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Artificial Ponds as Hotspots of Nitrogen Removal in Agricultural Watershed

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Abstract

Small waters, like ponds, are the most abundant freshwater environments, and are increasingly recognized for their function in ecosystem service delivery. In agricultural watershed, artificial ponds play an essential role in reducing nitrogen pollution. However, until now artificial ponds remain the least investigated part of water environments. The importance of microbial activities has seldom been discussed, which makes the microbial pathways and processes rates in nitrogen removal poorly understood. To illustrate the role of artificial ponds in microbial nitrogen removal in agricultural watersheds, 21 pond sediments and 11 soils are collected in an agricultural watershed of China. Results show that surface sediments in ponds carry significantly higher dissolved inorganic nitrogen (9.1-21.9 mg/kg) and total organic matter (64.8-113.0 g/kg) compared to the surrounding agricultural soils. High rates of microbial nitrogen removal in ponds (12.4-25.5 nmol N g⁻¹ h⁻¹) are observed, which are 2-9 times higher than those in dryland soils. In pond sediments, denitrification dominates (> 90% N-loss) the microbial nitrogen removal process with only a minor contribution of anaerobic ammonium oxidation. A high

potential of N₂O production (up to 9.4 nmol N g⁻¹ h⁻¹) occurs in ponds along with the rapid nitrogen removal. For denitrifier genes, *nir* gene are always more abundant than *nosZ* gene. Additionally, the *nirS* gene is more abundant under flooded conditions, while *nirK* gene prefers higher dissolved oxygen and NO₃⁻ in drylands. These findings present essential information on the mechanisms of microbial nitrogen removal in ponds, and highlight the ecosystem function of ponds in agricultural watersheds.

Keywords: Artificial pond, Agricultural watershed, Nutrient, Nitrogen removal, Nitrous oxide

1. Introduction

Ponds are primarily defined as artificial or natural water bodies between 1 m² and 2 ha in area, with a maximum depth of no more than 8 m (Oertli et al. 2005). The number of ponds is enormous globally, with an estimated number of 277 million ponds less than 1 ha in size (Downing et al. 2006). These small water bodies account for more than 90% of the global 304 million standing waterbodies, or 30% of global standing water by surface area (Downing et al. 2006). Ponds provide a number of vital ecosystem services including hydrological regulation, conservation of biodiversity and pollution mitigation (Brazier et al. 2021; Chen et al. 2019; Mushet et al. 2020). In nutrient cycling, the burial rates for organic carbon in ponds were 20-30 times higher than that reported for other habitats on a global scale (Downing et al. 2008). Moreover, small ponds (< 0.1 ha) make up 8.6% of the global lakes and ponds area, but contribute 15.1% of CO₂ and 40.6% of CH₄ emissions (Holgerson & Raymond 2016).

Many regions are known for large numbers of small waterbodies, including the vernal pool systems in North America, Mediterranean temporary ponds in southern Europe and North Africa, ponds of arid and desert areas in South Africa and Australia (Boix et al. 2016; Dahl 2014; Hobbie 1980; Wissinger et al. 2016). The agricultural activities in Asian countries particularly benefit from pond construction (Chen et al. 2017), especially for China with approximately 42% of the population living in rural areas (Chen et al. 2019). Due to the monsoon climate, the distribution of water resources in China is extremely imbalanced spatially and temporally (Wang & Zhang 2011), and the construction of ponds is a common mean to reserve water resources for

agricultural irrigation and rural life ([Chen et al. 2017](#)). The history of ponds construction dates back to 3,000 years ago ([Yin et al. 1993](#)), and the number of ponds (< 1 ha) has reached 4 million in China to date ([Lü et al. 2021](#)).

The construction of ponds in agricultural watershed altered the landscape as well as the ecological processes. For example, artificial ponds significantly mitigated the nutrients output by intercepting and degrading the diffuse pollutants from agricultural fields and rural life sources ([Capps et al. 2014](#)). It had been reported that the traditional multi-pond systems retained about 98% of total nitrogen and phosphorus output carried by rainfall runoff in an agricultural watershed in China ([Yan et al. 1998](#)). In the United States of America, 64% of NO_3^- and 36% of total nitrogen output was abated through farm ponds in an agricultural watershed ([Brunet et al. 2021](#)). On a global scale, approximate 25% nitrogen removal of watersheds occurred in ponds and such small waterbodies ([Harrison et al. 2009](#)).

To dates, the removal of nitrogen by artificial ponds was mostly attributed to their interception of pollutants, plant uptake and N burial in sediment ([Lü et al. 2019](#); [Verhoeven et al. 2006](#); [Xue et al. 2020](#); [Youn Chi & Pandit 2012](#)). However, microbial nitrogen transformation was seldom discussed in pond ecosystems, although it is essential for nitrogen removal. In nitrogen cycling, denitrification is generally considered as the dominant process in the freshwater ecosystems ([Hernandez & Mitsch 2007](#); [Seitzinger 1988](#); [Wang et al. 2019a](#)). Denitrification processes reduce NO_3^- to NO_2^- , NO, N_2O , and eventually to N_2 , in a stepwise manner, under anaerobic conditions ([Seitzinger et al. 2006](#)). N_2O and N_2 are usually the two main end products in microbial nitrogen removal. Nitrite reduction catalyzed by nitrite reductase (NIR, encoded by the *nirS* or *nirK* genes) followed by NO reduction by nitric oxide reductase (NOR, encoded by *nor* genes) is the main source of N_2O production ([Kuypers et al. 2018](#)), and N_2O can be reduced to N_2 via N_2O reductase (encoded by the *nosZ* gene) ([Hallin et al. 2018](#)). The release of N_2O which is an intermediate of denitrification is regulated by functional genes of both nitrite reductase and N_2O reductase. Anammox (anaerobic ammonium oxidation) is an alternative nitrogen removal pathway to denitrification, in which NH_4^+ is oxidized by NO_2^- by autotrophic anammox bacteria yielding N_2 as end product ([Jetten et al. 2003](#); [Wang et al. 2012](#)). Unlike denitrification, the anammox process is not restricted by the organic matter content and is more straightforward without emission of N_2O ([Cai et al. 2020](#); [Kartal et al. 2011](#); [Strous et al. 2006](#)).

Identification of the reaction rates, relative contribution, and microbial communities of the two pathways is crucial to understand the end products, environmental drivers, and processes dynamics in the nitrogen removal of artificial ponds.

In this study, a typical mountainous agricultural watershed in southwest China was selected. The land of the agricultural watershed was mainly composed of drylands, paddy fields and artificial ponds. We collected 21 sediment samples from the artificial ponds of different uses and 11 soil samples from dryland and paddy fields. By investigating the nutrient contents, microbial activities, and microbial community composition, we aim to show the roles of artificial pond (i) as nutrient reserve, (ii) in nitrogen removal of the agricultural watershed, and (iii) the major contributor in microbial nitrogen removal.

2. Materials and methods

2.1. Study area and sample collection

The study area, a mountainous agricultural watershed characterized by abundant and scattered artificial ponds, is located in Liuyin Town, Chongqing, southwest China (29° 56' 56"-29° 57' 43" N, 106° 37' 12"-106° 38' 13" E) (Fig 1). The study area was approximately 13.60 km², and the altitude was from 280 to 496 m. It was roughly estimated that 300 artificial ponds (< 1 ha, ~ 0.8 km² in total) existed in this area for irrigation and aquaculture. In this study, sediment samples were collected from seven ponds, including four irrigation ponds (P1, P2, P5 and P6) and three aquaculture ponds (P3, P4 and P7) (Table S1). At each pond three parallel sediment columns (30 cm depth) and the overlying water were collected. The sediment columns were segmented into three sections (0-10 cm, 10-20 cm, and 20-30 cm). Three parallel surface soil samples (0-10 cm) in dryland or paddy fields around the ponds were collected. The crop types in dryland include corn, cowpea fields and orchard gardens.

Fig. 1 Location and distribution of sampling sites in study area

Field samples were stored in sterile plastic bags and transported to the laboratory in a

cooler box (4°C) for subsequent analysis. One subsample was immediately incubated to determine microbial nitrogen removal activity, a second subsample was used for physicochemical analyses, and a small portion was stored at -80°C for molecular analysis. All samples were analyzed separately, and the values were averaged to represent site conditions.

2.2. Physicochemical analysis

Sediment/soil ammonium (NH_4^+), nitrite (NO_2^-), and nitrate (NO_3^-) were extracted from 5 g of fresh sediment/soil with 25 ml of 2 M KCl (1:5 wt./vol). The supernatant was filtered through a 0.22 μm membrane filter and the compounds were determined via a spectrophotometric detection assay (Wu et al. 2016). Moisture content was measured by oven-drying at 105 °C until a constant weight was achieved. The pH was determined in 1:2.5 sediment/water (wt./vol) suspensions after shaking and centrifugation, with a Mettler Toledo pH analyzer (S220, Switzerland). Total organic matter (TOM) was measured as loss on ignition at 550 °C (LOI 550) using a Muffle furnace. The TN and TP were determined with the potassium persulfate oxidation-ultraviolet spectrometry method (Apha 1998), using a UV spectrophotometer (UVmini-1240, Japan).

2.3. Measurements of potential denitrification and anammox rates

The denitrification and anammox rates of sediment/soil samples were measured using the slurry incubation and isotope pairing technique (Risgaard-Petersen et al. 2004). Fresh sediments/soils were mixed with water in the ratio of 1:7 (sediment: water) and flushed with ultrahigh purity He for 30 min to make anaerobic sediment slurries. These slurries were darkly pre-incubated at in-situ temperature for 36-48 h to remove background NO_x^- (NO_3^- and NO_2^-) and DO. After pre-incubation, the slurries were transferred into 12.5 mL tubes (Exetainers, Labco, UK) via injectors, these tubes were divided into two groups. The first group were used to analyze Fn (fraction of $^{15}\text{NO}_3^-$ in NO_x^- pool), and the second group of tubes were injected with $^{15}\text{NO}_3^-$ (99.6 atom%) solution to 100 μM final concentration. The tubes were incubated in the incubator at in-situ temperature and were stopped by adding 200 μL of 50% ZnCl_2 at 0 and 2 h from the beginning of incubation. $^{29}\text{N}_2$ and $^{30}\text{N}_2$ produced in the tubes were determined with a membrane inlet mass spectrometry (MIMS, HPR40, Hiden, UK), and the rates of denitrification and

anammox were calculated (Thamdrup & Dalsgaard 2002). The calculation equations are as follows:

$$R_D = D_{29} + 2 \times P_{30} \quad (1)$$

$$D_{29} = P_{30} \times 2 \times (1 - F_n) \times F_n^{-1} \quad (2)$$

where R_D ($\text{nmol N g}^{-1} \text{ h}^{-1}$) represents the total rate of $^{15}\text{NO}_3^-$ - based denitrification, D_{29} is the $^{29}\text{N}_2$ production rate from denitrification, P_{30} ($\text{nmol N g}^{-1} \text{ h}^{-1}$) is the total $^{30}\text{N}_2$ production rate; F_n represents the fraction of ^{15}N in total NO_3^- . The potential rates of anammox were estimated by the following equation (Wu et al. 2021; Xiao et al. 2018):

$$R_A (A_{29}) = P_{29} - D_{29} \quad (3)$$

where R_A (A_{29}) and P_{29} ($\text{nmol N g}^{-1} \text{ h}^{-1}$) represent the potential rate of $^{15}\text{NO}_3^-$ - based anammox (or $^{29}\text{N}_2$ production rate from anammox) and total $^{29}\text{N}_2$ production rate, respectively.

2.4. Determination of potential N_2O production rate

N_2O production rates were measured with headspace equilibrium gas chromatography using the samples prepared as describes in 2.3 of Methods and Materials section (Hou et al. 2015). Specifically, the supernatant in the tubes which were incubated for 0 h and 2 h was replaced by 5 mL of ultrahigh purity He to create 5 mL of headspace gas. After that, the tubes were shaken violently for 1 h to make gas-liquid equilibrium. The concentration of N_2O in the headspace gas was measured with a gas chromatograph (GC-2014C, Shimadzu, Japan) which was equipped with electron capture detector (ECD).

N_2O concentrations in the headspace after equilibrium were calculated according to equation:

$$C_G = P C_g / 1013.25 R T$$

where C_G (nmol L^{-1}) is the concentration of N_2O in the headspace after equilibrium, C_g (ppb: nmol mol^{-1}) is the volumetric concentration of N_2O in the headspace, R ($0.082057 \text{ L atm mol}^{-1} \text{ K}^{-1}$) is the ideal gas constant, T (K) is the temperature of the water samples after equilibrium, and P (hPa) is the pressure of laboratory.

The dissolved N_2O concentrations were determined according to equation:

$$C_L = C_G \times (K_0 R T + \alpha) \quad (5)$$

where C_L (nmol L⁻¹) is the concentration of dissolved N₂O in the incubated water, R (0.082057 L atm mol⁻¹ K⁻¹) is the ideal gas constant, α is the ratio of gas to liquid after replacement, and K_0 (mol L⁻¹ atm⁻¹) is the equilibrium constant which is calculated from Weiss formula (Weiss & Price 1980), as follow:

$$\ln K_0 = -62.7062 + 97.3066 \times 100/T + 24.1406 \times \ln(100/T) + S \times [-0.05842 + 0.033193 \times 100/T - 0.0051313 \times (100/T)^2] \quad (6)$$

The accumulation of N₂O per unit of incubation time is the rate of N₂O production which was calculated according to equation:

$$D_{N_2O} = (C_{L2} - C_{L0}) / (T_2 - T_0) \quad (7)$$

where D_{N_2O} (nmol N g⁻¹ h⁻¹) is the rate of N₂O production, T_0 and T_2 represent 0 and 2 incubation time respectively, C_{L0} and C_{L2} represent the concentration of N₂O at T_0 and T_2 respectively.

2.5. DNA extraction, sequencing, and data processing

Total genomic DNA was extracted from sediment/soil samples (approximately 0.5 g) using the FastDNA SPIN Kit for Soil (MP Biomedicals, USA) following the manufacturer's instruction. The concentration of extracted DNA was measured with a NanoDrop Lite (Thermo Fisher Scientific, Wilmington, DE, USA), and the DNA quality examined by 1% (wt./vol) agarose gel electrophoresis.

The *nirS* gene (performing NO₂⁻ reduction) was used to study the denitrification community, and was amplified by PCR using primers cd3aF and R3cd (Yergeau et al. 2007). PCR amplification of Anammox - specific 16S rRNA genes was based on primers A438f and A684r (Han & Gu 2013). More details about the conditions of PCR amplification are presented in Table S2. Prior to high-throughput sequencing, the PCR products were purified using the MiniBEST Agarose Gel DNA Extraction Kit (TaKaRa Bio, Japan).. Subsequently, purified amplicons were pooled in equimolar and paired-end (PE) sequenced (2×300) on an Illumina MiSeq PE300

platform. The raw sequences were merged and quality filtered in Quantitative Insights in Microbial Ecology (QIIME) (Caporaso et al. 2010) and Mothur (Schloss Patrick et al. 2009). OTUs with identity thresholds (93% for *nirS* and 97% for anammox 16S rRNA) were defined using Usearch (v. 7.0 <http://drive5.com/uparse/>). Rare OTUs with less than 0.01% of the total sequences were removed. To avoid biases arising from sequencing depth and to make samples comparable, sequences were rarified to a uniform sequencing depth based on the sample with the lowest sequences. The diversity indices (Shannon, Simpson, Ace and Chao1) and rarefaction curves were calculated also in Mothur referring to previous studies (Liu et al. 2020; Ye et al. 2021). The raw sequences of *nirS* and Anammox-specific 16S rRNA genes used in this study were deposited in the Sequence Read Archive (SRA, <https://submit.ncbi.nlm.nih.gov/subs/sra/>) of NCBI under the accession numbers PRJNA780407 and PRJNA780073.

2.6. Quantitative PCR analysis

The abundance of bacterial 16S rRNA genes, *-nir* gene (*nirS* and *nirK*), *nosZ* gene (*nosZ* I and *nosZ* II) and anammox - specific 16S rRNA genes in sediments/soil samples were quantified by a LightCycler ® R480 II Real-Time PCR (Roche, Switzerland). Each sample was analyzed in triplicate. The standard curves used for calculation were achieved with plasmid DNA with known concentrations and copy numbers. qPCR results with high amplification efficiency (90%-110%) and correlation coefficient values of the standard curve ($r^2 > 0.97$) were integrated into the analysis. The specificity of PCR amplifications was defined by melting curve analysis and gel electrophoresis. The primers, reaction systems, and programs are shown in Table S2.

2.7. Statistical analysis

Multifactor ANOVAs were used to determine the significance between samples. For factors that were statistically significant ($P < 0.05$), a Tukey's post hoc analysis was conducted to determine significant differences using the 'multcomp' package in v. R 4.1.0. The plots were conducted with Prism 8 software (version 8.0.2). Principal co-ordinates analysis (PCoA) ordination and non-metric multidimensional scaling (NMDS) was used to reveal differences in denitrification and anammox bacteria community structures based on Bray - Curtis dissimilarities. Significant

differences among the different samples were tested by an analysis of similarities test (ANOSIM). To identify the environmental factors likely to affect the composition of denitrification and anammox bacteria communities, Canonical Correlation Analysis (CCA) was used in Canoco 5 software. Partial least squares pathway modelling (PLS-PM) was conducted in R with the 'plspm' package (Sanchez & Trinchera 2012) to infer the effects of basic parameters (pH and moisture), nutrients (TN, TP, and TOM) and DIN (NH_4^+ , NO_3^- , and NO_2^-) on nitrogen removal rates and associated microorganisms. 1000 bootstraps were performed on model pathways to evaluate pathway coefficients and coefficients of determination (R^2).

3. Results

3.1. Physicochemical properties

In total, 32 samples including 21 pond sediments (7 each for 0-10 cm, 10-20 cm, and 20-30 cm depths), 7 dryland soils and 4 paddy soils were collected (Fig 2). Pond sediments were determined with the highest values of NH_4^+ concentrations (14.2 ± 2.4 mg/kg), followed by paddy soils (7.8 ± 3.2 mg/kg) and dryland soils (1.5 ± 0.7 mg/kg) (ANOVA, $P < 0.001$) (Fig. 2d). On the contrary, the concentrations of NO_3^- in dryland soils was significantly higher than the other two types of samples (0.2 ± 0.1 and 1.1 ± 0.7 mg/kg for sediments and paddy soils respectively, Tukey's post hoc, $P < 0.001$ for both) (Fig. 2e). The highest Dissolved Inorganic Nitrogen (DIN) concentrations were observed in ponds (14.8 ± 2.5 mg/kg), followed by the soils (6.0 ± 1.9 and 9.3 ± 2.7 mg/kg for dryland and paddy soils, Tukey's post hoc, $P < 0.001$ and $P = 0.009$, respectively) (Fig. 2f). TOM presented high concentrations in pond sediments (92.2 ± 19.9 g/kg). The TOM in dryland soils (56.5 ± 16.1 g/kg) were lower than those in sediments (Tukey's post hoc, $P < 0.001$) (Fig. 2i). The pond sediments ($54.2 \pm 8.8\%$) had higher moisture contents than the dryland and paddy soils ($17.3 \pm 2.4\%$ and $39.4 \pm 4.3\%$, Tukey's post hoc, $P < 0.001$ and $P = 0.002$, respectively) (Fig. 2a). The pH of dryland soil samples (6.3 ± 1.3) was lower than those of ponds and paddy soils (Tukey's post hoc, $P = 0.02$ and $P = 0.24$, respectively) (Fig. 2b). No significant difference on NO_2^- concentrations was observed among different samples ($0.1 - 0.6$ mg/kg, ANOVA, $P = 0.11$) (Fig. 2c). TN and TP (2.8 ± 1.2 and $0.5 \pm$

0.3 g/kg respectively) showed little noticeable difference in this study (ANOVA, $P = 0.42$ and $P = 0.11$, respectively) (Fig. 2g-h).

Fig. 2 Physicochemical properties of sediment and soil samples. (a) Moisture content, (b) pH, (c) NO_2^- , (d) NH_4^+ , (e) NO_3^- , (f) Dissolved inorganic nitrogen (DIN was composed with NH_4^+ , NO_3^- and NO_2^-), (g) TN, (h) TP, (i) TOM. Different lowercase letters above the boxes indicate significant differences between different groups based on one-way ANOVA with Tukey's test ($P < 0.05$)

3.2. Potential denitrification, anammox and N_2O production rates

The potential rates of denitrification, anammox and N_2O production were determined by slurry incubation and isotope pairing technique (Fig. 3). Surface sediments (0-10 cm) in ponds showed comparable denitrification rates with paddy soils (19.7 ± 4.5 versus $19.4 \pm 3.4 \text{ nmol N g}^{-1} \text{ h}^{-1}$), which were significantly higher than those in deeper sediments (11.6 ± 4.4 and $6.9 \pm 6.4 \text{ nmol N g}^{-1} \text{ h}^{-1}$ in 10-20 and 20-30 cm, Tukey's post hoc, $P = 0.02$, $P < 0.001$, respectively) and dryland soils ($7.9 \pm 4.1 \text{ nmol N g}^{-1} \text{ h}^{-1}$, Tukey's post hoc, $P < 0.001$) (Fig. 3a). Anammox rates were one magnitude lower than denitrification rates and showed no significant difference between samples ($0.04\text{-}1.5 \text{ nmol N g}^{-1} \text{ h}^{-1}$, ANOVA, $P = 0.16$), (Fig. 3b). The relative contributions of anammox to nitrogen removal was higher in sediments of 20-30 cm depth ($6.2 \pm 4.0\%$) and dryland soils ($8.8 \pm 4.2\%$) than those of 0-10, 10-20 cm layer sediments and paddy soils (less than 5%) (Fig. 3c). Denitrification dominated the nitrogen removal process in all samples. Based on the denitrification and anammox reaction rates and soil / sediment density ($1.4\text{-}1.9 \text{ g cm}^{-3}$), the N loss by surface sediments showed the highest intensity at $206 \pm 51 \text{ g N m}^{-2} \text{ yr}^{-1}$, and dryland soils were discovered with the lowest values at $99 \pm 48 \text{ g N m}^{-2} \text{ yr}^{-1}$. The N loss intensity of paddy soils ($202 \pm 38 \text{ g N m}^{-2} \text{ yr}^{-1}$) were comparable with those in pond surface sediments.

Fig. 3 Potential rates of sediment and soil samples in this study. (a) Potential denitrification rate, (b) potential anammox rate, (c) the contribution of anammox (ra%), (d) potential N_2 production

rate, (e) potential N₂O production rate, (f) the ratio of (N₂O/ N₂O + N₂). Different lowercase letters above the boxes indicate significant differences between different groups based on one-way ANOVA with Tukey's test ($P < 0.05$)

The surface sediments showed the highest potential N₂ production at $13.2 \pm 3.6 \text{ nmol N g}^{-1} \text{ h}^{-1}$, which were comparable to those in paddy soils of $10.0 \pm 0.8 \text{ nmol N g}^{-1} \text{ h}^{-1}$ (Tukey's post hoc, $P = 0.12$) (Fig. 3d). The deeper sediments (10-20 and 20-30 cm) and dryland soils showed lower potential N₂ production rates at 6.6 ± 2.2 , 5.0 ± 2.8 and $3.1 \pm 1.2 \text{ nmol N g}^{-1} \text{ h}^{-1}$ (Tukey's post hoc, $P = 0.002$, $P < 0.001$ and $P < 0.001$, respectively). The highest N₂O production rates were observed in paddy soils ($9.4 \pm 3.0 \text{ nmol N g}^{-1} \text{ h}^{-1}$, ANOVA, $P = 0.09$) (Fig. 3e). The ratios of N₂O/ (N₂O+N₂), which reflected the potential of N₂O release during nitrogen removal, showed high values up to 54.4% and 47.6% in dryland and paddy soils, respectively. In the pond sediments, samples taken at different depths showed little variances (ANOVA, $P = 0.22$) (Fig. 3f).

3.3 Abundance of denitrification and anammox bacteria

Functional genes involved in denitrification and anammox 16S rRNA gene were determined with qPCR assays. For *nir* gene, surface sediments in ponds were discovered with higher *nirS* gene abundances at $(7 \pm 4) \times 10^8 \text{ copies g}^{-1}$ than deeper sediments (10-20 and 20-30 cm) and dryland soils (Tukey's post hoc, $P = 0.002$, $P < 0.001$ and $P < 0.001$, respectively). The paddy soils showed the highest abundance of *nirS* genes at $(1.2 \pm 0.1) \times 10^9 \text{ copies g}^{-1}$ (Fig. 4a). The pond sediments showed the lowest *nirK* gene abundances from 0.1 to $2.0 \times 10^8 \text{ copies g}^{-1}$ (Fig. 4b). The paddy soils had the highest values of $(5.5 \pm 1.0) \times 10^8 \text{ copies g}^{-1}$ followed by dryland soils at $(2.7 \pm 1.3) \times 10^8 \text{ copies g}^{-1}$. Moreover, *nirS* genes were more abundant than *nirK* genes in all samples.

Fig. 4 Abundances of nitrogen removal related genes in this study including (a) *nirS* gene, (b) *nirK* gene, (c) anammox 16S rRNA gene, (d) bacteria 16S rRNA gene, (e) *nosZ* I gene, (f) *nosZ* II gene, (g)

the ratio of *nir/nosZ*. Different lowercase letters above the boxes indicate significant differences between different groups based on one-way ANOVA with Tukey's test ($P < 0.05$)

For N_2O -reducers, the abundances of *nosZ* II gene were higher than *nosZ* I gene. The abundances of *nosZ* I varied from 8.4×10^5 to 8.3×10^6 copies g^{-1} with no noticeable difference between samples (ANOVA, $P < 0.01$) (Fig. 4e). The *nosZ* II genes maintained at low abundance ($0.1 - 15.4 \times 10^7$ copies g^{-1}) across layers in ponds sediment without significant difference (ANOVA, $P = 0.12$) (Fig. 4f). The abundance of *nosZ* II genes in dryland and paddy soils showed high abundance at $(1.9 \pm 0.7) \times 10^8$ copies g^{-1} and $(3.7 \pm 0.9) \times 10^8$ copies g^{-1} than pond sediments (Tukey's post hoc, $P = 0.007$ and $P < 0.001$, respectively). The ratios of (*nirS* + *nirK*) / (*nosZ* I + *nosZ* II) were above 1 in all samples suggesting a higher abundance of *nir* genes (Fig. 4g).

Additionally, the abundance of anammox bacterial 16S rRNA genes was low with no significant difference between samples (8.2×10^3 to 5.8×10^4 copies g^{-1} , ANOVA, $P = 0.06$) (Fig. 4c). Total bacteria 16S rRNA gene numbers ($0.4\text{-}3.6 \times 10^{10}$ copies g^{-1}) showed highest abundance (ANOVA, $P < 0.001$) in the surface sediment samples of ponds. (Fig. 4d).

3.4 Diversity and composition of denitrification and anammox bacteria

The diversity of the key players in nitrogen removal was determined by amplicon sequencing. For *nirS*, after filtering the 330,501 raw sequences, 165,568 high-quality sequences were obtained which resulted in 1,443 OTUs. Shannon, Simpson, Chao1 and ACE indexes were calculated to determine the alpha diversity (Table S3 and Fig S2a). Paddy soils showed the highest Shannon diversity (4.81-5.46) and Chao richness (471.12-760.17) than those in pond sediments (2.62-4.38 and 296-587, respectively) and dryland soils (3.18-4.56 and 265-476, respectively).

Fig. 5 (a) Principal co-ordinates analysis (PCoA) of the *nirS* gene based on Bray-Curtis distances. (b) Relative abundance of *nirS*-type denitrifiers at the genus level

The most abundant 50 OTUs, covering 62.5% of the *nirS* sequences, were all affiliated to Proteobacteria. At the genus level, significant differences were discovered in pond sediments, paddy, and dryland soils. Most sequences identified in pond sediments were affiliated to *Azoarcus* ($59.45 \pm 5.14\%$), and followed by *Steroidobacter* and *Dechloromonas*. *Rhodanobacter* was the dominant genus in dryland soils ($58.1 \pm 16.2\%$), and *Steroidobacter* had the highest relative abundance in paddy soils ($44.1 \pm 2.1\%$) (Fig 5b and S3a). The PCoA showed that the first two axes explained 50.4% of *nirS* community (Fig 5a). The *nirS* community showed a significant separation between samples of sediment, dryland soil and paddy soil (ANOSIM, $P = 0.01$), and low variance was discovered in different layers of pond sediments. According to the CCA analysis, pH, NH_4^+ and DIN were the main factors significantly related to *nirS* community structure ($P < 0.01$, Fig S6a).

After filtering the 343,183 raw anammox 16S rRNA sequences, a total of 171,330 high-quality sequences were clustered into 204 OTUs. The Shannon diversity indexes of pond sediments, paddy soils and dryland soils (Table S4 and Fig S2b) were 0.69-2.94, 1.33-1.75 and 0.59-1.91, and little significant difference was observed between samples (ANOVA, $p = 0.17$). The pond sediments showed the highest Chao1 richness at 59.86-104.73 versus 41.00-77.10 in paddy soil and 41.80-86.19 in dryland soil (Tukey's post hoc, $p = 0.002$ and $p = 0.01$, respectively).

The dominant OTUs (30 OTUs, covering 94.2% of the sequences) were all affiliated to Planctomycetes. Only 9 OTUs were affiliated to anammox genus *Ca. Brocadia*, and other sequences were divided into two unknown clusters (cluster 1 and 2) (Fig. S3b). Cluster 1 was close to the uncultured bacterium from freshwater wetland and lake sediments, and cluster 2 was more similar with samples from South China Sea sediment and paddy soil. In pond sediments, less sequences were affiliated with anammox bacteria ($12.4 \pm 1.9\%$), while sequences in dryland and paddy soils were dominated by genus *Ca. Brocadia* (82.7% and 67.2%, respectively) (Fig. S4). The distribution of the bacteria for all samples were further analyzed by NMDS (Fig S5, explained by 80.81% of the variances). The community was significantly separated into two groups (pond sediments versus paddy/dryland soils, ANOSIM, $P = 0.002$). The community of sediment in different depths showed little variation. The CCA analysis indicated that TOM and

DIN significantly affected the bacterial community structure ($P < 0.01$, Fig S6b).

3.5 Drivers of nitrogen removal

PLS-PM analysis was used to access the direct and indirect effect of a single factor or the combination of interacting factors on potential nitrogen removal rates (Fig 6). Moisture content and pH showed significantly positive effects on nutrients (TN, TP and TOM), DIN and denitrifier community (coefficients = 0.665, 0.741 and 0.546 respectively; $P < 0.001$). DIN had a negative effect on denitrifiers gene abundance (coefficients = -0.552, $P < 0.05$). By contrast, DIN showed positive effects on denitrifier community composition (coefficients = 0.427, $P < 0.001$) and potential nitrogen removal rates (coefficients = 0.616, $P < 0.05$). Denitrifiers gene abundances also had a significantly positive effect on potential nitrogen removal rates (coefficients = 0.483, $P < 0.05$).

Fig. 6 Directed graph of the Partial Least Squares Path Model (PLS-PM). Each box represents an observed variables or latent variables. Path coefficients are calculated after 1000 bootstraps. Coefficients differ significantly from 0 are indicated by $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$. The model is assessed using the Goodness of Fit statistic, a measure of the overall prediction performance (The GoF index is 0.58)

4. Discussion

4.1. Ponds as nutrient pool of the watershed

Ponds are often overlooked freshwater ecosystems due to their relatively small area (Oertli 2018). However, the ponds appear to be a nutrient pool within the studied agricultural watershed. The dissolved inorganic nitrogen (DIN) in pond sediments (up to 21.9 mg/kg) was significantly higher than those of surrounding agricultural soils (6.0-9.3 mg/kg in surface soil), and so was the TOM which was 105.0 g/kg in surface sediments versus 56.5-76.5 g/kg in agricultural soils. Taking into consideration the respective areas of ponds and agricultural fields, the ponds may contain 18.5% of the total DIN and 14.9% of the TOM, while only covering 7.1% of the total

area in the targetted research region.

Additionally, the DIN in ponds was at a high level in comparison with other types of waterbodies, such as freshwater rivers (4.75-7.80 mg/kg) ([Arce et al. 2018](#); [Kim et al. 2016](#)), lakes (0.01-8.54 mg/kg) ([Cao et al. 2009](#); [Zimmer-Faust et al. 2017](#)) and reservoirs (1.5-11.55 mg/kg) ([Li et al. 2021a](#); [Shen et al. 2017](#)). The TOM content of ponds was also higher than those of freshwater rivers (6.2-85.0 g/kg) ([Mata et al. 2020](#); [Pisani et al. 2013](#)), lakes (32-79 g/kg) ([Gu et al. 2017](#); [Hickey & Gibbs 2009](#)) and reservoirs (13.8-61.8 g/kg) ([Han et al. 2020](#); [Trojanowska & Izydorczyk 2010](#)). The ponds were mostly located close to human settlements, and thus were apt to receive pollution discharged from daily life and farming ([Aguilar et al. 2012](#)). The high content of TOM and high ratio of NH_4^+ in DIN (93.3%-98.3%) also suggested that the nutrients in ponds probably originated from human activities ([Li et al. 2013](#)).

4.2. High rate of nitrogen removal in ponds

The nitrogen removal that occurred in pond sediment is a considerable rapid process when comparing with other agricultural units. The rates of nitrogen removal (denitrification plus anammox) in pond surface sediments ($12.4\text{-}25.5 \text{ nmol N g}^{-1} \text{ h}^{-1}$) were 2-9 times higher than those in dryland soils ($3.0\text{-}15.7 \text{ nmol N g}^{-1} \text{ h}^{-1}$), and were comparable with those of paddy field soils ($16.1\text{-}24.5 \text{ nmol N g}^{-1} \text{ h}^{-1}$). The nitrogen removal rates were also higher than those in estuaries influenced by extensive human activity ($3.1\text{-}7.5 \text{ nmol N g}^{-1} \text{ h}^{-1}$) ([Li et al. 2020](#)), rivers ($0.05\text{-}1.14 \text{ nmol N g}^{-1} \text{ h}^{-1}$) ([Zhang et al. 2021b](#)), wetlands ($0.30\text{-}4.12 \text{ nmol N g}^{-1} \text{ h}^{-1}$) ([Coban et al. 2015](#); [Gao et al. 2016](#); [Wang et al. 2019b](#)). The high rates of nitrogen removal in ponds were largely caused by the nutrient enrichment which was proven to have a positive effect on denitrification, mineralization processes ([Doroski et al. 2019](#)) and the overall geochemical dynamics ([Cheng & Basu 2017](#)). It was further confirmed using PLS-PM and Pearson correlation analysis that the DIN and TOM both showed positive correlations with the nitrogen removal rates (Fig 6 and S1).

In this study, denitrification dominated (> 90%) the nitrogen removal process in ponds, which was consistent with data from several natural habitats (tidal rivers, marine, reservoirs) ([McCarthy et al. 2015](#); [Tall et al. 2011](#); [Zhou et al. 2019](#)). In previous studies, the high proportion of denitrification is often associated with high level of organic matter or carbon content

([Ballantine & Schneider 2009](#); [Bruesewitz et al. 2011](#); [Small et al. 2016](#)), and it was ascribed to the physiology of many denitrifiers consuming organic matter in their metabolism ([Baker et al. 2000](#); [Jones Jr 1995](#)). It was also identified in this study that lower denitrification rates were detected in deeper sediments and dryland soils with lower organic matter content. Pearson correlation analysis further confirmed the significant correlation ($r = 0.437$, $P < 0.05$) between TOM and denitrification rates (Fig S1). The DIN (mostly NH_4^+) also had a positive effect (coefficients = 0.616, $P < 0.05$) on denitrification rates (Fig 6). In addition, a significant negative correlation between NO_3^- and denitrification rates was observed, which was different from previous studies ([Bruland et al. 2006](#); [Hunt et al. 2004](#)). It was probably because the denitrification rates in dryland soils, which had higher NO_3^- concentrations, were significantly lower than those of pond sediments and paddy soils. The NO_3^- and TOM act as electron acceptor and donor respectively, to regulate the denitrification process. TOM is more decisive compared to NO_3^- in freshwater ecosystems ([Burgin et al. 2010](#); [Hill & Cardaci 2004](#)). Hence, the higher denitrification rates in the sediments were more likely to be regulated by the high content of TOM in sediment. In general, the human nutrients input (DIN and TOM) not only turns the ponds into a nutrient pool, but also shapes the pond into a hotspot of microbial nitrogen removal.

By contrast, the anammox process only contributed $4.3 \pm 3.0\%$ of nitrogen removal in ponds, which was consistent with the levels reported in other natural ecosystems (river estuaries, inland rivers) ([Dale et al. 2009](#); [Kessler et al. 2018](#); [Zhou et al. 2014](#)). However, anammox bacteria were also shown to be widely abundant and responsible for up to 41% of the total N loss in other areas such as estuaries ([Dalsgaard et al. 2003](#)), paddy fields ([Shen et al. 2014](#); [Zhu et al. 2011b](#)) and constructed wetlands ([Zhu et al. 2011a](#)). Anammox is more active in environments where NO_3^- is readily available ([Engström et al. 2005](#); [Trimmer et al. 2003](#)). However, anammox as an autotrophic process is usually more competitive than denitrification in low organic carbon environments ([Plummer et al. 2015](#)). Thus, in surface sediments and paddy soils, which had higher organic matter, anammox was responsible for only 5% of the nitrogen removal. By contrast, higher anammox contributions (up to 13.4% of total nitrogen removal) were detected in dryland soils with higher content of NO_3^- and lower content of TOM.

On the basis of the nitrogen removal rates and sediments/soils density data ($1.41\text{-}1.89 \text{ g cm}^{-3}$), an estimated total loss by the combination of denitrification and anammox processes was 47.8

and 25.0 t N yr⁻¹ in dryland and paddy soils, while the pond surface sediments in the study area contributed 14.0 t N yr⁻¹. Thus, 16.2% of the nitrogen was removed from the ponds with only 7.12% of the area. The pond is a neglected hotspot for nitrogen removal in agricultural watershed.

Strong potentials of N₂O production appeared along with the nitrogen removal in ponds. The N₂O production rates from pond sediments (6.5 ± 2.2 nmol N g⁻¹ h⁻¹) were higher than many habitats like sea (0-0.09 nmol N g⁻¹ h⁻¹), estuary (0.03-3.40 nmol N g⁻¹ h⁻¹), riparian zones (0-0.04 nmol N g⁻¹ h⁻¹) and wetlands (0-1.60 nmol N g⁻¹ h⁻¹) (David et al. 2013; Li et al. 2021b; Lin et al. 2017; Liu et al. 2016; Teixeira et al. 2010). The N₂O/ (N₂O + N₂) percentages in pond sediments (34.2 ± 13.2%) were comparable or higher than those of a nitrogen-enriched subtropical estuaries and stormwater ponds (3.38-51.00%) (Błaszczak et al. 2018; Li et al. 2021b; Su et al. 2021). Despite the high rates of denitrification in ponds, the terminal product of nitrogen removal in pond sediment was more likely to be N₂ compared to dryland soil (N₂O/ (N₂O + N₂) up to 86.8%). Previous study demonstrated that the ratio of N₂O/ (N₂O + N₂) was negatively correlated with pH (Samad et al. 2016), matching the result of Pearson correlation analysis in our study (r = -0.558, *P* < 0.001). Under lower pH (< 7.0) conditions the *nosZ* genes (particularly *nosZ* II genes) and activity of the N₂O reductase may be restrained, which would increase N₂O emission and the N₂O/(N₂O + N₂) ratio in dryland soils (Čuhel & Šimek 2011; Jones et al. 2014; Pan et al. 2012). Thus, more than half of the N₂O was emitted as an intermediate product of denitrification processes in dryland soils.

4.3 Contributors to microbial nitrogen removal

The high abundance of *nirS*, *nirK*, *nosZ* I and *nosZ* II genes also supported that denitrifiers were the main contributors to nitrogen removal in ponds, in which these denitrifier gene abundances were 2-5 orders of magnitude higher than those of anammox bacteria. Despite of the frequent exchange of water/soil between ponds and surrounding agricultural fields, the denitrification community in pond sediments, which was mainly composed by genus *Azoarcus* (59.5 ± 21.0%), was significantly different from that in the surrounding agricultural soils. By contrast, the denitrification community was more similar with those reported in other freshwater ecosystems

(i.e. lake and river sediments) (Hong et al. 2020; Kim et al. 2011; Zhang et al. 2021a). Hence, it could be concluded that environmental properties like pH, DIN and TOM rather than geological distance were stronger determinants for the community composition of denitrifier in ponds.

The *nirS* and *nirK* genes were detected in high copy numbers in pond surface sediments ($(7.2 \pm 4.0) \times 10^8$ and $(1.2 \pm 0.6) \times 10^8$ copies g^{-1} , *nirS* and *nirK* respectively), being higher than those reported for marine (Lee & Francis 2017; Lindemann et al. 2016; Zheng et al. 2021) and freshwater sediments (Jin et al. 2020; Wang et al. 2019a; Zhu et al. 2018). This suggested extensive denitrification processes in ponds. It was noted that *nirS* genes were more abundant than *nirK* genes in pond sediments while *nirK* genes dominated over *nirS* gene in dryland soils. This is consistent with the properties of *nirK* type denitrifiers which prefer high DO and NO_3^- (Desnues et al. 2007; Knapp et al. 2009). It was reported that a higher frequency of co-occurrence of *nosZ* with *nirS* than with *nirK* was reported before, suggesting that *nirS* type denitrifiers are more likely to perform complete denitrification (Clark et al. 2012; Hallin et al. 2018). Taken this into account, our results would point to lower production of N_2O in the ponds compared to dryland soils and this supports the rate measurements.

In this study, the abundance of *nosZ* II gene (10^7 - 10^9 copies g^{-1}) was greater than that of the *nosZ* I gene (10^5 - 10^7 copies g^{-1}), and similar results were found in other terrestrial ecosystems with the ratio of 1.5-10 (Jones et al. 2013; Juhanson et al. 2017; Pascazio et al. 2018; Su et al. 2021; Tsiknia et al. 2015). It was reported that *nosZ* II played a greater role in reducing N_2O emission in terrestrial habitats (Jones et al. 2014; Xu et al. 2020). However, high *nosZ* II gene abundance together with high N_2O production potential ($N_2O / (N_2O + N_2)$) were observed in dryland soils in this study. It was probably because the activity of N_2O reductase was restrained by the low pH in dryland soils (Čuhel & Šimek 2011; Jones et al. 2014; Pan et al. 2012).

The *nir/nosZ* ratio had been widely used as indicator of N_2O production potential observed with positive correlation (Domeignoz-Horta et al. 2015; Saarenheimo et al. 2015; Zhao et al. 2020). Nevertheless, we observed a negative correlation between the *nir/nosZ* and $N_2O / (N_2O + N_2)$ ratio in the pond sediments ($r = -0.452$, $P < 0.05$), which coincided with previous studies (Linton et al. 2020; Mafa-Attoye et al. 2020). These results implied that the *nir/nosZ* ratio only is not a good indicator for N_2O production in natural habitats.

5. Conclusions

This study investigated the nutrients distribution, microbial nitrogen removal capacities and microbial key players in artificial ponds, dryland, and paddy soils in an agricultural watershed.

The following conclusions can be drawn from the results:

- The ponds sediment had significantly higher DIN and TOM content comparing with those in soil of dryland and paddy fields, which evidenced that ponds acted as a nutrients pool in the agricultural watershed.
- High rates of microbial nitrogen removal make the ponds a hotspot of nitrogen removal with denitrification as the absolutely dominant (> 90%) pathway. It was estimated that about 16% of nitrogen was removed in ponds covering only 7% of the area in the watershed. High potential of N₂O production occurred in ponds coinciding with the rapid nitrogen removal.
- The *nir* genes were always more abundant than *nosZ* genes. The *nirS* gene was more abundant under flooded conditions, while *nirK* gene prefers higher DO and NO₃⁻ in drylands. The nitrogen removal bacterial communities showed significant differences between sediment and soil samples.

Declaration of competing 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.

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Data availability

Data collected and used in this manuscript are available in supplementary material file.

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Figures

Figure 1

Location and distribution of sampling sites in study area

Figure 2

Physicochemical properties of sediment and soil samples. (a) Moisture content, (b) pH, (c) NO_2^- , (d) NH_4^+ , (e) NO_3^- , (f) Dissolved inorganic nitrogen (DIN was composed with NH_4^+ , NO_3^- and NO_2^-), (g) TN, (h) TP, (i) TOM. Different lowercase letters above the boxes indicate significant differences between different groups based on one-way ANOVA with Tukey's test ($P < 0.05$)

Figure 3

Potential rates of sediment and soil samples in this study. (a) Potential denitrification rate, (b) potential anammox rate, (c) the contribution of anammox (ra%), (d) potential N₂ production rate, (e) potential N₂O production rate, (f) the ratio of (N₂O/ N₂O + N₂). Different lowercase letters above the boxes indicate significant differences between different groups based on one-way ANOVA with Tukey's test ($P < 0.05$)

Figure 4

Abundances of nitrogen removal related genes in this study including (a) *nirS* gene, (b) *nirK* gene, (c) anammox 16S rRNA gene, (d) bacteria 16S rRNA gene, (e) *nosZI* gene, (f) *nosZII* gene, (g) the ratio of *nir*/*nosZ*. Different lowercase letters above the boxes indicate significant differences between different groups based on one-way ANOVA with Tukey's test ($P < 0.05$)

Figure 5

(a) Principal co-ordinates analysis (PCoA) of the *nirS* gene based on Bray-Curtis distances. (b) Relative abundance of *nirS*-type denitrifiers at the genus level

Figure 6

Directed graph of the Partial Least Squares Path Model (PLS-PM). Each box represents an observed variables or latent variables. Path coefficients are calculated after 1000 bootstraps. Coefficients differ significantly from 0 are indicated by * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$. The model is assessed using the Goodness of Fit statistic, a measure of the overall prediction performance (The GoF index is 0.58)

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