

Article

Early Metabarcoding Detection of Eukaryotic Putative Pathogens Nearby Wastewater Effluents of Ría de Vigo (NW Spain)

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Abstract

From a One Health perspective, the Ría de Vigo (NW Spain) represents a complex socio-ecological system where urban, industrial, and aquaculture activities converge, creating vulnerabilities due to the interconnection between human, animal, and environmental health. This study evaluates the utility of a multi-marker environmental DNA (eDNA) metabarcoding approach as an early warning tool to detect potentially harmful eukaryotic pathogens near wastewater discharge points in three distinct municipalities of the Ría de Vigo. Water and sediment samples were analyzed using the V4 and V9 regions of the 18S rRNA gene and the ITS-2 region for fungi. Bioinformatics analysis was performed with DADA2 and taxonomic assignments were based on SILVA and UNITE databases. Eukaryotic diversity varied by site and marker, with the highest richness found in inner estuary sediments. The V9 region provided broader taxonomic coverage, while V4 produced higher read abundances. Putative pathogens, including human, zoonotic, and aquaculture-related taxa, were more prevalent in inner estuarine zones. Pathogens such as *Rhodotorula mucilaginosa*, *Acanthamoeba* sp., *Cryptosporidium* sp., and harmful algae (HA) were detected. The findings emphasize the importance of a multi-marker strategy, sediment inclusion, and landscape-scale variables to improve early pathogen detection, contributing to effective eDNA-based surveillance systems for coastal health management.



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1. Introduction

Coastal ecosystems harbor an extensive diversity of eukaryotic microorganisms, each fulfilling distinct ecological roles and participating in biological interactions critical to maintaining ecosystem functionality. Furthermore, they provide services that benefit humanity, underpinning global economic stability by serving as resources for fisheries, raw materials, compounds with medical applications and recreational areas [1]. However, coastal zones are well-known as natural sinks for anthropogenic activities (e.g., inefficient wastewater discharges, industrial wastes, runoff of farms), leading to substantial environmental impacts, from habitat destruction and eutrophication to the release of potential pathogens, multidrug-resistant bacteria, and pharmaceutically active compounds (PhACs) [2–4]. Such pressures may reduce eukaryotic diversity, trigger pathogenic or harmful algal blooms, and disrupt trophic interactions, with cascading effects that compromise ecosystem functioning and services. In wastewater treatment plants (WWTPs), the microbial community is predominantly composed of prokaryotes, which have been extensively studied over the years compared to the non-bacterial community, which has been less investigated [5]. Within WWTPs and adjacent coastal zones, fungi contribute

to organic matter degradation, protists act both as grazers that regulate bacterial populations and as potential pathogen vectors, and metazoans can influence microbial food webs. These groups thus represent both essential ecosystem players and potential health hazards, as some are pathogens with risks for human, animal, and environmental health [5,6]. In addition, WWTPs and coastal sediments can act as reservoirs and dissemination hubs for emerging pathogens, antibiotic resistance genes, and even invasive species, raising concerns for One Health frameworks. Therefore, a critical task in wastewater treatment is to monitor the human sewage microbiome to document its fate and effective removal [7]. While culture-dependent methods and molecular techniques (e.g., qPCR and in situ hybridization) are commonly used for monitoring, recent advances in next-generation sequencing (NGS) have enabled a more comprehensive exploration of microbial diversity [8]. There is an ongoing debate about the type of target gene chosen for sequencing on the efficiency of the metabarcoding eDNA technique. Examination of short hypervariable regions of the 18S rRNA gene (V9 and V4) or the full-length gene with universal primers involves trade-offs between sequencing cost, taxonomic resolution, and amplification bias [9]. Although fungi can be detected through the amplification of the 18S rRNA gene, this approach can lead to an underestimation of their proportion and diversity. Therefore, for fungal detection, the nuclear ribosomal internal transcribed spacer (ITS) regions are the universal DNA markers [10]. Beyond primer bias and incomplete databases, eDNA metabarcoding is also limited by DNA persistence, PCR inhibition, low taxonomic resolution, and its inability to distinguish viable from non-viable organisms.

This study focused on the Ría de Vigo (Galicia, NW Spain), an area bordered by nine municipalities with a combined population of approximately 413,000 inhabitants, distributed from the outer to the inner parts of the Ría. The region contains several WWTPs that discharge effluents into the Ría [11]. Additionally, this coastal ecosystem also maintains high fish and shellfish production values chains. The area has also experienced the presence of pathogens affecting the aquaculture sector and occurrences of potentially toxic harmful algal blooms (HABs) [12,13]. Most studies focused on describing biodiversity and detecting pathogens in the Ría de Vigo have relied on traditional methods, with limited application of high-throughput sequencing, which has mainly been used to explore microbial diversity and associations in general environments [14–16] rather than in discharge zones [17].

This study aimed to compare eukaryotic diversity through high-throughput amplicon sequencing in the effluent discharge areas of WWTPs from three municipalities in the Ría de Vigo located in the middle (Vigo, Cangas) and inner (Redondela) zones of the Ría. Given the mixing of effluents at discharge points, the study specifically focused on detecting DNA from anthropogenic putative pathogens, as well as those that may impact animal species and ecosystems. To enhance the detection of eukaryotic diversity and eukaryotic pathogens and minimize biases associated with specific target regions, the study employed a multi-marker approach using two commonly used regions for eukaryotic biodiversity assessment (V9 and V4) along with the universal fungal marker, the internal transcribed spacer (ITS). In this context, our work is framed as an environmental diagnosis, providing a timely snapshot of local community structure and putative pathogen diversity, while also laying the groundwork for future monitoring strategies in coastal ecosystems. By applying a multi-marker eDNA metabarcoding approach to WWTP discharge zones in the Ría de Vigo, this study provides one of the first comprehensive assessments of eukaryotic pathogen diversity in these environments, contributing to early detection capacity and advancing the application of high-throughput sequencing within a One Health framework.

2. Materials and Methods

2.1. Sampling

The diversity of micro-eukaryotic communities in both water and sediment was analyzed at three sampling sites within the Ría de Vigo (Galicia, NW Spain) in July 2023.

Sampling was conducted once during this campaign with one site positioned near WWTPs outfall of each of the three municipalities: Vigo, Cangas, and Redondela. Notably, Vigo is the most populous city in the Ría de Vigo, whereas Cangas and Redondela are smaller towns. Cangas is located directly opposite Vigo, while Redondela is situated in the innermost part of the estuary, influenced by Alvedosa and Pexegueiro rivers, making it representative of the estuarine environment at the river's mouth (Figure 1). Although the three selected sampling sites shared a common potential source of contamination (i.e., effluent discharges), they were also chosen to represent contrasting estuarine zones with markedly different environmental and ecological conditions, including riverine influence, hydrodynamics, and organic matter loads. These were one-off sampling locations selected for this diagnostic study rather than permanent monitoring stations.



Figure 1. Geographical location of the sampling sites within the Ría de Vigo (Galicia, NW Spain). Water (W) and sediment (S) samples were collected at three stations: Vigo, Cangas, and Redondela. Sampling coordinates were as follows. Redondela: 42.28752° N, −8.61216° W; Cangas: 42.2491° N, −8.79076° W; Vigo, 42.21108° N, −8.77636° W. The red frames indicate the sampling locations.

At each site, one water sample (3 L) and one surface sediment sample (upper ~10 cm) were collected. In addition, in situ temperature and salinity of water were measured at each site, yielding the following average values: Cangas (21.35 °C, 19.36 PSU), Redondela (20.41 °C, 0.75 PSU), and Vigo (13.29 °C, 35.55 PSU). In total, six samples were obtained (three water and three sediment), each processed independently. For clarity, and hereafter throughout the manuscript, samples are referred to as WC and SC (Cangas water and sediment), WR and SR (Redondela water and sediment), and WV and SV (Vigo water and sediment). Water samples were pre-filtered through a 200 µm mesh to remove zooplankton and large particles and then filtered using sterile 0.22 µm pore size filters (Millipore, Merck, Burlington, MA, USA). Filters were stored at −20 °C until DNA isolation, while sediment samples were stored in sterile containers and kept frozen at −20 °C until further processing.

2.2. DNA Isolation, Amplification and Sequencing

DNA from sediments and water was isolated using DNeasy PowerSoil Pro and DNeasy PowerWater Kits (Qiagen, Hilden, Germany), respectively, following manufacturer's indications. Negative isolation controls were performed to discard any contaminations in downstream analysis. DNA concentration and purity were verified with the DS-11FX Spectrophotometer/Fluorometer (Denovix, Wilmington, NC, USA).

The V9 and V4 regions of the 18S SSU rRNA gene, and the ITS-2 region of the nuclear internal transcribed spacer (ITS), were amplified using previously described primers: 1380F/1510R for V9 [18], TAREuk454FWD1/TAREukREV3 for V4 [19], and the fungal-specific primers ITS86F/ITS4 for ITS-2 [20]. Library preparation and paired-end sequencing

(Illumina NovaSeq PE250, San Diego, CA, USA) were performed by AllGenetics (A Coruña, Spain) and Biology SL (Manilva, Spain). The resulting reads were deposited in the Sequence Read Archive (SRA) <http://www.ncbi.nlm.nih.gov/sra> (accessed on 15 May 2025) under BioProject accession number PRJNA1262121.

2.3. Bioinformatic Analysis

Raw paired-end reads were trimmed (adapter dimers removal) using Cutadapt v3.5 [21] and quality-checked with FastQC [22]. Bioinformatics analysis was conducted using the Microbial Genomics Module of Qiagen CLC Workbench 25 (Qiagen, <https://digitalinsights.qiagen.com> accessed on 3 March 2025).

Reads were quality filtered ($Q > 20$, quality limit = 0.05, maximum of 2 ambiguous nucleotides), trimmed to remove adapter and primer sequences, and length filtered (≤ 220 bp for V9; ≤ 250 bp for V4 and ITS-2). Reads with more than one expected error (maximum expected error = 1) were discarded. Amplicon Sequence Variants (ASVs) were inferred using the DADA2 algorithm [23], which includes dereplication of unique sequences, denoising (iterative estimation of a sample-specific error model and generation of candidate ASVs), chimera removal (consensus method), and merging of paired reads (minimum overlap of 12 bp, no mismatches allowed).

Taxonomic assignment was performed using the SILVA 99% SSU 18S database (v138.1) for V9/V4 ASVs [24] and the UNITE 99% database for ITS (v138.1) [25]. Unassigned fungal ASVs were further identified via BLASTn against the NCBI fungal ITS database. Singletons were excluded. Rarefaction plots were assessed from rarefied ASV samples.

2.4. Diversity and Community Composition

To compare the diversity and community composition across different locations and environmental compartments and for downstream analysis, ASV tables were subsampled to the sample with the lowest read count: 142,406 (V9), 267,595 (V4) and 40,124 (ITS-2). Alpha-diversity indices (Shannon–Wiener, Pielou and richness) were estimated using the Vegan R package 2.6.8 and UpSet plots were generated with UpSetR [26,27].

Putative pathogenic taxa were identified at the genus and/or species level, focusing on those associated with anthropogenic sources or marine environments. Classification was based on peer-reviewed scientific literature and two legal frameworks: Directive 2000/54/EC [28] and its Spanish transposition, Real Decreto 664/1997 [29]. Harmful algae were identified using the IOC-UNESCO Taxonomic Reference List of Harmful Micro Algae [30]. Taxonomic assignments were made using to the lowest possible rank based on sequence similarity. Identified genera were validated through BLASTn analysis, achieving sequence similarity values greater than 97%. The term “sp.” indicates assignment only at the genus level due to insufficient resolution, without implying detection of a single species. Additionally, several putative pathogens were detected at the species level and confirmed through BLASTn analysis, with similarity scores exceeding 99%. For V9 and V4 regions, species-level assignments were considered tentative (“closest match”) and only retained when supported by such high similarity values, providing greater confidence in those cases.

3. Results

3.1. Overall Sequencing Data

After sequencing, trimming and ASV detection, a total of 2,118,788 (V9), 2,472,531 (V4) and 499,846 (ITS-2) reads were obtained. These reads corresponded to 6039 (V9), 6679 (V4) and 4885 (ITS-2) taxonomically assigned ASVs. Following subsampling, 1,646,724 (V9) and 1,865,868 (V4) eukaryotic reads, along with 40,124 total fungal reads (ITS-2) were retained for further analysis (Table 1).

Table 1. Summary of sequencing results for each sample and target region (V9 and V4 18S rRNA gen and ITS-2). The table includes the number of reads before (BT) and after trimming (AT), average read length before and after trimming, percentage of reads retained, number of reads used in ASV (Amplicon Sequence Variant) generation, number of unique sequences, ASVs assigned taxonomy, and the total reads in ASVs with taxonomy. The number of reads after subsampling were also included. C-neg-W and C-neg-S refer to negative control samples of water and sediment, respectively. Sampling codes: WC: water from Cangas; SC: sediment from Cangas; WR: water from Redondela; SR: sediment from Redondela; WV: water from Vigo; SV: sediment from Vigo. NA: not applicable.

Target	Name	Reads BT	Avg. Length BT	Reads AT	Retained Reads (%)	Avg. Length AT	Reads in ASVs	Unique Sequences	ASV with Taxonomy	Reads in ASV with Taxonomy	Total ASV with Taxonomy	Total Reads ASV
V9	WC	724,544	250	720,142	99.39	133	657,268	10,304	2447	328,634	6039	1,646,724
	SC	762,576	250	757,774	99.37	136	711,936	7043	1031	355,968		
	WR	1,302,636	250	1,289,876	99.02	132	1,152,146	12,908	3095	576,073		
	SR	681,264	250	672,756	98.75	133	616,402	14,073	4342	308,201		
	WV	620,302	250	616,648	99.41	135	550,780	9281	1597	275,390		
	SV	615,656	250	612,126	99.43	134	548,908	8834	2002	274,454		
	C-neg-W	40	250	34	85.00	137	28	5	3	14	NA	NA
	C-neg-S	178	250	140	78.65	138	108	12	10	54		
	Total	4,707,196	NA	4,669,496	NA	NA	4,237,576	62,460	NA	2,118,788		
V4	WC	1,068,608	250	1,067,386	99.89	231	714,460	26,643	2388	357,230	6679	1,865,868
	SC	885,248	250	884,204	99.88	231	698,978	16,031	614	349,489		
	WR	1,779,298	250	1,778,564	99.96	231	1,469,394	34,231	2363	734,697		
	SR	968,022	250	966,652	99.86	231	754,382	28,880	3162	377,191		
	WV	961,382	250	960,270	99.88	231	685,738	29,211	2090	342,869		
	SV	865,048	250	864,228	99.91	231	621,956	25,644	1945	310,978		
	C-neg-W	68	250	56	82.35	237	36	4	3	18	NA	NA
	C-neg-S	254	250	244	96.06	247	118	9	8	59		
	Total	6,527,928	NA	6,521,604	NA	NA	4,945,062	160,653	NA	2,472,531		
ITS-2	WC	481,222	250	480,380	99.83	227.79	368,624	9877	630	40,124	4885	40,124
	SC	618,584	250	616,790	99.85	229.08	496,138	8667	165	3325		NA
	WR	2,153,728	250	2,147,658	99.86	228.39	1,766,638	25,375	1915	169,673		
	SR	658,958	250	657,288	99.87	227.34	509,194	16,849	2462	123,820		40,124
	WV	502,398	250	501,206	99.88	229.27	330,594	7404	120	6173		
	SV	594,614	250	593,596	99.83	228.46	461,186	14,223	1401	156,731		NA
	C-neg-W	306	250	306	100	229.48	214	19	13	107	NA	NA
	C-neg-S	68	250	68	100	230.04	62	3	3	31		
	Total	5,009,878	NA	4,000,320	NA	NA	3,932,650	82,417	6709	499,846		

3.2. Microeukaryote Diversity

Rarefaction curves for the V9 and V4 regions reached a plateau in all samples, indicating that sequencing depth was sufficient to capture eukaryote diversity. The highest eukaryotic diversity was observed within the first 100,000 sequences for both V9 and V4 sequencing (Figure 2A). Differences in richness, Shannon and Pielou indices were detected between the V9 and V4 regions, with a higher number of ASVs generally observed in the V9 region, particularly at the Redondela sampling point. In terms of richness and evenness, the highest diversity indices were found in Redondela sediment samples, while the lowest diversity was detected in sediment of Cangas samples, where the V9 region showed higher diversity indices compared to the V4 region (Figure 2B). Additionally, rare taxa (i.e., those representing less than 1% of total reads) were better represented in the V9 region than in the V4 region.

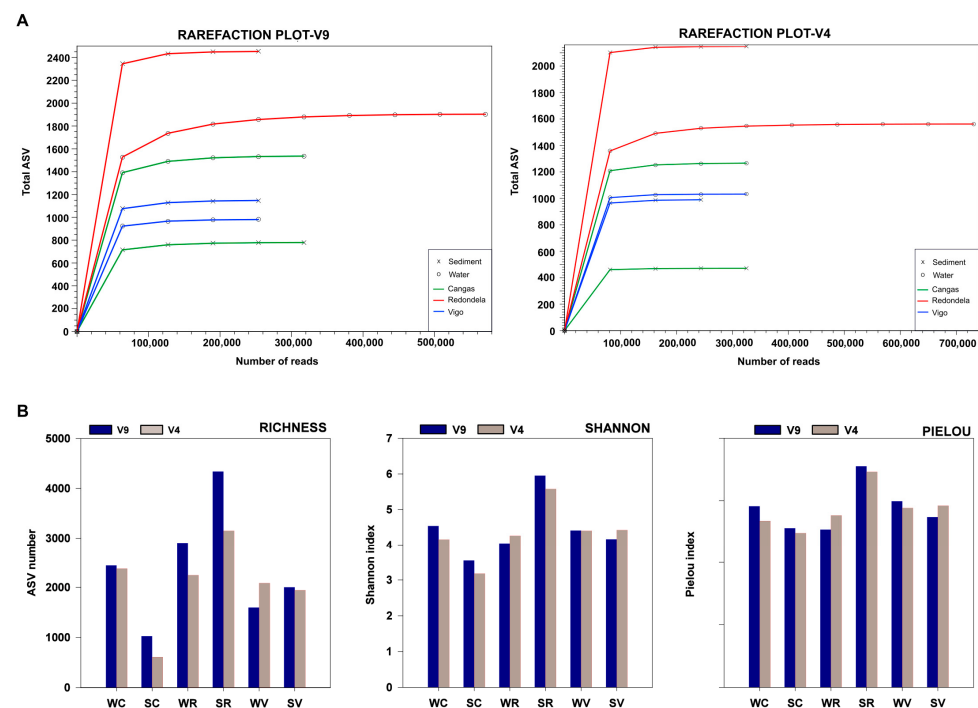


Figure 2. Eukaryotic diversity patterns based on multi-marker metabarcoding using V9 and V4 regions. (A) Rarefaction plots for each sample sequenced with the V9 and V4 regions, obtained for each environmental compartment across the sampling locations: Cangas, Redondela, and Vigo. (B) Alpha diversity indices (richness, Shannon, and Pielou) derived from eukaryotic sequencing of the V9 and V4 regions.

For the ITS-2 region, rarefaction curves revealed that samples from the sediment of Cangas and the water of Vigo revealed a low number of total reads. Including these samples in downstream analysis would have required rarefying the entire dataset to an impractically low threshold, leading to a substantial loss of information from other samples. Their low read counts may result from either technical limitation during sequencing or genuinely low microbial biomass in these environments. To ensure robust and comparable diversity analyses, we excluded these samples. For the remaining samples, rarefaction curves for the ITS-2 region reached a plateau across all sequencing efforts, confirming that sequencing depth was adequate to capture the full extent of fungal diversity. The initial 20,000 sequences yielded the highest fungal diversity (Figure 3A). Among the samples, sediment from Cangas (165 ASVs with 3325 total reads) and water from Vigo (120 ASVs with 6123 total reads) exhibited the lowest diversity, explaining their minimal representation in the rarefaction plot (Table 1, Figure 3A).

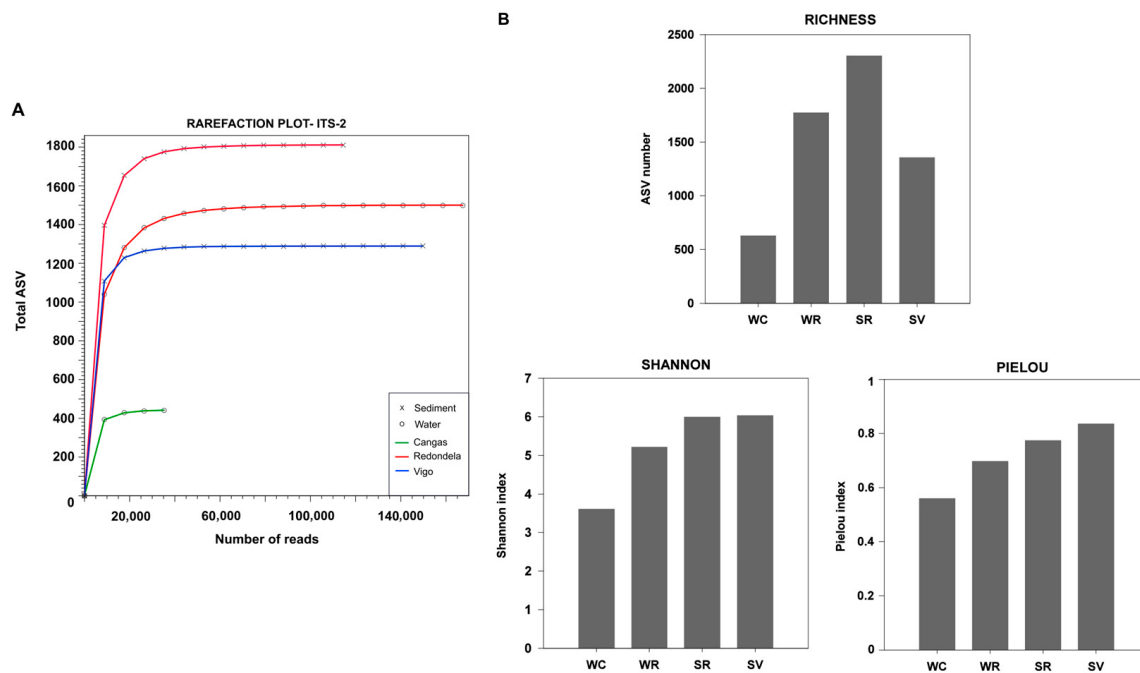


Figure 3. Fungal diversity overview obtained with the ITS-2 sequencing region in each sampling location and environmental compartment. (A) showing sequencing depth (total reads) and number of amplicon sequence variants (ASVs) detected. (B) alpha diversity indices (richness, Shannon and Pielou). Abbreviations: WC: water of Cangas, WR: water of Redondela, SR: sediment of Redondela, SV: sediment of Vigo.

Diversity indices indicate that sediment from Redondela consistently had the highest species richness, diversity, and evenness, followed closely by sediment from Vigo. Water from Redondela exhibited moderate values across all diversity metrics, while water from Cangas ranked the lowest, reflecting lower species richness, reduced biodiversity and a more uneven species distribution. These findings highlight significant differences in fungal community structure among sites, with sediment from Redondela and sediment from Vigo supporting the most diverse and balanced fungal communities (Figure 3B).

3.3. Exclusive/Common ASVs

Analysis of the V9, V4, and ITS-2 gene regions showed a strong location-specific pattern in microeukaryotic diversity, with the majority of ASVs being exclusive to each sampling region. Across all markers, specific ASVs accounted for 58% to 87% of the total detected per location.

For the V9 region, 79%, 78%, and 58% of ASVs were unique to Redondela, Vigo, and Cangas, respectively. In contrast, only 86 ASVs were shared among the three sites, representing a small fraction of the total: 5% in Cangas, 3% in Redondela, and 4.5% in Vigo (Figure 4A). A similar pattern was observed for the V4 region, with high levels of site specificity: 84% of ASVs in Redondela, 87% in Vigo, and 67% in Cangas. The number of shared ASVs dropped to 63, comprising just 3% in Redondela, 2% in Vigo, and 3% in Cangas (Figure 4B). For the ITS-2 region, 76% of ASVs in Redondela, 59% in Vigo, and 41% in Cangas were found to be exclusive. A total of 103 ASVs were shared across all sites, accounting for 3% in Redondela, 7.6% in Vigo, and 16% in Cangas (Figure 4C).

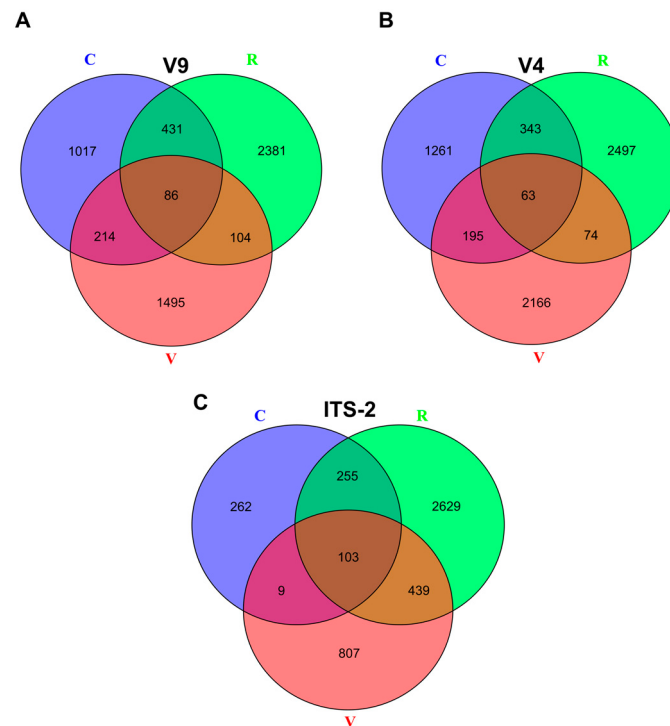


Figure 4. Venn diagrams illustrating the number of shared and unique ASVs among Cangas (C), Redondela (R), and Vigo (V), based on: (A) the V9 region of the 18S rRNA gene, (B) the V4 region of the 18S rRNA gene, (C) the nuclear marker internal transcribed spacer 2 (ITS2).

3.4. Putative Biohazards

Using the multi-marker approach, we identified genetic material from a wide range of genera and species associated with putative human and zoonotic pathogens, as well as those affecting marine organisms, terrestrial plants, and HA. As species-level resolution was not always achieved (highest identity $\geq 99\%$), we reported genera with pathogenic species. Since not all species of the same genera are pathogenic, pathogenicity should be interpreted with caution and would require further confirmatory analyses.

3.4.1. Exclusive/Common Putative Biohazards

Across the three genetic regions analyzed (V9, V4, and ITS-2), the distribution of putative biohazards revealed both shared and unique taxa, with clear patterns of exclusivity evident not only between sampling sites but also across environmental fractions (water and sediment).

For the V9 region, a substantial number of putative biohazards were consistently detected across all sites, representing 42% of the total taxa in Cangas, 32% in Redondela, and 50% in Vigo (Figure 5A).

However, a considerable proportion of these taxa were exclusive to individual locations: 30% were found only in Redondela, 25% only in Vigo and 11% only in Cangas. Notable differences were also observed between fractions (sediment vs. water). For instance, ten genera/species exclusively found in sediment of Vigo, whereas Redondela's sediment included 8 unique taxa (Figure 5A). Overall, only 3 putative biohazards were commonly detected in all samplings.

The V4 region displayed a similar pattern, although with slightly lower overall proportions. Putative biohazards were detected across all locations, comprising 20% of the total taxa in Cangas, 15% in Redondela, and 35.7% in Vigo. However, location-specific taxa were also observed: 40% were exclusive to Redondela, 43% to Vigo and 20% to Cangas.

Environmental fractions again showed distinct profiles, with 18 unique genera/species in sediment of Redondela and 16 in sediment of Vigo (Figure 5B).

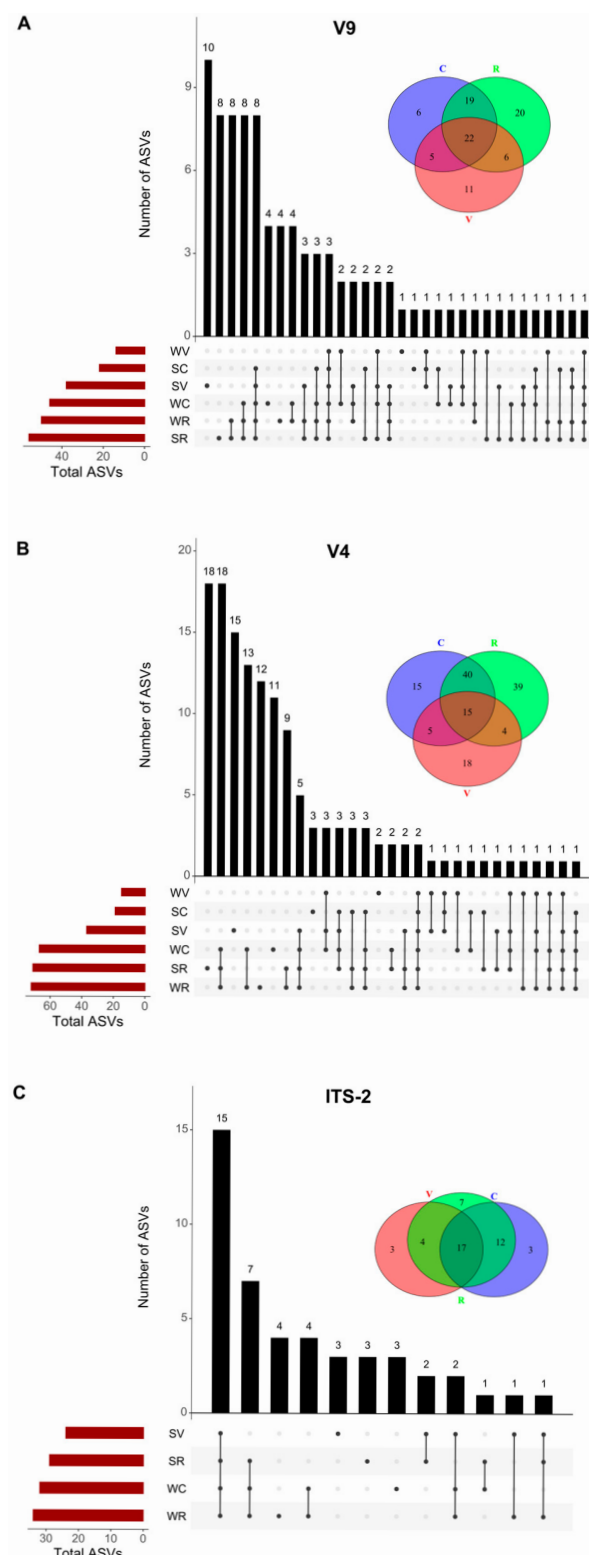


Figure 5. Venn diagrams illustrating the number of exclusive and shared putative biohazard ASVs among the three sampling regions: Cangas (C), Redondela (R), and Vigo (V). An accompanying Upset plot shows the number of exclusive and shared ASVs across both sampling locations and environmental compartments. Results are presented for: (A) V9 region, (B) V4 region, and (C) ITS-2 region. Abbreviations: WC: water from Cangas; SC: sediment from Cangas; WR: water from Redondela; SR: sediment from Redondela; WV: water from Vigo; SV: sediment from Vigo.

In contrast, the ITS-2 region exhibited a high level of overlap across locations, with most genera/species shared among them (Figure 5C).

3.4.2. Biohazards of Human/Zoonotic Origin

A total of 25 (V9), 31 (V4), and 26 (ITS-2) genera/species associated with human and/or zoonotic diseases were detected, with several taxa differentially identified across the three genetic markers. Figure 6A illustrates these differences, highlighting the distinct detection profiles of putative human/animal pathogens across ITS-2, V9, and V4. While some genera/species were consistently detected by all markers (e.g., *Rhodotorula* sp., *Cutaneotrichosporon* sp.), others were exclusive to one or two regions (e.g., *Penicillium* sp., *Cladosporium* sp.) (Figure 6A).

Using the V9 and V4 target regions, human and zoonotic putative pathogens represented only a small fraction of the total eukaryotic diversity, accounting for less than 0.2–2% of total reads across all analyzed samples (Figure 6B,C). In both regions, fungal pathogens were the most prevalent, followed by protists.

Analysis of the V9 region revealed significant local differences in the prevalence of human pathogens. In Redondela, putative human pathogens represented 0.67% of total reads in water samples and 2% in sediment samples. Comparatively, in Cangas, the proportions were 0.27% in water and 0.22% in sediment. Vigo exhibited the lowest prevalence, with 0.18% in water and 0.07% in sediment (Figure 6B). Redondela exhibited a diverse array of putative pathogen-associated genera in both sediment and water samples. Notable fungal genera included *Rhodotorula* (average 233 reads, 0.16% of total reads) and *Cutaneotrichosporon* (average 373 reads), which were detected in both matrices. Additionally, sediment samples were dominated by *Alternaria* (769 total reads), *Geotrichum* (285 reads), *Aspergillus* (232 reads), and *Penicillium* (143 reads), along with the putative pathogenic protist *Acanthamoeba* (183 reads). Several genera were exclusively identified in sediment from Redondela, including the fungi *Exophiala*, *Malassezia*, *Cryptococcus*, *Acremonium*, and *Sporothrix*, with less than 30 reads each (Figure 6B, Table S1). Other notable sediment-associated genera in Redondela included *Naegleria* (94 reads) and *Talaromyces* (80 reads), which are known to contain putative pathogenic species. Pathogen-associated taxa such as *Blastocystis* (54 reads) and Trypanosomatida (53 reads) were primarily identified in water samples. In Cangas, the most prevalent potential pathogens included the fungal genera *Rhodotorula* and *Cutaneotrichosporon* and the protist *Blastocystis*. Notably, *Blastocystis* was exclusively detected in sediment samples, accounting for 57 total reads. Vigo demonstrated the lowest diversity of human and zoonotic putative pathogen-associated genera. *Rhodotorula* was the predominant genus detected in water samples, with a total of 252 reads. Minor contributions from genera such as *Alternaria*, *Aspergillus*, *Penicillium*, and *Talaromyces* were also observed in water, each represented by 11–30 reads (Figure 6B, Table S1).

Using the V4 region, a lower pathogen abundance was observed compared to the V9 region. However, Redondela remained the area with the highest abundance of putative pathogens. The overall pathogen distribution pattern in this region is related with water sampling point instead of sediment observed with the V9 region. As with the V9 region, the V4 region revealed a higher abundance of putative pathogens in Redondela, followed by Cangas and Vigo, with slightly lower overall abundances compared to V9 (Figure 6C). In Redondela, *Rhodotorula* continued to be detected in both water (94 reads) and sediment (326 reads). Additionally, the genus *Cutaneotrichosporon jirovecii* was identified, in water (430 reads) and sediment (319 reads). Unlike the V9 region, the V4 region detected multiple species of the genus *Apiotrichum* in sediment, primarily *A. laibachii* (935 total reads) and *A. scarabaerum* (741 total reads), as well as the genus *Cryptosporidium* (80 total reads). However, the V4 region did not detect the genera *Alternaria*, *Penicillium*, or *Aspergillus* (Table S1).

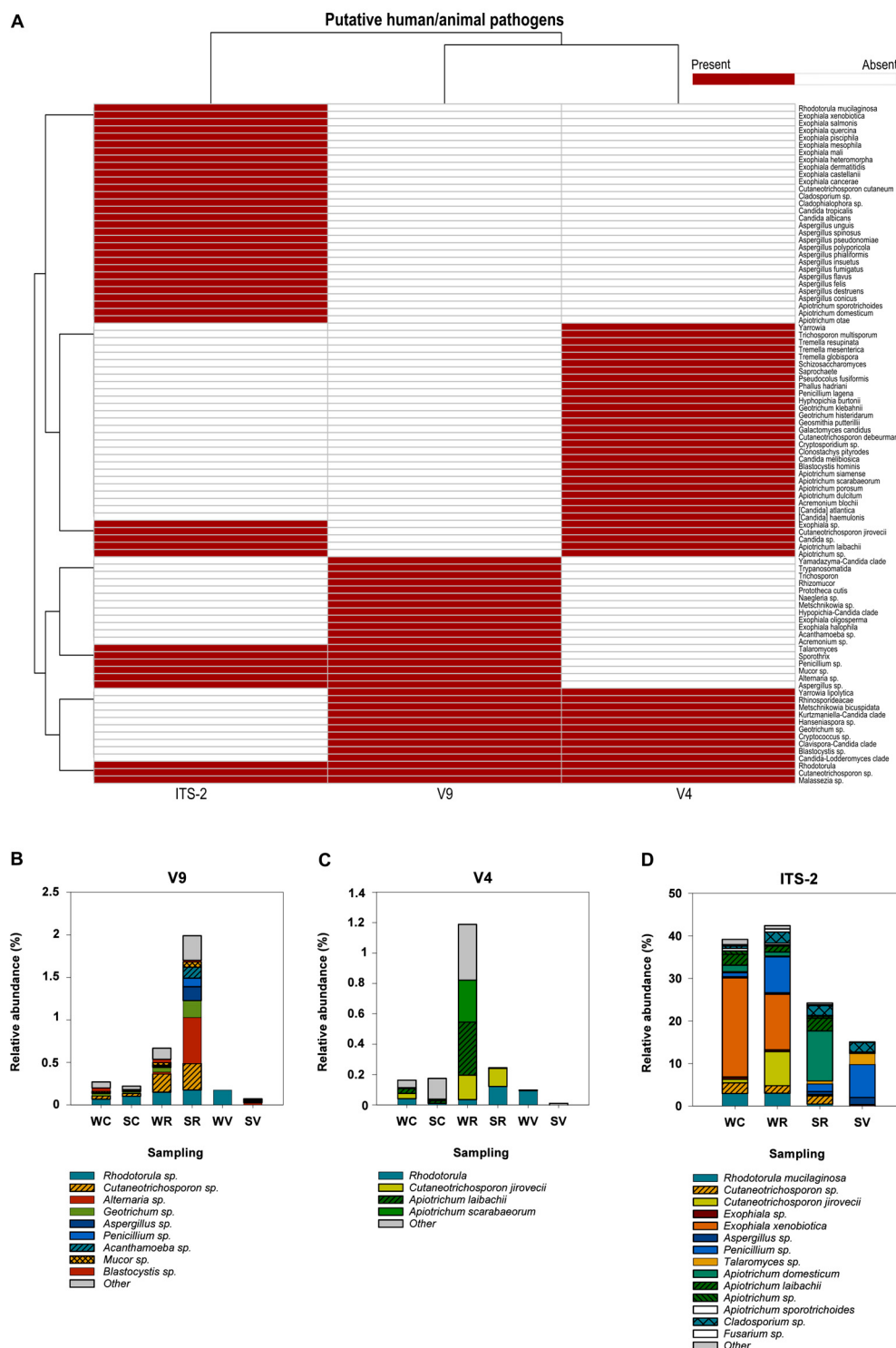


Figure 6. Detection and relative abundance of putative human and zoonotic pathogens across sampling sites and genetic markers. (A) Presence/absence heatmap of putative human and/or zoonotic pathogens detected across three genetic markers (ITS-2, V9, and V4). Relative abundance (%) of different potential human pathogens taxa across sampling sites based on (B) V9 region of 18S rRNA gene, (C) V4 region of 18S rRNA gene, and (D) Internal Transcribed Spacer 2. Taxa with a relative abundance below 0.01% are grouped under “Other putative pathogens”. Abbreviations: WC: water from Cangas; SC: sediment from Cangas; WR: water from Redondela; SR: sediment from Redondela; WV: water from Vigo; SV: sediment from Vigo.

The ITS-2 region identified several putative pathogenic fungi that were either undetected or present at low levels when using the universal eukaryotic V9 and V4 regions. Notably, *Exophiala xenobiotica*, along with the genera *Cladosporium* and *Penicillium*, were detected. *E. xenobiotica* accounted for 23% and 13% of total fungal reads in Cangas (water) and Redondela (water), respectively, and was exclusively found at these sampling points. The genus *Cladosporium* was detected in all samples, representing 0.5% to 2% of total fungal reads (Figure 6D). *Penicillium* sp. was another dominant genus, primarily associated with water from Redondela (WR) and sediment from Vigo (SV), comprising 8% of total fungal diversity at both sites. Different species of the genus *Apiotrichum* were also detected, with *A. domesticum* being the most dominant, accounting for 11% of total fungal diversity (4718 reads). Additionally, minor genera such as *Talaromyces* sp., *Candida* sp., *Aspergillus* sp., *Fusarium* sp., *Mucor* sp., *Malassezia* sp., and *Apiotrichum* sp. were detected across nearly all sampling points (Figure 6D).

3.4.3. Pathogens of Aquatic Organisms

DNA originating from putative pathogens typically associated with aquatic organisms was identified, revealing marked differences in detection efficiency among the three genetic markers. The V4 region identified the greatest number of taxa (39 genera/species), followed by V9 with (33), while in ITS-2 none was detected. Remarkably, 19 taxa were exclusively detected by V9, and 25 were unique to V4, highlighting the complementary nature of these two markers (Figure 7A). Although some pathogens were metazoans or fungi, the majority were protists, predominantly within the TSAR supergroup.

In line with patterns observed for zoonotic and human-associated taxa, the greatest presence of putative pathogen DNA was recorded in the Redondela samples, followed by those from Cangas and Vigo. Using the V9 marker, the relative abundance of these organisms remained below 2% in both Cangas and Vigo. Conversely, in Redondela, putative pathogens comprised 35% of water sample reads (WR) and 10.4% in sediment (SR). With the V4 marker, relative abundance was below 5% in Redondela and Cangas, and under 2% in Vigo (Figure 7B). Among the most representative pathogenic taxonomic groups identified using both V9 and V4 regions were *Rhogostoma* sp. (Rhizaria), *Amoebophyra* sp. (Alveolata), *Scuticociliata* sp. (Alveolata), *Monocystis* sp. (Alveolata); *Anurofeca* sp. (Opisthokonta), *Aplanochytrium* sp. (Stramenopila) and *Chilodonella uncinata* (Alveolata) (Figure 7A, Table 2). *Rhogostoma* sp. was predominantly detected in both water and sediment samples from Redondela, where it accounted for 35% (48,675 reads) of total eukaryotic reads in water using V9, compared to only 3% (6589 reads) with V4, which enabled species-level identification as *Rhogostoma minus*. The Scuticociliata (Philasterida) taxonomic group, was detected in water from Cangas (WC), representing 0.2% (V9) and 0.3% (V4) of total eukaryote reads. Other minority pathogens of the Philasterida order were detected: *Miamiensis avidus*, *Pseudocohnilembus hargisi* and *Uronema* sp., some of which were exclusively detected with V9, while other appeared only with V4 (Figure 7A, Table S2). The amphibian larval pathogen of the genus *Anurofeca* was also detected in Cangas and Redondela, predominantly in sediment from Redondela (SR), where it represented 7.5% (10,685 total reads) and 3.7% (10,005 total reads) of the total eukaryotic reads for V9 and V4, respectively (Table 2).

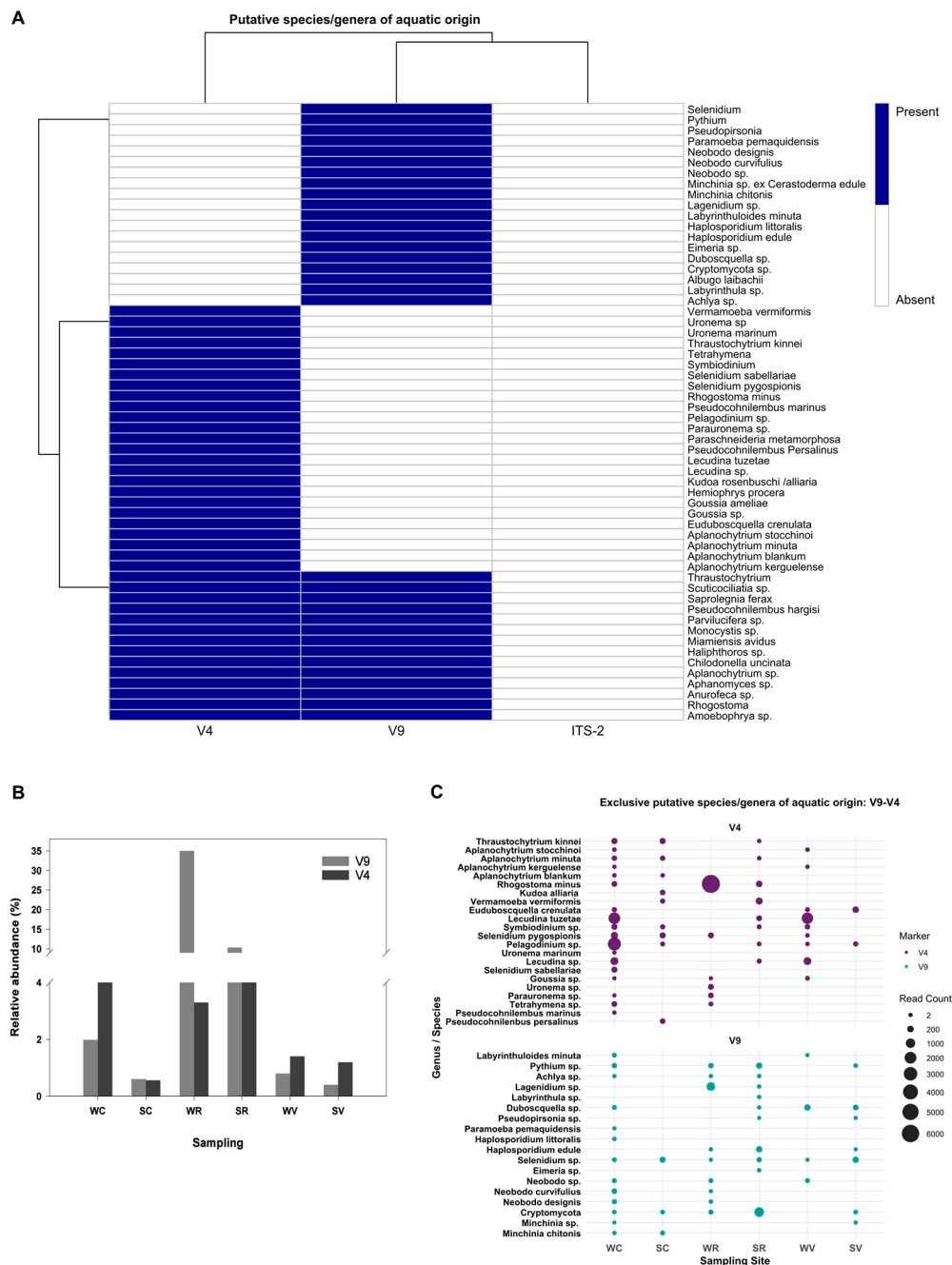


Figure 7. Detection and marker-specific distribution of putative aquatic pathogens across sampling sites. **(A)** Heatmap showing the presence or absence of putative pathogens affecting aquatic organisms, detected using three genetic markers: V9, V4, and ITS-2. **(B)** Relative abundance (%) of these putative pathogens at each sampling point. **(C)** Dot plot representing the number of sequencing reads corresponding to putative pathogens exclusively detected using the V9 and V4 regions of the 18S rRNA gene. Abbreviations: WC: water from Cangas; SC: sediment from Cangas; WR: water from Redondela; SR: sediment from Redondela; WV: water from Vigo; SV: sediment from Vigo.

Table 2. Representative Putative Aquatic-Origin Pathogens Detected by V9 and V4 Regions of the 18S rRNA gene. Values include the number of reads and their corresponding relative abundance (%) calculated from total eukaryotic reads per sampling site. Taxonomic classifications are shown by group, phylum/order, and genus/species. Known hosts and relevant references are provided for each taxon. WC: water from Cangas; SC: sediment from Cangas; WR: water from Redondela; SR: sediment from Redondela; WV: water from Vigo; SV: sediment from Vigo.

Group	Phylum/ Order	Genus/ Specie	V9 Number of Reads (%)						V4 Number of Reads (%)						Host	References
			WC	SC	WR	SR	WV	SV	WC	SC	WR	SR	WV	SV		
Alveolata	Gregarinasina	<i>Monocystis</i> sp.	NA	350 (0.24)	67 (0.05)	1369 (0.96)	NA	NA	1880 (0.70)	NA	636 (0.24)	68 (0.03)	1176 (0.44)	NA	Earthworm	[31]
	Chlamydodontida	<i>Chilodonella uncinata</i>	54 (0.04)	NA	147 (0.10)	NA	NA	NA	155 (0.05)	NA	66 (0.02)	NA	NA	NA	Farmed freshwater fishes	[32]
	Philasterida	<i>Miamiensis avidus</i>	16 (0.01)	NA	NA	NA	NA	NA	7 (0.002)	NA	NA	NA	NA	NA	Farmed fishes	[33–35]
		<i>Pseudocohnilembus hargisi</i>	NA	NA	266 (0.18)	12 (0.01)	NA	NA	NA	NA	422 (0.16)	6 (0.002)	NA	NA		
		<i>Pseudocohnilembus persalinus</i>	NA	NA	NA	NA	NA	NA	NA	79 (0.03)	NA	NA	NA	NA		
		<i>Scuticociliatia</i> sp.	476 (0.33)	NA	45 (0.03)	NA	NA	NA	613 (0.23)	NA	49 (0.02)	NA	NA	NA		
Opisthokonta	Syndiniales	<i>Amoebophrya</i> sp.	37 (0.03)	NA	NA	NA	692 (0.48)	12 (0.01)	2454 (0.91)	59 (0.02)	NA	NA	NA	2330 (0.87)	Dinophyceae	[36]
	Fungi	<i>Cryptomycota</i> sp.	12 (0.01)	16 (0.01)	33 (0.0232)	1053 (0.73)	NA	18 (0.01)	NA	NA	NA	NA	NA	NA	Phytoplankton	[37]
	Ichthyophonida	<i>Anurofeca</i> sp.	897 (0.63)	367 (0.26)	202 (0.14)	10,685 (7.5)	NA	20 (0.01)	97 (0.04)	570 (0.21)	265 (0.1)	10,005 (3.73)	NA	78 (0.03)	Anuran larvae	[38]
Rhizaria	Haplosporida	<i>Haplosporidium edule</i>	NA	NA	5 (0.003)	172 (0.12)	NA	2 (0.01)	NA	NA	NA	NA	NA	NA	Bivalves (<i>C. edule</i>)	[39]
	Thecofilosea	<i>Rhogostoma</i> sp.	900 (0.63)	8 (0.006)	48,675 (34.18)	1319 (0.93)	NA	NA	39 (0.01)	NA	444 (0.16)	33 (0.01)	NA	NA	Fish. NGD	[40]
		<i>Rhogostoma minus</i>	NA	NA	NA	NA	NA	NA	99 (0.037)	NA	6589 (2.46)	200 (0.07)	NA	NA		
Stramenopiles	Labirinthulomycetes	<i>Aplanochytrium</i> sp.	31 (0.02)	4 (0.003)	16 (0.01)	58 (0.04)	273 (0.19)	171 (0.12)	918 (0.340)	49 (0.02)	36 (0.01)	17 (0.01)	72 (0.02)	543 (0.2)	Seastar	[41]
	Lagenidiales	<i>Lagenidium</i> sp.	NA	NA	676 (0.47)	6 (0.004)	NA	NA	NA	NA	NA	NA	NA	NA	Mammals	[42]
	Oomycetes	<i>Aphanomyces</i> sp.	NA	NA	6 (0.004)	6 (0.004)	NA	NA	234 (0.09)	124 (0.05)	NA	78 (0.03)	32 (0.01)	NA	Crustaceans	[43]

Selecting of genetic marker had a substantial impact on the detection of putative pathogens across environmental compartments and study areas. Clear discrepancies were observed between the V9 and V4 regions, highlighting the limitations of relying on a single molecular region for assessing microeukaryotic microbial diversity. For instance, *Monocystis* sp. was not detected in either sediment or water from Vigo when using the V9 region, while it was detected in water from the same location (WV: 1176 reads) using the V4 region. Conversely, in sediment from Cangas (SC), this genus was detected with V9 but not with V4 (Table 2). *Aphanomyces* sp. further illustrated this contrast, since it was detected in both water and sediment from Cangas and Vigo with the V4 region, but was not detected with V9 (Table 2). Similarly, *Amoebophyra* sp. showed high read counts in water from Cangas (2454 total reads) and sediment from Vigo (2330 reads) with V4, whereas with V9, it was detected only in water from Vigo (692 reads) (Table 2).

A number of putative aquatic pathogenic taxa were detected exclusively by either the V9 (19 ASV) or V4 (25 ASV) genetic marker, each exhibiting distinct taxonomic profiles and spatial patterns (Figure 7C). Using the V9 region, several taxa were prominent in terms of detection and site-specificity. *Cryptomycota* and *Selenidium* was among the few taxa consistently detected across multiple sites and compartments, indicating a broad environmental distribution. In contrast, some taxa displayed more restricted patterns, such as *Eimeria* sp. exclusive in sediment, and *Lagenidium* sp. mainly detected in water, both from Redondela. Other exclusively taxa detected with V9 included the mollusc pathogens *Minchinia* spp. and *Haplosporidium* spp., *Achlya* (Stramenopiles), *Pythium* (Stramenopiles), *Neobodo* spp. (Excavata) and *Duboscquella* sp. (Alveolata). Similarly, certain genera were detected exclusively with the V4 region, such as *Rhogostoma minus* (Rhizaria), *Lecudina* spp. (Apicomplexa), and *Pelagodinium* sp. (Alveolata), strongly represented in water samples from Redondela (WR), Cangas (WC) and Vigo (WV). The V4 region also enabled species-level resolution for several taxa only classified to genus level with V9, including *Aplanochytrium minuta*, *Thraustochytrium kinnei*, *Selenidium pygospionis*, and *Rhogostoma minus* (Figure 7C, Table 2). Other exclusive taxa detected with V4 included: *Vermamoeba vermiformis* and *Kudoa alliarina*.

3.4.4. Harmful Algae (HA)

HA, as listed in the IOC-UNESCO *Taxonomic Reference List of Harmful Micro Algae*, were detected using both the V9 (14 genera) and V4 (18 genera) primer sets. Notably, 7 taxa were exclusively detected by V9, and 11 were unique to V4, highlighting the complementary nature of these two markers (Figure 8A).

Overall, in both regions, sediment samples from Vigo showed the highest diversity (number of genera and species) and relative abundance of HA. In this location, harmful taxa collectively accounted for 28% and 18% of total eukaryotic reads, based on the V9 and V4 regions, respectively. In the remaining samplings, HA represented only approximately 1% of total eukaryote reads, and in Redondela, they were scarcely detected. Several species of the genus *Alexandrium* (Dinophyceae) were identified with both the V9 and V4 regions, with *Alexandrium minutum* being the most dominant HA in the sediment of Vigo. This species accounted for 23% (33,232 reads) of total eukaryotic reads with the V9 region (Figure 8B) and 13.28% with the V4 region (Figure 8C). Other species, such as *A. margaleffii* and *A. tamarensense*, were exclusively detected using the V4 region; however, they represented less than 0.5% of total eukaryote reads (Figure 8A,C; Table S3). *Gonyaulax spinifera* (Dinophyceae) and *Gonyaulax* sp. were the second most abundant HA in the sediment of Vigo. *G. spinifera* accounted for approximately 2% of total eukaryotic reads, with 3207 reads using the V9 region and 7730 reads using the V4 region (Figure 8B,C). Several *Pseudo-nitzschia* spp. (Bacillariaceae) were detected with both target genes, though notable differences were

observed depending on the amplified region. Using the V9 region, the highest number of total reads of this genus was found in water from Cangas (1788 reads), followed by the water from Vigo (901 reads), although species-level classification was not achieved (Figure 8B). In contrast, the V4 region enabled species-level identification, revealing *P. australis* (266 total reads) and *P. fraudulenta* (428 total reads), both associated with water from Vigo (Figure 8C).

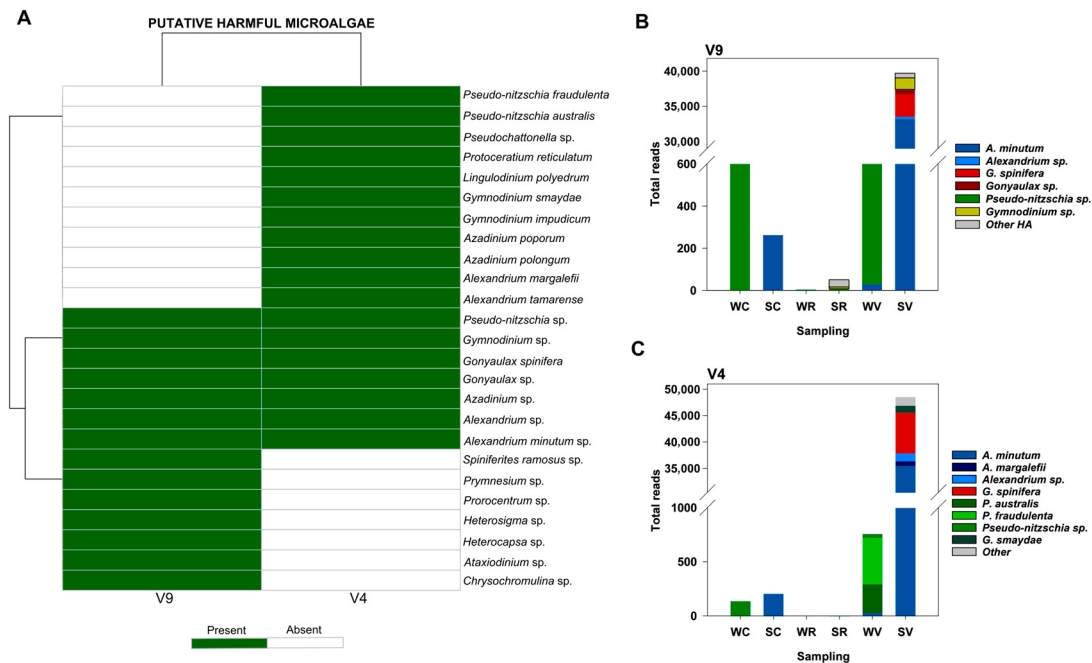


Figure 8. (A) Heatmap showing the presence or absence of HA, detected using V9 and V4 markers. Read counts and distribution of HA taxa across sampling sites based on two genetic markers: (B) V9 and (C) V4 region of the 18S rRNA gene. Abbreviations: WC: water from Cangas; SC: sediment from Cangas; WR: water from Redondela; SR: sediment from Redondela; WV: water from Vigo; SV: sediment from Vigo.

Discrepancies between amplicon sequencing results were also observed for *Gymnodinium* spp. and *Azadinium* spp., which were exclusively detected in sediment from Vigo using both regions Figure 8A. However, while the V4 region enabled the identification of two *Gymnodinium* species (*G. impudicum*, 380 reads; *G. smaydae*, 1286 reads) and two *Azadinium* species (*A. poporum*, 84 reads; *A. polongum*, 85 reads), the V9 region only allowed classification at the genus level (1644 reads) (Figure 8, Table S3). Other less abundant HA species were detected exclusively with the V9 region, including *Heterosigma* sp., *Heterocapsa* sp., and *Chrysochromulina* sp., while *Lingulodinium* sp. and *Protoceratium reticulatum* were identified with both target regions. Most of these genera were also dominant in the sediment of Vigo (Figure 8A, Table S3).

4. Discussion

4.1. Microeukaryotic Diversity

The analysis of microeukaryotic diversity provided essential baseline information on microbial communities in embayment impacted by wastewater effluents in the Ría de Vigo. The three sampling sites exhibited strikingly different environmental and ecological conditions, offering a valuable framework to examine how local factors modulate microbial eukaryotic community structure at the landscape scale.

Given the lack of consensus on optimal amplification targets and their associated taxonomic biases, we adopted a multi-marker approach (V9, V4, ITS2) for broad eukary-

otic detection. This strategy captured complementary taxonomic profiles and revealed how marker choice influences biodiversity patterns, with V4 yielding more reads and V9 capturing more taxa [44,45]. Such discrepancies largely reflect primer bias and amplification efficiency rather than true biological absence or abundance, which is why the use of multiple markers was essential to obtain a more balanced and comprehensive picture of community diversity. Low fungal detection with ITS-2 suggest either true scarcity or technical limits, highlighting the need for greater sampling or sequencing depth.

Despite differences in sequencing outputs, all markers consistently identified the same sites as having the highest and lowest diversity, confirming the robustness of the spatial patterns detected. Redondela showed the highest richness and community uniqueness, linked to its intertidal estuarine setting with low hydrodynamics, high riverine inputs and muddy, organic-rich sediments [46]. Conversely, Cangas, with coarse, gravel-dominated sediments, exhibited the lowest diversity with all markers. These patterns were consistent with in situ measurements of temperature and salinity, which also revealed marked contrasts among sampling sites, tentatively suggesting that local physicochemical conditions may contribute to shaping community composition.

High proportions of site-exclusive ASVs (58–87%) across all sites, mainly in Redondela, indicate strong environmental filtering, with sediment type, nutrient availability, and hydrodynamic conditions shaping distinct communities despite their proximity.

This pattern shows that environmental filtering, shapes both taxonomic richness and spatial differentiation, and that fine-scale habitat heterogeneity within a single estuary can strongly structure microbial eukaryotic communities, underscoring the need to integrate microscale habitat features into biodiversity assessments.

4.2. Putative Pathogen Diversity

A diverse array of putative pathogenic taxa was recorded across sites and habitats in the Ría de Vigo, including eDNA signatures relevant to human, animal, and environmental health. It is important to note that DNA detection alone does not confirm organism viability or infectivity; therefore, all statements regarding potential human, animal, or aquaculture risk should be considered tentative. Nonetheless, these findings provide valuable baseline information to guide further investigations of these environments. Although less spatially exclusive than the overall microeukaryotic community, likely due to their lower relative abundance, distinct location-specific patterns emerged, with up to 45% of pathogenic taxa unique to a single site, indicating that local environmental conditions influence their distribution.

Using multiple markers, we detected 18 (V9) to 27 (ITS-2) fungal taxa, 3–5 protistan taxa, and one Ichthyosporean pathogen with zoonotic potential, some listed under the European Directive 2000/54/EC. Clinically relevant fungi, including the genera *Rhodotorula*, *Cutaneotrichosporon* and *Exophiala* are known to cause opportunistic infections [47–49], while toxin and allergen producing genera such as *Aspergillus*, *Penicillium*, *Sporothrix* (V9, ITS-2) *Alternaria*, *Talaromyces*, and *Cladosporium* (ITS-2) were also found, several of them regulated under the European Directive 2000/54/EC [28]. Notable detections also included *Apiotrichum domesticum* (ITS-2, V4), associated with human summer-type hypersensitivity pneumonitis (SHP) [50]. Protozoan pathogens regulated under the European Directive 2000/54/EC were also identified. *Acanthamoeba* sp. (V9) can cause granulomatous amoebic encephalitis and keratitis [51], while *Cryptosporidium* sp. (V4) is associated with zoonotic cryptosporidiosis and waterborne outbreaks [52]. *Blastocystis* sp. (V4 and V9) is common in vertebrates including humans, with debated pathogenicity but growing evidence of waterborne transmission [53].

Detection patterns of zoonotic pathogens varied between markers, with some taxa appearing only in one or two targets. Certain markers also resolved multiple species within a genus, such as *Rhodotorula mucilaginosa* and *Cutaneotrichosporon cutaneum* with (V4 and ITS-2). This highlights the value of multi-marker approaches to improve coverage in pathogen detection. It is therefore likely that taxa detected exclusively by one marker reflect primer-specific bias rather than true absence, which reinforces the need for caution in interpreting marker-specific results. However, most findings were at genus level, and pathogenicity cannot be assumed for all members of a genus. Species-level confirmation with complementary techniques (e.g., targeted PCR, culturing, or sequencing of additional loci) is essential to validate pathogenic potential and avoid overestimation of health risks.

Pathogens of major concern for the aquaculture sector were detected, revealing the susceptibility of the Ría de Vigo to diseases affecting both bivalves and fish. The detection of *Haplosporidium edule* (V9) [39], exclusively in inner estuarine sediments (Redondela), highlights the potential for sediment to act as an environmental reservoir for benthic pathogens. This protozoan infects the cockle *Cerastoderma edule*, and its occurrence in muddy, low-hydrodynamic habitats suggests favourable conditions for maintaining infective stages near suitable hosts. Regarding fish pathogens, several taxa within the order Philasterida (*Pseudochonilembus* spp., *Uronema* sp., *Uronemella* sp., and *Miamiensis avidus*), all associated with scuticociliatosis, were recorded using both V9 and V4 regions [33,34], with *Pseudochonilembus hargisi* being unexpectedly prevalent. Although no outbreaks involving this species have been documented in Galicia, its repeated detection in metabarcoding surveys in Ría de Vigo [54] and its documented pathogenicity in Asian and American Aquaculture [34,35] suggest that its presence merits close monitoring. Pathogens associated with Nodular Gill Disease (NGD), including *Vermamoeba vermiformis* and *Rhogostoma* sp., were detected [40], with marker-specific differences (*Rhogostoma* sp., V9; *Rhogostoma minus*, V4) contributing to discrepancies in Rhizaria detection. *Rhogostoma* sp., common in WWTPs, and capable of harbouring *Legionellales* bacteria [55], and *Vermamoeba vermiformis*, a known host of pathogenic bacteria and viruses [56] were found exclusively in Cangas and Redondela, suggesting anthropogenic contamination. Other fish pathogens were detected at low abundance, including *Chilodonella uncinata*, which causes significant losses in freshwater fish farming [32], *Goussia* sp., a coccidian previously found in Galician waters, and *Kudoa alliiaria*, a myxosporean parasite that infects the musculature of commercial fish.

Several marine pathogens with important ecological roles were identified, indicating that sediment type, hydrodynamics, and host availability shape pathogen persistence in the Ría de Vigo. The anuran larvae pathogen *Anurofecia* sp. [38], was highly abundant (7% relative abundance) in muddy low-energy sediments of Redondela, while gregarines (*Selenidium* spp., *Lecudina tuzetae*) and Cryptomycota (*Rozella* sp.) in Cangas and Redondela pointed to sedimentary “seed banks” of invertebrate parasites [57,58]. The detection of the genus *Aphanomyces* spp. in both sediment and water from Cangas highlight the potential for harmful oomycetes to persist across the habitats. This genus includes species of high ecological and economic impact, such as *A. astaci*, a notifiable pathogen under OIE regulation and causative agent of crayfish plague [43]. Although species-level resolution was not achieved in this study, our findings underscore the need for targeted, high-resolution monitoring at species-level in future analyses.

Harmful Algae Blooms are a recurring issue in the Galician Rías, impacting aquaculture, fisheries, and public health [14]. Our metabarcoding survey detected 14 harmful algal taxa listed in the IOC-UNESCO Taxonomic Reference List of Harmful Microalgae [30], predominantly in sediment samples from Vigo. Saxitoxin-producing *Alexandrium* spp. (*A. minutum*, *A. margalefii*, and *A. tamarense*), along with *Gymnodinium* spp. were especially relevant. Those HA are responsible for paralytic shellfish poisoning (PSP) in humans following

the consumption of contaminated shellfish. Notably, *A. minutum* representing up to 20% of the total eukaryotic community. This high abundance likely reflects the accumulation of resistant cysts in muddy, aphotic sediments following the upwelling-driven bloom season. Such cyst beds are known to act as inoculant for future blooms, consistent with past events in Ría de Vigo [59] and the major bloom reported in the neighbouring Ría de Pontevedra in April 2023 [60] three months prior to sediment sampling. Other detected taxa included *Pseudo-nitzschia* spp., domoic acid producers, and *Azadinium* spp. responsible for amnesic shellfish poisoning (ASP) and azaspiracid shellfish poisoning (AZP), respectively [14,61]. While these HA taxa are not directly discharged from wastewater outfalls, nutrient enrichment from effluents may enhance bloom potential by fueling phytoplankton growth under favourable hydrodynamic conditions. Together, these findings reinforce the role of sediments as reservoirs of toxic microalgae and highlight the value of metabarcoding for early detection in integrated HAB monitoring strategies.

As a whole, this study provides valuable insights into the eukaryotic pathogen composition across three distinct outfall zones in the Ría de Vigo, encompassing outer, middle and inner sectors, and reflecting varying levels of urban pressure from small (Cangas, Redondela) to large (Vigo) municipalities. Surprisingly, Vigo recorded the lowest eukaryotic diversity and pathogens, despite the effluents from the WWTP of Vigo are responsible for 86% of the sewage dumping of a total of 12 WWTPs operating in the Ría. This finding agrees with that obtained from much less urbanized outer area in the Ría (Baiona) in which an efficient removal of prokaryotic putative pathogens from WWTP was confirmed in wastewater effluents and in mussels, acting as bioindicators of marine environment [17]. Conversely the inner estuarine zone of Redondela exhibited the highest overall eukaryotic diversity and the greatest abundance of putative pathogenic taxa, particularly in sediment samples. The spatial heterogeneity observed in pathogen distribution likely reflects difference in hydrodynamics and global circulation of water bodies and the continuous subsurface currents associated with river mouths, which affect the ecological processes and sediment typology in the Ría. Additionally, unlike other typical shallow estuaries, seasonal wind-driven coastal upwelling events promote short water renewal (3–4 days) in the Ría [62], mostly in outer-middle zones, therefore the suspended eukaryotic communities in the water column at those areas may be intermittently washed out or diluted. At this point, the strongest contribution to the eukaryotic putative pathogen community in the Ría was recorded in areas controlled by the land–sea interaction, in sediments of the inner estuarine area with a mixed impact of anthropogenic nitrogen-fertilization and biohazard inputs from wastewater and river mouths. This finding emphasizes the need to incorporate sediment analysis and integrate landscape characteristics into metabarcoding studies to achieve a more comprehensive assessment of environmental coastal health. In addition, this study was conceived as a diagnostic snapshot in a single summer sampling event, which provides a timely assessment of local community structure and putative pathogen diversity. However, this design does not capture potential seasonal or interannual variability, and future studies with temporal replication will be needed to validate and expand the spatial patterns observed.

This study demonstrates that a multi-marker eDNA metabarcoding approach is a powerful, non-invasive tool for earlier detection and simultaneously diagnosis of environmental pathogens in complex coastal socio-ecological systems. By improving taxonomic resolution, and capturing both abundant and rare taxa, this method expands the scope of marine pathogen diagnosis, despite inherent limitations such as genus-level resolution, PCR amplification, incomplete databases and the inability to distinguish viable from non-viable organisms. Given the incomplete depuration of wastewater withdrawal by the EU over the last years, both in large and small treatment plants, wastewater discharges were expected to

be major sources of eukaryotic pathogens. Contrary to expectations, wastewater discharges accounted for only a small fraction of detected genera, with pathogen communities instead shaped by local biogeochemical conditions, particularly in inner estuarine zones where fluvial inputs and organic-rich sediments promoted up to tenfold higher pathogen eDNA concentrations. These findings highlight the need to integrate sediment analyses, landscape features, and temporal replication into *One Health*-oriented monitoring strategies to better identify contamination hotspots and manage health risks in dynamic coastal environments.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/d17100671/s1>, Table S1: Relative abundance of total reads corresponding to human and/or zoonotic pathogens detected using the 18S rRNA gene (V9, V4) and ITS-2 markers across sampling sites. Sampling types included water and sediment from Cangas, Redondela, and Vigo. Total reads: V9–142,406 reads; V4–267,592 reads; ITS-2–40,124 reads.; Table S2: Number of reads and relative abundance (%) of minor potential aquatic pathogens (defined as those with relative abundance < 0.1%) detected at each sampling point using the 18S rRNA gene (V9 and V4 regions). Sampling codes: WC: water from Cangas; SC: sediment from Cangas; WR: water from Redondela; SR: sediment from Redondela; WV: water from Vigo; SV: sediment from Vigo; NA: no data available. Total read counts per marker: V9 = 142,406; V4 = 267,592. Table S3: Number of reads and relative abundance (%) of HA across different sampling sites. Sampling codes: WC: water from Cangas; SC: sediment from Cangas; WR: water from Redondela; SR: sediment from Redondela; WV: water from Vigo; SV: sediment from Vigo; NA: no data available. Total, read counts per marker: V9 = 142,406; V4 = 267,592.

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Data Availability Statement: The resulting raw sequence reads were deposited in the Sequence Read Archive (SRA) <http://www.ncbi.nlm.nih.gov/sra> (accessed on 15 May 2025) under BioProject accession number PRJNA1262121.

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