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*Original Contribution*

# Increase in Antimicrobial Resistance in Bacteria Isolated from Stranded Marine Mammals of the Northwest Atlantic

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**Abstract:** Studies on marine mammals can inform our understanding of the environmental health of the ocean. To evaluate the potential for changes in antimicrobial resistance, we analyzed a database spanning 2004–2010 that consisted of bacterial isolate identity and antimicrobial sensitivity for stranded pinnipeds in the Northwest Atlantic. Samples ($n$ = 170) from treated animals yielded 310 bacterial isolates representing 24 taxa. We evaluated changes in antimicrobial class resistance from 2004 to 2010 for eight taxa. *Escherichia coli* displayed a significant increase in resistance to several antimicrobial classes. Other taxa displayed significant increases in resistance to aminoglycosides, and/or fluoroquinolones. In addition, we observed a significant increase in multiple antimicrobial resistance in cultures from untreated animals. These results demonstrate an increase in resistance among common bacterial pathogens of marine mammals over a time span of 6 years.

**Keywords:** temporal variation, antimicrobial resistance, rehabilitation, marine mammal, MAR index, one health

## Introduction

Recognition of the link between animal and human health occurred early in the nineteenth century and has been promoted by both medical and veterinary professionals over the last 50 years (Kahn et al. 2007). The concept of ''one medicine'' or ''one health'' is important because it recognizes not only the value of collaborative efforts among various medical practitioners but also collaborations among public health officials and biological or physical scientists. Recently, Bogomolni et al. (2008) conducted a survey of zoonotic pathogens in the Northwest Atlantic that reported high levels of waterborne pathogens and pathogenic bacteria in marine mammals, seabirds, and sharks that either stranded along the New England coast or were by-catch in fisheries of the Gulf of Maine. Most importantly, these zoonotic pathogenic bacteria displayed a wide range of antimicrobial resistance (AMR) (Rose et al. 2009).

Antimicrobial resistance can be the result of use or overuse of antimicrobials in clinical and veterinary settings and is a concern to humans and animals as it limits the treatment of infectious diseases and other pathologies.

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Despite efforts to reduce the dissemination of antimicrobials into the environment, AMR continues to be of significant concern and has prompted further calls for research into "natural" sources of AMR (Allen et al. 2010). The development of AMR occurs normally in the aquatic environment as bacteria are constantly exposed to antimicrobials selecting for resistance (Zhang et al. 2009). Rapid changes in AMR in aquatic systems are facilitated by the ability of bacteria to transmit resistance genes in a horizontal fashion (Taylor et al. 2011). In addition to antimicrobial exposure, other factors such as metal pollutants and human waste contamination can affect AMR (Baker-Austin et al. 2006; Martinez 2008) and anthropogenic sources of pollution are hypothesized as a major cause of AMR in marine microbes (Martinez 2009). While the heavy use of prescribed antimicrobials in clinical and veterinary settings is widely known to select for resistant bacteria, recent study indicates that minimal concentrations of antimicrobials, such as those found in water and soil samples, can also select for resistance (Gullberg et al. 2011). As the acquisition and exchange of antimicrobial-resistant genes can increase resistance in pathogens, create new pathogens, and alter natural microbial populations, it is important that we develop an understanding of the dynamics of AMR in the marine environment. One step towards the understanding of AMR dynamics is through the use of data collected from sentinel species such as marine mammals (Reddy et al. 2001).

The AMR patterns in microbes associated with marine animals in the Northwest Atlantic vary widely among host taxonomic groups (Rose et al. 2009). Surveys of AMR in microbes associated with marine animals have been conducted on cetaceans along the Florida coast (Schaefer et al. 2009), sea turtles in the Mediterranean (Foti et al. 2009), and pinnipeds admitted to rehabilitation centers along the California coast (Johnson et al. 1998; Lockwood et al. 2006). Samples taken from lesion sites revealed that it is common for bacteria isolated from pinnipeds to exhibit resistance to multiple antimicrobials. While the effects of antimicrobial treatments on rehabilitated marine animals have been evaluated for the bacterium *Escherichia coli* (Stoddard et al. 2009), we have found no reports on temporal comparisons (spanning $>$ 5 years) of AMR in marine species.

The multiple antimicrobial resistance (MAR) index is a tool utilized to identify anthropogenic sources of fecal contamination in water (Krumperman 1983). It also provides an indication of the overall degree of AMR in enteric bacteria and can be used to assess changes in AMR over time. Frequently, the index is applied to studies of areas that are subject to contamination from activities such as combined sewer overflows, leaking septic systems, ineffective sewage treatment facilities, and/or agricultural related activities (Kelsey et al. 2003; Chitanand et al. 2010; Shah et al. 2012). In addition, the MAR index has also been used to compare AMR in marine animal tissues both exposed and not exposed to the environment (Rose et al. 2009).

The National Oceanographic and Atmospheric Administration (NOAA) authorized Marine Animal Rehabilitation Center (MARC) at the University of New England's (UNE) Marine Science Center has sampled microbes from marine animals and tested their resistance profiles since 2004. Not only were these data collected to support real-time treatment decisions but the database also constitutes a time series of microbial abundance and associated AMR from stranded marine animals along Northwest Atlantic coastlines. We retrospectively analyzed this database focusing solely on pinnipeds with the following goals: (1) compile a list of the most prevalent bacteria in rehabilitated pinnipeds, (2) provide a temporal comparison of AMR in common bacteria of pinnipeds, and (3) test for temporal changes in AMR of enteric bacteria in untreated pinnipeds.

## Materials and Methods

### Animals and Sampling

Data on samples from marine animals admitted for rehabilitation from Jan 1, 2004 to Dec 31, 2010 were used for this study. Of the 544 animals admitted during that time, 100 were sampled for bacterial identification and sensitivity testing. Of the sampled animals, 84 were pinnipeds (*Phoca vitulina*, *Pagophilus groenlandicus*, and *Halichoerus grypus*). Samples were typically taken immediately upon admission or later during the animals' rehabilitation stay.

Because some pinnipeds were sampled from multiple locations on the body, a total of 170 samples were submitted for antimicrobial testing. Samples used in this analysis were collected from areas of concern as identified in veterinary assessments (e.g., swelling or discharge). Sampled sites included abscesses, lung, ears, eyes, nasal, oral, and anal cavities, umbilical discharges, vaginal/penile openings, and various integumentary wounds (Table 1). Blood samples were taken if a systemic infection was suspected. No samples used in this analysis were collected without a clear clinical indication of problems in the affected site; therefore, some sampling bias is inherent. Samples were collected postmortem under three conditions: (1) when the animal died in-house prior to treatment, (2) when an animal died with a

**Table 1.** Bacterial isolates identified from 2004 to 2010. Identification at the most specific level possible is provided along with the total number of isolates identified. Only isolates that underwent antimicrobial sensitivity testing are included.

| | Internal cavities | | Integumentary system | | | Digestive system | | Circulatory system | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Abdominal | Thoracic | Wound | Abscesses | Umbilical | Anal | Oral | Pericardial | Lymph | Blood |
| Gram-positive isolates | | | | | | | | | | |
| *Enterococcus* spp. | 2 | 1 | 4 | 2 | 2 | 5 | 1 | | | 4 |
| *Staphylococcus* spp. | | | 1 | | | | | | | 3 |
| *Streptococcus* spp. | | | 1 | | 1 | | | | | |
| Gram-negative isolates | | | | | | | | | | |
| *Acinetobacter* spp. | 1 | | 2 | 2 | | 1 | 1 | | | 1 |
| *Aeromonas* spp. | | | 1 | | | | | | | |
| *Alcaligenes* spp. | | | 1 | | | | | | | |
| *Bordetella bronchiseptica* | | | | 1 | | | | | | |
| *Brevundimonas vesicularis* | | | | | | | | | | |
| *Citrobacter* spp. | 4 | 1 | 5 | 1 | 3 | 2 | | | | |
| *Comamonas* spp. | | | | | | | | | | |
| *Enterobacter* spp. | 1 | | 2 | 1 | 1 | 2 | | | | |
| *Escherichia coli* | 5 | 2 | 5 | 4 | 3 | 4 | | 1 | 1 | 3 |
| *Klebsiella* spp. | 2 | | 5 | 6 | 1 | 1 | | | | |
| *Morganella morganii* | 4 | | 2 | 2 | | | | | | 1 |
| Non-enteric Gram-neg rod | | | | 1 | | 2 | | | | 1 |
| *Pantoea* spp. | | | | | | | | | | |
| *Proteus* spp. | | 1 | 8 | 1 | 1 | 1 | | | | 1 |
| *Providencia rettgeri* | | | | | | | | | | |
| *Pseudomonas* spp. | 1 | 2 | 6 | 3 | 6 | 1 | 1 | 2 | | 2 |
| *Raoultella ornithinolytica* | | | | | | | | | | |
| *Serratia* spp. | | | 1 | 1 | | | | | | |
| *Shewanella* spp. | | | 3 | | | 2 | | | | 1 |
| *Stenotrophomonas maltophilia* | | | 2 | | | | | | | |
| *Vibrio* spp. | | | | | | | | | | 1 |
| | 20 | 7 | 49 | 25 | 18 | 21 | 3 | 3 | 1 | 18 |

| | Respiratory system | | Reproductive system | Urinary system | Nervous system | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Lung | Nasal | Genital | Multiple[a] | Ear | Eye | Brain | Misc | Total |
| Gram-positive isolates | | | | | | | | | |
| *Enterococcus* spp. | 1 | | 1 | 4 | 3 | 1 | | 1 | 32 |
| *Staphylococcus* spp. | | | | | 4 | | | 1 | 9 |
| *Streptococcus* spp. | | | | | 1 | | | | 3 |
| Gram-negative isolates | | | | | | | | | |
| *Acinetobacter* spp. | | | 1 | | | | | 1 | 10 |
| *Aeromonas* spp. | | | | | | | | | 1 |
| *Alcaligenes* spp. | | | | | | | | | 1 |
| *Bordetella bronchiseptica* | 2 | 2 | | | | | | | 5 |
| *Brevundimonas vesicularis* | | | | | 1 | | | | 1 |
| *Citrobacter* spp. | 1 | 5 | 1 | | | | | 1 | 24 |

**Table 1.** continued

| | Respiratory system | | Reproductive system | Urinary system | Nervous system | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Lung | Nasal | Genital | Multiple[a] | Ear | Eye | Brain | Misc | Total |
| *Comamonas* spp. | 1 | | | | | | | | 1 |
| *Enterobacter* spp. | 1 | 1 | 1 | | 4 | | | | 14 |
| *Escherichia coli* | 1 | 3 | 3 | 1 | 13 | | | | 49 |
| *Klebsiella* spp. | 1 | 2 | 2 | | 9 | 1 | 1 | | 31 |
| *Morganella morganii* | 1 | | 1 | 1 | 5 | 1 | | | 18 |
| Non-enteric Gram-neg rod | | | | | | 1 | | | 5 |
| *Pantoea* spp. | | | | | 1 | | | | 1 |
| *Proteus* spp. | 1 | 1 | 2 | 3 | 6 | | 1 | | 27 |
| *Providencia rettgeri* | | | 1 | | | | | | 1 |
| *Pseudomonas* spp. | 4 | 7 | | 1 | 23 | 1 | 2 | 1 | 63 |
| *Raoultella ornithinolytica* | | | | | 1 | | | | 1 |
| *Serratia* spp. | | | | | | | | | 2 |
| *Shewanella* spp. | 1 | | | | | | | | 7 |
| *Stenotrophomonas maltophilia* | | | | | | | | | 2 |
| *Vibrio* spp. | | 1 | | | | | | | 2 |
| | 15 | 22 | 13 | 10 | 71 | 5 | 4 | 5 | 310 |

[a]Urine, kidney, urachus, urogenital

wound or known infection, and/or (3) when an internal wound or infection was discovered during necropsy. Postmortem sample locations included brain, lung, abdominal and thoracic cavities, ear canals, pericardial and lung fluids, urachus, kidney, and bladder.

## Bacterial Identification and Sensitivity Profiles

Samples were collected using sterile aerobic and anaerobic culture swabs and stored in the appropriate transport medium prior to shipment. Samples from necropsied animals were collected using sterile cotton swabs and stored in sterile cryogenic tubes. In 2004 *only*, samples were sent on ice overnight and processed at either the University of New Hampshire Veterinary Diagnostic Laboratory (Durham, New Hampshire, USA) or the Connecticut Veterinary Medical Diagnostic Laboratory (Storrs, Connecticut, USA). From 2005 to 2010, samples were processed *only* at the IDEXX Reference Laboratory (North Grafton, Massachusetts, USA). Swabs were analyzed according to guidelines set by the Clinical and Laboratory Standards Institute (CLSI 2009). The majority of the data from 2005 to 2010 was generated by the IDEXX laboratory. However, the other two labs utilized in 2004 applied the same identification methods and any inconsistencies were related to the taxonomic level reported. Individual bacterial isolates were identified from each sample by use of standard methods such as biochemical tests, gram stains, microscopic morphologies, and growth in various media. Antimicrobial susceptibility was determined using the Kirby–Bauer disk diffusion technique (Bauer et al. 1966), in which the vendor chose the antimicrobials used in susceptibility testing. Only isolates that were tested against a panel of antimicrobials were used for this study, and only antimicrobials that were assayed in multiple years were evaluated in the temporal comparisons. These included amikacin, gentamicin, tobramycin, amoxicillin/clavulanic acid, amoxicillin, ampicillin, penicillin, piperacillin, ticarcillin, ceftiofur, ceftazidime, cephalexin, cephalothin, ciprofloxacin, enrofloxacin, marbofloxacin, chloramphenicol, trimethoprim/sulfamethoxazole, and tetracycline.

## Database Management and Statistical Analysis

For each bacterial isolate, information regarding test date, animal and tissue type, isolate identification, and antimicrobial panel sensitivity was entered into a spreadsheet and

combined with information regarding antimicrobial treatment periods extracted from the animals' medical files. This spreadsheet was then sorted to provide antimicrobial information by isolate type, year, and timing of sample collection relative to in-house antimicrobial treatment. We were not able to analyze the dataset by tissue type due to sample size limitations. If the exact same resistance profiles were found for bacteria from multiple sampled sites in an individual animal, then only one profile from that animal was included in the analysis.

We excluded the profiles of any antimicrobials considered ineffective due to natural or inherent resistance in a particular bacterium. We took a conservative approach in the removal of inherently ineffective antimicrobials so that we did not bias our analyses and consulted multiple sources for natural resistance data (Stock and Wiedemann 1998, 1999, 2001, 2002; Merck Veterinary Manual Online: http://www.merckmanuals.com/vet/index.html). In all cases, where naturally ineffective antimicrobials were used, the removal of antimicrobials resulted in a decrease in sample size and complete removal of a class for that bacterium. For *Proteus* spp., tetracycline resistance profiles were removed and for *Pseudomonas* spp. the chloramphenicol resistance profiles were also removed from the analysis. In addition, the beta-lactam resistance profiles were removed from the analysis for both *Klebsiella* spp. and *Morganella morganii*. The removal of some of the cephalosporin AMR profiles also resulted in a decrease in sample size for *Morganella morganii*. *Escherichia coli*, *Enterobacter* spp., and *Enterococcus* spp. were not intrinsically resistant to any of the antimicrobials used in the sensitivity tests.

Due to changes in the use of antimicrobials within a class, such as replacement of ampicillin by amoxicillin, antimicrobials assayed across time periods were grouped by class: aminoglycosides (amikacin, gentamicin, tobramycin), beta-lactams (amoxicillin, amoxicillin/clavulanic acid, ampicillin, penicillin, piperacillin, ticarcillin), cephalosporins (ceftiofur, ceftazidime, cephalexin, cephalothin), fluoroquinolones (ciprofloxacin, enrofloxacin, marbofloxacin), amphenicols (chloramphenicol), the carbapenem imipenem, sulfonamides (trimethoprim/sulfamethoxazole), and tetracycline. While we recognize that not all members of an antimicrobial class act in the same manner, we wanted a more concise means of synthesizing the degree of *multiple* antimicrobial resistance. Because classes are commonly referenced in veterinary manuals and peer-reviewed literature, we felt a class approach would not be misleading as we are not addressing the specific actions or mechanisms of AMR, but rather general trends. If there were not enough values within the antimicrobial class to meet the minimum statistical criteria, no data were reported for that antimicrobial class.

Fisher's Exact Test (Sokal and Rohlf 2012; www.langsrud.com) was used to evaluate whether the proportion of bacterial isolates reported as susceptible versus resistant varied between the paired time periods. We also tested for variation in the timing of sample collection (i.e., pre-, during-, or post-treatment) between time periods for each bacterium using *G* tests of independence (Sokal and Rohlf 2012). To minimize the family wide error rate when conducting multiple tests, we also applied a Bonferroni correction to adjust *P* values within a given taxa of bacteria (Sokal and Rohlf 2012). Specifically, if more than 2 Fisher's exact tests were conducted for a bacterium, then the correction was applied. If less than or equal to 2 tests were conducted for a bacterium, then no correction was applied.

## Temporal Comparisons

For temporal comparisons, only taxa with sufficient sample sizes for statistical comparisons were examined including *Citrobacter* spp., *Escherichia coli*, *Enterobacter* spp., *Enterococcus* spp., *Klebsiella* spp., *Morganella morganii*, *Proteus* spp., and *Pseudomonas* spp. Because the database contains more data points in recent years, data from earlier years were combined to meet a minimum statistical criterion of ten values per microbe and time period. The years were grouped to create two distinct time periods for each bacterial taxon. In all cases, data from at least 1 year were omitted to create a gap between time periods. This step ensured that at least 1 year elapsed between samples classified in different time periods and prevented our temporal comparison from being affected by samples collected only 1 month apart (i.e., December of 1 year compared to January of the following year).

## Multiple Antimicrobial Resistance Index

The multiple antimicrobial resistance (MAR) index (Krumperman 1983) was utilized to test for changes in enteric bacteria. For this comparison, we focused on a subset of individuals that received no antimicrobial drug treatment prior to sensitivity testing or during their rehabilitation period. During 2004–2010, a total of 18 pinnipeds had no antimicrobial drug treatment prior to testing and 16 of these individuals had enteric bacteria cultured and tested. We tested two hypotheses using this subset of

individuals: (1) MAR index values are significantly different over time and (2) correlated with the number of days in the facility. For the first hypothesis, profiles from enteric bacteria were divided into two distinct groups: isolates from 2004 to 2006 (temporal period one) and 2009 to 2010 (temporal period two). The MAR indices were calculated according to Krumperman (1983) using the following equation:

$$\mathrm{MAR} = a/(b \times c),$$

where $a$ is the aggregate resistance or sum of the resistance values, $b$ is the maximum number of antimicrobial drugs tested, and $c$ is the total number of isolates. We used this method to generate a composite MAR index for all animals to conduct the temporal comparison of enteric bacteria. Individual MAR indices for the 16 animals were used to test the hypotheses regarding enteric bacteria and rehabilitation duration. The MAR index defines values $<0.200$ as indicative of non-point sources, whereas values $>0.250$ are indicative of point sources (Krumperman 1983). Fisher's exact test was used to determine if sensitivity and resistance values were significantly different among the time periods and Spearman's coefficient of rank correlation was used to test for a significant correlation between the days in the facility and the MAR index (Sokal and Rohlf 2012).

## Results

### Isolate Identification

We identified 310 isolates of bacteria that conformed to initial inclusion criteria from a total of 84 pinnipeds with 24 taxa identified among the 170 swabs collected (Table 1). Because of inconsistency in the level of identification, i.e., sometimes at the species and other times genus level, most isolates were grouped at the generic level and species identity ignored. The majority (85%) of isolates identified were Gram-negative. *Pseudomonas* spp. isolates were the most frequent (24%) of the identified Gram-negative bacteria with *Escherichia coli* the second most commonly identified at a frequency of 15% (Table 1).

### Temporal Comparison of AMR

For the temporal comparison of all bacteria, a total of 79 pinnipeds were included in the analysis regardless of treatment (2004, $n = 12$; 2005, $n = 2$; 2006, $n = 5$; 2007, $n = 13$; 2008, $n = 8$; 2009, $n = 13$; 2010, $n = 26$). Three species of pinnipeds were represented: *Phoca vitulina* ($n = 74$), *Pagophilus groenlandicus* ($n = 3$), and *Halichoerus grypus* ($n = 2$). Eight of 24 bacterial taxa identified had sufficient sample sizes to support a temporal comparison and 10 of 32 comparisons exhibited significant differences in AMR over time after Bonferroni correction (Fig. 1). *Escherichia coli* exhibited significant increases ($P < 0.007$) in AMR to three of the six antimicrobial classes tested between the 2004–2006 and 2010 time periods. *Morganella*

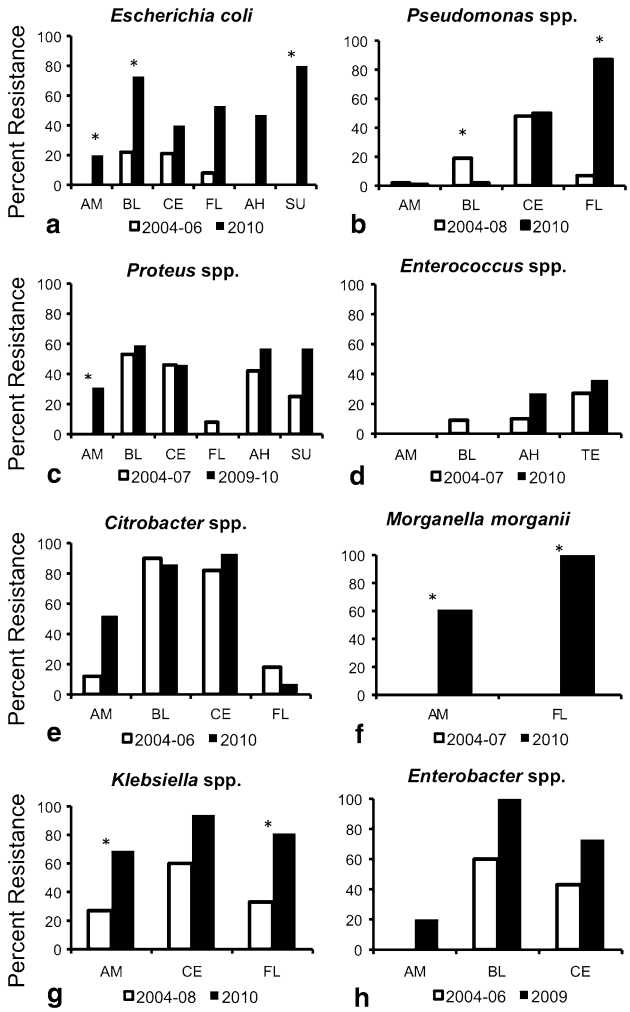


**Figure 1.** Temporal variation in antimicrobial resistance. Antimicrobial resistance percentages for eight taxa against 2–6 antimicrobial classes during two different time periods are shown. Time periods vary among taxa and correspond to the years displayed in each legend. Statistically significant differences in susceptibility and resistance after Bonferroni correction are indicated with *asterisks*. *AM* Aminoglycosides, *BL* beta-lactams, *CE* cephalosporins, *FL* fluoroquinolones, *AH* amphenicol, *SU* sulfonamide, *TE* tetracycline. Alphabetical designations (**a**–**h**) were added to the individual graphs to facilitate the discussion on observed trends.

*morganii* and *Klebsiella* spp. showed a significant increase ($P < 0.001$ and $P < 0.017$, respectively) in resistance to two classes of antimicrobials, the aminoglycosides and the fluoroquinolones. In only four comparisons did the AMR percentage decrease, but the apparent decrease was significant in only one of the four comparisons (Fig. 1b).

Overall, four of eight taxa (*Escherichia coli*, *Proteus* spp., *Morganella morganii*, and *Klebsiella* spp.) displayed a significant increase ($P < 0.007$, $P < 0.008$, $P < 0.001$, and $P < 0.017$, respectively) in resistance against aminoglycosides within the past 6 years (Fig. 1). In addition, three of six taxa (*Pseudomonas* spp., *M. morganii*, and *Klebsiella* spp.) exhibited a significant increase ($P < 0.013$, $P < 0.001$, and $P < 0.017$, respectively) in resistance to the fluoroquinolones. *Escherichia coli* displayed a significant increase in resistance to sulfonamide classes ($P < 0.008$; Fig. 1a). None of the antimicrobial classes tested for *Enterococcus* spp. displayed a significant difference in resistance between time periods ($P > 0.013$).

The *G* tests revealed no significant differences in the frequency of pre-, during-, and post-treatment sampling among time periods for any taxa, suggesting that temporal differences in resistance are not an artifact of variation in the timing of sampling.

### MAR in Untreated Seals

For the assessment of multiple antimicrobial resistance in enteric bacteria, resistance profiles were analyzed from a total of 16 individual seals, including *Phoca vitulina* ($n = 14$), *Pagophilus groenlandicus* ($n = 1$), and *Halichoerus grypus* ($n = 1$). Of these 16 individuals, 12 were classified as neonates, two as weanlings, with one juvenile and one adult rounding out the number (Table 2). *Escherichia coli* was the most prevalent of the enteric bacteria with *Aeromonas* spp., *Citrobacter* spp., *Enterobacter* spp., *Enterococcus* spp., *Hafnia* spp., *Morganella morganii*, *Pantoea* spp., *Proteus* spp., *Providencia rettgeri*, *Raoultella ornithinolytica*, *Serratia* spp., and *Streptococcus* spp. also present. There was a significant increase in resistance between the temporal periods ($P < 0.001$) and higher MAR index, 0.311, during the 2009–2010 temporal period (Table 2). To determine whether exposure time within the facility (as measured in days) was correlated to the observed differences in MAR values between temporal groups, we tested for a correlation in number of days in the facility and the MAR index for individuals. The average number of days in the facility prior to sampling was 4.4 days (range 1–26). The Spearman's coefficient of rank correlation was not significant at the 0.05 level ($r = 0.027$, df = 14), nor did we find a significant difference (two-tailed Fisher's exact test, $P = 0.5846$) in the age class distribution between the temporal periods. Thus, any patterns between time periods are not likely to have been generated by differences in time in treatment or the frequency of different aged hosts.

**Table 2.** Numbers of individuals, age classes, swabs, isolates, and antimicrobial drugs from the 16 pinnipeds not exposed to antimicrobial drug treatment prior to sensitivity testing on associated enteric bacteria.

| | 2004–2007 | 2009–2010 |
|---|---|---|
| Total # of animals | 9 | 7 |
| Neonates* | 6 | 6 |
| Weanlings, juveniles, adults* | 3 | 1 |
| Swabs | 13 | 10 |
| Isolates | 28 | 34 |
| Antimicrobial drugs | 18 | 19 |
| Susceptibility counts^ | 316 | 254 |
| Resistance counts^ | 42 | 201 |
| %Antimicrobial resistance | 11.7 | 44.2 |
| MAR index | 0.083 | 0.311 |

* No significant difference, ^ significant difference ($P < 0.001$).

## Discussion

### Overall Patterns of Diversity

The bacterial isolates identified in our study are similar to those detected in pinnipeds in previous studies (Johnson et al. 1998; Thornton et al. 1998; Lockwood et al. 2006; Bogomolni et al. 2008), with a few exceptions. In these previous studies, *Escherichia coli* and *Enterococcus* spp. were the most common bacteria isolated, whereas in our study *Pseudomonas* spp. were the most common. In addition, bacteria such as *Listeria* spp. and *Edwardsiella* spp. previously found in pinnipeds (Thornton et al. 1998; Bogomolni et al. 2008) were not detected in our samples.

Enterobacteriaceae comprised 54% of the isolates from 2004 to 2010 (Table 1) and members of the family are typical of the gut flora in mammals. Anal swabs could have biased the overall isolate composition, but only six anal swabs were taken during this period. Consequently, we suspect that some members of the Enterobacteriaceae are

likely common in wounds and infections of marine animals. These findings are consistent with the previous studies on rehabilitated pinnipeds (Johnson et al. 1998; Thornton et al. 1998; Lockwood et al. 2006). It is likely that these bacteria are non-pathogenic and opportunistic secondary invaders of wounds. We detected a significant increase in AMR over time in at least four taxa of Enterobacteriaceae (Fig. 1).

## Temporal Variation in AMR from 2004 to 2010

In 90% of the temporal comparisons in which we detected significant differences between time periods, AMR increased (Fig. 1). *Escherichia coli* displayed a significant increase in resistance to three antimicrobial classes and for *Morganella morganii* two antimicrobial classes exhibited a significant increase. *Escherichia coli* was the only taxon to exhibit significant increases in resistance to the beta-lactams and the sulfonamides (Fig. 1a). The one case in which a significant decrease occurred was beta-lactams in *Pseudomonas* spp. (Fig. 1b). This was likely due to changes in types of antimicrobials tested against *Pseudomonas* spp., e.g., penicillin and ampicillin were commonly used from 2004 to 2007 and more effective antimicrobials such as piperacillin and ticarcillin were consistently used from 2004 to 2010. These factors resulted in the apparent decrease in resistance percentage across the time periods. Multi-drug resistance has been well documented in *Pseudomonas* spp. and especially *Pseudomonas aeruginosa* with intrinsic resistance initially attributed to the outer membrane (Nikaido 1989). Subsequent research demonstrated the importance of multi-drug efflux pumps to the prevention of antimicrobial activity by most beta-lactams, older quinolones, chloramphenicol, tetracycline, macrolides, trimethoprim-sulfamethoxazole, and the fluoroquinolones (Poole 2001). More recently, Schaible et al. (2012) have reported increases in antibiotic resistance in *P. aeruginosa* under hypoxic conditions. These latter findings highlight the threats to both marine organisms and humans in coastal marine environments, which are increasingly subject to hypoxic events (Diaz and Rosenberg 2008; Rabalais et al. 2010).

The two classes of antimicrobials that exhibited the greatest number of significant increases were the aminoglycosides and the fluoroquinolones. From 1995 to 2002, the fluoroquinolones were the most prescribed antimicrobials in hospital settings in the United States, increasing threefold during that time (Linder et al. 2005). In 1999, 81% of patients examined were inappropriately prescribed fluoroquinolones (Lautenbach et al. 2003), leading to warnings that continued overuse would ultimately result in an increase in resistance. Recent study has established that only a minimal concentration of ciprofloxacin (100 pg/ml) is needed to trigger ciprofloxacin resistance in *E. coli* (Gullberg et al. 2011), a concentration now common in soils and water (Kemper 2008). Based on our analyses, the earlier warnings regarding fluoroquinolones were justified as three taxa displayed a significant increase in resistance to this antimicrobial class over time (Fig. 1b, f, g).

While aminoglycosides are very effective antimicrobial agents, the presentation of adverse effects in clinical settings led to their underuse (Durante-Mangoni et al. 2009). In 2008, an increase in Gram-negative bacterial infections resulted in the renewed use of these antimicrobial compounds by physicians (Falagas et al. 2008). Analysis of this database indicates that at least four Gram-negative taxa have significantly increased resistance to this class over the past 6 years (Fig. 1a, c, f, g).

During the periods of rehabilitation reviewed in this study, the impact of nosocomial infections was not tested; therefore, we caution that the presence of resistant bacteria in the facility could affect % resistance during the temporal periods. Our temporal results from a veterinary setting, however, correspond with other environmental and clinical longitudinal studies in demonstrating an increase in AMR. A significant increase in beta-lactam (includes cephalosporin and penicillin) resistant genes was found in soil samples from 1940 to present (Knapp et al. 2010), with the most significant increase occurring within the last 20 years. According to a 12-year study conducted in a clinical setting, *Klebsiella pneumoniae* strains have demonstrated a significant increase in resistance to trimethoprim/sulfamethoxazole, ciprofloxacin, and ceftazidime (van der Donk et al. 2011). We observed multiple bacterial taxa that exhibited a similar increase in AMR across multiple classes of antimicrobials during the most recent time periods (Fig. 1). These results suggest that the same patterns observed in terrestrial systems are also occurring in marine systems.

## Potential Sources of AMR

Although a large number of marine pathogens exist naturally (e.g., the *Vibrio* and *Mycobacterium* spp.), contaminant discharge through agricultural and municipal activity is thought to contribute significant additional pathogen loading to estuarine and coastal environments (Bartram

and Rees 2000). These same sources of pathogen contamination may also be the sources of antibiotics that are discharged in increasingly high quantities to our coastal environments, creating selection pressures on both pathogenic and non-pathogenic microorganisms. However, some marine aquaculture operations also utilize antimicrobials (e.g., oxytetracycline in the Atlantic salmon industry) and may be contributing to the selective pressures.

While we were concerned with the potential inflation of AMR after treatment with antimicrobials in rehabilitation, we were also interested in the status of animals not treated with antimicrobials in the facility and potential sources of AMR in the Gulf of Maine. In other words, can we infer what is happening to free-ranging animals by examining resistance profiles of animals not treated with antimicrobials in the rehabilitation center? To test this idea, we restricted our analysis to only enteric bacteria cultured from a subset of animals not exposed to a course of antimicrobials. Stoddard et al. (2009) determined that time, measured as days in a rehabilitation facility, was a significant risk factor for prevalence of resistant *Escherichia coli*; however, in our database, days at the facility were not correlated with multiple AMR in the 16 pinnipeds and the majority of these animals were tested 1–2 days after their arrival. Mirroring the larger database of pinnipeds and all bacteria, we again observed significant AMR differences on a temporal scale (Table 2). The MAR indices provide further support for significant increases in AMR over the 6-year time span. In 2004–2006, isolates had a MAR value of 0.083, suggesting that multiple AMR among isolates was acquired from non-point sources (Table 2; Krumperman 1983). In comparison, the overall MAR index for time period 2 (2009–2010) was $>0.250$, suggesting that a point source is a likely cause of the observed AMR. The significant increase in AMR coupled with the MAR index in non-treated pinnipeds between the two time periods suggests a temporal increase in AMR as a result of environmental processes (selection via anthropogenic point sources or natural reservoirs) in the Northwest Atlantic, and not simply an effect of time spent at the rehabilitation center. We interpret this subset of data with caution due to the findings of Rose et al. (2009) that higher levels of AMR are commonly found in sick or unhealthy marine animals and potentially related to differences in the overall health of animals. Rose et al. (2009) also reported that the MAR indices of exposed tissues of Gulf of Maine marine animals were indicative of point sources using Krumperman's metric (1983), but as in our study, no direct evidence of sources of AMR in the Gulf of Maine was determined. Without further studies we can only speculate that animals are acquiring AMR from either point sources, such as aquaculture or human wastewater streams, or natural reservoirs of AMR within the Gulf of Maine.

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## References

Allen HK, Donato J, Wang HH, Cloud-Hansen KA, Davies J, Handelsman J (2010) Call of the wild: antibiotic resistance genes in natural environments. *Nature Reviews Microbiology* 8:251–259

Baker-Austin C, Wright MS, Stepanauskas R, McArthur JV (2006) Co-selection of antibiotic and metal resistance. *Trends in Microbiology* 14:176–182

Bartram J, Rees G (2000) Monitoring bathing waters—a practical guide to the design and implementation of assessments and monitoring programmes. World Health Organization ISBN 0-419-24390-1

Bauer SAW, Kirby WM, Sherris JC, Turck M (1966) Antibiotic susceptibility testing by standardized single disc method. *American Journal of Clinical Pathology* 45:493–498

Bogomolni AL, Gast RJ, Ellis JC, Dennett M, Pugliares KR, Lentell BJ, et al. (2008) Victims of vectors: a survey of marine vertebrate zoonoses from coastal waters of the Northwest Atlantic. *Diseases of Aquatic Organisms* 81:13–38

Chitanand MP, Kadam TA, Gyananath G, Totewad ND, Balhal DK (2010) Multiple antibiotic resistance indexing of coliforms to identify high risk contamination sites in aquatic environment. *Indian Journal of Microbiology* 50:216–220

Clinical and Laboratory Standards Institute (2009) *Performance standards for antimicrobial disk susceptibility tests; approved standard*. Wayne, PA

Diaz RJ, Rosenberg R (2008) Spreading dead zones and consequences for marine ecosystems. *Science* 321:926–929

Durante-Mangoni E, Grammatikos A, Utili R, Falagas ME (2009) Do we still need the aminoglycosides? *International Journal of Antimicrobial Agents* 33:201–205

Falagas ME, Grammatikos AP, Michalopoulos A (2008) Potential of old-generation antibiotics to address current need for new antibiotics. *Expert Review of Anti-Infective Therapy* 6:593–600

Foti M, Giacopello C, Bottari T, Fisichella V, Rinaldo D, Mammina C (2009) Antibiotic resistance of gram negatives isolates from loggerhead sea turtles (*Caretta caretta*) in the central Mediterranean Sea. *Marine Pollution Bulletin* 58:1363–1366

Gullberg E, Cao S, Berg OG, Ilbäck C, Sandegren L, Hughes D, et al. (2011) Selection of resistant bacteria and very low antibiotic concentrations. *PLoS Pathogens* 7:e1002158

Johnson SP, Nolan S, Gulland FM (1998) Antimicrobial susceptibility of bacteria isolated from pinnipeds stranded in central and northern California. *Journal of Zoo and Wildlife Medicine* 29:288–294

Kahn LH, Kaplan B, Steele JH (2007) Confronting zoonoses through closer collaboration between medicine and veterinary medicine (as 'One Medicine'). *Veterinaria Italiana* 43:5–19

Kelsey RH, Scott GI, Porter DE, Thompson B, Webster L (2003) Using multiple antibiotic resistance and land use characteristics to determine sources of fecal coliform bacterial pollution. *Environmental Monitoring and Assessment* 81:337–348

Kemper N (2008) Veterinary antibiotics in the aquatic and terrestrial environment. *Ecological Indicators* 8:1–13

Knapp CW, Dolfing J, Ehlert PA, Graham DW (2010) Evidence of increasing antibiotic resistance gene abundances in archived soils since 1940. *Environmental Science and Technology* 44:580–587

Krumperman PH (1983) Multiple antibiotic resistance indexing of *Escherichia coli* to identify high-risk sources of fecal contamination of food. *Applied and Environmental Microbiology* 46:165–170

Lautenbach E, Larosa LA, Kasbekar N, Peng HP, Maniglia RJ, Fishman NO (2003) Fluoroquinolone utilization in the emergency departments of academic medical centers. *Archives of Internal Medicine* 163:601–605

Linder JA, Huang ES, Steinman MA, Gonzales R, Stafford RS (2005) Fluoroquinolone prescribing in the United States: 1995 to 2002. *The American Journal of Medicine* 118:259–268

Lockwood SK, Chovan JL, Gaydos JK (2006) Aerobic bacterial isolations from harbor seals (*Phoca vitulina*) stranded in Washington: 1992–2003. *Journal of Zoo and Wildlife Medicine* 37:281–291

Martinez JL (2008) Antibiotics and antibiotic resistance genes in natural environments. *Science* 321:365–367

Martinez JL (2009) Environmental pollution by antibiotics and by antibiotic resistance determinants. *Environmental Pollution* 157:2893–2902

Nikaido H (1989) Outer membrane barrier as a mechanism of antimicrobial resistance. *Antimicrobial Agents and Chemotherapy* 33:1831–1836

Poole N (2001) Multidrug efflux pumps and antimicrobial resistance in *Pseudomonas aeruginosa* and related organisms. *Journal of Molecular Microbiology and Biotechnology* 3:255–264

Rabalais NN, Diaz RJ, Levin LA, Turner RE, Gilbert D, Zhang J (2010) Dynamics and distribution of natural and human-caused hypoxia. *Biogeosciences* 7:585–619

Reddy ML, Dierauf LA, Gulland FMD (2001) Marine mammals as sentinels of ocean health. In: *Marine Mammal Medicine*, Dierauf L, Gulland F (editors), Boca Raton, FL: CRC Press, pp 3–13

Rose JM, Gast RJ, Bogomolni A, Ellis JC, Lentell BJ, Touhey K, et al. (2009) Occurrence and patterns of antibiotic resistance in vertebrates off the Northeastern United States coast. *FEMS Microbiology Ecology* 76:421–431

Schaefer AM, Goldstein JD, Reif JS, Fair PA, Bossart GD (2009) Antibiotic-resistant organisms cultured from Atlantic bottlenose dolphins (*Tursiops truncatus*) inhabiting estuarine waters of Charleston, SC and Indian River Lagoon, FL. *EcoHealth* 6:33–41

Schaible B, Taylor CT, Schaffer K (2012) Hypoxia increases antibiotic resistance in *Pseudomonas aeruginosa* through altering the composition of multidrug efflux pumps. *Antimicrobial Agents and Chemotherapy* 56:2114–2118

Shah SQA, Colquhoun DJ, Nikuli HL, Sorum H (2012) Prevalence of antibiotic resistance genes in the bacterial flora of integrated fish farming environments of Pakistan and Tanzania. *Environmental Science and Technology* 46:8672–8679

Sokal RR, Rohlf FJ (2012) *Biometry*, New York, NY: W.H. Freeman and Company

Stock I, Wiedemann B (1998) Identification of natural antibiotic susceptibility of *Morganella morganii*. *Diagnostic Microbiology and Infectious Diseases* 30:153–165

Stock I, Wiedemann B (1999) Natural antibiotic susceptibility of *Escherichia coli*, *Shigella*, *E. vulneris* and *E. hermannii* strains. *Diagnostic Microbiology and Infectious Diseases* 33:187–199

Stock I, Wiedemann B (2001) Natural antibiotic susceptibility of *Klebsiella pneumonia*, *K. oxytoca*, *K. planticola*, *K. ornithinolytica* and *K. terrigena* strains. *Journal of Medical Microbiology* 50:396–406

Stock I, Wiedemann B (2002) Natural antibiotic susceptibility of *Enterobacter amnigenus*, *Enterobacter cancerogenus*, *Enterobacter gergoviae* and *Enterobacter sakazakii* strains. *Clinical Microbiology and Infections* 8:564–578

Stoddard RA, Atwill ER, Conrad PA, Byrne BA, Jang S, Lawrence J, McCowan B, Gulland FMD (2009) The effect of rehabilitation of Northern Elephant Seals (*Mirounga angustirostris*) on antimicrobial resistance of commensal *Escherichia coli*. *Veterinary Microbiology* 13:264–271

Taylor NGH, Verner-Jeffreys DW, Baker-Austin C (2011) Aquatic systems: maintaining, mixing and mobilizing antimicrobial resistance? *Trends in Ecology & Evolution* 26:278–284

Thornton SM, Nolan S, Gulland FM (1998) Bacterial isolates from California sea lions (*Zalophus californianus*), harbor seals (*Phoca vitulina*), and northern elephant seals (*Mirounga angustirostris*) admitted to a rehabilitation center along the central California coast, 1994–1995. *Journal of Zoo and Wildlife Medicine* 29:171–176

Van der Donk CF, Beisser PS, Hoogkamp-Korstanje JA, Bruggeman CA, Stobberingh EE Antibiotic Resistance Surveillance Group (2011) A 12 year 1998–2009 antibiotic resistance surveillance of *Klebsiella pneumoniae* collected from intensive care and urology patients in 14 Dutch hospitals. *The Journal of Antimicrobial Chemotherapy* 66:855–858

Zhang XX, Zhang T, Fang HHP (2009) Antibiotic resistance genes in water environment. *Applied Microbiology and Biotechnology* 82:397–414