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# Different Denitrification Capacity in *Phragmites australis* and *Typha latifolia* Sediments: Does the Availability of Surface Area for Biofilm Colonization Matter?

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**Abstract:** Denitrification is a permanent nitrogen removal pathway; thus, it is a desirable ecosystem function in water bodies receiving agricultural runoff. Knowledge of denitrification capacity in response to vegetation type and varying $NO_3^-$ loads is essential for designing effectively constructed wetlands to control eutrophication. The aim of this study was to compare the nitrogen removal efficiency of two common wetland macrophytes, i.e., *Phragmites australis* and *Typha latifolia* in a $NO_3^-$ enrichment experiment (50−800 µM). Measurements of $NO_3^-$ consumption, and $N_2$ production were performed in vegetated and unvegetated mesocosms incubated in summer (26 °C) at biomass peak. Vegetated sediments demonstrated higher efficiency in converting $NO_3^-$ to $N_2$ via denitrification (<600–18,000 µmol N $m^{-2}$ $h^{-1}$) than bare sediments (300–3300 µmol N $m^{-2}$ $h^{-1}$). However, the denitrification stimulation effect from $NO_3^-$ pulsing differed significantly between plant types. It can be hypothesized that *P. australis* played a more beneficial role than *T. latifolia* due to its greater submerged surface area, which facilitated enhanced opportunities for contact between $NO_3^-$ and denitrifying bacteria. This ultimately resulted in an increased treatment performance. Understanding the interactions between plants and environmental drivers regulating denitrification is critical information for optimal wetland species selection. With an increasing global focus on sustainable water quality management, this research provides valuable insights into optimizing nature-based solutions.

**Keywords:** nitrate pollution; constructed wetlands; *P. australis*; *T. latifolia*; denitrification; biofilms; nature-based solutions



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## 1. Introduction

Eutrophication, resulting from the enrichment of aquatic environments with nutrients from land-based diffuse and point sources, remains a pressing environmental problem worldwide [1,2]. Nature-based solutions (NBSs) are increasingly being implemented to mitigate the impacts of anthropogenic activities on water quality, as they meet several priorities of national and international policy strategies (e.g., biodiversity conservation, human well-being) and may generate multiple benefits for nature and society [3,4]. Most of the NBSs aimed at tackling nutrient pollution in freshwater ecosystems (e.g., constructed wetlands, buffer strips, vegetated ditches), and, in particular, the mitigation of reactive nitrogen (N) excess, are based on the principle of phytoremediation, i.e., the use of macrophytes and their associated microbial communities to degrade, and/or uptake and store waterborne pollutants [5–7]. The primary application of phytoremediation is the building of constructed wetlands (CWs), which have been proposed as an environmentally sound tool

for N removal from agricultural drainage water since the early 1990s [8,9], and have recently received renewed attention due to their low energy requirements and low implementation and operational costs [10,11]. CWs are engineered systems designed and constructed to mimic the chemical, physical, and biological processes, and ecological functions occurring in natural wetlands, where pollutant removal is attributed to the cooperation between macrophytes and the bacterial community [6,12,13].

Macrophytes promote the biogeochemical transformation and processing of reactive N, and it is generally accepted that N mitigation capacity is primarily due to microbial removal rather than plant uptake [14–16]. Denitrification is the stepwise bacterial reduction of nitrate ($NO_3^-$) to dinitrogen gas ($N_2$). This process is of particular significance in aquatic ecosystems, where it plays a pivotal role in the permanent removal of $NO_3^-$, thereby ensuring the maintenance of good water quality [17,18]. This anaerobic respiration requires $NO_3^-$ and organic carbon as electron acceptors and electron donors, respectively, and takes place in sediments and biofilms on macrophyte surfaces under hypoxic–anoxic conditions [19,20]. Plant morphology determines the availability of surfaces for biofilm colonization, and physiological characteristics shaping oxygen release capacity or lability of organic matter (i.e., root exudates and decaying litter) can differentially affect the abundance and activity of N-cycling microbial communities, including denitrifying bacteria [21–23]. Such plant characteristics vary between different macrophytes, resulting in a species-specific effect on denitrification and ultimately on the effectiveness of NBSs relying on phytoremediation. A large body of mostly descriptive literature has demonstrated differences between plant species in nutrient removal and community structure of rhizosphere N-cycling bacteria [24–26], but there is a gap in understanding the causal mechanisms of macrophyte-mediated enhancement of denitrification. Furthermore, most studies on N removal in CWs (e.g., [14,27,28]) are based on a black-box approach that monitors the disappearance of the process substrate (i.e., the difference between $NO_3^-$ mass inflow and outflow), while fewer studies have measured $N_2$ production simultaneously (e.g., [29]).

The aim of the present study is to compare the efficiency of $NO_3^-$ removal via denitrification of sediments colonized by two wetland plants, *Phragmites australis* (Cav.) Trin. ex Steud. (common reed) and *Typha latifolia* L. (broadleaf cattail), commonly found in eutrophic water bodies. The macrophytes were selected because they are the most frequently used plant species in experiments treating different effluents at different levels (i.e., laboratory, pilot, or large scale) and for artificial vegetation coverage of CWs worldwide. Their ability to remove N is documented by several examples of successful application in CWs in the Mediterranean area and throughout Europe, often planted in single or mixed stands [5,30,31]. The $NO_3^-$ loading rate is a crucial operational parameter that affects the effectiveness of NBSs. Surprisingly, no attempt has yet been made to compare the denitrification performance of the two plants along a $NO_3^-$ concentration gradient by simultaneously measuring $NO_3^-$ consumption and $N_2$ production. This is a necessary condition to verify the hypothesis that the removed $NO_3^-$ is actually denitrified and permanently lost from the system. In the present study, benthic fluxes of $NO_3^-$ and $N_2$ were measured in open-air vegetated and unvegetated mesocosms incubated in summer, when the eutrophication risk is potentially higher. The tested $NO_3^-$ gradient (50−800 μM) reproduced the general availability found in lowland drainage waters receiving diffuse (agricultural runoff) and point source (wastewater treatment plant discharges) pollution, both in the study basin and in other human-impacted watersheds [2,15]. Under climate change scenarios, urban and agricultural runoff may be further exacerbated by intense rainfall events [32], and large pulses of bioavailable nutrients may be leached from soils and delivered to terminal water bodies, providing positive feedback for eutrophication.

## 2. Materials and Methods

### *2.1. Experimental Design: Sampling and Mesocosm Setup*

An outdoor experiment was carried out in an unshaded area at the University of Ferrara (Department of Environmental and Prevention Sciences) and involved nine experimental units, including three replicate mesocosms per plant species (*P. australis* and *T. latifolia*) and three unvegetated mesocosms serving as controls. In late May 2024, sediment, plants, and water were sampled from a drainage canal of the Po River lowland (North-eastern Italy, Emilia-Romagna Region; N 44.758841, E 11.803864) where the two macrophytes coexisted. Sediment samples were collected from the rhizosphere of monospecific stands of reed and cattail and in adjacent bare patches. Efforts have been made to exclude live plant material, large debris, and visible organisms (e.g., snails, macrofauna) from the mesocosms. Root fragments were carefully removed by hand from sediments used in bare mesocosms. Plants were sampled with a steel shovel, avoiding damage to the rhizomes to ensure new growth during the acclimation period. Plants similar in size and stem diameter and with a visually healthy rhizosphere were selected to limit the variability of macrophyte-related measurements. Laboratory-scale monospecific mesocosms simulating surface-flow wetlands (or slow-flowing lowland drainage canals) were constructed in flat-bottomed Plexiglas tubs (Ø 29 cm) filled with a 30 cm layer of muddy sediment with an average organic matter content of 6% as loss on ignition (Figure 1). A second Plexiglas liner (Ø 12 cm) was placed in the center of each mesocosm to create an annular sediment surface of 547 $cm^2$ onto which plant specimens were transplanted.

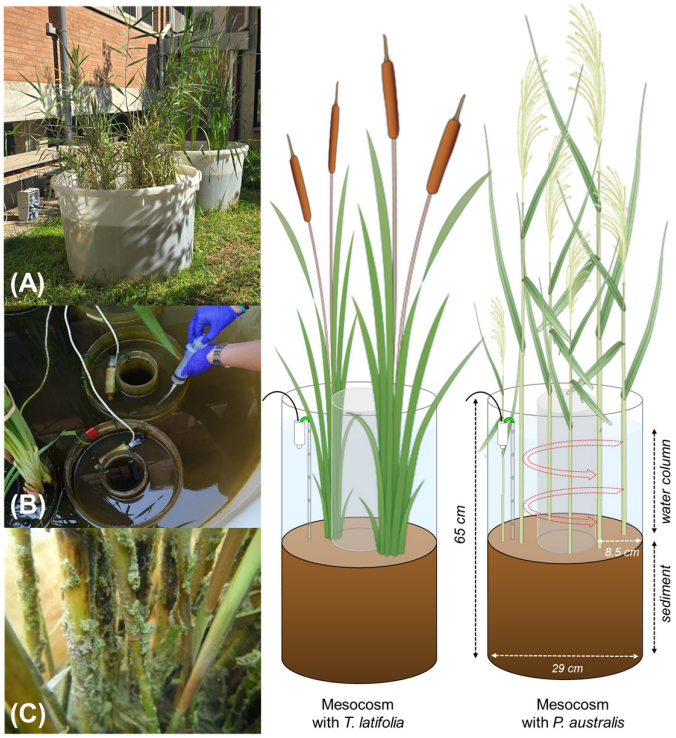


**Figure 1.** Scheme of the vegetated mesocosms (on the **right**) and some pictures of the experimental setup (on the **left**). (**A**) Tanks containing the mesocosms submerged by canal water, (**B**) water sampling procedure from a mesocosm during incubation, (**C**) detail of a biofilm colonizing the submerged stems of *P. australis*.

The stem density reproduced in the mesocosms (~60 plants $m^{-2}$ for *P. australis* and ~25 plants $m^{-2}$ for *T. latifolia*) approximated that which was measured in mid-summer in shallow agricultural drainage canals of the Po River plain [15]. Aboveground biomass (1800−2600 g of DM $m^{-2}$) overlapped with mean field values reported in the literature for

wetlands and canals with reed and cattail stands [30]. Prior to the start of the experiment, the mesocosms were acclimated for approximately two months. This period was considered to be sufficient for the plants to overcome the transplantation stress and for biofilms and bacterial communities to recover [33]. During the acclimation period, the mesocosms were regularly checked for the development of light brown halos surrounding the roots along their entire length against a darker sediment background. This was indicative of an active oxygen release mechanism to detoxify the anoxic sediment and recover from stress. The experimental units were maintained in natural weather conditions and fully submerged in tanks containing canal water, continuously recirculated, and aerated by aquarium pumps in order to keep the oxygen level close to saturation (Figure 1). The water level in the tanks was monitored daily and, if necessary, new canal water was added to compensate for evaporation losses. Each mesocosm was wrapped in aluminum foil to prevent exposure of the rhizosphere to ambient light and to keep the sediments in the dark.

### 2.2. Nitrate Enrichment Experiment

The enrichment experiment consisted of six incubations carried out over six consecutive days in mid-summer 2024 (end of July) under stable weather conditions. Water temperature was stabilized by a thermostat at 26 °C, the average mid-summer value usually recorded in shallow aquatic ecosystems of the Po River lowland [15,34]. Throughout the incubation time, a 12 V submersible electric pump (Whale®, Bangor, UK), connected to a multi-channel rheostat with voltage regulation provided a constant water flow (~3 cm $s^{-1}$) around the central Plexiglas tub of each mesocosm. This mesocosm configuration has previously been used in laboratory experiments to assess the effect of water velocity on benthic N fluxes and gas exchanges at the air–water interface in reed stands [33,35,36].

All the mesocosms with and without plants were exposed to six different levels of $NO_3^-$ in the water column (50, 100, 200, 300, 500, 800 μM) over six consecutive days and incubated according to the procedure described below. Before each incubation, approximately one-third of the water volume in the tank was removed and replenished with new water from the canal to maintain the chemical–physical parameters of the incubation medium as constant as possible, with the sole exception of $NO_3^-$ concentration. Indeed, the mesocosms were spiked with $NO_3^-$, each day a different concentration, from the lower (50 μM) to the higher (800 μM), by adding appropriate amounts of a 200 mM stock solution ($KNO_3$; Sigma-Aldrich, St. Louis, MO, USA) to increase the ambient concentration. Each target concentration was applied to the tank water in the late afternoon of the day before incubation to allow the mesocosms to equilibrate for at least 15 h. Several studies, both in the field and in pure cultures, have provided multiple lines of evidence that $NO_3^-$ availability acts primarily as a proximal control on denitrification rates, but has little effect on growth rates and abundance of denitrifiers, which are mostly controlled by long-term sediment conditions (e.g., organic carbon availability, water saturation) and competition among microbial communities [37]. Denitrifiers are ubiquitous, but the use of $NO_3^-$ as an electron acceptor remains energetically unfavorable compared to aerobic respiration [38]. The mesocosms used in the present study, although acclimatized to increasing $NO_3^-$ concentrations, were always kept fully submerged by continuously mixed and aerated canal water. It is, therefore, unlikely that the growth of denitrifiers between successive incubations has been stimulated by the $NO_3^-$ enrichment.

A batch-mode incubation approach was used, following a standardized procedure [39], with initial and final samples taken from the water phase of each open-top mesocosm [33,36]. Incubations were performed under conditions of constant temperature (26 °C) and natural light (800 W $m^{-2}$). The incubation time of 1.5 h was set as the minimum time required to detect significant deltas in water column solute concentrations while avoiding relevant

deviations from the starting conditions, i.e., keeping the variations in solute concentrations within 20–30% of the initial values [39]. The linearity of the concentration changes over time was verified in a series of preliminary tests, and the short incubation time chosen guaranteed the constancy of the N fluxes under investigation throughout the exposure at different $NO_3^-$ availability.

Before starting the incubation, the water in the tank was checked for $NO_3^-$ and volumes of $NO_3^-$ stock solution were added to yield the target $NO_3^-$ level. The water level in the tank was then lowered below the top of the mesocosms and each experimental unit was isolated from the others and the surrounding water. At the beginning and at the end of each incubation, water temperature and conductivity were measured with a multiparametric probe (YSI Model 85-Handheld Oxygen, Conductivity, Salinity, and Temperature System, YSI Incorporated, Yellow Springs, OH, USA), and water samples were collected from the water phase of each mesocosm using a 100 mL gas-tight glass syringe with an attached sampling tube. For dissolved $N_2$:Ar determination, an aliquot was transferred to 12 mL gas-tight glass vials (Exetainer®, Labco, High Wycombe, UK), filled from the bottom by overflowing the vial volume at least three times without headspace, and fixed with 100 µL of a saturated $ZnCl_2$ solution to stop microbial activity. As denitrification is of particular concern as a major source of nitrous oxide ($N_2O$), an important greenhouse gas, additional water aliquots for $N_2O$ measurements were sampled in a similar way to $N_2$. The remaining water volume sampled from each mesocosm was filtered (glass-fiber filters type GF/F, Whatman, Maidstone, UK) and transferred to 20 mL polyethylene vials for the determination of dissolved inorganic N species, ammonium ($NH_4^+$), nitrite ($NO_2^-$), and $NO_3^-$, by standard spectrophotometric techniques.

### *2.3. Analytical Methods*

Analyses of dissolved $N_2$:Ar were performed by Membrane Inlet Mass Spectrometry (MIMS) [40]. The instrument was equipped with a Bay Instruments S-50-65 membrane inlet (Easton, MD, USA) and a PrismaPlus® mass spectrometer (Pfeiffer Vacuum GmbH, Aßlar, Germany). A copper reduction column in an in-line furnace operating at 600 °C was used to remove $O_2$ and avoid the production of nitric oxide within the MIMS ion source, which can interfere with $N_2$:Ar determinations. $N_2$ was obtained by multiplying the measured $N_2$:Ar ratios by the saturated Ar at the sampling temperature and conductivity, derived from theoretical gas solubility tables [41]. Following the headspace equilibration technique, $N_2O$ was determined using a gas chromatograph (Trace GC, 2000 Series, Thermo Fisher Scientific, Waltham, MA, USA) equipped with a PoraPLOT Q column (length 32 m, inner Ø 0.32 mm) and an electron capture detector operated at 350 °C. There was no evidence of $N_2O$ production, as previously demonstrated by in situ measurements in the same canal network [33]. $NO_3^-$ was determined by using the cadmium reduction method on a Technicon Autoanalyzer [42]. $NO_2^-$ was measured using sulphanilamide and N-(1-naphthyl)ethylenediamine [43]. $NH_4^+$ was analyzed using salicylate and hypochlorite in the presence of sodium nitroprussiate [44]. For all incubations and treatments, $NH_4^+$ concentrations were always below the detection limit.

At the end of the incubations, the diameter of each stem in each mesocosm was measured with a caliber and used, together with the height of the water column, to calculate the total submerged plant surface potentially available for biofilm colonization. Aboveground plant biomass was then harvested from each mesocosm and oven-dried at 60 °C for 36 h. Plant N uptake was estimated from the measured biomass and average values of tissue N content (1.45%) and growth rates (3 mg $g^{-1}$ DM $d^{-1}$), previously measured in many agricultural canals colonized by monospecific stands of *P. australis* and *T. latifolia* belonging to the Po River lowland [15,33].

### 2.4. Calculation of Benthic Fluxes ($NO_3^-$, $NO_2^-$, and $N_2$)

Benthic fluxes of $NO_3^-$ and $NO_2^-$ (μmol N $m^{-2}$ $h^{-1}$) were determined according to the equation below [39]:

$$\text{Flux (X)} = \frac{([X]_f - [X]_i) \cdot V}{A \cdot t} \quad (1)$$

where $[X]_f$ and $[X]_i$ are the concentrations (μM) of the solute X at the end and at the beginning of the incubation, respectively; V (L) is the water volume inside each mesocosm, A ($m^2$) is the bioactive surface of each mesocosm (i.e., sediment surface or surface area available for biofilm colonization), and t (hours) is the incubation time. For vegetated treatments, benthic fluxes were calculated based on the sediment area (0.05 $m^2$) or the surface area potentially available for biofilm colonization in each mesocosm (i.e., submerged stems of reed or cattail). Positive fluxes indicate net solute production (release from the sediment to the water column), while negative fluxes indicate net consumption (removal from the water column). Net N removal was calculated by summing up the $NO_3^-$ and $NO_2^-$ fluxes.

In the present study, the terms "denitrification", "$N_2$ production", and "$N_2$ flux" have been used interchangeably. Denitrification is generally the dominant process responsible for the conversion of reactive N to $N_2$ in eutrophic freshwater environments, including CWs, with a negligible contribution of anammox, i.e., the anaerobic ammonium oxidation [45,46]. Preliminary anoxic slurry incubations performed with the sediment used for the mesocosm setup confirmed that anammox contributed a negligible amount (<0.2%) to total $N_2$ production.

Standard procedures for measuring and calculating benthic fluxes of $N_2$, commonly used for bare sediments [39] or in the presence of primary producers such as submerged macrophytes or microphytobenthos, are not applicable in sediments with emergent plants such as *P. australis* and *T. latifolia*. Indeed, mesocosms cannot be closed at the top; thus, the estimation of air–water gas exchange is required to correct for changes in water $N_2$ concentrations measured over the incubation time. Fluxes of $N_2$ were quantified according to the method proposed by Castaldelli and co-authors [33], which was previously applied to sediments colonized by *P. australis* [47]. The approach is an adaptation to open-top mesocosm incubations of the model developed by Laursen and Seitzinger [48] for estimating open-channel denitrification rates. The reaeration coefficient was measured by a tracer gas addition experiment, i.e., by quantifying the degassing of Ar, according to the approach developed by Soana and co-authors [35] for open-top mesocosms.

### 2.5. Statistical Analysis

To predict plant-specific benthic fluxes of N species across the tested gradient of $NO_3^-$ concentrations, data were fitted to the Michaelis–Menten equation to determine the kinetic parameters [49]:

$$R = \frac{R_{max} \cdot [NO_3^-]}{K_m + [NO_3^-]} \quad (2)$$

where R is the ambient rate, $R_{max}$ is the maximum rate, [$NO_3^-$] is the $NO_3^-$ concentration in the overlying water, $K_m$ is the affinity constant for $NO_3^-$ or half saturation constant, i.e., the $NO_3^-$ concentration at which the ambient rate is half of $R_{max}$. The analyses were performed in SigmaPlot 15.0 (Systat Software Inc., San Jose, CA, USA).

Benthic fluxes expressed in terms of sediment surface were analyzed with linear mixed-effects models, which were run with plant type (3 levels: *P. australis*, *T. latifolia*, and sediment) and $NO_3^-$ concentration (6 levels: 50, 100, 200, 300, 500, and 800 μM) as the main fixed factors, including interaction, with mesocosm as a random effect. The same analysis was used to test the effects of plant type and $NO_3^-$ concentration on benthic fluxes expressed in terms of surface area available for biofilm colonization. Model assump-

tions were checked using normality plots and residual plots [50]. Data were root-square transformed to meet the normality of residuals and homogeneity of variance. Significant differences between levels of fixed factors were identified by multiple comparisons of estimated marginal means [51]. Statistical significance was set at a $p$ level <0.05. Statistical analyses were performed with R Studio R-4.4.2 [52] using the lme function within the nlme package [53].

## 3. Results and Discussion

### 3.1. Macrophyte Selection as a Key Factor in Optimizing Water Treatment Performance

Macrophyte species selection is critical for the successful implementation of NBSs aiming to mitigate excess $NO_3^-$ in aquatic ecosystems. Although a good understanding of how plant functional traits affect N retention would lead to improvements in water treatment performance, this type of investigation is usually overlooked. Results from the present experiment demonstrated that not only the presence of plants but also the type of plant significantly influenced the benthic N fluxes when expressed in terms of sediment surface (Table 1).

**Table 1.** Effects of factors plant type (*P. australis*/*T. latifolia*/bare sediment) and $NO_3^-$ concentration on benthic N fluxes based on linear mixed-effects models. Degrees of freedom (df), F-test value (F), and significance level ($p$) are reported.

| Benthic Flux | Factor | Rate Expressed per $m^2$ of Sediment | | | Rates Expressed per $m^2$ of Surface Available for Biofilm Colonization | | |
|---|---|---|---|---|---|---|---|
| | | df | F-Value | $p$-Value | df | F-Value | $p$-Value |
| $NO_3^-$ removal | Plant type | 2 | 873.985 | <0.0001 | 1 | 0.0013 | 0.9715 |
| | $NO_3^-$ concentration | 5 | 212.769 | <0.0001 | 5 | 264.7168 | <0.0001 |
| | Plant type × $NO_3^-$ concentration | 10 | 24.347 | <0.0001 | 5 | 5.9449 | 0.0013 |
| $NO_2^-$ production | Plant type | 2 | 45.305 | <0.0001 | 1 | 36.8892 | <0.0001 |
| | $NO_3^-$ concentration | 5 | 147.246 | <0.0001 | 5 | 50.2283 | <0.0001 |
| | Plant type × $NO_3^-$ concentration | 10 | 2.831 | 0.0114 | 5 | 1.7726 | 0.1602 |
| Net N removal | Plant type | 2 | 761.83 | <0.0001 | 1 | 0.7952 | 0.3822 |
| | $NO_3^-$ concentration | 5 | 162.002 | <0.0001 | 5 | 209.451 | <0.0001 |
| | Plant type × $NO_3^-$ concentration | 10 | 21.677 | <0.0001 | 5 | 4.78 | 0.0042 |
| Denitrification | Plant type | 2 | 435.672 | <0.0001 | 1 | 2.6417 | 0.1183 |
| | $NO_3^-$ concentration | 5 | 196.673 | <0.0001 | 5 | 173.3046 | <0.0001 |
| | Plant type × $NO_3^-$ concentration | 10 | 12.117 | <0.0001 | 5 | 2.9863 | 0.0332 |

Nitrate removal, $NO_2^-$ production, net N removal, and denitrification rates were systematically higher in mesocosms with *P. australis* and *T. latifolia* compared to bare sediments; however, the effect of the two plants on N dynamics was different, with higher rates always detected in sediments colonized by *P. australis* (Figures 2 and 3). Along the gradient of $NO_3^-$ availability, $NO_3^-$ removal rates varied between $-2211 \pm 325$ and $-17{,}878 \pm 1479$ µmol N $m^{-2}$ $h^{-1}$ in *P. australis* mesocosms, between $-578 \pm 214$ and $-7647 \pm 988$ µmol N $m^{-2}$ $h^{-1}$ in *T. latifolia* mesocosms, and between $-416 \pm 198$ and $-1829 \pm 311$ µmol N $m^{-2}$ $h^{-1}$ in bare sediments (Figure 2). The presence of *P. australis* and *T. latifolia* boosted denitrification by a factor of ~5 and of ~2, respectively, compared to unvegetated sediment. The production of $N_2$ varied between $1818 \pm 752$ and $17{,}903 \pm 2243$ µmol N $m^{-2}$ $h^{-1}$ in *P. australis* mesocosms, between $505 \pm 25$ and $7824 \pm 1537$ µmol N $m^{-2}$ $h^{-1}$ in *T. latifolia* mesocosms and between $312 \pm 69$ and $3379 \pm 675$ µmol N $m^{-2}$ $h^{-1}$ in bare sediments (Figure 3). For each $NO_3^-$ level, the range

of $N_2$ production rates largely overlapped with the corresponding range of net N removal rates for both macrophytes.

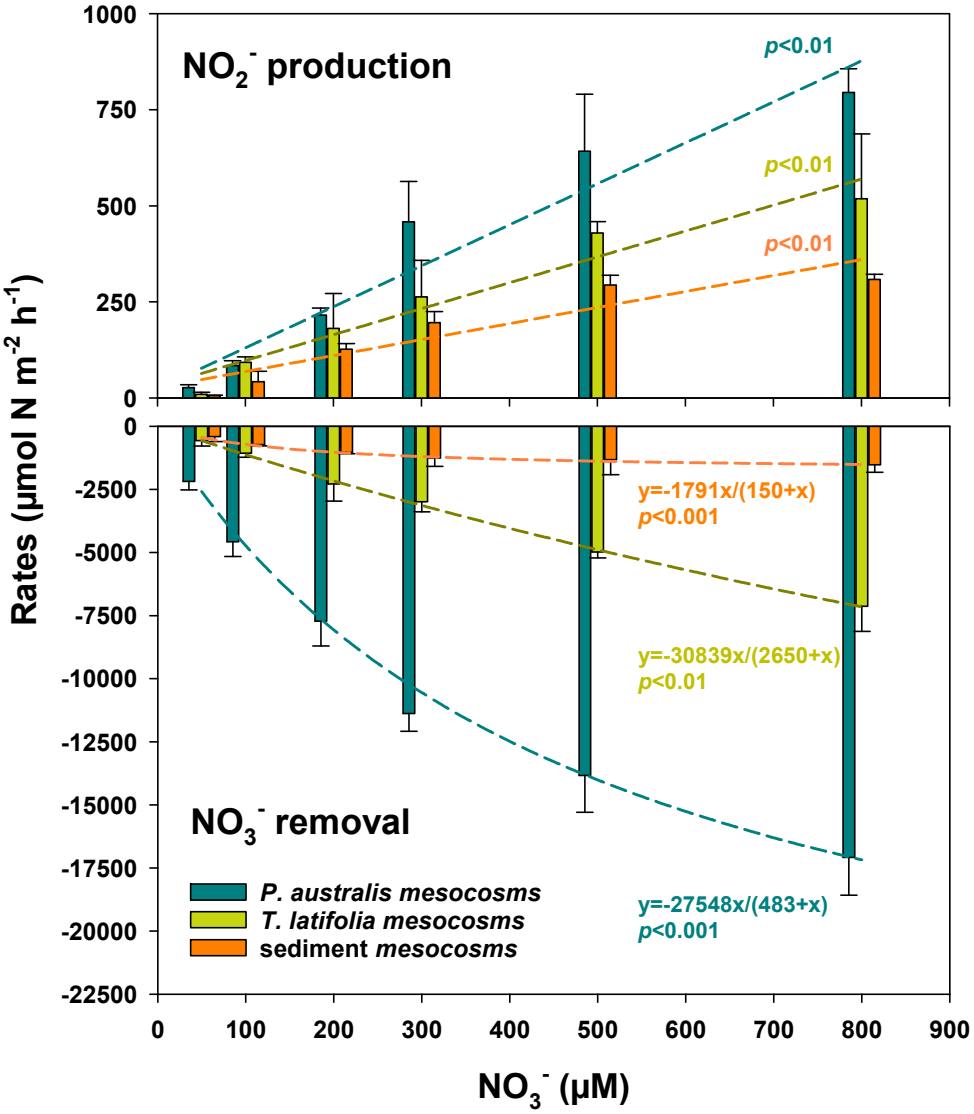


**Figure 2.** Rates of $NO_3^-$ removal and of $NO_2^-$ production measured in vegetated and bare mesocosms, as a function of $NO_3^-$ concentration. Note the different scales on the y-axis for the two panels. Rates are expressed per $m^2$ of sediment in the mesocosm (average ± standard deviation, $n$ = 3).

The correlation between denitrification and net N removal was in fact highly significant (Figure 4), and the slope represented the denitrification efficiency, i.e., the fraction of consumed N converted to $N_2$ by anaerobic $NO_3^-$ respiration. The one-to-one relation between net N removal and $N_2$ production in vegetated conditions proved that denitrification was the quantitative sink for $NO_3^-$, with an overall removal efficiency close to 100%. In support of this statement, the estimated N removal via assimilatory uptake averaged ~300 µmol N $m^{-2}$ $h^{-1}$ and accounted for <2–13% of the $NO_3^-$ consumption measured along the $NO_3^-$ gradient. These outcomes confirmed previous studies reporting that plant uptake was a small fraction of the total N removal [15,16,54] and suggested that emergent macrophytes play an important indirect role in supporting quantitatively relevant microbial processes responsible for N dissipation.

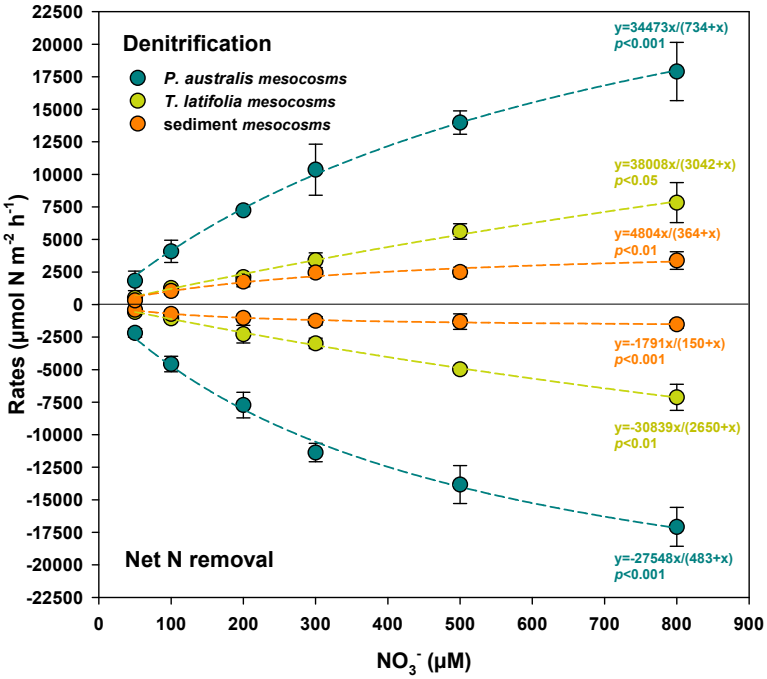


**Figure 3.** Rates of net N removal and denitrification measured in vegetated and bare mesocosms, as a function of $NO_3^-$ concentration. Rates are expressed per $m^2$ of sediment in the mesocosm (average ± standard deviation, $n$ = 3).

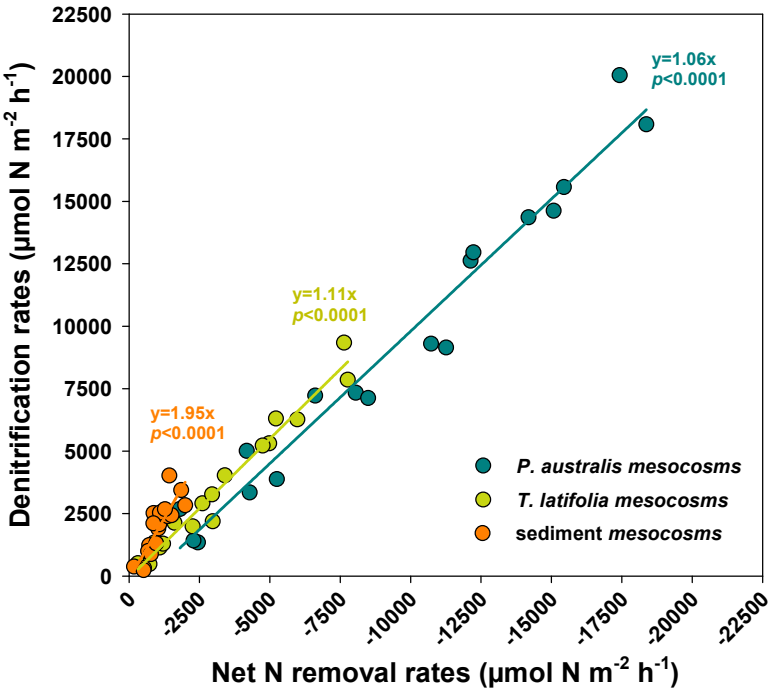


**Figure 4.** Correlation between net N removal and denitrification rates in vegetated and bare mesocosms. Rates are expressed per $m^2$ of sediment in the mesocosm.

Nitrification, i.e., the oxidation of $NH_4^+$ to $NO_3^-$, likely contributed to fuel denitrification in unvegetated sediments, supporting the quantitative discrepancy between $N_2$ production and net N removal rates, the former always being greater than the latter (Figure 4). Although not directly measured in the present study, photosynthetic oxygen production by microphytobenthos may have increased oxygen penetration into the sediment by expanding the oxic layer, thereby stimulating N removal via coupled nitrification–denitrification [55]. This effect was not observed in vegetated sediments possibly due

to competition for $NH_4^+$ between the nitrifying bacteria and the algal component of the biofilm having a higher growth rate and N uptake [56].

Denitrification capacity is known to vary across wetland plant species. Two main plant-mediated mechanisms have been shown to be implicated in increasing the N removal capacity of vegetated aquatic ecosystems, namely the release of oxygen and carbon in the rhizosphere [21–24], and the provision of surfaces for microbial attachment [20,22,36]. Due to distinctive morphological and physiological characteristics, it is likely that some species are more effective than others in mitigating $NO_3^-$ pollution. In the present experiment, all planted mesocosms performed better than bare mesocosms in converting $NO_3^-$ via the microbial-mediated denitrification pathway, confirming the positive influence of vegetation on net N removal, but *P. australis* showed the highest efficiency, which points toward a plant species dependence of the measured activity. Some studies have previously observed higher rates of N removal associated with *P. australis*-colonized sediments compared to *T. latifolia* [7,24,54]. Differential carbon availability and anoxic conditions at the sediment–water interface have been suggested to be responsible for the differences in performance, although the underlying mechanisms are not fully explored. Root exudates and litter from various plant species provide different amounts and qualities of organic carbon to act as electron donors for heterotrophic denitrifying bacteria and may ultimately determine the efficiency of denitrification in CWs [23,57]. Organic acids and sugars, more important components of *P. australis* root exudates than *T. latifolia*, were found to positively influence the abundance of denitrifying genes, which is likely to be related to the potential denitrification activity [13,58]. In addition, many more microbial species involved in the N cycling were observed in reed than in cattail rhizosphere [59], and the C:N ratio of *P. australis* detritus has been shown to shape the composition of the microbial community in a way that optimizes the removal of dissolved N [54].

### *3.2. Denitrification Capacity Along the $NO_3^-$ Gradient*

The present experiment provides the first description of the functional relationship between water $NO_3^-$ availability and denitrification rates in sediments colonized by *P. australis* and *T. latifolia*, by far the most common plants used in phytoremediation applications treating various effluents (e.g., agricultural runoff, industrial wastewater, landfill leachate, sewage, and mine drainage) [5,30,31]. Although there is great potential for macrophyte-based remediation of $NO_3^-$ pollution in CWs, published studies are generally limited by focusing on the efficiency of each individual plant species exposed to different $NO_3^-$ availability [28,47] or by comparing the two genera at fixed concentrations [14,25,26,60]. In the present experiment, $NO_3^-$ consumption differed significantly among all concentration levels (Figure 2; Table 1) and, passing from 50 µM to 800 µM, the rates were increased by a factor of 8, 13, and 4 in the presence of *P. australis*, in the presence of *T. latifolia*, and for unvegetated condition, respectively. Denitrification rates varied significantly with the level of $NO_3^-$ enrichment (Figure 3; Table 1), and an increase of more than 10-fold was measured, passing from 50 µM to 800 µM. Water $NO_3^-$ availability had a significant positive effect on denitrification rates in all conditions tested, but the stimulation was significantly higher in vegetated sediments likely due to the positive influence, in particular of *P. australis*, on the microbial communities. Higher $NO_3^-$ concentrations were the trigger for higher denitrification, suggesting that the process was limited by substrate availability and that the resident denitrifying community responded rapidly to the enrichment by adjusting its level of activity [17,37].

$NO_2^-$ production was detected in all tested conditions, with rates varying significantly between plant type and $NO_3^-$ enrichment level (Table 1). As $NO_3^-$ availability increased, $NO_2^-$ production rates followed a linear trend, ranging from 27 ± 7 to 795 ± 62, from

10 ± 5 to 519 ± 168, and from 6 ± 1 to 309 ± 13 μmol N $m^{-2}$ $h^{-1}$ in *P. australis*, *T. latifolia*, and bare sediment mesocosms, respectively (Figure 2). $NO_2^-$ production was increased by a factor of ~30 in *P. australis* mesocosms and of ~60 in *T. latifolia* and bare sediment mesocosms along the $NO_3^-$ gradient but was generally found to be a minor benthic flux, representing on average 1–7% and 1–17% of the corresponding $NO_3^-$ removal in vegetated and unvegetated conditions, respectively. The reduction of $NO_2^-$ to nitric oxide is the rate-limiting step in the denitrification process, and $NO_2^-$ can accumulate due to the lack of readily available organic substrates, especially at the highest $NO_3^-$ enrichments in unvegetated sediments [37,38].

$NO_3^-$ availability increased both net N removal and denitrification rates by approximately one order of magnitude for both macrophytes and in bare sediment conditions. A Michaelis–Menten curve was always observed, although $R_{max}$ was not reached along the investigated $NO_3^-$ gradient (Table 2; Figure 3). Rates of net N removal and $N_2$ production in *P. australis* mesocosms responded similarly along the $NO_3^-$ gradient, with $R_{max}$ up to about ~34,000 μmol N $m^{-2}$ $h^{-1}$ and a $K_m$ > 480 μM. The saturation trend observed in the *T. latifolia* mesocosms peaked at a similar $R_{max}$ (~38,000 μmol N $m^{-2}$ $h^{-1}$) but with a significantly higher $K_m$ (>2600 μM) than that detected in sediment colonized by *P. australis* (Table 2). These outcomes indicated that the denitrifying community associated with *P. australis* showed a higher affinity for $NO_3^-$ compared to *T. latifolia* [61]. By influencing the denitrification drivers (e.g., redox status, organic carbon availability) in multiple ways, different macrophyte species can select specific denitrifying communities in the rhizosphere and biofilms that respond in different ways to the variable $NO_3^-$ availability. Previous studies have observed species-specific differences in microbial community structure and carbon availability associated with the presence of *P. australis* and *T. latifolia* [21,54,57], and these are reasonably reflected in the $K_m$ values, which range by an order of magnitude between the two plants. The specific ability of reeds to support higher denitrification capacity has been documented and attributed to the physical and chemical properties of plant litter and root exudates that are more attractive to heterotrophic denitrifying bacteria, stimulating their growth [54,59].

**Table 2.** Michaelis–Menten model parameters, i.e., $R_{max}$ (maximum rate of net N removal or denitrification) and $K_m$ ($NO_3^-$ concentration in water at which the rate is half of $R_{max}$) (average ± standard error) in vegetated and bare mesocosms. Rates are expressed per $m^2$ of sediment in the mesocosm *** <0.001; ** <0.01; * <0.05.

| Treatment | Rate | $R^2$ | $R_{max}$ | $K_m$ |
|---|---|---|---|---|
| | | | μmol N $m^{-2}$ $h^{-1}$ | μM |
| *P. australis* mesocosms | Net N removal | 0.99 | −27,548 ± 2035 *** | 483 ± 69 *** |
| | Denitrification | 0.99 | 34,473 ± 1826 *** | 734 ± 66 *** |
| *T. latifolia* mesocosms | Net N removal | 0.99 | −30,839 ± 5079 ** | 2650 ± 535 ** |
| | Denitrification | 0.99 | 38,008 ± 11,064 * | 3042 ± 1062 * |
| Sediment mesocosms | Net N removal | 0.99 | 1791 ± 68 *** | 150 ± 17 ** |
| | Denitrification | 0.96 | 4804 ± 742 ** | 364 ± 121 * |

Bare sediments also showed a significant Michaelis–Menten model fit, with $R_{max}$ significantly lower than those obtained for the vegetated sediments for both net N removal (~1800 μmol N $m^{-2}$ $h^{-1}$) and denitrification rates (~4800 μmol N $m^{-2}$ $h^{-1}$) (Table 2; Figure 3). The rates measured at the highest $NO_3^-$ level accounted for 52–62%, 21–23%, and 70–85% of the $R_{max}$ for *P. australis*, *T. latifolia*, and bare sediment mesocosms, respectively, showing that saturation was far from being reached, especially in vegetated conditions along the tested $NO_3^-$ enrichment. The present outcome may reflect a non-limiting con-

dition of labile organic matter, provided by root exudates or leached from decaying plant material [54,57]. Although the role of emergent macrophytes as key components influencing the removal performance of aquatic ecosystems against $NO_3^-$ is widely recognized, the comparison of rates obtained here is limited to a few previous studies that have tested the denitrification potential of vegetated sediments along a $NO_3^-$ gradient under laboratory-controlled conditions. The $R_{max}$ values measured in vegetated mesocosms (up to ~38,000 μmol N $m^{-2}$ $h^{-1}$) were one order of magnitude higher than the range of values reported for a variety of emergent plants incubated at similar water temperature conditions (1600–3100 μmol N $m^{-2}$ $h^{-1}$) [29,61,62].

### *3.3. Biofilms as Hotspots of $NO_3^-$ Removal via Denitrification*

Aquatic plants create a unique microenvironment for the activity of N-cycling bacteria by offering surfaces for biofilm growth (i.e., submerged stems and leaves) [63,64], acting as biogeochemical hotspots and, in particular, as elective sites for denitrification, with rates comparable to or even higher than in sediments [36,65,66]. Biofilms are ensembles of autotrophs and heterotrophs whose synergistic metabolism leads to the establishment of oxic and anoxic micro-niches in close proximity, allowing the co-occurrence of contrasting redox reactions on a small spatial scale, e.g., mineralization coupled with nitrification and denitrification [20,67]. Although the present data do not conclusively explain why species-specific differences in denitrification capacity occurred, a reasonable hypothesis is that the two plants provided different surfaces available for biofilm colonization and, thus, for the potential attachment of denitrifying bacteria. This is highlighted by expressing the denitrification rates in terms of the surface available for biofilm colonization. Indeed, $NO_3^-$ availability had a significant effect on net N removal and denitrification rates even when reported per $m^2$ of surface available for biofilm attachment, whereas plant type was not significant (Table 1; Figure 5). Net N removal rates varied between $-253 \pm 26$ and $-2516 \pm 196$ μmol N $m^{-2}$ $h^{-1}$ in *P. australis* biofilms and between $-197 \pm 61$ and $-1991 \pm 268$ μmol N $m^{-2}$ $h^{-1}$ in *T. latifolia* biofilms (Figure 5). Similarly, denitrification rates ranged between $215 \pm 102$ and $2767 \pm 506$ and between $180 \pm 28$ and $2082 \pm 278$ μmol N $m^{-2}$ $h^{-1}$, for biofilms colonizing *P. australis* and *T. latifolia*, respectively (Figure 5).

Very few studies have provided quantitative estimates of the denitrification capacity of periphyton on emergent plants [65,68,69]; although epiphytic biofilms are usually implicated in most organic matter processing and biogeochemical cycles, they are thus responsible for the nutrient removal performance of CWs. Denitrification activity in biofilms can vary considerably and the range of previously reported rates, recorded for a variety of macrophytes, both submerged and emergent, spans three orders of magnitude, i.e., from 2 to 1860 μmol N $m^{-2}$ $h^{-1}$. The highest denitrification capacity was associated with emergent macrophytes, represented almost exclusively by *P. australis*, although an exhaustive comparison was not possible as the considered studies employed different methods to measure the process. The rates obtained for the two plants studied here were at the upper end of the range of values previously recorded, although in most cases measured at lower temperatures than in the present experiment (Table 3). However, comparisons with values reported in the literature must be treated with caution, as they are often obtained using acetylene inhibition-based techniques, which have been criticized for underestimating the process. Furthermore, in several cases, only potential denitrification rates were determined under unlimited or optimal conditions of $NO_3^-$ and organic carbon supply and, therefore, did not reflect in situ rates.

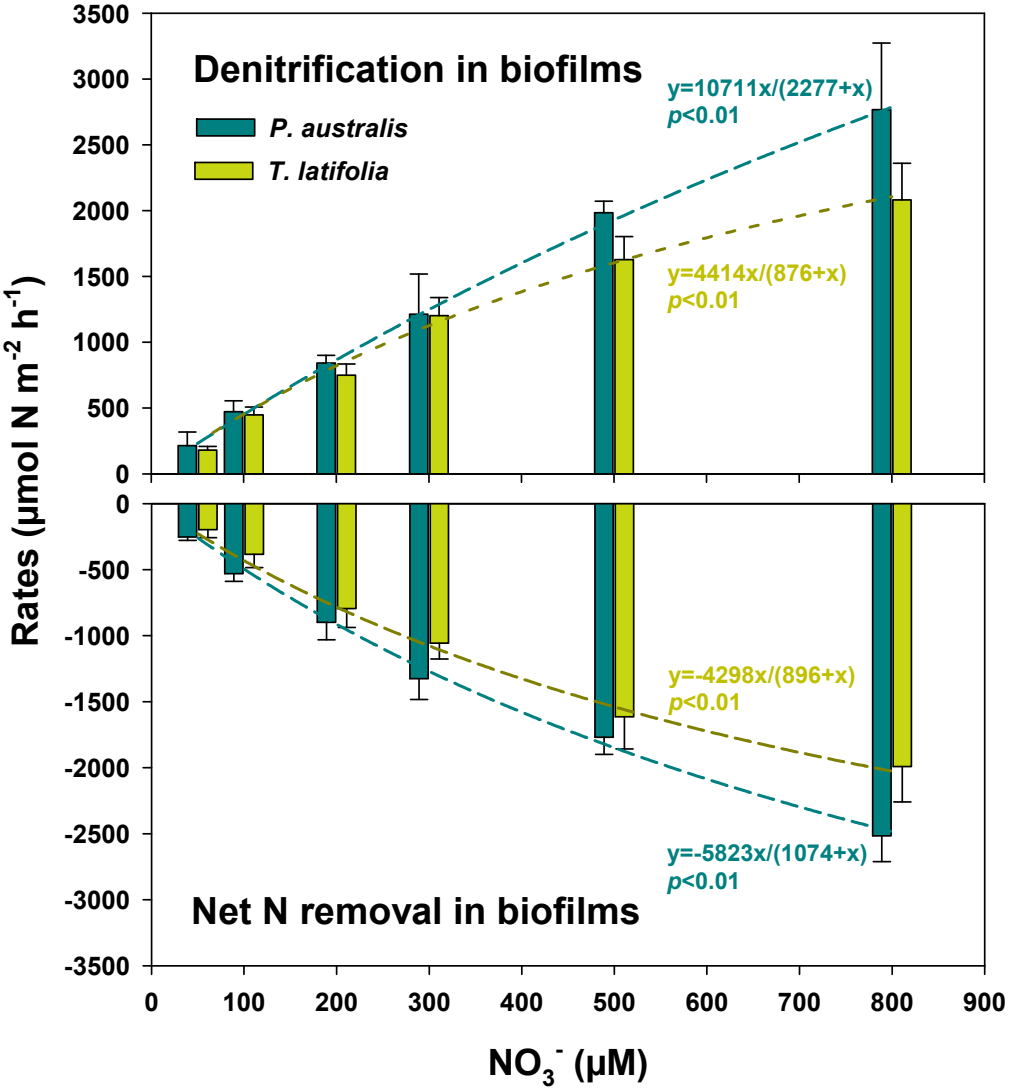


**Figure 5.** Rates of net N removal and denitrification in biofilms colonizing submerged stems of *P. australis* and *T. latifolia*, as a function of $NO_3^-$ concentration (average ± standard deviation, $n = 3$). Rates are expressed per $m^2$ of surface available for biofilm colonization.

**Table 3.** Literature review of denitrification rates measured in macrophyte biofilms. Rates are expressed per $m^2$ of the colonized surface.

| Macrophyte Species | T (°C) | $NO_3^-$ Concentration (µM) | Denitrification Rate (µmol N $m^{-2}$ $h^{-1}$) | Method | Reference |
|---|---|---|---|---|---|
| *Potamogeton pectinatus* | 18 | 295 | 15–500 | Acetylene inhibition technique | Eriksson and Weisner, 1997 [70] |
| *Potamogeton pectinatus* | 20 | 715 | 3–15 | Isotope pairing technique | Eriksson, 2001 [71] |
| *Myriophyllum spicatum* | 20 | 1000 | 300–450 | Acetylene inhibition technique | Bastviken et al., 2003 [72] |
| *Phragmites australis* | 14–18 | 70 | 100–350 | Acetylene inhibition technique | Toet et al., 2003 [65] |
| *Elodea nuttallii* | 14–18 | 60 | 50–70 | Acetylene inhibition technique | Toet et al., 2003 [65] |
| *Phragmites australis* | 16 | 200 | 5–150 | Acetylene inhibition technique | Venterink et al., 2003 [68] |

**Table 3.** *Cont.*

| Macrophyte Species | T (°C) | $NO_3^-$ Concentration (μM) | Denitrification Rate (μmol N $m^{-2}$ $h^{-1}$) | Method | Reference |
|---|---|---|---|---|---|
| *Potamogeton* spp., *Cladophora* spp. | 14−28 | 50 | 2−200 | Acetylene inhibition technique | Schaller et al., 2004 [73] |
| *Phragmites australis* | 20 | 1000 | 100–480 | Acetylene inhibition technique | Yamamoto et al., 2005 [69] |
| Emergent macrophytes (stand of *Phragmites australis* and other species) | 20 | 35−1200 | 980−1860 | Acetylene inhibition technique | Bourgues and Hart, 2007 [66] |
| *Actinoscirpus grossus*, *Nymphae* spp. | 23−28 | <15 | 135−250 | Isotope pairing technique | Adame et al., 2021 [74] |
| *Oryza sativa* | 16 | 1100 | 25−325 | Net $N_2$ flux measurement | Abulaiti et al., 2023 [75] |
| *Vallisneria natans*, *Hydrilla verticillata* | 24 | 50−80 | 5−170 | Isotope pairing technique | Deng et al., 2024 [64] |

These results suggest that *P. australis* plays a more beneficial role than *T. latifolia* due to its greater submerged surface area, which facilitates enhanced opportunities for contact between $NO_3^-$ and denitrifying bacteria and an overall greater treatment performance. When NBSs are designed and implemented to improve the quality of drainage waters receiving $NO_3^-$ polluted runoff, the critical factor controlling their efficiency is mainly the availability of submerged surfaces supporting denitrification hotspots fueled by $NO_3^-$ from the water column. In other words, the plant species themselves appear to be less important than their overall ability to provide submerged sites where denitrification can take place. On the other hand, if $NH_4^+$ is the major N form in the water, then the oxidizing capacity of the rhizosphere, which supports nitrifying activity, is important and species identity may have a relevant influence on N retention. *P. australis* oxygenates the sediments more than other aquatic macrophytes by actively transporting oxygen from leaves to roots through the aerenchyma, thereby favoring the establishment of proximate oxic and anoxic micro-niches that promote the co-occurrence of contrasting redox reactions, such as nitrification coupled to denitrification [60]. This points to the preferable use of reed as the best candidate plant species for N removal, even in systems with large amounts of organic matter and $NH_4^+$ loads. Due to its cosmopolitan distribution and tolerance to high organic and nutrient loads, *P. australis* has become by far the most widely utilized plant in CWs around the world. However, the use of *P. australis* has been restricted in the United States and New Zealand, where it is considered an exotic and problematic invasive species by natural resource and wildlife agencies, often forming monocultures in invaded wetlands and completely displacing other species [60,76].

Post hoc comparisons showed that at higher $NO_3^-$ enrichment, denitrification rates tended to be higher for *P. australis* biofilms than for *T. latifolia*. As denitrification rates also reflect patterns of dissolved organic carbon availability, it is reasonable to hypothesize that not only quality but also quantity of organic matter differed, with *P. australis* producing more biomass, potentially providing additional carbon for denitrifying bacteria [54,57,59]. Although they are not exhaustive in identifying the underlying causes of the difference in denitrification capacity between *P. australis* and *T. latifolia*, the present outcomes provide a solid starting point for future investigations into how the two plants shape microbial communities. *P. australis* and *T. latifolia* differ in their specific release of organic carbon, biomass accumulation, and timing of senescence, all of which may affect microbial communities and, thus, $NO_3^-$ removal rates. Differences in the composition and abundance of microbial

communities may be an important factor contributing to the differing effectiveness for phytoremediation in reed or cattail-dominated wetlands.

The present study concentrates on denitrification rates in summer, when eutrophication phenomena are most likely to occur. The sensitivity of denitrification to $NO_3^-$ has significant implications for water quality, so a better understanding of saturation kinetics and the complex interplay between rate-limiting factors is needed to optimize the design, restoration, and management of CWs aimed at enhancing suitable conditions for denitrifiers. Seasonal temperature fluctuations can directly influence microbial activity throughout the year, so replicating the experiment at other times during the growing season may increase knowledge of the mechanisms and drivers that regulate denitrification in vegetated sediments and support optimal vegetation management practices for use as NBSs.

## 4. Conclusions

Knowledge of the rate at which different plant species reduce $NO_3^-$ concentrations is crucial for the selection and maintenance of wetland plants and, thus, the basis for the successful large-scale application of phytoremediation for sustainable water quality management. This study provides the first comparative parametrization of *P. australis* and *T. latifolia* depuration potential along a $NO_3^-$ concentration gradient, capturing the range commonly found in nutrient-rich lowland drainage waters. In summer, when the eutrophication risk is higher, the presence of macrophytes plays a key role in improving denitrification capacity. If $NO_3^-$ is the main form of N in the water, then the biogeochemical processes in the biofilms are a major determinant of the overall treatment performance of the system. Therefore, the plants that perform better are those that provide more submerged surfaces for microbial attachment. The present results tend to support this statement, in fact, the increased provision of surfaces for biofilm colonization was likely responsible for the more beneficial role of *P. australis* in stimulating the denitrification of water column $NO_3^-$.

Understanding how plants and environmental variables interact in a complex milieu to modulate denitrification is essential to inform policymakers and land managers in the optimization of NBS design with the aim of improving treatment performance. Clarifying the relationships between the drivers of N removal via denitrification (i.e., $NO_3^-$ input, organic carbon lability, hydraulics) may help to select and manage effective plant species based on specific traits. A better insight into how the interplay between $NO_3^-$, temperature, and plant growth stage influence denitrification rates across seasons is critical to evaluating the potential use of phytoremediation as an NBS to effectively control N runoff.

Further research is needed to identify the mechanisms by which macrophytes influence the availability of labile carbon from root exudates and decaying plant litter, select specific microbial communities, and ultimately regulate denitrification. Combining biogeochemical (flux measurements) and molecular (i.e., metabarcoding, functional gene analysis) approaches has the potential to further explore the structural and functional composition of the microbial community associated with different aquatic plants and, thus, the intertwined dynamics of N cycling in vegetated sediments.

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