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No Evidence for Long-term Impacts of Oil Spill Contamination on Salt Marsh Soil Nitrogen Cycling Processes

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Abstract

Salt marshes are important sites of nitrogen cycling and removal that straddle the land/ocean interface, allowing them to intercept human-derived nitrogen before it reaches coastal waters where it causes problems like hypoxia and harmful algal blooms. In 2010, the Deepwater Horizon oil spill released an estimated five million barrels of crude oil into the Gulf of Mexico, significantly contaminating coastal wetlands over approximately 800 km of shoreline. We investigated microbial nitrogen cycling processes in soil from four salt marshes in Terrebonne Bay, Louisiana, USA that were either exposed or not exposed to Deepwater Horizon oil over the course of 1 year (2013–2014), 2.5–3.5 years post-spill. Specifically, we measured nitrification and denitrification potentials, nitrogen cycling functional gene abundances (*nir*S, bacterial and archaeal *amo*A), and soil physical and chemical properties. We show that variation in nitrification and denitrification potentials was independent of site oil exposure. Large year-to-year differences in springtime nitrification potentials were inversely related to plant live belowground biomass, indicating that competition for nitrogen is likely an important control on nitrification. There were positive correlations between nitrification potentials and both soil extractable nitrate concentrations and denitrification potentials, supporting the idea that denitrification is coupled with nitrification. We found no evidence that there was a long-term impact of oil exposure on salt marsh soil microbial nitrogen cycling processes and the nitrogen removal ecosystem service they provide. It is important to note, however, that these impacts could have been masked by high background variability in process rates or loss of oil exposed soil to coastal erosion.

Keywords Salt marsh · Nitrogen cycle · Oil spill · Nitrification · Denitrification

Introduction

Human activities such as the widespread application of industrial fertilizer, nitrogen fixation by crops, and fossil fuel combustion add a total of approximately 213 Tg N year^{-1} to the biosphere, nearly doubling the global natural rate of biological nitrogen fixation of 250 Tg N year^{-1} (Fowler et al. 2013; Peñuelas et al. 2013). Roughly 18% of this anthropogenic nitrogen leaches into rivers (Peñuelas et al. 2013), more than doubling the amount of nitrogen flowing from land to sea

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(Gruber and Galloway 2008). This anthropogenic nitrogen flux causes problems such as eutrophication (Ryther and Dunstan 1971), hypoxia (Diaz and Rosenberg 2008; Rabalais et al. 2014), and harmful algal blooms (Glibert et al. 2005) in the coastal ocean. Globally, the most important sink for this anthropogenic nitrogen is denitrification (Gruber and Galloway 2008), a microbial process that converts bioavailable nitrate (NO_3^-) into inert nitrogen gas (N_2), thereby removing it from the biosphere.

Salt marshes are globally distributed coastal wetlands that straddle the land/ocean interface, positioning them to intercept anthropogenic nitrogen that enters the coastal ocean via riverine or groundwater discharge (Hopkinson and Giblin 2008). Nitrogen removal occurs either through sedimentation and burial (2.6–5.7 Tg N $year^{-1}$) or denitrification (~ 1.3 Tg N $year^{-1}$), together accounting for one-third of riverine nitrogen loading to temperate coastlines occupied by salt marshes (Hopkinson and Giblin 2008). Denitrification is typically limited by the availability of NO_3^- substrate, which in marsh soil is primarily supplied by nitrification, the microbial oxidation of ammonium (NH_4^+) to nitrite (NO_2^-) and then NO_3^-. This coupling between nitrification and denitrification is necessary to maintain N_2 production in salt marshes (Reddy et al. 1989; Risgaard-Petersen and Jensen 1997).

Wetlands, and the microbial processes they support, are vulnerable to human disturbances such as oil pollution. In 2010, the Deepwater Horizon oil spill released an estimated five million barrels of crude oil into the Gulf of Mexico (Natter et al. 2012), contaminating coastal wetlands with oil over roughly 800 km of shoreline (Michel et al. 2013). Small-scale laboratory incubations aimed at understanding the immediate impacts of oil exposure on nitrogen cycling processes in coastal soils and sediments have so far been inconclusive, showing, for example, that denitrification rates were inhibited (Bonin et al. 1990; Levine et al. 2017), unaffected (Kleinhuizen et al. 2017; Shi and Yu 2014), or stimulated (Horel et al. 2014; Ribeiro et al. 2016) by oil exposure. Furthermore, these experiments are limited by their inability to account for the important influence that larger-scale processes such as root oxygenation (Koop-Jakobsen et al. 2017), plant nitrogen uptake (Buresh et al. 1981; Smith and Delaune 1985), and bioturbation (Dollhopf and Hyun 2005) can have on soil nitrogen cycling process rates.

Marton et al. (2015) used pairs of oil-exposed and unoiled reference sites at salt marshes in southern Louisiana to investigate the influence of oil on salt marsh nitrogen cycling processes within the context of the whole ecosystem. They found no evidence for "legacy" effects of oil exposure on nitrification potentials 1.5–2.5 years after the Deepwater Horizon spill. In this study, we extended these measurements at a subset of their field sites in Terrebonne Bay for another year and added denitrification potential measurements. There were two main objectives of this study. The first was to collect a longer time series of data over multiple years to determine whether there were any longer-term impacts of oil contamination on salt marsh soil processes. It also allowed us to quantify the seasonal and year-to-year variability in environmental conditions to determine whether this variability could have masked any oil-driven alterations of the nitrification potentials measured by Marton et al. (2015). The second objective of this study was to determine whether past oil exposure impacted denitrification potentials in salt marsh soils rather than focusing on nitrification alone. This allowed us to investigate a fundamentally different microbial process that may respond differently to oil exposure. It also provided the opportunity to explore nitrogen cycling and removal in salt marsh soils more generally. We hypothesized that there would be no difference between the nitrification and denitrification potentials measured at sites with a history of oil exposure and those measured at unoiled reference sites.

Methods

Site Description

Our study sites were located within the *Spartina alterniflora*–dominated salt marshes of Terrebonne Bay on the southern coast of Louisiana, USA and described in previous studies (Hill and Roberts 2017, Marton and Roberts 2014, Marton et al. 2015). This region experiences diurnal tides of low amplitude (~ 0.3 m; Hill and Roberts 2017), with inundation patterns often driven by wind rather than tide (Turner 2001). At each site, samples were collected along a transect at distances of 5, 10, 15, and 20 m from the edge of the marsh towards the interior. Marsh elevation increased gradually by ~ 12 cm along these transects from the marsh edge towards the interior (Marton et al. 2015). Water temperatures and salinity in the region ranged between 7 and 32 °C (mean: 23 °C) and 9–27 (mean: 18), respectively during 2013–2014 (CRMS 0355; https://www.lacoast.gov/crms_viewer2/Default.aspx#). One pair of sites located at Bay LaFleur (TB1 and TB2) was separated by around 6 km from the other pair of sites at Lake Barre (TB3 and TB4). Paired sites were separated from each other by less than 1.5 km.

Deepwater Horizon Oil Contamination History

One site from each pair was unoiled (TB1 and TB4) while the other site was oiled (TB2 and TB3). Oiled sites received oil in late summer and fall 2010 following the Deepwater Horizon oil spill. Our study took place over the course of 1 year from spring 2013 to spring 2014, ~ 2.5–3.5 years following the oiling event. Data from 2012 (~ 1.5–2.5 years post-oiling; Marton et al. 2015) are also included in our analysis. Oiled marshes were identified using Shoreline Cleanup and

Assessment Technique (SCAT) maps (http://www.noaa.gov/deepwaterhorizon/maps/traj_maps.html) and were verified as having detectable quantities of Macondo oil in the top 5 cm, whereas no Macondo oil was detected in the unoiled marshes (Turner et al. 2014). In 2011, oiled marsh sites had target polycyclic aromatic hydrocarbon (PAH) concentrations of $19{,}524 \pm 2158$ μg kg^{-1} (Turner et al. 2014). However, by April 2014, PAH concentrations at oiled sites had decreased substantially (183 ± 24 μg kg^{-1}), making them indistinguishable from those at unoiled sites (164 ± 21 μg kg^{-1}) (Ashton-Meyer 2017).

Field Sampling

Samples were collected approximately every other month starting in March 2013 and concluding in April 2014 for a total of seven sampling events, approximately 2.5–3.5 years following exposure to oil from the Deepwater Horizon spill. This sampling frequency was chosen to capture sub-seasonal temporal variability over a complete annual cycle. At each location along the transects at each of the four sampling sites, two 6.7 cm diameter cores were collected to a depth of 5 cm using a clear acrylic tube with beveled edges. The entire top 5 cm of soil collected in each core was used in all analyses described below. The first core was used to measure soil chemical and physical properties. The second core was used to measure nitrification and denitrification potentials and microbial abundances. Soil samples were stored in Whirl-Paks and kept cool and in the dark until they were returned to the laboratory where they were processed within 24 h. Soil temperature and redox potential (Eh) were measured using a handheld thermometer and probe (Orion Star A221 pH meter with Orion Sure-Flow Combination Redox/ORP electrode, Thermo Fisher Scientific), respectively, that were inserted directly into the soil. The salinity and temperature of the adjacent bay water were measured using a handheld meter (YSI 30). Two hundred and fifty milliliters bay water samples were collected, stored in the dark on ice, and filtered through acid-washed (10% v/v HCl) 0.2 μm filters (Pall Life Sciences Supor®-200) within 2 h of collection. Filtrate was stored frozen until nutrient analysis.

Water Analyses

Dissolved inorganic nutrient concentrations were measured in bay water and all other water samples described below using a Lachat Instruments QuickChem® FIA+ 8000 Series Automated Ion Analyzer with an ASX-400 Series XYZ Autosampler as detailed in Marton et al. (2015). Samples were analyzed simultaneously for dissolved NO_x (nitrate + nitrite) using Cu-Cd reduction followed by azo colorimetry and for PO_4-P using the automated ascorbic acid reduction method. NH_4-N was analyzed separately using phenate colorimetry to prevent contamination of the samples by fumes from the NH_4Cl buffer used in NO_x analysis (Greensberg et al. 1992). Standard curves were prepared using standard PO_4-P, NO_3-N, and NH_4-N stock solutions (Hach, Loveland CO) and yielded r^2 values of ≥ 0.99.

Soil Analyses

Soil water content was measured by weighing a field-moist soil subsample (~ 5.0 g), placing the soil subsample into an aluminum weigh boat, drying the soil to a constant weight at ~ 90 °C, and reweighing the soil. For extractable nutrient analyses, 2–3 g of soil was added to each of two 50-mL centrifuge tubes, one tube for extractable dissolved inorganic nitrogen (DIN), the other for dissolved inorganic phosphorus (DIP). Thirty milliliters of 2 N KCl was added to the DIN tube, and it was shaken at 250 rpm for 2 h. The DIN tube was then centrifuged and the supernatant was 0.2 μm filtered and stored frozen until analysis. Thirty milliliters of 0.5 M $NaHCO_3$ was added to the DIP tube and it was shaken at 250 rpm for 16 h. The DIP tube was then centrifuged, and the supernatant was 0.2 μm filtered and stored frozen until analysis. The remaining soil was dried, ground with a mortar and pestle, and passed through a 2-mm mesh sieve. Subsamples of dried soil were placed in a glass desiccator with fuming HCl for 24 h to remove inorganic carbon. Total organic C and total N were analyzed using a Flash 1200 Elemental Analyzer (CE Elantech, Lakewood, New Jersey). Concurrently measured sediment standards (National Institute of Standards and Technology, Buffalo River Sediment, 2704) yielded mean ± standard error organic carbon recoveries of $99.8 \pm 0.3\%$ (n = 10).

Nitrification Potentials

Nitrification potential incubations were carried out as described previously by (Marton et al. 2015). Briefly, the incubation medium was prepared by diluting NO_x-free, filtered seawater with deionized water to match the salinity of bay water measured in the field. NH_4Cl and a 1:1 mix of K_2HPO_4 and KH_2PO_4 were added to final concentrations of 300 μmol L^{-1} N and 60 μmol L^{-1} P. The soil was then homogenized within each Whirl-Pak and 2–3 g of soil from each bag was placed into three 50-mL centrifuge tubes. Thirty milliliters of salinity-adjusted estuarine-water medium was added to each centrifuge tube. Tubes were incubated on an orbital shaker at 325 rpm in the dark at a constant laboratory temperature of 21 °C. One tube from each set of three was sacrificed at 24, 48, and 72 h. These samples were centrifuged, filtered through 0.20 μm syringe filters (Corning, #431224, Corning, NY), and stored frozen until analysis for NO_x-N concentration as described above. Nitrification potentials were calculated as the linear slope of NO_x-N concentration regressed against

time. These potentials were corrected for incubation water volumes and soil dry weight and are expressed in units of nmol N gdw^{-1} d^{-1} (where gdw is grams dry weight).

Denitrification Potentials

An estuarine water incubation medium was prepared by diluting filtered seawater with deionized water to match the salinity of bay water measured in the field. D-glucose ($C_6H_{12}O_6$), potassium nitrate (KNO_3), and potassium phosphate (KH_2PO_4) were added to the medium to final concentrations of 15 mmol C L^{-1}, 2000 μmol N L^{-1}, and 200 μmol P L^{-1}. Twenty grams of field-moist soil was weighed into a 125 mL flask. Twenty milliliters of the estuarine water medium was added to each flask and flasks were sealed with Suba-Seals. The flask headspace was then flushed with ultra-high purity He and 10 mL of rinsed acetylene (C_2H_2) was added to the headspace. Flasks were incubated on a shaker table at 250 rpm in the dark and at a constant laboratory temperature of 21 °C. Flask headspace subsamples were collected at 0.5, 1, 2, and 4 h. At each time point, 10 mL of headspace gas was collected into a syringe and injected into a 5.9 mL Exetainer (Labco, UK) that had been pre-purged with helium. Nine milliliters of helium and 1 mL of C_2H_2 was injected into the flask to maintain constant headspace composition and pressure throughout the incubation. Nitrous oxide (N_2O) concentrations were measured using a Shimadzu GC-2014 equipped with electron capture detector. Denitrification potentials were calculated as the linear slope of N_2O-N concentration regressed against time. These potentials were corrected for incubation headspace volumes and soil dry weight and are expressed in units of nmol N gdw^{-1} d^{-1}.

Microbial Abundances

DNA was extracted from sediments using the PowerSoil DNA Extraction kit (MoBio) following the manufacturer's directions. DNA quality and quantity were assessed by spectrophotometric measurements using a Nanodrop (ThermoFisher, Waltham, MA). Ammonia-oxidizing archaea (AOA) and bacteria (AOB) were quantified by QPCR of archaeal and betaproteobacterial *amo*A genes as previously described (Bernhard et al. 2016). Denitrifying bacteria were quantified by QPCR of *nir*S genes as described in Bernhard et al. (2015). Methane-oxidizing bacteria (MOB) were quantified by QPCR of the *pmo*A gene using primers pmo189F and mb661R (Tavormina et al. 2008). Methane-oxidizing bacteria were included in this analysis because of their ability to oxidize ammonium (Bédard and Knowles 1989), making them relevant to our study of nitrogen cycling processes in salt marsh soil. All 20 μl reactions contained 10 μl of 2× iQ Sybr Green Mix (Bio-Rad, Hercules, CA), 10 μM of each primer, 0.008% BSA, and ca. 1–10 ng of template DNA. The following cycle conditions were used to amplify the *pmo*A genes: 45 cycles of 95 °C for 15 s, 58 °C for 20 s, 72 °C for 45 s. All QPCRs were run on either an iCycler (Bio-Rad) or a CFX Connect (Bio-Rad). Standard curves were generated using a plasmid DNA containing a copy of the *pmo*A gene with concentrations ranging from 1 pg to 0.1 fg. QPCR efficiencies for each gene were: 105% for archaeal *amo*A, 85.7% for betaproteobacterial *amo*A, 85.5% for *nir*S, and 88.7% for *pmo*A. Specificity of each reaction was assessed by melt curve analysis and run in duplicate to control for pipetting errors.

Additional Data Sources

Nitrification potentials, microbial abundances, and soil chemical characteristics were measured at the same sampling sites in 2012 (Marton et al. 2015). We compare our 2013–2014 data with these 2012 data and include the 2012 data in our analyses in every analysis described below where sufficient, comparable data was collected in 2012. Live plant belowground biomass data were reported by Hill and Roberts (2017) in a separate study focused on interannual variability in plant primary production. The study periods and purpose did not align perfectly, so plant data are only available from May 2013 on and were not collected at the same time as the soil data reported in this study. Plant data were collected 20 m from the marsh edge at a location ~ 15–35 m away from our transects at each of the four sampling sites. As such, plant data cannot be directly compared with soil parameters and processes reported here. Rather, they provide valuable context about what was happening with the plant communities in the region during a portion of our study period. Hourly hydrographic data from the Coastwide Reference Monitoring System (CRMS) site 0355 were retrieved from the Coastal Protection and Water Quality Authority website (https://cims.coastal.louisiana.gov/monitoring-data/default.aspx) on December 19, 2017. This site is located ~ 1 km east of the TB4 sampling site. Continuous Mississippi River monitoring data from USGS station 07374525 in Belle Chase, Louisiana was retrieved from the USGS website (https://waterdata.usgs.gov/la/nwis/uv/?site_no=07374525&PARAmeter_cd=00065,72020,63160,00060) on March 28, 2018. This station was selected because it is nearest the discharge point of the main channel of the Mississippi River.

Relative Elevation

Marton et al. (2015) measured the relative elevation of the marsh surface at each of our four sampling sites. Relative elevation was calculated on each of ten visits to each site by measuring the height of water above each sampling location along a transect when all locations were inundated and then setting the lowest site to a relative elevation of zero. It is important to note that these are not absolute elevations. Because relative elevation measurements were made at different times at each of the four sites, they cannot be directly compared between sites. They can

be used to assess how other variables change with respect to elevation within the same marsh, however.

Statistical Analyses

Statistical analyses were performed using the stats package in base R (R Core Team 2017). Because the environmental variables measured in this study are bounded by zero on the low end, they had non-normal distributions that were positively skewed with varying degrees of intensity. Nonparametric statistical analyses were used for this reason. The Mann-Whitney U test (wilcox.test function from the R stats package) was used to test whether two groups of data (i.e., potential nitrogen cycling rates at oiled versus unoiled sites) were significantly different. Correlations between two variables were tested using Kendall's tau rank correlation coefficient. Significance values for all tests were set to the $p < 0.025$ level.

A generalized linear model was used to visually express correlations between variables and was implemented with the "geom_smooth" function of the ggplot2 (Wickham 2016) R package (method = glm, family = inverse.gaussian). A multivariate approach was also used to test for differences in soil properties between sites. Specifically, a principle component analysis (PCA) was performed on a subset of soil data (temperature, Eh, water content, organic carbon, total nitrogen, extractable NO_x and NH_4^+, nitrification and denitrification potentials, and AOA, AOB, and nirS abundances) in which missing values were replaced with the average value for that parameter to allow use of all samples collected without skewing the results. An analysis of similarity (ANOSIM) was then performed on the same soil dataset using the vegan R package (Oksanen et al. 2019) to determine how different sites were from one another with respect to these soil properties.

Results

Within-Site Spatial Variability in Soil Properties

At both Bay LaFleur sites and the oiled Lake Barre site, relative elevation increased steadily by ~ 10 cm over the distance of 5 to 20 m from the marsh edge (Fig. 1). The rate of increase in relative elevation was nearly uniform across these sites. The exception was the unoiled site at Lake Barre. There, relative elevation increased by 10 cm between 5 and 15 m from the marsh edge, and the 20 m plot had a relative elevation of 28 cm, far higher than the ~ 10 cm observed at the other three sites at that distance from the marsh edge (Fig. 1). This high elevation resulted in soil that was visibly and chemically different from the other sampling locations; the soil texture appeared more peat-like at this location than the more typical silty salt marsh soil observed elsewhere. The highest nitrification potentials measured in this study were found at this high-

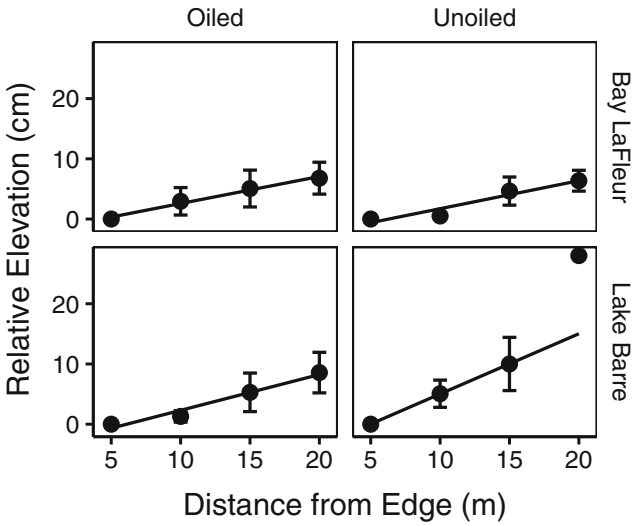

Fig. 1 Mean relative elevation (plus or minus one standard deviation) versus distance from the marsh edge at each of the four sampling locations. The lines represent a simple linear regression. The highest elevation location at the unoiled Lake Barre site was excluded from the regression to show that this site was similar to the other three sites except for one very different sampling location

elevation location, along with the highest soil Eh and organic carbon concentrations (Fig. 2). At the unoiled Lake Barre site, there were significant positive correlations (Kendall's tau rank correlation coefficient) between elevation and nitrification potential, microbial abundances, soil Eh, soil organic carbon, and soil extractable nitrate and ammonium concentrations (Fig. 2; see τ and p values on each panel). At the other sites, and at Lake Barre when the highest elevation location was excluded, soil organic carbon and extractable ammonium concentration were the only variables that increased significantly with increasing elevation.

Looking at these data from a broader perspective, a PCA showed that the highest elevation location at the unoiled Lake Barre sampling location clustered separately from the four primary sampling sites (Supplementary Fig. 1) with respect to a suite of soil properties. An ANOSIM corroborated that there was a substantial difference in soil properties between the high elevation sampling location and all the other lower elevation locations ($R = 0.467$, $p = 0.001$). The same PCA, with results grouped by site oiling history, showed that oiled and unoiled sites did not cluster separately (Supplementary Fig. 2). An ANOSIM showed that these two groups of data were very similar, even when the high elevation unoiled Lake Barre location is included ($R = 0.04$, $p = 0.005$).

Nitrogen Cycling Process Rates

There were no clear seasonal patterns in denitrification potentials (Fig. 3a), though potentials were much higher in March 2013 than they were in the other months sampled. The visual similarity in denitrification potentials between oiled and unoiled sites

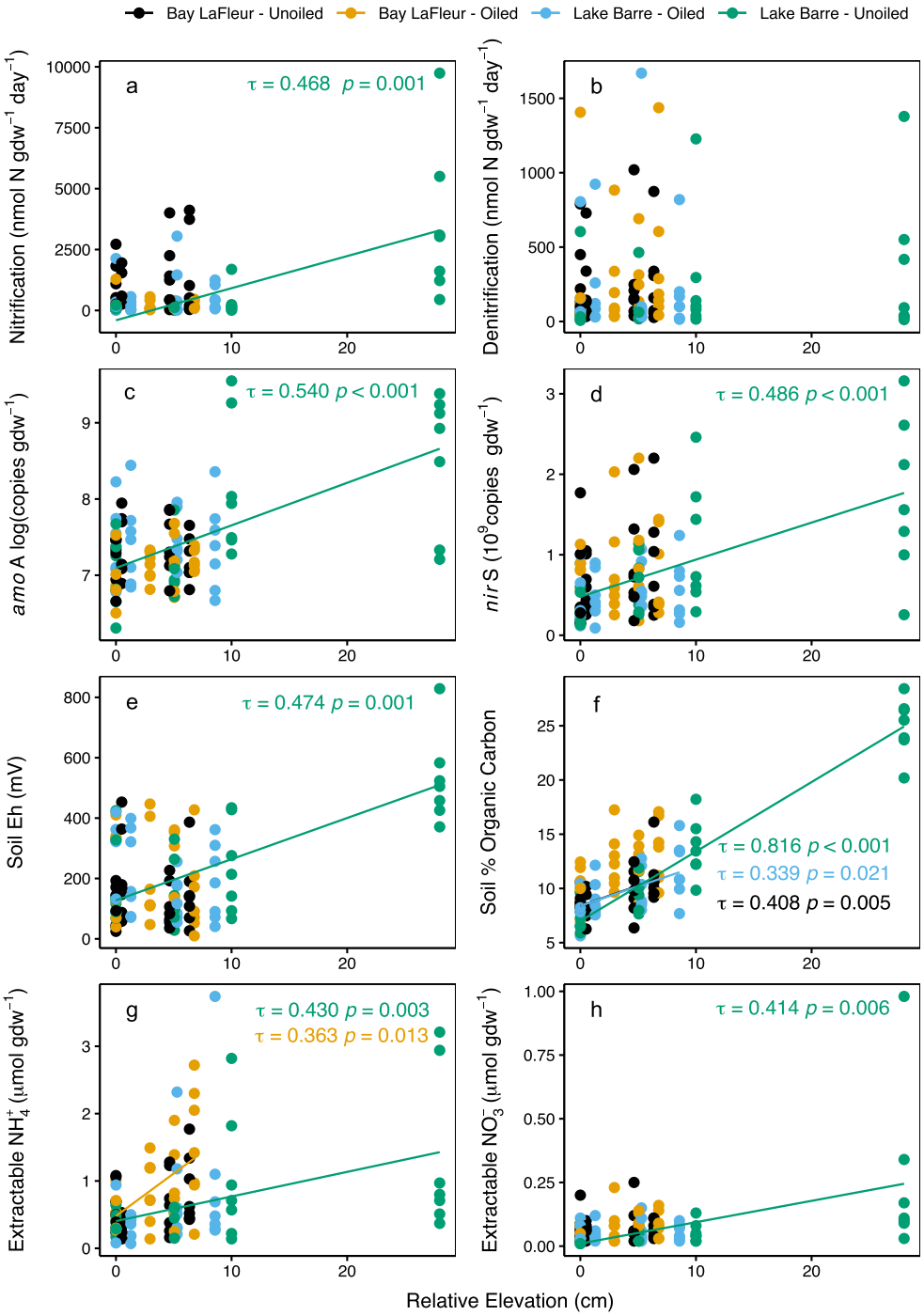

Fig. 2 Nitrification and denitrification potentials, archaeal *amo*A and *nir*S abundances, and select soil chemical characteristics plotted against relative elevation at all four sites during 2013–2014. Lines show statistically significant ($p < 0.025$) correlations

was supported by Mann-Whitney U tests that showed no significant difference between denitrification potentials at oiled versus unoiled sites when data were tested either by month (minimum $p = 0.08$ in September while $p > 0.2$ for other months) or with all months combined ($p = 0.50$). Nitrification potentials in July 2013 were indistinguishable from those in July 2012 (Fig. 3b,c). However, nitrification potentials in the spring (March–May) of 2013 were significantly higher ($p < 0.002$) than those in the springs of 2012 or 2014. The mean (± standard error) spring nitrification potential increased from 102 ± 22 nmol N gdw^{-1} d^{-1} in 2012 to 1060 ± 218 nmol N gdw^{-1} d^{-1} in 2013 before decreasing to 305 ± 71 nmol N gdw^{-1} d^{-1} in 2014. When the data were pooled across all sampling events, nitrification potentials were significantly higher (Mann Whitney U test; $p = 0.008$) at unoiled than at oiled sites. However, this difference was an artifact of the high nitrification potentials measured at the high elevation unoiled Lake Barre sampling location (open circles in Fig. 3c). When these data are removed from the analysis, the difference in nitrification potentials between oiled and unoiled sites was not significant

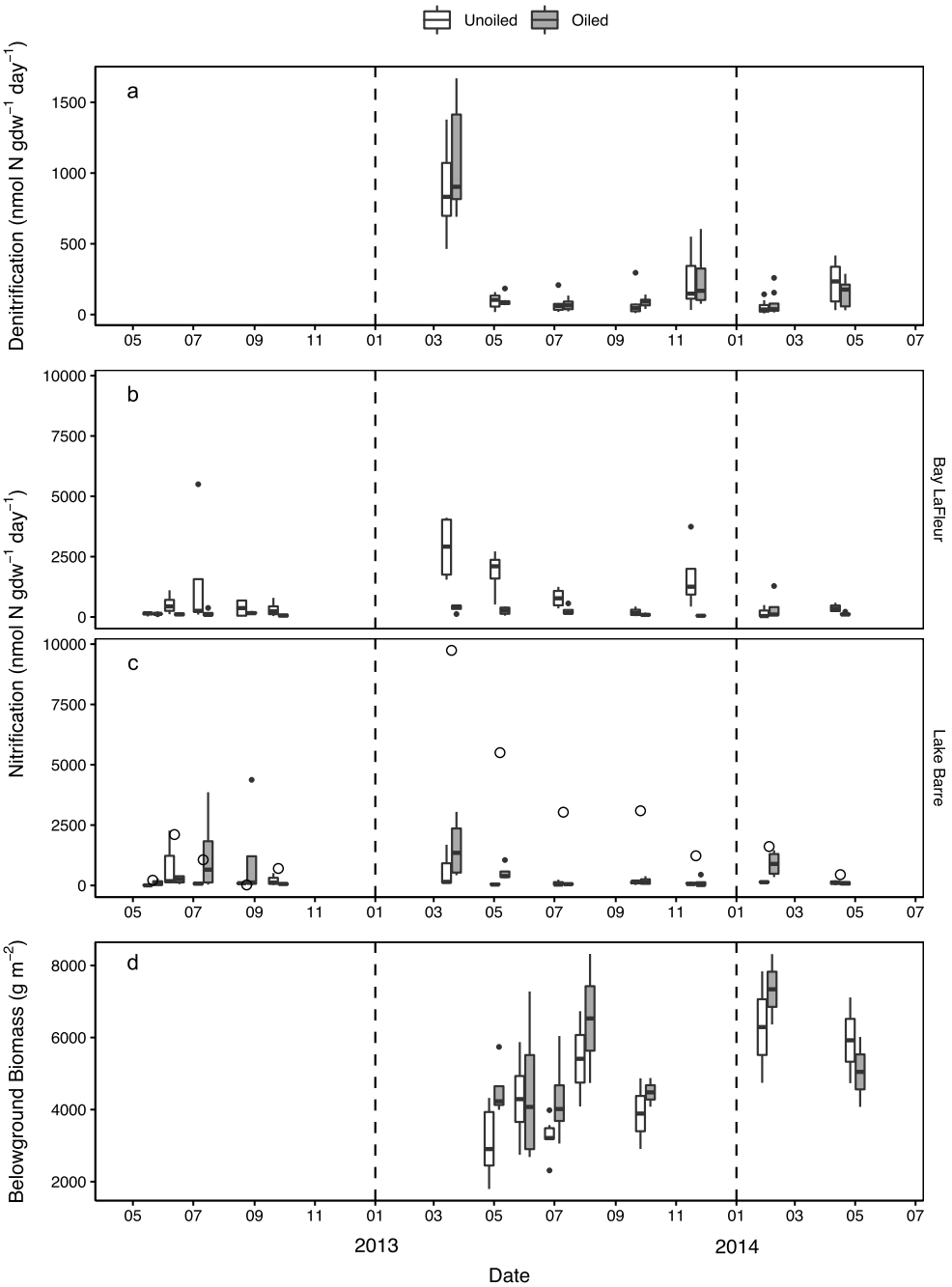

Fig. 3 Box plots of denitrification and nitrification potentials and of plant live belowground biomass at oiled and unoiled sites plotted as a time series from 2012 to 2014. Paired oiled and unoiled boxes are from the same sampling event and are centered on the date and time of sample collection. Nitrification potentials are split such that paired oiled and unoiled sites from Bay LaFleur (**b**) and Lake Barre (**c**) are displayed in separate panels. Open circles in panel c represent data from the 20 m site with the unusually high relative elevation

($p = 0.078$). When each month was analyzed separately (12 total over 2 years), November 2013 was the only time when nitrification potentials were significantly different between oiled and unoiled sites ($p = 0.017$). Moreover, when the four sampling sites are viewed separately, it becomes apparent that there was no consistent pattern in whether the oiled or the unoiled sites had higher nitrification potentials during any given sampling event. In Bay LaFleur, nitrification potentials at the unoiled site were often higher than they were at the oiled site (Fig. 3b). In contrast, nitrification potentials were more often higher at the oiled site in Lake Barre (Fig. 3c).

Microbial Abundances

Bacterial *amo*A abundances tended to be higher in the spring and summer than in the fall and winter (Fig. 4a), while there were no obvious seasonal patterns in archaeal *amo*A abundances (Fig. 4b). The pattern in *pmo*A abundance was inverted compared with bacteria *amo*A abundance during 2013–2014, but this relationship was not apparent in 2012 (Fig. 4c). There were no clear seasonal patterns in *nir*S abundances (Fig. 4d). There were no statistically significant differences (Mann Whitney U test) in microbial functional gene

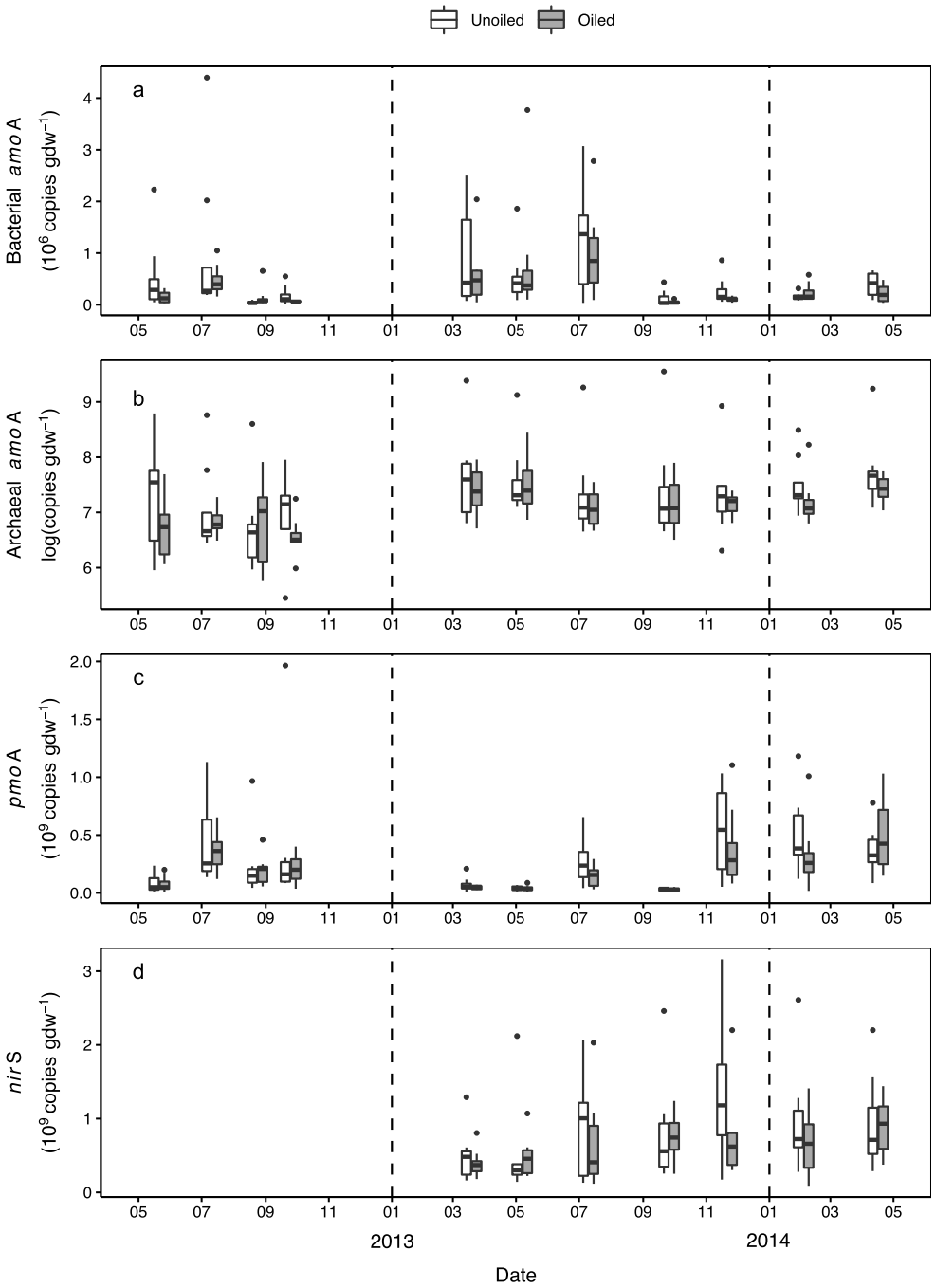

Fig. 4 Box plots of microbial abundances at oiled and unoiled sites plotted as a timeseries from 2012 to 2014. Paired oiled and unoiled boxes are from the same sampling event and are centered on the date and time of sample collection

abundances between oiled and unoiled sites ($p = 0.22$ for bacterial *amo*A, $p = 0.08$ for archaeal *amo*A, $p = 0.42$ for *pmo*A, and $p = 0.39$ for *nir*S). While it appears that *nir*S abundance was higher at unoiled than oiled sites in November 2013 (Fig. 4d), this difference was not statistically significant ($p = 0.13$).

Correlations between Nitrogen Cycling Potentials and Other Soil Properties

To investigate the underlying relationships between nitrogen cycling potentials and soil properties that were independent of oiling history, we combined data from all study sites and time periods for further analysis. When oiled and non-oiled sites were combined, nitrification potentials were positively correlated with archaeal *amo*A abundances (Kendall's tau rank correlation coefficient, $\tau = 0.251$, $p < 0.001$; Fig. 5a) and bacterial *amo*A abundances (not shown; $\tau = 0.306$, $p < 0.001$) but not *pmo*A abundances ($p = 0.66$) across all study sites during the 2013–2014 study period. Similar correlations did not exist between nitrification potentials and other potential drivers such as soil Eh (Fig. 5b; $p = 0.060$) or soil extractable ammonium concentration (Fig. 5c; $p = 0.67$). There was a positive correlation between denitrification

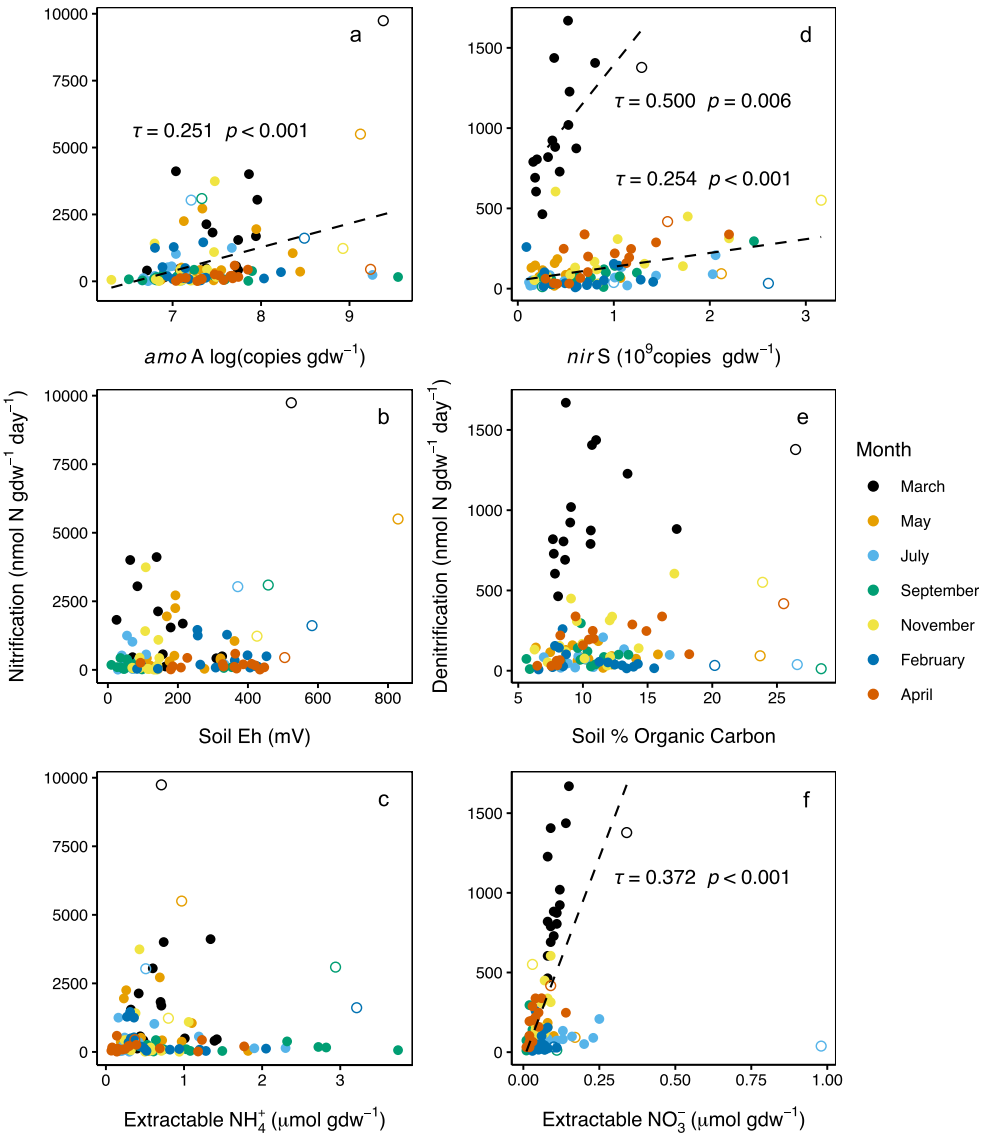

Fig. 5 Nitrification potentials plotted against archaeal *amo*A abundance (**a**), soil Eh (**c**), and soil extractable NH_4^+ concentration (**e**). Denitrification potentials plotted against nirS abundance (**b**), soil organic carbon content (**d**), and soil extractable NO_3^- concentration (**f**). Statistically significant correlations are displayed as dashed lines. Data from the high elevation, 20 m sampling location at the unoiled Lake Barre site are displayed as open symbols. Only data from the 2013–2014 study period are included in this plot

potentials and *nir*S abundances in every month except March 2013 (Fig. 5d; $\tau = 0.254$, $p < 0.001$). In March 2013, there was a different linear relationship between these two variables ($\tau = 0.500$, $p = 0.006$) that had a higher slope. No correlation existed between denitrification potentials and soil total organic carbon concentration (Fig. 5e; $p = 0.51$). Soil extractable nitrate concentrations were higher in July than they were in other months (Fig. 5f). When July data were excluded, there was a positive correlation between denitrification potentials and soil extractable nitrate content (Fig. 5f; $\tau = 0.372$, $p < 0.001$). Similarly, when July data were excluded, there was a positive correlation between soil extractable nitrate concentrations and nitrification potentials (Fig. 6a; $\tau = 0.400$, $p < 0.001$). These two variables were also positively correlated in July ($\tau = 0.701$, $p < 0.001$), albeit at a higher slope. A weaker correlation existed between soil extractable nitrate concentrations and bay water nitrate concentrations ($\tau = 0.376$, $p < 0.001$), but the slope of this correlation was effectively zero (Fig. 6b). There was a positive correlation between denitrification potentials and nitrification potentials (Fig. 6c; $\tau = 0.303$, $p < 0.001$). A weaker correlation between denitrification potentials and bay water nitrate concentrations ($\tau = 0.172$, $p = 0.008$), but again, the slope of this correlation was effectively zero (Fig. 6d).

Discussion

Influence of Oiling History on Salt Marsh Nitrogen Cycling Processes

Marton et al. (2015) previously showed that there was no significant difference in nitrification potentials between our oiled and unoiled sites during 2012, around 1.5–2.5 years following oil exposure. We extended this finding for another year and further showed no difference in denitrification potentials between oiled and unoiled sites. It is important to note that one

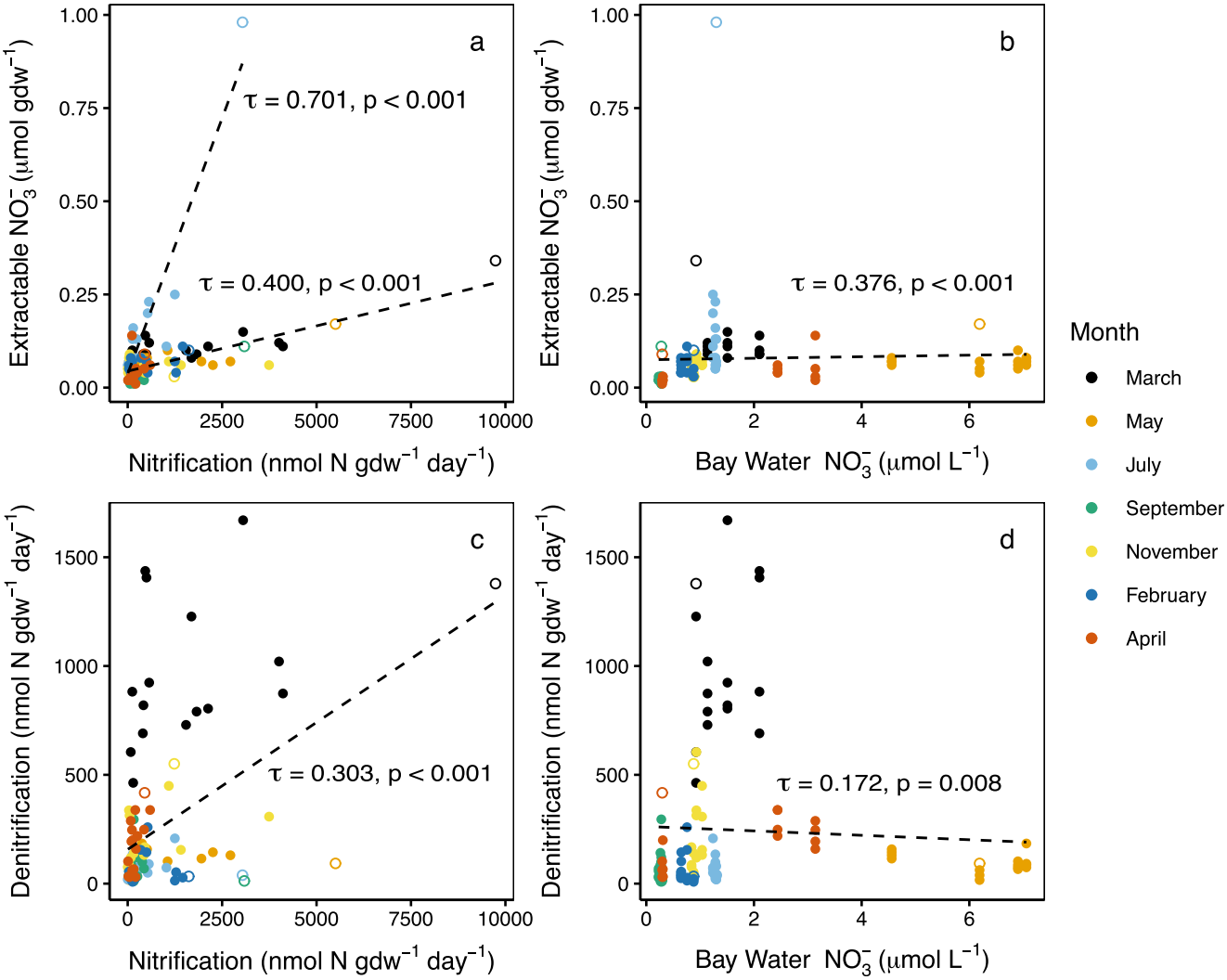

Fig. 6 Soil extractable NO_3^- concentration plotted against nitrification potential (**a**) and bay water NO_3^- concentration (**b**) and denitrification potential plotted against nitrification potential (**c**) and bay water NO_3^- concentration (**d**). Statistically significant correlations are displayed as dashed lines. Data from the high elevation, 20 m sampling location at the unoiled Lake Barre site are displayed as open symbols. Only data from the 2013–2014 study period are included in this plot

likely explanation for these observations is that the heaviest oiling was concentrated around the edges of the marsh as observed by Turner et al. (2014) and that the most heavily oil-impacted portion of our oiled sites were lost to rapid subsidence in the 1.5 years between initial oil exposure and the first measurements by Marton et al. (2015). In fact, on-site measurements demonstrated that the marsh edge was receding at a rate of 1.2–8.7 m per year during the study period. This observation fits within the broader context of rapid and extensive land loss in southern Louisiana (Couvillion et al. 2011) and is consistent with the larger-scale findings of Turner et al. (2016) who showed that oil contamination accelerated marsh loss. The result was that by the end of the study period in 2014, there were no longer detectable differences in oil-derived hydrocarbon concentrations in the top 5 cm of marsh soil between our oiled and unoiled sites (Ashton-Meyer 2017). Other studies that have shown that coastal microbial communities can recover back to a pre-spill state within months of oil being completely removed or degraded (Huettel et al. 2018; Rodriguez-R et al. 2015), so it is unsurprising that we would not see a difference in nitrogen cycling potentials between oiled and unoiled sites when there was no longer a difference in oil concentrations between these sites.

Even during 2012, though, when there was a detectable difference in oil content of the soil between oiled and unoiled sites, there were no detectable differences in nitrification potentials between these sites (Marton et al. 2015). This is consistent with the findings of Horel et al. (2014) who showed that salt marsh soil nitrification potentials were not influenced by the amount or type of oil added in laboratory soil slurry incubations. This lack of oil response is unexpected given that hydrocarbons are known to competitively inhibit ammonia

monooxygenase (Deni and Penninckx 1999; Keener and Arp 1994), making autotrophic nitrifying microorganisms 100 to 1000 times more susceptible to oil contamination than heterotrophic bacteria (Urakawa et al. 2012). A recent review of the response of nitrification to oil spills synthesized the results of multiple studies to suggest that nitrifying microorganisms are less susceptible to oil when they are in a soil matrix compared with an aqueous matrix (Urakawa et al. 2019). This is most likely because hydrophobic hydrocarbons tend to be associated with soil particles, making them less bioavailable and therefore less toxic to soil microorganisms (Alexander 2000). Furthermore, a history of oil exposure may make the microbial community more resilient to future acute oil exposure (Deni and Penninckx 1999; Kleinhuizen et al. 2017). This is particularly relevant in our study since the first data were collected more than a year after the original oil exposure, and in coastal Louisiana more generally where anthropogenic hydrocarbon discharges are commonplace (Asl et al. 2016).

However, a lack of oil response in a process rate does not necessarily indicate a lack of oil toxicity to the microorganisms mediating that process (Nyman 1999). For example, it is possible that nitrification carries on unimpeded due to a shift in microbial community composition towards more oil-tolerant nitrifiers (Newell et al. 2014; Urakawa et al. 2012). Bernhard et al. (2016) showed that there was no difference in the overall composition of the bacterial or archaeal ammonia-oxidizing communities between our oiled and unoiled study sites. However, they showed that different microbial populations correlated most strongly with nitrification potentials at oiled sites compared with unoiled sites. This suggests that oil exposure may have driven a shift in which components of the overall microbial community were actively nitrifying.

In general, we expect that denitrification potentials would show an even weaker response to oil than nitrification potentials (Urakawa et al. 2019) based on the assumptions that denitrifying and nitrifying microorganisms are more likely to be heterotrophic and autotrophic, respectively, and the observation that autotrophic nitrifying microorganisms are much more susceptible to oil exposure than heterotrophic bacteria (Urakawa et al. 2012). Our findings can neither support nor contradict this expectation since neither denitrification nor nitrification potentials were influenced by site oiling history. Other studies on salt marsh soils have also found that oil exposure did not alter denitrification potentials (Kleinhuizen et al. 2017; Shi and Yu 2014). In contrast, Horel et al. (2014) showed that salt marsh soil denitrification potentials were sometimes stimulated by oil addition, but the extent of this stimulation varied widely by oil type, oil concentration, and temperature, indicating that environmental conditions may play a substantial role in determining how and whether oil exposure alters denitrification or other microbial processes (Ortmann and Lu 2015). This provides valuable context for interpreting conflicting results from other studies that have shown, for example, that denitrification rates in estuarine sediments increased in response to oil exposure (Ribeiro et al. 2016) or that denitrification rates in marine sediments were inhibited by the addition of oil (Bonin et al. 1990; Levine et al. 2017).

While there was no difference in nitrification or denitrification potentials between oiled and unoiled sites in this study, there was a great deal of variability in these potentials across all sampling locations and dates. As Marton et al. (2015) pointed out, the nitrification potentials measured in these Louisiana marshes are higher than those reported from most other salt marsh environments, and the maximum potentials measured in 2013 (9740 nmol N gdw^{-1} d^{-1}; this study) were even higher than those reported in 2012. The range in denitrification potentials measured in this study was 8–1670 nmol N gdw^{-1} d^{-1} with a median value of 98 nmol N gdw^{-1} d^{-1}. Using a typical conversion factor for these soils of 0.3 gdw cm^{-3} and our sampling depth of 5 cm, it is possible to convert these values to approximate areal rates of 5–1043 μmol N m^{-2} h^{-1} (median = 61 μmol N m^{-2} h^{-1}) for the sake of comparison. This range and median are similar to those reported in many other studies in coastal Louisiana that used a comparable method (Rivera-Monroy et al. 2010). Moreover, few studies report both nitrification and denitrification potentials from salt marsh soils at less than seasonal temporal resolution over at least one annual cycle (Thompson et al. 1995). This study design provides the opportunity to look for environmental factors other than oil exposure that may help to explain some of the observed variability in these nitrogen cycling potentials.

Marsh Elevation as a Driver of Spatial Variability

Elevation is considered a master variable that affects other processes and parameters in salt marshes (Hopkinson and Giblin 2008). Within any given tidal regime, elevation determines the amount of time that a particular portion of marsh remains inundated or exposed by the tide and drives patterns of flooding and drainage. Combined with plant evapotranspiration (Wilson et al. 2015), these patterns drive plant zonation (Haines and Dunn 1976) and influence soil chemistry (Osgood and Zieman 1998) and redox potential (Howes et al. 1981). Morris et al. (2013) showed that salt marsh plant aboveground biomass reached a maximum slightly above sea level and decreased at both lower and higher elevations due to excessive flooding or exposure, respectively. In the extensive, low-relief salt marshes of Louisiana, where subsidence and land loss are rapid and the tidal range is very small (Turner 2001), small changes in elevation can make a large difference in plant health and marsh productivity. It has previously been shown that the relative elevation of the marsh platform increased by 12 cm from 5 to 20 m from the marsh edge at our study sites in Terrebonne Bay (Marton et al. 2015). We

observed increasing soil total organic carbon concentrations moving up this elevation gradient (Fig. 2) that are consistent with increased carbon availability in a more productive marsh. Spatial patterns in other soil parameters support those reported previously (Marton and Roberts 2014; Marton et al. 2015). Higher extractable ammonium concentrations probably resulted from increased ammonification of the more organic-rich soil, for example. Increased bacterial abundances may have resulted from greater availability of organic substrates. While, neither denitrification nor nitrification potentials increased significantly with elevation (Fig. 2a), nitrification potentials were uniformly highest at the highest elevation sampling location in Lake Barre compared with other sampling locations measured at the same time (Fig. 3b,c), suggesting that there is a break point in elevation/inundation above which nitrification potentials were higher. Samples from this location also had significantly higher (Mann Whitney U test; $p < 0.001$) soil redox potential and total organic carbon and total nitrogen concentrations than samples from all other locations. However, there was a great deal of variability in the nitrogen cycling potentials measured within and between the other sampling locations that could not be explained by changes in elevation or inundation.

Seasonal Patterns in Nitrogen Cycling Processes

There was no reproducible year-to-year seasonal pattern in nitrification potentials from 2012 to 2014. However, nitrification potentials measured in the summer (July) of each year were similar to one another (Fig. 3b,c). Against this backdrop, the most notable feature of this time series is that nitrification potentials measured in spring (March–May) 2013 (1060 ± 218 nmol N gdw^{-1} d^{-1}) were much higher than those measured in either spring 2012 (102 ± 22 nmol N gdw^{-1} d^{-1}) or 2014 (305 ± 71 nmol N gdw^{-1} d^{-1}). This roughly coincides with seasonal shifts in microbial gene abundances: bacterial *amo*A abundances were significantly higher and *pmo*A abundances significantly lower (Mann Whitney U test; $p < 0.001$) in March–July 2013 than they were in August 2013–May 2014 (Fig. 3). The result is that nitrification potentials were positively correlated with archaeal *amo*A abundances (Fig. 5a) and bacterial *amo*A abundances but not *pmo*A abundances across all study sites during the 2013–2014 study period.

Interestingly, there were also broad shifts in plant belowground live biomass at around these same times (Fig. 3d). Belowground biomass in spring 2013 (3670 ± 337 g m^{-2}) was only around half what it was in spring 2014 (6150 ± 316 g m^{-2}). Belowground biomass was measured at locations near (within 20 m) our sampling locations and was originally reported by Hill and Roberts (2017) over a 3-year period from 2013 to 2015. They did not observe consistent seasonal patterns in belowground biomass. Rather, biomass was typically relatively low (2000–5000 g m^{-2}) across sites in 2013 and late 2015. During the intervening ~ 1.5 years, total live belowground biomass was typically higher (5000–11,000 g m^{-2}). This overall range in belowground biomass is typical of what Hill and Roberts (2017) observed at three more frequently sampled salt marsh sites near Cocodrie, Louisiana, USA.

Plants can influence the structure and function of the soil microbial community in many ways. For example, close relatives of *Spartina alterniflora*, the dominant marsh grass at our study site, are known to leak oxygen from their roots into the surrounding soil (Koop-Jakobsen et al. 2017; Koop-Jakobsen and Wenzhöfer 2015). Oxygen leakage is likely from *S. alterniflora* roots as well, as evidenced by oxygen loss from roots suspended in gas (Howes and Teal 1994), oxidized metal crusts observed around individual roots (Mendelssohn and Postek 1982), enhanced sulfide oxidation activity associated with roots (Lee et al. 1999), and higher transcription of sulfur-oxidizing functional genes on roots relative to bulk marsh soil (Thomas et al. 2014). It is probable that methane-oxidizing bacteria occupied a niche in the soil in a thin film around plant roots where their limiting substrate, oxygen, was available. Low root biomass in spring 2013 resulted in a smaller colonizable area for methane-oxidizing bacteria as indicated by lower *pmo*A abundance.

While ammonia-oxidizing bacteria also require oxygen, they are likely limited by nitrogen availability in these marsh soils where extractable ammonium concentrations were 0.1–3.7 μmol gdw^{-1} with a median value of 0.5 μmol gdw^{-1}. Furthermore, up to 95% of NH_4^+ generated by ammonification can be taken up by marsh plants (Buresh et al. 1981; Smith and Delaune 1985). It is therefore likely that low belowground biomass in spring 2013 was indicative of low plant growth and nitrogen uptake, which allowed the ammonia-oxidizing microbial community to flourish. It is possible that there was not a corresponding temporal pattern in archaeal *amo*A abundances because at least some of these organisms are mixotrophic (Qin et al. 2014), allowing them to use organic carbon substrates to support their growth when ammonia is unavailable. The inverted seasonal pattern between nitrification potentials and plant live belowground biomass also suggests that ammonium uptake by plants may competitively inhibit nitrification potentials during the growing season. It is important to note that we only have these data from two growing seasons and nitrification potentials (this study) and belowground biomass (Hill and Roberts 2017) measurements were not made at exactly the same times and locations. However, this mechanism of competitive inhibition is the most plausible explanation of the observed temporal variability in our data and was previously invoked by Moin et al. (2009) to explain high nitrification rates early in the spring that tapered to a minimum in late summer.

Other important differences in environmental conditions were observed between the spring of 2013 and the springs of 2012 and 2014. For example, water levels were

substantially lower near our study sites in 2014 than they were in 2012–2013 (Fig. S3a). There was likely a confluence of favorable conditions that occurred around the same time in March 2013 that allowed soil nitrogen cycling potentials to be so much higher than they were at any other time point measured (Fig. 3). Surface soil temperatures measured at our site tended to be similar to the weekly running average of surface water temperatures (Fig. S3c) with the exception of March 2013 when the mean soil temperature was 21.6 °C and the weekly running average water temperature was only 17.7 °C. Mid-March, when these samples were collected, is early in the growing season in southern Louisiana, and plants have typically not yet reached their maximum growth rates (Hill and Roberts 2017). Bay water nutrient concentrations were also at a seasonal maximum in March 2013 (Fig. S3e), though this was not the case for soil nutrients or organic content (Fig. S4). All of these factors combined with the multi-year minimum in live belowground plant biomass discussed above may have created conditions favorable to microbial nitrogen cycling activity and stimulated the brief window of very high potentials observed across our study sites.

Coupled Nitrification-Denitrification in Salt Marsh Soils

Nitrogen budgets of a specific salt marsh (Valiela and Teal 1979) and of a generalized salt marsh ecosystem (Hopkinson and Giblin 2008) both show that denitrification is an important sink for bioavailable nitrogen. Following a comprehensive review of the literature, Hopkinson and Giblin (2008) estimated a net nitrogen loss from salt marshes of 1.3 Tg N $year^{-1}$ as N_2 gas, the equivalent of around 8% of total riverine nitrogen loading to latitudes occupied by these marshes. However, denitrification requires nitrate, and little nitrate from surrounding estuarine waters is able to penetrate into marsh soil, severely limiting its availability to the denitrifying microbial community in many salt marsh ecosystems. This makes nitrate production through nitrification the dominant nitrate source for salt marsh denitrification. This coupling between nitrification and denitrification is indicated by the positive correlation between their potential rates (Dollhopf and Hyun 2005; Thompson et al. 1995) and has been demonstrated experimentally by measuring $^{15}N\text{-}N_2$ production following $^{15}N\text{-}NH_4^+$ addition (Reddy et al. 1989; Risgaard-Petersen and Jensen 1997).

Our data further support this model of salt marsh nitrogen cycling. While there was a positive correlation between denitrification potentials and *nir*S abundances (Fig. 5d), the highest denitrification potentials in this study were observed in March 2013 (Fig. 3a) when *nir*S abundances were among the lowest measured (Fig. 4d), indicating that denitrification potentials were not limited by microbial abundance. There was no correlation between denitrification potentials and soil organic carbon content (Fig. 5e), which may simply reflect that excess organic carbon was added to these slurry incubations such that carbon availability did not limit these potential rate assays. Taken together with the carbon-richness of this soil, however, these data suggest that it is unlikely that denitrification potentials were limited by organic carbon availability. There was a positive correlation between denitrification potentials and soil extractable nitrate concentration except in July 2013 (Fig. 5f), however, and the fact that soil extractable nitrate concentrations were so low in these soils (typically less than 0.25 μmol gdw^{-1}) suggests that denitrification was likely limited by nitrate availability. There was a positive correlation between soil extractable nitrate concentrations and nitrification potentials (Fig. 6a), but not bay water nitrate concentrations (Fig. 6b), indicating that nitrification was the most important source of nitrate to these salt marsh soils. The positive correlation between denitrification and nitrification potentials at our study sites (Fig. 6c) provides evidence that these two processes were coupled.

Conclusions

Our results demonstrate that, 2.5–3.5 years following oil exposure, there was no detectable difference in nitrification or denitrification potentials between oiled and unoiled salt marshes. This indicates that the capacity of these salt marshes to remove bioavailable nitrogen via coupled nitrification-denitrification had recovered to pre-oil spill levels. These findings fit within a broad and emerging narrative in the literature that salt marsh soil nitrogen cycling processes are only weakly impacted by oil exposure and can recover quickly from this type of disturbance (Urakawa et al. 2019). However, it is important to interpret these results within the overall context of ongoing, rapid coastal land loss in this region that was accelerated substantially by the Deepwater Horizon oil spill (Turner et al. 2016). When salt marshes are lost to this, or any other disturbance, the nitrogen removal ecosystem service that they provide is lost as well (Hinshaw et al. 2017).

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