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Temporal Changes in Antibiotic Resistance Among Bacteria Isolated from Common Bottlenose Dolphins (*Tursiops truncatus*) in the Indian River Lagoon, Florida, 2003-2015

Adam M. Schaefer,[1] Gregory D. Bossart,[2] Tyler Harrington,[1]
Patricia A. Fair,[3] Peter J. McCarthy,[1] and John S. Reif[4]

[1]*Harbor Branch Oceanographic Institute at Florida Atlantic University, 5600 U.S. 1 North, Fort Pierce, FL 34946, USA*
E-mail: aschaef3@fau.edu
[2]*Georgia Aquarium, 225 Baker Street, NW, Atlanta, GA 30313, USA*
[3]*Department of Public Health Sciences, Medical University of South Carolina, Charleston, SC 29245, USA*
[4]*Department of Environmental and Radiological Health Sciences, College of Veterinary Medicine and Biomedical Sciences, Colorado State University, Fort Collins, CO 80523, USA*

Abstract

Increases in resistance to commonly used antibiotics have been reported globally in isolates from humans, wildlife, and the environment. To date, few studies have examined long-term trends in antibiotic resistance in organisms isolated from marine mammal populations. The objective of this study was to examine temporal trends in resistance to antibiotics among pathogens isolated from common bottlenose dolphins (*Tursiops truncatus*) between 2003 and 2015. Dolphins were captured and released in the Indian River Lagoon, Florida, an ecosystem with a large coastal human population and significant environmental impacts. Swab samples for microbiology were taken from the blowhole, gastric fluid, and feces and cultured on standard media under aerobic conditions. Isolates were identified using gram stain morphology and growth on selective media. Antibiotic resistance was measured using disc diffusion on Mueller Hinton agar and the Multiple Antibiotic Resistance (MAR) index calculated for each pathogen. A total of 733 isolates was obtained from 171 individual dolphins. The most commonly cultured pathogens included *Aeromonas hydrophila, Escherichia coli, Edwardsiella tarda*, and *Vibrio alginolyticus*. The overall prevalence of resistance to at least one antibiotic for the 733 isolates was 88.2%. The MAR index increased significantly between 2003 and 2007 and 2010 and 2015 for *Pseudomonas aeruginosa* and *V. alginolyticus*. For all bacterial isolates, resistance to cefotaxime, ceftazidime, and gentamicin increased significantly between sampling periods. This is one of few studies to use the MAR index for bacterial isolates from a marine mammal. The significant increases in resistance for some bacterial species likely reflect shared environmental exposures to antibiotics and transfer of resistance to dolphins from terrestrial sources or from animal or human populations.

Key Words: common bottlenose dolphins, *Tursiops truncatus*, antibiotic resistance, Multiple Antibiotic Resistance index, public health, Indian River Lagoon

Introduction

Resistance to antibiotics used to treat bacterial infections is a major public health issue worldwide (World Health Organization [WHO], 2014). As resistance to antibiotics increases, the probability of successfully treating infections caused by common bacterial pathogens continues to decrease, resulting in increased morbidity and mortality (Hawkey & Jones, 2009). Once primarily confined to healthcare settings, resistant strains are now commonly acquired in the community and from the environment (Berendonk et al., 2015). The Centers for Disease Control and Prevention (CDC; 2014) estimate that over two million antibiotic resistant infections occur in the United States annually, resulting in approximately 23,000 human deaths.

Aquatic environments have the potential to play a significant role in the emergence of antibiotic resistance, especially in areas highly impacted by human activities. Environmental concentrations of antibiotics in aqueous environments create a selective pressure which increases the prevalence of resistance among pathogenic bacteria (Tello et al., 2012). These ecosystems serve as reservoirs for antibiotic resistant genes (ARGs) by accumulating pathogens and facilitating the exchange of these

genes between bacterial species (Baquero et al., 2008; Rizzo et al., 2013). Further, environmental contamination and poor water quality, characterized by high concentrations of total nitrogen, nitrates, and phosphates, increase the dissemination of ARGs (Chitanand et al., 2010). Resistance can arise from direct exposure but can also be co-selected for in the environment. For example, co-selection for ARGs has been observed in waters contaminated with heavy metals due to the shared Merc-A gene (Stepanauskas et al., 2006; Seiler & Berendonk, 2012). As a result, aquatic organisms may act as vectors for the translocation of resistant bacteria and their genes between habitats (Grossart et al., 2010). For example, genes for antibiotic resistance were found to be transferable between multiple strains of *Enterococcus*, enteric bacteria frequently found in brackish and marine environments (Di Cesare et al., 2014).

The identification of temporal trends in resistance to antibiotics from studies of wildlife and domestic animal populations has the potential to inform hypotheses regarding environmental sources and reservoirs of resistant bacteria (National Academies of Sciences, 2017). To accomplish this goal, the Multiple Antibiotic Resistance (MAR) index can be used to provide a summary measure of resistance across antibiotics rather than for an individual drug (Krumperman, 1983). Several studies have documented increases in the MAR index in rivers and coastal waters which receive inputs from terrestrial sources (Edge & Hill, 2005; Chitanand et al., 2010; Rizzo et al., 2013; Rodriguez-Mozaz et al., 2015).

Sentinel species such as marine mammals (Bossart, 2011) have the potential to serve as indicators for transfer of antibiotic resistance from terrestrial sources that can be monitored over time (Radhouani et al., 2014; Vittecoq et al., 2016). Antibiotic resistance in bacteria cultured from bottlenose dolphins (*Tursiops truncatus*) in the southeastern U.S. has been documented previously (Greig et al., 2007; Schaefer et al., 2009; Morris et al., 2011; Stewart et al., 2014). These studies reported resistance to multiple antibiotics for human pathogens, including *Staphylococcus aureus* (*S. aureus*), *Escherichia coli* (*E. coli*), and *Pseudomonas aeruginosa* (*P. aeruginosa*). (Schaefer et al., 2009; Stewart et al., 2014). Bacteria cultured from estuarine bottlenose dolphins in the Indian River Lagoon (IRL), Florida, have shown high levels of resistance to multiple antibiotics used in human and veterinary medicine (Schaefer et al., 2009). Among all isolates analyzed in this earlier study, resistance was found most commonly to erythromycin (91%), clindamycin (87%), ampicillin (77%), and cephalothin (53%) (Schaefer et al., 2009). The objective of the current study was to determine whether the prevalence of resistance to antibiotics in bacteria cultured from bottlenose dolphins inhabiting the IRL has changed over time. A second objective was to use the MAR index to evaluate the source(s) of resistance.

Methods

Study Site

The IRL is a 260-km shallow water estuary that ranges from 1 to 8 km wide along the east coast of Florida and connects three distinct water bodies: the Mosquito Lagoon, the Indian River, and the Banana River. It was designated as an "Estuary of National Significance" due to its biological diversity and economic value (East Central Florida Regional Planning Council [ECFRPC] and the Treasure Coast Regional Planning Council, 2016). The IRL's northern region is characterized by shallow waters, limited tidal exchange, and long water residency times that concentrate microbiological and chemical agents (Woodward-Clyde Consultants, 1994). The southern extent of the IRL contains multiple inlets and freshwater inputs from man-made drainage canals and Lake Okeechobee (Woodward-Clyde Consultants, 1994). Substantial human residential development and agricultural activity exists along the entire border of the IRL. This has resulted in terrestrial inputs that have impacted water quality, decreased salinity, introduced large amounts of nitrates and phosphates, and reduced seagrass habitat as a result of eutrophication (Sime, 2005).

Dolphin Sampling

Common bottlenose dolphins from the IRL were captured, sampled, and released between 2003 to 2015 as a part of the Bottlenose Dolphin Health and Risk Assessment (HERA) Project, a collaborative multidisciplinary effort to assess health in two estuarine regions along the eastern coast of the U.S. (Bossart et al., 2017). Sampling took place during June and July each year. All research was approved by the Florida Atlantic University Institutional Animal Care and Use Committee and conducted under National Marine Fisheries Permit Numbers 14352 and 998-1678. Dolphins were captured, restrained, examined, and released according to established protocols (Fair et al., 2006). Swabs for bacterial isolation were collected from the blowhole, gastric fluid, and feces of each dolphin for culture and antibiotic sensitivity testing. Sterile Aimes culturettes (Fischer Scientific, Pittsburgh, PA, USA) were inserted into the blowhole during a breath, gently rotated along the wall of the nares, and removed during the subsequent breath. Gastric fluid was collected

by inserting a well-lubricated, soft, flexible, plastic stomach tube past the oropharynx to the first stomach, followed by gentle aspiration and placement of fluid into a 15-ml conical vial. A sterile Aimes culturette was then inserted in the gastric fluid and placed in transport media. Fecal samples were collected opportunistically in sterile, 15-ml conical vials as the animal defecated, or by insertion of a swab into the anus (Fair et al., 2006). All swabs were placed in transport media and held on ice packs for storage and overnight shipment.

Microbial Identification and Antibiotic Sensitivity Testing

Microbiologic testing was conducted in a single commercial laboratory (Micrim Laboratories, Ft. Lauderdale, FL, USA). Swabs were streaked initially on selective media, including Sheep blood agar, MacConkey, and CNA agar and incubated at 37˚C for 24 to 48 h under aerobic conditions. Standard methods used for organism identification included growth on selective and differential media, macroscopic appearance, and gram stain morphology according to the National Committee for Clinical Laboratory Standards (1997). After identification of each organism, screening for antibiotic resistance was performed for isolates that exhibited moderate to heavy growth. Antibiotics tested included amikacin, ampicillin, augmentin (amoxicillin/clavulanate), cefotaxime, ceftazidime, cephalothin, chloramphenicol, ciprofloxacin, enrofloxacin, erythromycin, furadantin, gentamicin, marbofloxacin, penicillin, piperacillin, sulfamethoxazole/trimethoprim, and tetracycline. Antibiograms were constructed using standardized concentrations of each antibiotic. Antibiotic resistance was determined by using a Kirby-Bauer disk diffusion test (Bauer et al., 1966). The protocol used standardized inocula of bacteria swabbed onto Mueller-Hinton agar plates. Impregnated antimicrobial discs were placed on the plates which were then incubated at 37˚C. Zones of inhibition were measured, and the Clinical and Laboratory Standards Institute antimicrobial susceptibility testing standards M2-A9 and M7-A7 were applied (National Committee for Clinical Laboratory Standards, 1997). Only microbes and antibiotics with > 5 isolates cultured and screened for resistance were included in subsequent statistical analysis.

The MAR index was calculated as the number of antibiotics to which an isolate was resistant/total number of antibiotics against which the isolate was tested (Krumperman, 1983). The MAR index has been used to reflect potential human impacts and the degree of antibiotic exposure for an environmental isolate; a value of > 0.20 is interpreted as an indicator of anthropogenic pollution (Kaspar et al., 1990; Chitanand et al., 2010). Values < 0.20 represent low-risk environments for contamination; the range between 0.20 and 0.25 is considered ambiguous (Krumperman, 1983).

Statistical Analysis

The year of sampling was categorized into two periods, 2003-2007 and 2010-2015, due to the lack of annual data for all years during the period. Statistical analysis was not performed for organisms with > 5 isolates in either sampling period. The overall difference in proportions of bacterial species isolated between sampling periods was evaluated using a chi-square test. A z-test of proportions was used to compare the proportions of isolation for each pathogen between sampling periods. For analysis of resistance, the proportion of all isolates resistant to each antibiotic between sampling periods was first compared using a chi-square test. In the next step, the percent resistance for each bacterium to each antibiotic between sampling periods was tested using chi-square.

A chi-square test was used to compare the percent resistance for each antibiotic for each organism between sampling periods. A Kolmogorov Smirnov test was used to test the normality of the MAR index for each bacterial species. The normality assumption was not met; therefore, a nonparametric Mann-Whitney U Test was used to compare the mean MAR index between sampling periods for each pathogen. Results were considered statistically significant at $p < 0.05$. All analyses were completed using *SPSS*, Version 22 (IBM Corp., Armonk, NY, USA).

Results

A total of 733 bacterial isolates from 171 dolphins were analyzed. Of those, 394 (53.7%) came from the blowhole, 199 (27.2%) from fecal swabs, and 140 (19.1%) from gastric fluid swabs. Fifty-nine percent of the isolates (n = 435) were obtained between 2003 and 2007, while the remaining 41% (n = 298) were cultured between 2010 and 2015. The most frequently isolated pathogens were *Aeromonas hydrophila* (*A. hydrophila*), *E. coli*, *Edwardsiella tarda*, *Vibrio alginolyticus* (*V. alginolyticus*), and *S. aureus* (Table 1). The overall composition of total isolates was statistically different between sampling periods ($p < 0.01$). Specifically, the proportions of *E. coli, Edwardsiella tarda*, and *S. aureus* increased significantly ($p < 0.01$) between 2010 and 2015 compared to the earlier sampling period.

The overall prevalence of resistance to at least one antibiotic for the 733 isolates was 88.2%. The prevalence of resistance was highest to erythromycin (91.6%), followed by ampicillin (77.3%) and cephalothin (61.7%). The MAR index ranged from 0.06 for *Edwardsiella tarda* to 0.63 for *P. aeruginosa*.

Table 1. Comparison of the proportion of potential pathogens cultured from Indian River Lagoon (IRL) dolphins between two time periods: 2003-2007 and 2010-2015 (total isolates, $N = 733$)

Organism	2003-2007 *n* (%)	2010- 2015 *n* (%)	Total *n*
Acinetobacter baumannii	25 (5.7)	10 (3.4)	35 (4.8)
Aeromonas hydrophila	76 (17.5)	53 (17.8)	129 (17.6)
Escherichia coli	23 (5.3)*	37 (12.4)*	60 (8.2)
Edwardsiella tarda	20 (4.6)*	30 (10.1)*	50 (6.8)
Klebsiella pneumoniae	7 (1.6)	6 (2.0)	13 (1.8)
Pseudomonas aeruginosa	13 (3.0)	6 (2.0)	19 (2.6)
Staphylococcus aureus	11 (2.5)*	26 (8.7)*	37 (5.0)
Vibrio alginolyticus	28 (6.4)	10 (3.4)	38 (5.2)
Other species	232 (55.3)*	120 (40.3)*	352 (48.0)

**p* value for differences in distribution of isolates between sampling periods, < 0.05

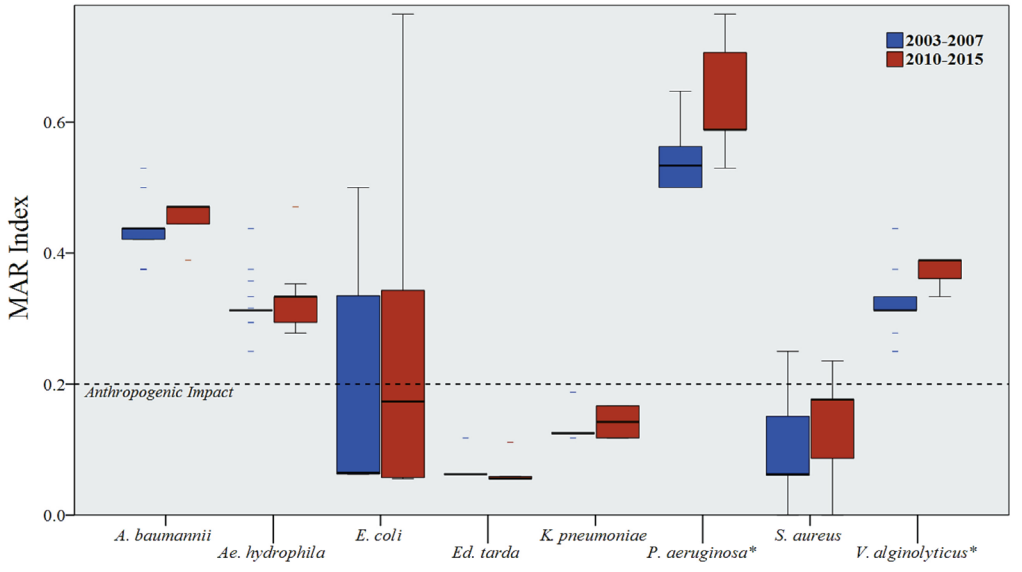

Figure 1. Multiple Antibiotic Resistance (MAR) index for dolphin isolates between sampling periods (2003-2007 and 2010-2015) from the Indian River Lagoon (IRL), Florida

(Figure 1). Two of the eight pathogens analyzed (*P. aeruginosa* and *V. alginolyticus*) showed statistically significant increases in the index between sampling periods. MAR indices > 0.20 were calculated for *Acinetobacter baumannii* (0.42, 0.45), *A. hydrophila* (0.31, 0.32), *P. aeruginosa* (0.54, 0.63), and *V. alginolyticus* (0.32, 0.37) in both sampling periods.

The significant differences in MAR indices between sampling periods were driven by the patterns observed for specific classes of antibiotics (Table 2). These included increases in resistance to 3rd-generation cephalosporins, cefotaxime and ceftazidime, for three and four pathogens, respectively. Resistance to ciprofloxacin, a fluoroquinolone, increased for *Acinetobacter baumannii*, *E. coli*, and *P. aeruginosa* between sampling periods. Similarly, large increases in resistance to gentamicin, piperacillin, and erythromycin were observed for *A. hydrophila*, *E. coli*, and *P. aeruginosa* (Table 2).

Table 2. Temporal comparison of percent resistance for each antibiotic by pathogen collected from dolphins in the IRL between 2003-2007 and 2010-2015. AM = ampicillin, AK = amikacin, AU = augmentin, C = cephalothin, CE = cefotaxime, CF = ceftazidime, CH = chloramphenicol, CI = ciprofloxacin, EN = enrofloxacin, ER = erythromycin, FU = furadantin, GE = gentamicin, PI = piperacillin, TE = tetracycline, and ST = sulfamethoxazole/trimethoprim.

Antibiotic	AM	AK	AU	C	CE	CF	CH	CI	EN	ER	FU	GE	PI	TE	ST
Acinetobacter baumannii															
2003-2007	80.1	0.3	67.1	3.2	3.2	40.0	17.4	1.5	1.5	87.4	18.9	0.3	66.0	12.9	6.7
2010-2015	69.4	0.0	52.0	6.7*	8.3*	35.0	12.8	2.2*	1.7	92.2*	13.9	1.7*	50.0	8.9	8.9*
Aeromonas hydrophila															
2003-2007	100.0	0.0	100.0	0.0	0.0	25.0	1.3	0.0	0.0	0.0	1.3	0.0	98.7	0.0	0.0
2010-2015	98.1	0.0	90.4	0.0	0.0	59.5*	1.9	0.0	0.0	0.0	1.9	0.0	100*	0.0	0.0
Escherichia coli															
2003-2007	40.0	0.0	30.0	0.0	5.0	100.0	25.0	0.0	0.0	0.0	25.8	0.0	15.0	10.0	15.0
2010-2015	48.0	0.0	48.0*	20.0*	16.0*	35.0	20.0	12.0*	8.0	0.0	24.0	4.0*	32.0*	24.0*	28.0*
Edwardsiella tarda															
2003-2007	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	5.0	0.0	0.0	0.0	0.0
2010-2015	0.0	0.0	0.0	0.0	0.0	0.0	3.4	0.0	0.0	96.6	0.0	0.0	3.4	0.0	0.0
Klebsiella pneumoniae															
2003-2007	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0	14.3	0.0	0.0
2010-2015	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	16.7*	0.0	0.0	0.0	0.0
Pseudomonas aeruginosa															
2003-2007	100.0	0.0	100.0	38.5	7.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	7.7	0.0	0.0
2010-2015	100.0	0.0	83.3	83.3*	16.7*	0.0	0.0	16.7*	16.7	0.0	0.0	33.3*	16.7*	0.0	0.0
Vibrio alginolyticus															
2003-2007	100.0	3.6	0.0	0.0	0.0	0.0	3.6	0.0	0.0	96.4	3.6	0.0	96.4	0.0	0.0
2010-2015	100.0	0.0	0.0	0.0	10.0*	0.0	0.0	0.0	0.0	100.0*	0.0	0.0	100*	10.0	10.0
Staphylococcus aureus															
2003-2007	63.6	0.0	0.0	0.0	36.4	0.0	0.0	9.1	9.1	9.1	0.0	0.0	18.2	0.0	9.1
2010-2015	65.4	0.0	0.0	0.0	34.6	0.0	0.0	0.0	0.0	53.8*	0.0	0.0	3.8	7.7	3.8

*Statistically significant difference from 2003-2007 sampling period by chi square

Of the 15 antibiotics with ≥ 5 isolates screened in each sampling period, three demonstrated statistically significant increases in the prevalence of resistance to all isolates between periods (Table 3). The proportion of all isolates resistant to cefotaxime was significantly higher in 2010-2015 (21.9%) compared to 2003-2007 (7.3%). Similarly, a significant increase in resistance to ceftazidime from 3.8% in 2003-2007 to 10% in 2010-2015 was observed ($p = 0.02$). Resistance to gentamycin also increased significantly from 1.1% in 2003-2007 to 4.2% in 2010-2015 ($p = 0.01$). Over 50% of all isolates were resistant to ampicillin, augmentin, cephalothin, and erythromycin in the latter sampling period (Table 3).

Discussion

The overall diversity and frequency of pathogenic bacteria isolated from IRL dolphins were similar to those previously reported by Schaefer et al. (2009). The most commonly cultured organisms in this study, *A. hydrophila*, *E. coli, Edwardsiella tarda*, and *V. alginolyticus*, are pathogens frequently associated with aquatic environments. These bacteria have been identified in samples from several

Table 3. Prevalence of resistance among all isolates from IRL dolphins by antibiotic, 2003-2007 and 2010-2015

	2003-2007		2010-2015		
Antibiotic	No. resistant/ No. tested	Percent resistant	No. resistant/ No. tested	Percent resistant	*p* value
Amikacin	4/369	1.08	7/259	2.70	0.21
Ampicillin	307/385	79.74	196/266	73.68	0.07
Augmentin	214/392	54.59	138/263	52.47	0.59
Cefotaxime	27/368	7.34	57/260	21.92	< 0.01*
Ceftazidime	14/370	3.78	26/260	10.00	0.02*
Cephalothin	246/392	62.76	160/265	60.38	0.54
Chloramphenicol	112/392	28.57	71/265	26.79	0.62
Ciprofloxacin	10/392	2.55	13/265	4.91	0.11
Enrofloxacin	12/391	3.07	13/265	4.91	0.23
Erythromycin	356/392	90.82	246/265	92.83	0.36
Furadantin	140/369	37.94	80/260	30.77	0.07
Gentamicin	4/370	1.08	11/260	4.23	0.01*
Marbofloxacin	8/359	2.23	11/259	4.25	0.15
Piperacillin	184/371	49.60	120/260	46.15	0.39
Tetracycline	98/392	25.00	66/263	25.10	0.98

*Statistically significant difference in percent resistant between sampling periods

marine mammal species, including bottlenose dolphins (Buck et al., 2006; Greig et al., 2007; Johnson et al., 2009; Morris et al., 2011). To date, few studies have evaluated temporal changes in bacterial resistance to antibiotics in marine mammal populations, and none have examined this trend over a time span as long as 13 years. Stewart et al. (2014) reported an increase in the prevalence of antibiotic resistant bacteria isolated from bottlenose dolphins from Sarasota Bay, Florida, between 2004 and 2009 during a single year, 2005, compared to 2004, 2006, and 2009, but no temporal trend was detected.

MAR indexing has been used to differentiate sources of bacteria and as an indicator of anthropogenic pollution in aquatic ecosystems by multiple investigators (Kaspar et al., 1990; Parveen et al., 1997; Kelsey et al., 2003; Webster et al., 2004; Sayah et al., 2005; Watkinson et al., 2007). The MAR index can also be used to differentiate between urbanized and non-urbanized sources of *E. coli* (Webster et al., 2004) and point and non-point sources of enterobacteria (Parveen et al., 1997).

Wallace et al. (2013) found increases in multiple antibiotic resistance to aminoglycosides and fluoroquinolones in 79 stranded pinnipeds admitted for rehabilitation from the Northwest Atlantic between 2004 and 2008. Increases in antibiotic resistance to the beta lactams and sulfonamides were demonstrated for *E. coli*. In a group of 16 untreated pinnipeds in the facility, the MAR index for enteric bacteria increased from 0.08 between 2004 and 2007 to 0.31 in 2009-2010. The authors hypothesized that a point source of contamination was responsible for the increase in the latter period. Similarly, in a study of marine vertebrates recovered along the New England coast (USA), the overall prevalence of resistance in marine mammals was 50%. The MAR index was ≥ 0.2 for 38% of the resistant bacteria, which was interpreted as suggesting exposure to contaminated sites (Rose et al., 2009).

MAR indices above 0.20 in isolates from estuaries may be influenced, in part, by local environmental conditions such as terrestrial runoff and water quality (Parveen et al., 1999; Webster et al., 2004). The IRL is impacted by freshwater discharges and fecal contamination with limited tidal flushing (Smith, 1993). Inputs from human sources such as septic tanks have been associated with an increase in the prevalence of resistant *E. coli* cultured from IRL dolphins (Schaefer et al., 2011) and nutrient enrichment (Lapointe et al., 2015). In a previous study, dolphins with home ranges that bordered areas with the highest density of septic tanks were 6.6 times more likely to be colonized with antibiotic resistant *E. coli* compared to areas with the lowest density (Schaefer et al., 2011).

Several of the organisms isolated from bottlenose dolphins are important human pathogens. *P.*

aeruginosa is an important cause of nosocomial infections worldwide (Morrison & Wenzel, 1984). In 2013, 13% of all healthcare-associated infections in the U.S. were caused by antibiotic resistant *P. aeruginosa* (CDC, 2014). Multiple drug resistant strains are associated with severe, adverse clinical outcomes in hospital settings worldwide (Aloush et al., 2006). The MAR indices for *P. aeruginosa* isolated from bottlenose dolphins were the highest recorded for any organism and increased during the study period. The indices of 0.54 and 0.63 for the two study periods are comparable to those reported from human healthcare settings (Paul et al., 1997). Based on these indices, it is likely that these isolates from dolphins originated from a source where antibiotics are used regularly and that they entered the marine environment through human activities or discharges from terrestrial sources such as septic tanks. Alternatively, dolphin organisms may have become antibiotic resistant through gene transfer as described above (Baquero et al., 2009; Rizzo et al., 2013).

Multiple *Vibrio* spp. are associated with human disease and aquatic environments (Baumann & Schubert, 1984). *V. alginolyticus* can cause wound and soft-tissue infections in humans (Chang-Chien et al., 2007). The incidence of *V. alginolyticus* infections in the U.S. increased between 1996 and 2010 (Newton et al., 2012). *V. alginolyticus* with resistance to β-lactam antibiotics have been cultured from seafood and show higher levels of multiple antibiotic resistance than those described for other *Vibrio* spp. (Ottaviani et al., 2001; Oh et al., 2011). In the current study, resistance to macrolide and 3rd-generation cephalosporins increased over the two sampling periods for this organism.

The MAR index for *Acinetobacter baumannii* ranged between 0.42 and 0.45 during the study period—levels thought to represent point sources of human pollution (Krumperman, 1983). The nationwide human health impact of this pathogen is of substantial concern. As a gram-negative bacillus with resistance to a wide range of antibiotics, it is a significant nosocomial pathogen with increasing infection rates over the past 10 years (Dijkshoorn, 2007). In addition to nosocomial infections, resistant strains associated with fish and fish farming have been reported globally (Huys et al., 2000). The high MAR index for *Acinetobacter baumannii* in bacteria isolated from dolphins in the IRL represents a significant public health concern. Similarly, while there were no significant increases in antibiotic resistance observed over time in *A. hydrophila*, *Klebsiella pneumoniae*, and *S. aureus*, their presence is significant due to the role of these organisms as human pathogens. In particular, isolation of methicillin-resistant *S. aureus* from common dolphins in estuarine locales along the southeastern U.S. coast (Schaefer et al., 2009; Stewart et al., 2014) raises concern regarding dissemination of this virulent pathogen in marine environments.

Increases in resistance to several classes of antibiotics were demonstrated during the 13-year period of observation in this study. Resistance to ciprofloxacin among *E. coli* isolates more than doubled between sampling periods, mirroring recent trends in human clinical infections. In hospital intensive care units, multidrug resistance to cephalosporin and ciprofloxacin increased among the gram-negative isolates of *Acinetobacter* spp., *P. aeruginosa*, and *K. pneumoniae* between 1997 and 2003 (Lockhart et al., 2007).

Fluoroquinolones are the most widely prescribed class of antibiotics for human use for which the number of outpatient prescriptions tripled between 1999 and 2005 (Linder et al., 2005). A clear, significant relationship between antibiotic usage in the community and bacterial resistance was demonstrated in a meta-analysis of 243 studies conducted worldwide (Bell et al., 2014). A concomitant increase in resistance to fluoroquinolones among coagulase-negative staphylococci paralleled the increase in usage of this class of antibiotics in the U.S. between 1999 and 2012 (May et al., 2014). In isolates from IRL dolphins, resistance to fluoroquinolones increased during the 2010-2015 sampling period for *Acinetobacter baumannii*, *E. coli*, and *P. aeruginosa*. This finding is consistent with a recent report describing increases in resistance to fluoroquinolones and aminoglycosides in the same bacterial taxa isolated from pinnipeds and stranded cetaceans in the Northwest Atlantic over a 6-year period (Wallace et al., 2013).

Similarly, cephalosporin consumption in the U.S. has also increased recently (Van Boeckel et al., 2014). Resistance to the 3rd-generation cephalosporins cefotaxime and ceftazidime increased significantly for all bacterial isolates from dolphins in the latter time period, possibly reflecting their increased usage in adjacent human communities.

Although the results of this study are restricted to bacteria culturable under aerobic conditions and do not capture the entire microbiome of the dolphin (Johnson et al., 2009; Jaing et al., 2015), our findings appear to reflect the usage and disposal of antibiotics from terrestrial sources into the aquatic environment. The discharge of antibiotics commonly used in human and veterinary medicine into the IRL directly via septic tanks and effluents or indirectly through canals and man-made drainage systems is a plausible explanation for the results reported. The estuarine environment of the IRL may be a reservoir of genes acquired and released by pathogens, or it may facilitate the exchange of genes among bacteria (Baquero et al., 2008; Rizzo et al., 2013; Vaz-Moreira et al., 2014). Given the high phylogenic diversity of bacterial species in

aquatic environments, there is a risk that other clinically relevant bacteria will acquire resistance. The relationship between the propagation of antibiotic resistance among pathogenic bacteria and the aquatic environment needs to be further elucidated. However, the similarity in the trends of increased resistance to fluoroquinolones and 3rd-generation cephalosporins in dolphin and human isolates highlights the connections between terrestrial and aquatic ecosystems.

The increase in the use of antibiotics in humans has clearly impacted resistance patterns for multiple pathogens over time (Fridkin et al., 2002). The current analysis provides insight into the changing patterns of antibiotic resistance in a marine environment. The results fill an important gap in integrating animal, environmental, and human data, and demonstrate the usefulness of studying coastal marine mammal populations as environmental sentinels of ecosystem and environmental health.

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