INTRODUCTION

Bacteriocins are one of a variety of antimicrobial substances produced by bacteria (12). Usually these antibiotic peptides are either strain or species specific, but some can target a broader range of species (3, 13). They can range from large, structured proteins with high molecular weight (like colicin) to simple, small peptides (like nisin) (4). Since the proteolytic enzymes of the gastrointestinal tract degrade bacteriocins, they are non-toxic and non-antigenic to animals, including humans (5, 7). These qualities brought about the suggestion of their clinical use as a replacement for antibiotics in the 1950's but because of their specificity they proved impractical (21). They have also been used as natural food preservatives (4, 21). In the US, starter cultures of bacteriocin producing bacteria are used to make cheeses and yogurts (20, 21). Bacteriocins are also used in such things as pasteurized egg production (19).

The genes that code for bacteriocins can be either chromosomally or plasmid coded (3). All major lineages of Archae (21) and Bacteria, including both gram-negative and gram-positive species of bacteria, have been found to produce them (3). Bacteriocin-producing bacteria have been isolated from a variety of habitats including soil, food, and the human body (4). Bacteriocins are often produced during stressful conditions such as nutrient depletion or overcrowding where their production helps the bacteria provide space to search for food and to multiply (1,22). Almost all bacteriocins are produced during post-logarithmic growth, when further growth may depend on the elimination of competitors (1). The production of bacteriocins gives the producing strain an advantage in invading and maintaining their numbers (21), however, the production of bacteriocins does incur a metabolic cost on the producing cell (9).

Past studies have shown 5 to 8% of marine bacteria tested to produce antimicrobial substances (11, 20). In 2001, Long and Azam found that 50% of the marine isolates tested produced antagonistic activity. This study also showed that antagonistic activity was more common by bacteria attached to particulate material than by those that are free-living (11). It has been suggested that these inhibitory compounds may be used to prevent other bacteria from colonizing the particulates (11). Studies have shown that attachment may be beneficial since organic matter adsorbs to particulate material creating a supply of possible nutrients and attachment may be affected by the quality of the particulate material present (8). Bacteria attached to particulate material may not be competing for the same resources as free-living bacteria (17). The attachment to particulate material has been found to increase the metabolism of bacteria 2 to 3 times higher than that of free-living bacteria (8, 10, and 11). A higher production of antimicrobial compounds by freshwater isolates than saltwater isolates also has been shown in previous studies (10). The water column in freshwater environments often has more interaction with the shore and benthos than saltwater environments and may contribute to the abundance of particulate material in freshwater. Furthermore, bacteria attached to particulate material found in waters that did not get flushed regularly by tides had significantly higher uptake of organic compounds than in waters that were flushed regularly. The uptake of organic carbon may be lower in the open ocean because the production of new particulate material is low in these waters (10).

While many bacteriocins and the bacteria that produce them are well known, bacteriocins produced by members of the genus *Vibrio* have not been well studied. *Vibrio* spp. are among the most common bacteria in the world's oceans (27). The first isolation and description of a bacteriocin produced by a *Vibrio* spp was by McCall and Sizemore in 1979 (14) and Hoyt and Sizemore in 1982 (6). The bacteriocin was isolated from *Benecka harvey* that is now recognized as *Vibrio harveyi*. Recently the bacteriocin produced by *V. vulnificus* was isolated and partially characterized from the Wilmington, NC area (25). Many other *Vibrio* spp may also produce anti-microbial substances that may be used commercially.

Todar (2002) states that in aquatic habitats the ecology of *Vibrio* spp. and *Pseudomonas* spp. overlap but are unique in that *Vibrio* spp. prefer saltwater while *Pseudomonas* spp. prefer freshwater (27). *Pseudomonas* spp. are widely distributed in soil and water and can be found in 10% of healthy people. Many *Pseudomonas* spp. are broadly resistant to antibiotics (27). The production of bacteriocins has been well studied in *P. syringae* and *P. aeruginosa* strains, while little information has been gathered on bacteriocin production by other *Pseudomonas* spp (18).

The objectives of this study were to isolate from freshwater and saltwater sites near Wilmington, NC *Vibrio* and *Pseudomonas* spp. They will be separated into free-living and those attached to particulate material. They will then be screened for the production of antimicrobial activity against a range of related and unrelated strains. These presumptive bacteriocins may provide a replacement to antibiotics or may be useful as food preservatives.

METHODS

Water samples were collected once a month in sterile glass bottles from August 2002 through July 2003 in sites around Wilmington in Southeastern North Carolina. Samples were taken from three freshwater sites (all man-made ponds that are susceptible to run-off from urban environments): Greenfield Lake (Site 1), the retention pond on Randall Parkway (Site 2), and the pond at Hugh McRae Park (Site 3); and three estuarine tidal creeks (all near the intracoastal and subject to tidal flushing): Hewlett's Creek (Site 4), Whiskey Creek (Site 5), and the Intracoastal Waterway off the pier at the Center for Marine Science (Site 6) (Figure 1). After collection, particulate material was precipitated from the water column by gentle centrifugation at 1500 x g for 5 minutes (23). To isolate the attached bacteria, the precipitated particulate material was resuspended, vortexed vigorously for two minutes, and then plated directly onto agar plates (2). Free-living bacteria were isolated from the water samples by plating the supernatant water after the particulate material had been precipitated by centrifugation. Saltwater samples were plated onto seawater yeast extract (SWYE) agar while freshwater samples were plated onto freshwater yeast extract (FWYE) agar plates and both were incubated at room temperature for 48 hours. SWYE consists of 1% proteose peptone, 0.3% yeast extract, and 2% agar in artificial seawater solution, pH 7.2-7.4. Freshwater yeast extract agar is the same as SWYE except distilled water is used instead of artificial seawater. The colonies growing on the SWYE and FWYE plates were transferred onto thiosulfate-citrate-bile salts (TCBS) agar plates, a selective/differential medium for *Vibrio* spp. (25), and pseudomonas isolation agar (PIA) plates, a selective media for *Pseudomonas* spp. (24). Both the TCBS and PIA plates were incubated at room temperature for 24 hours.

The colonies that grew on TCBS (presumptive *Vibrio*) and PIA (presumptive *Pseudomonas)* plates were screened for antimicrobial activity against a range of bacteria.

Figure 1. Sample sites along Cape Fear River Estuary in Southeastern North Carolina

The strains tested against the presumptive *Vibrio* spp. bacteriocin included *Vibrio vulnificus, Pseudomonas fluorescens, Escherichia coli, Vibrio harveyi* and *Vibrio parahaemolyticus*. The strains tested against the presumptive *Pseudomonas* spp. bacteriocin included *Vibrio vulnificus, Pseudomonas fluorescens, Escherichia coli, and Pseudomonas aeruginosa*. Lawns of the indicator strains were made on SWYE agar and allowed to air dry. Presumptive *Vibrio* and *Pseudomonas* colonies were spotted onto the surface of the spread plates and incubated at room temperature for 24 hours (18). Colonies that produce zones of clearing were marked as having antimicrobial activity and saved for further analysis.

Regression analyses were run using SAS to determine if there were any significance differences in the presence of antimicrobial activity due to environmental factors. T-tests were run using SAS to determine if there were any significance differences in the presence of antimicrobial activity between the different groups of bacteria (8).

RESULTS

During the 12-month sampling period, 12,615 viable colonies were isolated. Approximately half (6,565) of the colonies were isolated from freshwater sites and half (6,050) from saltwater sites. More of the colonies collected from freshwater sites were isolated from particulate material (3,531) than free-floating (3,034). More of the saltwater isolates were also isolated from particulate material (3,550) than free-floating (2,500).

When the freshwater and saltwater isolates were combined, the genus *Pseudomonas* made up 27% (3378) of the total viable population while the genus *Vibrio* made up 14% (1799). In freshwater samples *Pseudomonas* spp. dominated and comprised an average of 40% of the total microbial population (18%-83% range) while *Vibrio* spp. averaged 3% (0-35% range) of the population. In saltwater samples *Vibrio* spp. were the dominant genus and comprised an average of 24% (7%-53% range) of the total population while *Pseudomonas* spp. averaged 8% (0-19% range).

Of the *Pseudomonas* colonies isolated, 84% came from freshwater samples. Likewise, most of the *Vibrio* colonies isolated (87%) came from saltwater samples. Of the *Pseudomonas* colonies that were isolated 60% were attached to particulate material, while 75% of the *Vibrio* colonies isolated were attached to particulate material.

Of the 5,177 presumptive *Vibrio* or *Pseudomonas* colonies collected during this study, 1,283 (25%) showed antimicrobial activity. Production of antimicrobial activity was more common by *Pseudomonas* colonies than by *Vibrio* colonies regardless of the sampling date (Figure 2). The total number of *Pseudomonas* colonies with activity (1148) was significantly more than the number of *Vibrio* colonies with activity (140) ($p<0.01$).

More antimicrobial activity was seen by bacteria collected from freshwater sites than by bacteria from saltwater sites (Figure 3). Of the *Pseudomonas* colonies showing activity, 82% came from freshwater samples. Salinity significantly affected the production of bacteriocins by *Pseudomonas* spp. isolated from saltwater sites with isolates collected from sites with low salinity showing more production than isolates collected from sites with high salinity ($p<0.01$). Of the *Vibrio* colonies with antimicrobial activity, only 1 came from a freshwater sample while most came from saltwater samples.

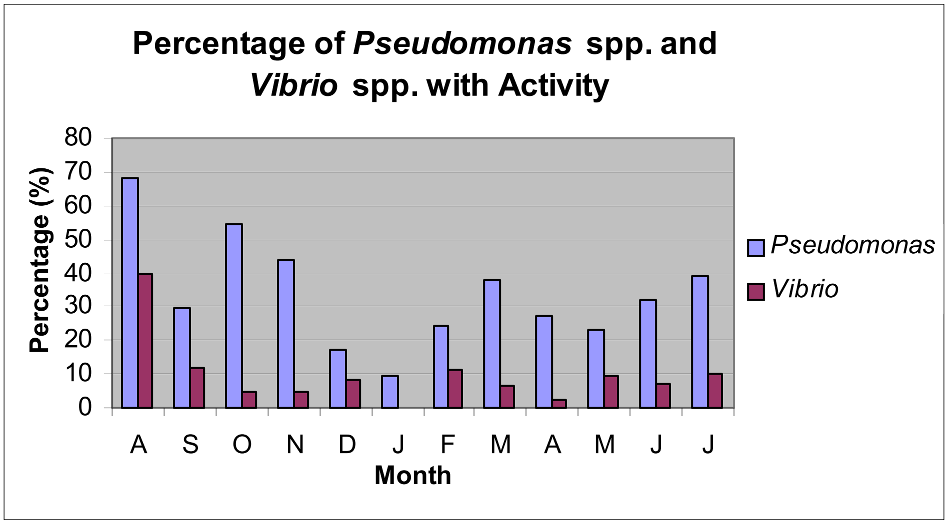

Figure 2: Percentage of *Pseudomonas* spp. and *Vibrio* spp. possessing antimicrobial activity from all sites for the sampling period.

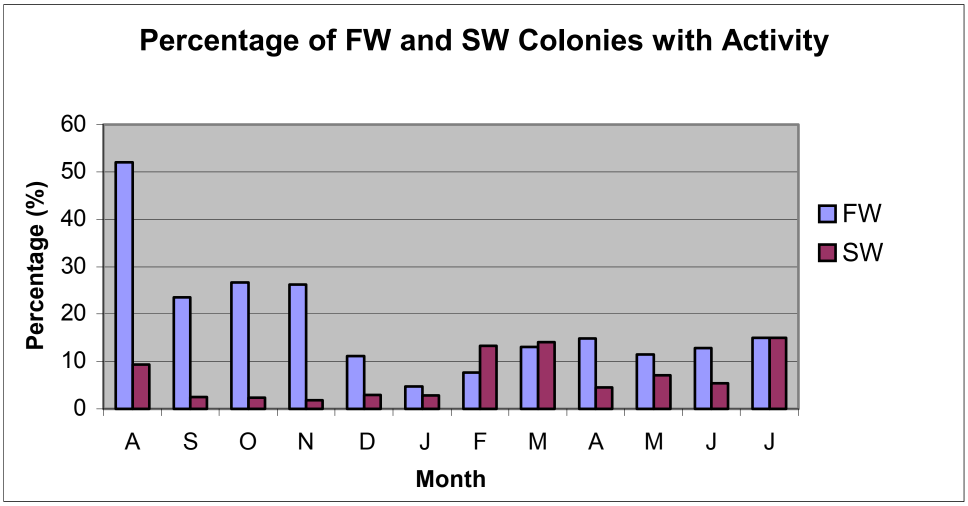

Figure 3: Percent of colonies possessing antimicrobial activity from saltwater samples and freshwater samples for a 12-month time period, 2002-2003.

Antimicrobial activity was also more common in isolates collected from particulate material in both freshwater and saltwater samples regardless of sampling date (Figure 4). This trend was still seen when the freshwater and saltwater isolates with activity were broken down by attached verses free-living (Table 1). Among *Pseudomonas* spp. isolated from freshwater and saltwater those that produced antimicrobial activity mostly came from particulate material (Table 2). The total number of *Pseudomonas* spp. with activity that came from particulate material was significantly different than those that were free-living ($p<0.01$). Most of the *Vibrio* spp. isolated with activity also came from particulate material (Table 3). The total number of *Vibrio* spp. with activity that came from particulate material was significantly different than those that were free-living ($p<0.01$).

Over the 12-month period the water temperature ranged from 5 to 30°C but there was little difference in the pattern of the average water temperatures of the FW and SW sites collected on the same date. The amount of dissolved oxygen (DO) was similar in the FW and SW sites with a marked increase in DO during the colder months, which peaked at 28 mg/L in January and March (Figure 5). Salinity during the sampling period remained constant for the FW sites (0.1 ppt). The salinity of the SW sites varied during the sampling period with a range of 8-34 ppt and an average of 22.3 ppt. Except for salinity, there was little difference between the freshwater and saltwater sites in their physical parameters.

The number of bacteria collected at all sites and the number of bacteria that produce antimicrobial activity both dropped during the colder months (Figure 6). Higher

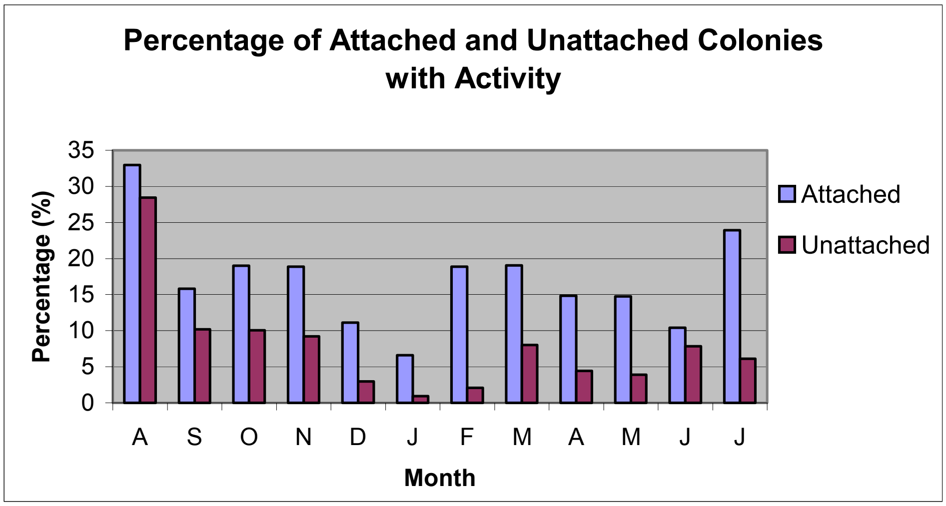

Figure 4: Percent of colonies possessing antimicrobial activity from bacteria adhered to particulate matter and free floating bacteria for the sampling period.

Table 1. The percentage of freshwater and saltwater isolates that are attached versus unattached

Source	# with Activity	Attached %	Unattached %
FW	937	66	34
SW	346	78	22

Table 2. The percentage of *Pseudomonas* spp. with activity that are freshwater versus saltwater and attached versus unattached

Pseudomonas	with Activity	Attached	Unattached
FW	82%	66%	34%
SW	18%	81%	19%

Table 3. The percentage of *Vibrio* spp. with activity that are freshwater versus saltwater and attached versus unattached

Vibrio	with Activity	Attached	Unattached
FW	0.7% (1)	100%* (1)	0
SW	99.30%	75%	25%

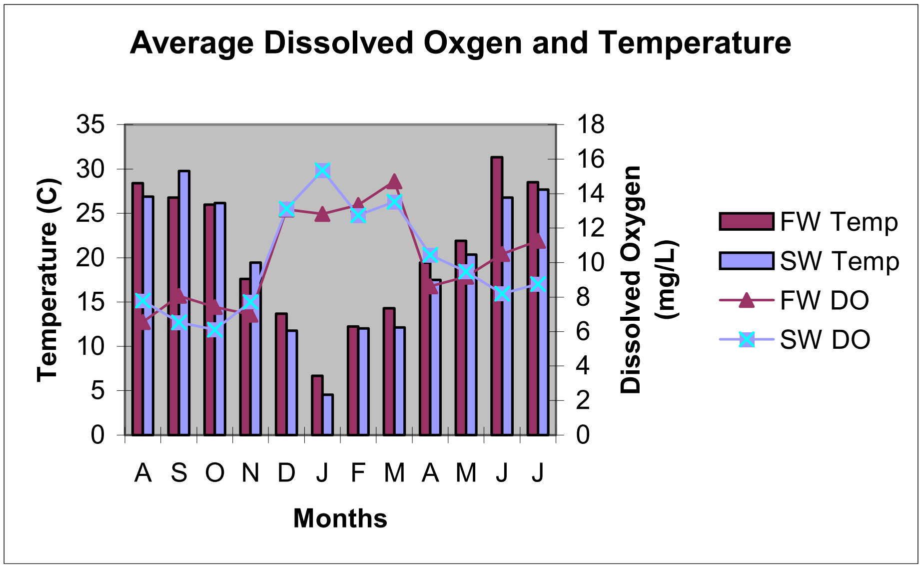

Figure 5: Average dissolved oxygen and temperature of freshwater and saltwater sites for the sampling period.

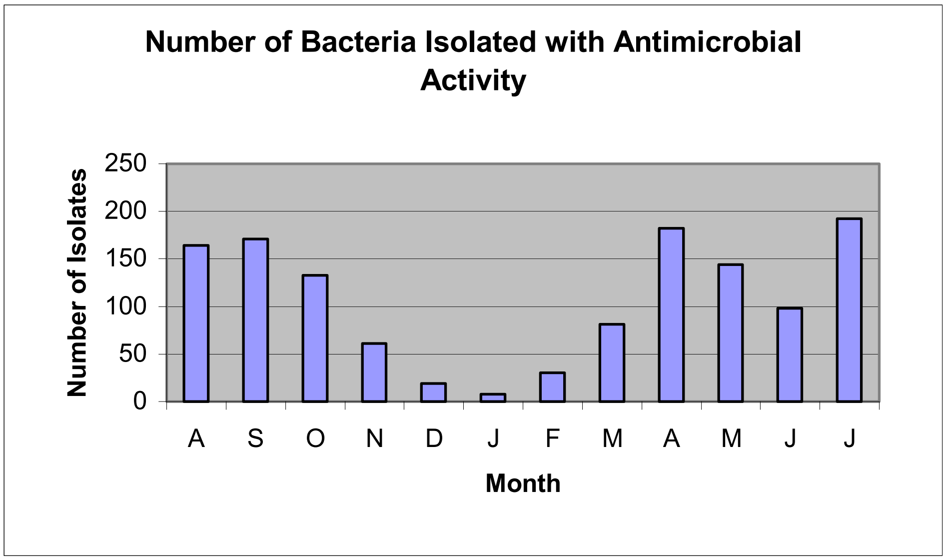

Figure 6: The number of bacteria with antimicrobial activity for the sampling season.

temperature significantly increased the production of bacteriocins by *Pseudomonas* spp. isolated from freshwater sites ($p<0.01$). Significantly more presumptive bacteriocins by *Vibrio* spp. were isolated from saltwater sites with high temperatures than by those isolated from waters with low temperatures ($p<0.01$).

Many of the antimicrobial colonies produced by *Pseudomonas* spp. in the study displayed activity against only one test organism, 68% against only *E. coli*, 17% against only *V. vulnificus,* 4% against only *P. aeruginosa* and .5% against only *P. fluorescens.* Only 8% of the *Pseudomonas* spp. that produced antimicrobial activity affected 2 bacteria (one being *E. coli*) and 3% affected 3 bacteria (one being *E. coli*). The presumptive bacteriocins produced by *Vibrio* spp. showed a broader range of antimicrobial activity with 42% showing antimicrobial activity against *V. parahaemolyticus,* 25% showing activity against *V. vulnificus*, 13% against *V. harveyi* and 10% against *E.coli*. Only 14 (10%) of the presumptive bacteriocins produced by *Vibrio* spp. affected 2 bacteria.

All of the freshwater sites had similar percentages of bacteria producing antimicrobial activity (approximately 14%) with freshwater site 2 having the highest. Almost all of the bacteria producing antimicrobial activity from the freshwater sites were *Pseudomonas* spp. The number of bacteriocins from bacteria isolated from freshwater sites was significantly higher in higher temperatures ($p<0.01$). Saltwater site 4 had the highest percentage of *Vibrio* colonies and *Pseudomonas* colonies displaying antimicrobial activity of all of the sites but only slightly higher than saltwater sites, site 5 & 6. The number of bacteriocins isolated from saltwater sites was significantly higher in higher temperatures ($p<0.01$). Saltwater Site 6 is the only site during the study that had more *Vibrio* colonies displaying antimicrobial activity than *Pseudomonas* colonies displaying antimicrobial activity (Figure 7). There were more bacteriocins produced by bacteria attached to particulate material than by free-living bacteria at all sites (Table 4).

DISCUSSION

Many of the viable bacteria isolated in the study (41%) were either presumptive *Vibrio* or *Pseudomonas* spp., as expected since both are common in aqueous environments. As predicted, *Pseudomonas* colonies were more abundant in freshwater samples than saltwater while *Vibrio* colonies were more abundant in saltwater samples than freshwater (27).

This study found more bacteria were associated with particulate material than free-living. The presence of particulate material would allow bacteria to occupy and exploit different niches and different resources. The high numbers of bacterial attachment may be explained by the access to a possible food source and the metabolic gain (8, 10). When bacterial metabolism increases, growth and reproduction rates also increase.

Pseudomonas spp. and *Vibrio* spp. exhibiting antimicrobial activity were common in our local estuarine and freshwater microbial assemblages. Antimicrobial activity was more common in *Pseudomonas* spp. (34%) than *Vibrio* spp. (8%). These data were similar to findings in the Suruga and Sagami Bays where the two most abundant bacteria that produced antimicrobial activity were *Pseudomonas* or *Vibrio* spp. with the *Pseudomonas* spp. producing more antimicrobial activity than *Vibrio* spp. (12).

Our data shows a higher percentage of bacteriocin production from bacteria attached to particulate material than those that are free-living. This supports the

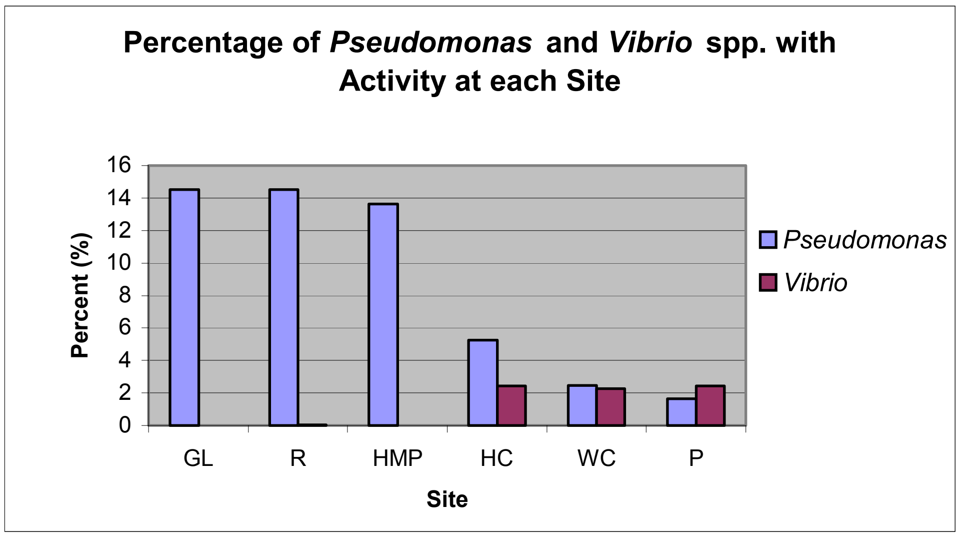

Figure 7: The percentage of *Pseudomonas* species and *Vibrio* species possessing antimicrobial activity at each site for the sampling period.

Table 4. Percentage of attached and unattached bacteria showing antimicrobial activity by site.

Site	Average Salinity	Total # Colonies	% with Activity	% Attached with Activity	% Unattached with Activity
1	0	994	31	22	9
2	0	1193	30	19	11
3	0	902	30	19	11
4	13	923	20	16	4
5	26	603	16	12	4
6	28	562	12	10	2

findings by Long and Azam (11). Regardless of the genus, most of the bacteria isolated in the freshwater and saltwater samples that produced antimicrobial activity were isolated from particulate material. The bacteria attached to particulate material have a more defined space to protect than free-floating bacteria. This means particulate material could be a very intense competitive environment where bacteria must struggle to survive and bacteriocins could provide a competitive edge. If attached bacteria are metabolically more active than free-living bacteria (8, 10) the attachment to particulate material may offset the cost associated with producing bacteriocins. This would allow those that produce bacteriocins to compete with the growth and reproduction of sensitive and resistant bacteria that do not have the metabolic cost of bacteriocin production.

Twice as many bacteria isolated from freshwater sites than from saltwater sites produced antimicrobial activity suggesting that antimicrobial activity may be more ecologically important in freshwater environments. This agrees with the findings in other studies (10). Freshwater sites had a higher percentage of *Pseudomonas* spp. with antimicrobial activity than saltwater sites. While the saltwater sites had more *Vibrio* spp. with activity than freshwater sites this percentage was comparatively low. The freshwater sites used in the study were not subjected to tidal flushing, increasing the abundance of available particulate material in freshwater environments. The man-made ponds were created to retain runoff water from the local environment. We think this collection of water from urban areas may help increase the concentration of particulate material and may provide bacteria with unique and more appealing particulate material than what is found in the SW sites. The abundance of available particulate material in freshwater environments, as suggested by Kirchman and Mitchell, provides bacteria with a solid substance on which they can grow (10). Since there is a metabolic advantage to being attached, the metabolic gain from attachment of bacteria may counter the cost of bacteriocin production making production a feasible and likely weapon.

In this study, a seasonal variation was seen and there was a drop in the number of bacteria isolated at each site during the colder months. This was expected for *Vibrio* spp. since the genus grows best in warm water. The genus *Pseudomonas*, however, has some psychrotrophs that can survive at temperature below 7°C. There was also a drop in the number of bacteria producing antimicrobial activity during colder months in free-floating and attached bacteria. The affect of temperature on bacterial activity is well known, with colder temperatures impeding bacterial activity (8). This could have inhibited the production of bacteriocins.

Most of the bacteriocins isolated in this study showed a narrow activity spectrum and very few attacked the *Pseudomonas* spp., which are known to be antibiotic resistant (27). Most of the bacteriocins isolated from *Pseudomonas* spp. only attacked *E. coli.* This may be due to the fact that both are commonly found in the human gut and are thus may be likely to vie for common resources. *E. coli* is considered a fecal coliform while *Pseudomonas* spp. commonly looked at in water quality studies are considered to be closely related to coliforms (15, 16). Bacteriocins produced by *Vibrio* spp. showed a broader range of activity mostly targeting other *Vibrio* spp. with a few affecting *E. coli* but none affecting *Pseudomonas* indicator strains. The presumptive *Vibrio* spp. bacteriocins targeted their close relatives since they mainly targeted members of the same genus and *E. coli*, which is in a family on the same level as *Vibrio* spp. in Bergey's Manual (27).

Freshwater site 2 seemed to have more of an optimum environment for the growth and multiplication of freshwater bacteria perhaps due to the highest average percentage of dissolved oxygen (DO) and lowest average salinity than any of the other sites. Saltwater site 4 had the lowest average salinity (13 ppt) and had the highest average percent of dissolved oxygen of the saltwater sites, which may contribute to the site having more *Vibrio* and *Pseudomonas* spp. producing activity than the other saltwater sites. The site was also further away for the intracoastal waterway than the other saltwater sites, which could indicate less effect from tidal flushing. Saltwater site 6 had the highest salinity (28 ppt) of all three saltwater sites and experienced the most tidal flushing effect, which may contribute to the small percentage of *Pseudomonas* spp producing activity at that site.

Overall, bacteria producing presumptive bacteriocins were common in our local estuarine and freshwater microbial assemblages. Local freshwater sites may provide optimum environments to isolate bacteriocin producing presumptive *Pseudomonas* spp. The fact that all three freshwater sites are man-made and are modified to retain runoff from urban environments may play a role in the abundance of presumptive bacteriocin production. Along with the lack of tidal flushing, the abundance of particulate material may be due to high particulate concentrations in local run-off water (26). The run-off water may provide the bacteria in the freshwater sites unique and more appealing particulate material than what would naturally be found in the waters. The saltwater sites have the advantage of tidal flushing which would allow the concentration of particulate material to be controlled by physically removing the particulate material that may be used by the bacteria. While presumptive bacteriocins produced by the *Pseudomonas* spp. are more common in our local waters, it may be more beneficial to look closer at the bacteriocins produced by the *Vibrio* spp. since they target a broader range of bacteria. Both species of bacteria isolated are gram-negative and were found to kill other gram-negative bacteria. These strains may be commercially useful since gram-negative bacteria are typically harder to kill with existing antibiotics and many food borne illness are caused by gram-negative bacteria. The strains that produced antimicrobial activity were saved for further testing.