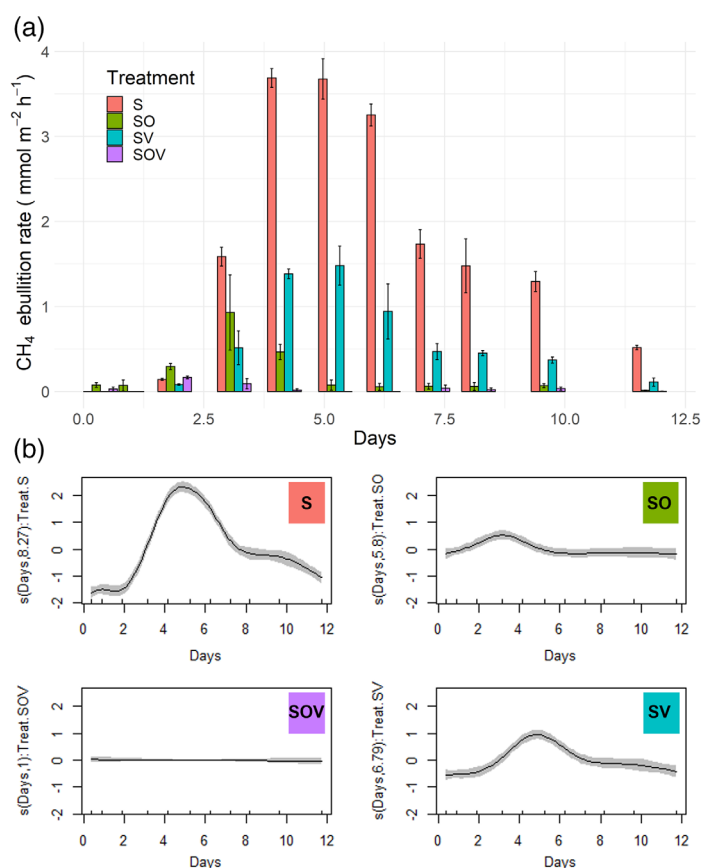


Fig. 2. (a) Hourly CH_4 ebullition rates (averages \pm standard errors, $n = 3$) measured in the four treatments in the 12-d experiment. (b) Generalized additive mixed model results of time effect on methane ebullitive emissions in each treatment. The continuous line is an estimate of the smooth function of the partial residuals (thus the y-axis is centered on zero) and indicates the x-axis covariate effects on the CH_4 emission deviation for each level of factor treatment. The shaded areas indicate the 95% confidence interval. The number on each y-axis caption is the effective degrees of freedom for the term being plotted. Tick marks on the x-axis are sampled data points.

SOV, in which rates were below $0.2 \text{ mmol m}^{-2} \text{ h}^{-1}$ (Fig. 2b; Tables S1, S2). Integration of rates to produce cumulative emission suggests that $\sim 10 \text{ L m}^{-2}$ of CH_4 bubbles, equivalent to $470 \pm 22 \text{ mmol } CH_4 \text{ m}^{-2}$, were released from S during the 12-d incubation (Fig. 3). In SV, SO, and SOV, such emission was reduced by 67%, 88%, and 97%, respectively.

Dissolved gas and nutrient fluxes

On a daily basis, all treatments were net heterotrophic, with O_2 demand ($-41 < x < -146 \text{ mmol } O_2 \text{ m}^{-2} \text{ d}^{-1}$; Fig. 4a) increasing in the presence of *S. tamesis* (i.e., SO > S and SOV > SV). Differences among treatments were highly significant but depended upon the incubation time ($p = 0.003$; Table 1). Daily O_2 demand in S and SO peaked at day 5, whereas in the presence of *V. spiralis* it decreased along the incubation period.

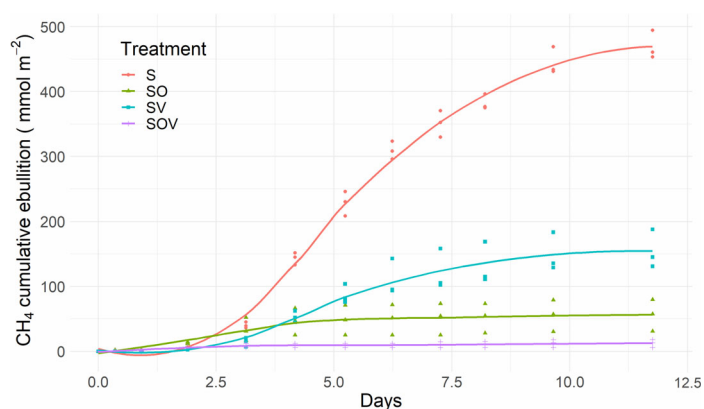


Fig. 3. Cumulative amount of CH₄ emitted via ebullition from the four treatments during the 12-d experimental period ($n = 3$ per treatment).

Fluxes of dissolved CH₄ ($-1 < x < 16$ mmol CH₄ m⁻²d⁻¹) were predominantly positive (Fig. 4b); differences among treatments depended upon the incubation time ($p < 0.001$; Table 1). Bare sediments displayed the highest rates of dissolved CH₄ release at days 5 and 10, followed by SO and SV, whereas in SOV fluxes were negligible. Bare sediments released 113 ± 48 mmol CH₄ m⁻² incubation period⁻¹, equivalent to 19% of the total CH₄ released by S via both ebullition and diffusion. As compared to bare sediments, dissolved CH₄ fluxes were reduced by 10%, 66%, and 84% in SO, SV, and SOV treatments, respectively.

Net N₂ fluxes ($-14 < x < 30$ mmol N m⁻²d⁻¹) included both positive and negative values, suggesting the co-occurrence of denitrification and N-fixation (Fig. 4c). Differences among treatments depended upon the incubation time ($p = 0.003$; Table 1). Excluding day 1, negative N₂ fluxes were measured during light incubations in SV and SOV, peaking in SV at day 10. Sediments with only macrophytes exhibited lower N₂ effluxes (day 5) or N₂ uptake (day 10) as compared to vegetated and bioturbated sediments (SOV). During the whole incubation period, the integrated N₂ fluxes were estimated at 62 ± 71 , 262 ± 47 , -10 ± 46 , and 200 ± 60 mmol N m⁻² incubation period⁻¹ in S, SO, SV, and SOV, respectively. In SO and SOV, the presence of oligochaetes increased by ~ 200 mmol N m⁻² incubation period⁻¹ net denitrification as compared to S and SV, respectively. In SV and SOV, the presence of macrophytes reduced by ~ 60 – 70 mmol N m⁻² incubation period⁻¹ benthic N₂ effluxes as compared to S and SO treatments.

Daily NO₃⁻ fluxes were mostly negative and significantly different among treatments ($p < 0.001$; Table 1; Fig. 4d). Nitrate uptake in SO and SOV exceeded by 20 ± 9 mmol N m⁻²d⁻¹ NO₃⁻ uptake measured in S and SV, which overlaps the increase of N₂ production measured in the presence of *S. tamesis* (15 ± 8 mmol N m⁻²d⁻¹). The comparison between treatments with and without plants indicated lower NO₃⁻ uptake in the presence of *V. spiralis*. Integrated NO₃⁻ fluxes resulted in

-90 ± 28 , -373 ± 101 , 42 ± 22 , and -168 ± 64 mmol N m⁻² incubation period⁻¹ in S, SO, SV, and SOV treatments, respectively.

Phosphate fluxes were significantly different at day 1 as compared to days 5 and 10 ($p < 0.001$; Table 1) and among treatments ($p < 0.001$; Table 1). They were positive only at day 1 in SO and SOV treatments, coinciding with active burrowing by oligochaetes, whereas at days 5 and 10 they were negative, in particular in treatments with macrophytes (Fig. 4e). Phosphate fluxes, integrated over the 12 d, resulted in -5.37 ± 1.08 , -1.62 ± 1.28 , -8.37 ± 1.00 , and -8.17 ± 0.68 mmol P m⁻² incubation period⁻¹ in S, SO, SV, and SOV treatments, respectively.

Discussion

In this study, methane release in bare organic sediments was nearly set to zero in the presence of submersed rooted plants and burrowing macrofauna. Oxygen transport to sediments via roots reduces methanogenesis (Sorrell et al. 2002) and increases CH₄ consumption by aerobic methane-oxidizing bacteria by providing them a substrate for their growth (Yoshida et al. 2014). ROL by *V. spiralis* roots was measured with planar optodes in organic sediments (Marzocchi et al. 2019) and was inferred in other studies (Soana and Bartoli 2013). Comparison of pore-water chemistry in vegetated and bare sediments demonstrated higher redox potential, higher NO₃⁻, and lower reduced metals and CH₄ concentrations in the presence of roots (Ribaud et al. 2011; Soana et al. 2012). *V. spiralis* displays also adaptive responses to seasonally decreasing sediment redox, via increased ROL during summer (Soana and Bartoli 2013). Such short-term adaptive responses (i.e., ethylene-induced aerenchyma increase, favoring larger oxygen transport to roots) might represent a common strategy for the submersed macrophytes that retain large amounts of particulate matter with the canopy and turn sediments organic (Sand-Jensen 1998; Mermillod-Blondin and Lemoine 2010). In late summer, the basal part of *V. spiralis* leaves lays within recent sediments, as evidenced by a 10–20-cm-long reddish leaf segment which is devoid of chlorophyll (Fig. 1d). Ferric iron coating is a consequence of oxygen leaking from leaves in ferrous iron-rich organic sediments (Soana et al. 2012; Marzocchi et al. 2019). ROL by *V. spiralis* calculated independently by Soana and Bartoli (2013) and Marzocchi et al. (2019) ranged between 2.5 and 6.0 mmol O₂ m⁻² h⁻¹. Such oxygen input to sediments has the potential to oxidize 1.3–3 mmol CH₄ m⁻² h⁻¹ and may explain significantly lower CH₄ fluxes in vegetated vs. bare sediments.

Bioirrigation and ventilation by *S. tamesis* inject O₂- and NO₃⁻-rich bottom water into sediments (Benelli et al. 2019). As such, these processes change the sediment and pore-water chemistry and favor the oxidation of anaerobic mineralization end products as metals, NH₄⁺, H₂S, and CH₄. Kajan and Frenzel (1999) demonstrated that macroinvertebrates burrows

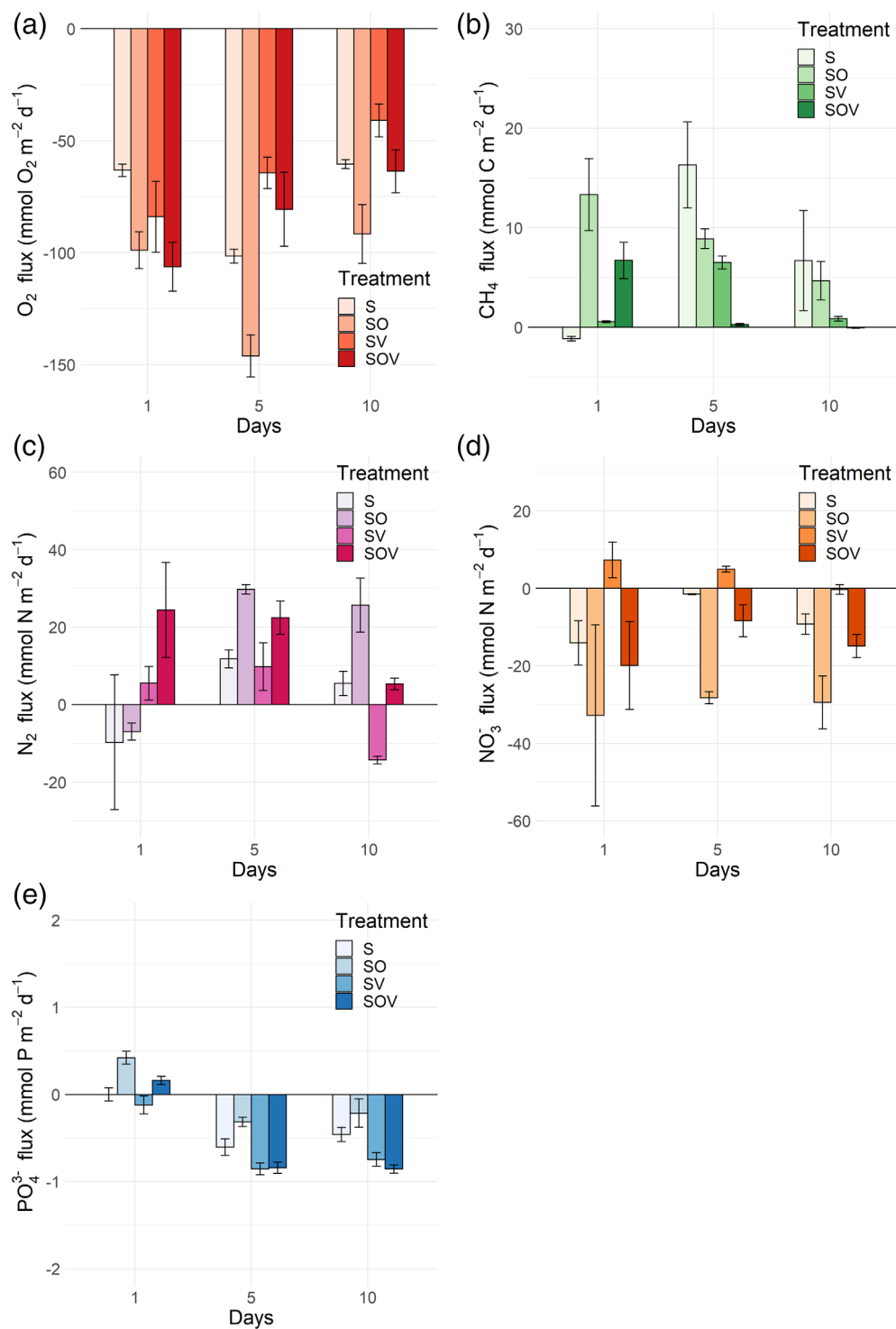


Fig. 4. Daily fluxes (mmol m⁻²d⁻¹) measured at days 1, 5, and 10 of dissolved (a) O₂, (b) CH₄, (c) N₂, (d) NO₃⁻, and (e) PO₄³⁻ in the four treatments. Averages ± standard errors (n = 3) are reported.

are important microsites for CH₄ oxidation. The presence of *S. tamesis* significantly increased O₂ and NO₃⁻ consumption measured in bare sediments, by up to 2 and 1 mmol m⁻² h⁻¹, respectively. As discussed for ROL by *V. spiralis*, bioturbation

has therefore a large potential to decrease CH₄ fluxes via aerobic oxidation. In nitrate-rich ecosystems as the study area, it may additionally stimulate anaerobic mechanisms (à Norðri and Thamdrup 2014). The combined activity of macrophytes

Table 1. Results of the mixed effects models applied to dissolved gas and nutrient fluxes. The table shows the effect of factors treatment (S, SO, SV, and SOV) and time (days 1, 5, and 10) on solute fluxes. Replicates were implemented as random part in the models. Significant values are marked in bold.

	Source of variation	SS	MS	Num df	Den df	F	Pr(>F)	Post hoc multiple comparison
O ₂	Treatment	11,803.6	3934.5	3	22	17.345	<0.001	
	Time	7305.7	3652.8	2	22	16.103	<0.001	
	Interaction	6634.2	1105.7	6	22	4.874	0.003	S:1-SOV:1, S:5-SO:5, S:5-SV:5, S:1-S:5, S:5-S:10, SO:1-SO:5, SO:5-SO:10, SV:1-SV:10, SOV:1-SOV:10
CH ₄	Treatment	299.76	99.92	3	22	6.066	0.004	
	Time	151.07	75.53	2	22	4.585	0.021	
	Interaction	575.15	95.86	6	22	5.819	<0.001	S:1-SO:1, S:5-SV:5, S:5-SOV:5, S:1-S:5
N ₂	Treatment	2155.0	718.35	3	22	6.044	0.003	
	Time	1597.5	798.77	2	22	6.721	0.005	
	Interaction	3212.2	535.36	6	22	4.505	0.003	S:1-SOV:1, SO:1-SO:5, SO:1-SO:10
NO ₃ ⁻	Treatment	5441.6	1813.87	3	22	9.2630	<0.001	
	Time	286.9	143.43	2	22	0.7325	0.492	
	Interaction	278.8	46.47	6	22	0.2373	0.960	
PO ₄ ³⁻	Treatment	1.55	0.52	3	22	22.663	<0.001	S:1-SO:1, S:10-SOV:10
	Time	4.30	2.15	2	22	94.132	<0.001	S:1-S:5, S:1-S:10, SO:1-SO:5, SO:1-SO:10, SV:1-SV:5, SV:1-SV:10, SOV:1-SOV:5, SOV:1-SOV:10
	Interaction	0.27	0.04	6	22	1.963	0.115	

SS, sum of squares; MS, mean of squares; Num df, numerator degrees of freedom; Den df, denominator degrees of freedom; F, F-statistics; Pr(>F), p-values.

and oligochaetes likely inhibits methanogenesis in the sediment layer where roots and burrows are present, deepens the redox boundaries where methanogenesis occurs, or favors CH₄ oxidation (Van der Nat and Middelburg 1998).

Results of this and previous studies suggest that the presence of roots and oligochaetes always stimulated nitrification and denitrification, and enhanced water column NO₃⁻ consumption and net N₂ production (Racchetti et al. 2017; Benelli et al. 2019). *S. tamesis* is generally associated to the roots of aquatic plants, where its cocoons are generally anchored. This worm is always associated to *V. spiralis* and both organisms may take advantage from such association (Benelli et al. 2019). In the rhizosphere, the worm may in fact reduce energy costly ventilation and irrigation activities to improve water chemistry in burrows (i.e., to increase O₂ levels and decrease ammonia concentrations). The contact between the worm thin skin and the root surfaces may theoretically allow O₂ diffusion in the blood circulation system of the oligochaete. This is supported by lower increase of O₂ demand in vegetated sediments added with the oligochaete (0.7–1.3 mmol O₂ m⁻² h⁻¹) as compared to sediments added with the oligochaete (1.3–1.9 mmol O₂ m⁻² h⁻¹). Moreover, *V. spiralis* roots decrease pore-water toxicity via NH₄⁺ uptake or by favoring nitrification (Racchetti et al. 2010; Soana and Bartoli 2013; Marzocchi et al. 2019).

The interaction between benthic primary producers and microbial communities involved in N cycling is complex. In N-poor environments macrophytes rely on sediments for N uptake and compete with microbial communities (Benelli et al. 2020). The stimulation of coupled nitrification–denitrification in vegetated sediments reported by Risgaard-Petersen and Jensen (1997) and Racchetti et al. (2017) is an indirect effect of ROL. In N-rich environments, NO₃⁻ is in large excess in the water column (100 < [NO₃⁻] < 200 μM) and macrophytes may assimilate N via the leaves (Cedergreen and Madsen 2003; Viaroli et al. 2018). This would mean lower plant-bacteria competition in sediments, supporting simultaneously elevated N uptake and denitrification in the rhizosphere (Racchetti et al. 2019). In SV treatment, NO₃⁻ fluxes were slightly positive, supporting a tight coupling between ROL and nitrification in excess to denitrification.

Light incubations suggest that macrophytes or their associated epiphytes may reverse net N₂ fluxes due to a larger stimulation of N-fixation than of denitrification (Finke and Seeley 1978; Zuberer 1982). Such stimulation was likely due to N-limitation in the rhizosphere, caused by uptake or coupled nitrification–denitrification (Marzocchi et al. 2019). Nitrogen fixation was also measured in freshwater macrofauna holobionts and in bioturbated sediments to compensate N-losses from denitrification (Marzocchi et al. 2021).

Interestingly, the dependency of vegetated sediments from N-fixation decreased in the presence of oligochaetes. Deposit feeders indeed increase NH_4^+ availability via sediment reworking, enhanced ammonification and direct excretion (Magri et al. 2018; Benelli et al. 2019).

Sediment sieving and mixing destroy the vertical zonation of microbial communities, that is re-established after 3–6 weeks (Stocum and Plante 2006; Mermillod-Blondin et al. 2018). In this experiment, treatments did not undergo preincubation as similar amendments of labile OM produced a fast stimulation of microbial metabolism (Soana et al. 2012, 2015). For this reason, measurements started the day after microcosms setup. The absence of adequate preincubation can result in absence of steady state, which may explain the large variability of dissolved gas and nutrient fluxes among replicates and treatments measured at day 1. We acknowledge that we might have underestimated the effect produced by slow growing microbes (e.g., nitrifiers) in the treatments with plants and/or worms and that our results rely on the vast metabolic repertoire and rapid metabolic shift of microbes to changing environmental conditions. It may also result in overestimated role of macrofauna at the beginning of the experiment, due to active burrowing. However, with a preincubation of several weeks, the high ebullitive rates occurred shortly after the organic amendment would have been unaccounted. A longer preincubation would have favored the growth of nitrifiers or methanotrophs or increased the solid-phase pools of oxidized metals; all these expected changes would have reinforced and not contrasted our results.

The potential of vegetated and bioturbated canals in supporting biogeochemical services needs to be further verified expanding this kind of studies to other sediment typologies, macrophytes, and macrofauna species, including different growth forms (e.g., emergent plants) or functional groups (e.g., filter feeders). In situ studies under natural, unmanipulated conditions would also be important to validate our results. This topic seems central in a period of ecological transition and restoration of impacted ecosystems. The conversion of floodplains into arable lands has resulted in loss of biodiversity and ecosystem services and in the creation of extended networks of artificial canals (Foley et al. 2005; Peacock et al. 2021). Their chemical and biological quality is generally poor, due to generalized N and P excess from fertilizers and organic enrichment (Leip et al. 2015). In the Po River Plain (Northern Italy), which is an intensive agriculture European hotspot, drainage and irrigation canals develop over a network of > 50,000 km and undergo frequent maintenance operations, mostly consisting of mechanical removal of accumulated sediments and vegetation, to maintain or improve discharge capacity. This sets to zero aquatic vegetation, associated macrofauna, and the key biogeochemical services these organisms provide, with detrimental effects on downstream aquatic ecosystems (Soana et al. 2018; Viaroli et al. 2018).

References

- á Norði, K., and B. Thamdrup. 2014. Nitrate-dependent anaerobic methane oxidation in a freshwater sediment. *Geochim. Cosmochim. Acta* **132**: 141–150. doi:10.1016/j.gca.2014.01.032
- Ávila, M. P., and others. 2019. The water hyacinth microbiome: Link between carbon turnover and nutrient cycling. *Microb. Ecol.* **78**: 575–588. doi:10.1007/s00248-019-01331-9
- Bates, D., M. Maechler, B. Bolker, S. Walker, R. H. B. Christensen, H. Singmann & J. Fox 2019. lme4: Linear mixed-effects models using ‘eigen’ and S4 (version 1.1-27). Available from <https://cran.r-project.org/web/packages/lme4/index.html>
- Benelli, S. & M. Bartoli 2021. Data from: “Worms and submersed macrophytes reduce methane release and increase nutrient removal in organic sediments”, Dryad, Dataset. doi:10.5061/dryad.ngf1vhht4
- Benelli, S., C. Ribaud, V. Bertrin, M. Bartoli, and E. A. Fano. 2020. Effects of macrophytes on potential nitrification and denitrification in oligotrophic lake sediments. *Aquat. Bot.* **167**: 103287. doi:10.1016/j.aquabot.2020.103287
- Benelli, S., M. Bartoli, C. Ribaud, and E. A. Fano. 2019. Contrasting effects of an alien worm on benthic N cycling in muddy and sandy sediments. *Water* **11**: 465. doi:10.3390/w11030465
- Cedergreen, N., and T. V. Madsen. 2003. Nitrate reductase activity in roots and shoots of aquatic macrophytes. *Aquat. Bot.* **76**: 203–212. doi:10.1016/S0304-3770(03)00050-0
- Dalsgaard, T., and others. 2000. Protocol handbook for NICE-nitrogen cycling in estuaries: A project under the EU Research Programme: Marine Science and Technology (MAST III). Ministry of Environment and Energy National Environmental Research Institute, Denmark, Department of Lake and Estuarine Ecology.
- Egertson, C. J., J. A. Kopaska, and J. A. Downing. 2004. A century of change in macrophyte abundance and composition in response to agricultural eutrophication. *Hydrobiologia* **524**: 145–156. doi:10.1023/B:HYDR.0000036129.40386.ce
- Finke, L. R., and H. W. Seeley. 1978. Nitrogen fixation (acetylene reduction) by epiphytes of freshwater macrophytes. *Appl. Environ. Microbiol.* **36**: 129–138.
- Foley, J. A., and others. 2005. Global consequences of land use. *Science* **309**: 570–574. doi:10.1126/science.1111772
- Golterman, H. L., R. S. Clymo, and M. A. M. Ohnstand. 1978. *Methods for physical and chemical analysis of fresh waters. Issue 8 of Handbooks (International Biological Programme)*. Blackwell.
- Hilt, S., S. Brothers, E. Jeppesen, A. J. Veraart, and S. Kosten. 2017. Translating regime shifts in shallow lakes into changes in ecosystem functions and services. *Bioscience* **67**: 928–936. doi:10.1093/biosci/bix106

- Hölker, F., and others. 2015. Tube-dwelling invertebrates: Tiny ecosystem engineers have large effects in lake ecosystems. *Ecol. Monogr.* **85**: 333–351. doi:[10.1890/14-1160.1](https://doi.org/10.1890/14-1160.1)
- Hothorn, T., F. Bretz, P. Westfall. 2008. Simultaneous inference in general parametric models (version 1.4-17). *Biom. J.* **50**: 346–363. Available from <http://multcomp.R-project.org>
- Kajan, R., and P. Frenzel. 1999. The effect of chironomid larvae on production, oxidation and fluxes of methane in a flooded rice soil. *FEMS Microbiol. Ecol.* **28**: 121–129. doi:[10.1111/j.1574-6941.1999.tb00567.x](https://doi.org/10.1111/j.1574-6941.1999.tb00567.x)
- Kosten, S., M. Piñeiro, E. de Goede, J. de Klein, L. P. Lamers, and K. Ettwig. 2016. Fate of methane in aquatic systems dominated by free-floating plants. *Water Res.* **104**: 200–207. doi:[10.1016/j.watres.2016.07.054](https://doi.org/10.1016/j.watres.2016.07.054)
- Kristensen, E., G. Penha-Lopes, M. Delefosse, T. Valdemarsen, C. O. Quintana, and G. T. Banta. 2012. What is bioturbation? The need for a precise definition for fauna in aquatic sciences. *Mar. Ecol. Prog. Ser.* **446**: 285–302. doi:[10.3354/meps09506](https://doi.org/10.3354/meps09506)
- Laanbroek, H. J. 2010. Methane emission from natural wetlands: Interplay between emergent macrophytes and soil microbial processes. A mini-review. *Ann. Bot.* **105**: 141–153. doi:[10.1093/aob/mcp201](https://doi.org/10.1093/aob/mcp201)
- Leip, A., and others. 2015. Impacts of European livestock production: Nitrogen, sulphur, phosphorus and greenhouse gas emissions, land-use, water eutrophication and biodiversity. *Environ. Res. Lett.* **10**: 115004. doi:[10.1088/1748-9326/10/11/115004](https://doi.org/10.1088/1748-9326/10/11/115004)
- Lewandowski, J., C. Laskov, and M. Hupfer. 2007. The relationship between *Chironomus plumosus* burrows and the spatial distribution of pore-water phosphate, iron and ammonium in lake sediments. *Freshw. Biol.* **52**: 331–343. doi:[10.1111/j.1365-2427.2006.01702.x](https://doi.org/10.1111/j.1365-2427.2006.01702.x)
- Magri, M., S. Benelli, C. Bondavalli, M. Bartoli, R. R. Christian, and A. Bodini. 2018. Benthic N pathways in illuminated and bioturbated sediments studied with network analysis. *Limnol. Oceanogr.* **63**: S68–S84. doi:[10.1002/lno.10724](https://doi.org/10.1002/lno.10724)
- Marzocchi, U., and others. 2021. Zebra mussel holobionts fix and recycle nitrogen in lagoon sediments. *Front. Microbiol.* **11**: 610269. doi:[10.3389/fmicb.2020.610269](https://doi.org/10.3389/fmicb.2020.610269)
- Marzocchi, U., S. Benelli, M. Larsen, M. Bartoli, and R. N. Glud. 2019. Spatial heterogeneity and short-term oxygen dynamics in the rhizosphere of *Vallisneria spiralis*: Implications for nutrient cycling. *Freshw. Biol.* **64**: 532–543. doi:[10.1111/fwb.13240](https://doi.org/10.1111/fwb.13240)
- Mermillod-Blondin, F., and others. 2018. Influence of tubificid worms on sediment structure, benthic biofilm and fauna in wetlands: A field enclosure experiment. *Freshw. Biol.* **63**: 1420–1432. doi:[10.1111/fwb.13169](https://doi.org/10.1111/fwb.13169)
- Mermillod-Blondin, F., and D. G. Lemoine. 2010. Ecosystem engineering by tubificid worms stimulates macrophyte growth in poorly oxygenated wetland sediments. *Funct. Ecol.* **24**: 444–453. doi:[10.1111/j.1365-2435.2009.01624.x](https://doi.org/10.1111/j.1365-2435.2009.01624.x)
- Oliveira-Junior, E. S., and others. 2020. Water hyacinth's effect on greenhouse gas fluxes: A field study in a wide variety of tropical water bodies. *Ecosystems* **1**: 1–17. doi:[10.1007/s10021-020-00564-x](https://doi.org/10.1007/s10021-020-00564-x)
- Oliveira-Junior, E. S., Y. Tang, S. J. van den Berg, S. J. Cardoso, L. P. Lamers, and S. Kosten. 2018. The impact of water hyacinth (*Eichhornia crassipes*) on greenhouse gas emission and nutrient mobilization depends on rooting and plant coverage. *Aquat. Bot.* **145**: 1–9. doi:[10.1016/j.aquabot.2017.11.005](https://doi.org/10.1016/j.aquabot.2017.11.005)
- Peacock, M., and others. 2021. Global importance of methane emissions from drainage ditches and canals. *Environ. Res. Lett.* **16**: 044010. doi:[10.1088/1748-9326/abeb36](https://doi.org/10.1088/1748-9326/abeb36)
- R Core Team. 2018. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. Available from <http://www.r-project.org/>
- Racchetti, E., and others. 2019. Is flood irrigation a potential driver of river-groundwater interactions and diffuse nitrate pollution in agricultural watersheds? *Water* **11**: 2304. doi:[10.3390/w11112304](https://doi.org/10.3390/w11112304)
- Racchetti, E., D. Longhi, C. Ribauda, E. Soana, and M. Bartoli. 2017. Nitrogen uptake and coupled nitrification-denitrification in riverine sediments with benthic microalgae and rooted macrophytes. *Aquat. Sci.* **79**: 487–505. doi:[10.1007/s00027-016-0512-1](https://doi.org/10.1007/s00027-016-0512-1)
- Racchetti, E., M. Bartoli, C. Ribauda, D. Longhi, L. E. Q. Brito, M. Naldi, P. Iacumin, and P. Viaroli. 2010. Short term changes in pore water chemistry in river sediments during the early colonization by *Vallisneria spiralis*. *Hydrobiologia* **652**: 127–137. doi:[10.1007/s10750-010-0324-6](https://doi.org/10.1007/s10750-010-0324-6)
- Ribauda, C., M. Bartoli, E. Racchetti, D. Longhi, and P. Viaroli. 2011. Seasonal fluxes of O₂, DIC and CH₄ in sediments with *Vallisneria spiralis*: Indications for radial oxygen loss. *Aquat. Bot.* **94**: 134–142. doi:[10.1016/j.aquabot.2011.01.003](https://doi.org/10.1016/j.aquabot.2011.01.003)
- Risgaard-Petersen, N., and K. Jensen. 1997. Nitrification and denitrification in the rhizosphere of the aquatic macrophyte *Lobelia dortmanna* L. *Limnol. Oceanogr.* **42**: 529–537. doi:[10.4319/lo.1997.42.3.0529](https://doi.org/10.4319/lo.1997.42.3.0529)
- Rudd, J. W., R. D. Hamilton, and N. E. R. Campbell. 1974. Measurement of microbial oxidation of methane in lake water. *Limnol. Oceanogr.* **19**: 519–524. doi:[10.4319/lo.1974.19.3.0519](https://doi.org/10.4319/lo.1974.19.3.0519)
- Sand-Jensen, K. A. J. 1998. Influence of submerged macrophytes on sediment composition and near-bed flow in lowland streams. *Freshw. Biol.* **39**: 663–679. doi:[10.1046/j.1365-2427.1998.00316.x](https://doi.org/10.1046/j.1365-2427.1998.00316.x)
- Schielzeth, H., and others. 2020. Robustness of linear mixed-effects models to violations of distributional assumptions. *Methods Ecol. Evol.* **11**: 1141–1152. doi:[10.1111/2041-210X.13434](https://doi.org/10.1111/2041-210X.13434)

- Soana, E., A. Gavioli, E. Tamburini, E. A. Fano, and G. Castaldelli. 2018. To mow or not to mow: Reed biofilms as denitrification hotspots in drainage canals. *Ecol. Eng.* **113**: 1–10. doi:[10.1016/j.ecoleng.2017.12.029](https://doi.org/10.1016/j.ecoleng.2017.12.029)
- Soana, E., M. Naldi, S. Bonaglia, E. Racchetti, G. Castaldelli, V. Brüchert, P. Viaroli, and M. Bartoli. 2015. Benthic nitrogen metabolism in a macrophyte meadow (*Vallisneria spiralis* L.) under increasing sedimentary organic matter loads. *Biogeochemistry* **124**: 387–404. doi:[10.1007/s10533-015-0104-5](https://doi.org/10.1007/s10533-015-0104-5)
- Soana, E., and M. Bartoli. 2013. Seasonal variation of radial oxygen loss in *Vallisneria spiralis* L.: An adaptive response to sediment redox? *Aquat. Bot.* **104**: 228–232. doi:[10.1016/j.aquabot.2012.07.007](https://doi.org/10.1016/j.aquabot.2012.07.007)
- Soana, E., M. Naldi, and M. Bartoli. 2012. Effects of increasing organic matter loads on pore water features of vegetated (*Vallisneria spiralis* L.) and plant-free sediments. *Ecol. Eng.* **47**: 141–145. doi:[10.1016/j.ecoleng.2012.06.016](https://doi.org/10.1016/j.ecoleng.2012.06.016)
- Sorrell, B. K., M. T. Downes, and C. L. Stanger. 2002. Methanotrophic bacteria and their activity on submerged aquatic macrophytes. *Aquat. Bot.* **72**: 107–119. doi:[10.1016/S0304-3770\(01\)00215-7](https://doi.org/10.1016/S0304-3770(01)00215-7)
- Stocum, E. T., and C. J. Plante. 2006. The effect of artificial defaunation on bacterial assemblages of intertidal sediments. *J. Exp. Mar. Biol. Ecol.* **337**: 147–158. doi:[10.1016/j.jembe.2006.06.012](https://doi.org/10.1016/j.jembe.2006.06.012)
- Valderrama, J. C. 1977. Methods used by the hydrographica department of the national board of fisheries. Goteborg, Sweden.
- Van Der Nat, F. J. W., and J. J. Middelburg. 1998. Effects of two common macrophytes on methane dynamics in freshwater sediments. *Biogeochemistry* **43**: 79–104. doi:[10.1023/A:1006076527187](https://doi.org/10.1023/A:1006076527187)
- Viaroli, P., E. Soana, S. Pecora, A. Laini, M. Naldi, E. A. Fano, and D. Nizzoli. 2018. Space and time variations of watershed N and P budgets and their relationships with reactive N and P loadings in a heavily impacted river basin (Po river, Northern Italy). *Sci. Total Environ.* **639**: 1574–1587. doi:[10.1016/j.scitotenv.2018.05.233](https://doi.org/10.1016/j.scitotenv.2018.05.233)
- Wickham, H. others. 2019. ggplot2: Create elegant data visualisations using the grammar of graphics (version 3.3.4). Available from <https://cran.r-project.org/web/packages/ggplot2/index.html>
- Wood, S. N. 2017. *Generalized additive models: An introduction with R*, 2nd Edition. Chapman and Hall/CRC.
- Yoshida, N., H. Iguchi, H. Yurimoto, A. Murakami, and Y. Sakai. 2014. Aquatic plant surface as a niche for methanotrophs. *Front. Microbiol.* **5**: 30. doi:[10.3389/fmicb.2014.00030](https://doi.org/10.3389/fmicb.2014.00030)
- Zuberer, D. A. 1982. Nitrogen fixation (acetylene reduction) associated with duckweed (Lemnaceae) mats. *Appl. Environ. Microbiol.* **43**: 823–828. doi:[10.1128/aem.43.4.823-828.1982](https://doi.org/10.1128/aem.43.4.823-828.1982)

Acknowledgments

Daniele Longhi and Elisa Soana are acknowledged for precious help during laboratory incubations. We thank Stefano Leonardi for his support in data analysis.

Submitted 15 February 2021

Revised 29 July 2021

Accepted 31 July 2021