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Research Article

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Inhibition of Environmental Bacterial Growth from a Saltwater Tidal Creek by Ozone Impregnated Nanobubbles

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Abstract

Ozone impregnated nanobubbles (OINBs) have been studied as a disinfectant for aquaculture, but few studies have investigated the bacteriostatic effect of OINBs on environmental water samples. This study examined whether there is a dose dependent effect of OINBs on environmental water samples collected from Filbin Creek, South Carolina, USA, to determine whether OINBs can inhibit growth across a natural community of bacteria. Water samples were exposed to different fractions of OINBs from 0-50% and plated onto marine or TCBS agar. At a dose of 10% OINBs, there was a 97% and 98% decrease in bacterial colony number for TCBS and Marine Agar plates, respectively. Metagenomic analysis indicated a high prevalence of Vibrio spp. which were susceptible to OINB treatment. Resistant colonies at the highest volumetric treatment were sequenced and found to belong to the genus Bacillus. OINBs appear to be an effective method to reduce potential pathogenic bacterial growth in environmental samples.

Keywords: Metagenomics, Bacteriostatic, Antibiotic Resistance, Bacillus, Vibrio

Abbreviations

OINB: Ozone Impregnated Nanobubbles Asw, Artificial Sea Water
TCBS: Thiosulfate Citrate Bile Salts Sucrose Agar

1. Introduction

Ozone nanobubbles (OINB) are neutrally buoyant, less than 1µm in diameter bubbles that have been studied as a disinfectant for the aquaculture, wastewater, shipping, floral, and produce industries [1-5]. OINB technology is an attractive alternative to antibiotic treatment, especially relevant to pathogenic agents that acquire antibiotic resistance [6]. Due to the extended lifetime of air nanobubbles in water (up to 14 days), generation of nanobubbles using ozone should permit the oxidative effect of ozone to occur over a longer time compared to the short half-life of dissolved ozone in water (~30 minutes) [7,8]. In one study, Seki et al., report antimicrobial effects of stored OINBs after a year suggesting that OINBs are persistent, and the lifetime can be extended at low temperature [9].

Increased interest in scalable ecosystem level treatments with OINBs as a control for eutrophication, algal blooms, and pollutants has led to questions of efficacy and persistence in estuarine/marine environments and selection pressure on the ecosystem. While many studies have focused on the control of viral and bacterial pathogens in marine aquaculture, wastewater treatment, or clinical settings using OINBs, fewer studies have systematically assessed

the efficacy of OINBs in a natural system [3, 6, 10-16]. Further, outside of the clinical research arena, very little is known regarding the potency of OINBs, stored for days, on bacterial growth inhibition [9]. Understanding the effect of storage time on OINBs potency could allow production of OINBs at a central facility to enable treatment at remote locations.

This study examined inhibition of bacterial growth from water samples extracted from a tidal creek estuary in South Carolina following the addition of OINB generated in artificial sea water (ASW). The objective of the study was to two-fold: 1) to define whether OINBs could suppress growth of natural microbial populations in a dose-response manner, and 2) to determine whether stored OINBs were effective at inhibiting bacterial growth a minimal dose that causes near total growth inhibition. Metagenomics was utilized to characterize natural populations grown on selective bacterial agar and implicate bacterial species that demonstrated resistance to OINB treatment. Analysis of antibiotic resistance genes (ARG) was conducted to observe the ARG landscape in the culturable microbial community and resistant community.

2. Materials and Methods

2.1 Samples

Environmental water samples were collected in triplicate from Filbin Creek, North Charleston, South Carolina (32.8932301°N, 79.9656422°W) on 10 May, 2023 during an ebbing tide (10 psu, 27ºC) in autoclaved 2L glass (Corning Ware, USA) bottles and held for 24 hours in the laboratory at room temperature. The sampling site is in close proximity to United States Geological Survey monitoring site USGS-021720677 (Cooper River at Filbin Creek at North Charleston, SC). Artificial sea water (ASW) was created by dissolving 1.36 kg of Instant Ocean®

Sea Salt in 136 L of deionized water in a 184 L acrylic tank. Light refractometry was used to verify the final salinity of 10 psu. To ozonate the ASW, a 7.5HP-30 NBOT system (NBOT Labs, Mount Pleasant, SC) was used to circulate the ASW through the system, generate the ozone, and inject the OINBs. Dissolved ozone in the ASW was monitored using DKK-TOA Corporation OZ-20 meter with the probe placed in the tank for the duration of the treatment. Similarly, dissolved oxygen, salinity, pH and Oxidation-Reduction Potential (ORP) were measured during the OINB treatment of the ASW using a Hanna Instruments model HI9829 multiparameter meter. OINB size and concentration were estimated by particle counting using a Nano sight Pro (Malvern Panalytical). OINB-treated ASW was collected in 50mL sterile conical tubes and held at room temperature for 1 hour prior to addition to environmental water samples.

2.2 Dose-Response

Environmental water samples were exposed to varying amounts of OINB-treated ASW: 0%, 1%, 5%, 10%, 15%, 25%, and 50%. Triplicate environmental water samples (500 µL) were aliquoted into 1.5mL microcentifuge tubes (Eppendorf, protein lo-bind 0030-108-115, Germany), followed by ASW, and then OINB-treated ASW to arrive at a final volume of 1mL per tube. Triplicate mixtures were vortexed for 5 seconds and incubated for 10 minutes at room temperature. Incubation time was selected based on a pilot experiment that demonstrated complete inhibition of growth on marine agar plates within 1 minute of exposure time. Following incubation, 50 µL of the OINB-treated mixture was spread onto pre-warmed marine agar (Millipore 76448, Germany) 100x15mm plates (VWR 25384-088, USA) until dry. A second 100µL aliquot was spread onto pre-warmed TCBS (Thiosulfate–citrate–bile salts–sucrose, HIMedia GM189, India) agar plates until dry. All reagents were prepared in ultra-pure water and using sterile techniques. Plates remained on the bench until the entire experiment was completed. Once complete, all the plates were incubated overnight at 37ºC. Colonies were counted using a light box (NXENT, A4 tracing light pad, China).

2.3 Multi-Day Response

A separate 2L bottle of OINB-treated ASW was held at room temperature in the laboratory for 5 and 7 days following the initial generation of OINB-treated ASW on Day 1 that was used for the dose-response experiment. A fresh environmental water sample was collected in triplicate from the same site (Filbin creek) five days after the generation of OINB-treated ASW (15 May 2023, 10 ppt, 27ºC). Environmental water samples were exposed to 5 day-old or 7 day-old, 15% OINB- treated ASW for 10 minutes as described above and samples plated and counted as described above.

2.4 DNA Extraction

For DNA extraction from agar plates, the plates were washed with 1.5mL marine broth and colonies were collected using a bent sterile glass rod. Agar washes were performed on marine and TCBS agar plates containing bacteria from Day 1, 0% OINB samples as well as agar plates from the 50% OINB-treated ASW marine plates and 10% OINB-treated TCBS plates. Marine broth was centrifuged at 3000xg for 5 minutes at room temperature to collect bacteria. Supernatant was decanted and DNA was extracted from the remaining pellets using the DNeasy PowerSoil Pro kit according to the manufacturer's protocol.

2.5 Metagenomics

Metagenomic DNA extracted from plate wash pellets was end repaired and dA-tailed using the NEBNext Ultra II End Repair/dA tailing Module (New England Biolabs, Ipswich, MA). End repaired and dA tailed DNA was then used to generate individual nanopore DNA sequencing libraries for each sample following the Ligation Sequencing Kit (SQK-LSK109) protocol using the long fragment buffer (Oxford Nanopore Technologies, UK). Each prepared library (40 fmol) was loaded onto a single R9.4.1 flow cell and all flow cells sequenced using a GridION DNA X5 Sequencer for 42-67 hours using MinKNOW (v.23.04.5). Base calling was performed using the

Guppy basecaller (v.6.5.7) with the high-accuracy model and a minimum read length cutoff of 200 bp and a quality score cutoff of 9. Sequences passing the quality and length filters were then analyzed using the Fastq Antimicrobial Resistance Workflow (v2023.04.26-1808834) available within the Nanopore EPI2ME platform. This workflow includes the What's In My Pot (WIMP) workflow for taxonomic classification of sequence reads using Centrifuge against the NCBI RefSeq database. Reads assigned a taxonomy are then passed to the antimicrobial resistance pipeline for identification of antibiotic resistance genes using minimap against the Comprehensive Antibiotic Resistance (CARD) database [17-19]. The relative abundance of each taxa or antibiotic resistance gene was calculated by dividing by the total number of identified taxa or the total number of identified antibiotic resistance genes. Information on sequence abundance, quality, and filtering is provided in Table 1.

QC Parameters	M-0	M-50	T-0
Estimated Bases (Gb)	19.55	10.6	11.14
Reads Generated (M)	1.84	1.66	1.93
Estimated N50 (kb)	12.81	13.23	13.08
Passed Bases Called (over Q score 9) (Gb)	9.44	6.15	5.56
Failed Bases Called (under Q score 9) (Gb)	7.77	3.66	4.69
Reads Analyzed for Taxonomy (#)	936,836	1,007,316	1,011,558
Avg Sequence Length (bp)	10,078	6,101	5,498
Avg Quality Score (Q)	12.29	12.25	12.35
Reads Classified (#)	909,242	922,681	957,783
Reads Unclassified (#)	27,594	84,635	53,775
Reads Analyzed for Antibiotic Genes (#)	909,242	922,681	957,783
Reads Aligned to CARD Database (#)	33,004	19,495	32,808
Average Accuracy of CARD Alignment (%)	81	77	82
CARD Genes (#)	161	69	108

Table 1: Sequence Abundance, Quality, and Filtering Data

2.6 Statistics

Dose-response and pairwise statistical metrics were calculated using Sigma Plot version 13.0 (Systat Software Inc.). Plate colony number versus dose were modeled using either five parameter logistic curves (marine agar) or four parameter logistic curves (TCBS agar) within the dynamic curve fitting module. T-test was used for pairwise comparisons between ASW and ONIB-ASW groups for day1, day 5, and day 7 if colonies were present. Otherwise, no test was conducted when one group displayed no growth.

3. Results

3.1 Effect of OINBs on Growth

Following ozone treatment, the pH of ASW was slightly lower than untreated ASW; pH = 8.5 (untreated) versus pH = 8.3 (treated). ASW in general was more basic than the untreated environmental water from Filbin creek, pH = 7.5. There was no change in salinity following ozone treatment. Oxidation-reduction potential of the OINB-treated ASW was 773.3 mV. Dissolved oxygen was 227.6 %. Mean ± SD particle size of the treated ASW was 169.5 ± 66.7 nm with an estimated concentration of $3.5x10^7 \pm 7.74x10^6$ particles per mL.

Bacterial growth was inhibited by the addition of NB-treated ASW in a dose dependent manner regardless of selective agar utilized (Figure 1 and 2). The EC50 for marine agar plates was estimated at 6.5% OINB-treated ASW and the EC50 for TCBS plates was estimated at 5.8% NB- treated ASW. Addition of OINB-treated ASW between 5% and 10% resulted in a sharp decline in bacterial growth. Bacterial growth on marine agar was inhibited 99±0.1% on Day 5 ($p<0.001$) but was not different from untreated environmental water samples on Day 7 (100 ±30% vs. 78±47%, untreated vs. 15% OINB-treated ASW, Figure 1 inset). Potency of 15% OINB-treated ASW was maintained for 5 days after the generation of OINBs in ASW regardless of media ($p<0.001$) and continued to inhibit bacterial growth on TCBS plates at Day 7. Complete inhibition of growth on TCBS plates was observed at Day 5 and growth continued to be inhibited by 84±14% on Day 7 ($p=0.03$, Figure 2 inset).

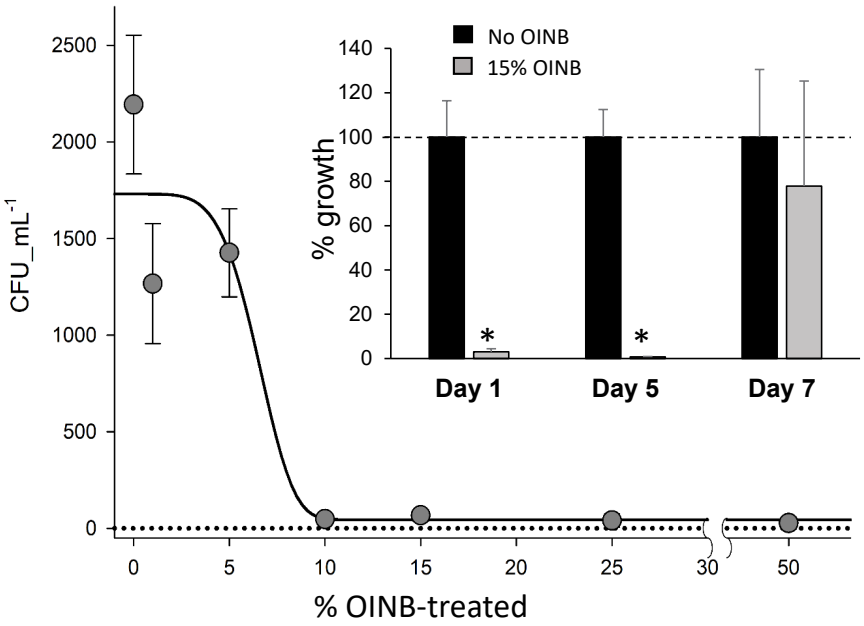

Figure 1: Dose response and effect of storage on bacterial growth from marine agar plates after exposure to different percentages of OINB-treated ASW. Data are plotted as mean ± SD colony forming units per mL. Data were fitted using a five-parameter logistic curve. Half maximal effective concentration (EC50) = 6.5%. Dashed horizontal line indicates the zero line. *Inset*. Potency of bacterial growth inhibition by 15% OINB-treated ASW over time, as assessed by marine agar plating. Untreated ASW (No OINB) growth was set as 100% (horizontal dashed line). Environmental water samples exposed to 15% ozone nanobubbles (15% OINB) for 10 minutes resulted in 97% and 99% growth inhibition at Day 1 and Day 5, respectively. On Day 7, there was no significant inhibition of growth observed on the 15% OINB plate. * denotes significance ($p<0.05$, T-test).

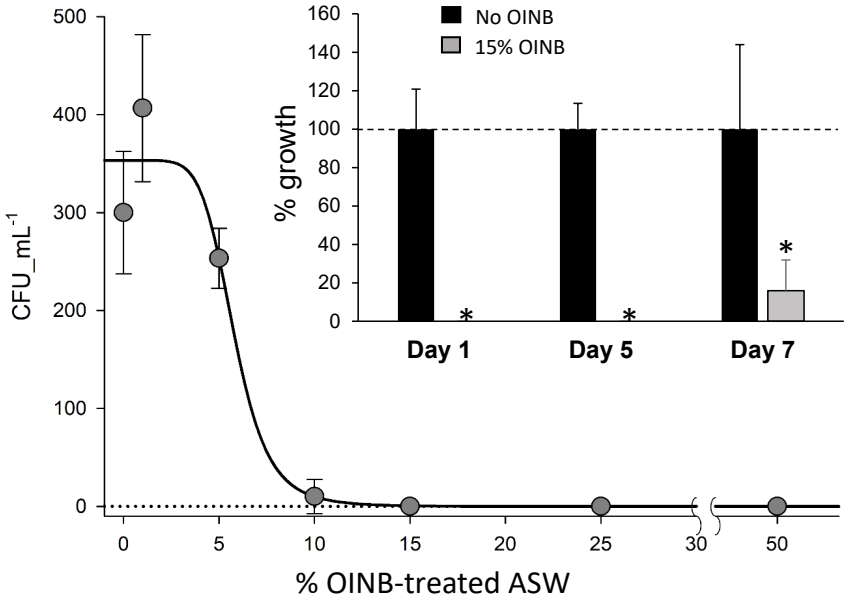

Figure 2: Dose response and effect of storage on bacterial growth from TCBS agar plates after exposure to different percentages of OINB-treated ASW. Data are plotted as mean ± SD colony forming units per mL. Data were fitted using a four-parameter logistic curve. Half maximal effective concentration (EC50) = 5.7%. Dashed horizontal line indicates the zero line. *Inset*. Potency of bacterial growth inhibition by artificial seawater impregnated with 15% ozone nanobubbles over time, as assessed by TCBS agar plating. Untreated ASW (No OINB) growth was set as 100% (horizontal dashed line). Environmental water samples exposed to 15% ozone nanobubbles (15% NB) for 10 minutes resulted in no growth at Day 1 and Day 5. On Day 7, there was slight growth observed on the 15% OINB plate indicating 84% inhibition. * denotes significance ($p<0.05$, T-test).

3.2 Plate Wash Taxonomic Classification

Taxonomic classification of bacteria contained in plate washes identified 2220 unique taxa in the non-OINB treated marine agar plate wash (M-0), with 44 taxa having greater than 0.1% relative abundance. Within this treatment, *Gammaproteobacteria* taxa identified as *Pseudoalteromonas shioyasakiensis* (37% relative abundance) and *Vibrio parahaemolyticus* (16% relative abundance) showed the greatest abundances, representing 53% of the identified taxa (Figure 3). TCBS plate washes from the non-OINB treatment (T-0) showed 1647 unique taxa, with 38 taxa having greater than 0.1% relative abundance. Within this more selective media, *Gammaproteobacteria* taxa identified as *Vibrio fluvialis* (50% relative abundance) and an unclassified *Vibrio species* (12%) showed the greatest abundances, representing 62% of the identified taxa. Following Filbin Creek water treatment with 50% OINB, plate washes from the marine agar (M-50) indicated a complete taxonomic shift, with taxa identified as *Bacillus pumilus* (68% relative abundance), *Bacillus cereus* (10% relative abundance), and *Bacillus thuringiensis* (10% relative abundance) becoming the most abundant taxa while previously abundant taxa were no longer detected. No colonies were observed on TCBS plates following 50% OINB treatment.

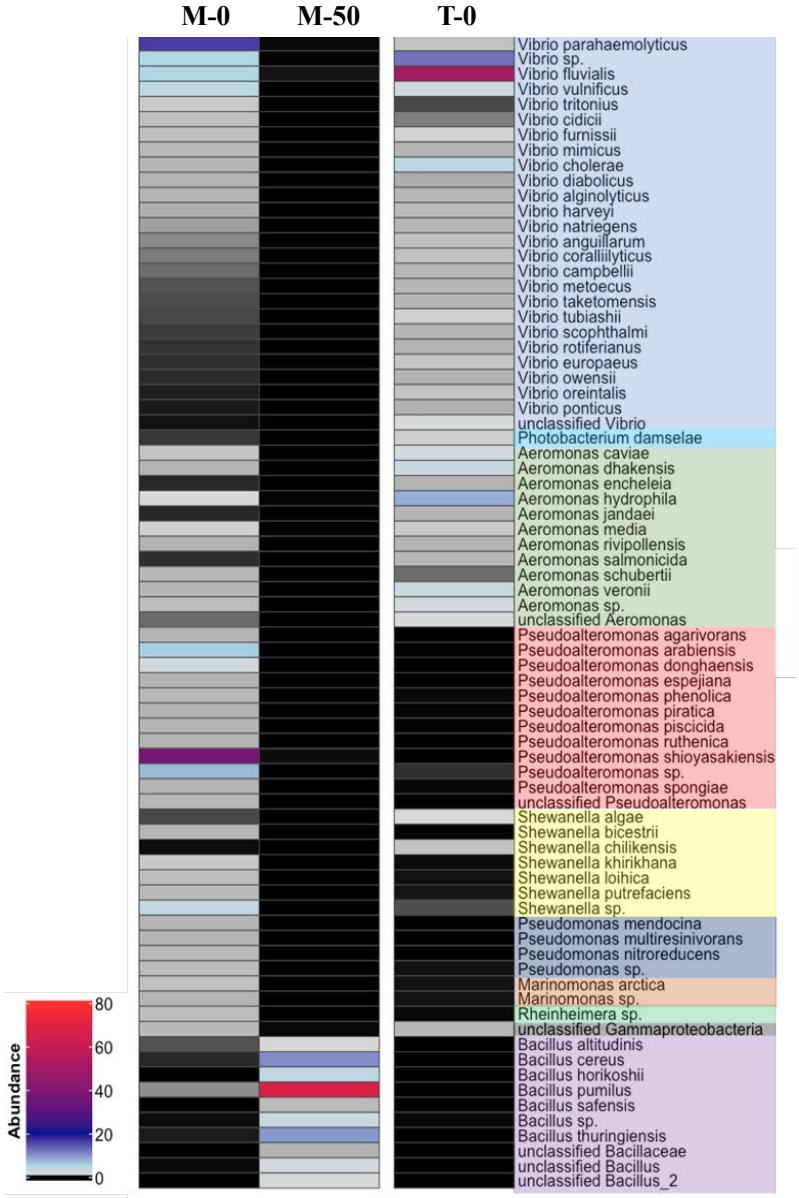

Figure 3: Relative abundance of bacterial species identified. Bacterial colonies from marine agar plates (M-0) or TCBS plates (T-0) not treated with OINBs were identified to determine the bacterial community observed on by agar plating. OINB resistant bacteria from marine agar plates treated with 50% OINB (M-50) were identified to determine which species were resistant to OINB. No bacteria grew at 50% OINB on TCBS plates and data were not gathered. Taxa were grouped at the genus level and are represented by different colors. Only taxa having a relative abundance greater than 0.1% in any condition are shown in the heatmap.

3.3 Antibiotic Resistance Gene Abundance

Classification of sequences against the CARD database showed a range of antibiotic resistance genes harbored by bacteria isolated on marine agar from non-OINB treated Filbin Creek water (M-0; Figure 4). Among the beta-lactamase genes, *CARB-20* (15% relative abundance) was the most abundant ARG followed by the *OXA-12* (3% relative abundance) and *OXA-181* (3% relative abundance) genes. Among the efflux pump type mechanisms of antibiotic resistance, the *tet34* gene (11% relative abundance) was the most abundant followed by the *tet35* (8% relative abundance), and *acrB* (6% relative abundance) genes. The *alaS* gene, conferring resistance to novobiocin was also abundant in the M-0 treatment marine agar plate wash. The most abundant ARGs identified in the TCBS plate washes from the non-OINB treatment (T-0) were the *OXA-12* beta-lactamase (7% relative abundance), the *acrB* (12% relative abundance) efflux pump gene, and *alaS* gene (23% relative abundance). Coinciding with the taxonomic shift observed following treatment with 50% OINB, a similar shift was observed in the M-50 bacterial ARG profile, with the *Bacillus cereus* beta-lactamase genes, *BcI* and *BcII*, and the *Bacillus pumilus* chloramphenicol resistance gene, *cat86*, becoming the most abundant ARGs.

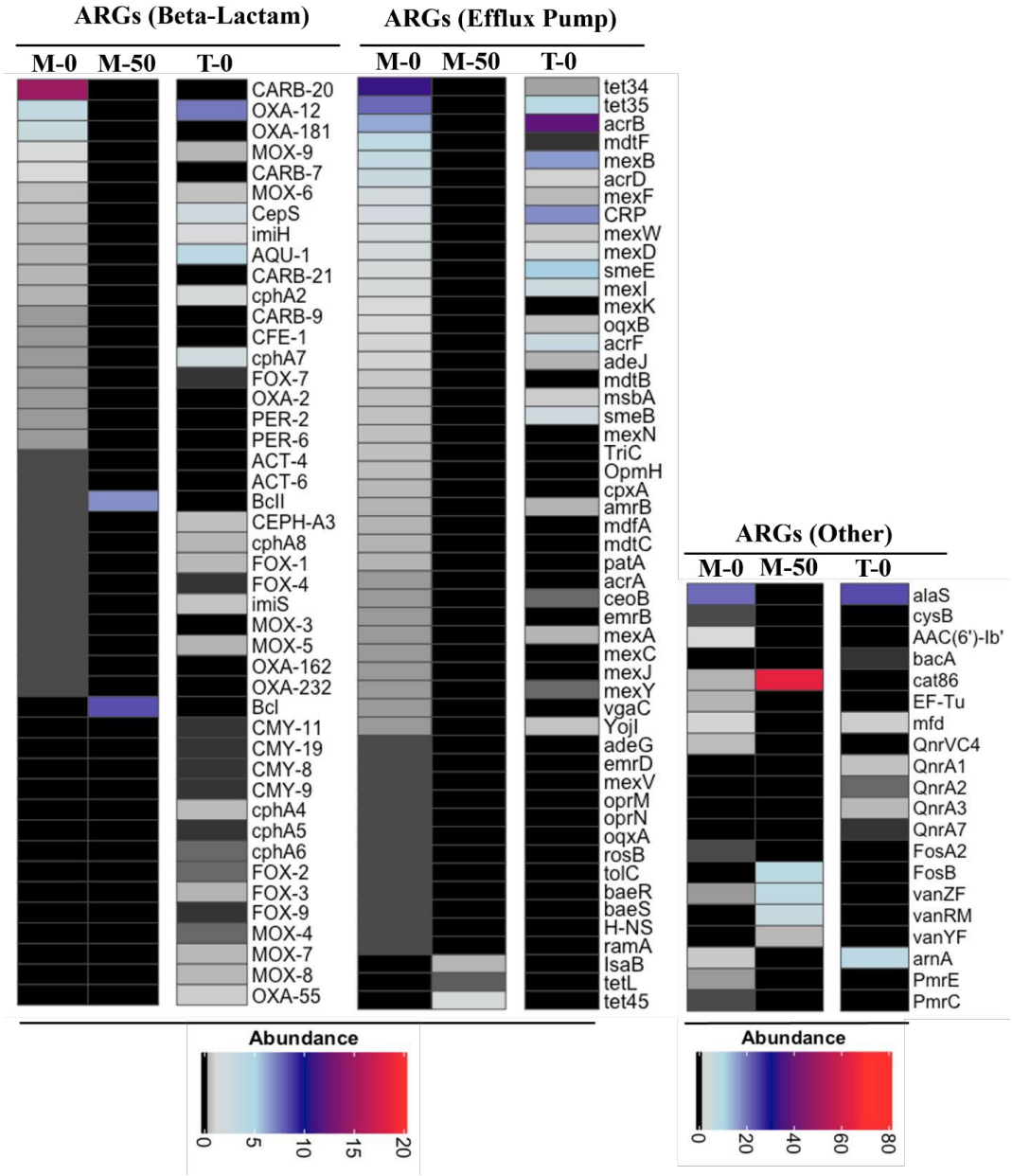

Figure 4: Relative abundance of antibiotic resistance genes (ARGs). ARGs were identified from marine agar plates (M-0) or TCBS plates (T-0) not treated with OINBs, and from marine agar plates treated with 50% OINB (M-50). ARGs were grouped based on the mechanism of resistance, beta-lactamases, efflux pumps, and other types of resistance.

4. Discussion

Consistent with previous reports regarding the bacteriostatic or bactericidal effect of OINB , similar inhibitory effects on the microbial community were seen for environmental water samples from an estuarine tidal creek [4,6,9,12,14,20]. Inhibition of bacterial growth was complete for bacteria grown on selective TCBS agar and nearly complete for bacteria grown on less selective marine agar at fractions equal to or greater than 15% OINB-treated ASW. Although culture-based approaches likely under-represent the complete microbiome, a complete shift in the culturable majority was still observed. Results from this study suggest that OINBs generated in lower salinity water (10 psu), are more potent compared to OINBs in higher salinity sea water (30 psu) [11]. Imaizumi et al., reported that dilution of OINB below 20% in ASW was ineffective at inhibiting growth of *Vibrio parahaemolyticus* which may reflect differences between the ozone generating systems or sea water components [11]. Because OINBs are difficult to quantify due to the fact that colloids or impurities may not be discernable from OINBs, comparisons between dosing studies should be interpreted with caution [1,21]. However, there were similarities with the Imaizumi et al study in that environmental bacterial growth inhibition was witnessed beyond 24 hours, which in this study was 5 days and 7 days after generation and storage of OINBs at room temperature, suggesting OINBs in the presence of dilute sea salts survive for at least a week [11]. Regardless, it should not be overlooked that treating sea water with OINBs leads to the generation of secondary oxidants such as bromates and chlorates [1,4]. Because bromates and other secondary oxidants were not measured in this study, it is possible that some long-term bacteriostatic observations may be due to the persistence of these oxidants.

Because most studies using OINBs are focused on monocultures or several specific microbes there is a gap in knowledge regarding which microbes are susceptible and which microbes are resistant to OINB treatment [6,10-12,14]. Selection pressures, such as OINB, should result in population changes in the microbiomes to enhance populations that can resist high oxidative environments [15]. Microbes play a key role in nutrient cycling and it is possible that treatment of waterbodies such as ponds, lakes, and estuarine tidal creeks could cause a temporary dysbiosis impacting mineral cycling [22,23]. In large ecological systems that routinely turn-over, e.g. tidal creeks, OINB treatment will result in gradients throughout the water column and across the water body, where regions of selection will be higher than others. To that end, we conducted a pilot microbiome analysis of the environmental water samples to determine which bacteria are sensitive or resistant to OINBs.

Microbes identified in this study were dominated by several genera, some of which contain species know to be pathogenic to humans (*Vibrio parahemolyticus, Vibrio vulnificus, Vibrio fluvialis, Vibrio cholerae, Aeromonas hydrophila*). OINBs were effective in inhibiting growth of these bacteria; however, the dominant culturable species shifted to members within the genus Bacillus following the most rigorous treatment (50% OINB) used in this study. *Bacillus pumilus* is known to be resistant to high levels of oxidative stress, which is consistent with monogeneric growth observed at 50% OINB [24,25]. Members of this genus are also known spore forming bacteria, so it is possible that these species survived the increased oxidative stress supplied by the OINBs by forming spores that subsequently germinated during plate culturing. Additionally, several species within this genus are important nitrogen fixers and phosphorus solubilizes in soil, which could result in increased nitrogen and phosphorus in the water depending on abundance and relative contribution of these microbes to the aquatic nutrient cycle [26]. The shift in community structure was also evident in the shift in the dominant type of ARGs found in the different treatments. In the non-OINB treated conditions, a wide range of ARGs previously identified in *Vibrio* and *Aeromonas spp* were observed while following OINB treatment, the ARG profile shifted to one dominated by ARGs associated with *Bacillus spp.*

5. Conclusions

OINBs are effective at inhibiting growth of a wide range of environmental bacteria, including known pathogens. OINBs are not completely effective at limiting growth of all bacteria and likely serve as a strong selection pressure based on the persistence of *Bacillus sp*. at the highest treatment strength in this study. Nonetheless, the results observed during this study demonstrate that OINBs are a potential potent disinfectant for the treatment of pathogens in contaminated waters that arise from natural disasters such as hurricanes or flooding events. Moreover, OINBs ability to impact microbial communities either by eliminating a microbe or dramatically slowing its grow represents a powerful tool in enhancing and global public health by providing a process to reduce potential bacterial pathogens without the use of antibiotics in the case of aquaculture or blunting acts of bioterrorism involving water-borne pathogens.

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