

1 Occurrence and patterns of antibiotic resistance in vertebrates  
2 off the Northeastern United States coast  
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6 Julie M. Rose<sup>1\*</sup>, Rebecca J. Gast<sup>1</sup>, Andrea Bogomolni<sup>1</sup>, Julie C. Ellis<sup>2</sup>, Betty J.  
7 Lentell<sup>3</sup>, Kathleen Touhey<sup>4</sup>, Michael Moore<sup>1</sup>  
8

9 <sup>1</sup>Woods Hole Oceanographic Institution, Woods Hole MA, USA

10 <sup>2</sup>Cummings School of Veterinary Medicine, Tufts University, 200 Westboro Rd, North Grafton,  
11 MA, USA 01536

12 <sup>3</sup>National Marine Fisheries Service, Northeast Fisheries Observer Program, 166 Water Street,  
13 Woods Hole, MA 02543, USA

14 <sup>4</sup>Cape Cod Stranding Network, a project of IFAW, 290 Summer Street, Yarmouthport, MA  
15 02675, USA

16  
17 \*corresponding author. Biology Department, MS #32 Redfield 324, Woods Hole Oceanographic  
18 Institution, Woods Hole, MA 02543. Phone: 508-289-3786, Fax: 508-457-2134, Email:  
19 jrose@whoi.edu

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## **Abstract**

The prevalence of antibiotic-resistant bacteria in the marine environment is a growing concern, but the degree to which marine mammals, seabirds and fish harbor these organisms is not well documented. This project sought to identify the occurrence and patterns of antibiotic resistance in bacteria isolated from vertebrates of coastal waters in the northeastern United States. 472 isolates of clinical interest were tested for resistance to a suite of 16 antibiotics. Fifty-eight percent were resistant to at least one antibiotic, while 43% were resistant to multiple antibiotics. A multiple antibiotic resistance (MAR) index value greater than or equal to 0.2 was observed in 38% of the resistant pathogens, suggesting exposure of the animals to bacteria from significantly contaminated sites. Groups of antibiotics were identified for which bacterial resistance commonly co-occurred. Antibiotic resistance was more widespread in bacteria isolated from seabirds than marine mammals, and was more widespread in stranded or bycaught marine mammals than live marine mammals. Structuring of resistance patterns based on sample type (live/stranded/bycaught) but not animal group (mammal/bird/fish) was observed. These data indicate that antibiotic resistance is widespread in marine vertebrates, and they may be important reservoirs of antibiotic resistant bacteria in the marine environment.

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## Introduction

The promise of bacterial disease control through the discovery and use of antibiotics has been dramatically undermined by the appearance of resistant strains and the spread of resistance genes. A background level of antibiotic resistance occurs naturally in any environment, and as such, antibiotic resistance genes and antibiotic resistant microorganisms have been documented in areas with little to no obvious anthropogenic impact or influence, and in environmental samples obtained before the use of antibiotics in disease treatment (Graves *et al.*, 1988; Magee and Quinn, 1991; McKeon *et al.*, 1995; Boon and Cattanach, 1999; Pei *et al.*, 2006; Singer *et al.*, 2006; Sjolund *et al.*, 2008). However, the wide-spread use of these drugs in human disease treatment and agriculture has resulted in a significant increase in the spread and persistence of antibiotic resistance in the environment (Smith *et al.*, 2002).

Steps have been taken to reduce the dissemination of antibiotics into the environment, such as limiting the amount and types of antibiotics used (Shlaes *et al.*, 1997). However, recent work has shown that even after the removal of the selective pressure of antibiotics in an environment, resistance levels have been slow to decline (Heuer *et al.*, 2002; Sørnum *et al.*, 2006) or have even increased (Enne *et al.*, 2001). The dogma that maintenance of resistance genes in the absence of selection is energetically costly for an organism has become less accepted as studies have shown that some organisms carrying resistance genes in a population are actually quite robust (Salyers and Amabile-Cuevas, 1997; Singer *et al.*, 2006). Additionally, metal pollution has been demonstrated to indirectly aid in the long-term persistence of antibiotic resistance in bacterial communities due to a combination of the stability of metals in terrestrial and aquatic environments and commonly occurring co- and cross-resistance to metal toxicity and

1 antibiotics (Rasmussen and Sorenson, 1998; Baker-Austin *et al.*, 2006; Wright *et al.*, 2006; De  
2 and Ramaiah, 2007).

3         The persistence and spread of bacteria resistant to antibiotics in the environment is of  
4 concern because of the potential increase in community acquired resistances. This is in contrast  
5 to the traditional antibiotic-resistance hot spots of hospitals and nursing homes where close  
6 physical proximity and people highly susceptible to infection are thought to contribute to the  
7 spread of resistant bacteria. Antimicrobial use in treatment of humans and food-animal  
8 husbandry (terrestrial and aquatic) results in the release of wastes that carry both antibiotics and  
9 antibiotic resistant bacteria (ABR bacteria) into the terrestrial and coastal marine environment  
10 (Silbergeld *et al.*, 2008). Recent analysis of soil and aquatic metagenomic databases suggest that  
11 antibiotic resistance is diverse and widespread in environmental bacteria (D'Costa *et al.*, 2007).  
12 There are only a few reports of antibiotic resistance in marine animals compared to terrestrial  
13 animals, but it has significance with regard to marine mammal stranding and rehabilitation  
14 activities, and dissemination of resistant bacteria in the environment. To date, studies that  
15 examined the prevalence and types of antibiotic resistance in bacteria isolated from marine  
16 organisms have included surveys of seabirds in rehabilitation facilities (Ziegerer *et al.*, 2002;  
17 Steele *et al.*, 2005), stranded seals (Johnson *et al.*, 1998) and sharks (Blackburn, 2003). All of  
18 these studies have shown that ABR bacteria were consistently recovered from a variety of  
19 animals, and that usually more than half of the isolates (sometimes as many as 75%) were  
20 resistant to at least one antibiotic.

21         While those studies were successful in identifying and describing the presence of ABR  
22 bacteria in marine animals, the number of isolates and samples tested were not usually large  
23 enough to allow statistical evaluation of the data. With the potential for marine animals to serve

1 as reservoirs for both pathogenic and commensal ABR bacteria, a more comprehensive study is  
2 needed to thoroughly document the occurrence of ABR bacteria in marine mammals and  
3 seabirds, and determine whether there are patterns to the presence and persistence of resistant  
4 bacteria. We investigated differences in ABR bacteria type and occurrence among different  
5 mammals and seabirds, and whether different animal provenance affected the types of ABR  
6 patterns present. This project was designed to examine patterns associated with ABR in marine  
7 animals by surveying bacterial isolates recovered from stranded, bycaught and live marine  
8 mammals (seals, whales, dolphins and porpoises) and seabirds in the Northeastern US.

## 9 **Methods**

10 **Bacterial culture and antibiotic sensitivity testing.** As part of a broader survey of pathogens  
11 in coastal marine vertebrates (Bogomolni *et al.*, 2008), tissues/organs routinely sampled included  
12 fecal/cloaca swabs for live animals, and thorax and abdomen or coelom for those examined by  
13 necropsy. Swabs from nasal/blowhole/nares were collected as appropriate and practical on live  
14 animals and if contamination of the outside surface of dead animals was minimal. Other sites  
15 were chosen for bacterial isolation if lesions or infection were suspected. All samples were  
16 collected using sterile methods. Aerobic and anaerobic bacteria were collected using  
17 Fisherfinest™ Amies clear gel transport swabs (Fisher Scientific, Pittsburgh, PA). Swabs were  
18 shipped overnight to IDEXX Laboratories, Grafton, MA. Swabs sent to IDEXX were planted  
19 onto plates and incubated for 24 hours. Aerobic samples were plated on blood agar and  
20 MacConkey plates, while anaerobic samples were plated on blood agar, MacConkey and  
21 anaerobic blood agar plates. Gram stains were performed on all isolates. Samples were then  
22 placed in a Vitek system (Biomerieux, Durham, USA), which performed both bacterial  
23 identification and antibiotic susceptibility testing. The Vitek system uses biochemical testing to

1 identify bacterial isolates, and broth microdilution and the Kirby Bauer disk method to perform  
2 antibiotic susceptibility tests according to CLSI guidelines. IDEXX used ATCC control strains  
3 EC25922, PSA 27853, EC35218, SA 29213, EF 52199 and EF 29212. Antibiotics tested  
4 include: amikacin (AMK), ampicillin (AMP), augmentin (AUG), carbenicillin (CAR),  
5 ceftazidime (CAZ), ceftiofur (CEF), cephalothin (CEPH), chloramphenicol (CHL), ciprofloxacin  
6 (CIP), gentamycin (GEN), tribrissen (TRI), piperacillin (PIP), enrofloxacin (ENR), tetracycline  
7 (TET), ticarcillin (TIC), and tobramycin (TOB).

8 **Stranded and Bycaught Mortality Samples.** Marine mammals were collected with the  
9 assistance of the New England Aquarium, University of New England Marine Animal  
10 Rehabilitation Center, the NOAA NEFSC Observer Program and the authors. Large whale cases  
11 were necropsied at the site of stranding (usually beach) while other animals were necropsied in a  
12 laboratory within 4-48 hours post mortem (stored at 4°C overnight). Full necropsies of marine  
13 mammals were conducted under protocols described in Pugliares (2007). Stranded and bycaught  
14 birds were collected by the staff at the Seabird Ecological Assessment Network (SEANET,  
15 <http://www.tufts.edu/vet/seanet/>), Massachusetts Audubon Society, National Oceanographic  
16 Atmospheric Administration (NOAA) Northeast Fisheries Science Center (NEFSC) Observer  
17 Program and the authors. Necropsies of marine birds were conducted using protocols as  
18 described by SEANET with tissue samples at Tufts University. Details of the sample source  
19 locations, species sampled, bacteria isolated, and pathobiological analyses have been published  
20 (Bogomolni *et al.*, 2008). Sample collection methods are briefly summarized below.

21 **Live Animal Samples.** Fecal samples from seals and birds were collected from beaches at the  
22 Isles of Shoals, NH/ME; Great Island in Wellfleet, MA; Muskeget Island, Nantucket Sound,  
23 MA; Monomoy National Wildlife Refuge; and Chatham Harbor, Chatham, MA. Visual

1 identifications and photographs of the species present at each beach were made before  
2 approaching the animals and collecting feces. Animals were identified as harbor seal (*Phoca*  
3 *vitulina*), grey seal (*Halichoerus gryphus*), Double-crested Cormorant (*Phalacrocorax auritus*),  
4 and Herring (*Larus argentatus*) and Great Black-backed Gulls (*L. marinus*). If a seal haul-out  
5 site was not > 90% of one species, samples were recognized as *seal species 1* or *seal species 2*.  
6 Bacterial swabs were collected on site.

7 Fecal samples were collected from live-caught gulls at Appledore Island, ME, and  
8 Monomoy National Wildlife Refuge, MA. Adult Great Black-backed Gulls, Herring Gulls, and  
9 Laughing Gulls (*Larus atricilla*) were captured during egg incubation using either a walk-in nest  
10 trap (a chicken wire cage with an opening on the bottom and an entrance on one side) or a drop-  
11 down trap (chicken wire cage propped up on one side by a wooden peg attached to a line). Once  
12 a bird was trapped in either trap type, it was immediately approached and the bird gently  
13 removed and placed into a cloth cone for restraint and to prevent injury. Each bird was banded,  
14 measured, and pharyngeal and cloacal swabs were collected to obtain samples of bacteria.

15  
16 **Data management and statistical analysis.** For each bacterial isolate, information was  
17 collected about animal and tissue of origin, animal provenance (live, stranded or bycaught),  
18 location coordinates of sample collection, taxonomic identification of isolate by IDEXX and  
19 sensitivity to each of the 16 antibiotics listed above. The dataset was then manipulated to obtain  
20 a variety of general information, including the prevalence of single and multiple antibiotic  
21 resistances across all isolates, the occurrence of antibiotic resistance within taxonomic groups of  
22 bacterial isolates, the effectiveness of each antibiotic against all bacterial isolates, and the  
23 prevalence of multiple antibiotic resistances within different tissue groups across all animals.

1 The proportion of drugs to which a particular isolate was resistant generated the Multiple  
2 Antibiotic Resistance Index (MAR: range 0 to 1) (Krumperman, 1983). A single MAR value  
3 was calculated for each tissue sampled in each animal by averaging MAR values for bacterial  
4 isolates from multiple swabs of the same tissue or MAR values for multiple bacteria isolated  
5 from a single swab. By averaging MAR values for multiple bacterial isolates from single tissues,  
6 we sought to minimize the potential bias of repeated sampling of single tissues and equalize the  
7 contribution of individual animals to our tissue-specific analysis. This method should also have  
8 reduced the potential bias of bacterial isolates that possess innate resistance to many antibiotics  
9 (e.g. *Chryseobacterium*, *Pseudomonas*) (Fraser and Jorgensen, 1997). Fortunately,  
10 *Chryseobacterium* and *Pseudomonas* isolates also made up a very small proportion of the dataset  
11 (20 out of 472 isolates), so this potential bias should not have affected our analysis.

12 The prevalence of antibiotic resistance in bacterial isolates of different groups (birds vs.  
13 mammals; live vs. stranded vs. bycaught animals) was compared using the Storer-Kim method  
14 for comparing binomials (Storer and Kim, 1990; Wilcox, 2003). The similarity of resistance  
15 patterns across all bacterial isolates was compared for the 16 antibiotics with a cluster analysis  
16 combined with the similarity profile (SIMPROF) test using the ecological statistical software  
17 program PRIMER v6 (Clarke and Warwick, 2001; Clarke and Gorley, 2006). The Bray-Curtis  
18 coefficient was used to create a similarity matrix, and then a hierarchical agglomerative  
19 clustering method with group-average linking was used to generate a dendrogram illustrating  
20 similarities among antibiotics. The significance of clustering levels was determined using the  
21 SIMPROF test for null structure. The Bray-Curtis coefficient is particularly useful for  
22 comparing patterns of antibiotic resistance and detecting the presence of shared resistances in  
23 this study because it is independent of joint absence, in other words, similarity between two

1 bacterial isolates is only increased if both exhibit resistance to the same antibiotic(s) (Clarke *et*  
2 *al.*, 2006). Similarity is not affected by two isolates both exhibiting sensitivity to the same  
3 antibiotic.

4 The Bray-Curtis coefficient was also used to generate a similarity matrix among the  
5 subset of bacterial isolates that exhibited some antibiotic resistance. The ANOSIM test for  
6 differences between groups of samples was used to determine the significance of similarity  
7 between antibiotic resistance profiles of bacterial isolates grouped according to animal type  
8 (birds vs. mammals) and according to animal provenance (live vs. stranded vs. bycaught  
9 organisms) (Clarke and Green, 1988). This procedure uses ranked similarities and a permutation  
10 test to compare the overall similarity of samples within groups that were created based on animal  
11 type or animal provenance to the overall similarity of samples between groups to determine the  
12 significance of differences in antibiotic resistance profiles between groups.

## 13 **Results**

14 The dataset consisted of 472 isolates of clinical interest from 149 animals tested for  
15 resistance to a suite of 16 antibiotics. 287 were isolated from 79 seabirds, 174 from 64 marine  
16 mammals and 11 from 6 sharks. In birds, 22 isolates were found from 5 bycaught, 109 from 34  
17 live and 156 from 40 stranded. In marine mammals, 28 isolates were found from 12 bycaught,  
18 56 from 31 live and 90 from 21 stranded. Fifty-eight percent of the total isolates were resistant  
19 to at least one antibiotic, while 43% were resistant to multiple antibiotics. A multiple antibiotic  
20 resistance (MAR) index value greater than or equal to 0.2 was observed in 38% of the resistant  
21 pathogens. While most isolates demonstrated resistance to at least one antibiotic, many also  
22 were resistant to multiple antibiotics (Figure 1). The total amount of antibiotic resistance  
23 observed within individual isolates ranged from 0-13 antibiotics. Fourteen percent of the total

1 isolates were resistant to a single antibiotic, 10% were resistant to two antibiotics and 33% were  
2 resistant to three or more antibiotics. Three isolates were resistant to 10 or more antibiotics.

3         The prevalence of antibiotic resistance within individual taxonomic groups of bacterial  
4 isolates was determined for a subset of the data. Taxa for which there were more than 20 isolates  
5 were grouped and the percentage of isolates demonstrating antibiotic resistance within each of  
6 these groups was determined (Table 1). This method yielded nine total groups and a wide range  
7 of total antibiotic resistance was observed among these groups. Six of these groups had greater  
8 than 70% of their isolates demonstrating antibiotic resistance, which was substantially higher  
9 than the average of 57% of isolates from the whole dataset that demonstrated antibiotic  
10 resistance. However, the remaining three groups (*Edwardsiella* spp., *Escherichia coli* and a  
11 group of non-enteric gram negative rods) had much lower antibiotic resistance on average,  
12 ranging from 14 to 30% of isolates demonstrating any antibiotic resistance.

13         Detailed information about the 18 bacterial isolates that demonstrated resistance to eight  
14 or more antibiotics is listed in Table 2. These isolates represented 10 different bacterial taxa and  
15 were isolated from marine mammals, seabirds and sharks. The provenance of these isolates  
16 included bycaught and stranded marine mammals and live and stranded seabirds. The isolates  
17 did not include any representatives from live marine mammals or bycaught seabirds. The 9  
18 tissues from which isolates were obtained also varied considerably, including both internal and  
19 external tissues.

20         The percentage of total bacterial isolates demonstrating resistance to each of the 16 tested  
21 antibiotics is shown in Figure 2. The percentage of resistant isolates ranged from 1 (for  
22 ciprofloxacin and enrofloxacin) to 39 (cephalothin). Four antibiotics were ineffective against  
23 >25% of tested isolates: carbenicillin, augmentin, ampicillin and cephalothin. Seven antibiotics

1 were ineffective against <5% of tested isolates: amikacin, ceftazidime, ciprofloxacin,  
2 enrofloxacin, gentamicin, tobramycin and tribrissen.

3         Antibiotics were clustered into groups according to similarity in resistance patterns across  
4 all tested bacterial isolates (Figure 3). Solid lines in the cluster diagram indicate significant  
5 differences between groups of antibiotics and dotted lines with asterisks at the node insignificant  
6 differences in resistance patterns between two antibiotics. The degree of similarity between two  
7 antibiotics increased only if resistance to both was observed in individual isolates. Similarity  
8 estimates were not affected by an isolate that demonstrated sensitivity to two or more antibiotics.  
9 In general, antibiotics from the same class tended to group together, i.e. aminoglycosides such as  
10 amikacin and gentamicin, penicillins such as ampicillin and carbenicillin, quinolones such as  
11 ciprofloxacin and enrofloxacin, and  $\beta$ -lactams such as augmentin and cephalothin showed  
12 similar patterns of resistance across all bacterial isolates. However, we did observe two pairs of  
13 unrelated antibiotics with similar patterns of resistance across isolates: ceftiofur (a  $\beta$ -lactam  
14 cephalosporin) vs. chloramphenicol and also ceftazidime (another  $\beta$ -lactam cephalosporin) vs.  
15 tribrissen (a sulfonamide).

16         Isolates were also grouped based on the marine mammal or seabird tissue from which  
17 swabs were taken (Figure 4). Only tissues with greater than 5 isolates were included in this  
18 analysis; this included oral, cloacal, blowhole, fecal, coelom, spleen and thorax samples. The  
19 multiple antibiotic resistance (MAR) index was calculated for each of these isolates, and isolates  
20 were further grouped based on whether MAR indices were 0, <0.2 (amount of antibiotic  
21 resistance typical of nonpoint sources of pollution) and >0.2 (amount of antibiotic resistance  
22 considered characteristic of point-source pollution) (Krumperman, 1983). Figure 4 illustrates the  
23 percentage of bacterial isolates within each of these three groups for each tissue. In general,

1 tissues in contact with the environment (oral, cloaca, blowhole) had higher percentage of isolates  
2 with a MAR >0.2 than did isolates from internal tissues (fecal, spleen, thorax). Additionally, a  
3 greater percentage of isolates from internal tissues demonstrated no antibiotic resistance than  
4 isolates from external tissues.

5         The analysis of similarity (ANOSIM) statistical test yielded no significant groupings of  
6 resistance patterns based on either animal provenance or animal type (both  $p > 0.05$ ). A  
7 binomial comparison of the total amount of antibiotic resistance in birds vs. mammals indicated  
8 that on average a significantly higher percentage of bacterial isolates from seabirds demonstrated  
9 resistance to at least one antibiotic than did bacterial isolates from marine mammals (61 vs. 50%,  
10  $p = 0.02$ ). Isolates were next subdivided into two groups based on whether they were taken from  
11 mammals or birds (the number of shark samples was too small to constitute a reasonable group).  
12 An ANOSIM test based on animal provenance yielded no significant results for seabirds ( $p >$   
13  $0.05$ ), and binomial comparisons of seabirds grouped according to animal provenance yielded no  
14 significant differences in the occurrence of antibiotic resistance among bacterial isolates from  
15 live, stranded or bycaught birds (all  $p > 0.05$ ). An ANOSIM test based on animal provenance  
16 was significant for marine mammals, however ( $p = 0.02$ ), indicating significant differences in  
17 antibiotic resistance patterns between live and bycaught marine mammals ( $p = 0.04$ ) and between  
18 live and stranded marine mammals ( $p = 0.02$ ), but no significant differences in resistance  
19 patterns between stranded and bycaught marine mammals ( $p = 0.09$ ). These results indicate that  
20 live mammals as a group were resistant to different types of antibiotics than were stranded and  
21 bycaught marine mammals. Additionally, a binomial comparison of live vs. bycaught or  
22 stranded marine mammals indicated that the percentage of bacterial isolates demonstrating  
23 antibiotic resistance from live mammals was significantly lower than the isolates from bycaught

1 or stranded marine mammals ( $p < 0.001$  for both comparisons). The percentage of antibiotic  
2 resistant bacterial isolates from bycaught marine mammals was not significantly different than  
3 was observed in stranded marine mammals ( $p = 0.56$ ).

## 4 **Discussion**

### 5 **Comparison to other studies of antibiotic resistance in terrestrial and aquatic animals.**

6 Our study contains one of the largest and most diverse datasets in terms of both the variety of  
7 marine animals and tissues sampled and the bacterial groups isolated. In general, most studies of  
8 bacteria isolated from mammals and birds have reported relatively high prevalence of antibiotic  
9 resistance and similar patterns of effectiveness across antibiotics as those observed here. In  
10 bacteria isolated from stranded harbor seals over a 12 year period, Lockwood (2006) observed  
11 widespread antibiotic resistance, with only one antibiotic capable of killing or inhibiting growth  
12 of all isolates tested. The authors did not report results for overall percentage of isolates resistant  
13 to one or multiple antibiotics, but did observe similar patterns in antibiotic-specific results to  
14 ours, with cultures exhibiting resistance most frequently to ampicillin (74% resistant) and  
15 cephalothin (64% resistant). A study of sharks from a variety of locations including waters off  
16 Belize, Florida, coastal and offshore Louisiana and Massachusetts found a high prevalence of  
17 antibiotic resistance in bacteria isolated from cloacal swabs (75, 86.5, 62, 52 and 87.5% for the  
18 five locations, respectively) (Blackburn, 2003). Dolejska et al. (2007) reported lower total  
19 occurrence of antibiotic resistance in isolates from Black-headed Gulls (30% vs. 58% observed  
20 in this study), but all of their isolates were *E. coli* and in our study this taxon had the lowest  
21 antibiotic resistance when compared to other bacterial groups (14% of our *E. coli* were  
22 susceptible to all antibiotics vs. 58% of all isolates tested). Another study of seabirds from  
23 rehabilitation centers on the Pacific coast of the United States had a much smaller sample size

1 (19 isolates from 15 birds) but saw high occurrence of antibiotic resistance, with 68% of isolates  
2 demonstrating resistance to at least one antibiotic (Steele *et al.*, 2005). These authors reported  
3 high levels of resistance to ampicillin, augmentin and cephalothin, which was consistent with our  
4 results, but also observed a high degree of resistance to ceftiofur (37% resistant), which was  
5 relatively effective against our bacterial isolates (10% resistant). Even higher levels of antibiotic  
6 resistance were reported by Bass *et al.* (1999) in a study of antibiotic resistance in *E. coli* isolates  
7 from diseased poultry at the Poultry Diagnostic and Research Center, University of Georgia.  
8 Virtually all isolates were resistant to at least one antibiotic, and 64% were resistant to five or  
9 more antibiotics. In contrast, a study of bacterial isolates from zoo animals in Japan found that  
10 21% of isolates tested for resistance to a wide spectrum of antibiotics demonstrated resistance to  
11 two or more antibiotics, which is approximately half the occurrence of multiply-resistant isolates  
12 we observed here (43%) (Ahmed *et al.*, 2007). Overall, reports on ABR bacteria from animals,  
13 and marine animals in particular, indicate not only the widespread presence of these microbes,  
14 but often a significant percentage of the bacteria demonstrating resistance to multiple antibiotics.

#### 15 **Antibiotic resistance in our samples.**

16 We observed widespread occurrence of antibiotic resistance and multiple antibiotic resistance in  
17 our samples (Fig. 1), which was consistent with many of the studies discussed in the previous  
18 section. A multiple antibiotic resistance (MAR) index value greater than or equal to 0.2 was  
19 observed in 38% of the resistant pathogens, suggesting exposure of the animals to bacteria from  
20 significantly contaminated sites. High MAR index values have been shown to be indicative of  
21 environments with high enteric disease potential (Krumperman, 1983). However, we noted that  
22 this high occurrence of resistance was not evenly distributed across all antibiotics, bacterial  
23 taxonomic groups, or all tissues sampled. The 16 antibiotics tested showed a wide range of

1 effectiveness against the bacterial isolates, from 99% effective (ciprofloxacin and enrofloxacin)  
2 to 61% effective (ampicillin). The four least effective antibiotics were ineffective against >25%  
3 of tested isolates and included cephalothin, ampicillin, augmentin and carbenicillin. Relatively  
4 high occurrence of antibiotic resistance against ampicillin, augmentin and cephalothin has been  
5 reported previously in environmental isolates (Boon and Cattanach, 1999; Miranda and  
6 Zemelman, 2001; Steele *et al.*, 2005; Lockwood *et al.*, 2006; Dolejska *et al.*, 2007; Lima-  
7 Bittencourt *et al.*, 2007; Watkinson *et al.*, 2007), although this is not always the case (Bass *et al.*,  
8 1999; Edge and Hill, 2005). To our knowledge, high occurrence of resistance to carbenicillin  
9 has not been reported previously. While bacterial resistance to ciprofloxacin, enrofloxacin and  
10 ceftazidime was low within the overall group, the demonstration of resistance by some isolates to  
11 these front-line antibiotics is noteworthy for its illustration of the diversity and widespread nature  
12 of antibiotic resistance within our environmental samples.

13         Among groups of bacterial isolates commonly sampled, occurrence of antibiotic  
14 resistance ranged from 14% of isolates (*E. coli*) to 92% (*Enterobacter* spp.) (Table 1). We were  
15 surprised to see such a range of resistance across different taxonomic groups, in particular to see  
16 such low occurrence of antibiotic resistance in *E. coli* when compared to other taxonomic groups  
17 and to the overall average for the data set as a whole. *E. coli* is often used exclusively to  
18 determine occurrence of antibiotic resistance in an environment in general, and for source  
19 tracking of fecal pollution based on antibiotic resistance patterns (e.g. (Krumperman, 1983;  
20 Parveen *et al.*, 1997; Bass *et al.*, 1999; Kelsey *et al.*, 2003; Edge and Hill, 2005; Dolejska *et al.*,  
21 2007; Kaneene *et al.*, 2007; Sjolund *et al.*, 2008). Our results are consistent, however, with two  
22 studies examining antibiotic resistance across a variety of bacterial taxa. Boon and Cattanach  
23 (1999) compared antibiotic resistance in *E. coli* and native heterotrophic bacteria isolated from

1 the Yarra River, Australia. This study reported significantly greater incidence of antibiotic  
2 resistance in native heterotrophic bacteria than in *E. coli* isolated from the same sites. Lima-  
3 Bittencourt et al. (2007) also reported much lower incidence of multiple antibiotic resistance in  
4 *E. coli* isolates relative to 9 other enterobacterial taxa. These results highlight the importance of  
5 examining a range of bacteria in order to determine an accurate representation of antibiotic  
6 resistance in an environment.

7 We did not observe any consistent trends in the group of isolates resistant to the largest  
8 number of antibiotics (Table 2). The isolates spanned a wide range of animal types, animal  
9 provenance, tissue types and bacterial taxonomic groupings. We did observe differences in  
10 occurrence of multiple antibiotic resistances across animal tissues sampled (Fig. 4). Most studies  
11 of bacteria isolated from animals have used swabs of single tissues or feces to characterize the  
12 incidence of single and multiple antibiotic resistance in the animal as a whole. Our results are in  
13 contrast, however, with one study that found no differences in the incidence of antibiotic  
14 resistance between bacteria isolated from swabs of gills and intestinal content in pelagic and  
15 demersal fish (Miranda and Zemelman, 2001). In our study, tissues that came into direct contact  
16 with the environment (oral, cloacal, blowhole) had higher incidence of bacteria with resistance to  
17 multiple antibiotics, and lower incidence of no resistance, than internal tissues (spleen, thorax)  
18 and fecal samples. Most of the bacteria isolated from fecal samples were *E. coli*, which had a  
19 relatively low incidence of antibiotic resistance compared to other bacterial taxonomic groups. It  
20 is not possible in our data set to determine whether it was the sample type (feces), bacterial  
21 group, or both, that had low occurrence of antibiotic resistance. However, bacteria isolated from  
22 the spleen and thorax belonged to a range of different taxonomic groups, suggesting results for  
23 these two internal tissues were tissue-specific rather than bacterial group-specific (data not

1 shown). The animals themselves may not be harboring large internal pools of antibiotic-resistant  
2 bacteria. High levels of antibiotic resistance have been reported to occur in biofilms (Stewart  
3 and Costerton, 2001; Gilbert *et al.*, 2002). It may be possible that the oral, cloacal and blowhole  
4 swabs sampled biofilms that are commonly present on external tissues.

#### 5 **Implications for animal health.**

6 The occurrence of antibiotic resistance was higher in bacteria isolated from seabirds than from  
7 marine mammals. Within marine mammals, there were also significant differences between the  
8 occurrence of antibiotic resistance in live vs. stranded and live vs. bycaught animals.

9 Additionally, there were differences in the patterns of antibiotic resistance, or the groups of  
10 antibiotics to which bacteria demonstrated resistance, between live vs. stranded and live vs.  
11 bycaught animals. We did not observe these differences among live, stranded or bycaught  
12 seabirds in either the incidence of antibiotic resistance or the patterns of antibiotic resistance.

13 The differences between marine mammals and seabirds may be due to different diet and habitat.  
14 Seabirds live and forage largely in nearshore coastal environments, which may result in  
15 increased exposure to either highly impacted sites (sewage treatment ponds, landfills) or bacteria  
16 brought to the marine environment in runoff from highly impacted sites (Nelson *et al.*, 2008).

17 These results may also indicate that live marine mammals are generally healthier than their  
18 stranded or bycaught counterparts. It has been a tacit assumption that bycaught animals  
19 represent a subsample of the ‘healthy’ population. This needs further examination given our  
20 findings in this study. A confounding factor in the analysis is that most of the samples from live  
21 animals were fecal swabs, and as described above, most of the bacteria isolated from the fecal  
22 swabs were *E. coli*. Both fecal samples and *E. coli* both showed relatively low incidence of  
23 antibiotic resistance relative to other tissues and other bacterial groups. Our data set

1 unfortunately does not contain a significant number of non-live-mammal fecal or *E. coli*  
2 samples. Thus, it is possible that the differences between the live vs. stranded and live vs.  
3 bycaught mammals in terms of both incidence of antibiotic resistant bacteria and patterns of  
4 antibiotic resistance may have been due to the sample types collected.

#### 5 **Implications for antibiotic resistance in the environment.**

6 We observed large variability in the incidence of antibiotic resistance among taxonomic groups  
7 of bacterial isolates (Table 1). These results indicate the need for expanding the scope of studies  
8 that seek to characterize antibiotic resistance in an environment based on a single indicator  
9 organism. This needed expansion of common current methodology was also suggested by the  
10 variability in multiple antibiotic resistances observed in different tissues sampled from the  
11 marine mammals and seabirds examined in this study. It may be that accurate characterization of  
12 antibiotic resistance in bacterial isolates from animal hosts should include multiple swabs from a  
13 range of tissues when possible.

14 This study examined the occurrence of antibiotic resistance in bacteria isolated from  
15 marine mammals and seabirds, and did not include samples from the coastal marine environment  
16 itself. The results of the tissue-specific multiple antibiotic resistance analysis suggested the  
17 possibility for relatively high incidence of antibiotic resistance in the surrounding environment.  
18 We do not, however, have direct evidence that the origin of antibiotic resistance in our samples  
19 was the coastal environment itself. There have been a few studies that compared the occurrence  
20 of antibiotic resistance in both animals and their surrounding environment, with mixed results.  
21 Edge and Hill (2005) reported slightly higher incidence of multiple antibiotic resistance in fecal  
22 swabs from seabirds (average MAR 0.07) than samples of surface waters (average MAR 0.059).  
23 Parveen et al. (1997) reported much lower incidence of antibiotic resistance among fecal samples

1 from terrestrial wild animals (27.6% of bacterial isolates resistant to at least one antibiotic) than  
2 from local estuarine surface waters (82% resistant isolates). However, Watkinson et al. (2007)  
3 found that the incidence of antibiotic resistance among bacterial isolates from oysters exposed to  
4 wastewater treatment plant discharge was much lower than from the discharge itself. Resistant  
5 isolates were found in 4% of the oysters vs. 31% of the discharge samples. Based on these  
6 discrepancies, it is thus unclear whether the high levels of antibiotic resistance observed are  
7 reflective of the larger coastal environment.

## 8 **Conclusions**

9 In summary, we observed widespread antibiotic resistance in bacterial isolates from a range of  
10 marine mammals and seabirds, and the high incidence of single and multiple antibiotic  
11 resistances in this study was consistent with other studies of bacterial isolates of animal origin.  
12 The source of antibiotic resistance in bacterial isolates from these marine mammals and seabirds  
13 is not clear. Some of these animals live in nearshore waters and/or come into regular contact  
14 with human populations, but multiple antibiotic resistance was also observed in marine mammals  
15 that inhabit offshore, deep water far from the presumed impact of coastal human populations.  
16 However, the widespread occurrence of single and multiple antibiotic resistant bacterial isolates  
17 from these marine mammals, as well as the relatively high occurrence of antibiotic resistance on  
18 external tissues sampled may reflect a large environmental pool of antibiotic resistant bacteria in  
19 coastal waters of the northeastern United States. We found large variability in the occurrence of  
20 antibiotic resistance both across bacterial taxonomic groups and animal tissues sampled,  
21 highlighting the potential need for the expansion of current common practices of single tissue  
22 samples and single indicator organisms to assess the incidence of antibiotic resistance in an  
23 animal or the environment. In terms of the animals sampled themselves, the observed

1 differences in incidence of antibiotic resistance between marine mammals and seabirds may be  
2 caused by differences in behavior and lifestyle and reflect the greater general exposure of  
3 seabirds to sources of human pollution. Additionally, the differences in both incidence and  
4 patterns of antibiotic resistance among live, stranded and bycaught marine mammals may be  
5 indicative of differences in overall animal health.  
6

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17

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33  
34

1 Table 1. Occurrence of antibiotic resistance across the nine most commonly isolated  
 2 bacterial taxonomic groups.

3

Bacterial Group (Total Number of Isolates)	Isolates sensitive to all antibiotics	Isolates resistant to one antibiotic	Isolates resistant to multiple antibiotics
<i>Aeromonas</i> spp. (20)	3	2	15
<i>Edwardsiella</i> spp. (20)	17	2	1
<i>Enterobacter</i> spp. (27)	2	5	20
<i>Escherichia coli</i> (117)	101	4	12
Non-enteric gram negative rod (27)	19	3	5
<i>Proteus</i> spp. (24)	2	9	13
<i>Shewanella</i> spp. (43)	12	17	14
<i>Vibrio</i> spp. (36)	4	2	30
<i>Vibrio alginolyticus</i> (21)	2	1	18

4

5

1 Table 2. Bacterial isolates demonstrating resistance to 8 or more antibiotics. Details include  
 2 the common name of the bird, mammal or shark from which each bacteria was isolated, the  
 3 provenance of the source animal (live, stranded or bycaught), the animal tissue swabbed, the  
 4 taxonomic affiliation of the bacterial isolate, the number of antibiotics to which each isolate  
 5 demonstrated resistance and the resistance profile. Antibiotic abbreviations as follows:  
 6 amikacin (AMK), ampicillin (AMP), augmentin (AUG), carbenicillin (CAR), ceftazidime  
 7 (CAZ), ceftiofur (CEF), cephalothin (CEPH), chloramphenicol (CHL), ciprofloxacin (CIP),  
 8 gentamycin (GEN), tribrissen (TRI), piperacillin (PIP), enrofloxacin (ENR), tetracycline  
 9 (TET), ticarcillin (TIC), and tobramycin (TOB).

10  
11

Animal Source (Common Name)	Provenance	Swab Location	Bacterial Isolate	# Resist	Resistance Profile
Harp Seal	Mammal Bycatch	Thorax	<i>Chryseobacterium indologenes</i>	13	AMK, AUG, AMP, CAR, CAZ, CEF, CEPH, CHL, GEN, TET, TIC, TOB, TRI
Harbor Porpoise	Mammal Bycatch	Thorax	<i>Sphingomonas multivorium</i>	12	AMK, AMP, CAR, CEF, CEPH, CHL, CIP, GEN, PIP, TET, TIC, TOB
Minke Whale	Mammal Strand	Prescapular Lymph	<i>Vibrio alginolyticus</i>	10	AMK, AMP, CAR, CEF, CEPH, CIP, ENR, GEN, PIP, TIC
Great Black-backed Gull	Bird Strand	Coelom	<i>Pseudomonas sp.</i>	9	AUG, AMP, CAR, CAZ, CEF, CEPH, CHL, TIC, TRI
Common Dolphin	Mammal Strand	Thorax	<i>Pseudomonas sp.</i>	9	AUG, AMP, CAR, CEF, CEPH, CHL, CIP, ENR, TIC
Herring Gull	Bird Live	Oral	<i>Proteus mirabilis</i>	9	AMP, CAR, CEPH, CHL, GEN, PIP, TET, TIC, TOB
Hooded Seal	Mammal Strand	Lymph	<i>Pseudomonas sp.</i>	9	AUG, AMP, CAR, CEF, CEPH, CHL, ENR, TET, TIC
Great Black-backed Gull	Bird Strand	Coelom	<i>Pseudomonas sp.</i>	8	AUG, AMP, CAR, CEF, CEPH, CHL, TIC, TRI
Atlantic White-sided Dolphin	Mammal Bycatch	Abdomen	Non-Enteric Gram Negative Rod	8	AMK, CEF, CEPH, CIP, ENR, GEN, TET, TOB
Herring Gull	Bird Live	Cloaca	Non-Enteric Gram Negative Rod	8	AMK, AUG, AMP, CAR, CEPH, TET, TIC, TRI
Pygmy Sperm Whale	Mammal Strand	Roof of Mouth	<i>Providencia rettgeri</i>	8	AUG, AMP, CAR, CEPH, CHL, PIP, TET, TIC
Pygmy Sperm Whale	Mammal Strand	Oral	<i>Pseudomonas sp.</i>	8	AUG, AMP, CAR, CEF, CEPH, CHL, TET, TIC
Herring Gull	Bird Live	Cloaca	<i>Burkholderia cepacia</i>	8	AUG, AMP, CAR, CAZ, CEF, CEPH, TET, TIC
Herring Gull	Bird Live	Oral	<i>Proteus vulgaris</i>	8	AUG, AMP, CAR, CEF, CEPH, PIP, TET, TIC
Herring Gull	Bird Live	Oral	<i>Pseudomonas sp.</i>	8	AUG, AMP, CAR, CEF, CEPH, CHL, PIP, TIC
Herring Gull	Bird Live	Oral	<i>Proteus penneri</i>	8	AMP, CAR, CEPH, CHL, PIP, TET, TIC, TRI
Herring Gull	Bird Live	Cloaca	<i>Escherichia coli</i>	8	AUG, AMP, CAR, CEF, CEPH, PIP, TET, TIC
Thresher Shark	Shark	Nares	<i>Pseudomonas sp.</i>	8	AUG, AMP, CAR, CEF, CEPH, CHL, TET, TIC

1 **Figure Legends**

2 1. Incidence of antibiotic resistance in bacterial isolates from marine mammals and seabirds.  
3 Isolates were plotted as a percentage of the total dataset based on the number of antibiotics to  
4 which they demonstrated resistance.

5  
6 2. Effectiveness of each antibiotic tested against the entire group of bacterial isolates. The  
7 percentage of total isolates demonstrating resistance is plotted for each antibiotic.

8  
9 3. Cluster dendrogram illustrating similarities among antibiotics in terms of which of the  
10 bacterial isolates demonstrated resistance. Dotted lines and asterisks indicate insignificant  
11 differences between pairs of antibiotics.

12  
13 4. Incidence of multiple antibiotic resistances among bacteria isolated from different animal  
14 tissues. Isolates were grouped into three categories according to whether they were sensitive  
15 to all antibiotics Multiple Antibiotic Resistance Index = 0, MAR less than 0.2 but greater  
16 than 0, or MAR greater than 0.2. The percentage of isolates in each category is plotted for  
17 each tissue.

18

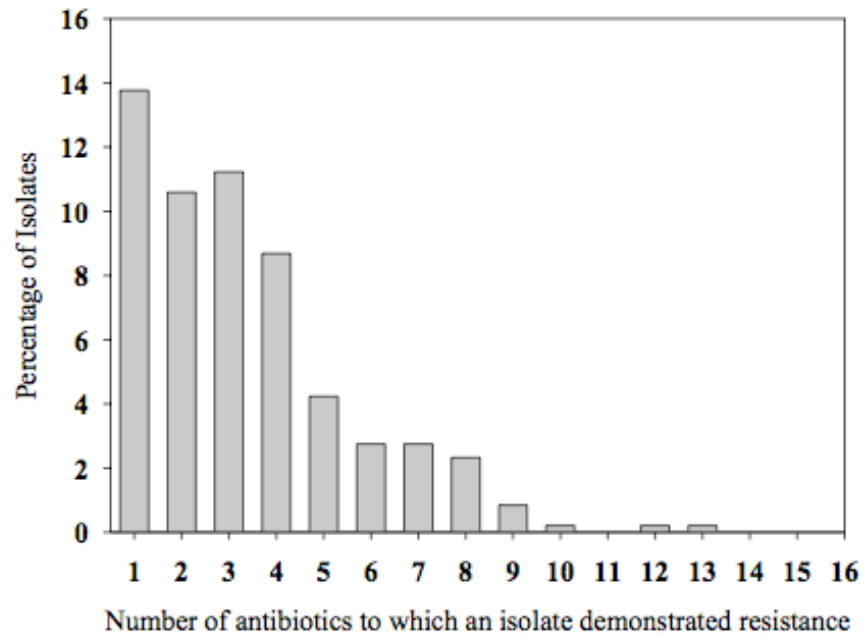
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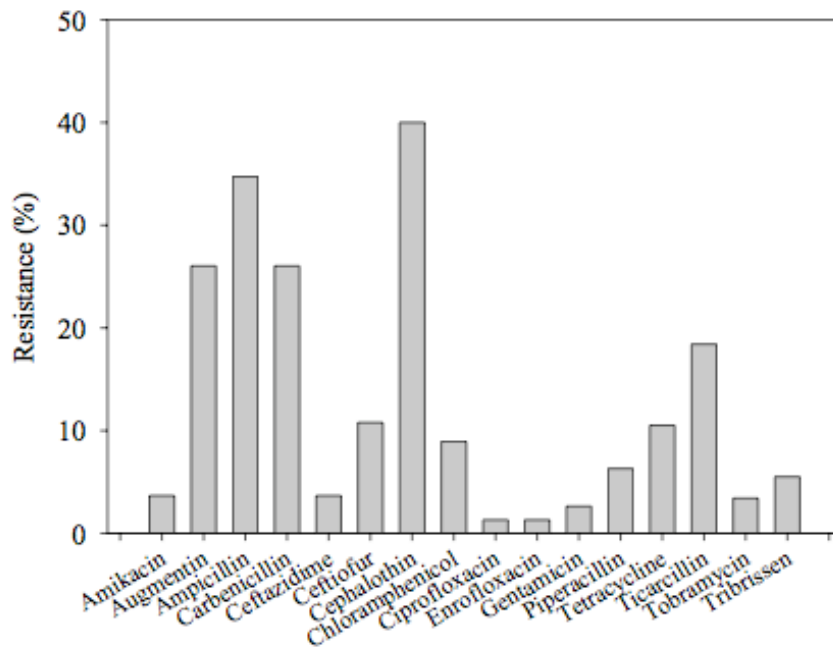
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Rose et al. Figure 1

1

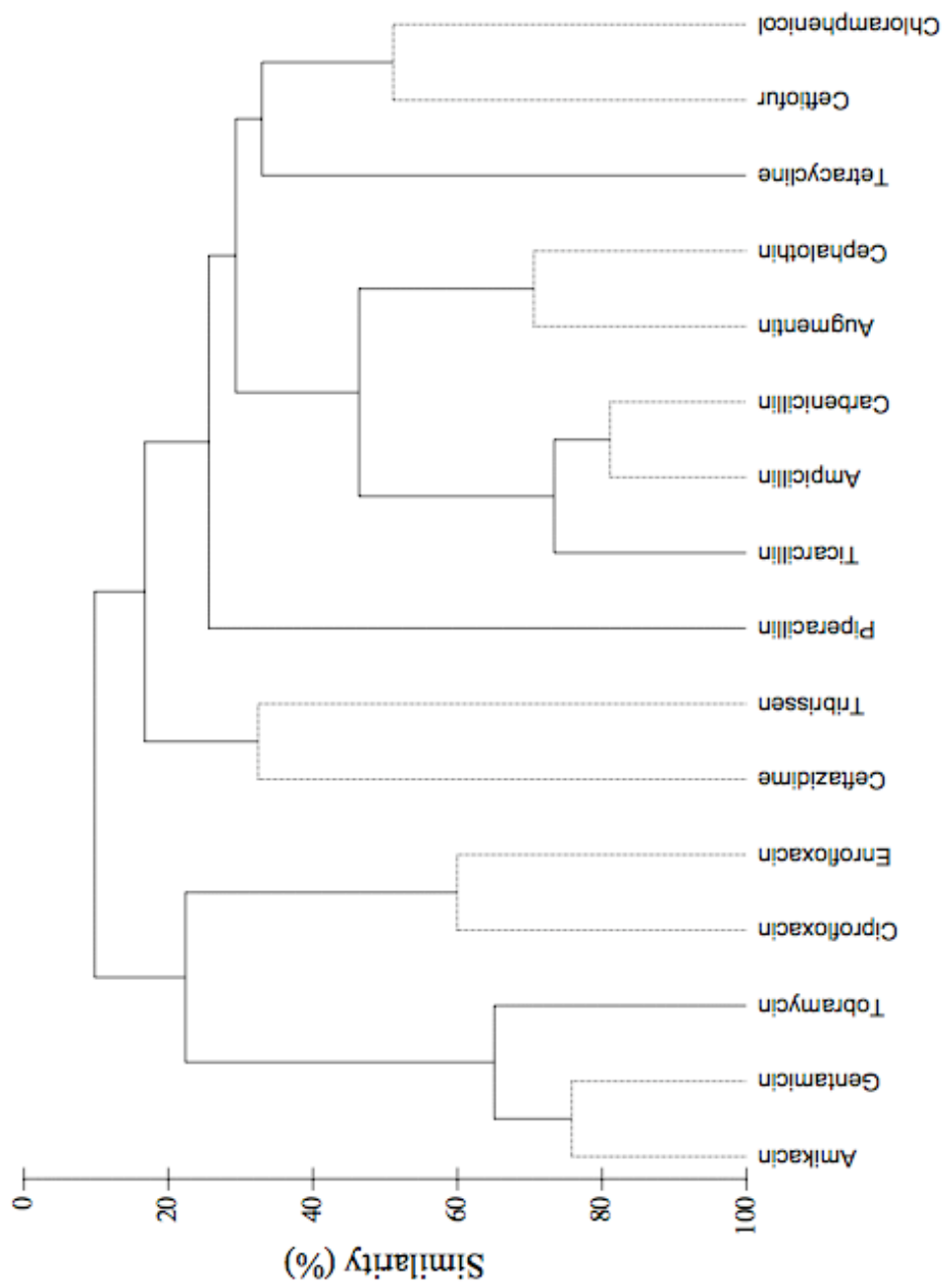
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Rose et al Figure 3

1

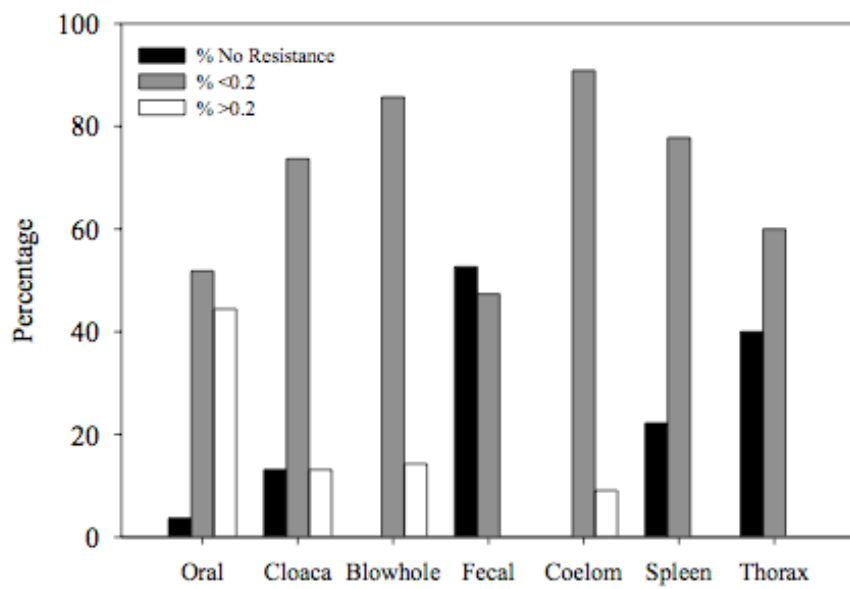
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Rose et al. Figure 4

1

2



Rose et al Figure 5