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Subcuticular and biofilm microbiomes in *Holothuria tubulosa* and their potential for denitrification

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ABSTRACT: Holothurians, as benthic invertebrates inhabiting marine ecosystems, have a crucial function in that they actively process organic detritus in the sediments. Previous works have provided evidence of the capability of holothurians to reduce nitrate and ammonium concentrations in aquaculture tanks. However, the mechanisms underlying this nitrogen decrease still need to be elucidated and might be related to bacterial symbionts in the holothurians. Here we characterize the community of bacterial symbionts in the biofilm and subcuticle of *Holothuria tubulosa* and explore the presence of nitrification and denitrification genes. To characterize these bacterial symbionts, we extracted DNA and amplified the V3-V4 hypervariable region of the 16S rRNA gene. We obtained a notable contribution of *Bacteroidota*, *Alphaproteobacteria* (mostly *Rhodobacterales*), and *Gammaproteobacteria* (mostly *Pseudomonadales*) both within the biofilm and subcuticle of *H. tubulosa*. Subsequently, we tested the presence of specific genes encoding enzymes involved in nitrification (i.e. archaeal *amoA* and bacterial *amoA*) and denitrification (i.e. *nirS* and *nosZ)*. Our results confirm the presence of denitrification genes in the holothurian biofilms. These findings indicate that the holothurians house a diverse community of bacterial symbionts, which includes species with the potential for nitrogen removal. Therefore, holothurian holobionts may play a multifaceted ecological role, both processing organic detritus and reducing nitrogen levels in coastal areas. These roles could be extended to sustainable aquaculture, making them valuable ecosystem engineers with significant implications for ecosystem and aquaculture health.

KEY WORDS: Echinoderm microbiome · Biofilm · Subcuticular bacteria · Denitrification · Holothurian · Holobiont · Sea cucumber

1. INTRODUCTION

Holobionts are entities formed by the association of a host and its symbiotic microbes (Theis et al. 2016). This term has gained importance in recent years due to the evidence of the ubiquitous nature of animal-associated prokaryotes and their central role in biology, ecology, and evolution (Bordenstein & Theis 2015, Simon et al. 2019). A first step in holobiont research requires the characterization of symbiont microorganisms in the hosts, and the current development of massive sequencing techniques has made this approach feasible (Schuster 2008). Interdisciplinary approaches that combine molecular tools with biogeochemical processes can effectively explore unknown functions emerging from holobionts. In fact, recent research has shown the pivotal role of the symbiotic association between invertebrates and bacteria in driving several biogeochemical processes at the ecosystem level. For example, corals, sponges, and oysters, as holobionts, participate in the carbon and nitrogen cycles (de Goeij & van Duyl 2007, de Goeij et al. 2013,

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Rädecker et al. 2015, Arfken et al. 2017, Pogoreutz et al. 2017, Pita et al. 2018, Glaze et al. 2022). Siboni et al. (2008) suggested a coupling of nitrification and denitrification, ultimately producing dinitrogen to remove nitrogen from the coral holobiont. Further, Arfken et al. (2017) found bacteria carrying functional denitrification genes in oysters, facilitating nitrogen removal in oyster reefs. Other associations, such as those between echinoderms and bacteria, have been studied in the search for new antimicrobial compounds (Kiani et al. 2014, León-Palmero et al. 2018), but little is known about the role of the echinoderm holobionts in biogeochemical processes in marine ecosystems.

Holothuria tubulosa Gmelin, 1790 is an echinoderm of the class Holothuroidea, commonly known as sea cucumbers. Holothurians are benthic organisms usually found at the rock–sand interface of coastal zones (Ocaña et al. 2000). They are sediment-eaters, playing an important role in the recycling of organic matter and bioturbation (Uthicke 1999, Purcell et al. 2013). Recent studies have shown that holothurians can serve as extractive species in integrated multitrophic aquaculture systems, effectively reducing ammonium, nitrate, transparent exopolymer particles, bacteria, and chromophoric dissolved organic matter in seawater (Sadeghi-Nassaj et al. 2018 a,b). Several echinoderm species possess a subcuticular layer of bacteria (Kelly & McKenzie 1995). These subcuticular bacteria have been visualized using fluorescence confocal and electron microscopy in various species of echinoderms, demonstrating that it is a widespread phenomenon, present in approximately 60% of the studied echinoderms (McKenzie & Kelly 1994, Kelly & McKenzie 1995). Regarding their diversity, Lawrence et al. (2010) were pioneers in applying molecular tools to characterize these bacteria, identifying 4 different groups through phylogenetic analysis of the 16S rRNA genes, all belonging to the *Alpha-* and *Gammaproteobacteria*. Later, massive sequencing techniques were applied to the study of the subcuticular bacteria of the brittle star *Amphipholis squamata*, showing that 70–80% of these bacteria belong to the *Roseobacter* clade and that the symbiont community is much less diverse than the neighboring water communities of sea and sediment (Morrow et al. 2018). More recently, Wada et al. (2020) showed that a single operational taxonomic unit (OTU) exists at high densities in the subcuticular space in *Acanthaster planci*, forming a biofilm-like structure between the cuticle and the epidermis.

Although the symbiotic relationships between echinoderms and subcuticular bacteria have been recognized for over 4 decades (Holland & Nealson 1978), the potential functions of these bacteria remain largely unexplored. For instance, they could provide direct nutrients to the host, based on the observation of partially digested bacteria within phagosomes of epidermal cells in various holothurians, as well as in asteroid larvae (Roberts et al. 1991, Bosch 1992). They appear to translocate amino acids from seawater to the host for protein synthesis (Lesser & Walker 1992). Additionally, they could play a relevant role in the early stages (larval development) of the host, helping to absorb labile dissolved organic matter (Fontaine & Chia 1968, Walker & Lesser 1989). On the other hand, holothuroids secrete mucus that coats the cuticle (Ocaña et al. 2000) and can contain associated bacteria that ultimately form a microbial biofilm. This kind of biofilm has been studied in corals, and Siboni et al. (2008) reported the presence of archaea, including marine group II, marine group III, anaerobic methanotrophs, anaerobic nitrate reducers, and ammonia-oxidizing archaea. Furthermore, it is possible that the ammonia-oxidizing archaea as coral symbionts play a role in nitrogen recycling through nitrification and denitrification processes (Siboni et al. 2008). Rädecker et al. (2015) and Pogoreutz et al. (2017) also demonstrated the regulation of environmental nitrogen by the coral holobionts. However, the current knowledge of the composition and the role of subcuticular and biofilm bacteria of holothurians is very limited.

In this study, we delve into the composition of the biofilm and subcuticle microbiomes of *H. tubulosa* by partial amplification and massive sequencing of the 16S rRNA genes. To explore the potential participation of subcuticular and biofilm bacteria in the nitrogen cycle, we tested the presence of key genes associated with nitrification (i.e. bacterial *amoA* and archaeal *amoA*) and denitrification (i.e. *nirS* and *nosZ*). This investigation advances our understanding of the echinoderm symbionts and highlights the broader significance of holobionts in shaping nutrient cycling at the ecosystem level.

2. MATERIALS AND METHODS

2.1. Sample collection

We collected 8 individuals of *Holothuria tubulosa* from the coast of Granada, Spain (36° 42′ 10.1″ N, 3° 24′ 42.7″ W), by scuba diving at depths from 5 to 15 m. Once collected, we transferred them to an aquarium until sampling. We collected biofilm samples using sterile swabs to scrub the bivium (dorsal zone) and subcutaneous samples using a biopsy punch in

areas where the biofilm had previously been removed to avoid the transference of biofilm bacteria to subcuticle samples. From each holothurian, we collected a single biofilm sample and 2 replicate samples from subcutaneous punch biopsies. From the aquarium, we also collected a sample of sediment and a sample of water that was subsequently filtered through a 0.22 µm polycarbonate filter for comparison purposes. We preserved all samples in saline buffer (0.75 M saccharose, 0.05 M Tris, 0.02 M EDTA, and 0.4 M NaCl) until DNA extraction. DNA extractions were performed using a DNeasy Powersoil Kit (Qiagen).

2.2. Sequencing and data analysis

We used Illumina Next Generation Sequencing to obtain sequences of the V3-V4 hypervariable region of the 16S rRNA gene amplified with primers 341F 'CCT ACG GGG GNG GGG CWG CAG' and 805R 'GAC TAC HVG GGG GTA TCT AAT CC' using Macrogen facilities. All 8 biofilm samples passed the quality control, and 11 out of the 16 subcuticle samples passed the quality control. The 5 samples that did not pass the quality control were replicates, so we had at least 1 biofilm and 1 subcuticular sample from each individual. The water and sediment samples also passed the quality control. The sequences are available in GenBank Bioproject PRJNA1003448 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1003448 with accession numbers 36890840 to 36890860). We processed the data using the R package 'DADA2' v.3.10 (Callahan et al. 2016) installed in R v.4.4.2 (R Core Team 2023). The 'DADA2' protocol clusters reads into unique sequences termed amplicon sequence variants (ASVs). Primers were removed using the 'Cutadapt' v.2.10 package (Martin 2011). Raw sequences underwent quality control following standard filtration parameters and chimera removal. We performed taxonomic assignment of the ASVs using the 'assignTaxonomy' function of the 'DADA2' package and using 'SILVA SSU' v.138.1 (Quast et al. 2012) as a reference database. We rarefied the data to the minimum number of reads and randomizing 100 times using the function 'rrarefy' of the R package 'vegan' v.2.6.4 (Oksanen 2013).

2.3. Statistical analysis

To compare the dissimilarities in the composition of ASVs within subcuticular, biofilm, water, and sediment communities, we employed a non-metric multidimensional scaling (NMDS) ordination model based on the Bray-Curtis index in the R package 'vegan' v.2.6.4 (Oksanen 2013). Given that the NMDS model ordination is contingent on the initial configuration of communities in multidimensional space, we iteratively executed the model through 1000 permutations to identify the ordination with the optimal goodness of fit (Oksanen 2013). Lastly, we determined the location of the centroid associated with the subcuticular communities and the biofilm, and we examined the 95% confidence interval (CI) tied to their location in the multidimensional space. To ascertain the 95% CI around the centroids, we employed the 'ordihull' function in 'vegan' v.2.6.4 (Oksanen 2013).

Finally, we compared the diversity between communities associated with the subcuticle and the biofilm using Chao1 and Simpson's inverse indices with the functions 'chao1' and 'diversity' in the R packages 'fossil' v.0.4.0 (Vavrek 2011) and 'vegan' v.2.6.4 (Oksanen 2013), respectively. During our data exploration, we observed that the richness and diversity estimates (Chao1 and Simpson's inverse) did not conform to the normality and homogeneity of variance assumptions required for parametric tests. We used the Shapiro test for the assumption of normality, Levene's test for homogeneity of variance, and the conventional alpha of 0.05 for all tests. Therefore, for comparisons between subcuticular and biofilm samples, we implemented a Mann-Whitney-Wilcoxon test to compare the medians of each group using the 'wilcox.test' function with the R v.4.2.2 software (R Core Team 2023).

2.4. Testing the occurrence of functional genes

First, we used the Functional Annotation of Prokaryotic Taxa (FAPROTAX) software (Louca et al. 2016) to envisage the functions that the holothurian symbionts could be performing. Secondly, we explored the functional genes involved in the nitrogen cycle using PCR tests. We tested the functional genes (bacterial *amoA* and archaeal *amoA*) that encode the catalytic subunit A of ammonia monooxygenase, which catalyzes the oxidation of ammonium to nitrite in the nitrification process. Furthermore, we tested the functional genes *nirS* and *nosZ* involved in denitrification. The *nirS* gene encodes nitrite reductase, which catalyzes the transformation of nitrite into NO, and the *nosZ* gene encodes the nitrous oxide reductase, which reduces N_2O to N_2.

The PCR reactions contained 12.5 µl of OneTaq® 2× Master Mix with Standard Buffer (NewEngland

BioLabs), 1 µl of each primer at 10 µM, 1 µl of template DNA (ca. 40 ng), and 9.5 µl of Milli-Q water. Thermocycling parameters were as follows: initial denaturation at 45°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing (temperatures for each gene are given in Table 1), elongation at 68°C for 30 s, and finally, elongation at 68°C for 5 min. Table 1 also includes the primer details and positive controls. We visualized the PCR results by electrophoresis on 2% agarose gels.

3. RESULTS

3.1. Description of subcuticular and biofilm microbiomes

The number of reads of the V3-V4 16S rRNA gene amplicons per sample ranged from 35 876 to 60 648, obtaining a total of 9172 amplicon sequence variants (ASVs). The rarefaction curves show that a normalization to 35 876 reads was appropriate to determine the ASV richness (Fig. A1 in the Appendix). After the random normalization, the number of ASVs ranged from 269 to 910 in the subcuticle samples, and from 266 to 1854 in the biofilm. The number of ASVs in the water and sediment samples were 149 and 205, respectively (Table A1).

The relative abundances of the most prevalent taxonomic groups in the subcuticle (Fig. 1a) and biofilm (Fig. 1b) samples were substantially different from those in water and sediment samples. The most relevant taxonomic groups found in the subcuticle and biofilm samples were the phylum *Bacteroidota*, and the classes *Alphaproteobacteria* and *Gammaproteobacteria*. Within the *Alphaproteobacteria*, ASVs of the order *Rhodobacterales* contributed about 10% of the total abundance. Within the *Gammaproteobacteria*, the order *Pseudomonadales* was prevalent, accounting for 14% on average.

To discern the specificity level of the biofilm and subcuticle microbiomes, we analyzed the ASVs present exclusively in each sample type. We found more exclusive ASVs in the subcuticle than in the biofilm (Fig. 2). In the subcuticle samples, the exclusive ASVs usually accounted for more than 25% of the total (Fig. 2a), whereas in the biofilm samples, the exclusive ASVs accounted for less than 25% of the total (Fig. 2b).

The NMDS ordination model based on the ASV composition across sampled communities showed a high goodness of fit between the distances in the ordination against the original data (linear fit R^2 = 0.9, nonmetric fit R^2 = 0.9). The NMDS model showed clustering that clearly discriminates between subcuticle and biofilm communities, which, in both cases, were also different from sediment and seawater communities (Fig. 3). Therefore, these results support those derived from our taxonomic analyses, underscoring differences in the microbiomes associated with distinct sections of the holothurian body.

We estimated the ASV richness using the Chao 1 index (Fig. 4a) and diversity using Simpson's inverse index (Fig. 4b). The median value of the Chao 1 index for the subcuticle samples was 602 ASVs, whereas for the biofilm samples it was 1606 ASVs. The biofilm samples showed a significantly higher richness than the subcuticle samples (Mann-Whitney-Wilcoxon test W = 13, p = 0.009). The median value of Simp-

Table 1. Description of primers used to study functional genes involved in the nitrogen cycle, including the annealing temperature, the bacterial DNA used as positive controls, and their corresponding references

Process	Functional genes	Primers	Annealing temp. (°C)	Positive control	References
Denitrification	*nirS*	*nirS*-1F 5′-CCTAYTGGCCGCCRCART-3′ *nirS*-3R 5′-GCCGCCGCCGTCRTGVAGGAA-3′	62	*Escherichia coli* transformed with a constructed plasmid containing the *nirS* gene	Braker et al. (1998)
	nosZ	*nosZ*1F 5′-WCSYTGTTCMTCGACAGCCAG-3′ *nosZ*1R 5′-ATGTCGATCARCTGVKCRTTYTC-3′	63	*Paracoccus denitrificans* (ATCC 17741)	Henry et al. (2006)
Nitrification	bacterial *amoA*	*amoA*-1F 5′-GGGGTTTCTACTACTGGTGGT-3′ *amoA*-2R 5′-CCCCTCKGSAAAGCCTTCTTC-3′	60	*Nitrosomonas europaea* (ATCC 25978)	Rotthauwe et al. (1997)
	archaeal *amoA*	arch-*amoA*F 5′-STAATGGTCTGGCTTAGACG-3′ arch-*amoA*R 5′-GCGGCCATCCATCTGTATGT-3′	53	*Nitrososphaera viennensis* (EN76T)	Francis et al. (2005)

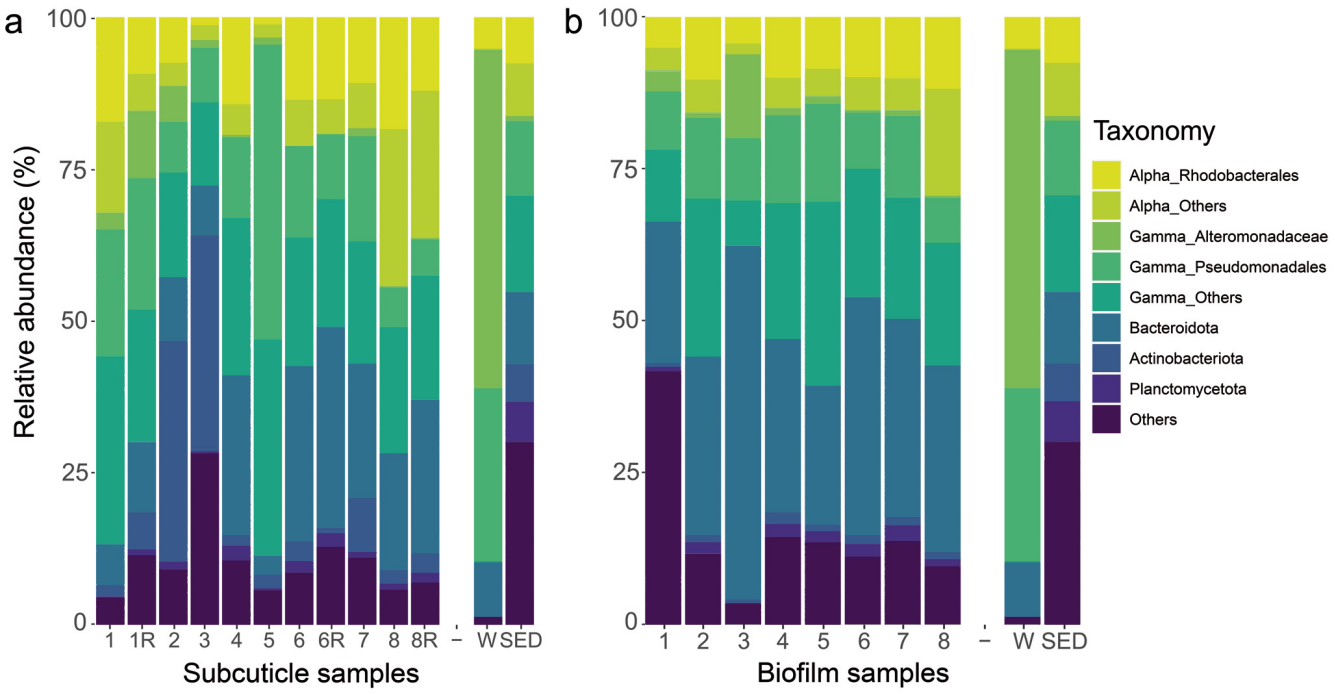

Fig. 1. Relative abundance of the most relevant taxonomic groups in *Holothuria tubulosa* (a) subcuticle and (b) biofilm samples. The *y*-axis shows the relative abundance (% of total abundance) of the taxonomic groups for each sample (*x*-axis). In terms of taxonomy, *Alpha_Rhodobacterales*, *Alpha_*others, *Gamma_Alteromonadaceae*, *Gamma_Pseudomonadales*, and *Gamma_*Others, all belong to the phylum *Pseudomonadota*. The remaining taxa, *Bacteroidota*, *Actinobacteriota*, and *Planctomycetota* are phyla with a relevant relative abundance in the samples. Those phyla with low relative abundance are included in Others. W: water sample, SED: sediment sample, R: replicate samples

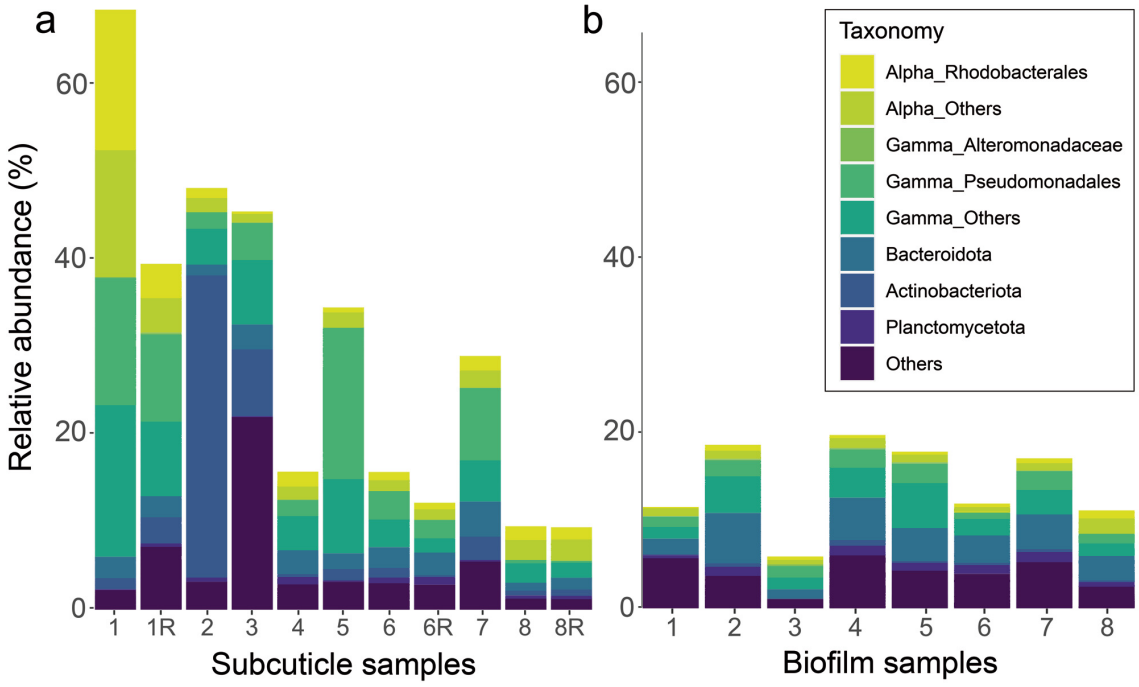

Fig. 2. Relative abundance of the taxonomic groups of the exclusive amplicon sequence variants (ASVs) in (a) subcuticle and (b) biofilm samples from *Holothuria tubulosa*. The *y*-axis shows the relative abundance (% of total abundance) of these ASVs for each sample (*x*-axis). In terms of taxonomy, *Alpha_Rhodobacterales*, *Alpha_*Others, *Gamma_Alteromonadaceae*, *Gamma_Pseudomonadales*, and *Gamma_*Others, all belong to the phylum *Pseudomonadota*. The remaining taxa *Bacteroidota*, *Actinobacteriota*, and *Planctomycetota* are phyla with a relevant relative abundance in the samples. Those phyla with low relative abundance are included in Others. R: replicate samples

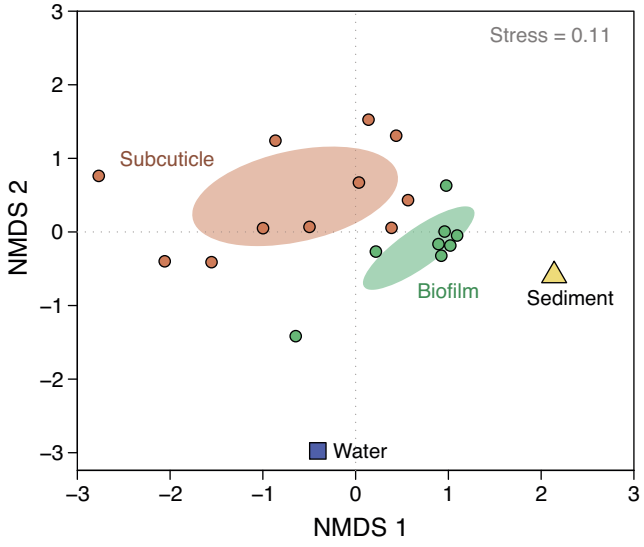

Fig. 3. Non-metric multidimensional scaling (NMDS) ordination model based on Bray-Curtis index comparing the dissimilarities in composition of bacterial communities of *Holothuria tubulosa* subcuticle, biofilm, water, and sediment. Ellipses show the 95% credible intervals on the location of centroids for the subcuticular and biofilm communities

son's inverse index was also higher in the biofilm samples (141) than in the subcuticle (53) samples, although we did not find statistically significant differences (Mann-Whitney-Wilcoxon test $W = 25$, $p = 0.129$). This lack of statistical significance might be related to the wide variability of diversity values found in the biofilm samples.

3.2. Functional profile

We visualized the putative functional profiles of the holothurian symbionts based on the taxa identified in the samples using FAPROTAX (Louca et al. 2016). Comprehensively, the predicted functions are chemoheterotrophy, functions related to the nitrogen cycle, degradation of aromatic compounds in subcuticle samples, dark oxidation of sulfur, fermentation, photoheterotrophy, and photoautotrophy. In the case of the functions related to the nitrogen cycle, we observed the potential for denitrification of nitrate, nitrite, and nitrous oxide both in subcuticular and biofilm samples (Fig. A2). Given that FAPROTAX suggests that holothurian microbiomes have taxa with the potential to be involved in the nitrogen cycle, we performed a search of well-known taxa associated with nitrogen cycling in the subcuticular and biofilm data set (Table 2).

To corroborate the presence (or absence) of key functional genes involved in the nitrogen cycle, we used PCR amplifications of the functional genes for nitrification (bacterial a*moA* and archaeal *amoA*) and denitrification (*nirS* and *nosZ*). We were unable to detect the presence of the functional genes bacterial

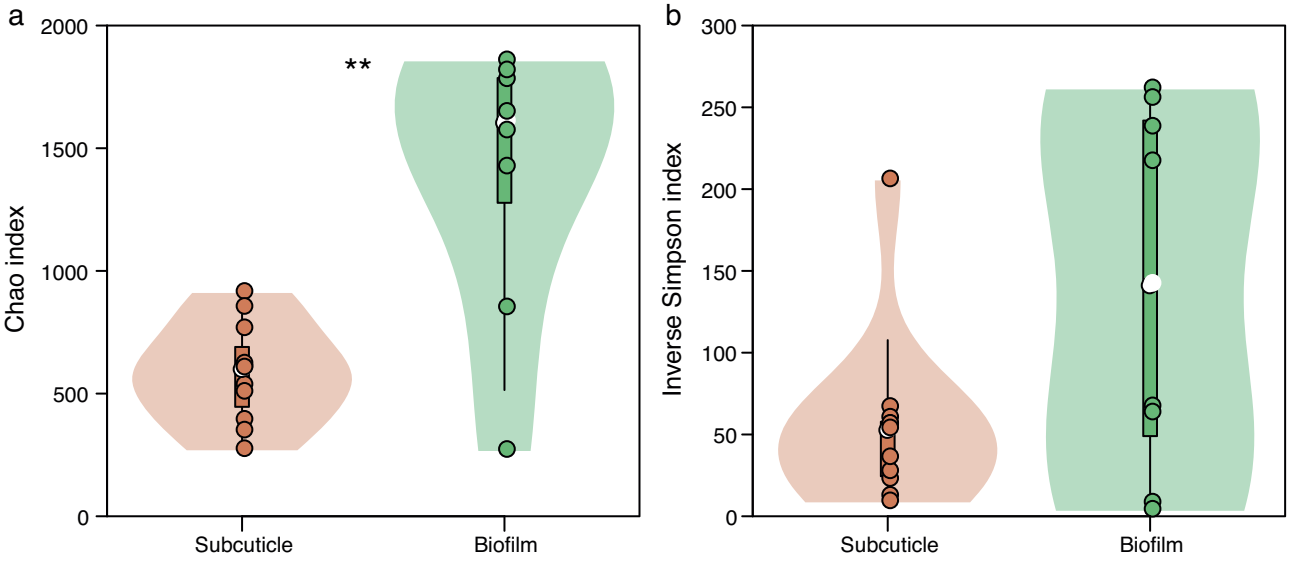

Fig. 4. Data distribution of the (a) Chao1 richness index and (b) the inverse Simpson diversity index for the *Holothuria tubulosa* subcuticle and biofilm samples. The white dots represent the median value of the data distribution. The box within the violin represents the interquartile range, from the first quartile (25th percentile) to the third quartile (75th percentile) of the data. The whiskers indicate the minimum and maximum values of the data, excluding the outliers. Differences in the Chao 1 and Inverse Simpson index were tested using the Mann-Whitney-Wilcoxon test. Asterisks indicate significant differences (**$p < 0.01$)

Table 2. Nitrogen cycle functions associated with the bacterial taxa found in the subcuticle and biofilm samples. We show the relative abundance of each of the taxa found in the subcuticle and biofilm samples

Function	Bacteria	Relative abundance (%), mean ± SD		References
		In subcuticle	In biofilm	
Ammonia-oxidation	*Nitrosospira* spp.	0.0109 ± 0.0064	0.0254 ± 0.0064	Prosser et al. (2014)
	Nitrosomonas spp.	0.0950 ± 0.0617	0.0136 ± 0.0082	Prosser et al. (2014)
	oc32	0.0081 ± 0.0081	0.0593 ± 0.0389	Prosser et al. (2014)
	DSSD61	0.0041 ± 0.0330	0.0000 ± 0.0000	Prosser et al. (2014)
	AqS1	0.0258 ± 0.0111	0.0561 ± 0.0167	Semedo et al. (2021)
	CM1-21	0.0383 ± 0.0251	0.0324 ± 0.0080	Semedo et al. (2021)
Nitrite-oxidation	*Nitrospina* spp.	0.0205 ± 0.0111	0.0533 ± 0.0130	Park et al. (2020)
	Nitrospira spp.	0.1201 ± 0.0487	0.1917 ± 0.0382	Park et al. (2020)
	Nitrolancea spp.	0.0081 ± 0.0081	0.0000 ± 0.0000	Sorokin et al. (2014), Park et al. (2020)
Denitrification	*Methylophaga nitratireducenticrescens*	0.0000 ± 0.0000	0.0007 ± 0.0007	Auclair et al. (2010)
	Pseudomonas spp.	1.0541 ± 0.2953	0.0174 ± 0.0127	Hargreaves (1998)
	Bacillus spp.	0.0849 ± 0.0395	0.0098 ± 0.0078	Hargreaves (1998)
	Woeseia spp.	0.5813 ± 0.1591	0.8503 ± 0.1774	Mußmann et al. (2017)
	Pseudohongiella spp.	0.0099 ± 0.0074	0.0233 ± 0.0068	Xu et al. (2016)
	Pseudohongiella nitratireducens	0.0098 ± 0.0098	0.0000 ± 0.0000	Xu et al. (2016)

amoA and archaeal *amoA*. We did not observe a clear amplification of the *nirS* gene in the subcuticle samples (Fig. 5a,b), but we observed an evident amplification in 7 out of 8 biofilm samples (Fig. 5c), and in water and sediment samples. As with *nirS*, we did not observe amplification of the *nosZ* gene in any of the samples from the subcuticle or their respective replicates (Fig. 5d,e). However, we observed an evident amplification of the *nosZ* gene in the 8 biofilm samples (Fig. 5f) and in the sediment sample.

4. DISCUSSION

Currently, there is evidence that all classes of the phylum Echinodermata host subcuticular bacteria. However, the presence of these bacteria in the class Holothuroidea is thought to be less common, according to the study by Kelly & McKenzie (1995), where only 39% of the surveyed holothurian species hosted subcuticular bacteria. With the present study, we confirm the presence of these subcuticular bacteria in *Holothuria tubulosa*, a fact that had not been reported previously. Remarkably, we found higher ASV exclusivity and lower richness and diversity in the subcuticle samples than in the biofilm samples. In the biofilm samples, we found evidence of denitrification genes, suggesting a potential function of the holothurian holobionts in the nitrogen cycle, thus providing a service in coastal marine ecosystems.

4.1. Subcuticular and biofilm microbiomes

Chiarello et al. (2020) studied the surface microbiomes of some echinoderms, among other taxa, and concluded that more than 90% of their prokaryotic phylogenetic richness was unique. In the case of the phylum Porifera, the microbiome richness varies widely among different host species, and the complexity (assessed by the number of OTUs) ranges from 50 to 3820 genetically distinct symbionts per host (Thomas et al. 2016). Recent studies show that different species of echinoderms harbor rich and diverse microbiomes and that the phylum *Bacteroidota* and the class *Alphaproteobacteria* predominate (Crowley 2022), as we also saw in our samples. Our ASV exclusivity and ordination analyses confirm that the bacteria associated with distinct parts of the body are different from each other, as well as different from the bacteria from the water and sediments (Figs. 2 & 3). Hence, we can assert that there is a site-specific (subcuticle vs. biofilm) selection of the bacterial community. The process by which echinoderms acquire subcuticular bacteria remains largely unknown. There is evidence of vertical transmission from the parent to the incubated embryo in brittle stars (Morrow et al. 2018), and transmission from the environment was also previously proposed by Walker & Lesser (1989). In fact, Schuh et al. (2020) indicated that larvae acquire most of their bacterial load after the onset of feeding. We observed that the subcuticle

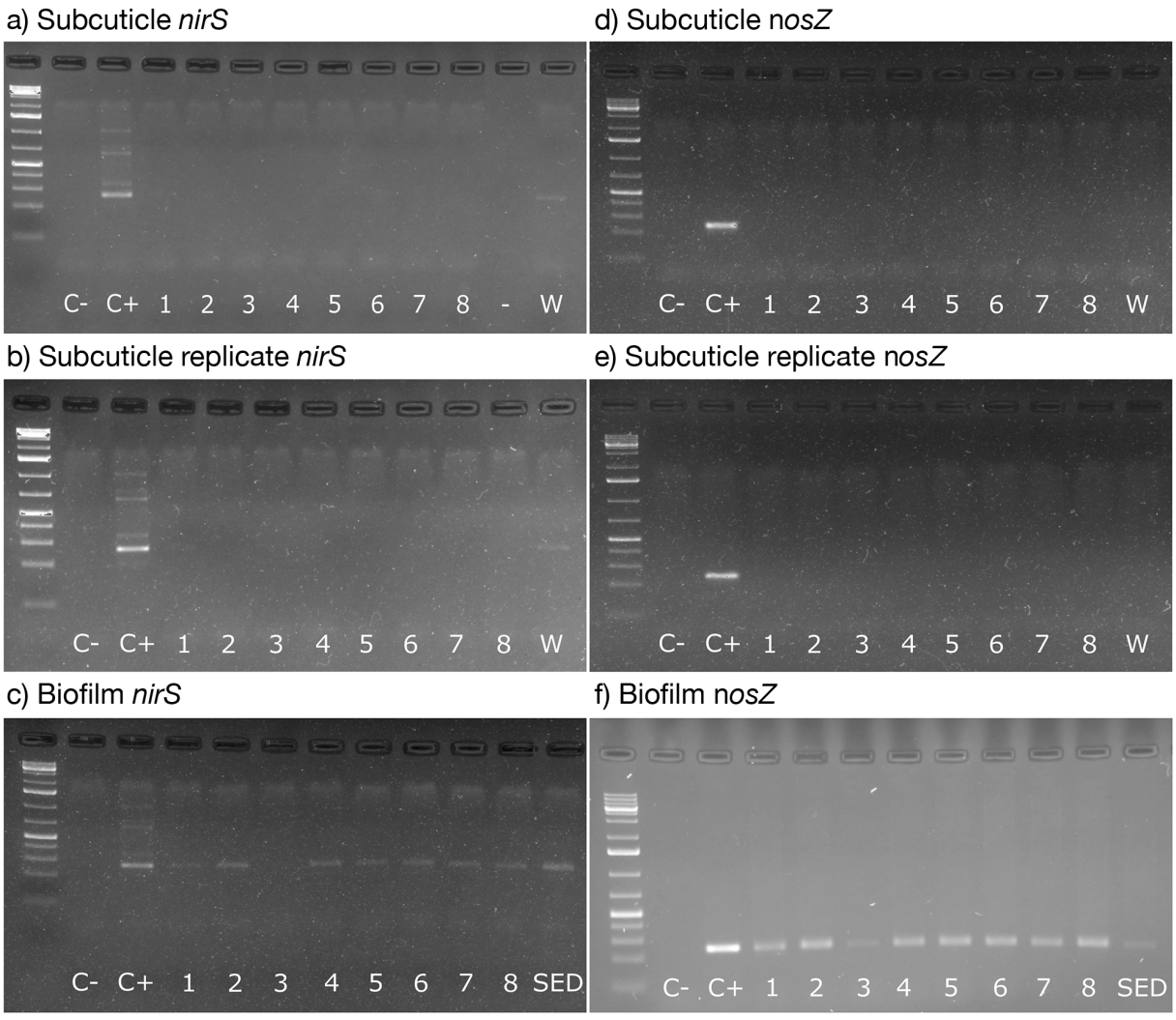

Fig. 5. Gel electrophoresis images showing the PCR results to test the occurrence of the (a–c) *nirS* and (d–f) *nosZ* genes. Gels a, b (replicate), d, and e (replicate) correspond to the subcuticular samples, and the last sample in each of these gels is water (W). Gels c and f correspond to biofilm samples, and the last sample in these gels is sediment (SED). In all the gels, the DNA ladder appears on the left, followed by a negative control (C–) and a positive control (C+)

samples of *H. tubulosa* presented richness and diversity values higher than those reported in other echinoderms such as sea stars. Wada et al. (2020) found that in *Acanthaster planci*, there was a 61.8% predominance of a single OTU (COTS27 affiliated to the phylum *Spirochaetota*). Concerning the biofilm microbiome, it remains uncertain whether bacterial acquisition is related to the surrounding environment or if there is an active selection by the host. Our NMDS ordination (Fig. 3) indicates that the biofilm and subcuticular communities are closer to each other compared to the sediment and water communities. On the other hand, the sediment community is closer to the biofilm community than to the subcuticle communities, suggesting a more relevant environmental influence on the biofilm.

4.2. Potential functions at the holobiont and ecosystem levels

The microbiomes associated with marine invertebrates can perform different functions or services both from the perspectives of the host and the ecosystem. The relationship between host and microbiome can be nutritional through the supply of certain nutrients or, in the case of strains with antimicrobial capacity, defensive (Rosenberg et al. 2007, León-Palmero et al. 2018). For instance, indirect evidence suggests that the endosymbiont bacteria of some echinoderms can take up dissolved amino acids (Siebers 1979, Manahan et al. 1983, Davis et al. 1985, Brothers et al. 2015). In the case of the brittle star *Amphipholis squamata*, subcuticular bacteria can absorb dissolved

amino acids for the synthesis of proteins that are later translocated to the host (Lesser & Walker 1992). Some investigators have also suggested that subcuticular bacteria can metabolize dissolved organic matter (Holland & Nealson 1978, Walker & Lesser 1989).

At the ecosystem level, the symbiosis between bacteria and marine invertebrates such as corals, sponges, and oysters can participate in the nitrogen cycle (Yang & Li 2012, Rädecker et al. 2015, Arfken et al. 2017, Moeller et al. 2019). However, the role of echinoderm microbiomes in this cycle is unknown. Our results using FAPROTAX (Fig. A2) suggest that the echinoderm microbiomes include taxa with putative functions associated with the nitrogen cycle (Table 2). By analyzing the data obtained from 16S rRNA gene sequencing, we observed that several genera of the family *Nitrosomonadaceae*, whose cultivated representatives are ammonia-oxidizing bacteria (AOB), appeared in these samples (Prosser et al. 2014). Among the representatives of this family, we found *Nitrosospira*, *Nitrosomonas*, oc32, DSSD61, and unclassified ASVs belonging to the *Nitrosomonadacea*. Concerning AOB, we identified the presence of 2 genera (AqS1 and Cm1-21) and unclassified ASVs belonging to the *Nitrosococcaceae* (Semedo et al. 2021). We also detected nitrite-oxidizing bacteria, such as *Nitrospina*, *Nitrolancea*, and *Nitrospira* (Sorokin et al. 2014, Park et al. 2020). Some members of the genus *Nitrospira*, in addition to oxidizing nitrite, can carry out complete nitrification (comammox bacteria) (Daims et al. 2015, van Kessel et al. 2015, Palomo et al. 2016). We also detected denitrifying bacteria such as *Methylophaga nitratireducenticrescens* in the biofilm. Mauffrey et al. (2017) also detected these bacteria in aquaria and determined that they carry out complete denitrification (Auclair et al. 2010). We also detected bacteria of the genera *Pseudomonas* and *Bacillus*. It is known that these genera can reduce nitrate in microzones with low oxygen concentrations in the upper layer of sediments (Hargreaves 1998). Furthermore, we observed the presence of the genus *Woesia*. Mußmann et al. (2017) studied the metagenome of some *Woesia* strains and observed that they possibly perform denitrification producing N_2O. Finally, we also observed *Pseudohongiella nitratireducens*, which can reduce nitrate (Xu et al. 2016).

Despite the results obtained by FAPROTAX and the detailed study of the sequences, we were unable to confirm the occurrence of the nitrification archaeal *amoA* and bacterial *amoA* genes using PCR. This could be due to these functional genes falling below our detection threshold and their low prevalence in the total ASVs. However, we successfully confirmed the presence of the denitrification genes *nirS* and *nosZ* (which encode nitrite reductase and nitrous oxide reductase, respectively) in the biofilms of *H. tubulosa* for the first time, using PCR (Fig. 5). We detected *nir*S and *nos*Z only in the biofilm samples but not in the subcuticle. This fact suggests more of an environmental than physiological function even though nitrate, nitrite, and nitric oxide reductases appear to have evolved for detoxification of nitric oxide in invertebrate and vertebrate hosts (Vázquez-Torres & Bäumler 2016).

Future studies should aim to quantify the abundance of these genes (by quantitative PCR or droplet digital PCR) and denitrification rates. In addition, the conditions (i.e. nitrogen and oxygen concentrations) in which denitrification occurs could be key to determine the balance between denitrification products (i.e. N_2O and N_2). Quantifying this nitrogen removal by the holothurian holobionts can be of great interest to the conservation of marine ecosystems. Despite ammonium, nitrite, and nitrate being present in nature, human activities have significantly altered the nitrogen cycle, increasing their availability (Gruber & Galloway 2008, Battye et al. 2017) and inducing coastal eutrophication (Zhou et al. 2020). Overall, anthropogenic nitrogen represents a severe problem in coastal ecosystems since this element is usually the limiting resource, producing phytoplankton blooms (Howarth & Marino 2006). One of these human activities is related to aquaculture discharges (Vizzini et al. 2005). Our data suggest the ability of the *H. tubulosa* microbiome to reduce nitrogen concentrations, emphasizing its high intrinsic value as an extractive species in integrated multitrophic aquaculture (Neofitou et al. 2019, Grosso et al. 2021). Moreover, knowing more about the microbiomes of these organisms could be relevant for aquaculture and ecosystem services due to their multiple functionalities beyond denitrification and nitrification.

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Appendix. Additional data on rarefaction curves of all samples (Fig. A1), the total number of ASVs obtained in each sample (Table A1), and the bubbleplots obtained from the results of Functional Annotation of Prokaryotic Taxa (FAPROTAX) for the functions related to the nitrogen cycle (Fig. A2)

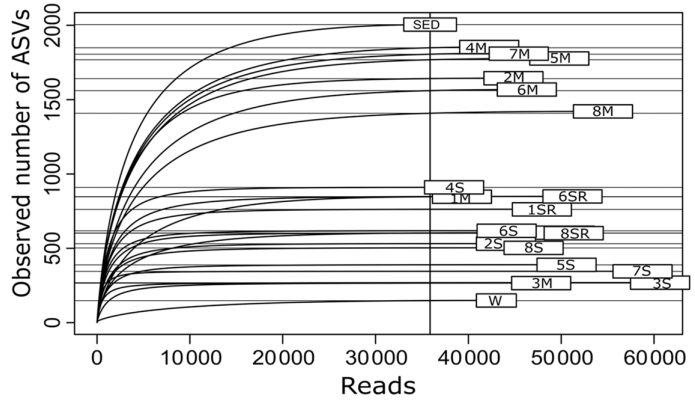

Fig. A1. Rarefaction curves of all samples. The *x*-axis represents the number of reads per sample and the *y*-axis represents the number of amplicon sequence variants (ASVs) per sample. All samples reach a plateau. The vertical line indicates the minimum number of reads in the samples. M: biofilm samples; R: replicate samples, S: subcuticle samples, SED: sediment; W: water

Table A1. Number of amplicon sequence variants (ASVs) obtained per sample. Samples are numbered from 1 to 8; R: replicate samples; W: water; SED: sediment

Sample	Number of ASVs	
	Subcuticle	Biofilm
1	605	847
1R	762	Not applicable
2	531	1644
3	269	266
4	910	1854
5	389	1777
6	618	1568
6R	849	Not applicable
7	345	1813
8	503	1421
8R	602	Not applicable
W		149
SED		205

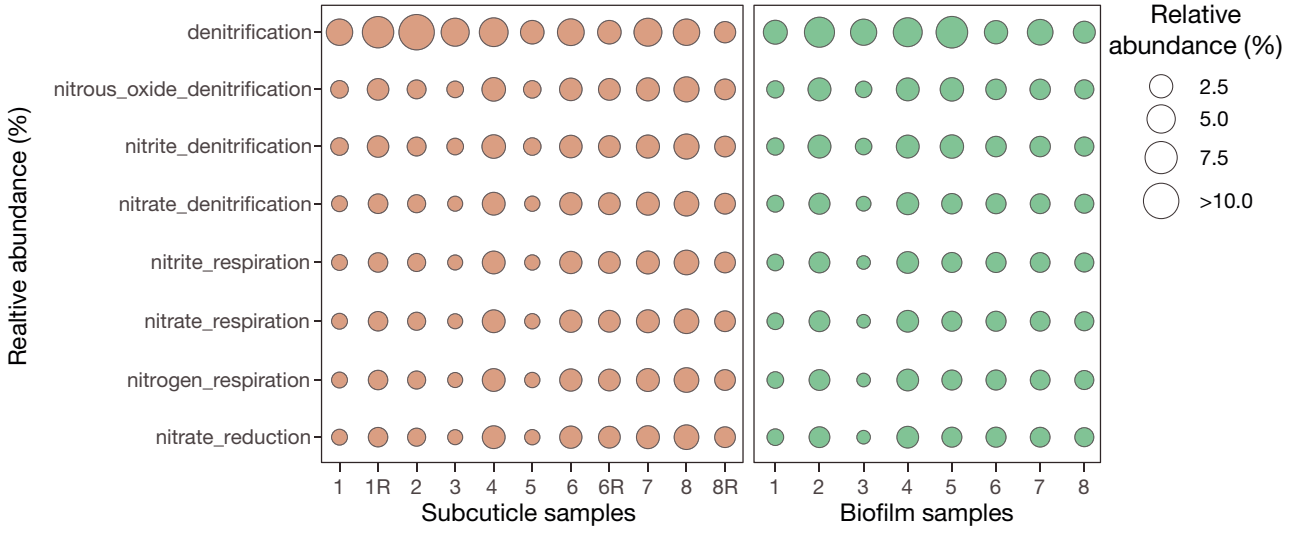

Fig. A2. Results of FAPROTAX related to the nitrogen cycle functions. The size of a circle represents the relative abundance of that function in the sample. R indicates replicate samples

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