

Effect of heavy metal pollution on the incidence of antibiotic resistance in *Aeromonas hydrophila* isolates obtained from the surface of fish

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ABSTRACT: A large collection of aquatic *Aeromonas* obtained from the surfaces of 5 species of fish in 2 streams with different contamination histories were examined to test hypotheses on the effects of contaminant history, stream habitat and longitudinal location on the incidence of antibiotic resistance (AR) towards 6 antibiotics and levels of multiple antibiotic resistance (MAR). Fish species included an open-water species (*Micropterus salmoides*), 2 mid-water species of centrarchids (*Lepomis auritus* and *L. punctatus*) and 2 species of bottom-dwelling fish (*Ameiurus natalis* and *A. platycephalus*). Metal analysis of the sediments indicated that there was a strong downstream contamination gradient in one stream but not in the other. However, we found that the average MAR level was similar between the 2 streams. Comparisons among fish species found the highest levels of resistance in bacteria isolated from bottom-dwelling fish in the least-contaminated stream. However, there were clear differences in levels of resistance between bacteria isolated from the 2 bottom-dwelling fish, with those isolated from *A. natalis* having significantly higher levels than those isolated from *A. platycephalus*. We suggest that these differences relate to higher concentrations of metals and other contaminants in habitats where *A. natalis* is normally found. For some antibiotics, there was a clear pattern of decreasing resistance among bacteria isolated from bottom-dwelling, mid-water and open-water fish. Unlike culturable sediment bacteria from the same streams in previous studies, these commensal aeromonads did not show the same expected patterns of increasing antibiotic resistance in metal-contaminated vs. uncontaminated streams.

KEY WORDS: Antibiotic resistance · *Aeromonas* · Commensal · Fish · Industrial pollution · Habitat partitioning

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INTRODUCTION

Antibiotic resistance (AR) in bacteria is increasing. A number of studies have suggested a model wherein environmental reservoirs of pathogenic bacteria such as *Campylobacter*, *Aeromonas*, *Escherichia coli* or *Salmonella* acquire AR determinants by an unknown mechanism possibly mediated by plasmids and/or transposons (Levy 2001, Wilson & Salyers 2003, Taylor et al. 2011). Understanding the ecology and spread of antibiotic resistant bacteria could contribute to better

control and management of subsequent human infections (Levy & Marshall 2004, Allen et al. 2010, Taylor et al. 2011).

One environmental mechanism that seems to have contributed to the increase in AR is an indirect or co-selection response through exposure to heavy metals (McArthur & Tuckfield 2000, Stepanauskas et al. 2005, 2006, Baker-Austin et al. 2006, Wright et al. 2006). These studies have shown either through field correlation or through controlled laboratory experiments that exposure to heavy metals (e.g.

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Hg, Cd or Ni) above background concentrations increases both the incidence of heavy metal resistance and the incidence of AR in planktonic-, biofilm- and sediment-associated aquatic microbial communities.

Native bacterial flora may act as environmental reservoirs of resistance genes in aquatic systems (Taylor et al. 2011). Perhaps more importantly, natural environmental bacteria are an unlimited source of genes that might act as or be modified into resistance genes that can be transferred to pathogenic bacteria (Baquero et al. 2008, Allen et al. 2010). These genes may not function primarily as resistance genes but can under selection be converted into AR genes. Such genes are considered part of the resistome, the set of potential genes that can confer resistance (D'Costa et al. 2006).

Fish are highly mobile organisms that can move into or out of contaminated areas. However, within a given reach of contaminated stream, not all fish will receive the same exposure to a toxicant, especially if the toxicant is primarily associated with the sediments of the stream. Some fish spend nearly all of their time in the water column, with most feeding and daily activities for these fish occurring above stream sediments. In contrast, benthic fish species spend much of their time hiding or feeding among sediments. Based on our previous studies of sediment-associated bacteria, we hypothesized that commensal bacteria obtained from fish in a heavy metal-contaminated stream would have higher proportions of AR and higher proportions of multiple antibiotic resistance (MAR) than similar bacteria collected from fish in a less disturbed stream.

Moreover, within each stream, we hypothesized that commensal bacteria associated with bottom-dwelling fish (ictalurids) would have higher incidences of AR than similar bacteria that are associated with water column fish (centrarchids). Finally, because the contamination in our polluted stream was from point sources, we expected there to be a decreasing incidence of resistance further from these sources in the contaminated stream and no such pattern in the reference stream. To test these hypotheses, we collected fish from a heavy metal-contaminated stream and a relatively undisturbed stream, and isolated a large library of *Aeromonas hydrophila* that were then screened against 6 different antibiotics.

MATERIALS AND METHODS

Study sites

We collected fish from 2 extensively studied stream systems found on the US Department of Energy's Savannah River site (SRS) in South Carolina, USA. The first stream, Fourmile Branch (FMB), received thermal effluents for 30 yr but had been recovering from thermal stress for over 15 yr. In addition, FMB has had inputs of heavy metals (Cd, Hg) either directly from plant operations or from leachates arising from settling basins in the watershed (Tuckfield & McArthur 2008). These basins received effluent that also contained nitrate, radioactive Cs-137, other heavy metals and tritium. During thermal flows, discharge was an order of magnitude higher than historic base flows. These high flows resulted in widening of the channel and removal of most of the instream structure. Subsequent to reactor operations, the stream has begun a trajectory of recovery (Lakly & McArthur 2000). FMB is a low-gradient sandy bottom stream that drains a watershed of approximately 57 km². The stream empties into a riverine swamp adjacent to the Savannah River that forms the border between South Carolina and Georgia. Water quality and physical parameters for our study streams are provided in Table 1.

Meyers Branch (MB) is a blackwater low-gradient stream on the SRS that drains 50 km². MB has received minimal impacts from site operations and for the past 50 yr has had no continual anthropogenic inputs. However, during wet years, the headwaters of MB connect to stormwater runoff from 2 industrial areas, one of which has an associated coal ash basin within its drainage that has contributed metals

Table 1. Average stream water chemistry for Meyers Branch and Fourmile Branch. NTU: nephelometer turbidity units; NA: not available. Source: Halverson et al. (1997)

	Meyers Branch		Fourmile Branch	
	Mean	Range	Mean	Range
Water temperature (°C)	15.7	0.1–25.5	17.5	4.2–31
pH	6.9	5.80–8.30	6.5	3.1–8.5
Stream maximum depth (cm)	49.3	23–111	NA	NA
Stream velocity (cm s ⁻¹)	28.3	5–82	NA	NA
Dissolved oxygen (mg l ⁻¹)	7.7	4.20–12.4	8.4	5.0–15.9
Specific conductivity (µS cm ⁻¹)	48.0	25.0–72.4	57.9	0.15–135
Turbidity (NTU)	6.0	1.50–47.0	7.0	1.1–42
Total suspended solids (mg l ⁻¹)	5.2	0.25–93.2	4.9	<1–47
Drainage area (km ²)	50		57	
Stream length (km)	10		24	

(Tuckfield & McArthur 2008). Additionally, MB flows into a contaminated stream, Steel Creek; thus, fish in the lowest portions of MB may be at risk of impact from Steel Creek. Prior to the establishment of the SRS, the stream drained primarily agricultural lands. However, extensive floodplain forests were maintained and continue today. These floodplain forests are dominated by hardwood trees such as water tupelo *Nyssa aquatica* and bald cypress *Taxodium distichum*.

Study organisms

We selected fish species based on their abundance, distribution breadth and habitat use. Selection of both bottom-dwelling and mid-water species allowed us to contrast bacteria inhabiting fish from these different habitat-use guilds. Two species of ictalurids represented our bottom-dwelling taxa, yellow bullhead *Ameiurus natalis* and flat bullhead *A. platycephalus*. These nocturnal species spend their daylight hours hiding on or near the sediments and commonly feed among the sediments at night. They are moderate-sized ictalurids generally less than 250 mm standard length (SL) on the SRS. The ictalurids also differ from our other study species by not having scales covering their bodies. Though frequently syntopic and both bottom dwelling, these 2 species generally differ in habitat utilization. Because of this habitat specificity, both species were not necessarily collected from each location (Table 2). Flat bullheads are typically found in or adjacent to

faster water with sand or mixed sand and silt substrates (Meffe & Sheldon 1988) and are often associated with cover such as wood debris or root masses. In contrast, yellow bullheads are associated with slower water with muddy and leafy substrates (Meffe & Sheldon 1988). In SRS streams, the diet of the yellow bullhead consists of crayfish and a variety of other aquatic and terrestrial arthropods and fish (Sheldon & Meffe 1993). The diet of the flat bullhead is similar but differs in having a prevalence of snails in its diet (Sheldon & Meffe 1993), a predominance also noted in other areas (Yerger & Relyea 1968).

We selected 2 species of centrarchids, redbreast sunfish *Lepomis auritus* and spotted sunfish *L. punctatus*, as our inhabitants of mid-water. Redbreast sunfish are a larger species commonly reaching 150 mm SL, whereas spotted sunfish are usually less than 120 mm SL in SRS tributary streams (Marcy et al. 2005). These 2 sunfish species are generally syntopic, but spotted sunfish are more broadly distributed in SRS tributary streams; redbreast sunfish are typically more abundant in intermediate-size streams where they inhabit deeper and broader waters (Meffe & Sheldon 1988, Marcy et al. 2005). Both are primarily invertivorous species feeding on a variety of aquatic and terrestrial insects, microcrustaceans, smaller decapods and snails (Sheldon & Meffe 1993, Wiltz 1993, Marcy et al. 2005).

We collected at least 25 adult redbreast sunfish and 25 spotted sunfish from each of the 11 locations, but collections of the ictalurids was always more variable with fewer individuals collected (Table 2). Largemouth bass *Micropterus salmoides* are more typical of lentic or larger riverine waters than the tributaries that we examined, but this species was also fortuitously collected for comparison.

Table 2. Numbers of fish collected from each location. FMB: Fourmile Branch; MB: Meyers Branch; *La*: *Lepomis auritus*; *Lp*: *L. punctatus*; *An*: *Ameiurus natalis*; *Ap*: *A. platycephalus*; *Ms*: *Micropterus salmoides*

Site	Creek	<i>La</i>	<i>Lp</i>	<i>An</i>	<i>Ap</i>	<i>Ms</i>
F-RD3	FMB	25	25	4	0	0
F-CA	FMB	27	25	2	0	1
F-BCA	FMB	25	25	12	5	6
F-6.1	FMB	25	25	15	15	10
F-12.2	FMB	25	25	1	10	2
F-BDK	FMB	30	25	1	5	11
M-B6.2	MB	25	25	6	4	0
M-ARR	MB	25	26	1	7	0
M-BRR	MB	25	25	14	6	0
M-RD9	MB	25	25	3	8	0
M-G22	MB	25	25	4	2	1
M-BDK	MB	25	25	3	11	4
Total		307	301	66	73	35

Sediment sampling

Previous studies have indicated a decreasing longitudinal gradient in contaminant concentration for 10 metals (Fe, Al, Mn, Hg, Zn, Cu, Pb, Ni, Cr and Cd) in FMB sediments progressively downstream from Castor Creek, a tributary to FMB, with lowest concentrations occurring near the streams entering into the riverine swamp (Tuckfield & McArthur 2008). We collected sediment samples from 5 locations on FMB and 6 locations on MB. At each location, samples were collected at a central location and then at 3 contiguous 100 m distances upstream and downstream. At each stream sampling location, one 10 cm × 2.5 cm diameter core was taken from near-bank bottom sed-

iments, placed on ice in a sterile bag and transported to the laboratory. Concentrations for 10 metals (ppm; Hg in ppb) were obtained from all sediment samples by inductively coupled plasma methods from services provided by the inorganic chemistry laboratories of the University of Georgia. Metal concentration measurements reported below the laboratory detection limit (DL) were replaced by DL/2 before data analysis, a typical environmental data analysis practice (Newman et al. 1989).

Fish collections

To capture the contamination gradient in our comparison, fish collections were made at 5 locations along an 8.8 km section of FMB (Fig. 1). Fish were captured with a backpack electrofisher by walking upstream and collecting around known habitat for our target species. At each of the 5 locations, the starting point was marked and the stream marked into 100 m contiguous sections. The capture location of each fish was recorded by noting from which 100 m reach of stream the fish was collected.

For comparison with the contaminated stream, we also made collections at 6 locations along a 7 km segment of the reference stream, MB (Fig. 1). Collections in the lowest reach began 300 m above the MB confluence with Steel Creek to lessen the potential influences of Steel Creek.

Bacterial isolations

Commensal bacteria of several taxa can be found associated with external surfaces of most species of fish. Some of these bacteria can be pathogenic and debilitating to fish under various circumstances. *Aeromonas hydrophila* is the primary cause of motile aeromonas septicemia (MAS), formerly referred to as red sore disease among a number of other synonyms (Esch & Hazen 1978, Shotts 1994). Isolates of *A. hydrophila* can be obtained from both diseased and healthy fish, indicating that lesions are secondary to some other weakening of the fishes' immune or protective systems. We isolated *A. hydrophila* from each fish collected.

On capture, the presence of lesions on the fish was recorded in the field and each fish was individually transferred from the net into a sterile bag. The fish were kept on ice until delivery to the lab, refrigerated overnight and processed the next morning. A sterile cotton swab wetted with sterile 0.85% saline was

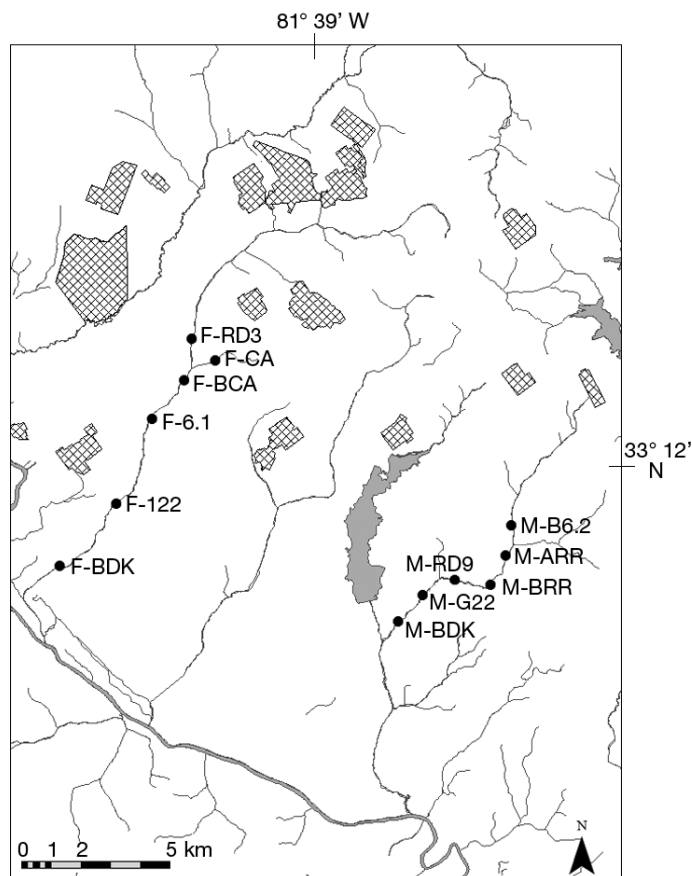


Fig. 1. Location of the streams and sampling sites. F: Fourmile Branch sites; M: Meyers Branch sites

used to swab a 2 cm × 2 cm square area posterior to and above the fish's left pectoral fin. The swab was then streaked onto an *Aeromonas* medium base (Ryan) plate (Oxoid) prepared according to the manufacturer's instructions to include 50 µg ml⁻¹ ampicillin, which that selects for *A. hydrophila* (Henriques et al. 2006). The plates were incubated at 30°C for 24 h. After incubation, the presence of *Aeromonas*-type colonies (opaque green with a darker green center) was noted. Master plates were created by randomly removing up to 10 colonies per plate and individually transferring them with a sterile toothpick to a fresh nutrient broth agar plate (8 g nutrient broth (Difco) and 15 g bacto-agar l⁻¹). The colonies were dotted onto the master plate in a 50-unit grid pattern. The plates were then sealed with parafilm and incubated at 25°C for 2–3 d. The master plates were then replicated using sterile velveteen squares and a replicating tool (Bel-Art Products) onto a series of nutrient broth agar plates each containing one of the following antibiotics: kanamycin (100 µg ml⁻¹), streptomycin (100 µg ml⁻¹), tetracycline (30 µg ml⁻¹), ciprofloxacin (30 µg ml⁻¹), chloramphenicol (30 µg

ml⁻¹) and gentamicin (30 µg ml⁻¹). These antibiotics and concentrations were used based on our previous studies (McArthur & Tuckfield 2000) and represent 4 distinct classes of antibiotics. These plates were incubated at 25°C and counted 1 wk later. Each isolate was scored for resistance or susceptibility to each antibiotic and the level of MAR. MAR scores indicated the number of antibiotics each isolate was resistant to and ranged from 0 to 6.

A total of 785 fish were collected, of which we were able to collect *Aeromonas* from 654 individuals. These 654 fish yielded 4468 presumptive *A. hydrophila* colonies that were collected and screened for each of the 6 different antibiotics. Isolates from both species of centrarchids and both species of ictalurids were collected from each stream in sufficient numbers to allow meaningful tests of our hypotheses. Large-mouth bass *M. salmoides* were primarily caught in FMB and so these specimens were only used for within FMB comparisons.

Statistical analyses

Each isolate was screened for resistance to 6 different antibiotics and the resulting score for each isolate was summed for all isolates from a specific fish. This produces a score from 0 to the maximum number of isolates obtained for each antibiotic and fish (range 6–10). This score was converted to a proportion of isolates tolerant to a specific antibiotic, which in turn was transformed using $y = \arcsin(\sqrt{p})$ where p is the proportion of resistant isolates per fish. Differences in response y between streams, species and the interaction between streams and species were determined using ANOVA.

Each fish sample produced a multivariate response vector $\mathbf{y} = \{y_1, y_2, y_3, y_4, y_5, y_6\}$ among the 6 antibiotics. Principal component analysis (PCA) was used as a dimensionality reduction technique to produce a summarized univariate measure of AR among isolates that explained the largest amount of variation among the 6 transformed responses. A PCA was also performed among the metal concentration measurements in sediment samples collected from the same streams (Tuckfield & McArthur 2008). Stream data were combined separately for metal and AR responses and PCs generated from the correlation matrix, which preserves the relative contribution (i.e. eigenvector coefficients) of each univariate response measure to the information contained in each AR or metal PC. Therefore, the eigenvalue corresponding to each PC also represents the number of dimensions sum-

marized in that PC. The first PC scores are the dimensionality reduced summary responses used in our analyses.

RESULTS

Based on 2-way ANOVAs (Stream, Species, Stream × Species) using the response to each of the 6 antibiotics and MAR, we found no significant differences in the levels of resistance between the 2 study streams (ANOVA Stream $p > 0.1$ in all analyses). In other words, the level of resistance within these 2 streams was not significantly different across all fish species examined. However species-specific patterns were observed.

Habitat use of the fish species did affect the occurrence of antibiotic resistance (Fig. 2). For chloramphenicol, streptomycin and MAR, bacteria isolated from the bottom-dwelling fish had significantly higher levels of resistance than those isolated from all other fish species (Fig. 2). However, primarily bacteria isolated from *Ameiurus natalis* drove this pattern. Levels of resistance in bacteria from the other bottom-dwelling species, *A. platycephalus*, were generally lower than those obtained for either of the 2 *Lepomis* species but higher than those from *Micropterus salmoides*. MAR followed a similar pattern with *A. natalis* > *L. auritus* > *L. punctatus* > *A. platycephalus* > *M. salmoides*. No significant species patterns were

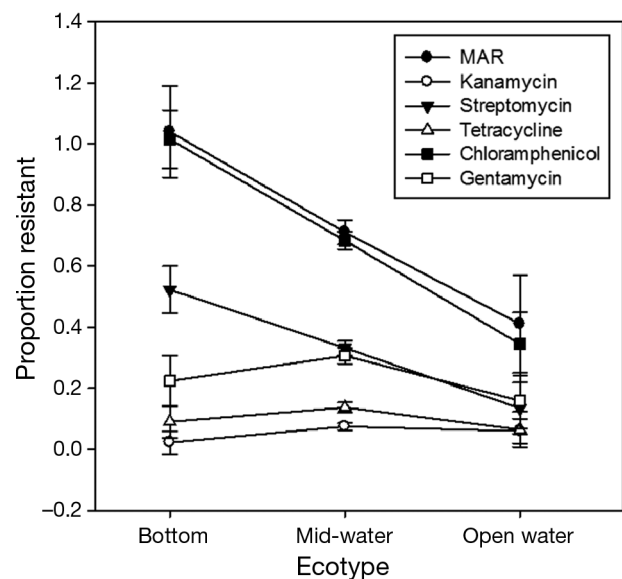


Fig. 2. Ecotypic difference in the proportion of the bacterium *Aeromonas hydrophila* tolerant to various antibiotics and the mean multiple antibiotic resistance (MAR) for each ecotype. Error bars are SE

found for kanamycin, gentamicin or tetracycline resistance. Thus, for some antibiotics and for MAR, these results support our hypotheses with the noted exception of *A. platycephalus*, which was very different from the other bottom-dwelling species.

Habitat use or other biological aspects of the bottom-dwelling fish species did affect the occurrence of AR of bacteria isolated from their surfaces (Fig. 3), where 7 out of 12 possible pairwise comparisons by antibiotic and MAR between bacteria from the 2 bottom-dwelling species of fish averaged across streams were significantly different ($p < 0.05$). Significantly higher levels of resistance were observed towards chloramphenicol, streptomycin and MAR in

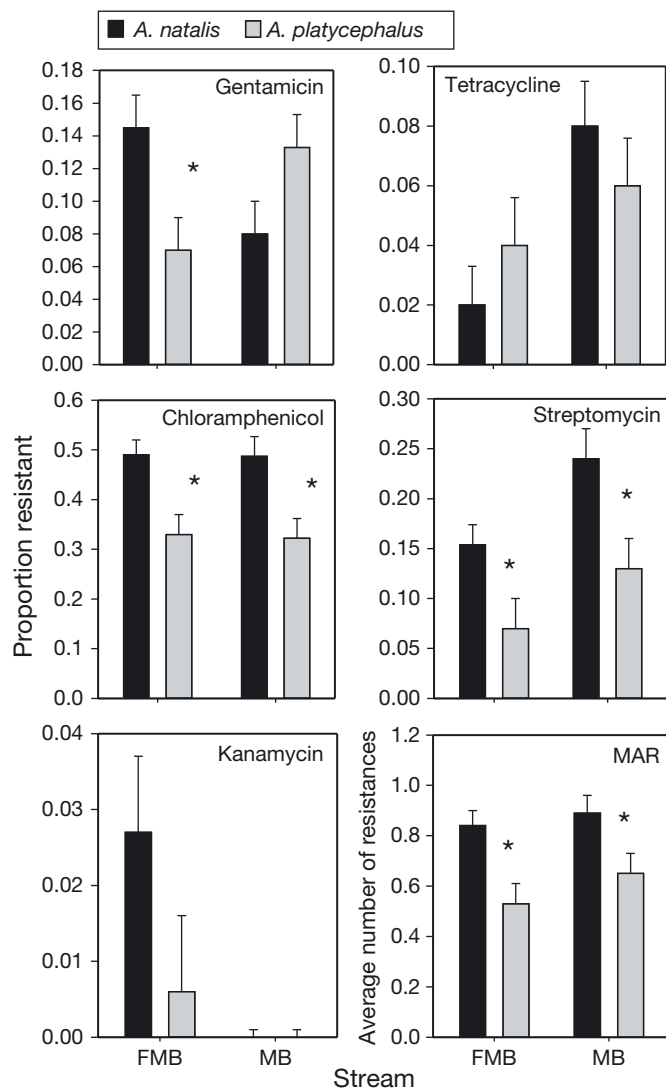


Fig. 3. Comparisons within streams between levels of resistance of the bacterium *Aeromonas hydrophila* collected from *Ameiurus natalis* and *A. platycephalus*. *: significant difference between species within a stream. FMB: Fourmile Branch; MB: Meyers Branch; MAR: multiple antibiotic resistance

bacteria isolated from *A. natalis* than bacteria from *A. platycephalus* in both streams. Higher levels of resistance were only found towards gentamicin in bacteria from *A. natalis* collected in MB, but no differences were observed in bacteria collected in FMB. In every other significant case, higher levels of resistance towards the 5 antibiotics and levels of MAR were observed in bacteria from *A. natalis*. In contrast, there were only 3 out of 12 possible pairwise comparisons that were significantly different in levels of resistance in bacteria collected from the 2 mid-water species between the 2 stream systems (data not shown).

The first PC for the metals ($PC1_{Met}$) reduced the dimensionality of the metals data from 10 variables to a single metric that explained 78 and 41.5% of the variability among sites in FMB and MB, respectively. The values of $PC1_{Met}$ for each stream cannot be directly compared between streams as the values are computed from a correlation matrix within each stream. Relative differences between species within a stream were compared. $PC1_{Met}$ indicated that there was a distinct longitudinal gradient of metals in FMB (Fig. 4). $PC1$ of metals in MB also indicated a decrease in metal concentrations along the upstream 3 sites, but this pattern did not continue further downstream along the stream course.

The within-stream longitudinal patterns (Figs. S1 to S5 in the Supplement at www.int-res.com/articles/suppl/a079p197_supp.pdf) of $PC1$ scores based on the 6 antibiotics ($PC1_{AR}$) were highly variable among fish species (Fig. 4). Of the 4 fish species plotted per stream, only *A. natalis* and to some degree *A. platycephalus* in MB (Fig. 4b) demonstrated a decreasing pattern of resistance among the 6 antibiotics with distance downstream. This observation was not expected. No consistent longitudinal gradient of resistance was observed in FMB for either of these 2 species (Fig. 4a). However, AR was considerably lower in bacteria on *A. natalis* from the most-downstream site. Interestingly, the $PC1_{AR}$ explained less variation among the data within each stream than the $PC1_{Met}$ with 33.9 and 37% for MB and FMB, respectively. Thus, variation in patterns of AR across the 6 antibiotics is generally not related to stream location for most of the fish examined.

DISCUSSION

The relationship between industrial metal contamination and levels of AR, while complex and varied, has increasingly been shown to be important in a

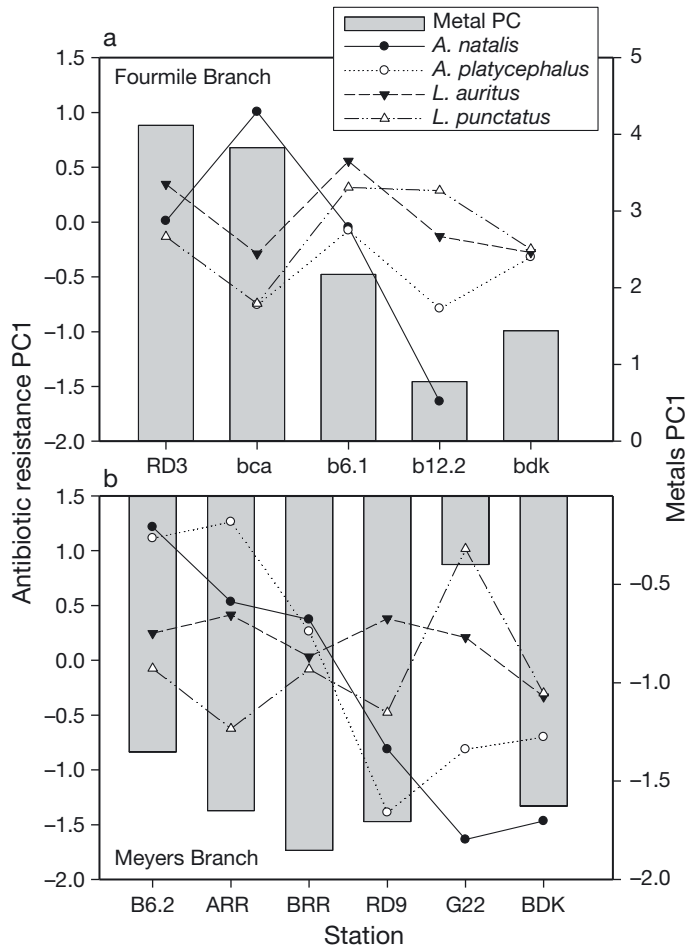


Fig. 4. Longitudinal pattern of PC1 scores for antibiotic resistance (lines) by fish species and metals (bars) for each stream and sampling location. Full species names as in Table 2

variety of ecosystems and for a diverse suite of contaminants (Alonso et al. 2001, Berg et al. 2005, Baker-Austin et al. 2006). However, there is not a clear one-to-one correspondence between levels of heavy metal and industrial contamination and the proportion of bacteria resistant to some antibiotics. Various metals are positively correlated with AR in a variety of habitats (Sabry et al. 1997, De Souza et al. 2006). For example, Berg et al. (2005) found that Cu exposure in soils increased levels of Cu resistance and concurrent levels of AR. Miranda & Castillo (1998) demonstrated that easily isolated aquatic aeromonads from source waters in Chile demonstrated resistance to both antibiotics and metals and warned that such a finding could indicate a serious public health problem. Aeromonads isolated from rainbow trout *Oncochrynychus mykiss* from fish farms in Australia demonstrated high levels of both metal resistance and AR (Akinbowale et al. 2007).

We have previously shown that there is a spatially explicit distribution of AR in sediment-associated bacteria decreasing along a downstream gradient in FMB but not in MB (McArthur & Tuckfield 2000, Tuckfield & McArthur 2008). Based on those patterns, we hypothesized that bacteria attached to the surface of fish might have differential exposure to industrial contamination between stream systems, among locations in FMB and among species of fish based on their typical habitat.

Our first hypothesis predicted a higher proportion of antibiotic-tolerant bacteria on the surfaces of fish collected from the contaminated stream than from the less-impacted stream system. The results from our 4400+ isolates found some significant differences in the incidence of AR between the same species of fish collected from the 2 stream systems. However, average levels of resistance across all species of fish did not differ between the 2 streams. Higher levels of resistance, when observed, occurred in MB but not in FMB, the highly disturbed stream. This lack of a pattern of resistance between the 2 streams was unexpected given the previous studies conducted in these systems that demonstrated differences in the frequency of resistant bacteria associated with the sediments (McArthur & Tuckfield 2000). In those earlier studies, spatial patterns in resistance traits were only found in FMB and not MB (Tuckfield & McArthur 2008). However, although resistance in sediment bacteria was observed in MB, there was no downstream longitudinal pattern (Tuckfield & McArthur 2008); rather, the incidence of resistance was fairly constant along the stream course averaging around 20% of the isolates showing resistance. In contrast, the bacteria in FMB showed a clear pattern of increasing resistance (up to 100% of isolates) and subsequent decrease.

FMB has been impacted by point-source pollution of metals and other contaminants from a variety of sources, including polycyclic aromatic and chlorinated hydrocarbons that have been shown to influence levels of AR in some bacteria in other studies (Kadavy et al. 2000). Thus, the selective pressures in the 2 systems are different but have resulted in similar levels of AR in bacteria isolated from the fish species sampled in this study. Given that the pollution history of these streams is extremely different, we are surprised by this result, and the observation further complicates understanding of the mechanisms that develop and maintain gene nurseries in the environment.

Our second hypothesis was that levels of bacterial AR would be higher in isolates taken from fish associated with sediments than in bacteria from fish

found in mid- or open waters (Fig. 2). Further, our third hypothesis predicted these differences in AR of bacteria collected from the different ecotypes of fish within a stream system to follow the patterns of distribution of contaminants in sediments (Fig. 3). These 2 hypotheses are not mutually exclusive. Since contaminants are often stored in the sediments, especially in depositional zones (Fletcher et al. 2014), we expected that bacteria isolated from bottom-dwelling fish would have higher incidences of AR than those isolated from open-water-dwelling fishes because of their close association with contaminated sediments. Sediments also have orders of magnitude higher densities of bacteria than the overlying water. Our results generally support these hypotheses.

Our resistance data support the contention that a higher concentration of metals in the sediments is indicative of higher exposure or bioavailability (Fig. 2). When difference in resistance did occur, the highest levels were associated with bacteria isolated from bottom-dwelling fish species. Bacteria isolated from *Ameiurus natalis* had the highest levels of resistance (Figs. 2 & 3) to some antibiotics. However, bacteria isolated from the other bottom-dwelling fish, *A. platycephalus*, had lower or similar levels of resistance to these same antibiotics than bacteria isolated from the mid-water *Lepomis* species. At first glance, this observation seems to refute hypothesis 2. However, when combined with results of hypothesis 3, it is apparent that not only proximity to the substrate but also the type of substrate present is important in determining risk of exposure of commensal bacteria to contaminants in those substrates. Previous studies on the SRS have found *A. natalis* and both *Lepomis* species to be typical of habitat with slower water and more silty and leafy substrates, whereas the habitat of *A. platycephalus* is faster running water with coarse sandy substrates (Meffe & Sheldon 1988).

Even though fish are relatively long-lived highly mobile organisms that can traverse long distances over relatively short periods of time, some species of fish have preferential habitats that limit their exposure to different types of sediment. If heavy metal deposition differs among the specific habitats, we might expect differences in the response of the bacteria found on their host surfaces. We have found that metal concentrations in sandy reaches are significantly lower than those found in depositional finer sediments (Fletcher et al. 2014). Moreover, contaminant levels can be highest in the slowest depositional zones. *A. natalis* often inhabits particularly slow lotic or lentic waters. Local stream studies have found *A. natalis* in slower water with more silt

or even muddy and leafy substrate than in habitat used by *A. platycephalus* (Meffe & Sheldon 1988). Thus, this use of slower depositional zones by *A. natalis* may result in bacteria on its surfaces receiving greater contaminant exposure and thus greater indirect selection than those on *A. platycephalus*. Indeed for some antibiotics we screened and for MAR, there was a strong relationship with fish habit and habitat preferences (Figs. 2 & 3) even when averaged across streams (Fig. 2). Similarly, the intermediate resistance of the mid-water *Lepomis* may be influenced by their use of slower water and more silty and leafy substrates than that of *A. platycephalus* (Meffe & Sheldon 1988).

Interestingly, bacteria from the open-water fish *Micropterus salmoides* had the lowest level of resistance of all other bacterial isolates from the other species of fish. Largemouth bass spend nearly all of their time in open water and thus bacteria on their surfaces are only exposed to dissolved metals. The concentration of dissolved metals in similar streams found on the SRS is much lower than that found in the sediments (Fletcher et al. 2014, J. V. McArthur unpubl. data). Use of cover by *Lepomis* or *Ameiurus* species could be further influenced by use of woody debris and root mats in these streams.

When all AR traits and metal concentrations were considered together using PCA, we observed a complex relationship between the various species (Fig. 4). Tuckfield & McArthur (2008) have previously shown that the concentrations of 10 heavy metals in the sediments of these same 2 streams decrease with distance in FMB. Our PCA of all 10 elements showed that again concentrations decreased with distance downstream in FMB but not MB. However, there were no general longitudinal patterns of AR among any isolates (Fig. 4). Resistance in *A. hydrophila* did not show any significant relationship with metal concentrations in either stream with a few exceptions.

Our data support the contention that higher exposure to stream sediments results in higher proportions of AR bacteria (Fig. 3). However, since we did not specifically sample slow depositional zones, we can only suggest that higher exposure occurred in these zones. Microbial biofilms are an effective barrier against antimicrobials (Mah 2012); however, we have demonstrated that biofilms in similar streams can have elevated concentrations of some metals relative to the sediments or water column (Fletcher et al. 2014). As metals accumulate in biofilms, the associated bacteria are exposed to increasing concentrations of metals and the co-selection response takes over (Wright et al. 2006).

The mucus layer of fish has been shown to be a protective barrier to infections from a variety of pathogens (Bols et al. 2001). Various pollutants, at fairly high concentrations, can weaken the prophylactic effects of fish mucus and thus increase physiologic stress and susceptibility to infections (Bols et al. 2001). In some cases, the mucus layer actually increases in thickness in response to stress (Bols et al. 2001). However, there is little research on the impact of low-level chronic exposure to pollutants such as heavy metals on fish mucus. Although fish mucus is not bacterial derived, it is a complex carbohydrate medium that does have a number of bacteria associated with it. Indeed, numerous bacteria can be isolated from surfaces of healthy fish.

Fish mucus in healthy fish has been shown to have a bactericidal effect on human and fish pathogens, including *A. hydrophila*, the species used in our investigations (Bols et al. 2001, Subramanian et al. 2008). Isolates of *A. hydrophila* can be obtained from both diseased and healthy fish, indicating that lesions are secondary to some other weakening of the fishes' immune or protective systems.

Does fish-produced biofilm offer protection to commensal bacteria from toxicants dissolved in the water and associated with the sediments? In essence, we ask whether chronic heavy metal exposure selects for antibiotic-resistant bacteria on the surface of different fish species. Since the AR profiles of commensal bacteria from these 2 streams are very similar, we may be seeing effects moderated by the interaction between the antimicrobial activity of fish mucus and the increased levels of metals binding to this slime.

Potential human health problems

Pathak & Gopal (2005) observed significant occurrences of bacterial populations in fish organs with high levels of resistance for both antibiotics and metals and suggested that their observations are an indicator of potential fish fauna and public health risks. Because of the widespread occurrence of resistance traits and the seeming maintenance of these traits under a wide variety of habitats, AR is being seen as an ecological problem (Kümmerer 2004). To understand the factors that control the acquisition and dissemination of resistance traits, we need to understand the selective pressures acting on bacteria in diverse habitats (e.g. Martinez 2009).

Hassen et al. (1998) observed high levels of metal resistance and AR in sites with naturally high metal concentrations, indicating that the mechanisms where-

by metals select for AR traits are old and probably were a general trait under primitive earth conditions (Pawlowski et al. 2016). Microbes evolved in metal-rich environments, and their novel genetic exchange mechanisms would have allowed sharing of resistance elements among diverse bacterial groups. It only required the emergence of the wide-scale use of heavy metals in our modern world to create environments where either metals indirectly select for AR or where direct selection by antibiotics released into the environment from clinical and agricultural settings are subsequently maintained in the environment under metal selection.

Our study demonstrates that for some AR traits, likelihood of exposure to heavy metals increases the proportion of resistant bacteria found associated on the surfaces of various fish species. MAR aeromonad infections have been observed in individuals catching fish from the Savannah River (Haburchak 1996). The prevalence of antibiotic-resistant bacteria associated with fish creates a public health issue, especially for certain people, e.g. subsistence fisherman who likely spend more time handling fish. This group of people is also less likely to seek medical help if an infection occurs from handling tackle or fish. Studies conducted on the Savannah River (Burger et al. 2001, 2002) found significant differences between ethnic groups in the likelihood of ingestion of Hg and radium from subsistence fishing and that groups at risk were least likely to hear, understand or follow a fishing advisory. These same individuals would be those most likely to handle fish with resistant bacteria associated with their surfaces.

Our data suggest that potential exposure to antibiotic-resistant bacteria is dependent on the species of fish and where the fish was caught. Based on our data in intermediate-sized streams, fish most closely associated with sediments in the slowest depositional zones would have a higher likelihood of harboring resistant bacteria. For mid-water species that may generally harbor lower rates of resistant bacteria, likelihood for exposure to humans would be lower.

CONCLUSIONS

These data reinforce the linkage between potential metal exposure and AR in bacteria. We have shown that *Aeromonas* found on the surface of native freshwater fishes have variable levels of AR. For some species, the patterns are spatially defined while for others there appears to be no relationship with stream location or the pattern may be mediated by

local habitat conditions. Since neither of the streams used in this study have had antibiotics released into them either from clinical or agricultural practices, the observed patterns are likely the results of co-selection of resistance traits (Baker-Austin et al. 2006). Additional studies are needed to determine what combination of contaminants is driving the observed patterns in our study species. Regardless of whether a downstream longitudinal gradient in sediment was observed, a gradient appeared to occur in several resistance traits. The effects of local habitat may be adding much 'noise' to large-scale gradient patterns and at times may even override them. Indeed, the effects of habitat were apparent with inhabitants of slower water with finer substrates and more organic matter having higher levels of resistance. Moreover, within the slow habitats, those living near the bottom showed the highest levels. Future broad-scale studies should directly compare metal concentrations with resistance of bacteria from sediments and fish from diverse lotic and lentic habitats.

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