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Distribution and potential activity of aerobic denitrifying bacteria isolated from sediments of a coastal lagoon system in northwestern Mexico

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ABSTRACT. Denitrification is the primary pathway by which fixed nitrogen is lost from marine systems and consists of the sequential respiration of nitrate into molecular nitrogen by diverse heterotrophic microorganisms. Since estuarine sediments serve as important niches for denitrification, this study assessed the distribution and potential activity of aerobic denitrifying bacteria isolated from sediments at 2 depths and 2 habitats (with *Zostera marina* seagrass and without seagrass) along a transect in Bahía de San Quintín (Mexico), and their relationship with various environmental parameters. A total of 1,611 bacteria were isolated, of which 85.1% carried denitrifying genes (*nirK*, *nirS*, or *nosZ*). Their distribution was heterogeneous in the bay and was primarily influenced by sediment texture, pH, total organic carbon, and total nitrogen, with the highest abundance of denitrifying bacteria in surface sediments (66.2%). The denitrifying isolates were classified into 23 species belonging to γ-Proteobacteria (82.4% of isolates), α-Proteobacteria (7.9%), Bacilli (5.7%), and Actinobacteria (4%). Denitrifying activity in aerobic conditions was confirmed in 7 species of bacteria carrying denitrifying genes: *Paracoccus marcusii*, *Planococcus maritimus*, *Planococcus rifietoensis*, *Pseudomonas songnenensis*, *Psychrobacter alimentarius*, *Psychrobacter celer*, and *Psychrobacter piscatorii*. The results suggest that these sediments harbor a high abundance of culturable bacteria with *nirK*, *nirS*, or *nosZ* genes, although denitrification activity in aerobic conditions could not be confirmed in most cases, possibly due to the inactivity of these genes or those involved in nitrate reduction (nas, nar, or nap). This study represents the first step towards understanding the ecology of microorganisms involved in aerobic denitrification in Bahía de San Quintín and in coastal lagoons in general.

Key words: aerobic denitrification, estuary, sediments, isolation, denitrifying genes.

INTRODUCTION

Denitrification is an important process in the cycle of marine nitrogen (N) (Hutchins and Capone 2023). It is a modular respiratory pathway performed by various microorganisms, mainly heterotrophic facultative anaerobes. In a complete pathway, it consists of the sequential reduction of nitrate (NO_3^-), nitrite (NO_2^-), nitric oxide (NO), and nitrous oxide (N_2O) to molecular N (N_2), remaining as N_2O in an incomplete pathway (Zumft 1997). This process can produce N_2O, a greenhouse gas 300 times more potent than carbon dioxide (Ravishankara et al. 2009), and participates in the recycling of organic matter and eliminates reactive N in oxygen (O_2)-limited environments, such as marine sediments (Devol 2015).

The metalloenzymes involved in denitrification are cytoplasmic (Nas), membrane (Nar), and periplasmic (Nap) molybdenum-dependent nitrate reductases; nitrite reductases with copper (NirK) or heme iron (NirS) active sites; nitric oxide reductases (Nor); and copper-dependent nitrous oxide reductases (Nos) (Zumft 1997). The denitrifying genes most commonly used as markers in the study of denitrifying communities are *nirK*/*nirS* because they generate NO, the first gaseous product, and the *nosZ* gene because it participates in the reduction of N_2O (Damashek and Francis 2017, Pajares and Ramos 2019).

Certain microorganisms can also denitrify by simultaneously using O_2 and NO_3^- as electron acceptors (e.g., Lu et al. 2019, Cheng et al. 2020, Feng et al. 2020). Aerobic denitrification was formally proposed by Robertson and Kuenen

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(1984) and has been reported in permeable coastal sediments (Gao et al. 2010, Marchant et al. 2017). This pathway follows the same sequence of reductions as anaerobic denitrification, differing in that it features a more branched electron transport chain and a Nap-dominated NO_3^- reduction and facilitates the co-occurrence of nitrification and denitrification (Yang et al. 2020a, Zhang et al. 2023). Furthermore, microorganisms that carry out aerobic denitrification have the advantage of their metabolic activity being uninterrupted by the presence of O_2.

Coastal areas account for 44% of global denitrification (Seitzinger et al. 2006). In particular, denitrification in upwelling-influenced inverse estuaries, such as Bahía de San Quintín (Mexico), contributes to the removal of excess NO_3^- and organic matter from the ocean (Camacho-Ibar et al. 2003). The main environmental factors regulating denitrification in coastal sediments are dissolved O_2, NO_3^-, and organic matter (Devol 2015). Moreover, seagrasses tend to enhance denitrification rates by facilitating the availability of NO_3^- and organic matter in the form of plant tissues (Eyre et al. 2016).

Proteobacteria are the most abundant microbial group carrying out this metabolism in marine environments, particularly the genera *Pseudomonas*, *Alcaligenes*, and *Paracoccus* (Sousa and Bhosle 2012). However, the taxonomy and activity of denitrifying bacteria from sediments of reverse estuaries are unknown. Furthermore, there are very few studies on aerobic denitrifying bacteria isolated from marine sediments (Zheng et al. 2011, Zhang et al. 2023) and even fewer on those from coastal systems (Chen et al. 2020). Therefore, the objective of the present work was to evaluate the distribution of aerobic bacteria with denitrifying genes isolated from the sediments of Bahía de San Quintín and their relationship with different environmental parameters, as well as the potential denitrifying activity under aerobic conditions in those isolates with the marker genes *nirK*, *nirS* or *nosZ*.

Materials and methods

Study site and sampling

San Quintín is a hypersaline coastal lagoon with an average depth of ~2 m situated in a basaltic terrain of cineritic volcanic cones on the northwest coast of the Baja California Peninsula, Mexico (Fig. 1). *Zostera marina* meadows cover 45% of the bay area (Ward et al. 2003). The bay is Y-shaped and comprises a pristine eastern arm known as Brazo San Quintín and a western arm with oyster farming activity known as Bahía Falsa. Terrestrial inputs of water and nutrients into the bay are minimal; circulation is dominated by tidal exchanges with the Pacific Ocean and coastal upwelling is the main source of nutrients (Camacho et al. 2003). The mean residence time of water at the entrance to the bay is less than 2 days, whereas at the innermost part it is greater than 12 days (Canu et al. 2016).

In October 2021, 4 sediment cores were collected in areas with *Z. marina* meadows (seagrass sediments) and another 4 cores in areas without seagrass (bare sediments) ~25 m from the meadow edges in a 3-station transect (mouth, middle, and head) at Brazo San Quintín, avoiding the channel area (Fig. 1). Cores were collected by SCUBA with PVC corers (5 cm diameter and 25 cm depth), which were sealed in sterile plastic bags, stored at 4 °C and transported to the laboratory within 5 h. In the *Biogeoquímica de Nutrientes* Laboratory of the Oceanological Research Institute of the Autonomous University of Baja California (UABC, Ensenada), the upper strata (surface: 0–5 cm) and lower strata (bottom: 15–20 cm) of 3 cores per area were obtained and their physicochemical properties were characterized. Denitrifying bacteria were isolated from composite and homogenized samples from 3 cores per zone, which were frozen at –80 °C until processed in the *Ecología Microbiana Acuática* Laboratory of the Institute of Marine Sciences and Limnology of the National Autonomous University of Mexico (UNAM, Mexico City). At each station, in situ measurements of temperature, salinity, and dissolved O_2 in the water column above the sediments were taken with a YSI 6-Series multiparameter probe (Yellow Spring Instruments, Yellow Springs, USA).

Physicochemical analyses

Physicochemical analyses were done in triplicate (except for O_2 profiles, which were determined in one intact core per season and habitat) as described in Samperio-Ramos et al. (2024). Briefly, the dissolved O_2 profile was measured with a Unisense microsensor (Aarhus, Denmark) in the nuclei equilibrated by constant bubbling for 6–12 h. Sediment texture was determined with an LA-910 particle analyzer (Horiba Instruments, Irvine, USA) and pH was analyzed with a Starter 2100 pH meter (Ohaus, Parsippany, USA). The concentrations of Fe(II) and Fe(III) were obtained by the extraction method with HCl and ferrozine (Lovley and Phillips 1987, Samperio-Ramos et al. 2016) using a FLAME-S UV-Vis spectrophotometer (Ocean Insight, Orlando, USA). Total organic carbon (TOC) and total N (TN) were determined on an Elementar Vario analyzer (Elementar Americas, Inc., Ronkonkoma, USA). Total carbohydrate content was determined by acid hydrolysis with H_2SO_4 at 105 °C for 25 h (Myklestad et al. 1997). NO_3^- and NO_2^- were extracted with KCl (Wheatley et al. 1989) and analyzed with an AA3-HR segmented flow autoanalyzer (Seal Analytical Ltd, Mequon, USA).

Isolation of denitrifying bacteria

With 100 mg of 12 composite sediment samples (corresponding to 3 stations, 2 habitats, and 2 depths) from 3 cores per sampling area, 100-µL aliquots of serial dilutions (10^{-1}, 10^{-2}, and 10^{-3}) were seeded in 2 selective culture media for denitrifying bacteria (marine medium: 5 g of casein peptone, 1 g KNO_3, 1.5 g of meat extract, 1.5 g of yeast extract, 15 g of bacteriological agar, and 35 g of marine salts per liter of distilled water; Braker medium: 37.35 g of commercial marine medium, 0.51 g KNO_3, 0.5 g of yeast extract,

and 15 g of bacteriological agar per liter of distilled water), which were incubated under aerobic conditions for 2–4 days at 28 ± 1 °C. The colonies obtained were morphologically characterized. Subsequently, a representative colony was reseeded in the culture medium where it was isolated, under aerobic conditions for 1–2 days at 28 ± 1 °C to obtain sufficient biomass.

Amplification of denitrifying genes, 16S rRNA gene sequencing, and phylogeny

DNA was extracted from each bacterial representative using the 2% hexadecyltrimethylammonium bromide (CTAB) technique (Stewart and Via 1993) and its quality and concentration were verified on a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, USA). The *nirK*, *nirS*, and *nosZ* genes were amplified by polymerase chain reaction (PCR) using 2 different primer pairs per gene (Table 1); a positive amplification was considered with at least one of the primer pairs. Given the high variability of denitrifying genes, degenerate primers were used to detect as much of the denitrifying community as possible, which is phylogenetically very diverse (Wei et al. 2015). The final concentration of the reagents used in the PCRs contained 0.9× Buffer supplemented with 2.7 mM $MgCl_2$ and 0.9 mM dNTPs, 0.2 µg/µL of molecular grade bovine serum albumin (BioLabs, San Diego, USA), 0.3–0.5 µM of each primer, 0.025 U/µL of MyTaq (Bioline, London, United Kingdom), 1.7 ng/µL of DNA, and molecular grade water. The presence of *nirK*, *nirS*, or *nosZ* was checked by agarose gel electrophoresis.

The 16S rRNA gene was amplified by PCR in the DNA of representatives with one or more denitrifying genes (Table 1) and sequenced by the Sanger method at the Institute of Biology of UNAM (Mexico City). Sequences were taxonomically classified using the online tools Classifier from the Ribosomal Database Project (Cole et al. 2014) and BLASTn from the National Center for Biotechnology Information (NCBI) (Altschul et al. 1990). Subsequently, sequences were aligned with the ClustalW algorithm in BioEdit (Hall 1999), and a phylogenetic tree was made with the neighbor-joining method and a bootstrap of 10,000 repetitions in MEGA 11 (Tamura et al. 2021); this was edited in the Tree of Life program (Ciccarelli et al. 2006).

NO_3^- and NO_2^- reduction tests

To determine the potential denitrifying activity under aerobic conditions of the isolated bacteria with the *nirK*, *nirS*, or *nosZ* genes, the modified Holding and Colle (1971) test was carried out in tubes with marine broth or Braker at 28 ± 1 °C and a Durham bell until reaching its exponential phase (~48 h). We took 5-mL aliquots and added 5 drops of reagent β (0.4 g of sulfanilic acid and 50 mL of 5N acetic acid) and 5 drops of reagent α (0.25 g of α-naphthylamine and 50 mL of 5N acetic acid). In cases where a red shift did not occur (absence of NO_2^-), ~0.1 g of zinc powder was added to check the presence or absence of NO_3^-. The presence of gas in the Durham bell indicated the production of gaseous N.

Data analysis

Data were analyzed in RStudio v. 2023.3.0 (Posit team 2023). Alpha diversity indices (Chao1, Simpson, and Shannon) of the denitrifying community were calculated

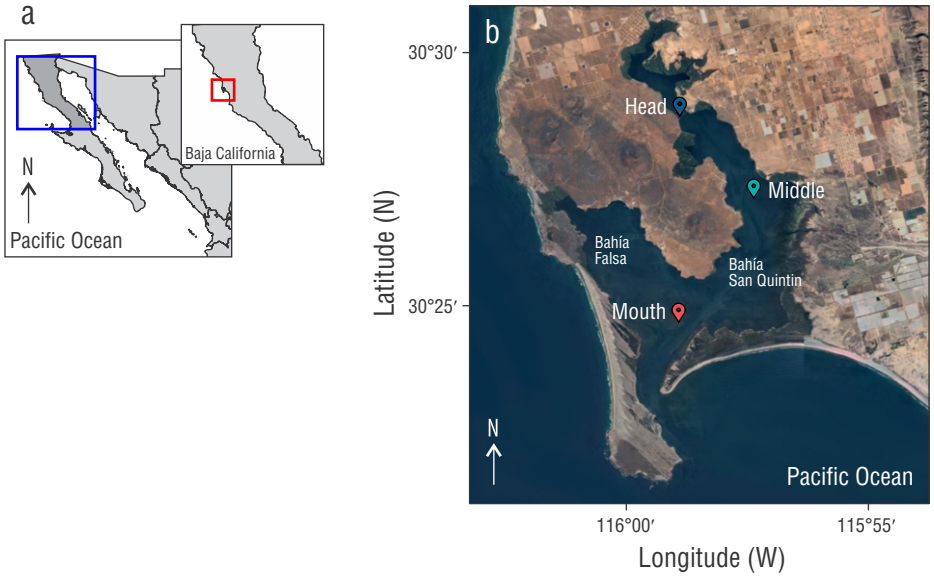

Figure 1. Location of Bahía San Quintín (red box) in Baja California (blue box), Mexico (**a**). Location of sampling stations in Bahía San Quintín (head [dark blue marker], middle [turquoise marker], and mouth [coral marker]) (**b**).

Table 1. Primers used to amplify the *nirK*, *nirS*, *nosZ*, and 16S rRNA genes, and their thermocycling conditions. Abbreviations: touchdown (TD), cycle (c), base pairs (bp).

Gene	Primer	Primer sequence (5'–3')	Thermocycling conditions	Length	Reference
nirK	nirK1F	GG(A/C)ATGGT(G/T)CC(C/G)TGGCA	95 °C × 5 min, 10 TD (95 °C × 30 s, 56–51 °C × 30 s, 72 °C × 45 s), 25 c (95 °C × 30 s, 51 °C × 30 s, 72 °C × 45 s), 72 °C × 7 min	510 bp	Braker et al. (1998)
	nirK5R	GCCTCGATCAG(A/G)TT(A/G)TGG			
	F1aCu	ATCATGGT(C/G)CTGCCGCG	95 °C × 5 min, 6 TD (95 °C × 15 s, 63–58 °C × 30 s, 72 °C × 30 s), 34 c (95 °C × 15 s, 58 °C × 30 s, 72 °C × 30 s), 72 °C × 5 min	473 bp	Hallin et al. (1999)
	R3Cu	GCCTCGATCAG(A/G)TTGTGGTT			
nirS	cd3aF	GT(C/G)AACGT(C/G)AAGGA(A/G)AC(C/G)GG	95 °C × 5 min, 6 TD (95 °C × 30 s, 62–57 °C × 20 s, 72 °C × 40 s), 34 c (95 °C × 30 s, 57 °C × 20 s, 72 °C × 40 s), 72 °C × 5 min	425 bp	Michotey et al. (2000)
	R3cd	GA(C/G)TTCGG(A/G)TG(C/G)GTCTTGA			
	nirS1F	CCTA(C/T)TGGCCGCC(A/G)CA(A/G)T	95 °C × 5 min, 10 TD (95 °C × 30 s, 57–52 °C × 30 s, 72 °C × 1 min), 30 c (95 °C × 30 s, 52 °C × 30 s, 72 °C × 1 min), 72 °C × 7 min	890 bp	Braker et al. (1998)
	nirS6R	CGTTGAACTT(A/G)CCGGT			
nosZ	nosZl-2F	CGCRACGGCAASAAGGTSMSSGT	95 °C × 5 min, 35 c (95 °C × 30 s, 60 °C × 30 s, 72 °C × 30 s), 72 °C × 5 min	267 bp	Throbäck et al. (2004)
	nosZl-2R	CAKRTGACKSGCRTGGCAGAA			
	Nos661F	CGGCTGGGGGCTGACCAA	94 °C × 5 min, 35 c (94 °C × 30 s, 55 °C × 1 min, 72 °C × 1 min), 72 °C × 10 min	1,140 bp	Scala y Kerkhof (1998)
	Nos1773R	AACGA(A/C/G)CAG(T/C)TGATCGA(T/C)AT			
16S rRNA	27F	TACCTTGTTACGACTT	94 °C × 5 min, 30 c (94 °C × 1 min, 52 °C × 1 min, 72 °C × 1.20 min), 72 °C × 10 min	1,465 bp	Lane et al. (1985)
	1492R	AGAGTTTGATCMTGGCTCAG			

with the 'vegan' package (Oksanen et al. 2021). With the abundance data per species, the following were carried out: (1) a distribution analysis of the denitrifying species using a heat map with a cladogram using the Canberra dissimilarity matrix and the "average" grouping with the 'ComplexHeatmap' package (Gu et al. 2016); (2) a Spearman correlation matrix with the physicochemical data using the packages 'corrplot' (Wei and Simko 2021) and 'Hmisc' (Harrell 2021); and (3) an analysis of the denitrifying community structure by non-metric multidimensional scaling (NMDS) using the Bray–Curtis dissimilarity matrix with the 'vegan' package and plotted with the 'ggplot2' package (Wickham 2016), where vectors of significant environmental data ($P < 0.05$) were integrated.

Finally, the presence of the *nirK*, *nirS*, or *nosZ* genes found in the isolate species was contrasted with the NCBI BLASTn tool. To do this, we accessed the reference genomes of species with the highest percentage of identity alignment and searched the gene locus in the protein table.

Results

Physicochemical properties of water and sediments sampled

The water column of the bay showed an increase in temperature (from 20.8 to 22.7 °C), salinity (from 32.42 to 35.39‰), and dissolved O_2 (from 7.02 to 7.59 mg·L^{-1}) from the mouth to the head (Table 2). The sediments were anoxic from 2 mm depth (Fig. 2) and alkaline (pH from 7.89 to 8.2; Table 3). Sand content decreased from the mouth to the head in both habitats and depths (seagrass: from 62.4–70.4% to 21.4–29.6%; bare: from 82.9–90.9% to 23.4–37.3%), whereas silt increased (seagrass: from

29.6–37.6% to 70.3–78.0%; bare: from 9.1–17.2% to 62.4–76.4%) (Table 3). The concentrations of Fe(III) were higher than those of Fe(II), and both were higher in the middle zone (seagrass: 8.0–10.9 $mg \cdot g^{-1}$ and 0.2–0.7 $mg \cdot g^{-1}$; bare: 5.9–9.1 $mg \cdot g^{-1}$ and 0.2–0.6 $mg \cdot g^{-1}$, respectively). From the mouth to the head, TOC (seagrass: from 0.23–0.67% to 1.17–1.45%; bare: from 0.14–0.31% to 0.58–1.03%) and TN (seagrass: from 0.03–0.07% to 0.11–0.16%; bare: from 0.02–0.04% to 0.06–0.10%) increased. The concentrations of total carbohydrates, NO_3^-, and NO_2^- did not show a clear distribution pattern (Table 3).

Distribution and diversity of aerobic denitrifying bacteria isolated from sediments

A total of 1,611 isolates were obtained in the sediments of this bay, which were grouped into 155 morphological groups, of which 66 representatives (corresponding to 1,371 isolates) contained a *nirK*, *nirS*, or *nosZ* gene (85.1% of the total of the isolates).

The most abundant marker gene in the isolates was *nirK* (843 isolates), followed by *nosZ* (740 isolates) and *nirS* (573 isolates) (Fig. 3). Bacteria with only *nosZ* (20.3% of

Table 2. Physicochemical conditions of the water column at the 3 sampling stations.

Station	Temperature (°C)	Salinity (‰)	Oxygen ($mg \cdot L^{-1}$)
Mouth	20.8	32.42	7.02
Middle	21.7	34.45	7.11
Head	22.7	35.39	7.59

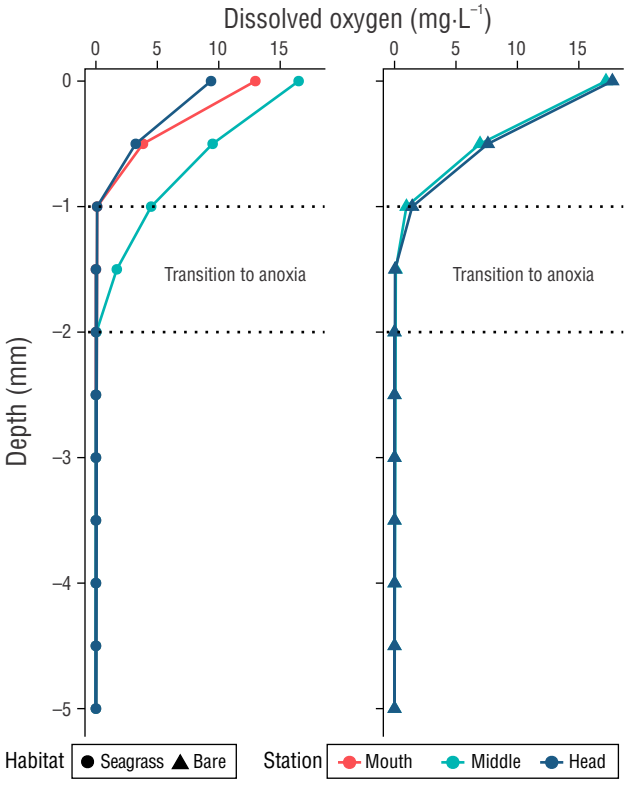

Figure 2. Vertical profiles (from the surface to 5 mm depth) of dissolved oxygen concentrations in cores of seagrass (right) and bare (left) sediments of Brazo San Quintín. Dissolved oxygen could not be measured in bare sediments at the mouth.

Table 3. Values (average ± standard deviation) of physicochemical parameters analyzed in triplicate in the sediment samples from Brazo San Quintín. Abbreviations: seagrass (P), bare (D), surface (S), bottom (F), total organic C (TOC), total N (TN), total carbohydrate (TCCHO), nitrate (NO_3^-), and nitrite (NO_2^-).

Sample	pH	Sand (%)	Silt (%)	Clay (%)	Fe(III) ($mg \cdot g^{-1}$)	Fe(II) ($mg \cdot g^{-1}$)	TOC (%)	TN (%)	TCCHO ($\mu mol\ C \cdot g^{-1}$)	NO_3^- ($\mu g \cdot g^{-1}$)	NO_2^- ($\mu g \cdot g^{-1}$)
Mouth.PS	8.04 ± 0.04	62.39 ± 0.67	37.62 ± 0.67	0.00 ± 0.01	5.16 ± 1.10	0.09 ± 0.10	0.67 ± 0.16	0.07 ± 0.02	27.0 ± 1.47	0.98 ± 0.13	0.08 ± 0.03
Mouth.PF	7.89 ± 0.07	70.36 ± 1.17	29.64 ± 1.16	0.01 ± 0.00	4.14 ± 0.24	0.47 ± 0.1	0.23 ± 0.0	0.03 ± 0.0	4.86 ± 1.04	0.70 ± 0.0	0.06 ± 0.03
Mouth.DS	8.01 ± 0.03	90.90 ± 1.47	9.10 ± 1.47	0.00 ± 0.00	5.24 ± 1.32	0.16 ± 0.1	0.31 ± 0.0	0.04 ± 0.0	6.51 ± 0.95	1.42 ± 0.25	0.10 ± 0.05
Mouth.DF	7.94 ± 0.09	82.85 ± 2.31	17.16 ± 2.31	0.00 ± 0.00	3.52 ± 0.51	0.31 ± 0.2	0.14 ± 0.0	0.02 ± 0.0	1.80 ± 0.33	0.55 ± 0.21	0.03 ± 0.01
Middle.PS	8.09 ± 0.09	57.27 ± 7.71	42.72 ± 7.71	0.01 ± 0.00	10.85 ± 1.82	0.20 ± 0.1	0.98 ± 0.2	0.11 ± 0.0	28.9 ± 3.51	1.41 ± 0.26	0.21 ± 0.01
Middle.PF	7.96 ± 0.06	63.79 ± 1.99	36.14 ± 2.08	0.07 ± 0.08	8.03 ± 1.35	0.74 ± 0.1	0.71 ± 0.0	0.07 ± 0.0	10.9 ± 4.06	0.46 ± 0.21	0.03 ± 0.01
Middle.DS	8.08 ± 0.04	67.16 ± 4.48	32.84 ± 4.48	0.00 ± 0.01	9.07 ± 1.07	0.18 ± 0.07	0.75 ± 0.23	0.08 ± 0.02	11.4 ± 0.75	0.40 ± 0.14	0.06 ± 0.01
Middle.DF	7.98 ± 0.04	53.35 ± 2.04	46.49 ± 2.04	0.16 ± 0.00	5.87 ± 0.99	0.59 ± 0.2	0.53 ± 0.0	0.05 ± 0.0	4.10 ± 0.55	0.13 ± 0.01	0.00 ± 0.00
Head.PS	8.15 ± 0.06	29.56 ± 3.87	70.25 ± 3.87	0.19 ± 0.00	8.07 ± 1.15	0.18 ± 0.1	1.45 ± 0.2	0.16 ± 0.0	48.3 ± 7.87	1.70 ± 0.37	0.09 ± 0.04
Head.PF	8.04 ± 0.05	21.40 ± 0.64	78.04 ± 0.74	0.57 ± 0.10	5.49 ± 1.34	0.59 ± 0.09	1.17 ± 0.06	0.11 ± 0.01	21.4 ± 2.36	0.85 ± 0.23	0.02 ± 0.00
Head.DS	8.20 ± 0.09	23.36 ± 0.41	76.37 ± 0.31	0.27 ± 0.10	8.70 ± 1.40	0.23 ± 0.1	1.03 ± 0.1	0.10 ± 0.0	16.4 ± 4.72	1.05 ± 0.36	0.06 ± 0.01
Head.DF	8.08 ± 0.03	37.26 ± 0.88	62.39 ± 0.91	0.35 ± 0.03	6.48 ± 0.33	0.32 ± 0.2	0.58 ± 0.0	0.06 ± 0.0	4.01 ± 0.68	0.40 ± 0.07	0.03 ± 0.00

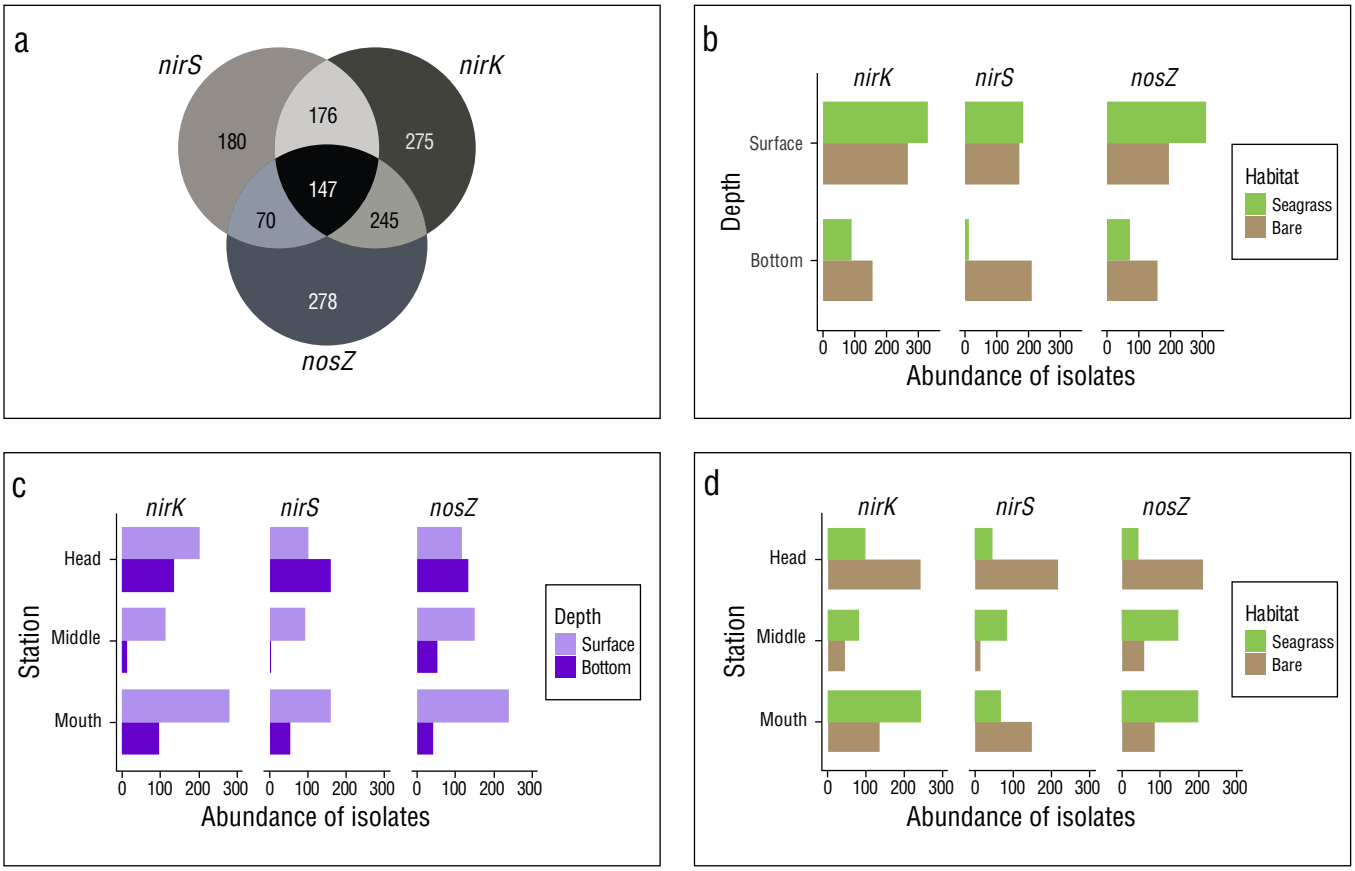

Figure 3. Distribution of marker gene (*nirK*, *nirS*, or *nosZ*) abundance in the isolates obtained from the sediments of Brazo San Quintín (**a**), by depth and habitat (**b**), by station and depth (**c**), and by station and habitat (**d**).

total isolates) and *nirK* (20.1%) were dominant, whereas bacteria with only *nirS* represented 13.1%. Among the isolates with 2 genes, those with *nirK* and *nosZ* were the most abundant (17.9%), followed by those with *nirK* and *nirS* (12.8%), whereas the least abundant were those with *nirS* and *nosZ* (5.1%). Only 10.7% of isolates had all 3 genes (Fig. 3a).

The distribution of the isolated denitrifiers in the bay was heterogeneous. By depth, surface sediments had a higher relative abundance of denitrifiers (66.2%) than bottom sediments (33.8%) (Fig. 3b). However, the relative abundance of isolates at these depths varied depending on the habitat, so that on the surface it was greater in seagrass sediments (38.2%) than in bare sediments (29.4%) and at the bottom it was greater in bare sediments (24.3%) than in seagrass sediments (8.1%) (Fig. 3b). Isolates with *nirS* were more abundant at the head (45.4%), whereas isolates with *nirK* and *nosZ* were more abundant at the mouth (44.7 and 38.4%, respectively). A lower proportion of denitrifiers was isolated at the middle station (*nosZ*: 27.6%; *nirS*: 16.9%; *nirK*: 15.2%) (Fig. 3c, d). This distribution was also influenced by the habitat, so that the relative abundance of these isolates at the head was greater in the bare sediments (78.7%), whereas at the middle and mouth stations it was greater in seagrass sediments (72.7% and 57.7%, respectively) (Fig. 3d).

In general, in these sediments there was a low dominance (Simpson: 0.83 to 0.88) and a high diversity (Shannon: 1.96 to 2.36; close to H_{max}: 2.71 to 3.04) of denitrifying isolates (Table 4). The environments with the highest richness of denitrifying isolates were the middle station, the bottom stratum, and the bare sediment (Chao1: 28.2, 31, and 30.1, respectively).

Taxonomy, phylogeny, and activity of bacteria with denitrifying genes

A phylogenetic tree was built with the 16S rRNA sequences of the 62 representatives corresponding to 1,338 isolates (4 representatives with poor quality sequences, belonging to 33 isolates, were discarded), classified into 4 classes (γ-Proteobacteria [82.4% of isolates], α-Proteobacteria [7.9%], Bacilli [5.7%], and Actinobacteria [4%]), distributed in 7 families, 11 genera, and 23 species (Fig. 4). The genera with the highest number of isolates were *Psychrobacter* (986 isolates), *Pseudomonas* (117 isolates), and *Paracoccus* (106 isolates). Furthermore, the species with the highest number of isolates were *Psychrobacter piscatorii* (267 isolates),

Psychrobacter celer (193 isolates), *Psychrobacter submarinus* (130 isolates), *Psychrobacter pacificensis* (124 isolates), and *Pseudomonas songnenensis* (117 isolates) (Fig. 4).

Of the 23 classified species, 18 had their genome represented in the NCBI, of which only 3 contained any of the marker genes found in this study: *Mesobacillus jeotgali* (*nosZ*; ID: WP_102264655.1), *Mesobacillus maritimus* (*nosZ*; ID: WP_251428094.1), and *P. songnenensis* (*nirK* and *nosZ*; ID: WP_239060843.1 and WP_015275732.1, respectively). In this study, *nirS* was also found in *P. songnenensis*, although it is not reported in its genome (NCBI reference sequence: NZ_RFFN01000004.1).

Relationship between the abundance of denitrifying bacteria species and environmental variables

The heat map showed that *P. songnenensis* and *Paracoccus marcusii* dominated at the bottom of the bare head sediment. *Psychrobacter piscatorii* and *P. submarinus* were abundant in several sediment samples, dominating at the surface of the bare sediment at the head and in the seagrass at the mouth. *Psychrobacter celer* and *P. pacifiensis* were most abundant at the surface of the bare sediment at the mouth (Fig. 5a). The abundances of the denitrifying species showed few significant correlations with the environmental variables studied, highlighting the significant positive correlations of *P. piscatorii* with pH, Fe(III), TOC, and carbohydrates; *Psychrobacter alimentarius* correlated with NO_3^- and NO_2^- and *Micrococcus endophyticus* with pH and Fe(III). Significant negative correlations were observed mainly for *P. celer* with silt, clay, and TN content; *Arthrobacter* sp. with pH, Fe(III), and TOC; and *P. marcussii* with NO_3^- and NO_2^-, although the latter also had a significant positive correlation with Fe(II) (Fig. 5b).

The NMDS (Fig. 6) showed 3 groupings based on the abundance of the isolated denitrifying species: (1) the species from surface sediments, except those from the bare sediment at the mouth, showed a slight positive correlation with the pH and were represented by *Metabacillus endolithicus*, *P. alimentarius*, *Psychobacter nivimaris*, *P. piscatorii*, and *P. submarinus*. (2) Bottom species of the middle and head stations showed a positive correlation with the clay texture and a high abundance of *Psychrobacter* sp., *P. celer*, *P. songnenensis*, *Planococcus maritimus*, and *Planococcus rifietoensis*. (3) Mouth species (except surface species with *Z. marina*) positively correlated with sandy texture and negatively correlated with TOC and TN, and were mainly associated with *M. maritimus* and *Arthrobacter enclensis*.

Verification of denitrifying activity in the isolated species

The denitrifying activity was verified under aerobic conditions in 7 species (Table 5): *P. marcusii* (2 representatives with *nirK*, 1 with *nirS*, and 1 with active *nirK*/*nirS* and *nosZ*), *P. maritimus* (2 representatives with active *nirS*), *P. rifietoensis* (1 representative with active *nirS*), *P. songnenensis* (1 representative with active *nirS* and *nosZ* and 1 with active *nirK*/*nirS* and *nosZ*), *P. alimentarius* (1 representative with active *nirK*/*nirS*), *P. celer* (4 representatives with *nirK* and 1 with active *nirS*), and *P. piscatorii* (1 representative with active *nirK*/*nirS* and *nosZ*).

Table 4. Alpha diversity indices: specific richness (number of species present per sample), Chao1, Simpson, and Shannon, calculated from the abundances of bacterial isolates with denitrifying genes per environment studied. H_{max}: maximum theoretical value of the Shannon index.

Environment	Specific richness	Chao1	Simpson	Shannon	H_{max}
Mouth	14	14.5	0.833	2.067	2.639
Middle	20	28.2	0.856	2.171	2.996
Head	15	17.3	0.831	1.957	2.708
Surface	17	18.0	0.854	2.169	2.833
Bottom	22	31.0	0.858	2.259	3.091
Bare	24	30.1	0.878	2.310	3.178
Seagrass	21	21.4	0.882	2.355	3.045
Average	19	22.93	0.856	2.184	2.927
Standard deviation	3.74	6.75	0.02	0.14	0.20

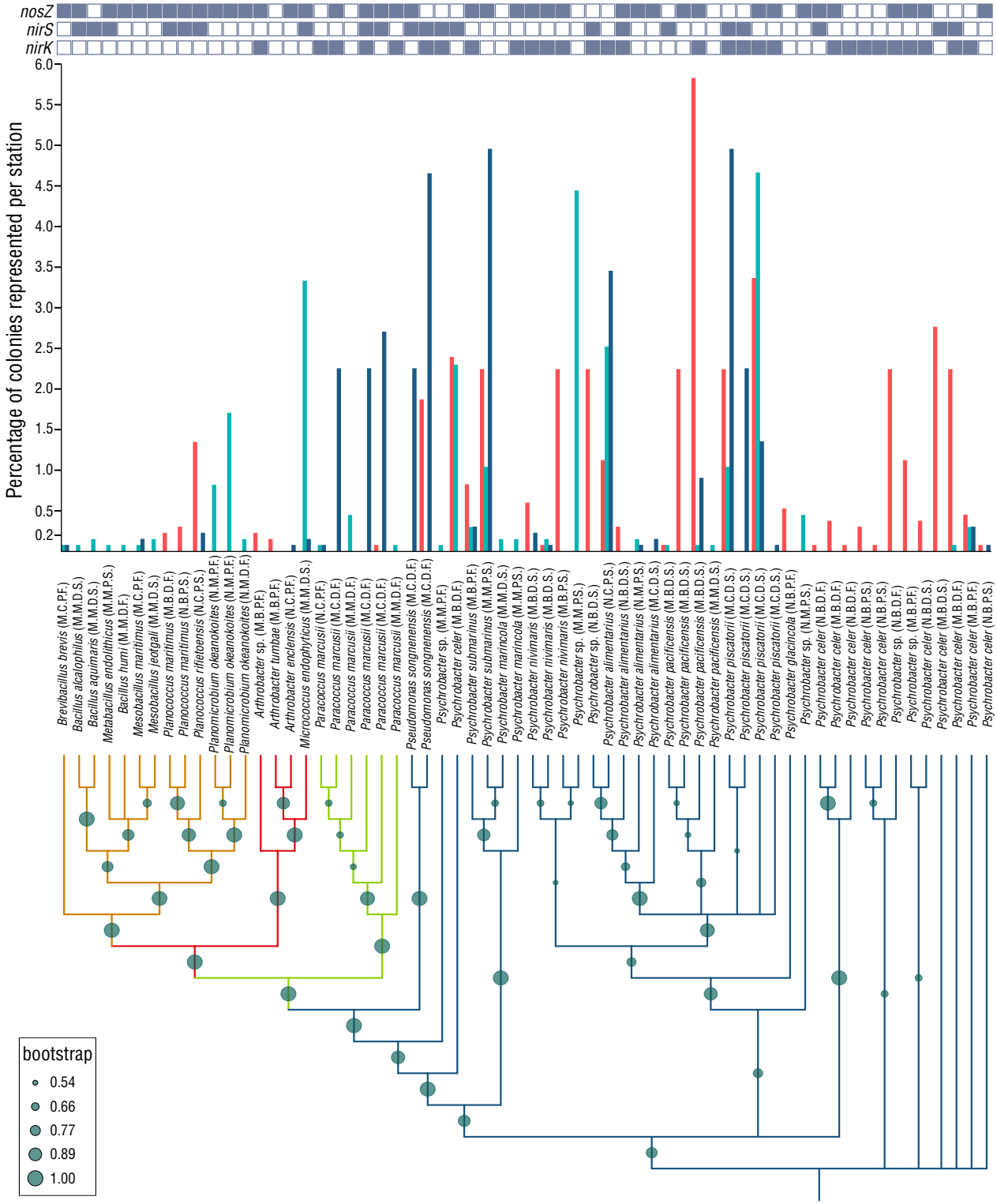

Figure 4. Phylogenetic tree of the 16S rRNA sequences of the denitrifying bacterial representatives isolated in the sediments of Brazo San Quintín. The bootstrap value is shown in blue circles on the nodes. The color of the branches represents the class to which each representative belongs: α–Proteobacteria (—), γ–Proteobacteria (—), Actinobacteria (—), and Bacilli (—). The origin of the representative is indicated in parentheses: culture medium (marine [M], nutritious [N]), station (mouth [B], middle [M], or head [C]), habitat (seagrass [P] or bare [D]), and depth (surface [S] or bottom [F]). The tree bar graph shows the relative abundance of the isolates associated with each representative in each station: mouth (■), middle (■), or head (■). In the binary system, the presence (■) or absence (□) of the *nirK*, *nirS*, or *nosZ* genes is shown.

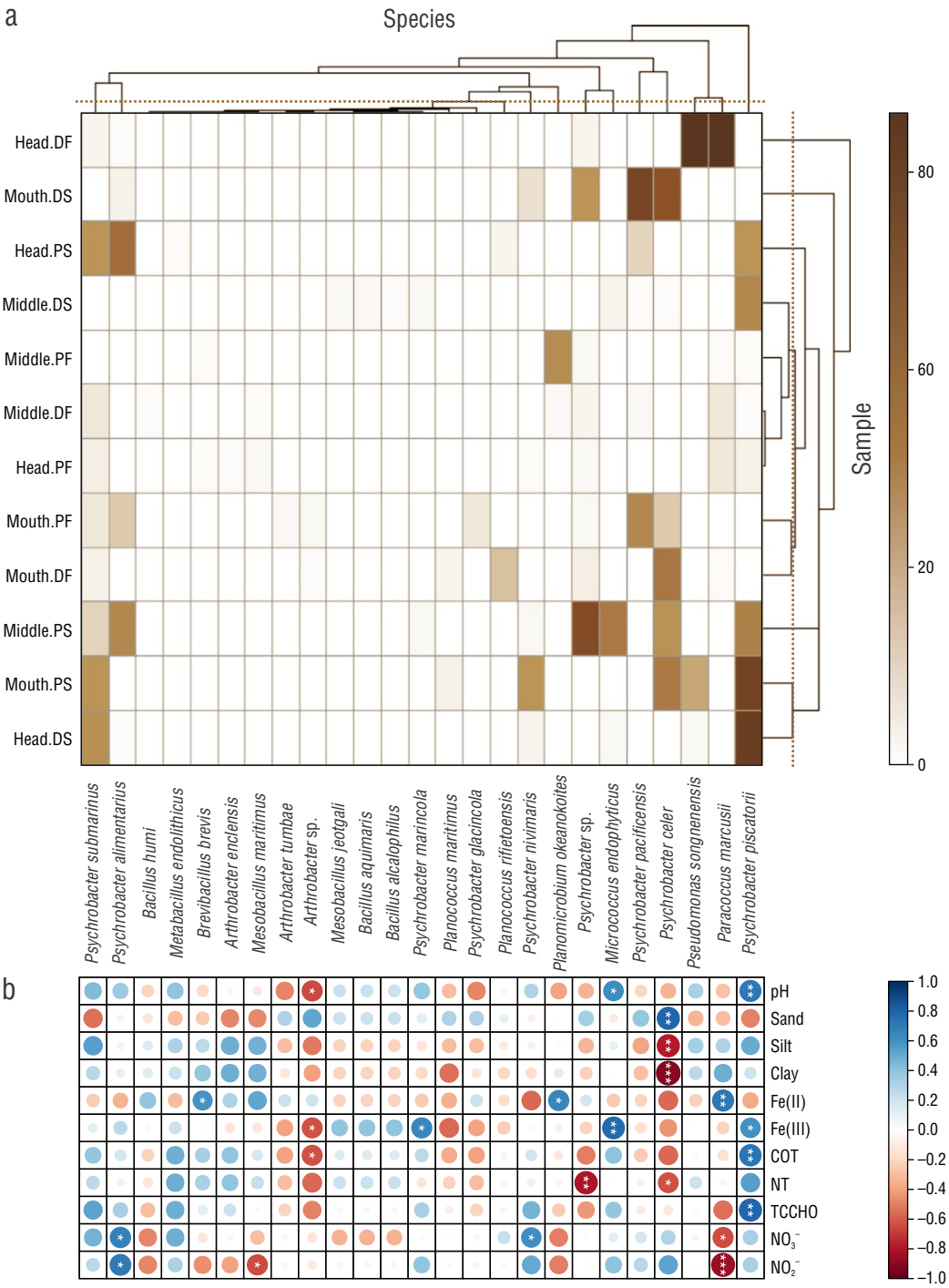

Figure 5. Heat map of the abundances of denitrifying species isolated per sample (color gradient from white to brown) (**a**). Dendrograms show hierarchical clustering between species (columns) and samples (rows), respectively. Spearman correlation matrix between abundances of denitrifying species and physicochemical parameters of sediments (**b**). The color gradient corresponds to the correlation coefficient values and the circle size to its absolute value. Significant correlations are represented by white asterisks inside circles (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). Abbreviations: seagrass (P), bare (D), surface (S), bottom (F), total organic C (COT), total N (NT), and total carbohydrates (TCCHO).

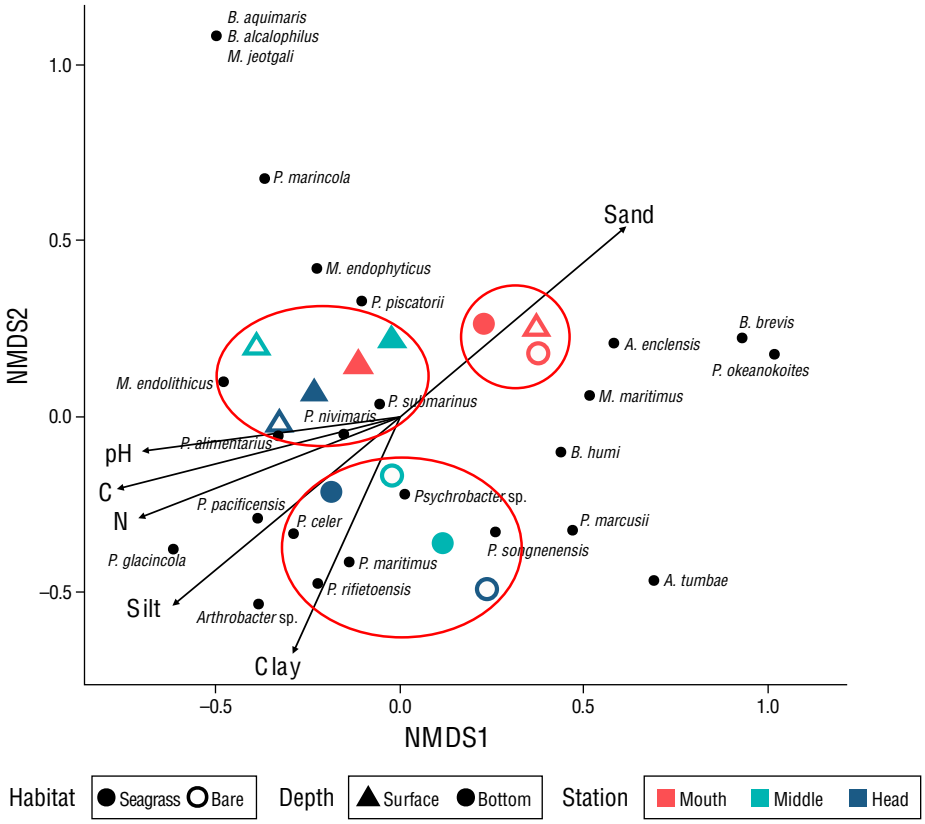

Figure 6. NMDS plot of sample distribution (symbols) as a function of the Bray–Curtis distance matrix of the isolated denitrifying species and their correlation with the significant environmental vectors ($P < 0.05$). Stress level: 0.069. Abbreviations: total organic C (C) and total N (N).

Discussion

Distribution of aerobic denitrifying bacteria isolated from Brazo San Quintín

To date, aerobic denitrifiers have been isolated in various marine systems (e.g., Zheng et al. 2011, Chen et al. 2020, Zhang et al. 2023) with the exception of reverse estuaries. Permeable coastal sediments, such as those in Bahía de San Quintín, are favorable environments for the development of aerobic denitrifying bacteria (Gao et al. 2010, Marchant et al. 2017). Furthermore, the proliferation of *Z. marina* and the shallow state of this bay promote large contributions of organic matter for denitrifying bacteria in these sediments (Eyre et al. 2016). Likewise, high concentrations of Fe in the sediment of the bay, derived from the erosion of basaltic rocks from the San Quintín volcanic field (Navarro et al. 2006), can stimulate the development of bacteria with *nirS* (Zumft 1997). In this study, a large number of denitrifying bacteria are isolated for the first time under aerobic conditions in the sediments of Brazo San Quintín (85.1% of 1,611 isolates had denitrifying genes).

Isolates with *nirK*, *nirS*, or *nosZ* genes belonged to the classes Actinobacteria, Bacilli, γ-Proteobacteria, and α-Proteobacteria, which have been associated with denitrification in coastal sediments (Zhu et al. 2018, Ming et al. 2021). The 3 most abundant genera were *Psychrobacter*, *Pseudomonas*, and *Paracoccus*. *Pseudomonas* and *Paracoccus* are considered the most abundant denitrifying bacterial genera in marine sediments (Sousa and Bhosle 2012) and have also been associated with aerobic denitrification (Chen et al. 2020, Zhang et al. 2023). Likewise, *Psychrobacter* has been related to anaerobic (Zhu et al. 2018, Cabezas et al. 2022) and aerobic (Zheng et al. 2011) denitrification in marine environments; furthermore, its genome reveals affinity for the aerobic denitrification pathway (Lasek et al. 2017).

There were bacterial species that simultaneously had the *nirK* and *nirS* genes, such as *P. songnenensis*, *Psychrobacter* sp., *P. alimentarius*, *P. celer*, and *P. piscatorii* (Fig. 4). These results contradict the traditional idea that such genes are mutually exclusive in genomes of denitrifying organisms (Zumft 1997, Jones et al. 2008), but are in line with observations of *nirK*/*nirS* co-occurrence found in NCBI genomes (Graf et al. 2014), anaerobic and microaerobic cultures of *Bradyrhizobium oligotrophicum* S58 (Sánchez and Minamisawa 2018), and aerobic cultures of *Pseudomonas stutzeri* (Wittorf et al. 2018). Therefore, a deeper study of the physiology and metabolism of bacteria with *nirK*/*nirS* in their genomes is needed.

Table 5. Representative isolates whose denitrifying gene activity, identified by PCR, could be verified under aerobic conditions. Origin of representatives: culture medium (marine [M] or nutritious [N]), station (mouth [B], middle [M], or head [C]), habitat (seagrass [P] or bare [D]), depth (surface [S] or bottom [F]), and number assigned in the isolation (abundance of isolates for each representative [N]).

Species	Representative	Main morphology (color, elevation, border)	N	Genes found by PCR			Proven genes
				nirK	*nirS*	*noZ*	
Paracoccus marcusii	M.M.D.F.5	Cream, flat, smooth	1	+	–	+	*nirK*
	M.C.D.F.2	Cream, elevated, smooth	37	–	+	–	*nirS*
	M.C.D.F.3	Light brown, flat, wavy	30	+	+	+	*nirK/nirS, nosZ*
	M.C.D.F.4	Orange, flat, smooth	30	+	–	–	*nirK*
Planococcus maritimus	M.B.D.F.7	Orange, elevated, lobed	3	–	+	–	*nirS*
	N.B.P.S.7	Orange, flat, smooth	4	–	+	–	*nirS*
Planococcus rifietoensis	N.C.P.S.2	Orange, elevated, smooth	3	–	+	–	*nirS*
Pseudomonas songnenensis	M.C.D.F.6	Orange, elevated, wavy	30	–	+	+	*nirS, nosZ*
	M.C.D.F.8	Orange, elevated, smooth	87	+	+	+	*nirK/nirS, nosZ*
Psychrobacter alimentarius	N.B.D.S.3	Orange, flat, wavy	4	+	+	–	*nirK/nirS*
Psychrobacter celer	N.B.P.S.6	White, flat, smooth	30	+	–	+	*nirK*
	N.B.P.S.8	Cream, protuberant, smooth	4	+	–	+	*nirK*
	N.B.P.S.9	Yellow, flat, smooth	1	+	–	+	*nirK*
	N.B.D.F.6	Cream, flat, lobed	1	+	–	+	*nirK*
	N.B.D.F.10	Cream, elevated, lobed	1	–	+	+	*nirS*
Psychrobacter piscatorii	M.C.D.S.3	Colorless, flat, wavy	30	+	+	+	*nirK/nirS, nosZ*

Strong physicochemical gradients occur along Brazo San Quintín (Canu et al. 2016, Samperio–Ramos et al. 2024), which could influence the distribution of denitrifying bacteria. However, the distribution of these isolates was heterogeneous in this arm, being more abundant at the mouth and head than in the middle zone, and without clear differences between bare sediments or those with *Z. marina*. Aerobic denitrifying activity has been demonstrated in permeable coastal sediments (Gao et al. 2010, Marchant et al. 2017). Therefore, the greater permeability of sandy sediments at the mouth and the arrival of nutrients through coastal upwellings associated with the California Current (Canu et al. 2016), intensified by an atypical La Niña phase durng the sampling time (Cheng et al. 2022), promoted greater development of aerobic denitrifying bacteria in this area. In addition, higher TOC contents in the sediments of the head, due to their silty content and the longer water confinement time (Ribas-Ribas et al. 2011, Canu et al. 2016), could promote higher abundances of aerobic denitrifying bacteria in this area of the bay. In general, surface sediments had higher relative abundances of aerobic denitrifying bacteria than bottom sediments, possibly due to the diffusion of O_2 and nutrients from the water to surface sediments (Ribas-Ribas et al. 2011) and their higher organic matter contents, with respect to the deeper sediments. On the other hand, bacteria with *nirS* were the least abundant in these sediments, despite the affinity of the *nirS* enzyme cd1 with Fe (Zumft 1997) and the high concentrations of this metal in Bahía San Quintín (Navarro et al. 2006, Samperio-Ramos et al. 2024). Therefore, a possible underestimation of the real abundance of

nirS-carrying bacteria due to the isolation technique cannot be discarded (Stewart 2012).

The diversity of denitrifying bacteria isolated in San Quintín was lower than that found in sediments from other estuaries where massive sequencing techniques (Ming et al. 2021) and clone libraries (Zheng et al. 2015) were used. Therefore, this could be a consequence of the technique used in this study, since isolation in agar does not allow the growth of the majority of bacteria present in a certain environment; these have been identified, without the need to culture, by means of PCR amplification and sequencing of marker genes such as 16S rRNA (Stewart 2012). Furthermore, the heterogeneous distribution of denitrifying bacteria in this bay, together with the low significant correlation found between denitrifying species and environmental parameters, could also be due to the isolation in agar. On the other hand, according to the NMDS, the distribution of these isolates was conditioned mainly by the texture, pH, and concentration of TOC and TN in these sediments; these results coincide with those obtained by quantitative PCR in sediments from various estuaries in China. (Wang et al. 2014, Zhu et al. 2018, Ming et al. 2021).

Denitrifying activity in bacteria isolated with denitrifying genes

Although denitrification is a modular process (Zumft 1997), which makes it difficult to observe the complete reduction of NO_3^- to N_2 in monocultures, complete aerobic denitrification was confirmed in 3 species (*P. marcusii*, *P. songnenensis*, and *P. piscatorii*). The reduction of NO_3^- and NO_2^- under aerobic conditions was also confirmed in 4 other species (*P. maritimus*, *P. rifietoensis*, *P. alimentarius*, and *P. celer*).

The verification of denitrifying activity under aerobic conditions in 3 *Psychrobacter* species supports the proposal that this genus can carry out aerobic denitrification (Lasek et al. 2017). The ability to carry out this process in the presence of O_2 could explain the dominance of *Psychrobacter* in the isolates obtained in San Quintín, since the isolation conditions were also aerobic. On the other hand, the denitrifying activity observed in *P. marcusii* and *P. songnenensis* could follow a complete denitrification route (with N_2 production), since the presence of the *nosZ* gene has been described in both genera (Jones et al. 2013), and the genome of *P. songnenensis* contains the genes *nirK* and *nosZ* (NCBI: NZ_RFFN01000004.1). Regarding the reduction of NO_3^- and NO_2^- verified in *P. maritimus* and *P. rifietoensis*, there are no previous records of such activity in this genus; however, its class (Bacilli) is known to be able to carry out aerobic denitrification (Yang et al. 2020b).

Conclusions

In the sediments of Brazo San Quintín there is a high abundance of aerobic bacteria with denitrifying genes. We were able to verify the ability to carry out denitrification in the presence of O_2 in at least 7 of these species. Although the agar isolation technique does not allow a real estimate of the abundance and diversity of denitrifying bacteria in this bay, it is the first work to isolate aerobic bacteria with denitrifying potential in the sediments of a coastal lagoon-type estuary. In future studies it would be of interest to compare the denitrifying community between the pristine arm (Brazo San Quintín) and that influenced by oyster farming activity (Bahía Falsa) to analyze the effects of this type of anthropogenic activities on the denitrifying community of Bahía San Quintín.

English translation by Claudia Michel-Villalobos.

Declarations

Supplementary material

This work does not include supplementary material.

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Conflict of interests

The authors declare that they have no conflict of interest.

Author contributions

Conceptualization: SP-M; Data curation: PP-Z; Formal analysis: PP-Z; Financing acquisition: GAS-R; Research: PP-Z, SP-M; Methodology: PP-Z, SP-M, GAS-R; Supervision: SP-M; Validation: SP-M; Writing—original draft: PP-Z, SP-M; Writing-review and editing: SP-M, GAS-R.

Data availability

The sequences are found in GenBank under accession numbers OR987974–OR988035.

Use of AI tools

The authors did not use any artificial intelligence tools for this work.

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