

## Article

# Oyster Reefs Are Reservoirs for Potential Pathogens in a Highly Disturbed Subtropical Estuary

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**Abstract:** Estuaries worldwide are grappling with deteriorating water quality and benthic conditions that coincide with the rising detection of pathogenic and potentially pathogenic microbes (PPM). Both indigenous PPM and those that enter estuaries through urban and agricultural runoff are funneled through suspension-feeding organisms and deposited onto the benthos, where they can be moved through food webs. This study explored PPM communities in the Indian River Lagoon system, a biodiverse but urbanized estuary in east central Florida (USA). PPM were surveyed in estuary water, at stormwater outfalls, and in biodeposits of a key suspension feeder, the eastern oyster *Crassostrea virginica*. A total of 52 microbial exact sequence variants, with per-sample relative abundances up to 61.4%, were identified as PPM. The biodeposits contained relatively more abundant and diverse PPM than the water samples. PPM community composition also differed between seasons and between biodeposits and water. The community differences were driven primarily by *Vibrio* and *Pseudoalteromonas* spp. This investigation provides evidence that, through biodeposition, oyster reefs in the IRL estuary are a reservoir for PPM, and it documents some taxa of concern that should be conclusively identified and investigated for their pathogenicity and potential to pervade food webs and fisheries.

**Keywords:** pathogens; microbes; oysters; *Crassostrea virginica*; biodeposits; Indian River Lagoon; Florida



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## 1. Introduction

Estuarine water quality and benthic conditions are deteriorating worldwide because of urbanization and climate change [1,2]. Of particular concern are increases in the detection of pathogens in estuary waters and sediments that threaten human and ecosystem health [3–7]. These increases are likely due, in part, to improved detection methods. But they also appear to be fueled by a combination of factors like wastewater seepage and stormwater runoff, which introduce new pathogens, along with eutrophication and warming, which stimulate the growth of both indigenous and introduced taxa [8,9]. However, the precise mechanisms by which virulent microbes enter, persist, and proliferate in estuarine systems are complex and still poorly understood [10,11].

The Indian River Lagoon (IRL) is a barrier island estuary system that spans approximately one-third (251 km) of Florida's east coast (USA). With a database of >9000 species of animals, plants, and protists at the time of writing this paper [12], the IRL watershed is a biodiversity hotspot, and it was designated as one of the 28 estuaries of national significance by the U.S. Environmental Protection Agency [13]. Over the past several decades, however, increasing eutrophication from population growth and climate change has reduced IRL water quality and led to harmful algal blooms, hypoxic events, habitat degradation, and biodiversity losses [14–18]. These disturbances likely induced changes to the estuary's microbiome, including the emergence and growth of pathogenic taxa. Studies on IRL microbial communities are limited, but they have detected pathogenic and potentially pathogenic microbes (PPM) that vary spatially and temporally [19–22]. This underscores the need for further investigations of PPM in estuaries, focusing on potential hot spots formed by pollution point sources (e.g., stormwater outfalls) and microbial aggregators like suspension-feeding organisms.

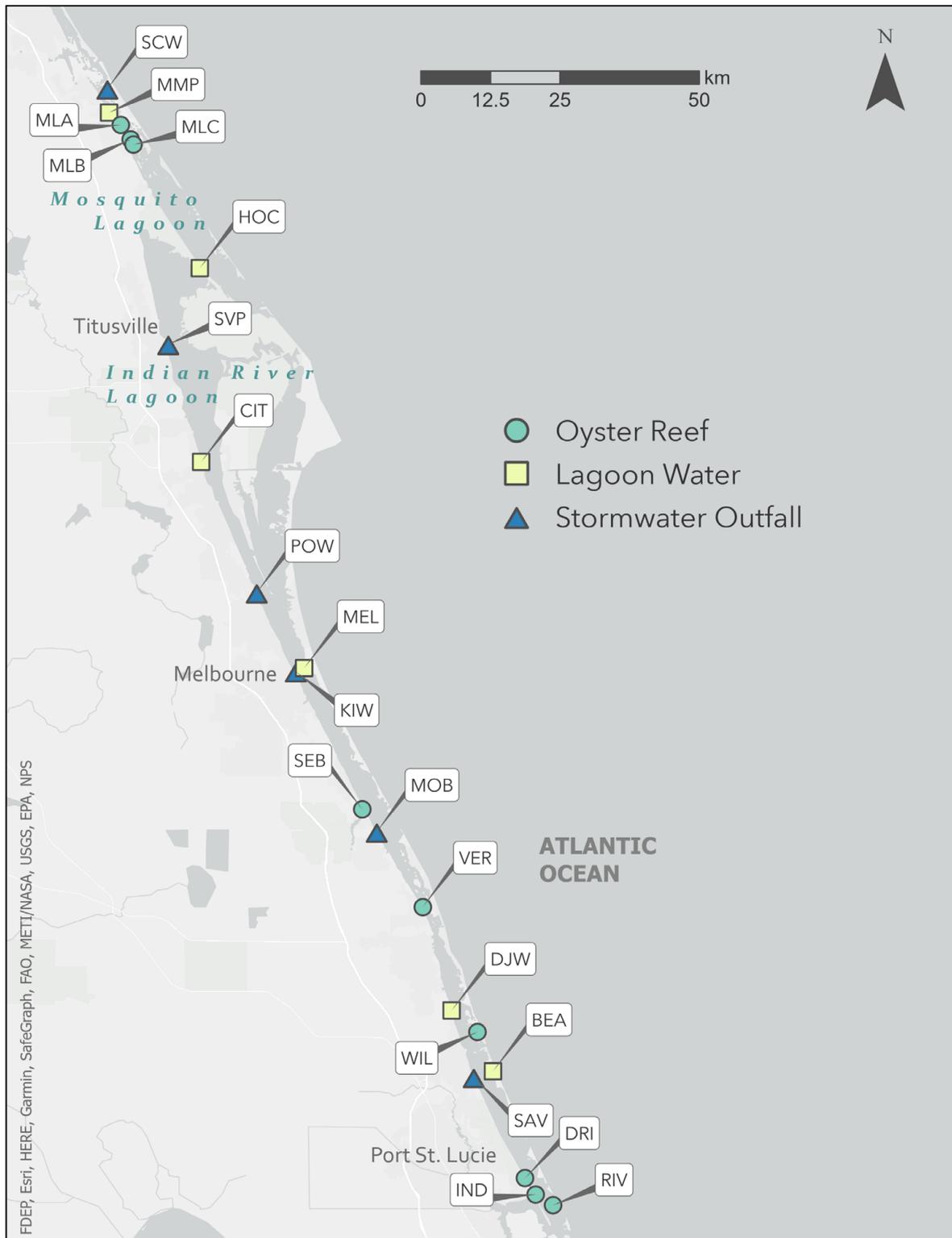
The eastern oyster, *Crassostrea virginica*, is an important suspension feeder in the IRL and other estuaries [23–25], wherein it filters a wide variety of organic and inorganic particles [26–28], including bacteria [25,29], from large volumes of water. Some particles are rejected prior to digestion in the form of pseudofeces, while others are ingested and either assimilated into biomass or excreted as feces [30–32]. Any or all of these particle fractions may contain bacteria that are pathogenic to a wide variety of species inhabiting coastal systems, including humans [2,5,8], animals [33–35], vascular plants [36,37], and macroalgae [38–40]. Pathogens that are ingested and remain in oyster guts and tissues present a direct health risk to oyster consumers. Human infections from consuming pathogen-contaminated oysters are well documented [19,41–43], and this is concerning since oysters are farmed for food on leased plots in the IRL [44] and can be recreationally harvested in certain areas [45]. But infections from contaminated oysters may occur in other predators such as fishes [46] and large crustaceans [47] as well. Oysters can also transport suspended pathogens to the benthos in their biodeposits [48,49], likely turning oyster reefs into PPM reservoirs as excreted biodeposits accumulate in reef crevices and sediments. From there, PPM may infect benthic organisms through direct contact, or be carried into trophic webs and distributed by myriad deposit feeders and their predators [50]. However, fully understanding this pathway is difficult because the composition and abundance of PPM in IRL oyster biodeposits is unknown.

This paper presents an examination of microbial communities in the IRL, with a particular focus on PPM from the water column, stormwater outfalls, and oyster biodeposits. The study's objectives were to identify potential PPM hotspots, document some taxa of concern that should be further identified and investigated, and assess the potential for oyster reefs to serve as PPM reservoirs.

## 2. Materials and Methods

### 2.1. Study Sites

Samples were collected from 21 sites throughout the IRL system (Figure 1, Table 1), including 9 oyster reef (OR), 6 lagoon water (LW), and 6 stormwater outfall (SO) sites. LW samples were collected  $\geq 1$  km from any stormwater outfall, and SO samples were collected within 1 m of an outfall mouth. The sites complement those used in a parallel study on IRL microplastics [51].



**Figure 1.** Locations and letter codes of oyster reef (OR, aqua circles), lagoon water (LW, yellow squares), and stormwater outfall (SO, blue triangles) sites throughout the Indian River Lagoon system. This color scheme continues throughout.

**Table 1.** Details of the collection sites (listed north to south by type): oyster reef (OR), lagoon water (LW), and stormwater outfall (SO).

Site	Name	City/Town	Type	Lat.	Long.	Temp. (°C)		Salinity (ppt)	
						Jan.	Jul.	Jan.	Jul.
MLA	Mosquito Lagoon Reef A	Edgewater	OR	28.9697	−80.8820	14	28	37	32
MLB	Mosquito Lagoon Reef B	Edgewater	OR	28.9460	−80.8660	13	29	37	29
MLC	Mosquito Lagoon Reef C	Edgewater	OR	28.9374	−80.8615	13	28	34	30
SEB	Saint Sebastian River	Sebastian	OR	27.8553	−80.4922	19	32	25	20
VER	Vero North Relief Canal	Vero Beach	OR	27.6967	−80.3947	19	31	5	32
WIL	Wildcat Cove	Fort Pierce	OR	27.4933	−80.3061	17	30	30	35
DRI	Driftwood Motel	Jensen Beach	OR	27.2551	−80.2295	18	30	30	30
IND	Indian Riverside Park	Jensen Beach	OR	27.2285	−80.2127	18	29	31	31
RIV	River Cove Park	Stuart	OR	27.2112	−80.1843	17	28	31	31
HOC	Haulover Canal	Mims	LW	28.7365	−80.7547	21	29	14	19
MMP	Menard-May Park	Edgewater	LW	28.9896	−80.9012	20	29	30	25
CIT	City Point Community Church	Cocoa	LW	28.4211	−80.7525	15	31	15	16
MEL	Melbourne Causeway	Melbourne	LW	28.0856	−80.5862	16	30	19	28
BEA	Bear Point Sanctuary	Fort Pierce	LW	27.4294	−80.2813	15	30	33	35
DJW	D.J. Wilcox Preserve	Fort Pierce	LW	27.5282	−80.3481	15	29	30	27
SCW	South Causeway	New Smyrna Beach	SO	29.0284	−80.9039	21	30	28	21
SVP	Space View Park	Titusville	SO	28.6126	−80.8056	24	28	0	0
KIW	Claude Edge Front Street Park	Melbourne	SO	28.0798	−80.5997	14	29	17	26
POW	POW/MIA Park	Melbourne	SO	28.2082	−80.6628	16	29	7	20
MOB	Mo Bay Grill	Sebastian	SO	27.8189	−80.4690	23	30	0	17
SAV	Savannah Road	Fort Pierce	SO	27.4194	−80.3124	17	28	0	14

## 2.2. Sample Collection and Processing

Samples were collected on 18–21 January and 19–22 July 2021 to capture temperature extremes in wet and dry seasons (January—typically cool, dry; July—typically warm, wet; see Table 1). At all 21 sites, water samples (500 mL,  $n = 3$ ) were collected just below the surface to avoid capturing floating debris and pollen and were immediately stored on ice in sterile bags awaiting laboratory processing. At each OR site, adult oysters ( $n = 3$ ) of comparable size (mean length =  $77 \pm 2$  mm) were collected. After scrubbing the shells with site water to remove sediment and biofouling, each oyster was transferred to a dry, sterile bag and stored on ice prior to laboratory processing. Surface water temperature (digital thermometer) and salinity (refractometer) were measured at each site.

In the laboratory, each water sample was thoroughly mixed and vacuum-filtered through a sterile nitrocellulose filter (47 mm diameter, 0.22  $\mu$ m pore size). Clogged filters were moved to individual sterile Petri dishes and replaced as needed until the entire sample volume was filtered (1–10 filters/sample). Using stereomicroscopy, each filter was quickly analyzed for the presence of microplastics for a parallel investigation. The filters were kept cool during analysis by placing an ice pack between the Petri dish and the microscope stage, and they were then preserved in 95% molecular-grade ethanol and stored at  $-20$  °C. Microbes were separated from the filters via the following process, adapted from Sneed et al. [52]: Filters from each sample were pooled, shredded into ca. 0.5 cm strips, submerged in 95% molecular-grade ethanol, and vortexed at 3200 rpm for 1 min. Filter strips were carefully discarded, the sample was centrifuged at 5000 rpm for 20 min, the supernatant was discarded, and the pellet was stored at  $-80$  °C awaiting DNA extraction. While traces of material remained on the filters, this protocol removed most of the sample biomass.

The outer shells of the live oysters were scrubbed again in the laboratory with filtered deionized water to remove as much biofouling as possible. Each oyster was placed in a separate, randomly assigned sterile tank filled with 500 mL of water from the region in which it was collected (water from MLC for northern sites MLA-MLC; water from VER for central sites SEB-WIL; water from IND for southern sites DRI-RIV). Tanks were covered and oysters were allowed to defecate for 12 h. Using sterile glass pipettes, oyster feces (OF) and oyster pseudofeces (OP)—collectively ‘biodeposits’ hereafter—were retrieved in separate samples (each <15 mL total volume) based on visual inspection [53], filtered

(1–3 filters/sample), and processed according to the same methods used for the water samples. All oysters appeared healthy and active at the end of the defecation period, with no apparent difference in depuration across the replicates.

### 2.3. DNA Extraction and Sequencing

DNA was extracted from all water and biodeposit samples using the Qiagen DNeasy PowerSoil HTP 96 Kit according to the manufacturer's protocol. Extracts were sent to Jonah Ventures (Boulder, CO, USA) for PCR amplification, library preparation, and sequencing following an adapted Earth Microbiome Project protocol (<https://earthmicrobiome.org>, accessed on 21 November 2023). In short, the V4 hypervariable region of the 16S rRNA gene (ca. 254 bp) was amplified from each DNA sample using the updated primer pair 515F (GTGYCAGCMGCCGCGGTAA) and 806R (GGACTACNVGGGTWTCTAAT) [54,55]. This region is too short to conclusively identify bacteria to the species level, but it is very effective for profiling community composition and classifying taxa at higher levels [56]. Reactions were visually inspected for amplicon size and PCR efficiency using 5 µL of PCR product per sample in 2% agarose gels. Amplicons were cleaned with Exo1/SAP for 30 min at 37 °C, followed by inactivation for 5 min at 95 °C, and were then stored at –20 °C. Samples underwent a second round of PCR for indexing. The final PCR products were cleaned again, normalized using the SequalPrep™ Normalization Kit (Applied Biosystems, Waltham, MA, USA) and pooled by combining 5 µL of each normalized sample. Library pools were then sequenced (10,000 reads/sample target depth) on the iSeq 100 platform (Illumina Inc., San Diego, CA, USA) with an iSeq i1 Reagent cartridge (Illumina Inc., San Diego, CA, USA).

### 2.4. Bioinformatics and Statistical Analyses

Taxonomic assignments were conducted by Jonah Ventures (Boulder, CO, USA) using the following protocol: Raw sequence data were demultiplexed using Phenix v2.1.0 [57], enforcing the strict matching of sample barcode indices. Cutadapt v3.4 [58] was used to remove gene primers from the forward and reverse reads and to discard any read pairs where one or both primers were not found at the expected 5' location, with an error rate < 0.15. Read pairs were merged using VSEARCH v2.15.2 [59], removing resulting sequences < 244 bp, >264 bp, or with a maximum expected error rate > 0.5 bp. Reads in each sample were then clustered using the UNOISE3 [60] denoising algorithm ( $\alpha = 5$ ), and unique raw sequences observed < 8 times were discarded. Counts of the resulting exact sequence variants (ESVs) were compiled, and chimeras were removed using the UCHIME3 [61] algorithm. For each ESV, a consensus taxonomy was assigned using a custom best-hits algorithm and the SILVA v138.1 [62] reference database. Finally, the consensus taxonomy and ESV count matrices were produced and delivered for downstream analyses.

Analyses were performed using the phyloseq [63], WRS2 [64], and VEGAN [65] packages in RStudio [66] v2022.02.3, and with the PRIMER v7 (PRIMER-e, Auckland, New Zealand) and XLSTAT 2023 v25.1.1408 (Lumivero, Denver, CO, USA) software packages. Samples were grouped by treatment across sites for statistical comparisons (for each season:  $n = 18$  for LW and SO;  $n = 27$  for OW, OF, and OP). A total of 7 samples (3% of total) were removed after examining ESV richness across the dataset and between biological replicates, retaining 227 samples for analysis. Removed samples had (1) no or extremely low ESV richness (<25% of the number of ESVs from the other replicates) and (2) no reads for ESVs that were relatively abundant in the other replicates, indicating possible PCR inhibition from clay, polysaccharides, or humic substances in the samples [67]. For the remaining samples, non-target sequences (i.e., eukaryotes, chloroplasts, and mitochondria) were removed, and read counts were transformed to relative proportions per sample [68].

The overall compositions of microbial communities were viewed across seasons and treatments at the order level [69]. Potentially pathogenic microbes (PPM) were identified as ESVs with a 100% match [70] to one or more known or suspected pathogens for humans, animals, vascular plants, and/or macroalgae based on a literature review [9,33–40,71–148] (Table S1). PPM proportions were square root transformed to reduce the influence of

dominant taxa [149], and differences in community composition were identified using a two-way crossed analysis of similarities (ANOSIM) [150] and visualized via non-metric multidimensional scaling (nMDS) plots based on Bray–Curtis similarities [151]. Groups were considered different via ANOSIM only when  $R \geq 0.2$  and  $p < 0.05$  [152]. A two-way similarity percentage analysis (SIMPER) was then used to determine the key taxa characterizing each group [152], which were defined as those contributing  $\geq 10\%$  to the overall similarity between replicate samples. Relationships between PPM composition and environmental variables were identified using distance-based linear models (DistLM, Best, adjusted  $R^2$ ). Raw and transformed relative abundance and richness data failed normality (Shapiro–Wilk test) and homogeneity (Levene’s test), which are assumptions of the parametric two-way ANOVAs that were intended to detect differences in both metrics among treatments and seasons. Therefore, non-parametric two-way mixed ANOVAs (bwtrim function) and subsequent post hoc tests (mcp2atm function) were used [64,153]. Mann–Whitney U tests were used to detect seasonal differences in temperature and salinity after those data also failed normality and homogeneity tests [154].

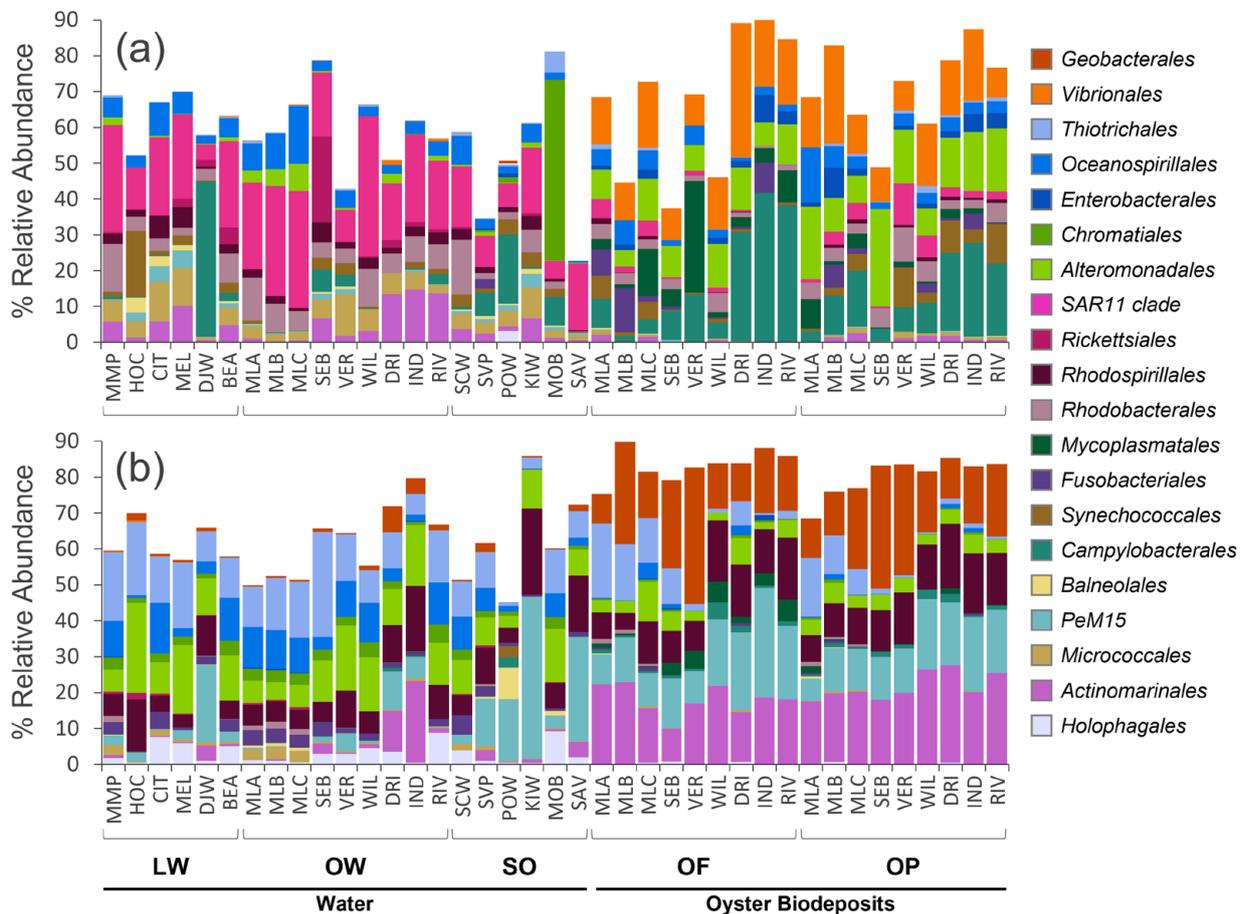
### 3. Results

The results tables for all statistical tests are reported in the Supplementary Materials (Tables S1–S5). Water temperatures were higher ( $p < 0.0001$ ) in July ( $29.4 \pm 0.2$  °C) compared to January ( $17.4 \pm 0.7$  °C). No significant difference in seasonal salinity was detected, but salinity varied widely across sites, from 0 to 37 ppt (Table 1). A total of 1830 microbial ESVs (1812 Bacteria and 18 Archaea) were found across the 227 samples. The average ESV richness was  $144 \pm 3$  per sample, ranging from 12 (OF, site MLC, July) to 265 (SO, site SVP, July). Water and biodeposit microbial communities differed overall during both seasons (Figure 2). In January, the dominant orders were the SAR11 clade in water and Vibrionales and Campylobacterales in biodeposits. In July, Thiotrichales and Alteromonadales were common in water, and biodeposits were dominated by Geobacterales, PeM15, and Actinomarinales.

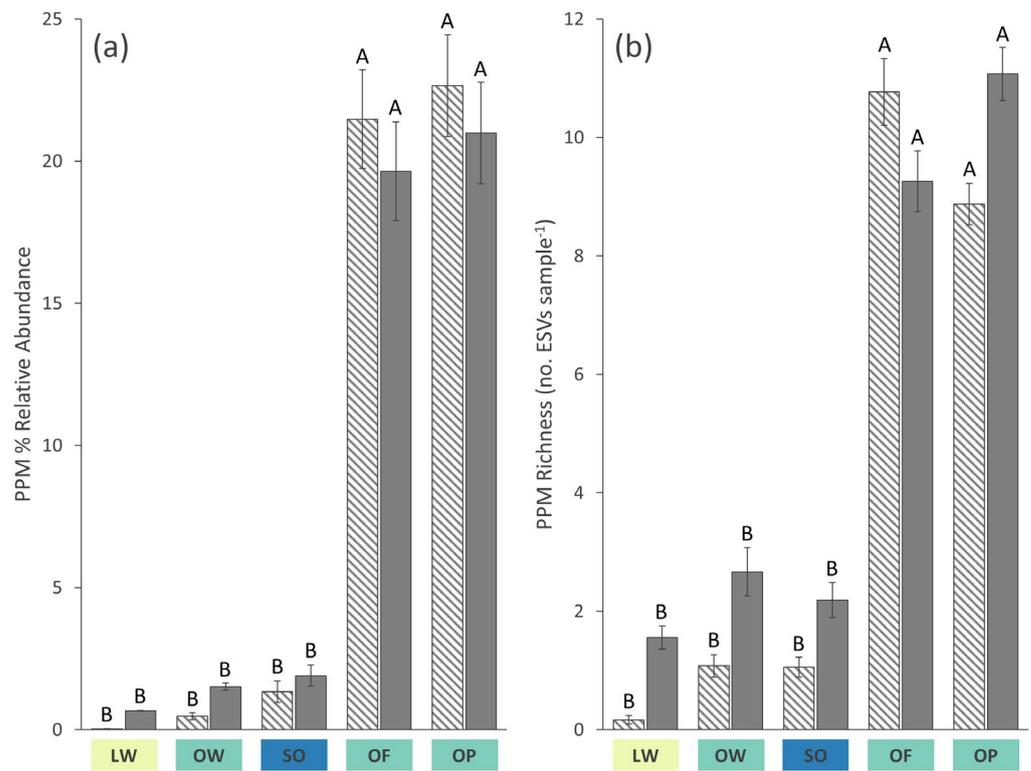
A deeper investigation of the microbial communities revealed 52 ESVs across 5 phyla and 20 families that were identified as PPM (Table S6), with per-sample relative abundances detected at up to 61.4%. In both January and July, the relative abundance and taxonomic richness of PPM were higher in OF and OP compared to all other treatments ( $p < 0.0001$ ; Figure 3). PPM community composition differed (Figure 4) between seasons ( $R = 0.734$ ,  $p = 0.001$ ) and among treatments ( $R = 0.443$ ,  $p = 0.001$ ). Pairwise comparisons following the two-way crossed ANOSIM revealed that the treatments were split into two groups based on PPM composition—oyster biodeposits (OF and OP) and water (LW, OW, and SO) ( $R \geq 0.494$ ,  $p = 0.001$ )—with no significant differences found within these two groups. There was little overlap in the PPM detected from the biodeposits and from the water used for the depuration tanks (MLC, VER, and IND), and relative abundances were generally higher in the biodeposits when PPM overlap did occur (Figure 5). While water temperature and salinity were both significant contributors to PPM community composition ( $p = 0.001$ ), temperature explained 19.8% of the variation compared to only 4.1% for salinity.

A few PPM ESVs were notably unique among sites or treatments, such as ESV\_016069 (Bacteroidaceae, *Bacteroides* sp.) and ESV\_072564 (Arcobacteraceae, *Arcobacter* sp.), which were only detected in samples from stormwater site POW (Figure 5, Table S6). However, the largescale differences in PPM composition between seasons and among treatments were primarily driven by prevalent and abundant taxa from the families Pseudoalteromonadaceae and Vibrionaceae, with five ESVs contributing most to those differences: ESV\_008978, ESV\_010267, ESV\_010681, ESV\_033709, and ESV\_069904 (Figure 5). ESV\_069904 (Vibrionaceae, *Vibrio* sp.) was the most abundant microbe in the January biodeposits, being found at all sites and reaching a relative abundance of 31.2% (OF, site DRI). Although it was also present in the water column in January, it was found primarily in OW samples, where it never exceeded a relative abundance of 0.6%. The January biodeposits were also characterized by ESV\_033709 and ESV\_010267 (both Pseudoalteromonadaceae, *Pseudoal-*

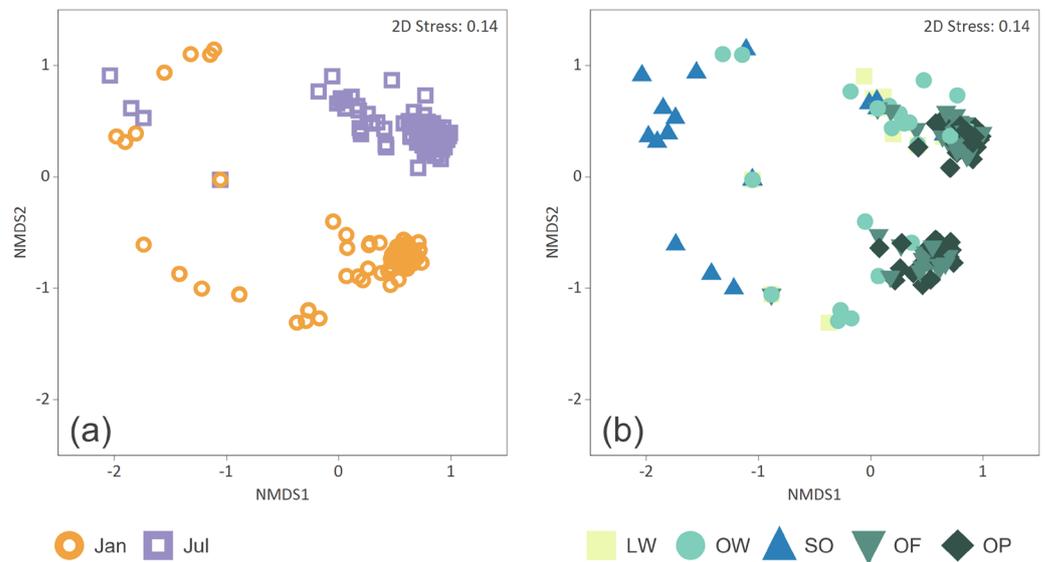
*teromonas* sp.). In July, ESV\_069904 was not detected in any samples, except OP from site MLC. Instead, the July PPM composition was largely characterized by ESV\_008978 (*Pseudoalteromonadaceae*, *Pseudoalteromonas* sp.) and ESV\_010681 (*Vibrionaceae*, *Vibrio* sp.). Both ESVs were found in biodeposits from nearly all oyster reef sites in both seasons but were more plentiful in July, with relative abundances up to 19.8% (OF, site MLB). In water samples, they were found almost exclusively in July, with only ESV\_010681 being detected in January (OW, sites MLC and RIV). In addition to these five major contributors, several other ESVs primarily found in biodeposits also exhibited seasonal patterns (Figure 5). ESV\_069856 (*Flavobacteriaceae*, *Tenacibaculum* sp.), ESV\_073069, and ESV\_073075 (both *Alteromonadaceae*, *Shewanella* sp.) were more prevalent in January, while ESV\_010878 (*Vibrionaceae*, *Vibrio* sp.) and ESV\_073954 (*Oceanospirillaceae*, *Oceanospirillum* sp.) were found more in July. Examples of the potential pathogenicity of each of the 52 ESVs from the literature review are listed in Table S1. Taxa matching the ESVs have been documented as known or suspected pathogens in humans, a variety of animals from cattle to corals to crustaceans, vascular plants, and macroalgae.



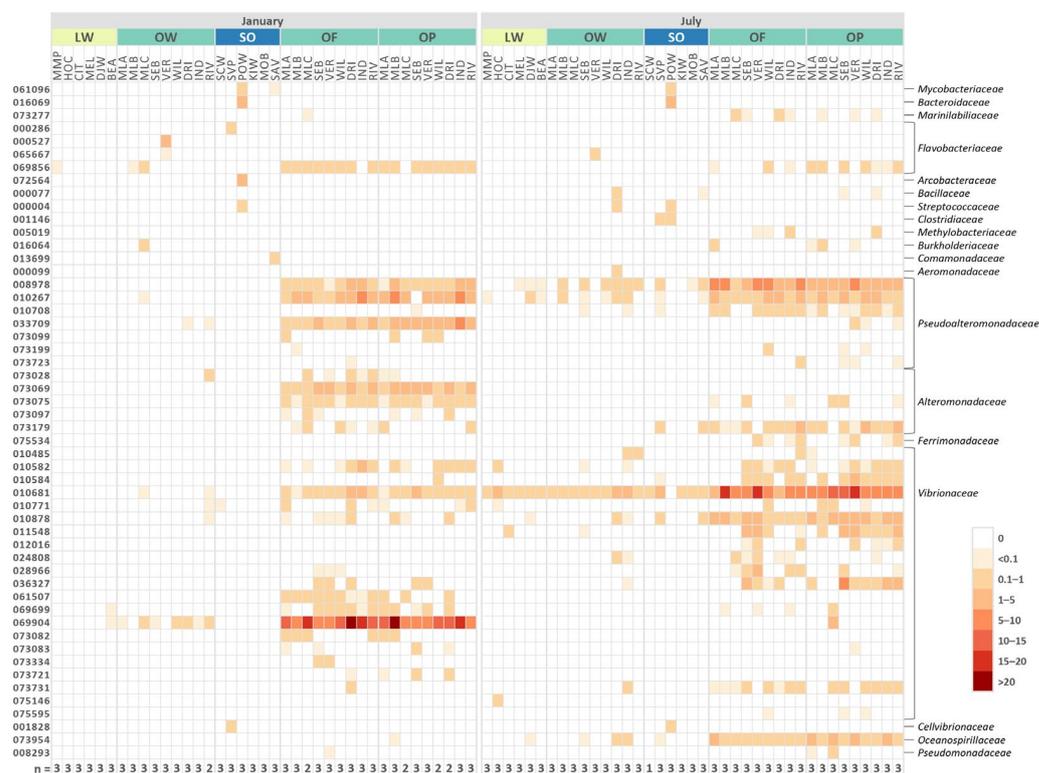
**Figure 2.** Average percent relative abundances per site in (a) January and (b) July for the 20 most abundant microbial orders from five treatments: lagoon water (LW), oyster reef water (OW), stormwater outfall (SO) water, oyster feces (OF), and oyster pseudofeces (OP).



**Figure 3.** Average PPM (a) percent relative abundance and (b) richness in January (diagonal lines) and July (solid) from five treatments: lagoon water (LW), oyster reef water (OW), stormwater outfall (SO) water, oyster feces (OF), and oyster pseudofeces (OP). Letters denote significant differences among groups in each panel (two-way mixed ANOVA and post hoc tests on trimmed means).



**Figure 4.** Non-metric multidimensional scaling (nMDS) plots of samples based on Bray–Curtis similarities of PPM communities, coded by (a) season and (b) treatment, from five treatments: lagoon water (LW), oyster reef water (OW), stormwater outfall (SO) water, oyster feces (OF), and oyster pseudofeces (OP).



**Figure 5.** Heatmap of the average percent relative abundance of the 52 PPM ESVs detected across sites, treatments, and sampling seasons. ESV number is denoted on the left. Identifications are listed at the family level. The number of samples in each group is indicated in the bottom row.

#### 4. Discussion

Microbial communities from water and biodeposits exhibited broad-scale differences in their composition during both seasons. Water samples contained high relative abundances of SAR11 in January and Thiotrichales and Alteromonadales in July. SAR11 dominates surface bacterioplankton communities [155,156]. Thiotrichales and Alteromonadales have been documented as two of the most abundant orders forming biofilms on suspended plastic particles [157]. Therefore, their abundance in water samples in this study was unsurprising. SAR11 is not a pathogen of concern, but both Thiotrichales and Alteromonadales contain several known and suspected pathogens [158,159]. The orders that dominated biodeposit samples also varied in their potential to contain pathogens. For example, Vibrionales and Campylobacterales, which were abundant in January, have been frequently associated with human and animal diseases [69,144,160–162]. Conversely, Geobacterales, which was abundant in the July biodeposit samples, is potentially quite beneficial. It is key in biogeochemical cycling and is being investigated for its use in bioelectrochemistry and bioenergy applications [163]. This order-level analysis illustrates that microbial communities are complex assemblages containing both beneficial and harmful taxa, and it emphasizes the need for a more detailed investigation to detect PPM.

Per volume, the oyster biodeposits contained more diverse and abundant PPM that produced a different community composition than what was detected in the water column (Figures 2–5). These results reinforce similar findings from other studies that have compared pathogen densities between oysters and water [164,165] and highlight the ability for oysters to concentrate PPM by filtering large water volumes. *Crassostrea virginica* is one of the most prolific suspension feeders in estuaries within its range. In a laboratory bacterial depletion study using water collected from the IRL, Galimany et al. [25] found that a single adult oyster can clear approximately 3 L of water and remove  $1.5 \times 10^5$  suspended bacterial cells per hour. Bacterial removal can increase further when multiple cells are clumped together or are aggregated with larger particles (e.g., sediment, detritus, plankton, microplastics)

that oysters filter more efficiently [27,166,167]. These aggregates can especially facilitate the uptake of the many surface- and sediment-associated PPM, like *Vibrio* spp. [26,168,169] and *Pseudoalteromonas* spp. [101,170,171], which were abundant in the present study. In fact, three common pathogenic vibrios—*V. parahaemolyticus*, *V. vulnificus*, and *V. cholerae*—have been detected in IRL sediments at concentrations three orders of magnitude higher than in the water column [19]. In addition to filtering these bacteria-laden sediments, the oysters may have also refiltered old biodeposits that are commonly resuspended [172–174], resulting in new, more concentrated biodeposit pathogen loads.

Strong seasonality was also detected for the microbial communities in this study, with the July samples containing a different community composition compared to those from January (Figures 2, 4 and 5). These results are not surprising, as it has been well established that many microbes grow better in warm waters [175–181]. Indeed, the relationship between pathogen emergence and sea surface temperature extends beyond individual systems to be recognized globally as a concerning consequence of climate change [182–186]. Conversely, work on congeners of taxa that were more prevalent in January, such as *Tenacibaculum* sp. and *Shewanella* sp. (Figure 5), suggests that their wide temperature tolerances may give them a competitive advantage in winter [187–189]. Although PPM richness and relative abundance did not differ between January and July, changes in their community composition highlight the need for further investigations of microbial seasonality. This is especially important as estuaries continue to warm, with water in the IRL reaching summer highs > 33 °C (IRLON: Indian River Lagoon Observatory Network of Environmental Sensors, Florida Atlantic University Harbor Branch, irlon.org) at the time of writing this paper. However, temperature accounted for just 20% of the total variation in PPM community composition herein, emphasizing that other factors were also important.

Salinity has been identified as an additional driver of pathogen composition in estuaries, especially among *Vibrio* spp. [19,190]. However, studies have demonstrated that this relationship is not linear when salinities vary widely [191,192]. In this investigation, salinity accounted for only 4% of PPM community variation. This may have been partly due to the wide salinity range among sites and seasons and the below average watershed precipitation during the 2021 wet season [193]. Lower precipitation surely also resulted in less PPM entering the IRL through stormwater outfalls than expected. Future investigations exploring the effects of salinity on estuarine PPM should sample before and after rain events and across multiple wet and dry seasons to account for annual variation.

Turbidity and chlorophyll are frequent drivers of pathogen composition in estuaries [164,165,191,194], and they were likely influential in this study as well. Although not measured during sample collection, both metrics differed in the IRL between the sampling seasons. A monitoring station located near the SEB oyster reef site (IRL-SB, maintained by Florida Atlantic University, hourly data) recorded higher chlorophyll and turbidity values in July than in January ( $p < 0.0001$ , Mann–Whitney U). Similar seasonal differences likely occurred at the other sites, especially those located away from inlets where tidal flushing is minimal. Pathogen composition can be altered by the suspended particles that are abundant during high-chlorophyll and/or high-turbidity events. For example, Johnson et al. [191] investigated the environmental factors associated with the abundances and distributions of pathogenic *Vibrio parahaemolyticus* and *V. vulnificus* in the northern Gulf of Mexico. The authors found that turbidity was a significant predictor of *V. parahaemolyticus* but not *V. vulnificus*. Moreover, the former species was more abundant in sediments than the latter, suggesting that the resuspension of the sediment grains to which it was attached was a driving factor behind its abundance in the water column during high-turbidity events. Similarly, positive relationships between chlorophyll a and pathogen concentrations could be caused by bacteria attached to the zooplankton that are feeding on developing phytoplankton blooms [195]. Such findings further support the potential for suspended particles to engineer differences in water column and biodeposit PPM communities by shifting the ratio of attached versus free-living bacteria, with attached bacteria being filtered more easily by oysters and other suspension feeders.

Although the biodeposit PPM loads detected in this study are concerning from the perspective of fisheries, the strong similarity between OP and OF PPM communities demonstrates that these microbes were present in both the ingested and rejected fractions and were not selectively taken up. The biodeposit PPM communities (Figure 5, Table S6) also appeared quite different overall from those commonly reported in *C. virginica* gut and muscle tissue [196–198], which may be more difficult for the oysters to excrete [199]. Hence, it is likely that recommended depuration methods [200,201] could be used to help purge harvested oysters of the microbes detected in this study, thereby reducing the human health risks associated with oyster consumption in the IRL.

Likely of greater concern is the potential for oyster reefs to function as hot spots for PPM by condensing pathogens onto the benthos [202], where they can both infect reef inhabitants and be moved through the ecosystem via trophic transfer. Oyster reefs are homes to diverse assemblages of polychaetes, crustaceans, molluscs, and other animals [203–206] that can be infected by contacting or consuming the pathogens potentially found in the biodeposit samples investigated in this study [114,120,135]. Infected animals often die, but they can also spread pathogens to higher-level predators such as wading birds [207] and several commercially and recreationally important fishery species, including juvenile groupers, snappers and flounders, and adult drum and sheepshead [179,208]. Many of these benthic-feeding fish tend to have higher pathogen concentrations than those feeding on pelagic prey [179], possibly causing diseases in the fish [121] and in the humans consuming them [209]. These connections establish a trophic pathway for pathogenic microbes that likely began, in part, with suspension feeding by oysters and will likely continue to intensify in increasingly populated coastal systems contending with eutrophication and climate change.

## 5. Conclusions

This study provides evidence that oyster reefs in the IRL estuary are a reservoir for PPM and documents some possible taxa of concern that should be positively identified and investigated further for their pathogenicity and potential to pervade food webs and fisheries. PPM were more diverse and abundant in oyster biodeposits than in the water column and were different in summer compared to winter. The seasonal differences were most likely due to a suite of drivers that included water temperature and, presumably, the turbidity and chlorophyll levels across the sites. The results also suggest that these environmental drivers helped to engineer PPM community structure in the treatments by regulating microbial growth and facilitating the uptake of certain taxa by oysters. The metabarcoding used in this study is not sufficient to conclusively identify pathogens from the many innocuous and beneficial taxa that exist. However, it is shown here as an effective tool to explore spatial and temporal differences in PPM communities and to highlight PPM hotspots that can be further investigated using other technologies designed for pathogen detection and virulence assessment. PPM monitoring in estuaries is becoming increasingly important as climate change and urbanization elevate water temperatures and concentrations of suspended particles that can serve as pathogen vectors.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/environments10120205/s1>, Table S1. Differences in temperature and salinity between seasons; Table S2. Differences in PPM relative abundance and richness between groups; Table S3. Differences in PPM community composition between groups; Table S4. Effects of temperature and salinity on PPM community composition; Table S5. Key PPM taxa defining each group; Table S6: Taxonomic hierarchy of the 52 ESVs with 100% identity match to one or more potentially pathogenic microbes (PPM).

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