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Spatial Partitioning of Microbial Functional Potential Suggests Complex Nitrogen Cycling in a Subterranean Estuary

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ABSTRACT

Subterranean estuaries (STEs) are key bioreactors regulating the quantity and chemical composition of groundwater-derived nitrogen (N) reaching coastal ecosystems. Yet, the microbial controls on N-cycling within these groundwater–seawater mixing zones remain poorly understood. We investigated the spatio-temporal variations in microbial communities and their N-cycling potential within an alluvial Mediterranean STE with high N concentration. We explored changes in microbial abundance, heterotrophic activity, taxonomic composition, and the abundance of N-cycling genes across groundwater samples collected at several depths and distances from the shoreline in winter and summer. Microbial abundance, activity, and diversity varied strongly across hydrochemical zones according to physicochemistry and aquifer depth but showed limited seasonality. Functional predictions suggested a complex, spatially structured suite of N pathways encoded by diverse taxa occupying different STE zones, and quantitative-PCR revealed niche partitioning between ammonia-oxidizing archaea, prevalent in fresh-groundwater, and bacterial denitrifiers enriched in deep-saline layers. Multiple linear model predictions showed a stronger fit for NO_2^- and NH_4^+ concentrations when using microbial properties than when using environmental variables, highlighting their importance for understanding N cycling in STEs. Our results suggest that the functional potential of the STE microbiome is complex and spatially structured across hydrochemical zones, explaining spatial variations in STE N-cycling.

1 | Introduction

Subterranean estuaries (STEs) are hidden transition zones where fresh groundwater flowing through coastal aquifers mixes with underground seawater and also feature intense biogeochemical transformations (Duque et al. 2020; Moore 1999). These STE reactions alter groundwater chemistry before it

enters the sea via submarine groundwater discharge (SGD), hence determining nutrient and trace elements fluxes to the coastal ocean (Moore and Joye 2021; Wilson et al. 2024). SGD-driven nutrients inputs can play key roles in sustaining coastal productivity and ecosystem functioning (Lecher et al. 2015; Lecher and Mackey 2018; Rodellas et al. 2015; Romano-Gude et al. 2025; Santos et al. 2021). However, when excessive, they

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may also promote eutrophication events (Hwang et al. 2005), stimulate harmful algal blooms (Montiel, Lamore, Stewart, and Dimova 2019), and cause degradation of overall coastal water quality (Alorda-Kleinglass et al. 2024; Amato et al. 2016).

Microorganisms drive many of these biogeochemical transformations in STEs, thus impacting the composition of groundwaters discharging to the coastal sea (Archana et al. 2021; Ruiz-González et al. 2021). Recent studies have shown that coastal subsurface microbial communities regulate organic matter degradation, redox dynamics and nutrient transformations across fresh groundwater-marine mixing areas (Abarike et al. 2024; Calvo-Martin et al. 2025), which can determine the quality and chemical composition of SGD fluxes. However, the functional potential of the STE microbiome has long been overlooked, as most studies to date have focused on taxonomic composition rather than on the potential role of these microbial communities in driving key biogeochemical processes (Archana et al. 2021; Ruiz-González et al. 2021).

Among microbial transformations in coastal aquifers, those regarding nitrogen (N) are of particular concern due to the role of N in coastal biogeochemistry, influencing nutrient dynamics and marine biota (Couturier et al. 2017; Lecher and Mackey 2018; Santoro 2010; Wilson et al. 2024). N pollution is a threat to groundwater quality, typically due to inputs from diverse anthropogenic sources such as industrial wastewater, domestic sewage, and most prominently, agricultural fertilizers (Galloway et al. 2013; Gao et al. 2024; Morrissy et al. 2021; Richardson et al. 2025). Microbial transformations within STEs determine the fate and form of N discharged to coastal waters, thus modulating the transfer of human-derived nutrients to the ocean (Santoro 2010; Santos et al. 2021). For instance, reductions of up to 50% in dissolved organic nitrogen (DON) concentrations were observed between a beach aquifer and its submarine discharge point (Euler et al. 2024), while permeable aquifers removed up to 84% of incoming anthropogenic nitrogen, markedly reducing nutrient loading to coastal waters (Chen et al. 2025). Conversely, other studies detected the production of nitrate in more pristine aquifers, likely due to DOM remineralization (Robinson et al. 2018; Santos et al. 2009). A deeper understanding of the metabolic processes involved in such transformations is thus required to elucidate the role of microbial communities on STE nutrient cycling. However, the vast phylogenetic novelty of coastal groundwater microbiomes (Brick et al. 2025; Ruiz-González et al. 2025), along with genetic evidence showing that complex microbial interactions are required to complete nitrogen transformation pathways in inland groundwater (Mosley et al. 2022), poses a challenge for studying microbial metabolisms within STEs, which remain much less explored than inland aquifers (Ruiz-González et al. 2021, 2025).

Studying the distribution of key genes involved in the transformation of nitrogen can provide insights into the relevance of distinct N metabolism capacities in STE (Santoro et al. 2008; Wilson et al. 2024). However, only a few studies have explored the genetic functional potential of the STE microbiome (Adyasari et al. 2024; Jiang et al. 2023; Rogers and Casciotti 2010; Santoro et al. 2006, 2008; Sun et al. 2024; Sun et al. 2022; Wilson et al. 2023; Wu et al. 2023; Xiao et al. 2018). These studies have provided evidence that metabolisms such

as ammonia oxidation, nitrate reduction via denitrification, anammox, and dissimilatory nitrate reduction to ammonium (DNRA) can be important in coastal groundwater. For example, several studies focusing on the *amoA* gene have shown that *amoA* abundance varies with oxygen and salinity gradients (Rogers and Casciotti 2010; Santoro et al. 2008; Wilson et al. 2023). Moreover, nitrite oxidation targeted through nitrite-oxidoreductase (*nxrB*) was found to vary with depth, following gradients in DOC, salinity, and ammonium (Sun et al. 2022). This suggests that accurately addressing the spatial variability is particularly relevant in STEs, as these systems are characterized by strong geological, hydrological, and physicochemical heterogeneity (e.g., Folch et al. 2020; Massmann et al. 2023). Also, most studies have focused on a single functional microbial group (Santoro et al. 2008; Sun et al. 2022; Wilson et al. 2023), providing an incomplete view of the potential complexity of N metabolisms hidden in these systems. Understanding nutrient transformations occurring in STEs, as well as their variability and drivers, thus requires detailed functional explorations of microbial communities at a resolution that adequately captures the spatial and physicochemical heterogeneity of these systems.

This study investigates the spatial dynamics of microbial communities and their role in nitrogen transformations within the Argentona coastal alluvial aquifer (Mataró, NE Spain), on two occasions (summer and winter). This is a unique instrumented site characterized by high nitrate concentrations (likely from agricultural sources, Diego-Feliu et al. 2022; Martínez-Pérez et al. 2022), where 19 installed piezometers allow for an exceptional high-resolution spatial characterization of the aquifer at different depths and distances from the shore, covering the entire salinity gradient that results from the mixing of fresh groundwater and seawater intrusion (Folch et al. 2020; Gutiérrez-Martín et al. 2023). Specifically, we aimed to: (i) characterize the spatio-temporal patterns in microbial abundance, taxonomic composition and bulk heterotrophic activity across the subterranean estuary; (ii) quantify the distribution of key N-cycling functional genes along the aquifer; and (iii) evaluate whether incorporating this microbial information improves understanding of the spatio-temporal variations in dissolved nitrogen species. We hypothesized that the steep vertical and longitudinal gradients in physico-chemistry would lead to the establishment of spatially heterogeneous microbial assemblages differing in their functional potential. We further expected that functional gene abundances and community activities would correlate predictably with nitrogen gradients.

2 | Material and Methods

2.1 | Study Area

The study site is located at the mouth of the Argentona ephemeral stream alluvial aquifer, ca. 40 km northeast of Barcelona, Catalonia, Spain (Folch et al. 2020). The region is characterized by a semi-arid Mediterranean climate with torrential events mainly in autumn (Palacios et al. 2020). The alluvial aquifer overlies a granitic basement and consists of unconsolidated quaternary gravels, sands and clays derived from granite (Folch et al. 2020; Martínez-Pérez et al. 2022). Fresh groundwater typically alternates with saline water towards the sea, while

landward flow can occur during prolonged droughts. Overall, salinity increases with depth, as shown by conductivity profiles and electrical resistivity surveys (Diego-Feliu et al. 2021; Folch et al. 2020; Palacios et al. 2020) (Figure 1a; Figure S1).

The Argentona experimental site was established in 2015 with the installation of a series of piezometers along a transect perpendicular to the shoreline, extending 20–113 m inland (Figure 1a). Most piezometers are clustered in nests (N1, N2, N3

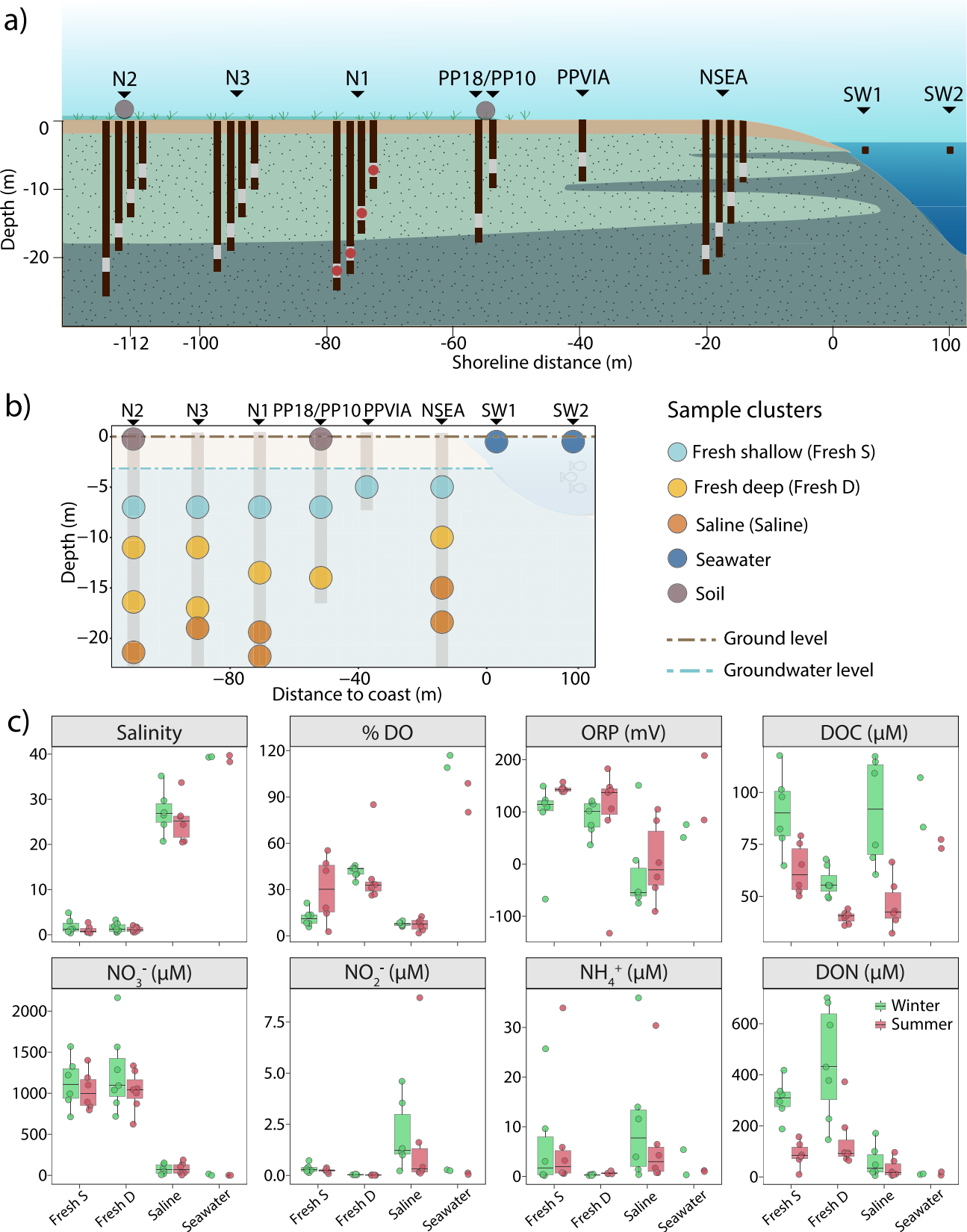


FIGURE 1 | Legend on next page.

FIGURE 1 | Sampling stations in the experimental site, sample clusters and physicochemical parameters. (a) Schematic representation of the sampling stations throughout the STE, including groundwater, soil and seawater sampling points, and highlighting in darker blue a simplified view of the seawater intrusion in the aquifer. Grey zone denotes the screened area. Red dots indicate the location of in situ sensors for continuous monitoring. Note that the X axis is non-linear for seaward values. (b) Sample locations coloured according to the four clusters defined from physicochemical parameters. Dashed lines indicate mean ground (brown) and water (blue) level. (c) Boxplots showing variations in salinity, dissolved oxygen (DO), oxidation–reduction potential (ORP), dissolved organic carbon (DOC), nitrate (NO_3^-), nitrite (NO_2^-), ammonium (NH_4^+) and dissolved organic nitrogen (DON) across the different clusters, with winter and summer campaigns represented in green and red, respectively.

and NSEA), consisting of 4 piezometers each, reaching depths from 10 to 24 mbs (meters below the surface) within the same location, while others are individual piezometers (e.g., PP10, PP18 and PPVIA, Figure 1a). Each piezometer has a 2-m screen that allows collecting water at specific depths ranging from 5 to 21 mbs (Folch et al. 2020; Gutiérrez-Martín et al. 2023; Palacios et al. 2020) (Figure 1a). In addition, piezometer N1 is equipped with sensors that allow continuous recording of pH, oxidation–reduction potential (ORP), conductivity, and temperature (Figure S1).

2.2 | Sampling and Physicochemical Parameters

Two field campaigns were conducted under contrasting hydrological conditions (Figure S2): one in winter (13–15 December 2021), after various precipitation episodes, and one in summer (26–28 July 2022), following a 3-month period of low precipitation and high temperatures. A total of 46 samples were collected, including 19 groundwater, 2 seawater, and 2 surface soil samples per campaign (Figure 1a).

Groundwater was collected from piezometers using submersible pumps placed at the middle of the 2m screened interval of the piezometer. Prior to sampling, each piezometer was purged with approximately three times its internal water volume of the piezometer to ensure complete renewal. Surface seawater (0.5m depth) samples were collected from a boat at 5 and 100m from the shore using submersible pumps. During sampling, temperature, dissolved oxygen (DO), pH, salinity, and oxidation–reduction potential (ORP) were measured in situ using a continuous flow cell coupled to a multiparametric YSI probe (YSI Professional Plus, with multiparametric Quatro Cable), except for seawater samples, which were measured directly after collection. All water samples for microbial analysis were prefiltered through a 20 μm mesh to remove large particles and avoid fast filter clogging, and were kept in acid-rinsed carboys (5–20L). Samples for inorganic nutrients, dissolved organic carbon (DOC), total dissolved nitrogen (TDN), and fluorescent dissolved organic matter (FDOM) were filtered in situ (see below). All water samples were maintained at 4°C in dark and processed in the laboratory within 2–8 h after collection. Finally, two surface soil samples (top 5cm) were manually retrieved at N2 and PP18 sites (Figure 1a) during each campaign, stored in sterile vials, and frozen at –80°C upon arrival at the laboratory until DNA extraction.

For measuring nitrate (NO_3^-), nitrite (NO_2^-) and ammonium (NH_4^+), 10mL of water were filtered in situ through 0.45 μm nylon filters and stored at –20°C in sterile polyethylene tubes until analysis on a CFA Bran-Luebbe autoanalyser. Water

samples for DOC, TDN and FDOM were also filtered in situ using a Masterflex L/S PTFE-Diaphragm Pump System connected to an acid-washed 47-mm filter PFA Tefzel Clamp, where a sequential filtration was performed using pre-burnt GF/D (2.7 μm), GF-A (1.6 μm) and GF/F (0.7 μm) glass fibre filters (Whatman, USA). DOC aliquots were fixed adding 50 μL of clean orthophosphoric acid at 25% until pH < 2. DOC and TDN were processed in a Shimadzu TOC-V organic carbon analyser. FDOM was characterized by Excitation-Emission matrices (EEMs) on a LS55 PerkinElmer Luminescence spectrometer following Romera-Castillo et al. (2022). Wavelengths fluorescence corresponding to peak-C (350 nm/450) and peak-T (290/350 nm) were extracted to compute the ratio of humic-like to protein-like fluorescence (FDOM T/FDOM C) (Coble 1996). Nutrients, DOC, TDN and FDOM were measured at the Chemistry service and the Observation and Characterization of the Marine Environment service, respectively, of the *Institut de Ciències del Mar* (Barcelona).

2.3 | Estimates of Deviations From Conservative Mixing of Nutrient

To explore nutrient behaviour within the STE, a simple two-endmember reactive mixing model was used to estimate the potential production or consumption of the different chemical elements by quantifying deviations in their concentrations from the theoretical conservative mixing line between fresh groundwater and seawater. Sample N2_15, which had the lowest salinity and highest nitrate among all groundwater samples, was used as the fresh groundwater endmember, and the most off-shore sample, SW2, was used as the seawater endmember (see Supporting Information).

2.4 | Prokaryotic Abundances and Heterotrophic Activity

The abundance of total prokaryotes and of high nucleic acid (HNA) content cells were quantified in duplicate on a CytoFLEX-S Flow Cytometer (Beckman and Coulter, Pasadena, CA) following the protocol of Pernice and Gasol (2023).

In winter, prokaryotic heterotrophic activity was estimated as protein synthesis assessed from incorporation of radioactively labelled leucine (^3H -leucine), whereas in summer we used BONCAT (biorthogonal non-canonical amino acid tagging) as an alternative method because of a defective batch of ^3H -leucine. ^3H -leucine incorporation (Revvity, 40 nM final conc.) was determined during 2–4 h incubations (duration adjusted for expected cell densities), following Kirchman et al. (1985)

with the modifications of Smith and Azam (1992). Incorporated radioactivity was measured on a Beckman liquid scintillation counter.

Single-cell transcriptional activity was estimated using BONCAT following Leizeaga et al. (2017) with minor modifications. Aliquots of 9 mL (seawater) or 45 mL (groundwater) were incubated for 3 h in the dark at in situ temperature with 1 μ M L-homopropargylglycine (HPG). Killed controls were fixed prior to HPG addition. Incubations were terminated with 3.7% (v/v) formaldehyde overnight at 4°C, filtered onto 0.2 μ m polycarbonate filters and stored at -80°C. Filters were permeabilized (lysozyme + achromopeptidase), subjected to a Cu(I)-catalysed click reaction with CR110 azide fluorochrome, counterstained with 4',6-diamido-2-phenylindole (DAPI), and examined by epifluorescence microscopy. BONCAT+ cells and DAPI counts were used to calculate the percentage of translationally active prokaryotes per sample.

2.5 | DNA Extraction and 16S rRNA Gene Sequencing

5–20 L of groundwater, and 2 L of seawater, were filtered through 0.2 μ m polycarbonate membrane filters (47 mm, PCTE, GVS) using a peristaltic pump. Filters were immediately frozen at -80°C. DNA was extracted from filters or from 0.7 to 1.4 g of soil using the DNeasy PowerSoil Pro Kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions. The V4-V5 region of the 16S rRNA gene was amplified with primers 515F (5'-GTGYCAGCMGCCGCGGTAA-3') and 926R (5'-CCGYCAATTMTTTRAGTTT-3') (Parada et al. 2016). Amplicons were pooled, purified, and libraries prepared by the Centre for Genomic Regulation (www.crg.eu, Barcelona, Spain). Sequencing was performed on an Illumina MiSeq platform (2 $\times$ 300 nt paired-end). Raw reads are available under European Nucleotide Archive (ENA) accession PRJEB98179.

Reads were processed and amplicon sequence variants (ASVs) inferred in R version 4.2.2 (R Core Team 2022) with DADA2 v1.26.0 (Callahan et al. 2016), using the Auladell and Obiol (2022) workflow available on GitHub. Briefly, primers were trimmed with CUTADAPT v3.5 (Martin 2011), reads were truncated to 230 nt (forward) and 180 nt (reverse) based on quality scores, and pseudo-pooling method was used to maximize retention of rare taxa. Chimeras were removed, and taxonomy was assigned via DADA2 *assignTaxonomy()* function, implementing the RDP naïve Bayesian classifier method (Wang et al. 2007), against SILVA 138.2 (McLaren and Callahan 2021). Chloroplasts and mitochondrial ASVs were discarded. To standardize sampling depth for richness analysis, the ASV table was rarefied to 38,402 reads per sample using the *rrarefy* function in the vegan 2.6-4 package (Oksanen et al. 2022).

2.6 | Prediction of Genes Involved in the Nitrogen Cycle

To explore the diversity of nitrogen-related pathways potentially encoded by the studied microbial communities, we employed PICRUST2 v2.6.1 (Douglas et al. 2020; Wright and Langille 2025)

to infer the abundances of genes related to the N-cycle from our ASVs. A nearest sequenced taxon index (NSTI) threshold of 2 was applied to assign each ASV to its nearest reference genome, resulting in the exclusion of 964 ASVs and the retention of 42,500 ASVs for downstream analysis. The retained ASVs had an average NSTI value of 0.30 ± 0.38 , indicating that most retained ASVs were relatively close to available reference genomes.

To compute the abundance of each gene, we calculated each gene's relative abundance from the percentage of each KEGG Ortholog (KO)'s raw count (from PICRUST2-derived output table *pred_metagenome_unstrat.tsv.gz*) with respect to the total number of NSTI-filtered reads retained in each sample (PICRUST2-derived output table *seqtab_norm.tsv.gz*). We then linked the stratified (per-ASV) KO predictions (combined_KO_predicted.tsv.gz) to our curated taxonomic table in order to associate each predicted KO with the corresponding ASV taxonomy. The 13 most abundant nitrogen-related KOs were chosen to explore their spatio-temporal pattern, while also ensuring representation across major N-cycling pathways: *nirK* (K00368), *nirS* (K15864), *nosZ* (K00376), *nifH* (K02588), *amoA* (K10944, same KO than *pmoA*), *hao* (K10535), *nirB* (K00362), *nirD* (K00363), *glnA* (K01915), *gltB* (K00265), *gltS* (K00284), *nasC/nasA* (K00372), and *nirA* (K00366) (see Table S1).

KO richness was calculated as the number of KOs present in each microbial community from a total of 10,106 KOs predicted by PICRUST2. KO diversity was calculated using the Shannon index, considering either the selected 13 KOs, or the diversity of KOs associated with the “Nitrogen metabolism” pathway as defined by the KEGG pathway database (<https://www.kegg.jp/kegg/pathway.html>), which in PICRUST2 includes 46 KOs (39 of which were detected in our dataset). Richness and diversity metrics were computed using the vegan package (version 2.6-10) in R.

2.7 | Quantification of *nirK*, *nirS* and *amoA* Genes Through Quantitative Polymerase Chain Reaction (qPCR)

Quantitative Polymerase Chain Reaction (qPCR) was used to validate PICRUST2 results, quantifying the abundance of three selected genes related to the nitrogen cycle: the ammonia monooxygenase gene (*amoA*) of ammonia-oxidizing archaea (AOA), involved in the first step of nitrification, and two nitrite reductase genes involved in denitrification (*nirK*, which encodes the copper-containing nitrite reductase, and *nirS*, which encodes the cytochrome cd1 nitrate reductase). Primer sequences, amplicon sizes and annealing temperatures are listed in Table S2. All reactions were performed on a CFX96 Touch Real-time PCR Detection System (Bio-Rad) with the iQ SYBR Green Supermix (Bio-Rad) containing a hot-start Taq DNA polymerase. We employed touchdown PCR protocols for *nirK* and *nirS*, in which the annealing temperature is progressively lowered to improve amplification specificity (see Table S2) (Saghāi et al. 2025). Prior to quantification, all environmental DNA extracts were screened for PCR inhibitors (e.g., humic substances, phenolics) by assessing the amplification efficiency of a synthetic internal positive control (IPC-A gBlock) in dedicated qPCR reactions containing the

extracts (Table S2). PCR inhibition was deemed unacceptable if the IPC-A Cq in the sample reaction exceeded that of the non-template control (NTC) by more than two cycles (Persson et al. 2026). In such cases, the affected samples were diluted tenfold prior to subsequent qPCR analyses of the N-cycling targets. Each 20 μ L reaction (see reagent details in Table S2) was run in duplicate alongside triplicate no-template controls and standard curve dilutions of gBlocks (see Table S3) to ensure accurate quantification. qPCR efficiencies for *nirK* and *nirS* ranged from 83% to 100%, demonstrating robust performance. For *amoA*, quantification was performed using a previously validated standard curve run on a separate plate, rather than plate-specific standards. Because *amoA* quantification was referenced to an external standard curve, absolute copy numbers should be interpreted with caution and not fully comparable quantitatively between campaigns (Bustin et al. 2009).

Melting-curve analysis (60°C to 95°C, 0.5°C increments) suggested amplification only of a single target in the majority of samples. Starting quantities (SQ) obtained by regression from qPCR software were converted to gene copies per litre by correcting for sample volume filtered and dilution factors. This normalization allowed direct spatial and seasonal comparisons of gene abundances.

2.8 | Statistical Analyses

All statistical analysis and graphical outputs were generated in R version 4.3.3 (R Core Team 2022). To identify hydrochemically different zones along the STE, samples were clustered based on physicochemical variables using a *k*-means clustering algorithm (*kmeans()*). The selected variables included temperature, salinity, pH, dissolved oxygen, oxidation–reduction potential, NO_3^- , NO_2^- , NH_4^+ , DOC, DON, as well as the spatial coordinates (distance from the shoreline and depth). These coordinates are included in the analysis because they are directly related to key variables such as groundwater transit times (distance) and the different aquifer layers (depth; see Martínez-Pérez et al. 2022). Variables were standardized (*z*-score), and soil samples were omitted from this analysis because physicochemical data were not available. To determine the optimal number of clusters we used diagnostic partitioning based on Calinski-Harabasz criterion, implemented through the *cascadeKM()* function (*cclust* package). Guided by these diagnostics and ecological interpretability, we imposed *k* = 4, and assigned each sample to one of four clusters: fresh shallow groundwater (hereafter “Fresh_S”, *n* = 12 samples, salinity 0.37–4.9), fresh deep groundwater (“Fresh_D”, *n* = 14, salinity 0.54–3.3), saline groundwater (“Saline”, *n* = 12, salinity 21–35) and seawater (*n* = 4, salinity 38–40, Figure 1b). For the three groundwater clusters, these salinity ranges corresponded to average total dissolved solids (TDS) values of 1.6 ± 1.4 , 1.5 ± 0.87 , and $24 \pm 3.7 \text{ g L}^{-1}$, respectively. These clusters were consistent across the two sampling campaigns.

Microbial community patterns were explored by non-metric multidimensional scaling (NMDS) based on Bray-Curtis distances, using *metaMDS()* on R vegan package v2.6.10 (Oksanen et al. 2022). Differences in microbial taxonomic composition among clusters and campaigns were assessed

using analysis of similarity (ANOSIM, R vegan). Links between physicochemical variables and functional gene abundances (qPCR or PICRUST2) were explored using principal coordinates analysis (PCoA) on Bray-Curtis distances of Hellinger-transformed gene abundance matrices via *cmdscale()*, followed by fitting nutrient variables using *envfit* (R vegan). Spearman correlations between physicochemical variables and gene abundances (qPCR and PICRUST2) were calculated using the *cor()* function. *p*-values were obtained via *cor.test()* and significant correlations (Bonferroni-corrected, *p* < 0.05 and *p* < 0.01) were indicated as a heatmap using the *pheatmap* R package. Given that none of the variables met normality assumptions (*p* < 0.05), Wilcoxon tests were applied. For comparisons between campaigns, paired Wilcoxon signed-ranks tests (*V*) were used, whereas comparisons between clusters or independent groups were performed using unpaired Wilcoxon rank-sum tests (Mann–Whitney, *W*). Test statistics and *p*-values are reported. Indicator ASVs of each hydrochemical cluster were identified using the *multipatt()* function and the group-equalized Indicator Value index (IndVal.g), implemented in the *indicspecies* R package v. 1.8.0 (Cáceres and Legendre 2009), based on the four clusters and soils samples (Soil, Fresh_S, Fresh_D, Saline, Seawater). Only significant indicator ASVs (*p* < 0.05) were retained for subsequent analyses.

3 | Results

3.1 | Physicochemical Variability Along the STE

The physicochemical parameters and nutrient concentrations featured pronounced spatial heterogeneity along the STE but little seasonal variation (Figure 1c), leading to the grouping of samples into three clearly different clusters representing three hydrochemically different areas of the aquifer (Figure 1a). Accordingly, continuous temperature and salinity measurements in N1 (Figure S1) showed pronounced vertical differences in conductivity and a general temporal stability of both parameters within each depth, with the exception of the shallowest depth (N1_10) where more pronounced variations in conductivity and temperature were detected. Saline groundwater samples (Saline cluster) were characterized by much higher salinity (avg. 26 ± 5), lower dissolved oxygen (avg. $7.5\% \pm 2.9\%$), ORP (avg. $-5 \pm 78 \text{ mV}$), NO_3^- (avg. $79 \pm 65 \mu\text{M}$) and DON (avg. $47 \pm 52 \mu\text{M}$), but higher levels of NO_2^- (avg. $1.9 \pm 2.6 \mu\text{M}$) and NH_4^+ (avg. $9 \pm 12 \mu\text{M}$) as compared to fresh groundwater samples (including both Fresh_S and Fresh_D; avg. 0.15 ± 0.17 and $3.5 \pm 8.1 \mu\text{M}$, respectively; Figure 1c). Despite similar salinity (avg. of 1.5 ± 1.4 in Fresh_S vs. 1.4 ± 0.83 in Fresh_D), and other environmental parameters (i.e., ORP, NO_3^- and NO_2^-), the two fresh groundwater clusters differed in dissolved oxygen (avg. of $21\% \pm 17\%$ in Fresh_S vs. $40\% \pm 14\%$ in Fresh_D), DOC (avg. of 76 ± 21 vs. $48 \pm 10 \mu\text{M}$) and NH_4^+ (avg. of 7 ± 11 vs. $0.50 \pm 0.26 \mu\text{M}$, Figure 1c).

Seasonal differences between campaigns were generally minor, with local shifts in specific parameters observed for some groundwater depths. For example, dissolved oxygen levels in Fresh_S samples showed different variability, ranging from 5.8% to 21% in winter to 2.7%–55% in summer, with no significant difference

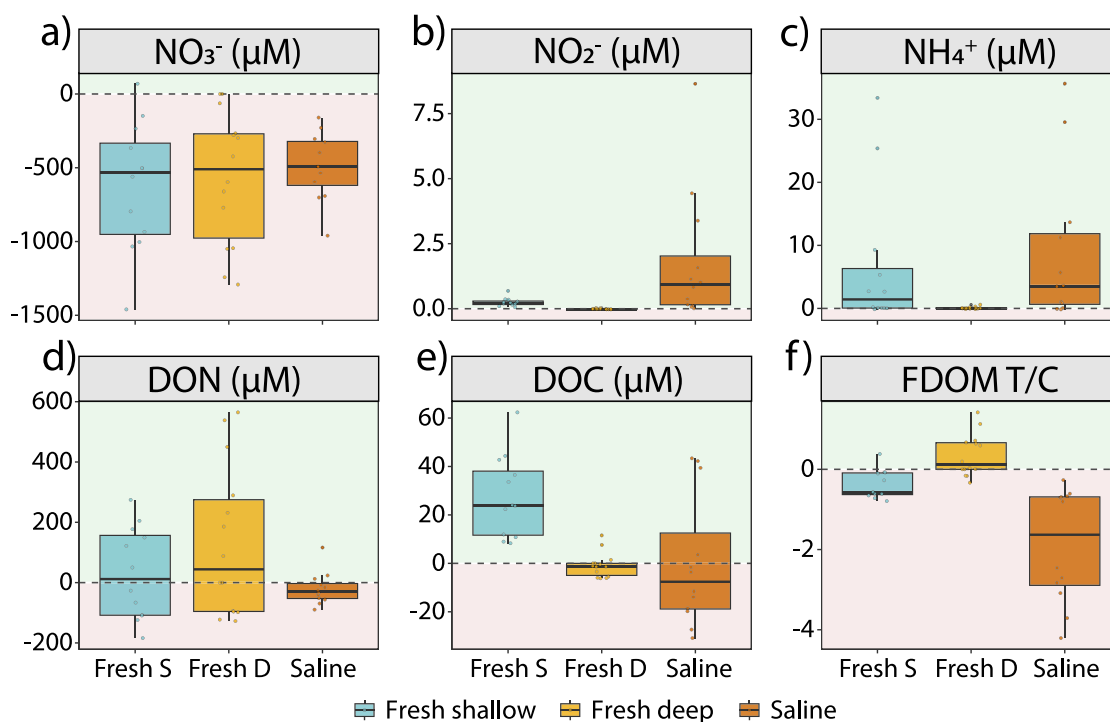


FIGURE 2 | Apparent consumption and production of chemical elements within the STE. Estimated deviations from the binary conservative mixing between fresh groundwater and seawater endmembers for nitrate (NO_3^- , a), nitrite (NO_2^- , b), ammonium (NH_4^+ , c), dissolved organic nitrogen (DON, d), dissolved organic carbon (DOC, e) and the ratio between peak T and peak C of fluorescent dissolved organic matter (FDOM T/C, f). Values are shown for each cluster. Positive values indicate apparent net production (green); negative values indicate apparent net consumption (red). Fresh_S and Fresh_D indicates Fresh-shallow and Fresh-Deep groundwater clusters, respectively.

between campaigns ($V=3$, $p=0.14$). Additionally, a general decline was observed throughout the STE from winter to summer in DOC (avg. 78 ± 24 to $49 \pm 14 \mu\text{M}$; $V=190$, $p<0.001$) and DON (avg. 280 ± 210 to $90 \pm 85 \mu\text{M}$; $V=180$, $p<0.001$).

The concentration of inorganic nutrients, TDN and DOM diverged markedly from a theoretical binary conservative mixing between fresh groundwater and seawater, indicating net production or consumption of certain elements within the STE (Figure 2). It should be noted, though, that this approach provides only a broad qualitative assessment of the behaviour in the STE (productive vs. consumptive), mainly because (i) the deviations highly depend on the selected endmembers and (ii) there are most likely additional endmembers. Consequently, the two-endmember approach is an oversimplification of the system and may introduce artefacts in the inferred production or consumption of certain solutes, and should therefore be interpreted only in a broad and qualitative manner.

Compared to the binary conservative mixing line, NO_3^- showed net removal across all groundwater clusters (Figure 2a). NH_4^+ exhibited net apparent production particularly within the Saline and Fresh_S clusters in both seasons (Figure 2c), and NO_2^- production was also detected in most saline groundwater samples (Figure 2b). Positive deviations in DOC concentration were found in all Fresh_S samples, and in certain saline groundwater sites (Figure 2e), and DON exhibited patterns of both positive and negative deviations (Figure 2d). The labile fraction of fluorescent dissolved organic matter (FDOM T/C peak ratio) suggested net consumption across the STE (Figure 2f), with the exception of Fresh_D, where a positive deviation was observed.

3.2 | Spatio-Temporal Variations in Prokaryotic Abundance and Heterotrophic Activity

Prokaryotic abundance, the percentage of HNA cells and prokaryotic heterotrophic activity varied markedly across the three groundwater sample clusters (Figure 3). The highest cell abundances were observed in saline groundwater locations (range $0.69\text{--}6.1 \times 10^5$ cells mL^{-1} ; $W=42$, $p<0.001$) followed by shallow freshwater samples ($0.47\text{--}1.8 \times 10^5$ cells mL^{-1} ; $W=210$, $p=0.09$), while deeper freshwater layers showed consistently the lowest cell abundances ($0.13\text{--}0.47 \times 10^5$ cells mL^{-1} ; $W=0$, $p<0.001$) (Figure 3a,b). The proportion of HNA cells tended to increase in saline groundwater (Figure 3c,d), coinciding with the highest heterotrophic activity (Figure 3e,f).

No clear seasonal variations were detected in the proportion of HNA cells throughout the STE (Figure 3c-f). However, in saline groundwater, prokaryotic abundance nearly doubled from winter to summer, although this increase was not statistically significant (avg. from 1.8 to 3.0×10^5 cells mL^{-1} ; $V=5$, $p=0.29$; Figure 3a,b).

3.3 | Microbial Community Structure and Taxonomic Composition

The NMDS revealed that microbial community composition was strongly differentiated according to the three groundwater sample clusters, and differed clearly from soils and seawater communities (ANOSIM $R_{\text{bycluster}}=0.82$, $p<0.0001$, Figure 4a). Samples within each cluster were grouped together, although

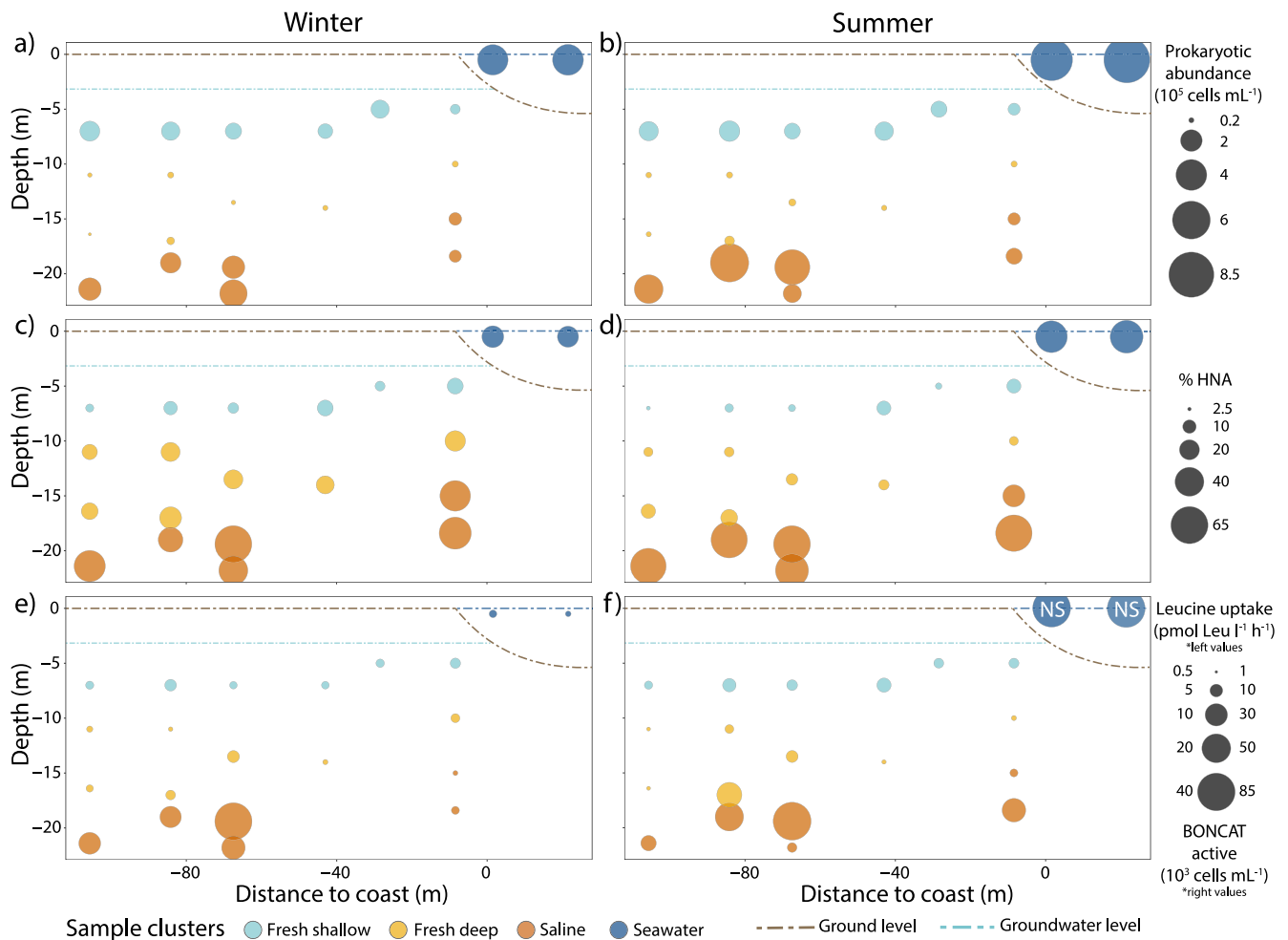


FIGURE 3 | Spatio-temporal variations in microbial properties along the STE. Total prokaryotic abundance (a, b), percentage of high nucleic acid content cells (HNA cells, c, d), and heterotrophic activity, measured as ^3H -leucine incorporation rates in winter (e) and as BONCAT active cells in summer (f) represented across the STE, in winter (a, c, e) and summer (b, d, f). Size is proportional to each variable's values, and colour denotes the different sample clusters. "NS" indicates BONCAT values outside the plotted scale, corresponding to 4.5×10^5 and 4.3×10^5 active cells mL^{-1} for the left and right seawater bubbles (f), respectively.

all groundwater samples closest to the sea (NSEA) showed the highest similarity to marine communities (Figure 4a). In contrast, season had no discernible effect on overall community structure (ANOSIM $R_{\text{bycampaign}} = 0.02$, $p = 0.17$).

ASV richness per sample was much higher in the STE than in seawater (avg. 2300 ± 730 vs. 740 ± 240 ASVs; $W = 1$, $p < 0.001$; Figure 4b). Within the aquifer, it declined from the highest richness in Fresh_S samples (avg. 2900 ± 360 ASVs) to the lowest in saline groundwater (avg. 1600 ± 690 ASVs). Fresh_D communities showed intermediate richness (avg. 2400 ± 480 ASVs), similar to that in surface soil samples (avg. 1900 ± 480 ASVs). In terms of taxonomic composition (Figure 4c), groundwater samples clearly differed from soils and seawater samples, showing a larger number of phyla (87 vs. 37) and a dominance of groups such as Patescibacteria, Nanoarchaeota, Thermoproteota, Verrucomicrobiota, and Nitrospirata, which were nearly absent in adjacent soils and seawater. The relative abundances of these phyla varied over depth: Fresh_D communities hosted lower proportions of Verrucomicrobiota and Patescibacteria, but higher proportions of Thermoproteota than the Fresh_S groundwater

cluster (Figure 4c). Saline groundwater was dominated by Pseudomonadota, Patescibacteria, and Verrucomicrobiota, but also Bacteroidota, Thermodesulfobacterota, Actinomycetota, and Nitrospirata. Indicator analysis further supported this spatial partitioning, showing that most indicator ASVs reached their highest abundance within their corresponding hydrochemical cluster, with only limited overlap between adjacent groundwater layers and a small contribution of soil indicators to the Fresh_D and Saline clusters (Figure S3a). At the order level, although a large fraction of indicator ASVs remained unclassified, highlighting the potential taxonomic novelty of the STE microbiome, roughly the same groups dominated the three groundwater indicator pools, with most classified sequences belonging to Omnitrophales (Verrucomicrobiota) and Woesearchaeales (Nanoarchaeota) (Figure S3b).

3.4 | Functional Prediction of Nitrogen Cycling Potential

We used PICRUST2 predictions based on 16S rRNA gene data to infer the potential metabolisms related to nitrogen cycling,

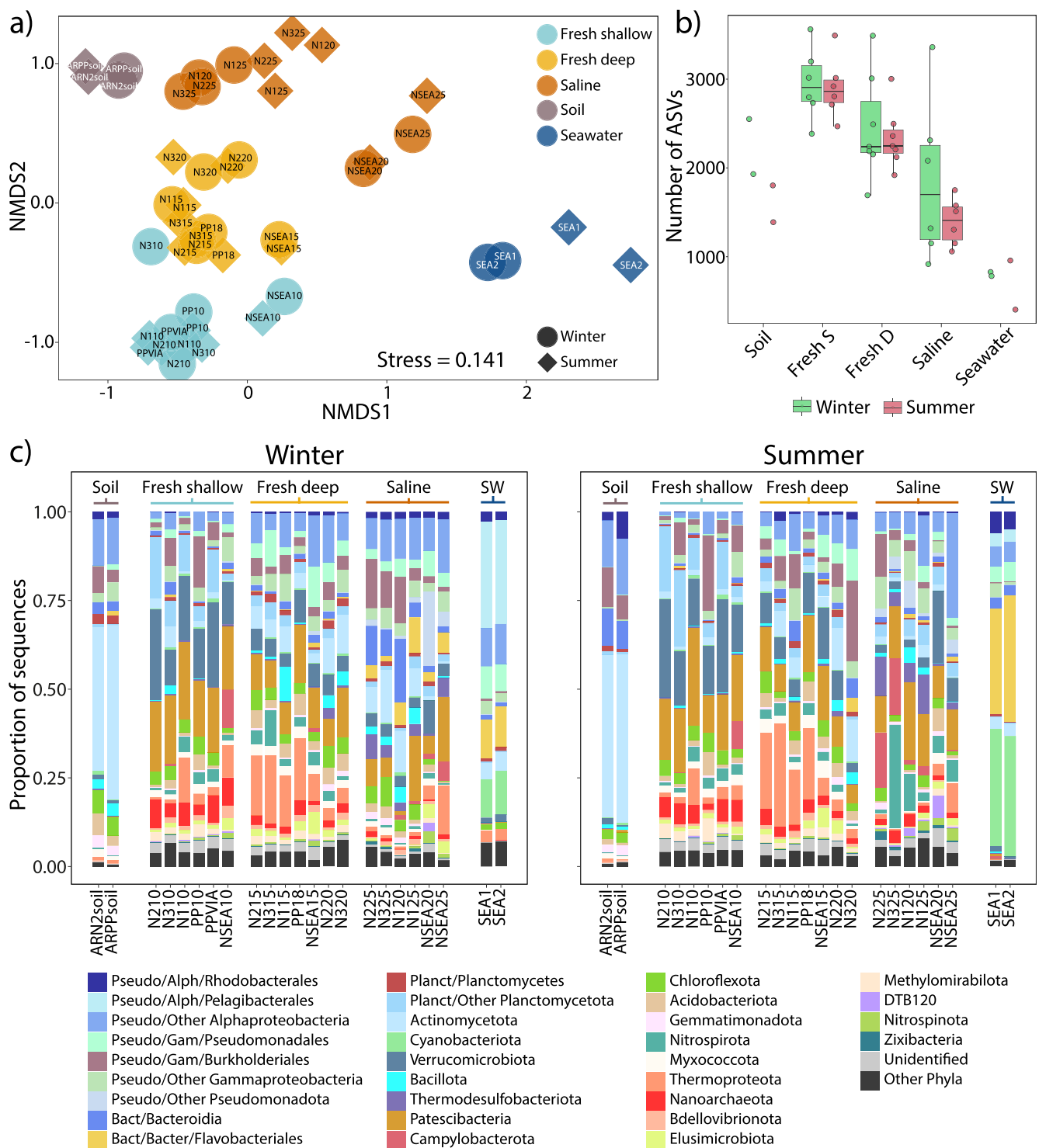


FIGURE 4 | Taxonomic structure, richness and composition of prokaryotic communities along the STE. (a) Non-Metric Multidimensional Scaling (NMDS) ordination of all microbial communities based on Bray-Curtis dissimilarities, colour-coded by cluster. Shape indicates sampling season. (b) Number of ASVs per sample (taxonomic richness) across different clusters and colour-coded by campaign. (c) Taxonomic composition of prokaryotic communities, ordered by cluster and differentiating between campaigns. Fresh_S and Fresh_D indicate Fresh-shallow and Fresh-Deep groundwater clusters, respectively. Note that the classification was performed at the phylum level, except in the case of Pseudomonadota, Bacteroidota, and Planctomycetota, where the main classes and even orders were distinguished.

identifying KEGG Orthologs (KOs) (Figure 5; Figure S4). The 964 ASVs that were excluded from the analysis for having NSTI > 2, were predominantly affiliated with some Nanoarchaeota and Patescibacteria (Figure S5a), yet they represented less than 3.4% of reads per sample (avg. 1.3%, Figure S5b), indicating that PICRUSt2 predictions captured the majority of the studied communities.

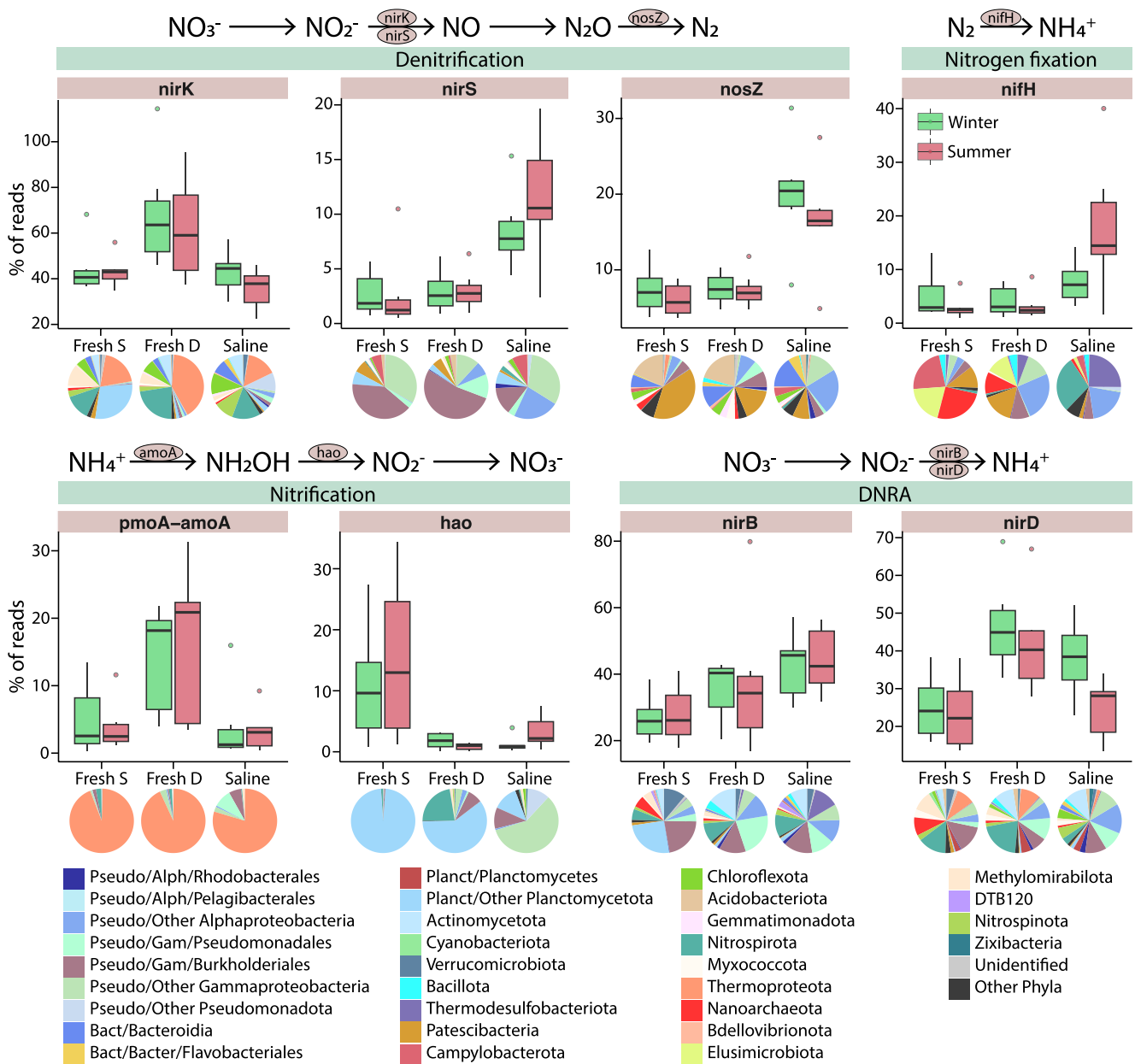


FIGURE 5 | Distribution of predicted nitrogen-cycle functional genes and their taxonomic contributors by cluster. Relative abundance of ASVs predicted to encode KOs inferred by PICRUSt2 (shown as % of PICRUSt2-retained reads) by cluster, color-coded by sampling campaign. Above each panel, the corresponding nitrogen transformation process is shown, with the targeted genes placed at the respective step. Pies show the taxonomic groups estimated to carry that gene in each cluster, combining the two campaigns. Fresh_S and Fresh_D indicate Fresh-shallow and Fresh-Deep groundwater clusters, respectively. Taxonomic assignments are shown at the phylum level, except for Pseudomonadota, Bacteroidota and Planctomycetota, where the main classes and even orders were distinguished.

From the suite of KOs predicted by PICRUSt2, we focused on those assigned to genes that mediate key steps in the nitrogen cycle (Table S1), and were predicted to be abundant in our dataset. Respiratory nitrogen transformations were assessed via genes involved in nitrification (*amoA*, *hao*), denitrification (*nirK*, *nirS* and *nosZ*), and dissimilatory nitrate reduction to ammonium (DNRA) (*nrfB* and *nirD*, Figure 5). Within assimilatory pathways, nitrogen fixation was tracked with *nifH* (Figure 5), heterotrophic ammonia assimilation with *glnA*, *gltB* and *gltS*, and assimilatory nitrate reduction with *nasC/nasA* and *nirA* (Figure S4). This set of 13 genes provides a comprehensive overview of both energy conserving

and biosynthetic nitrogen transformation potentials across the STE.

The relative abundance of PICRUSt2-predicted genes exhibited pronounced cluster specificity (ANOSIM $R_{\text{bycluster}} = 0.48$, $p < 0.001$, Figure 5; Figure S4). Among respiratory pathways, *nirK* was the most abundant denitrification marker predicted for the largest proportion of reads (avg. 50% of reads retained by PICRUSt2), with highest proportion in Fresh_D groundwater (avg. 65%). In contrast, *nirS* and *nosZ* reached their highest predicted relative abundances in saline groundwater clusters (avg. 10% and 18%, respectively). Predictions for nitrogen-fixation

gene *nifH* also peaked in deep saline layers (avg. 13%), and particularly in summer. Nitrification gene predictions showed comparable relative contributions (avg. of 8.0% vs. 5.3% of reads for *amoA* and *hao*, respectively) but with contrasting spatial patterns: *amoA* predominated in deeper freshwater areas (avg. 15%), whereas *hao* was more abundant in Fresh_S samples (avg. 13%). Finally, predictions of DNRA genes *nirB* (avg. 36%) and *nirD* (avg. 34%) were prevalent throughout the STE, with a tendency for higher predicted relative abundance with depth (Figure 5).

Genes from assimilatory nitrogen pathways were predicted to be ubiquitously abundant across the STE (Figure S4). Among these, *glnA*, involved in heterotrophic ammonia assimilation, was estimated to be the most abundant (180% of reads), with *gltB* and *gltS* being also prevalent (avg. 63% and 39%, respectively). Overall, most PICRUST2 predicted genes showed little seasonal variations, but the saline groundwater cluster exhibited notable shifts in a subset of genes, particularly *nirS*, *nifH*, *nirD*, *glnA* and *nirA* (ANOSIM $R_{\text{bycampaign}} = 0.25$, $p = 0.03$, Figure 5; Figure S4).

In most cases, functions were distributed across multiple phyla, with the exception of nitrification genes *amoA* (mostly assigned to Thermoproteota) and *hao* (dominated by Planctomycetota in Fresh_D or Gammaproteobacteria in Saline groundwater) (Figure 5; Figure S4). At the order level, *amoA* was mainly associated with Nitrosopumilales, whereas *hao* was dominated by Brocadiales in fresh groundwater but was mainly assigned to Chromatiales in the saline layer. *nirS* was strongly associated with Burkholderiales in the fresh clusters, while its contributors were more evenly distributed in saline groundwater. By contrast, *nirK* and the DNRA-related genes *nirB* and *nirD* were distributed among a broader range of orders, including Omnitrophales, Nitrosopumilales or Nitrospirales, with their relative contributions varying among clusters (Figure S6). In general, the identity of the taxa potentially carrying each function was similar across clusters, but differed largely among individual KOs (Figure 5; Figures S4 and S6).

Specific anammox KOs cannot be predicted by PICRUST2, so we surveyed ASV taxonomy for known anammox genera: *Candidatus Brocadia*, *Scalindua*, *Kuenenia*, *Jettenia*, *Anammoxoglobus*, and *Anammoximicrobium* (Ren et al. 2022). Only *Scalindua* and *Brocadia* were present (Figure S7), with the latter being the most abundant (up to 16%), particularly in Fresh_S samples.

Compared to adjacent systems (soils, seawater), the STE harboured the greatest functional KO-repertoire predicted by PICRUST2 (Figure S8). Considering the 10,106 predicted KOs, richness was highest in STE and soil samples, and lowest in seawater (Figure S8a). When focusing on the nitrogen metabolism, however, the STE harboured more diversity than soils and seawater, either when considering the 39 detected KEGG nitrogen metabolic pathways or the 13 nitrogen-related KOs used in this study (Figure S8b,c). At the KO level, several denitrification, nitrogen fixation and assimilatory nitrate reduction markers (e.g., *nirK*, *nirS*, *nifH*, *nirB*, *nirD* and *nirA*) showed richness values in the STE in between soils and seawater samples, whereas assimilation-related KOs such as *glnA*, *amoA* and *nasC/nasA* were notably richer within the STE (Figure S8d).

3.5 | Distribution of Ammonia Oxidizers and Denitrifiers Using qPCR

The quantification of *amoA*, *nirK* and *nirS* genes by qPCR was used to validate PICRUST2 predictions, revealing pronounced spatial heterogeneity in copy numbers across the STE (Figure 6; Figure S9). Gene abundances of *amoA* (involved in nitrification) were highest in fresh groundwater zones, showing an average 9.5×10^5 copies L^{-1} , compared to saline layers (avg. 4.0×10^5 copies L^{-1} , Figure 6a,b), however, the difference was not significant ($W = 211$, $p = 0.087$; Figure S9). In contrast, the denitrification genes *nirK* and *nirS* both peaked in saline groundwater (avg. 52×10^5 copies L^{-1} for *nirK* and 48×10^5 copies L^{-1} for *nirS*), exceeding abundances observed in fresh-groundwater (avg. 2.4×10^5 and 4.0×10^5 copies L^{-1} for *nirK* and *nirS*, respectively; $W = 55$, $p = 0.0016$ for *nirK* and $W = 58$, $p = 0.0022$ for *nirS*; Figure 6c–f; Figure S9). Overall, *amoA* copies were the most abundant in Fresh_S, and in Fresh_D, whereas *nirS* and *nirK* showed the highest copy numbers in saline groundwater samples (Figure 6g,h). qPCR results generally agreed with PICRUST2-derived functional predictions, although *nirK* reached its highest predicted abundance in the Fresh_D cluster rather than in saline groundwater.

Temporal variability was minor compared to spatial differences (Figure 6; Figure S9). For instance, *amoA* copy gene numbers fluctuated in certain freshwater locations, and there was a general, non-significant decline in *amoA* abundance in saline groundwater sites, from 6.4×10^5 in winter to 1.6×10^5 copies L^{-1} in summer ($V = 19$, $p = 0.093$). As stated in the Methods, *amoA* quantification relied on an external standard curve rather than plate-specific standards, therefore, absolute copy numbers should be interpreted cautiously in cross-campaign comparisons. Denitrification gene abundances of *nirK* and *nirS* also declined seasonally in saline groundwater, decreasing non-significantly from 48×10^5 and 67×10^5 copies L^{-1} in winter to 11×10^5 ($V = 16$, $p = 0.29$) and 29×10^5 ($V = 17$, $p = 0.20$) copies L^{-1} in summer, respectively (Figure 6c–f; Figure S9). In contrast, significant seasonal differences were detected in fresh-groundwater, with *nirK* showing a significant increase during summer in Fresh_D ($V = 0$, $p = 0.016$), and *nirS* decreasing in Fresh_S during the same season ($V = 21$, $p = 0.031$; Figure S9). Because the seasonal decline was more pronounced for *nirK* than for *nirS*, *nirS* became the dominant *nir* gene in saline groundwater during summer (Figure 6h).

3.6 | Links Between Microbial Functional Potential and Groundwater Physico-Chemistry

Pooling all samples together, different associations emerged between the abundance of individual genes (gene copies L^{-1}), measured by qPCR, relative abundances predicted by PICRUST2 (% of reads corresponding to ASVs predicted to carry the genes), and environmental variables (Figure S10a). For example, the qPCR-measured *amoA* gene copy abundance showed only a significant positive association with NO_3^- and a negative association with the DOC/DIN ratio. *nirK* and *nirS* (qPCR), instead, showed strong and significant correlations with most parameters, correlating negatively with %DO, ORP, pH, and NO_3^- and positively with DON, DOC/DIN, NO_2^- , and NH_4^+ . When considering the

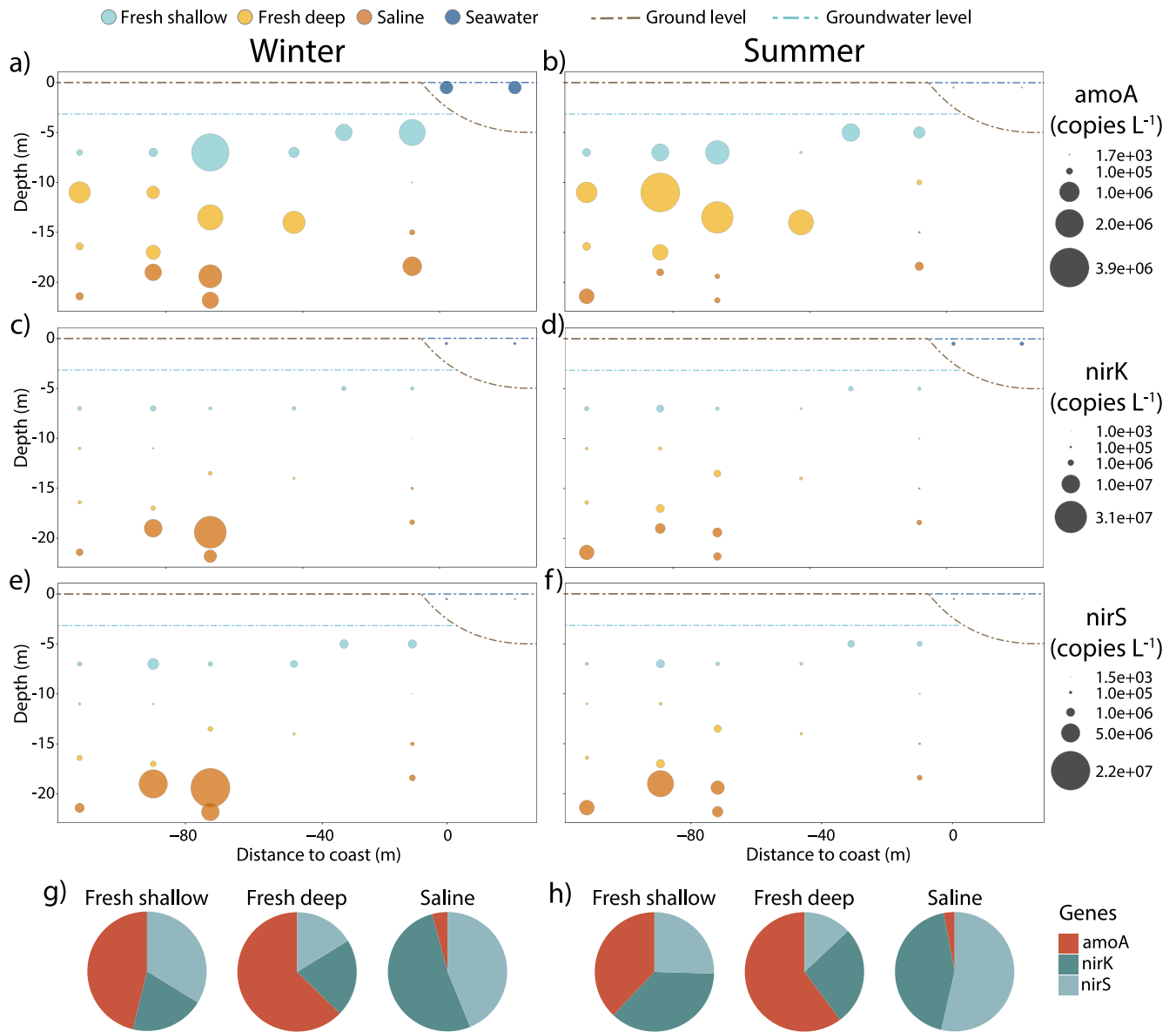


FIGURE 6 | Spatio-temporal variations in nitrification and denitrification genes along the STE. Gene copy numbers per liter for *amoA* (a, b), *nirK* (c, d) and *nirS* (e, f) in winter (a, c, e) and summer (b, d, f). Bubble size is proportional to gene copy number, and color corresponds to cluster assignment. Relative contribution of the three genes within each cluster, shown for winter (g) and summer (h) campaigns.

relative abundance of PICRUST2 predicted KOs, *nirA*, *nirK*, and *amoA* were negatively associated with DOC/DIN, salinity, DOC, NO₂⁻, and NH₄⁺, and positively with NO₃⁻, pH, ORP, DON, FDOM T/C, and %DO, whereas *nosZ*, *nirS*, *nifH*, *gltB*, and *nirB* showed the opposite pattern. *glnA*, *nasC/nasA*, *gltS*, and *hao* showed generally weaker associations (Figure S10a). Notably, while the patterns of *amoA* and *nirS* based on qPCR or PICRUST2 were broadly similar, those of *nirK* showed a completely opposite trend.

An ordination of communities based on qPCR data showed significant grouping by sampling cluster (ANOSIM $R_{\text{bycluster}} = 0.32$, $p < 0.001$) with DOC, NO₃⁻ and NO₂⁻ significantly associated with the gene abundance ordination (Figure S10b). Ordination based on PICRUST2 predicted relative gene abundances also showed significant grouping by cluster (ANOSIM $R_{\text{bycluster}} = 0.47$, $p < 0.001$, Figure S10c), and in addition to NO₃⁻, NO₂⁻, and

DOC, NH₄⁺ and DON were also significantly associated with the ordination of the predicted functional potential.

4 | Discussion

4.1 | Large Spatial Heterogeneity and Seasonal Stability of STE Microbial Communities Driven by Physicochemical Gradients

The hydrogeochemical structure of the Argentona aquifer was summarized by three hydrochemical sample clusters separating shallow fresh, deep fresh and saline groundwater. This contrasts with previous studies describing an intermediate brackish mixing zone (salinity range 6.2–25) at depths ranging from 13 to 17 m (Folch et al. 2020; Ruiz-González et al. 2022), which was not observed during the samplings of this study. This difference

may be due to dry years in which the study was performed and the relatively wet period prior to sampling that displaced saline and brackish waters seawards. In turn, the observed differences between the two fresh groundwater layers during our sampling, which largely differed in oxygen, NH_4^+ and DOC concentration, potentially reflect different fresh groundwater recharge pathways, water ages, or in situ transformation processes.

Our conservative-mixing analysis supports previous findings from reactive end-member mixing analysis (Goyetche et al. 2022) suggesting that nutrient dynamics in the Argentona STE are primarily driven by biogeochemical transformations that occur throughout the entire salinity gradient. Nitrate concentrations were much higher in fresh than in saline groundwater, in agreement with terrestrial anthropogenic inputs (Rufi-Salís et al. 2019). Net NO_3^- deviations were found across the three sample clusters, supporting NO_3^- attenuation throughout the aquifer, a biofiltering potential shown in STEs worldwide (Chen et al. 2025; Santos et al. 2021). Local NH_4^+ and NO_2^- production was mostly found in fresh shallow and saline groundwater, both of which showed lower dissolved oxygen concentrations than the intermediate fresh groundwater layer. NH_4^+ accumulation under low-oxygen conditions has been linked to organic matter remineralization and reductive nitrogen pathways such as DNRA in other STEs (Couturier et al. 2017; Rodellas et al. 2018), whereas NO_2^- may represent a transient intermediate generated during multiple nitrogen transformation pathways, including nitrification, denitrification, and DNRA (Daims et al. 2016). This supports localized microbial production of both NH_4^+ and NO_2^- in different sections of the Argentona STE.

The abundance, activity, and taxonomic richness and composition of the STE microbiome varied across the three hydrochemical clusters. Deeper saline groundwater contained higher prokaryotic abundances, a larger proportion of HNA cells, and elevated heterotrophic production compared with fresh groundwater. HNA cells are generally interpreted as larger and metabolically active bacteria (Morán et al. 2007), supporting the enhanced microbial activity observed in deeper saline zones. A similar spatial structuring in these properties was found during a previous summer sampling at the study site (Ruiz-González et al. 2022), indicating that this spatial partitioning of communities is relatively stable through time. However, these authors detected a brackish intermediate layer, which led them to the conclusion that salinity was the most important driver of microbial assemblages. Our results show instead a clear separation even between the two fresh groundwater clusters, indicating that factors other than salinity also contribute to shaping microbial communities.

The elevated microbial biomass, size and heterotrophic activity in deep saline layers co-occurs with higher apparent DOC consumption and local production of NO_2^- and NH_4^+ , suggesting active microbial transformation of organic matter and nitrogen compounds in this deep layer despite its expected stability and much older origin (Martínez-Pérez et al. 2022). This high biomass could be sustained, at least in part, by in situ chemolithoautotrophy fueled by the oxidation of reduced compounds, as inorganic carbon fixation is widespread in inland groundwater (Overholt et al. 2022; Ruiz-González et al. 2021). Such processes could also contribute to DOC release and hence support

heterotrophic growth (e.g., Sebastián et al. 2019). However, the extent and relevance of carbon fixation in STEs remains largely unexplored.

The taxonomic composition of microbial communities also mirrored the three clusters, supporting a vertical structuring of communities in this system following the pronounced physicochemical gradients. Similar depth-driven turnover of microbial communities has been reported in the few other cases where comparable high-resolution spatial studies have been conducted in STEs, showing that hydrogeochemical gradients strongly structure microbial assemblages (Brick et al. 2025; Hong et al. 2019; Sang et al. 2019). However, microbial communities from the Argentona aquifer appear to be highly site-specific, as they showed minimal taxa overlap even between close sites (Ruiz-González et al. 2025; Ruiz-González et al. 2022). This suggests the existence of fine-scale environmental heterogeneity not captured by our sampling strategy, and points to the presence of multiple, spatially discrete micro-niches capable of sustaining such elevated microbial diversity.

Regarding temporal changes, our data indicate only minor seasonal differences in microbial communities. However, as evidenced by previous studies, the multilayered aquifer is dynamic, both geochemically, through landward and seaward shifts of the fresh-saline interface, and microbiologically (Palacios et al. 2020; Ruiz-González et al. 2022). The absence of clear evidence for seasonal dynamics in our dataset may therefore reflect the lack of extreme precipitation events, storm surges, and the relatively calm and similar conditions during the two studied periods.

4.2 | Spatial Segregation of Microbial Functional Groups Suggests Complex Regulation of the Aquifer N Cycling

The spatial structuring of N-cycling genes underscored the role of hydrogeochemical zonation in shaping microbial metabolisms and STE functioning, and suggests a complex regulation of N-cycling within the aquifer. We observed differences in the predicted potential for denitrification (*nirK*, *nirS*, *nosZ*) and DNRA (*nirB*, *nirD*) across clusters, with relative increases in intermediate and deep layers, respectively. Indeed, although the ratio between predicted DNRA to denitrification (estimated as the ratio of *nirB* + *nirD* to *nirK* + *nirS*, Figure S11) was nearly 1:1 across the STE, it approached 2:1 in the deepest saline zone, where high NH_4^+ concentrations were observed, suggesting that DNRA may represent an important nitrate removal pathway in these systems. This aligns with results from the Yangtze STE (China), where DNRA accounted for over 35% of N transformation and surpassed denitrification (Chen et al. 2025), and with microbial evidence that denitrification and DNRA were the main processes removing and transforming, respectively, anthropogenic N inputs in Mobile Bay, USA (Montiel, Lamore, Stewart, Lambert, et al. 2019). The higher predicted DNRA potential in deeper layers agrees with the idea that under nitrate-limited and high organic carbon conditions, DNRA may become relatively more favourable than denitrification due to kinetic and enzymatic constraints (Liao et al. 2025), as well as with studies suggesting that increases in groundwater salinity

may favour DNRA in front of denitrification and nitrification in STEs (Santoro 2010).

Anammox represents another important nitrogen removal pathway in STEs, with high abundance detected at aerobic-anaerobic transition zones in the Gloucester Point STE, Virginia, US (Wu et al. 2021), although rates were found to be generally lower than denitrification in low-permeability intertidal sediments in Renshan, China (Jiao et al. 2018). At our site, anammox bacteria (*Brocadia*) were most abundant in fresh-shallow groundwaters, in agreement with decreases in anammox potential rates with increasing salinity in intertidal sediments (Jiao et al. 2018).

Assimilatory nitrate reduction (ANR; *nasC/nasA*, *nirA*) and heterotrophic ammonia assimilation (*glnA*, *gltB*, *gltS*) also emerged as potentially important N sinks in Argentona STE. Unlike denitrification or DNRA, these processes incorporate N in biomass, coupled with DOC acquisition by heterotrophs, thereby retaining N within the microbial food web. Experimental investigations showed that elevated DIN can accelerate particulate organic-matter turnover in a STE in SW Iberian Peninsula (Ibáñez et al. 2021), consistent with the link that we observed between DOC concentrations and genes for ANR or heterotrophic ammonia assimilation. Also, positive correlations between the relative abundances of *nasC/nasA*, *gltS* and *gltB* with DON:DIN ratios align with previous findings showing that heterotrophic uptake of NO_3^- and NH_4^+ is regulated by DOC:DIN in headwater streams (Peñarroya et al. 2023, 2024). These observations suggest that uptake of DIN by heterotrophs could influence N fate to a larger extent than denitrification in nitrate and carbon rich STEs like Argentona.

Net NO_2^- and NH_4^+ production in Argentona likely results from multiple processes, including organic matter mineralization, incomplete denitrification, DNRA or anammox, all of which can be modulated by redox conditions, DOC and salinity (Couturier et al. 2017; Wu et al. 2021). Organic carbon mineralization was likely most important in the deep STE layers, as evidenced by the elevated heterotrophic activity and apparent DOC depletion in these zones, likely explaining the observed accumulation of NH_4^+ . Signals for DNRA-related KOs (*nirB*, *nirD*) and NO_2^- accumulation in deep-saline layers are consistent with carbon-driven NO_3^- recycling (DNRA or incomplete denitrification) under low-oxygen transition zones where fresh and saline groundwater mix (Burgin and Hamilton 2007; Kelso et al. 1997). The co-occurrence of anammox *Brocadia* alongside high *amoA* relative abundances is consistent with the potential coexistence of nitrification and anammox across redox interfaces, as have been observed in coastal sediments and mangrove coastal aquifers (Lu et al. 2021; Wu et al. 2021).

Adding to this complexity, N-cycling functions were predicted to be distributed across multiple distant phyla, with many different taxa performing a given step of the same pathway. Functional redundancy among microorganisms complicates the prediction of biogeochemical outcomes, as different taxa may perform similar functions but respond differently to environmental drivers. This diversity in responses contributes to both the stability and resilience of ecosystem processes (Biggs et al. 2020; Louca et al. 2018; Yachi and Loreau 1999). Our results suggest that KO richness and diversity were higher in the

STE than in seawater, indicating a likely broader, more redundant, and versatile nitrogen-related functional potential and reflecting the higher niche heterogeneity of STE compared to seawater. Moreover, taxa predicted to be involved in different steps of a single metabolic pathway were often spatially segregated, contributing to high functional complexity, as shown in groundwater microbiomes (Mosley et al. 2022). In addition, the presence of anoxic microniches within oxic zones may explain the apparent coexistence of aerobic and anaerobic metabolisms within STEs (Calvo-Martin et al. 2022). Oxygen gradients in STEs have been shown to drive shifts from nitrification to denitrification, depending on redox conditions and microbial community structure (Wu et al. 2021). In the Argentona STE, some of the main chemical reactions have been inferred through the chemical identification of the fresh and saline end-members and the spatial distribution of their mixing ratios, pointing to significant denitrification in groundwaters from the mixing and deep saline zones (Goyetche et al. 2022; Martínez-Pérez et al. 2022). Moreover, a study conducted with flow-through reactor experiments at the site suggests that groundwater level fluctuations can modulate redox conditions and oxygen availability, stimulating ammonium oxidation during low water table periods and promoting denitrification when the water table rises into organic-rich layers, releasing DOC and limiting oxygen supply (Roumelis et al. 2025). This may explain some of the observed seasonal differences in gene abundances, as ORP increased and DOC decreased from winter to summer, likely due to the more intense precipitation preceding the winter sampling survey or increased biochemical activity at the very shallow levels (above the sampling depth) removing DOC. Altogether, although denitrification has long been considered the main process transforming nitrate from coastal aquifers, our results suggest that N-cycling within STEs is mediated by a larger diversity of microbial processes that occupy different niches within the aquifer.

Quantitative-PCR data were used to validate some of the PICRUST2 predictions, and indicated that ammonia-oxidizing archaea (AOA) prevailed in oxygenated fresh groundwater (mostly in the Fresh_D cluster), whereas *nirK* and *nirS*, markers of denitrification, were enriched in oxygen-depleted deep saline groundwater. Overall, qPCR and PICRUST2 showed broadly consistent abundance patterns, with the main discrepancy observed for *nirK*, likely reflecting inherent differences in how the two approaches capture functional diversity. To evaluate this, we extracted all genomes carrying *amoA*, *nirS*, and *nirK* linked by PICRUST2 to our ASVs, and checked the coverage of these functional genes by our qPCR primers (Figure S12). *AmoA* primers (Tournia et al. 2008) showed the highest coverage (21% of *amoA*-containing genomes, capturing up to 90% of the community carrying *amoA* in fresh groundwater sites, Figure S12b). Even though PICRUST2 cannot differentiate *amoA* from *pmoA* because they share the same KO (K10944), the dominance of Thermoproteota (archaeal ammonia oxidizers) suggests that the predicted monooxygenase activity corresponds to ammonia oxidation rather than methanotrophy, but this should be confirmed through metagenomics or metatranscriptomics.

For *nirS* primers (Throbäck et al. 2004), the primers captured 17% of *nirS*-containing genomes, mostly belonging to Burkholderiales and other Gammaproteobacteria, representing between 10% and 50% of the ASV reads predicted to carry *nirS*.

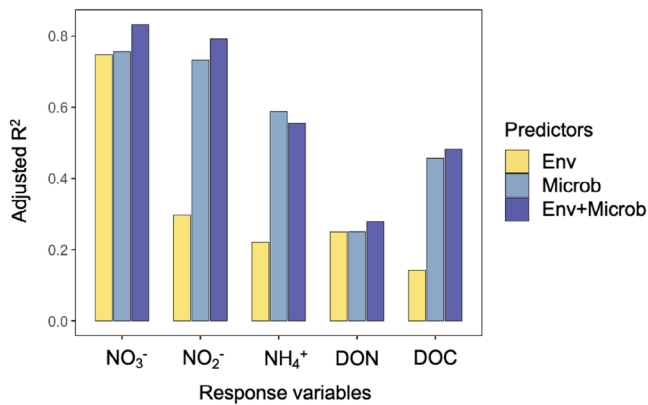


FIGURE 7 | Comparison of the adjusted R^2 values from multiple linear regression (MLR) models used to predict nutrient, DOC, and DON concentrations. Models included either physicochemical predictors (“Env”, pH, temperature, salinity, ORP, dissolved oxygen), microbial predictors (“Microb”, prokaryotic abundance, %HNA, and the relative abundance of 13 genes predicted by PICRUSt2), or both (“Env + Microb”). Details on the models and adjustment are shown in Table S5 and Figure S13, respectively.

In the case of *nirK* primers (Henry et al. 2004), the primers were able to capture only a very small fraction of predicted *nirK* taxa (mostly Alphaproteobacteria, which were not predicted to be the dominant denitrifiers according to PICRUSt2 in the study site).

Considering that total prokaryotic abundances varied substantially among clusters, and that PICRUSt2 provides predictions of relative gene abundances within the community whereas qPCR measures absolute gene concentrations per water volume, comparisons between the two methods should be interpreted with caution. To address this limitation, we examined the proportion of community reads (Figure S12c) represented by ASVs predicted by PICRUSt2 to carry *amoA*, *nirS*, or *nirK* and which were detectable by the corresponding qPCR primers *in silico*. These ASVs showed qualitative spatial trends consistent with those observed in qPCR patterns: *amoA*-associated ASVs were the most prominent in deep-freshwater sites, while both *nirK* and *nirS*-associated ASVs peaked in saline groundwater (especially in winter). Despite the known and inherent limitations of PICRUSt2, our findings support its utility for exploring potential patterns in functional diversity when more costly and time-consuming analyses such as metagenomics are not feasible (Adyasari et al. 2024; Hong et al. 2019).

Compared with primers commonly used in other environmental datasets, the *amoA* and *nirK* primer sets used here show the best coverage among the many alternatives (Table S4), while the *nirS* primers (*cd3aF/R3cdm*) showed lower coverage than two other primer pairs (*cd3F/cd4R* and *cd8F/cd2R*). This is relevant given that most of the functional information available on STEs to date is based almost exclusively on qPCR (Rogers and Casciotti 2010; Santoro et al. 2008; Wilson et al. 2023; Xiao et al. 2018), indicating that our current understanding of their functional complexity is likely biased and incomplete. Such limitations highlight the need for metagenomic and metatranscriptomic studies, which can reveal broader taxonomic and functional diversity and overcome the inherent biases of primer-based approaches (Kumar and Yadav 2024).

4.3 | Microbial Information Improves Understanding of Nitrogen Concentrations in the STE

To date, most studies of N-transformations in STEs rely primarily on chemical fluxes (Couturier et al. 2017), stable isotope approaches (Wong et al. 2020), transport modelling (Spiteri et al. 2008), reactive end-member mixing analysis (Goyetche et al. 2022), or detailed hydrogeochemical and geophysical characterizations (Martínez-Pérez et al. 2022), using reaction stoichiometries or rates, but rarely including microbial taxa, genes, or functional potential (e.g., Euler et al. 2024; Li et al. 2025; Wu et al. 2021).

We observed clear correlations between the abundance of individual microbial properties and the measured physico-chemical gradients, suggesting a strong link between the physicochemical environment and the presence of certain metabolisms. Hence, we used multiple linear regression (MLR) models (see Supplementary Methods) to evaluate the relevance of including microbial community data for improved understanding of N transformations occurring in the STE (Figure 7; Figure S13, Table S5). This method assumes that the effects of the predictors are linearly related to the response variables, and implicitly assumes that correlation implies causality. For instance, gene abundances may vary nonlinearly with salinity or other physicochemical parameters, because of thresholds or saturation effects; likewise, gene markers can be strongly correlated (e.g., *nirK* and *nirS*). Models including only microbial properties (i.e., %HNA, prokaryotic abundance, and PICRUSt2-predicted gene abundances), performed as well as those based solely on environmental data (temperature, salinity, ORP, oxygen, and pH) in predicting the variability in NO_3^- and DON concentrations. Remarkably, however, microbial-based models explained a much larger proportion of the variation in NH_4^+ , NO_2^- , and DOC than physicochemistry-based models alone, indicating that incorporating microbial community information substantially improves our ability to explain the distribution of these compounds. Since microbial communities respond to and influence local physicochemical conditions, these results highlight the complementary information provided by microbial variables rather than a separation between biological and physicochemical controls. In most cases, combining both environmental and microbial variables yielded slightly better model performance (Figure 7; Figure S13, Table S5).

Simple microbial properties such as total abundance or %HNA emerged as important predictors (Table S5), together with several predicted relative abundance of genes involved in denitrification (*nirK*, *nirS*, *nosZ*), DNRA (*nirB*, *nirD*), and heterotrophic assimilation of nitrate or ammonium (*gltS*, *nirA*). The fact that NO_3^- and DON were equally well explained by environmental variables than by microbial variables suggests that their concentrations are either mainly governed by mixing between NO_3^- and DON-rich fresh groundwater and more saline groundwater or influenced by gradients or reactions that affect environmental and microbial variables alike. In contrast, the variability in NH_4^+ , NO_2^- , and DOC, more sensitive to redox processes, appears to be primarily driven by microbial activity. Alternatively, this pattern may reflect the more integrative and smoothly varying nature of physicochemical gradients in STEs, which tend to

mask fine-scale variability that microbial indicators can better resolve due to their sensitivity to microzonation and niche differentiation. Thus, despite the limitations of both qPCR and PICRUST2, our results suggest that integrating microbial data can improve understanding of the N-cycling within STEs. The fact that we predicted the presence of KOs associated with anaerobic metabolisms in oxic conditions, and vice versa, might indicate either the presence of microniches within sediments cavities and pores, that a fraction of the microbial communities is dormant, not responding to current conditions, or that the detected microbiome integrates both present and past hydrogeochemical conditions in the system. Future STE studies should therefore aim to integrate molecular tools (e.g., qPCR, metagenomics/metatranscriptomics) with high-resolution biogeochemical modelling to better resolve the dynamic coupling between microorganisms and nitrogen fluxes (Louca et al. 2016; Reed et al. 2014). Also, coupling dynamic changes (spatial or temporal) in functional genes with direct process-rate measurements, targeted mRNA assays, isotope tracing, and including poorly explored abiotic transformations of N (i.e., chemodenitrification by nitrite-iron interactions, Buchwald et al. (2016)) would certainly help to better constrain the relative contribution of N-cycling processes in regulating nitrogen removal or recycling in coastal aquifers. This is a critical step towards improving predictive models and estimation of nutrient export to the ocean driven by SGD (Erler et al. 2014; Otwell et al. 2018), which in the study site can be a relevant source of N to the sea (Diego-Feliu et al. 2022). Indeed, median SGD-driven fluxes of DIN were found to be significantly higher than the median SGD-derived nutrient fluxes estimated worldwide, and increased up to 9-fold after extreme precipitation episodes mostly due to changes in the proportions of brackish and saline discharge (Diego-Feliu et al. 2022). This suggests that although the potential role of microorganisms in shaping the chemistry of groundwater likely spans the entire STE salinity gradient, their relative importance in determining SGD-driven nutrient fluxes will likely depend on the prevailing groundwater flow paths.

5 | Conclusions

Our study demonstrates that microbial controls on nitrogen cycling in subterranean estuaries are spatially structured along pronounced physicochemical gradients and remain seasonally consistent across sampling campaigns. We show that microbial N transformations within the aquifer likely encompass diverse metabolisms (including nitrification, denitrification, nitrogen fixation, DNRA, anammox, assimilatory nitrate reduction, or heterotrophic ammonia assimilation), which are spatially segregated within the aquifer and encoded by a wide diversity of taxa. Nitrification appears to dominate in fresher, oxic zones, whereas DNRA potential exceeds denitrification in deep saline groundwater, supporting a potentially important role of DNRA for N removal. The abundance of genes involved in heterotrophic assimilation of nitrate and ammonium further suggests a potential but mostly overlooked role in modulating coastal groundwater chemistry. Finally, we show that simple microbial descriptors (e.g., prokaryotic abundance, %HNA) and specific functional genes improved predictions of dissolved N speciation, highlighting the tight coupling between microbial communities and biogeochemical processes. Overall, this complexity underscores

the need for deeper microbial knowledge to better constrain nitrogen transformations and fluxes via submarine groundwater discharge.

Author Contributions

Daniel Romano-Gude: methodology, data curation, investigation, formal analysis, visualization, writing – original draft, writing – review and editing. **Cristina Romera-Castillo:** writing – review and editing, data curation. **Andrea G. Bravo:** investigation, writing – review and editing. **Catalina Mena:** writing – review and editing, investigation. **Bella Almillategui:** investigation, writing – review and editing. **Nestor Arandia-Gorostidi:** writing – review and editing, formal analysis. **Valentí Rodellas:** conceptualization, methodology, investigation, supervision, writing – original draft, writing – review and editing. **Anna J. Székely:** methodology, writing – review and editing. **Jordi Garcia-Orellana:** conceptualization, methodology, investigation. **Albert Folch:** writing – review and editing, conceptualization, methodology, investigation. **Clara Ruiz-González:** conceptualization, methodology, investigation, supervision, writing – review and editing, writing – original draft, formal analysis. **Marc Diego-Feliu:** investigation, writing – review and editing, conceptualization, methodology. **Jesus Carrera:** conceptualization, methodology, investigation, writing – review and editing. **Stefan Bertilsson:** methodology, writing – review and editing.

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Ethics Statement

This study did not involve human participants, vertebrate animals, or regulated invertebrates and therefore did not require ethical approval.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Raw sequence reads are deposited in the European Nucleotide Archive (ENA) under accession number PRJEB98179. The environmental meta-data and the nonrarefied ASV and taxonomic tables are provided as Tables S6–S8.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Figure S1:** Temporal variations between July 2021 and September 2022 in temperature (a) and electrical conductivity (b) recorded at different depths within nest N1 (64 m inland, Figure 1a) with permanent installed sensors. Orange shaded bands denote the periods during which the two sampling campaigns were performed. Note the different scales in the Y axes of conductivity plots. **Figure S2:** Precipitation and air temperature variations in the Argentona sampling site during the period 2021–2022. Vertical yellow areas indicate the two sampling campaigns conducted, each spanning 3 days. Meteorological data were obtained from the 'Xarxa d'Estacions Meteorològiques Autòniques' (XEMA) through the 'Portal de Dades Obertes de la Generalitat de Catalunya' (https://analisi.transparenciacatalunya.cat/Medi-Ambient/Dades-meteorol-giques-di-ries-de-la-XEMA/7bvh-jvq2/data_preview), using daily records from the Cabrils station, the closest to the sampling site. **Figure S3:** Distribution and taxonomic composition of cluster-indicator ASVs. (a) Relative contribution of ASVs identified as indicators of each groundwater cluster (Soil, Fresh_S, Fresh_D, Saline and Seawater) across all clusters. Bars show the percentage of reads assigned to the indicator ASVs of each cluster. (b) Taxonomic composition of indicator ASVs for each cluster at the order level. Donut charts show the percentage of indicator ASVs assigned to each order, with percentage labels displayed only for taxa representing more than 5% of the indicator ASVs within each cluster. The 25 most abundant orders are shown individually, whereas the remaining orders are grouped as "Other orders". Taxonomic groups are ordered clockwise from the most to the least abundant, with "Other orders" always displayed last. Numbers at the center of each donut indicate the total number of indicator ASVs (n) identified for each cluster. **Figure S4:** Predicted distributions of heterotrophic nitrogen assimilation genes. Predicted relative abundances (expressed as % of PICRUSt2-retained reads) of the KOs predicted by PICRUSt2 in each cluster, colour-coded by sampling campaign. Above each panel, the corresponding nitrogen transformation process is shown, with the targeted genes placed at the respective step. Pie charts indicate the predicted taxonomic carriers of each gene, combining winter and summer campaigns. Fresh_S and Fresh_D indicates Fresh-shallow and Fresh-Deep groundwater clusters, respectively. Taxonomic assignments are shown at the phylum level, except for Pseudomonadota, Bacteroidota and Planctomycetota, where the main classes and even orders were distinguished. Pseudo, Pseudomonadota; Alph, Alphaproteobacteria; Gam, Gammaproteobacteria; Bact, Bacteroidota; Bacter, Bacteroidia; Planct, Planctomycetota. **Figure S5:** Abundance and taxonomic assignment of ASVs excluded from the PICRUSt2 analysis. Read abundance (a) and relative contribution (b) of ASVs discarded by PICRUSt2 due to NSTI > 2. Percentage values are indicated above each bar. Samples are organized by cluster and

campaign. Grey indicates ASVs retained by the analysis. **Figure S6:** Taxonomic composition of PICRUST2-predicted nitrogen cycling genes. Taxonomic composition of the predicted microbial contributors to the selected nitrogen cycling and nitrogen assimilation genes (*nirK*, *nirS*, *nosZ*, *nifH*, *nasC/nasA*, *nirA*, *nirB*, *nirD*, *hao*, *amoA*, *gltB*, *gltS* and *glnA*) across the three groundwater clusters (Fresh_S, Fresh_D and Saline). Taxonomic assignment was performed at the order level. The 30 most abundant orders across all genes and groundwater clusters are shown individually, whereas the remaining orders were grouped as "Other orders". Percentage values are displayed only for orders representing more than 5% of the predicted gene abundance within each donut. Orders are arranged clockwise from the most to the least abundant, with "Other orders" always shown last. Numbers at the center of each donut indicate the total number of ASVs predicted to contribute to each gene within each groundwater cluster. **Figure S7:** Distribution of anammox-related genera. Relative contribution (% of total reads) of *Scalindua* (a) and *Brocadia* (b) genera (anammox-associated genera) of identified ASVs, separated by cluster and colour-coded by sampling campaign. Fresh_S and Fresh_D indicate Fresh shallow and Fresh-Deep groundwater clusters, respectively. **Figure S8:** Richness and diversity of KEGG Orthologs (KOs) across different systems. (a) Boxplot showing KO richness in Soil, STE, and Seawater samples, based on a total of 10,106 KOs predicted by PICRUST2. (b) KO diversity within nitrogen related KOs classified under the "Nitrogen metabolism" pathway in the KEGG database (46 KOs in total, of which 39 were detected in our dataset). (c) KO diversity and (d) number of ASVs (taxonomic richness) within 13 selected nitrogen-related genes included in this study (*nirK*, *nirS*, *nosZ*, *nifH*, *amoA*, *hao*, *nirB*, *nirD*, *glnA*, *gltB*, *gltS*, *nasA*, and *nirA*). Diversity in panels b and c was calculated using the Shannon index. Open-coloured circles indicate sample values, while dots represent statistical outliers. **Figure S9:** Distribution of nitrogen-cycle gene abundances quantified by qPCR. Abundance of *amoA*, *nirK* and *nirS* qPCR-based abundances, grouped by cluster and colour-coded by campaign. Fresh S and Fresh D indicate Fresh-shallow and Fresh-deep groundwater clusters, respectively. Statistical differences among clusters (blue) were assessed using pairwise Wilcoxon rank-sum test for independent samples. Differences between sampling campaigns within each cluster (red) were evaluated using paired Wilcoxon signed-rank test on samples collected from the same wells. Significance levels are indicated as $p < 0.05$ (*) and $p < 0.01$ (**). **Figure S10:** Relationship between groundwater physico-chemistry and nitrogen-cycle gene abundances. (a) Heatmap of Spearman's correlation coefficients (ρ) between gene abundances and measured variables, including nutrients, DOC and physicochemical parameters, considering all groundwater samples together. Positive correlations are indicated in blue and negative in red, with color intensity proportional to ρ values. Gene abundance data source (PICRUST2 or qPCR) is indicated in the bottom axis. Asterisks indicate significance levels: *** $p < 0.001$ Bonferroni-adjusted threshold; ** $p < 0.01$; * $p < 0.05$. (b) Principal coordinates analysis (PCoA) based on Bray-Curtis dissimilarities of qPCR-measured *amoA*, *nirK* and *nirS* copy numbers, with nutrient variables fitted using envfit. (c) PCoA based on Bray-Curtis dissimilarities of PICRUST2-predicted read abundances for the 13 most abundant nitrogen-cycle genes, overlaid with envfit-fitted nutrient vectors. In panels (b) and (c), points are colored by sample cluster and shape indicates sampling campaign. Nutrients marked with an asterisk (*) denote significant ($p < 0.05$) envfit correlations. **Figure S11:** Ratio of predicted DNRA to denitrification genes. The ratio between predicted DNRA (*nirB* and *nirD*, encoding nitrite reductases that reduce NO_2^- to NH_4^+) and denitrification (*nirS* and *nirK*, encoding nitrite reductases that reduce NO_2^- to NO) gene abundance, inferred using PICRUST2, across environmental clusters. Boxplots are coloured by sampling campaign. Fresh_S and Fresh_D indicates Fresh-shallow and Fresh-Deep groundwater clusters, respectively. **Figure S12:** Coverage and taxonomic analysis of primers targeting *amoA*, *nirS*, and *nirK* genes compared to predictions from PICRUST2. (a) Bar plots showing the number of genomes predicted by PICRUST2 encoding *amoA*, *nirS*, or *nirK* (left bars), compared to the number of those genomes detected by specific primers for each gene (right bars). (b) Stacked bar plots showing the percentage of ASV reads (coloured by phylum) captured by the qPCR primers over all the ASV reads predicted by PICRUST2 to carry *amoA*, *nirS*, or *nirK*,

across shallow fresh groundwater (Fresh_S), groundwater (Fresh_D), deep and fresh saline groundwater samples in winter and summer. Grey areas indicate predicted gene-carrying ASVs not detected by the primers. (c) Boxplots showing the relative abundance (% of total community reads) of ASVs associated with *amoA*, *nirS*, or *nirK* genes that were captured by the primers, for each habitat (Fresh_S, Fresh_D, Saline) and season (winter, summer). Only ASVs identified by PICRUST2 predictions were included to estimate total community reads. **Figure S13:** Observed versus predicted concentrations of dissolved nitrogen and carbon compounds in groundwater samples ($n = 38$), based on the best multiple linear regression (MLR) models indicated in Table S5. Models included only environmental predictors (a), microbial predictors (b) or a combination of both (c). Concentrations of NH_4^+ were log10 transformed. The dashed line represents the 1:1 relationship. Colours indicate the hydrogeochemical sample clusters. **Table S1:** List of the 13 nitrogen related KEGG Orthologs (KOs) selected for the spatio-temporal analysis, including the genes, full names, functional pathways, reaction, and corresponding KO identifiers. Note that the KO K10944 is shared between ammonia monooxygenase (*amo*) and particulate methane monooxygenase (*pmo*). Consequently, PICRUST2 cannot differentiate *amoA* from *pmoA* solely based on KO (but see Discussion). **Table S2:** Primers used to target nitrogen-cycle genes and the synthetic internal positive control (IPC-A). Columns lists: N-cycle process (biogeochemical step targeted), gene, primer name, primer sequence (5'-3'), amplicon size (bp, expected product length), final qPCR reaction mix (20 μL), qPCR cycles and reference (original publication source). Primer sequence share reported in 5'-3' orientation and employ standard IUPAC nucleotide ambiguity codes for degenerate positions. BSA refers to bovine serum albumin. **Table S3:** gBlock sequences used in this study. For each entry the table lists: Gene gBlock, sequence (5'-3'), size (bp) and clone reference (citation and accession number). Sequences are shown in 5'-3'-orientation. Uppercase letters correspond exactly to the clone sequence deposited in GenBank. Lowercase letters denote synthetic nucleotides appended to the 5' and 3' ends of the gBlocks to facilitate primer binding; these appended bases are not part of the reference clone sequence. **Table S4:** Coverage of commonly used PCR/qPCR primer pairs targeting *amoA*, *nirS*, and *nirK* genes, analysed using PrimerEvalPy (Vázquez-González et al. 2024). The table reports the performance of each primer pair (left column) in terms of gene coverage across predicted ASVs from PICRUST2. The primer-pairs used in this study are indicated in bold. The "Coverage by number of ASVs" (middle column) indicates the percentage of ASVs targeted by each primer pair relative to the total number of ASV predicted to carry the respective gene. The "Coverage by reads" (right column) reflects the proportion of total reads assigned to those ASVs captured by each primer pair, relative to the total reads from all the gene-containing ASVs retained by PICRUST2 (for details on the analysis, see Supplementary Methods). **Table S5:** Best multiple linear regression (MLR) models predicting the concentrations of dissolved inorganic and organic nitrogen and carbon within the STE ($n = 38$ groundwater samples). Models included only environmental predictors (top table), microbial predictors (middle table) and a combination of both (bottom table). Models were selected using forward stepwise selection based on the Akaike Information Criterion (AIC). The sign in parentheses denotes the sign of the coefficient in the MLR model. Prok. Abund., prokaryotic abundance; %HNA, % of HNA cells. Variables in bold indicate significant predictors; however, some non-significant predictors were also retained in certain cases when they improved the AIC. **Table S6:** Environmental, microbial and categorical variables used for most analyses in Romano-Gude et al. **Table S7:** ASV count table used for analyses in Romano-Gude et al. **Table S8:** Taxonomic categorization of the obtained ASVs in Romano-Gude et al. **Data S1:** Supplementary Methods.