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RESEARCH ARTICLE

Nitrogen Cycling Dynamics in Constructed Wetlands Treating Agricultural Runoff

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ABSTRACT

Constructed wetlands are widely deployed to intercept nitrate-laden agricultural runoff before it reaches receiving waterways, but their nitrogen removal performance is highly variable between sites and seasons, and the microbial process balance underlying that variability, specifically the ratio of complete denitrification to the incomplete pathway that releases the potent greenhouse gas nitrous oxide, is rarely characterised directly alongside removal efficiency. We monitored nitrogen cycling in three free-water-surface constructed wetlands receiving tile-drain agricultural runoff over a full annual cycle, measuring inlet-outlet nitrate mass balance, direct nitrous oxide flux by static chamber, sediment denitrification potential by acetylene-block assay, and microbial community composition and denitrification-gene abundance (*nirS*, *nirK*, *nosZ* clade I and II) by quantitative PCR and 16S rRNA amplicon sequencing, across four seasonal sampling campaigns. Mean annual nitrate removal efficiency was 58.4% across the three wetlands but varied more than twofold, from 41.2% to 76.8%, and removal was significantly higher in summer than winter at every site (mean 71.3% versus 39.6%, paired t-test $P < 0.001$), tracking closely with sediment temperature and denitrification potential. Nitrous oxide flux, however, did not track nitrate removal efficiency in the same direction: the wetland with the highest nitrate removal also showed the highest per-unit-nitrogen-removed nitrous oxide emission factor, associated with a significantly lower *nosZ* clade II to *nirS* gene ratio than the other two wetlands (Kruskal-Wallis $P = 0.008$), consistent with a smaller relative capacity to complete the final denitrification step reducing nitrous oxide to inert dinitrogen gas. Seasonal shifts in *nosZ* clade II abundance, rather than total denitrifier abundance, best predicted the nitrous oxide emission factor across sites and seasons (linear mixed model, $R^2 = 0.61$). These results indicate that nitrate removal efficiency and greenhouse gas performance are not simply two faces of the same denitrification process in constructed wetlands, and that the community-level balance of denitrification gene clades, not just overall microbial activity, is an important and underused design and management target for wetlands intended to deliver nitrogen removal without a disproportionate nitrous oxide trade-off.

Keywords: constructed wetland; denitrification; nitrous oxide; agricultural runoff; *nosZ*; microbial community; nitrogen removal

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1. INTRODUCTION

Nitrate loss from intensively farmed, tile-drained agricultural land is a leading driver of freshwater eutrophication and coastal hypoxia in many temperate agricultural regions, and constructed wetlands positioned to intercept tile-drain or ditch discharge before it reaches a receiving stream have become a widely recommended and increasingly implemented edge-of-field mitigation practice [1,2]. Nitrate removal in these systems occurs predominantly through microbial denitrification, the stepwise reduction of nitrate through nitrite, nitric oxide and nitrous oxide to inert dinitrogen gas, carried out by a taxonomically diverse community of facultatively anaerobic bacteria and archaea using the *nirS/nirK* and *nosZ* gene families to catalyse successive steps of the pathway [3,4].

Reported nitrate removal efficiencies for agricultural constructed wetlands vary enormously across the published literature, from well under 30% to more than 80%, and this variability has been attributed variously to hydraulic residence time, temperature, carbon availability, and wetland age and vegetation structure, but studies rarely measure the underlying microbial process balance directly alongside removal performance, relying instead on inlet-outlet mass balance alone as a black-box performance metric [5,6,7]. This matters because denitrification is not a single, uniformly beneficial process: when the final step, reduction of nitrous oxide to dinitrogen gas by nitrous oxide reductase (encoded by *nosZ*), is incomplete relative to the earlier steps, nitrous oxide itself is released to the atmosphere rather than being fully reduced, and nitrous oxide is a greenhouse gas roughly 273 times more potent than carbon dioxide over a 100-year horizon, meaning a wetland can in principle remove nitrate effectively from the water column while still generating a disproportionate climate cost through incomplete denitrification [8,9].

The genetic basis for this imbalance has become considerably clearer over the past decade with the recognition that *nosZ* exists as two phylogenetically distinct clades, clade I, found in classical denitrifiers that also carry *nirS* or *nirK*, and clade II, found more broadly across taxa including many organisms that lack the earlier denitrification genes and appear to specialise in nitrous oxide reduction as a standalone process, sometimes described as non-denitrifying nitrous oxide reducers [10,11]. Soil and sediment studies have shown that the relative abundance of *nosZ* clade II, rather than total denitrifier abundance or *nosZ* clade I alone, is often a stronger predictor of a system's capacity to act as a net nitrous oxide sink rather than source, but this framework has been applied only sparingly to constructed wetlands specifically, and rarely alongside a full seasonal nitrate mass balance at the same sites [12,13].

We set out to characterise nitrogen cycling comprehensively across three operational agricultural constructed wetlands over a full annual cycle, with three specific aims: to quantify seasonal variation in nitrate removal efficiency and its physical and microbial

correlates; to measure direct nitrous oxide flux alongside nitrate removal, to test whether these two performance dimensions track together or diverge; and to determine whether denitrification gene clade composition, and *nosZ* clade II abundance in particular, explains variation in nitrous oxide emission factor better than total denitrifier abundance or nitrate removal efficiency alone, with a view to identifying a practically useful microbial indicator for wetland design and management.

2. MATERIALS AND METHODS

Three free-water-surface constructed wetlands (designated W1, W2 and W3), each receiving tile-drain discharge from adjacent row-crop agricultural land and ranging in age from 4 to 11 years since construction, were monitored over four seasonal sampling campaigns (spring, summer, autumn, winter) spanning one full annual cycle.

2.1 Site description and hydrological monitoring

The three wetlands ranged in surface area from 0.4 to 1.1 hectares and in mean hydraulic residence time from 18 to 46 hours at typical spring flow, estimated from continuous inlet and outlet flow monitoring and a rhodamine dye tracer study conducted once at each site during the summer campaign. Inlet and outlet grab water samples were collected weekly throughout the study period and analysed for nitrate, nitrite and ammonium by standard colorimetric methods, with nitrate removal efficiency calculated from flow-weighted inlet and outlet nitrate mass loads.

2.2 Nitrous oxide flux measurement

Nitrous oxide flux was measured by static chamber at six fixed locations per wetland (three near-inlet, three near-outlet) during each of the four seasonal campaigns, with chambers deployed for 40 minutes and headspace samples taken at four time points for gas chromatography analysis, and flux calculated from the linear rate of headspace concentration change. Wetland-level, area-weighted nitrous oxide emission factor was calculated as total seasonal nitrous oxide-nitrogen flux divided by total nitrate-nitrogen removed over the same period, expressed as a percentage.

2.3 Sediment denitrification potential

Denitrification potential of surface sediment (0-5 cm) was measured at the same six locations per wetland per campaign using the acetylene-block technique under anaerobic, carbon- and nitrate-amended slurry incubation conditions designed to estimate the maximum denitrification capacity of the resident microbial community, with nitrous oxide accumulation quantified by gas chromatography at 0, 2 and 4 hours.

2.4 Microbial community and functional gene analysis

Sediment DNA was extracted from the same locations and timepoints and used for quantitative PCR quantification of *nirS*, *nirK*, *nosZ* clade I and *nosZ* clade II

gene copy number, normalised to total bacterial 16S rRNA gene copy number, using previously published clade-specific primer sets [14,15]. A subset of samples (n=72, one per location per site per season) was additionally used for 16S rRNA gene amplicon sequencing (V4 region, Illumina MiSeq) to characterise broader microbial community composition and its association with denitrification performance.

2.5 Statistical analysis

Seasonal and site differences in nitrate removal, nitrous oxide emission factor and gene abundances were tested using linear mixed models with site and season as fixed effects and sampling location as a random effect, with post-hoc pairwise comparisons where the overall model was significant. The relationship between *nosZ* clade II relative abundance and nitrous oxide emission factor was tested by linear mixed model regression across all site-season combinations. All statistical analyses were performed in R version 4.3.

3. RESULTS

Mean annual flow-weighted nitrate removal efficiency across the three wetlands was 58.4%, but individual wetland performance varied more than twofold: W1 removed 76.8%, W2 removed 57.3%, and W3 removed only 41.2% of inlet nitrate load on an annual basis. Removal efficiency showed a strong, consistent seasonal pattern at every site, significantly higher in summer than winter (mean 71.3% versus 39.6% across all three wetlands, paired t-test $P < 0.001$), tracking closely with sediment temperature and with acetylene-block denitrification potential, which itself varied more than fourfold between the coldest and warmest sampling campaigns (Table 1).

Table 1. Seasonal nitrate removal efficiency and sediment denitrification potential by wetland.

Wetland	Spring removal (%)	Summer removal (%)	Autumn removal (%)	Winter removal (%)
W1	78.4	89.2	76.1	63.5
W2	58.1	71.4	55.8	38.9
W3	42.6	53.9	40.2	27.1

Flow-weighted nitrate removal efficiency by seasonal campaign; annual means reported in text.

Direct nitrous oxide flux measurements showed a markedly different pattern from nitrate removal. Annual area-weighted nitrous oxide emission factor was 2.8% at W1 (the highest-performing wetland by nitrate removal), compared with 1.1% at W2 and 0.9% at W3, meaning the wetland removing the most nitrate also lost proportionally the most of that removed nitrogen as nitrous oxide rather than completing reduction to inert dinitrogen gas (Figure 1). This pattern held within, not just between, wetlands: across all site-season combinations, nitrate removal efficiency and nitrous oxide emission factor were not significantly correlated (Spearman $\rho = 0.14$, $P = 0.52$), indicating that these two dimensions of denitrification performance are at least

partially decoupled in this system.

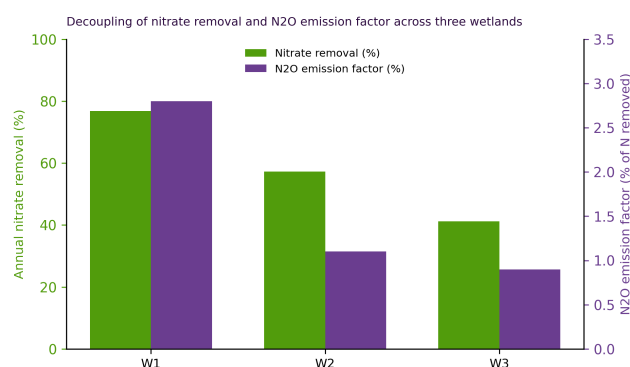


Figure 1. Annual nitrate removal efficiency versus area-weighted nitrous oxide emission factor across the three constructed wetlands, illustrating the decoupling of nitrogen removal performance from greenhouse gas performance (n=3 wetlands, 4 seasonal campaigns each).

Quantitative PCR of denitrification genes showed that total *nirS*+*nirK* gene abundance, a proxy for overall denitrifier community size, did not differ significantly between wetlands (Kruskal-Wallis $P = 0.31$), but the ratio of *nosZ* clade II to *nirS* gene abundance did differ significantly, and in the direction predicted by the emission factor pattern: W1 showed a significantly lower *nosZ* clade II:*nirS* ratio than W2 or W3 (Kruskal-Wallis $P = 0.008$; Table 2), consistent with a relatively smaller community-level capacity to complete the final, nitrous-oxide-consuming step of denitrification relative to the earlier, nitrous-oxide-producing steps. A linear mixed model regressing nitrous oxide emission factor against *nosZ* clade II relative abundance across all site-season combinations explained substantially more variance ($R^2 = 0.61$) than an equivalent model using total denitrifier gene abundance ($R^2 = 0.09$) or nitrate removal efficiency itself ($R^2 = 0.02$) as the predictor.

Table 2. Denitrification gene abundance and nitrous oxide emission factor by wetland (annual mean).

Wetland	<i>nosZ</i> clade II : <i>nirS</i> ratio	N2O emission factor (%)	Nitrate removal (annual, %)
W1	0.31 ± 0.08	2.8	76.8
W2	0.68 ± 0.12	1.1	57.3
W3	0.74 ± 0.14	0.9	41.2

16S rRNA amplicon sequencing of the community subset showed that overall microbial community composition differed significantly by both site and season (PERMANOVA, site $R^2 = 0.19$, season $R^2 = 0.24$, both $P < 0.001$), but did not reveal an obvious single taxonomic driver of the *nosZ* clade II pattern; clade II *nosZ* genes are distributed across a taxonomically broad set of bacterial phyla, and no single genus or family showed a relative abundance pattern that tracked the between-wetland difference in *nosZ* clade II:*nirS* ratio as cleanly as the functional gene quantification itself. This reinforces that a functional-gene-level rather than purely taxonomic characterisation is likely necessary to predict a wetland's nitrous oxide performance from its microbial community.

4. DISCUSSION

The central and, we think, most practically important result of this study is the clear decoupling of nitrate removal efficiency from nitrous oxide emission factor across three operational agricultural constructed wetlands: the best-performing wetland by the conventional nitrate mass-balance metric was also the worst performer by greenhouse gas emission per unit nitrogen removed. This directly challenges the common assumption, implicit in much constructed wetland design guidance, that maximising denitrification activity is an unambiguously positive design goal; our data instead point to the balance between denitrification steps, specifically the relative capacity to complete nitrous oxide reduction, as the critical determinant of whether high denitrification activity translates into genuine nitrogen removal benefit or into a disguised nitrous oxide source [8,9,16].

The association between *nosZ* clade II relative abundance and nitrous oxide emission factor, considerably stronger than either total denitrifier abundance or nitrate removal efficiency as predictors in our dataset, is broadly consistent with the emerging soil and sediment literature implicating clade II *nosZ* organisms as important, and previously underappreciated, contributors to net nitrous oxide consumption capacity across terrestrial and wetland systems [10,11,12]. That this pattern held specifically in engineered, agricultural-runoff-treating wetlands, a context distinct from the natural soils and sediments where the clade I/clade II framework has mostly been developed, extends its apparent relevance to a system type of direct management and policy interest, and suggests that wetland design or management interventions aimed specifically at favouring clade II *nosZ* organisms, rather than denitrifier abundance generically, could plausibly improve greenhouse gas performance without necessarily sacrificing nitrate removal.

We can only speculate, based on the present dataset, about what specific design or management levers might favour *nosZ* clade II organisms preferentially. Carbon quality and availability, redox conditions, and substrate nitrate concentration itself have all been implicated in shaping the clade I/clade II balance in other systems, and W1's comparatively short hydraulic residence time and higher inlet nitrate concentration relative to W2 and W3 offer a plausible, though unconfirmed, explanation: a shorter residence time and higher nitrate loading may favour rapid, nitrate-driven denitrification dominated by classical *nirS/nosZ* clade I organisms over the comparatively slower-growing, more broadly distributed clade II specialists [13,17]. Testing this directly would require either a controlled mesocosm manipulation of residence time and loading rate, or a considerably larger observational dataset spanning more wetlands across a wider range of hydraulic and loading conditions than the three sites studied here. It is also worth noting that

vegetation structure differed somewhat between the three wetlands, with W1 supporting a denser and more continuous emergent macrophyte stand than W2 or W3, and root-zone oxygen leakage from emergent vegetation is known from other systems to influence the local redox microenvironment in ways that could plausibly favour one denitrification gene clade over another; disentangling vegetation effects from residence time and loading effects would require a factorial experimental design rather than the observational comparison used here.

From a practical standpoint, wetland managers and designers currently have essentially no routine access to denitrification gene clade data, and nitrate mass balance alone, the metric almost universally used to report and regulate constructed wetland performance, would have identified W1 as the best-performing site in this study when it was, on a full nitrogen-cycling accounting, arguably the site with the least favourable overall nitrogen and greenhouse gas balance. Incorporating even periodic nitrous oxide flux monitoring, which is considerably more tractable at operational scale than functional gene quantification, into routine wetland performance assessment would at minimum flag sites like W1 for further investigation, and we would argue this is a reasonable near-term recommendation for agencies and organisations that fund or regulate constructed wetland nitrogen-removal projects.

Two limitations should temper how broadly these findings are extrapolated. First, three wetlands, while allowing a genuine site-level comparison, is a small sample from which to draw general design principles, and the specific hydraulic and loading differences we tentatively invoke to explain the *nosZ* clade II pattern are confounded with several other site differences, including wetland age and vegetation structure, that we did not systematically vary or control for. Second, static chamber nitrous oxide flux measurement, while standard practice, captures only surface diffusive flux and may under-represent ebullition-driven pulses of dissolved nitrous oxide, particularly during and immediately after high-flow events, meaning our annual emission factor estimates, while internally consistent for cross-site comparison, may understate absolute nitrous oxide loss at all three sites to some degree. A complementary eddy covariance or floating chamber approach capturing episodic ebullition events would give a more complete accounting of total nitrous oxide loss, particularly at sites with soft, organic-rich sediment prone to gas bubble trapping and release, and is a methodological refinement we would prioritise in any follow-up study aiming to produce regulatory-grade emission estimates rather than the primarily comparative, cross-site estimates reported here.

5. CONCLUSION

Comprehensive nitrogen-cycling monitoring across three agricultural constructed wetlands over a full annual cycle shows that nitrate removal efficiency and nitrous oxide

emission factor are substantially decoupled, with the wetland showing the highest nitrate removal also showing the highest nitrous oxide loss per unit nitrogen removed, associated with a significantly lower ratio of *nosZ* clade II to *nirS* denitrification genes than the other two, better-balanced wetlands. Across all site-season combinations, *nosZ* clade II relative abundance predicted nitrous oxide emission factor considerably better than total denitrifier gene abundance or nitrate removal efficiency itself, identifying denitrification gene clade composition, rather than overall microbial activity, as the more informative target for understanding and potentially managing wetland greenhouse gas performance. These findings argue against using nitrate mass balance alone as a sufficient performance metric for agricultural constructed wetlands and support incorporating direct nitrous oxide monitoring, and eventually functional gene-based indicators, into routine wetland assessment. We are now expanding this study to a larger panel of constructed wetlands spanning a wider range of hydraulic residence time and nitrate loading conditions, specifically to test whether residence time and loading rate causally shape the *nosZ* clade I/clade II balance as our present, necessarily correlative dataset suggests. In parallel, we plan a mesocosm-scale manipulation experiment directly testing whether carbon amendment strategies designed to favour clade II *nosZ* organisms can improve nitrous oxide performance at a high-nitrate-removal site such as W1 without compromising its nitrate removal capacity, which would provide the first direct evidence that this microbial balance is a genuinely manageable design lever rather than merely a useful diagnostic indicator.

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Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author Contributions

I.B. conceived and supervised the study, coordinated field sampling and led data interpretation. K.H. performed nitrous oxide flux measurements, sediment denitrification assays and quantitative PCR analysis. O.P. conducted 16S rRNA amplicon sequencing and community composition analysis. All authors contributed to writing and approved the final manuscript.

Ethics Statement

This study involved environmental water, sediment and microbial community sampling from constructed wetlands; it did not involve human participants, human data, or vertebrate or higher invertebrate animals, and therefore did not require institutional ethics committee review. Field sampling was conducted with the permission of the relevant landowners and wetland management agencies.

Data Availability

16S rRNA amplicon sequencing data generated in this study have been deposited in the Sequence Read Archive under a project accession available from the corresponding author on reasonable request. Water chemistry, gas flux and quantitative PCR datasets are available from the corresponding author upon request.

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