Title

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Restoring wetlands on intensive agricultural lands modifies nitrogen cycling microbial communities and reduces N_2O production potential

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Abstract

The concentration of nitrous oxide (N_2O), an ozone-depleting greenhouse gas, is rapidly increasing in the atmosphere. Most atmospheric N_2O originates in terrestrial ecosystems, of which the majority can be attributed to microbial cycling of nitrogen in agricultural soils. Here, we demonstrate how the abundance of nitrogen cycling genes vary across intensively managed agricultural fields and adjacent restored wetlands in the Sacramento-San Joaquin Delta in California, USA. We found that the abundances of *nirS* and *nirK* genes were highest at the intensively managed organic-rich cornfield and significantly outnumber any other gene abundances, suggesting very high N_2O production potential. The quantity of nitrogen transforming genes, particularly those responsible for denitrification, nitrification and DNRA, were highest in the agricultural sites, whereas nitrogen fixation and ANAMMOX was strongly

associated with the wetland sites. Although the abundance of *nosZ* genes was also high at the agricultural sites, the ratio of *nosZ* genes to *nir* genes was significantly higher in wetland sites indicating that these sites could act as a sink of N_2O. These findings suggest that wetland restoration could be a promising natural climate solution not only for carbon sequestration but also for reduced N_2O emissions.

Keywords: functional genes, land use change, land management, nitrogen fixation, denitrification, ammonia oxidation

1. Introduction

Nitrous oxide (N_2O) is a GHG with a 298-fold greater warming potential than carbon dioxide (CO_2) and is involved in the destruction of stratospheric ozone layer. High N_2O emissions mainly originate in soil ecosystems, particularly in drained agricultural soils. Pressure to the agricultural sector is increasing as the global population and the demand for food grows. In recent years, the excessive use of nitrogen-based fertilizers has greatly contributed to the elevated N_2O concentrations (Park et al., 2012), and it has been predicted that farmlands and fertilizer applications will increase 35-60% before 2030, and therefore it is expected that these agricultural soils will contribute up to 59% of total N_2O emission (US EPA, 2012).

Since the agricultural sector plays a crucial role in N_2O emissions (Tian et al., 2020), it is essential to understand how the underlying mechanisms in soil lead to N_2O productions and emission, as well as to potential consumption by soil microorganisms. Soil nutrient ratios and availability, soil moisture, vegetation species and density, and temperature are the most important factors for microbes performing nitrogen (N) transforming processes (Firestone et al., 1980; Liimatainen et al., 2018; Pärn et al., 2018). Therefore, it is crucial to understand how N transforming potential will vary among intensively managed arable lands and how these emissions change if these managed soils undergo land-use change, such as restoration to wetlands. Restored and natural wetlands have already proven to be effective to sequester carbon (C) from the atmosphere (Hemes et al., 2019), and since the N_2O emission from natural wetlands is considered negligible, this suggests that wetland restoration could lead to significantly lower N_2O production potential.

The Sacramento-San Joaquin Delta (hereafter referred to as the Delta) is California's most important agricultural area, which is highly vulnerable to inundation due to the subsided agricultural peat soils. Some of the areas in the Delta are now up to 9 m below sea level, and more than 1500 km of levees and dams are protecting the area from flooding (Drexler et al., 2009). To reverse the soil subsidence, wetland restoration activities have been underway for more than two decades, and many studies have shown restoration to be a highly efficient measure to build up lost soils (Chamberlain et al., 2018; Eichelmann et al., 2018; Hemes et al., 2019). However, most of the focus has been on reducing CO_2 emissions for intensively managed agricultural sites and better quantifying the effects of methane (CH_4) emissions on the wetland ecosystem greenhouse gas balance after re-wetting (Hemes et al., 2018a).

So far, no long-term N_2O measurements across the different land use types at the Delta are available. Short-term weekly ebullition chamber and dissolved N_2O measurements at a restored and a natural nearby wetland showed insignificant N_2O emissions that had negligible effect on the ecosystem GHG budget (McNicol et al., 2017; Pärn et al., 2018), while corn, alfalfa and pasture have shown to be moderate to large N_2O sources. For example, Hemes et al., (2019) and Anthony et al. (in prep) showed that the N_2O emissions detected with automated chambers from corn and alfalfa were 3.28 ± 0.12 g N_2O-N m^{-2} yr^{-1} and 0.51 ± 0.07 g N_2O-N m^{-2} yr^{-1}, respectively. Measurements at pasture sites by (Teh et al., 2011) and (Pärn et al., 2018) have shown a similar range, where the emission was 2.4 ± 1.3 g N_2O-N m^{-2} yr^{-1} and 0.61 ± 0.27 g N_2O-N m^{-2} yr^{-1}, respectively. These studies have clearly shown that N_2O emissions from different land use types at the Delta will vary substantially, being lower in wetland ecosystems and larger in agricultural systems.

Extensive research to understand the stoichiometric regulation of soil C cycling at a Delta rice fields has been carried out by Hartman et al., (2017). However, no analyses in terms of N cycling processes are available. The N cycle is driven by abiotic and biotic (i.e., decomposition, mineralization, assimilative and dissimilative) processes (Espenberg et al., 2018), which include different microbial pathways, such as N fixation, nitrification, denitrification, dissimilatory nitrate reduction to ammonium (DNRA) and anaerobic ammonium oxidation (ANAMMOX) (Kuypers et al., 2018). The abundance of functional genes of microbes involved in N cycling has been shown in many studies to be effective in predicting the potential of N cycling processes

(Jones et al., 2014) in various ecosystems, including agricultural soils (Long et al., 2013) and natural and restored wetlands (Han et al., 2013; Ligi et al., 2015). In denitrification, the abundance of *nirK* and *nirS* genes refers to the N_2O emission potential, and the abundance of *nosZI* and *nosZII* genes indicates the potential for N_2O reduction, which is the only known biological sink for N_2O (Spiro, 2012). The other organisms which may change the N balance between soil and atmosphere are ammonium oxidizing bacteria (AOB) and archaea (AOA), ANAMMOX-specific bacteria, N-fixers, and microbes carrying out DNRA.

The main aim of this study is to understand how the abundance of N cycling functional genes has changed after the restoration of wetlands from intensively managed agricultural sites in the Delta. Based on the previous knowledge, we hypothesized that: (i) organic-rich drained soils have higher potential for N_2O production and emissions; (ii) wetland restoration would increase the abundance of N_2O reducers and decrease the abundance of ammonia oxidizers, leading to less N_2O emission in restored wetlands due to the anaerobic conditions; and (iii) lower soil temperature at the wetlands reduce the potential for N_2O production. If true, these hypothesized drivers and land-use patterns of N_2O emissions reductions could provide further incentive for agriculture to wetland restoration.

2. Materials and Methods

2.1. Site description

The seven sites analyzed in this study are located on Twitchell, Sherman and Bouldin Islands and are composed of four restored wetlands and three agricultural sites. The mean annual air and soil temperature measured close to the flux tower from topsoil layer (<15 cm depth) is shown in Table 1. All sites experience similar mean annual precipitation of 338 mm. Although being in a proximity with similar annual air temperature and precipitation, the sites experience a wide variety of management practices and differences in the soil chemical composition as well as in GHG fluxes (Valach et al., 2021).

Table 1. Site characteristics of all studies sites in the Sacramento-San Joaquin Delta, California. Tower data with site information are publicly available through the AmeriFlux network.

Site (Ameriflux ID)	Year restored	Elevation (m)	Annual (2018) mean air temperature (°C)	Annual (2018) mean soil temperature (°C)
Sherman Wetland (US-Sne)	2016	-5	14.1	15.4
East End (US-Tw4)	2013	-5	14.9	13.4
Mayberry (US-Myb)	2010	-4	14.1	13.2
West Pond (US-Tw1)	1997	-5	14.2	12.4
pasture (US-Snf)	n.a.	-4	15.6	17.1
alfalfa (US-Bi1)	n.a.	-2.7	16.0	17.0
corn (US-Bi2)	n.a.	-5	16.3	17.6

Sherman Wetland (263 ha) was restored from a pasture in November 2016 and was still in the process of establishing a vegetated canopy at the time of the soil sampling in 2018. East End wetland (303 ha) was constructed in 2013 on fields previously intensively cultivated with corn. After flooding, the wetland was filled with cattail (*Typha* spp.) and tule (*Schoenoplectus acutus*) with tiny areas of open water. Mayberry wetland (121 ha) was constructed in 2010 with a varied bathymetry where deep channels (up to 2 m) alternate with shallow vegetated areas creating the most heterogeneous wetland of the four wetland systems. West Pond wetland (3 ha) is the oldest system and was constructed in 1997 on Twitchell Island with dense vegetation and a closed canopy (Miller et al., 2008; Miller and Fujii, 2010). Since intensive agriculture is causing elevated nitrate concentration in both drainage water and river water (Schlegel and Domagalski, 2015; Wang et al., 2019), all of these wetlands will receive N through the inflow water, which maintains the water level. In addition, all of these wetland sites will experience at least some growth of *Azolla* spp. (Valach et al., 2021; especially *Azolla filiculoides*), which is a native species (Ta et al., 2017) in the region and can fix large amounts of atmospheric N through their symbiotic relationship with the cyanobacterium *Anabaena azolae* (Carrapiço, 2010), providing additional N input to the system. Based on vegetation development, peat accumulation and soil C/N ratio we consider Mayberry and West Pond as mature wetlands and Sherman Wetland together with East End as young wetlands.

The agricultural sites include three of the most dominant agricultural land use types in the Delta region: corn, alfalfa, and pasture. Bouldin corn consists mainly of organic-rich peat that is drained for corn (*Zea mays*) cultivation, and the site experiences a short, intensive growing period followed by flooding of the fallow field in autumn and drainage in spring. Corn is intensively fertilized with potassium and N fertilizers (e.g., potassium thiosulfate and urea-ammonium nitrate) to ensure fast and large biomass production. Alfalfa that is located on the Bouldin island (*Medicago sativa* L.) is a perennial forage legume that is grown for cattle and harvested 5-7 times in a year. Sherman pasture has drained semi-organic rich soils and is primarily used for grazing cattle, and it receives N compounds to the soil mostly from excreta. In sum, all sites receive some N inputs that can contribute to N cycling, though the pathway varies.

2.2. Soil sampling and soil chemical analyses

We measured soil C, N, P, Mn, Al, Fe content, and pH at all wetland and agricultural sites in August 2018 from the top-soil layer (0-15 cm). Ten samples were collected using sediment cores at each site across two transects, with each sampling location at least 3 m apart. Soil samples for chemical analysis were immediately bagged, stored at 4 °C, and air-dried at room temperature in the laboratory, while samples for microbial analyses were immediately stored at -18 °C before DNA extraction. Air-dried samples were sieved to 2 mm, and all major visible roots were removed. These samples were then ground to a fine powder and analyzed in duplicate for total C and N using an element analyzer (CE Elantech, Lakewood, NJ, USA). Soil organic (P_o) and inorganic (P_i) pools were determined by sequentially extracting with 0.5 M sodium bicarbonate ($NaHCO_3$, 1 g organic dry weight fresh soil in 45 mL solution) and 0.1 M sodium hydroxide solution (NaOH, 45 mL solution following Tiessen & Moir, 1993). Total P in both extracts ($NaHCO_3$-P_t and NaOH-P_t) was determined by measuring PO_4 according to the standard colorimetric method of (Murphy and Riley, 1962) after autoclaving extracted solutions with ammonium persulfate ($(NH_4)_2S_2O_8$) following Tiessen & Moir (1993). Inorganic P was also determined in the NaOH extract (NaOH-P_i) following Murphy and Riley (1962) after acidifying and centrifuging the extractant (Tiessen & Moir, 1993). Organic P in the NaOH extract (NaOH-P_o) was estimated by subtracting inorganic P from total P.

Soil pH was determined by creating a 1:1 soil to water solution, vortexing for 1 minute, then measuring the solution pH after 10 minutes (McLean, 1982). A second, separate field extraction was performed utilizing a 0.2 M sodium citrate and 0.05 M ascorbic acid (citrate-ascorbate) with a pH of 6 to provide an estimate of reducible short-range order Fe and Mn oxides and substituted Al oxides (Reyes and Torrent, 1997). Approximately 1.5 g of soil (oven-dry equivalent) was added to 45 ml of solution within 1 minute of sampling. Upon return to the lab, samples were shaken for 16 h, centrifuged at 1000 rcf for 20 min, and then decanted. Citrate-ascorbate extracts were then analyzed for Fe, Mn, and Al in triplicate via inductively coupled plasma optical emission spectroscopy (ICP-OES; Perkin Elmer Optima 5300 DV).

2.3.DNA extraction and quantitative PCR

The DNA was extracted from 0.25 g of the wet soil samples using the PowerSoil DNA Isolation kit (MO BIO Laboratories Inc., Carlsbad, CA, USA) according to the described manufacturer's protocol. The homogenization step was performed at 5000 rpm for 20 s using Precellys® 24 homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France). The extracted DNA was stored at −20 °C until further analyses. The quantity and quality of the extracted DNA were determined using the spectrophotometer Infinite M200 (Tecan AG, Grodig, Austria). The qPCR assays were performed using RotorGene® Q equipment (Qiagen, Valencia, CA, USA). The qPCR reactions were performed in 10 µL volume containing 5 µL Maxima SYBR Green Master Mix (Thermo Fisher Scientific Inc., Waltham, MA, USA), an optimized concentration of forward and reverse primers, 1 µL of template DNA and sterile distilled water. The gene-specific primer sets, optimized primer concentrations, and thermal cycling conditions for each target gene are shown in Table S1. All qPCR measurements were performed in triplicates. Standard curves for each target gene were prepared from serially diluted stock solutions of target sequences (Eurofins MWG Operon, Ebersberg, Germany).

The quantification data were analyzed with RotorGene Series Software v. 2.0.2 (Qiagen) and LinRegPCR program v. 2018.0 (Ruijter et al., 2009). The gene abundances were calculated as a mean of fold differences between a sample and each 10-fold standard dilution in respective standard as recommended by (Ruijter et al., 2009). The abundance of each target gene was presented as gene copy numbers per gram of dry soil (copies/g dw). qPCR was used for 16S

rRNA gene amplification to evaluate the abundance of bacterial and archaeal communities. For estimation of nitrification (bacterial *amoA*, archaeal *amoA*), denitrification (*nirK*, *nirS*, *nosZI*, *nosZII*), DNRA (*nrfA*), anaerobic ammonium oxidation (ANAMMOX-specific 16S rRNA) and N fixation (*nifH*), the respective functional genes were quantified using qPCR (Table S1).

2.4. Statistical analyses

Principal component analysis (PCA) was performed on environmental and genetic parameters using the R package ade4 v. 1.7–15 (Dray and Dufour, 2007). Spearman's rank correlation coefficients were used to assess the relationships between and among soil chemical parameters and target gene abundances. The p-values were adjusted for the false discovery rate by the Benjamin-Hochberg method with significance at $p < 0.05$. Since soil N and P content was normally distributed, one-way ANOVA followed by Tukey HSD test was used to analyze the differences between sites. All other parameters were not normally distributed, and therefore, the Kruskal-Wallis Test followed by Dunn's Test was used. The data analysis and figures were created using R 3.6.3 (R Team, 2021).

3. Results

3.1. Soil properties

The various land management practices in the Delta resulted in significant differences in soil properties. The difference was observable among wetland sites but also between wetlands and agricultural sites. Soil C% and N% was highest in West Pond, Mayberry, and corn where the mean values were for C were 17.0 ± 2.6%, 15.6 ± 3.6%, and 15.4 ± 1.1% and 1.09 ± 0.1%, 1.0 ± 0.25%, and 1.12 ± 0.08% for N, respectively. East End, Sherman Wetland, alfalfa, and pasture had significantly lower values (Tukey's HSD, $p<0.001$). Although the C% and N% were highly variable, the C/N ratio has a similar value among all sites and the mean values were between 13.1 and 16.7. A clear correlation between C% and N% was observed at all sites (Figure 1). In addition, both C% and N% were generally higher ($R^2 = 0.81$ and $R^2 = 0.85$, respectively) in older wetlands as well as in corn when compared with younger wetlands.

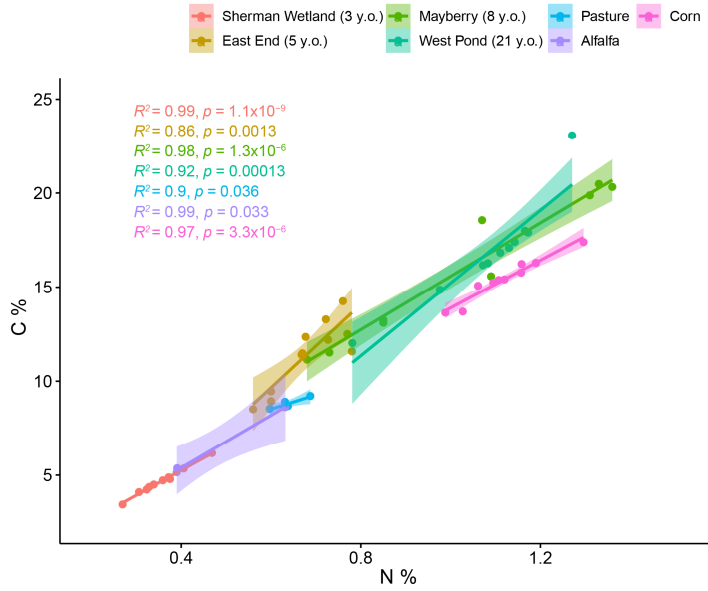

Figure 1. Percentage of soil C and N content at the restored wetlands, pasture, alfalfa and corn sites in the Delta determined from topsoil (0-15 cm) samples with 95% confidence intervals.

Soil pH varied across the wetlands and agricultural sites, where West Pond, East End, pasture, alfalfa, and corn were slightly acidic (mean values from 4.78 ± 0.16 to 5.78 ± 0.09) while Sherman Wetland and Mayberry were near-neutral (6.62 ± 0.25 and 6.93 ± 0.33, respectively; Figure S2-A). The Fe and Al concentrations divided the sites into two groups, where East End, pasture, alfalfa, and corn had almost two times higher concentrations than in Sherman Wetland, Mayberry, and West Pond (Figure S2-B, C). However, the concentration of Mn (Figure S2-D) was more variable, where the highest concentration was at the corn site (0.48 ± 0.1 mg g^{-1}) followed by Sherman Wetland (0.38 ± 0.03 mg g^{-1}). All other sites had mean concentrations between 0.17 and 0.34 mg g^{-1}.

Although there was no statistically significant difference in $NaHCO_3$-extractable P_i, the highest concentration was at the pasture (30.0 ± 6.0 µg g^{-1}), and younger wetlands showed slightly higher levels than older wetlands (Figure S1-A). $NaHCO_3$-P_o concentration was in a similar range at the pasture (31.2 ± 2.3 µg g^{-1}) and in East End (30.0 ± 9.4 µg g^{-1}), while all other sites showed significantly ($p<0.05$) lower concentrations (Figure S1-B). NaOH-P_i, associated with amorphous and crystalline Fe and Al minerals, was highest at East End (424.4 ± 105.8 µg g^{-1}), which is

253 partly situated on alluvium soil rich in Fe deposited by historic runoff from the northern Sierra
254 Nevada range (Graham and O'Geen, 2010). East End was followed by West Pond, which had
255 slightly lower concentrations (368.2 ± 161.2 µg g^{-1}). All other sites had significantly ($p<0.05$)
256 lower concentrations, staying between 152.2 ± 62.2 to 239.8 ± 82.6 µg g^{-1} (Figure S1-C). NaOH-
257 P_o was in a similar range at the corn site (248.8 ± 137.7 µg g^{-1}) and West Pond (263.0 ± 130.3 µg
258 g^{-1}), which were both significantly higher than all other sites, where mean values were between
259 69.4 ± 48.3 µg g^{-1} and 174.9 ± 160.6 µg g^{-1}. Also, it was notable that the concentration of NaOH-
260 P_o increased from younger wetlands to older wetlands (Figure S1-D).
261
262 **3.2. Abundance of soil prokaryotes**
263
264 The bacterial 16S rRNA gene abundance ranged from 4.89 x 10^9 to 7.98 x 10^{10} copies g^{-1} dry
265 weight (dw) across all studied sites (Figure 2-A) with significant differences between
266 ecosystems. Highest abundances were at the corn site (3.97 x 10^{10} ± 1.62 x 10^9 copies g^{-1} dw)
267 followed by Mayberry (2.73 x 10^{10} ± 1.45 x 10^9 copies g^{-1} dw) and West Pond (2.39 x 10^{10} ±
268 8.03 x 10^9 copies g^{-1} dw). The abundance of bacterial 16S rRNA genes was significantly
269 ($p<0.01$) lower in younger wetlands (Sherman Wetland and East End) and pasture. The archaeal
270 16S rRNA gene abundance (range from 1.81 x 10^8 to 1.28 x 10^{10} copies g^{-1} dw across all study
271 sites) was significantly higher in Mayberry (5.51 x 10^9 ± 3.13 x 10^9 copies g^{-1} dw), West Pond
272 (4.00 x 10^9 ± 1.01 x 10^9 copies g^{-1} dw) and corn (3.54 x 10^9 ± 1.99 x 10^9 copies g^{-1} dw) than in
273 East End, Sherman Wetland and pasture (4.13 x 10^8 to 1.51 x 10^9 copies g^{-1} dw; Figure 2-B).
274 Like the bacterial abundance, the abundance of archaeal communities was also highest in
275 Mayberry and West Pond wetlands and corn.
276
277 **3.3. Abundance of nitrogen cycling microbes**
278
279 The abundance of N transforming genes showed a lot of variation in different ecosystems. The
280 bacterial and archaeal *amoA*, *nirK*, *nirS*, *nosZI*, *nosZII,* and *nifH* genes were detected in all study
281 sites, whereas *nrfA* and ANAMMOX-specific 16S rRNA genes were absent at some sites (Figure
282 2 and 3).

283 The highest abundance of bacterial *amoA* genes was seen in Sherman Wetland ($1.94 \times 10^7 \pm 1.79$
284 $\times 10^7$ copies g^{-1} dw) and corn ($9.94 \times 10^6 \pm 8.53 \times 10^6$ copies g^{-1} dw). The abundance of archaeal
285 *amoA* genes was highest at the corn site ($1.63 \times 10^8 \pm 9.62 \times 10^7$ copies g^{-1} dw), where the
286 average abundance was up to three orders of magnitude higher than all other sites (Figure 2 C-D;
287 $p<0.001$). Overall, the abundance of archaeal *amoA* genes was significantly ($p<0.01$) higher than
288 bacterial *amoA* genes in all sites except Sherman Wetland where the abundance of bacterial
289 *amoA* genes was two orders of magnitude higher.
290 Of the genes coding for nitrite reductase in denitrification, *nirK* genes were overall more
291 abundant (2.07×10^8 to 5.87×10^9 copies g^{-1} dw) than *nirS* genes (2.02×10^7 to 3.59×10^8 copies
292 g^{-1} dw; Figure 2 E-F). In Figure 3-E, the abundance of *nirK* genes at the corn site significantly
293 ($p<0.001$) exceeded the abundance of *nirK* genes from other sites (Figure 2-E, Figure 3, Figure
294 S3) but also the abundance of all other N transforming target genes. Each of the *nir* genes
295 significantly ($p<0.01$) outnumbered *nrfA* genes that are responsible for the nitrate reduction to
296 ammonia. In addition, *nrfA* genes were not detected at East End and in more than half of the
297 West Pond soil samples (Figure 2-J). While *nir* genes were most abundant at the corn site, the
298 *nrfA* gene was most abundant at the Pasture site and then followed by the corn (Figure 2-J,
299 Figure 3). In wetland sites the abundance of *nrfA* gene was low and mostly associated with
300 Mayberry and Sherman Wetland (Figure 2-J). Both clades of N_2O reducers, harboring either
301 *nosZI* or *nosZII* genes, were present at all sites (Figure 2 G-H). However, the *nosZII* gene
302 abundance dominated over *nosZI* genes in all soil samples. It was also observed that the
303 abundance of *nosZII* genes showed an increasing trend among the wetlands from youngest to
304 oldest. When comparing the genes encoding N_2O reduction (*nosZI* + *nosZII*) to the genes
305 encoding denitrification nitrite reduction (*nirS* + *nirK*), the ratio was lower in Mayberry, West
306 Pond, alfalfa, and corn, whereas the ratio was significantly ($p<0.01$) higher at the Sherman
307 Wetland, East End, and pasture.
308 The abundance of *nifH* gene, which is the most widely used marker gene to identify N-fixing
309 bacteria and archaea ranged from 1.73×10^5 to 4.97×10^9 copies g^{-1} dw (0.004% to 6.23% of the
310 bacterial 16S rRNA bacteria) across the sites (Figure 2-I and Figure S3). The *nifH* gene was
311 significantly more abundant ($p<0.001$) at the wetland sites than in pasture, alfalfa, and corn
312 (Figure 3), however among the wetland sites, significant differences were only between
313 Mayberry and West Pond ($p<0.001$).

314 The abundance of the ANAMMOX-specific 16S rRNA genes from microbes responsible for
315 anaerobic ammonium oxidation (ANAMMOX) was low to absent at most sites except Mayberry
316 ($8.55 \times 10^4 \pm 1.30 \times 10^5$ copies g^{-1} dw). However, at Mayberry, the ANAMMOX-specific 16S
317 rRNA gene abundance was highest in the open water channels where the abundance was two
318 orders of magnitude higher (10^5 copies g^{-1} dw) than in the vegetated zone (10^3 copies g^{-1} dw). At
319 the corn site, one of the soil samples showed a very high abundance (2.87×10^5 copies g^{-1} dw)
320 that was comparable with the abundance seen in open water channel sediments at Mayberry.
321 While the average soil moisture at the corn site was 0.32 m^3 m^{-3}, the sample with the high
322 abundance of ANAMMOX-specific 16S rRNA genes showed moisture of 0.75 m^3 m^{-3}, which
323 probably created an anaerobic zone. From all other sites, there were two soil samples at the West
324 Pond and Sherman Wetland that also showed some abundance (in the range of 10^3 copies g^{-1}
325 dw), although all samples from these sites had similar moisture levels.
326

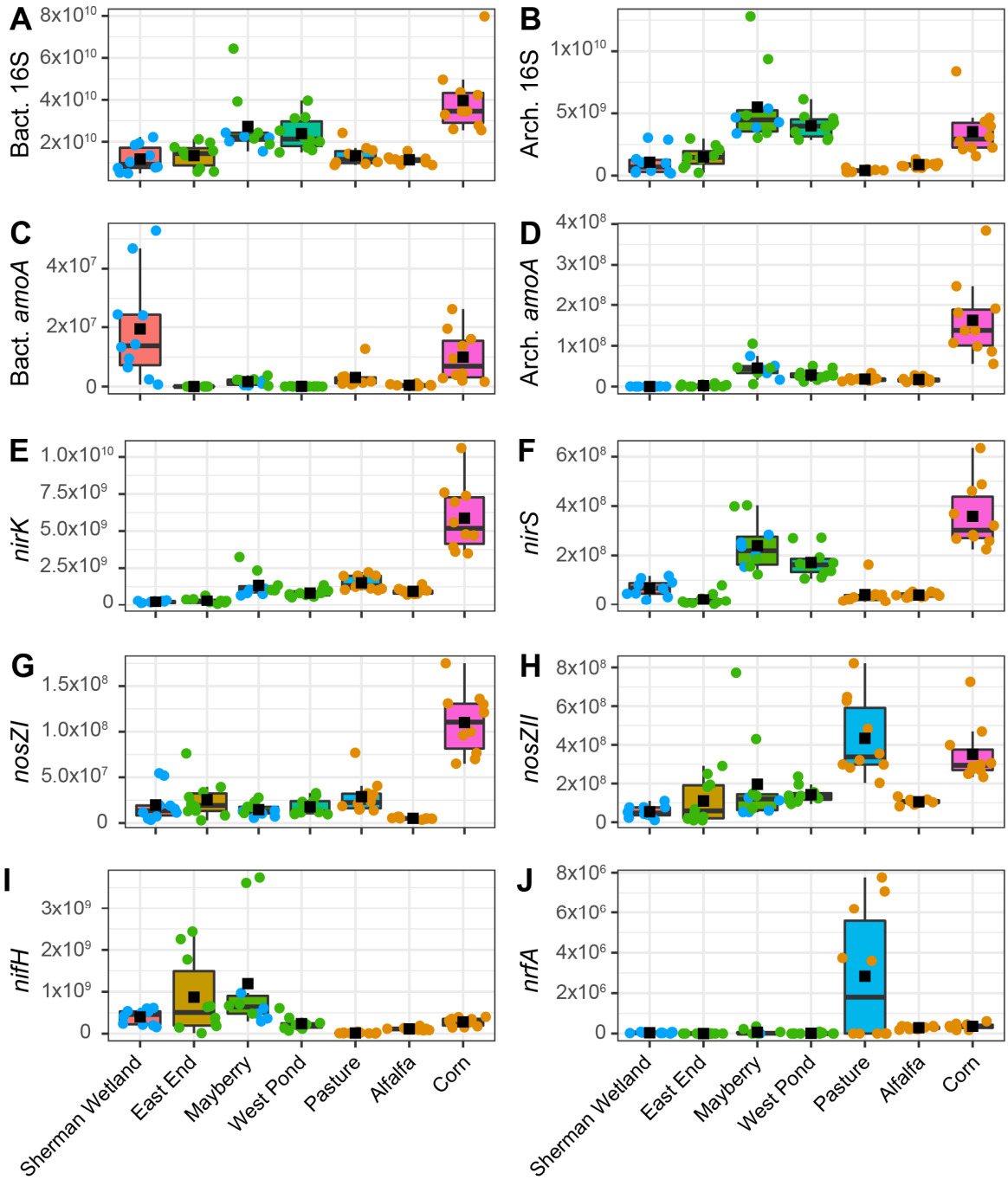

Figure 2. Box plots of the abundances (copies g^{-1} dw^{-1}) of the bacterial and archaeal 16S rRNA genes (A, B, respectively), as well as the measured functional genes (C-J) from the restored wetlands, pasture, and corn sites. The central line is the median, black square is the mean, edges of the box are the 25th and 75th percentiles and the whiskers represent the 95% confidence

interval. Blue, green and orange dots represent the environmental conditions where samples were collected.

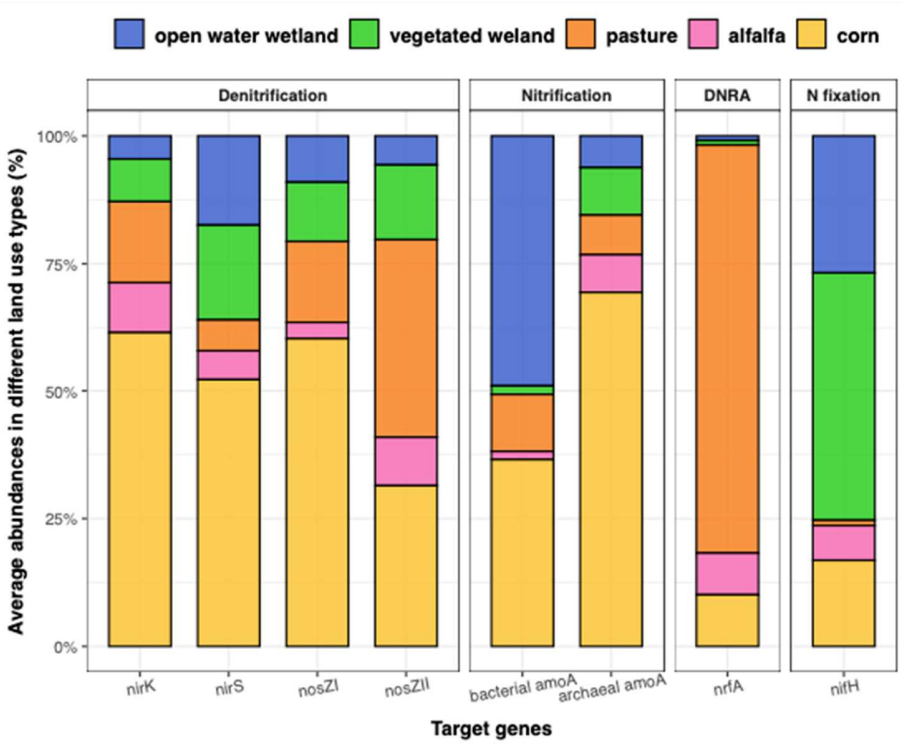

Figure 3. Average target gene proportions in percent in different land use types in the Delta.

3.3. Relationship between target genes and soil parameters

The principal component analysis (PCA) significantly differentiated pasture, corn, alfalfa, and wetland sites, while the difference between wetlands vegetated and open-water areas was less evident (Figure 4-A). However, differences were observable between older wetlands (8 and 21 y.o.) and younger wetlands (3 and 5 y.o.; Figure 4-B). Older wetlands and corn differed from pasture and younger wetlands mainly by soil C and N content and the abundance of bacterial and archaeal 16S rRNA, *nirS* and *nirK* genes. The abundance of *nosZI*, *nosZII*, bacterial *amoA* and *nrfA* genes were positively related with each other but at the same time negatively related to the C/N ratio and water table. The availability of Fe, Al and Mn was highest in the pasture site and showed negative relationships with the abundance of *nifH* genes and soil pH. Overall, the N transforming genes, particularly those responsible for denitrification, nitrification and DNRA

were highest in the agricultural sites, whereas N fixation and ANAMMOX was strongly associated with the wetland sites (Figure 4-C).

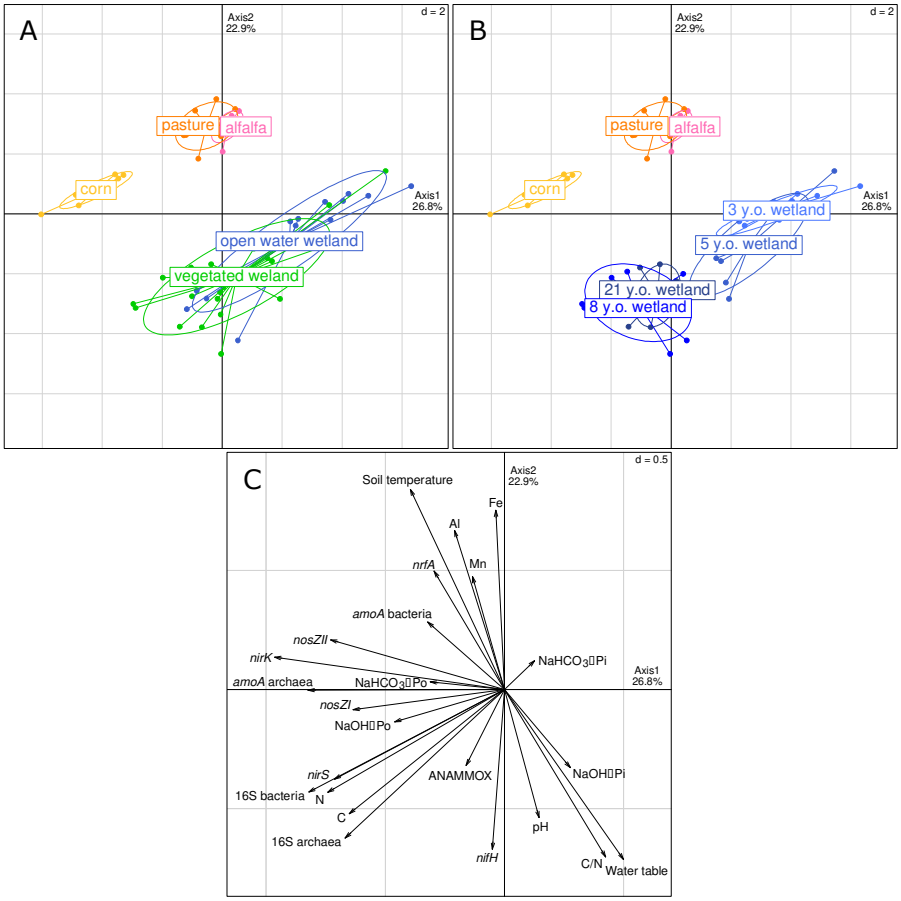

Figure 4. Ordination plots with 95% confidence ellipses based on principal component analysis (PCA) grouping sites and variables based on land management (A), the age of the restored wetlands (B), and soil physical, chemical and microbial parameters (C).

Spearman's rank correlation showed that bacterial *amoA*, archaeal *amoA*, *nirK*, *nirS*, *nrfA*, bacterial 16S rRNA and archaeal 16S rRNA genes had a significant positive correlation with soil pH in vegetated zones. In contrast, this correlation did not occur in open water zones, corn and pasture sites (Table S2). Soil C and N content had positive correlations with most of the N cycling genes in both vegetated and open water zones, but as like with pH, no correlation was observable in corn and pasture sites. In vegetated zones, Al had a significant negative correlation with *nirK*, *nirS*, bacterial *amoA*, archaeal *amoA* and archaeal 16S rRNA genes, while in open

364 water zones and agricultural sites, it did not affect the gene abundances. On the other hand, Fe
365 had a significant negative effect on the abundance of *nirK* and *nirS* genes in both vegetated and
366 open water zones. It also had a significant negative correlation with the abundance of bacterial
367 and archaeal 16S rRNA genes in vegetated zones. From analyzed P compounds, we detected that
368 $NaHCO_3$-P_i and NaOH-P_i had both significant negative correlations with archaeal *amoA* and
369 archaeal 16S rRNA gene abundances in vegetated zones. On the other hand, in open water zones,
370 both of these genes were positively correlated with NaOH-P_o (Table S2).
371

372 ## 4. Discussion

373 Nitrous oxide is the third most important well-mixed greenhouse gas, and its concentration in the
374 atmosphere is strongly related to land management. Here we analyzed how the abundance of N-
375 cycling genes changes from intensively managed agricultural sites to restored wetlands of
376 different ages in the Delta. Due to the extensive drainage, water table fluctuations, and fertilizer
377 use, many agricultural sites in the Delta have been revealed to be significant N_2O sources
378 (Deverel et al., 2017; Hemes et al., 2019; Teh et al., 2011). At the same time, restored and
379 natural marshes in the Delta have shown negligible N_2O emissions (McNicol et al., 2017; Pärn et
380 al., 2018) or even to be small sinks (Windham-Myers et al., 2018). Apart from management
381 practices, several studies have concluded that the most important physico-chemical factors
382 controlling N_2O emissions from drained organic soils are soil nitrate content, soil moisture, pH,
383 and temperature (Liang et al., 2018; Yang and Silver, 2016).

384 The abundance of denitrification genes had a clear correlation with soil C and N content, both of
385 which are important elements for denitrification. Another factor promoting denitrification in the
386 Delta soils is the mean air temperature, which was above 14°C in all studied sites in 2018. The
387 mean soil temperature even exceeded the mean air temperature at the dry and open water sites.
388 Braker et al., (2010) showed that denitrification activity increased linearly from 4 to 25°C in
389 incubated agricultural soils. They also noted that microbial activity did not increase after
390 incubation at 37°C compared with 25°C, indicating an optimum temperature between 25-35°C.
391 Other studies have also reported similar optimum temperatures (Kesik et al., 2006; Saad and
392 Conrad, 1993; Saleh-Lakha et al., 2009). While the mean annual temperature at the Delta is
393 below optimum, the summer and early autumn temperatures are significantly higher. For
394 example, topsoil temperature measurements before sampling from a weeklong period showed

that the average temperatures at the alfalfa, corn, and pasture were 25.5°C, 23.1°C and 22.9°C, respectively with a corresponding maximum temperature of 38.4°C, 48.5°C and 31.6°C. At the same time, wetland soils showed significantly lower temperatures where the average values were between 17.6°C to 20.5°C and maximum values between 18.1°C to 30.1°C. The highest temperature was recorded at Sherman Wetland with extensive open and shallow water areas. Therefore, the Delta agricultural soils provide ideal conditions for denitrification, while the wetland sites are below optimum. Similar temperature regimes have been shown by (Hemes et al., 2018b), where restored wetlands had the potential to cool daytime surface temperature by up to 5.1°C as compared to a dominant drained agricultural land use. Hence, the wetlands systems can inhibit denitrification activity with lower temperatures.

In addition to temperature, soil pH is another factor affecting successful denitrification (Baggs et al., 2010). Previous studies have shown that the optimal pH for denitrification is between 7.0 to 8.0 (Knowles, 1982; Saleh-Lakha et al., 2009). The pH at the Delta sites was lower, with the highest values at Mayberry and Sherman Wetland, where the mean was close to 7. On the other hand, agricultural sites showed a more acidic condition, where the mean pH values were between 4.78 to 5.55, being lowest at the pasture site. Many studies have reported that acidic soils have lower relative N_2O reduction activities, resulting in higher N_2O/N_2 product ratios and, therefore, promoting N_2O emission from soils (Liu et al., 2010; ŠImek and Cooper, 2002; Dörsch et al., 2012).

The abundance of denitrifying genes *nirK* and *nirS* was highest at the corn site, indicating a high N_2O production and emission potential. High N_2O emissions from corn and alfalfa sites at the Delta have been shown by Hemes et al., (2019), where the mean annual emissions were 3.28 ± 0.12 g N_2O-N m^{-2} yr^{-1} and 0.51 ± 0.07 g N_2O-N m^{-2} yr^{-1}, respectively. N_2O measurements over alfalfa and corn fields in Canada showed similar results where corn was almost four times higher source than alfalfa (Wagner-Riddle et al., 2011). On the other hand, study carried out by McNicol et al., (2017) showed that Mayberry wetland has negligible N_2O emissions. Overall, from the abundance *nir* genes, the abundance of *nirK* gene significantly outnumbered the abundance of most N cycling genes in studied ecosystems and was especially high at the corn site (Figure S3). At the corn site, even the abundance of *nosZI* and *nosZII* genes was also relatively high. Jones et al., (2013) also saw that both clades of *nosZ* genes were found in different environments, including lake sediments and drained agricultural soils. The high

quantity of denitrification genes at the corn site could be related to the irrigation systems and winter flooding practices that create occasionally or seasonally wet and anaerobic conditions. The specific corn cultivation cycle is often divided into three periods in the Delta: a short growing season followed by fallow and flooding (for wintertime bird habitat). Flooding has been shown to temporarily significantly increase N_2O emissions (McNicol and Silver, 2014; Anthony et al., in prep.). Therefore, flooding could provide suitable anaerobic conditions for microbes with *nosZI* and *nosZII* genes and potential N_2O reduction to N_2 for a third of the year. However, the ratio of *nosZ* (*nosZI* + *nosZII*) genes to *nir* (*nirK* + *nirS*) genes was very low at the corn site compared with all other sites, indicating that incomplete denitrification and high N_2O emissions are more likely to take place than complete denitrification (Ligi et al., 2014). Studies have also shown that the N_2O sink capacity increases with the ratio of *nosZII*/*nosZI* abundance (Jones et al., 2014), suggesting that the pasture and Mayberry sites might have the highest potential for N_2O reduction to N_2, while Sherman Wetland and corn probably have the lowest. Jones et al., (2014) also noted that *nos*Z clade II genes were influenced more by low pH than *nosZ* clade I genes, which was observable at the corn site where *nosZII* gene abundance was significantly lower than *nosZI* gene abundance.

The abundance of nitrification genes was also notable at the Delta sites. AOB had a lower abundance in most of the sites than AOA, except in the Sherman Wetland. The lower abundance of AOB in wetland sediments has been shown by many other studies (Cao et al., 2011; He et al., 2018), which could be related to the lower dissolved oxygen concentration in wetlands with low water flow rates (Kayee et al., 2011; Limpiyakorn et al., 2013). Sherman Wetland with large shallow open water areas is more aerated and provides a suitable habitat for AOB. In addition, the average ratio of AOB to AOA was also significantly higher in Sherman Wetland compared with other sites. Some authors have shown that AOA prefers low-nutrient soils, whereas AOB is dominant in high-nutrient soils (Pratscher et al., 2011; Verhamme et al., 2011). Surprisingly in our case, AOB was highly abundant in a Sherman Wetland, which had the lowest amount of N and P. On the other hand, AOB prefers soils with a lower C/N ratio, as was seen in Sherman Wetland, which was one of the first islands in the Delta that was drained and used for cultivation and has the lowest layer of organic soils. This has resulted in fast and extensive decomposition and loss of C. All other wetland and agricultural sites experienced higher C/N ratios and hence

456 lower AOB abundances. Similar results have been shown by Regan et al., (2017) in grassland
457 soils and by Truu et al., (2020) in forest soils.

458 The abundance of *nrfA* gene responsible for the DNRA process was highest at the pasture site
459 and almost absent at the West Pond and East End. The high abundance of *nrfA* gene in pasture
460 soils is recently described by Friedl et al., (2018). They showed that high labile C availability
461 under perennial pasture upon re-wetting increases heterotrophic soil respiration, reduces the soil
462 redox potential, and shifts NO_3^- consumption from denitrification to DNRA. Therefore, re-
463 wetting, which happens during late summer at the pasture, might be the main driver for the
464 DNRA. However, the pasture site also had a relatively high concentration of Fe, and a recent
465 study by Robertson et al., (2016) showed that DNRA might be linked to ferrous iron oxidation;
466 nevertheless, this process has not sufficiently studied in natural ecosystems. Re-wetting of the
467 fallow field in autumn could also explain the abundance of *nrfA* gene at the corn site, which also
468 has a high availability of C. On the other hand, the ratio of *nrfA*/*nir* was significantly higher at
469 the pasture than corn site, which indicates that DNRA could be more likely at the pasture site
470 following re-wetting. Putz et al., (2018) noted that DNRA was lower in more fertilized soils,
471 which would explain the difference at the nutrient-rich corn site compared to pasture.

472 The abundance of ANAMMOX bacteria was highest in the Mayberry open water channels,
473 which are much deeper than the surrounding vegetated zones. Deeper zones in the wetland could
474 provide more consistent anaerobic conditions as vegetated areas will transport oxygen to the soil.
475 Mayberry is also occasionally affected by saltwater intrusion, and since ANAMMOX bacteria
476 are adapted to saline conditions, this condition could also favor their abundance. For example,
477 Dapena-Mora et al., (2007) showed that higher concentrations of NaCl freshwater did not inhibit
478 ANAMMOX bacteria and Windey et al., (2005) indicated that freshwater ANAMMOX strains
479 could gradually adapt to high salt contents in water. Although the ANAMMOX bacteria were
480 almost absent at the corn site, they were found in one sampling spot with high soil moisture
481 content. This could indicate that when the corn site is flooded in the autumn, it can provide a
482 suitable environment for the ANAMMOX bacteria. Another possible cause for low abundance is
483 that only the top layer of the soils was sampled (0-15 cm), which would miss ANAMMOX
484 bacteria if more abundant in deeper layers. For example, Humbert et al., (2012) indicated that
485 ANAMMOX bacteria were few or absent in the topsoil (0-10 cm) but increased significantly
486 with soil depth. Previous studies have indicated that oxygenation of topsoil's, competition for

inorganic N by plants, or heterotrophic nitrate- and nitrite-reducing bacteria can limit the abundance of ANAMMOX bacteria in upper soil layers (Xu et al., 2011).

The high abundance of *nifH* genes in the wetlands indicated that microbial N-fixation is an important process for acquiring N. The abundance of *nifH* genes is often high in presence of *Azolla* spp. (mosquito fern) (Ekman et al., 2008), which is found extensively in the Delta wetlands (Miller and Fujii, 2010; Valach et al., 2021). The symbiotic relationship with the cyanobacterium *Anabaena azolae* and *Azolla* spp. can fix large amounts of atmospheric N (Carrapiço, 2010), providing additional input to the system. East End and Mayberry have shown the highest growth of *Azolla* and represent the highest abundance of the *nifH* marker gene used to identify N-fixers (Silva et al., 2013). At the time of sampling, all the open water patches at East End were covered with floating *Azolla* mats, while at Mayberry, the mats were more common in areas with slow-flowing water, such as within vegetation stands. Since *Azolla* are found in still water with little flow (Trindade et al., 2011), there were few mats at Sherman Wetland, as the large open water areas are frequently disturbed by the wind. On the other hand, West Pond has very dense vegetation, making it also a less preferred habitat for *Azolla*, consistent with lower *nifH* gene abundance. The corn site showed a lower abundance of *nifH* genes, most likely due to the already available N that is added to the field during fertilization as well as the lack of symbiosis with N fixing bacteria. Although the *nifH* gene abundance was also relatively low in alfalfa soil samples, we can still estimate that close to the root zone, the abundance is probably slightly higher as alfalfa is yielding 5 - 7 harvests in a single year in a very poor soil condition. As the soil is low in both C and N, then the N fixation by microbes and mineral fertilizer addition is essential for a high production rate. At the same time frequent harvesting does not allow plants to enrich the soil with C and N; therefore, growing any other crop is challenging. Low N content in the soil probably also limited denitrification and N_2O production, which is concordant with (Rochette et al., 2004). Another factor that could reduce the abundance of *nifH* gene abundance in alfalfa soils is relatively low pH. The major process leading to low pH during N cycling in soils is the imbalance of cation over anion uptake in the rhizosphere of plants that are actively fixing N_2 (Bolan et al., 1991). When pH decreases below 6.5, it will reduce the ability of alfalfa plants to fix N_2 (Rice et al., 1977). At the pasture site, the soil pH was even lower than at the alfalfa and corn site, with a mean value of 4.8. Therefore, the low *nifH* gene abundance at the

517 pasture site will show that atmospheric N fixation is negligible, and cattle grazing could be
518 currently the main N input source (Wang et al., 2019).

519 Another important factor controlling N_2O emissions is the soil C/N ratio. According to several
520 studies, the highest N_2O emissions occur when soil C/N ratio is between 10-20:1, indicating that
521 Delta soils, especially corn, have a high potential to emit N_2O (Klemedtsson et al., 2005; Mu et
522 al., 2014; Pärn et al., 2018). Drained soils provide ideal conditions for corn cultivation as C and
523 N content is high. However, due to the aerobic conditions, organic matter and N will be lost as
524 CO_2 (oxidation) and N_2O (incomplete denitrification), and what is left is acidic ecosystem with
525 low nutrient and C content, which can be used either for alfalfa cultivation or for pasture (e.g.
526 Sherman pasture before restored to Sherman Wetland; Baldocchi et al., (2012). Therefore, when
527 drained and still rich in C, the agricultural sites are significant sources of CO_2 and N_2O (Hemes
528 et al., 2019). On the other hand, based on the abundance of N transforming genes, we saw that
529 wetland restoration will reduce the N_2O production potential. Hence, the wetland restoration can
530 be suggested as an important climate mitigation measure to capture C and reduce the N_2O
531 emissions. However, once wetland restoration projects are completed, they must be maintained
532 to avoid potentially large N_2O emissions (Lugato et al., 2018), as these systems are also effective
533 to fix atmospheric N_2.
534

535 ## 5. Conclusions

536

537 Drained and fertilized agricultural sites are globally important N_2O sources, and their
538 contribution to emissions of this ozone-depleting greenhouse gas is increasing annually as more
539 land is converted for agricultural use. Our research has shown that the highest N_2O production
540 potential in the studied Delta ecosystems is from the intensively managed organic-rich
541 agricultural sites. The abundance of *nirS* and *nirK* genes at the organic-rich corn site
542 significantly exceeded the abundance at all other sites. At the same time, the abundance of *nosZ*
543 genes at the corn site were significantly outnumbered by *nir* genes. This clearly shows that Delta
544 agricultural sites rich in organic compounds have a high potential to be large N_2O sources,
545 exacerbating negative climate effects. Our results also confirmed the second hypothesis that
546 wetland restoration would increase the abundance of N_2O reducers and decrease the abundance
547 of ammonia oxidizers, leading to lower N_2O production potential. In addition, wetlands will help

keep the soil temperatures much lower than agricultural sites, reducing the potential N_2O production as temperatures are below optimum for denitrification and nitrification even during the hottest days in summer. Therefore, wetland restoration is a promising solution to mitigate N_2O emissions from intensively managed arable lands.

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All Delta sites used in this analysis are part of the Ameriflux network, with data available at http://ameriflux.lbl.gov/.

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