WHEN RIVERS BECOME RESERVOIRS OF ANTIBIOTIC RESISTANCE: INDUSTRIAL EFFLUENTS AND GENE NURSERIES

J Vaun McArthur[1], R. Cary Tuckfield[1], Angela H. Lindell[1], Craig Baker-Austin[2]

AUTHORS: [1]Savannah River Ecology Laboratory, University of Georgia, Aiken, South Carolina 29802, [2]CEFAS, Weymouth, UK.

Abstract. Industrially polluted streams and rivers are of great concern to public health officials and environmental regulators. A lesser known problem is that some water resources have become reservoirs of antibiotic resistance genes that can, under natural conditions, be transferred to water-borne pathogens. The current opinion in the scientific community is that the rapid and continuing increase in antibiotic resistance found in clinical settings is caused by the misuse and overuse of antibiotics in medicine and agriculture. We present here a summary of studies, primarily at the Savannah River Site (South Carolina), that demonstrate that bacteria exposed to heavy metal pollution show elevated levels of antibiotic resistance (AR) and multiple antibiotic resistance (MAR) without ever being directly exposed to antibiotics.

INTRODUCTION

The toxic effects of industrial pollution in waterways have been the basis of numerous studies. These toxins pose both wildlife and public health hazards. In the United States limits have been established to control the amount of toxic material entering streams and rivers. However, legacy pollution or that originating from goverment5 sites prior to the establishment of the EPA remains a significant problem in some areas. More importantly, certain industries continue to generate large amounts of toxic wastes that can pose serious problems for rivers and streams. For example, the recent collapse of a coal ash basin resulted in > 1 billion gallons of metal laden waste to enter tributaries of the Tennessee River (Ruhl et al., 2009.

The effects of human activity on the biosphere are primarily based on the study of higher organisms. Heavy metal laden pollution such as coal combustion products are known to impact higher organism development, growth, and fecundity (Rowe et al., 2002). Considering that the vast majority of life is microbial understanding the effects of environmental changes on the micro-biosphere should not be ignored (Martinez 2009). We know that pollution can alter environmental microbiota community structure and thus function. However, the ecology of specific strains and or their genes under environmental stress is still poorly understood.

To understand the effects of environmental stressors on microbial populations and processes it is necessary to briefly review microbial evolutionary mechanisms. Mobile genetic elements (MGE) are responsible for many phenotypic traits, such as resistance, that allow bacteria to survive in stressed environments. The genetic platforms containing resistance genes are self-replicating elements that might be ecologically stable (Martinez 2009). The evolutionary and ecological effects of gene transfer events are assumed to be directly related to and exacerbated by strong environmental selection (Ochman et al., 2000; van Elsas and Bailey 2002) especially when the transferred MGE confers a measureable advantage. One such condition is heavy metal pollution.

The central role that horizontal gene transfer (HGT) plays in the adaptation of bacterial communities to changing environmental selective pressures has been demonstrated through several diverse lines of evidence (e.g., Top *et al.*, 2002, Herron *et al.*1998). Transmissibility of MGE's in an infectious manner greatly accelerates adaptive responses. MGE mediated evolution in contrast to mutation-based changes is best evidenced by the rapid and global dissemination of antibiotic resistance (AR) genes (O'Brien 2002, Ochman *et al.*2000, Yurieva *et al.*1997). Significantly, both the presence of MGE and the evidence of MGE integration into microbial genomes have been identified in all major microbial taxonomic groups, including gram-positive, gram-negative bacterial as well as archaeal taxa (Sørensen et al., 2005, Boucher et al., 2003).

Bacterial MGE spread not only vertically, by inheritance, but also horizontally by involving phylogenetically distant cells (Davison 1999, Lorenz and Wackernagel 1994, Sobecky 1999). Bacterial cells in ecological proximity to each other may be more important in microbial adaptations and gene exchange than genetic relatedness alone. Microbes that are able to draw from the collective genetic resistome (D'Costa *et al.*2007, Wright 2007) should have higher chances of survival than those individuals that lack such ability.

Genetic vectors responsible for assembly and mobility of AR genes, such as transposons, integrons and R plasmids of similar or identical type are widespread in various niches of the environment (Sørum and L'Abe´e-Lund 2002). Integrons are MGE systems that contain ele-

ments necessary for site-specific recombination and expression of exogenous DNA. Integrons are significant from an evolutionary perspective in that they are both highly mobile as well as adaptive (Wright *et al.,* 2008).

Rapid changes in bacterial genetic composition occur primarily through the acquisition of exogenous DNA via HGT mechanisms such as conjugation, transformation or transduction. These novel genetic mechanisms are probably very ancient and arose in bacteria as a means of tolerating or resisting toxic or inhibitory substances, exploiting changes in food abundance or other environmental change that promoted gene exchange by MGE.

Gene content in a given bacterial community should reflect past and present selective pressures in that environment (Turner *et al.,* 2002). For example, AR genes are often more abundant in bacterial communities exposed to antibiotic contamination (Heuer and Smalla 2007, Pei *et al.*2006) and mercury resistance genes are more abundant in mercury contaminated sediments (Smalla *et al.,* 2006). However, in contrast to classic selection theory, previous studies have documented that additional
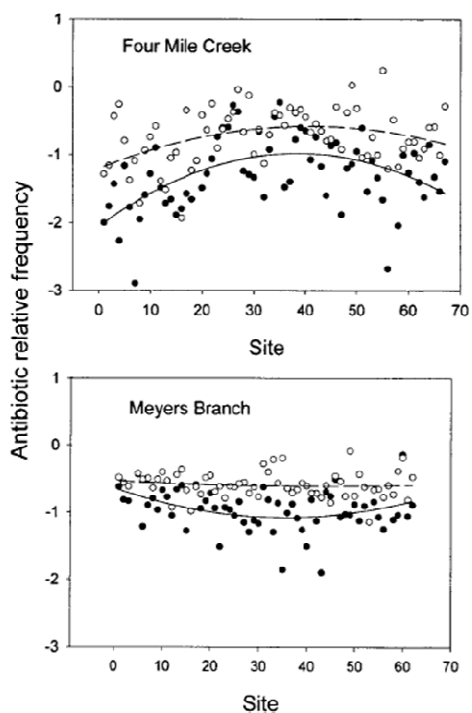

Figure 1. Spatial distribution of antibiotic resistance in stream sediment associated bacteria. Open circles streptomycin-resistance; closed circles kanamycin resistance

genotypes and phenotypes can be co-selected along with traits under direct selection including AR in metal-contaminated systems (Wright *et al.,* 2008) and as re-

viewed in (Alanso *et al.,* 2001, Baker-Austin *et al.,* 2006, Summers *et al.*1993).

Genotypes may persist in a bacterial community even after a given selective pressure no longer exists (Enne *et al.,* 2001). Turner *et al.,* (2002) predict that the more intense the selective pressure, the narrower the range of genotypes that can persist, thereby resulting in a more homogenous (less diverse) pool of MGE's.

Patterns and processes in riverine systems are primarily controlled by downstream water movement (Poole 2002, Ward, 1989; Townsend, 1996, Vannote *et al.*1980). The structure and ecological connectivity within stream systems are impacted by longitudinal, lateral and vertical vectors (Ward, 1989; Ward & Stanford, 1995). Natural variation within stream ecosystems may impose diverging selective pressures that act on host/MGE combinations and drive them to proliferate or form new associations. Because of the vertical and horizontal transmission of genes both within and among bacterial species, certain genes may have spatial distributions that are independent of the distribution of a specific bacterial species (McArthur and Tuckfield 1997). Thus, the length of stream over which certain gene functions are adaptive may vary for each gene.

Strong selection imposed by point source pollution along stream continua may create localized "hot spots" of microbial evolution via HGT. These "gene nurseries" may remain even after the selection is removed. These genes may be subsequently transferred into waterborne pathogens and disseminated downstream.

We present summary results of experiments demonstrating that indirect selection of bacteria by heavy metals can and does result in significant increases in antibiotic resistance and that these AR genes have unique spatial and temporal distributions.

RESULTS

Spatial Analysis of Resistance Traits in Contaminated Streams. We examined the spatial distribution of antibiotic resistant bacteria associated with stream sediments in samples collected every 200 m from > 10 km in two streams found on the Savannah River Site (McArthur and Tuckfield 2000). The Savannah River Site is a Department of Energy facility located in South Carolina on the border with Georgia near Augusta. Each bacterial isolate from Four Mile Creek (FMC), a highly contaminated stream, and Meyers Branch (MB), a relatively unimpacted coastal plain stream, were screened for the prevalence of AR to four antibiotics: chloramphenicol, tetracycline, kanamycin, and streptomycin.

A strong quadratic and consistent spatial pattern of resistance with downstream distance was observed in the contaminated stream for each antibiotic (Fig 1). For streptomycin, there was no statistically significant rela-

tionship between prevalence and downstream distance in MB, the uncontaminated stream (Fig 1). For kanamycin,
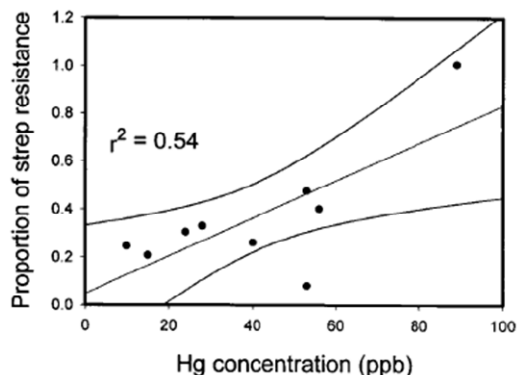

Figure 2. relationship between sediment Hg concentration and prevalence of streptomycin resistant bacteria.

however, we found a significant but inverted quadratic relationship with downstream distance in MB. Clearly, processes promoting these spatial prevalence patterns are different in the contaminated stream than in the uncontaminated stream raising the obvious follow-up research question – what is the cause?

Peak AR in FMC nearly coincided, spatially, with the confluence of another metal contaminated stream, Castor Creek (CC). In fact, for a selected group of 9 sediment samples collected upstream in CC from its confluence with FMC, we found a significant ($p < 0.05$) and positive relationship between Hg concentration in the sediments

Figure 3. Spatial variograms: Among all pairs of sediment samples separated h meters apart, spatial correlation declines with h for antibiotic and metal resistance (top and middle panels) in FMC. However, spatial covariance (cross-variogram) of the two measures is negative and declines with h. No similar patterns in MB. (Tuckfield and McArthur 2008)

and streptomycin resistance prevalence (Fig. 2).

These data were subjected to geospatial statistical analyses and a negative spatial correlation between metal concentration and the prevalence of antibiotic (aminoglycosides) resistance among stream bacteria in sediment samples was observed (Tuckfield and McArthur 2008). Results show a significant and negative correlation between metals scores versus aminoglycoside resistance scores and suggest that selection for metal tolerance among sediment bacteria may influence selection for antibiotic resistance differently than previously supposed. In addition, we used a spatial cross-correlation analysis that showed decreasing metal concentrations scores were associated with increasing aminoglycoside resistance scores as the separation distance between sediment samples decreases, but for contaminated streams only. The downstream vector of contaminant transport established a spatial gradient of metals from their point source, the strength of the correlation declined with separation distance between paired sediment samples (Fig. 3). These results were counterintuitive to our previous observations (Figs. 1 and 2) and may be influenced by metal bioavailability in the sediments and by a "cocktail effect" from complex combinations of pollution mediated selection agents.

Effect of Coal Ash Settling Basin Water on Bacterial Resistance Patterns. We examined the sensitivity of bacteria from ash settling basins of coal-fired power plants to a diversity of antibiotics to test the hypothesis that toxic metal exposure indirectly selects for antibiotic-resistant bacteria throughout the entire microbial community.

Coal-fired power plants represent a major source of global metal pollution, accounting for 10−60% of the anthropogenic emissions of As, Cd, Cr, Cu, Hg, Mn, Mo, Ni, Pb, Se, Sb, and V (Nriagu *et al.*1988). In the United States, coal-fired power plants generate about 10^{13} g of metal-rich ash annually, about a third of which is mixed with surface water and deposited to ASBs (Rowe *et al.*2002). The overflow of ASBs is usually discharged back to surface waters.

To avoid culturability biases, we employed fluorescent, single-cell viability probing and high-throughput (up to 100 samples h^{-1}) flow-cytometric analyses to study the tolerance of ASB intake and discharge bacterioplankton to diverse metals and antibiotics. Because no single physiological viability indicator is universally appropriate, we employed two viability probes in parallel: probing for cell membrane integrity with SYTO-9 and propidium iodide nucleic acid stains (Boulos *et al.*1999, Hoefel *et al.*2003, Gregori *et al.*2001) and probing for esterase activity with carboxyfluorescein diacetate (Hoefel *et al.*2003).
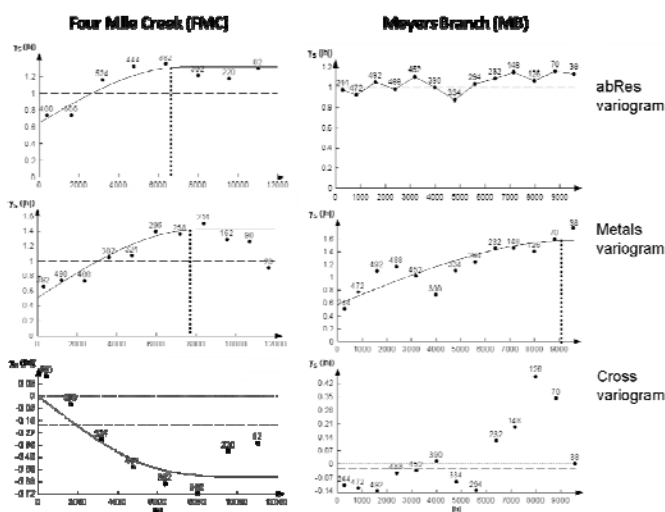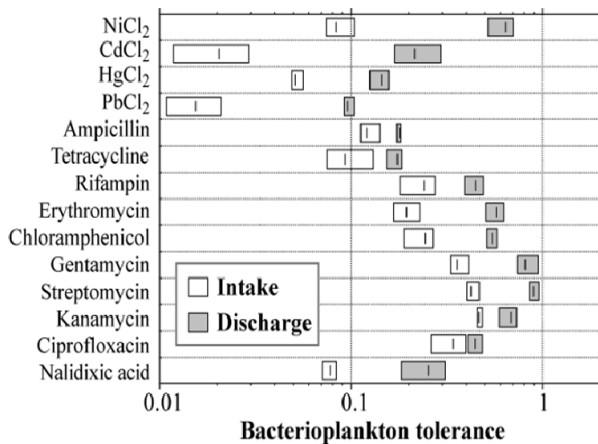
Figure 4. Tolerance of bacterioplankton to various metals and antibiotic in intake and discharge water from ash settling basins. (Stepanauskas et al. 2005)

Bacterioplankton collected in the discharge of three ASBs were significantly more tolerant to most tested antibiotics and metals than bacterioplankton simultaneously collected in their respective intakes, that is, a larger fraction of ASB discharge bacterioplankton remained viable after an experimental exposure to various metals and antibiotics, compared to ASB intake bacterioplankton ($p < 0.001$, ANOVA) (Fig. 4). Overall, discharge bacteria were more tolerant than intake bacteria in 69 out of the 84 linear contrasts (3 power plants × 14 toxicants × 2 viability probes; $p < 0.05$).

Probes for membrane integrity and esterase activity produced tolerance patterns that were strongly correlated, with the exception of tolerance to streptomycin. Because the two probes provide independent indications of cell viability, good agreement between the methods suggests that our measurements of the effects of diverse toxicants on mixed microbial communities were robust.

The most likely causes for the observed results are shifts in microbial community composition resulting from the selective pressure imposed by elevated metal concentrations or acquisition of resistance through HGT (Summers 2002, Alonso et al., 2001).

In addition to toxic metals, ASB bacteria may experience selective pressure from organic toxicants, including polycyclic aromatic hydrocarbons, polychlorinated dibenzo-p-dioxins, and polychlorinated dibenzofurans, which are present in trace amounts in coal ash (Gohda et al.1993). However, since no antibiotics are introduced into the ASBs that we studied during plant operations, the elevated frequency of antibiotic tolerance in ASB bacterioplankton cannot be attributed to direct selection by antibiotic exposure. This evidence supports the hypothesis that metal contamination directly selects for metal tolerant bacteria while co-selecting for antibiotic tolerant bacteria (Stepanauskas et al., 2005).

Coselection of Metal and AR. We tested the hypothesis that resistances to metals and antibiotics are co-selected in complex microbial assemblages by experimentally exposing naïve freshwater bacterioplankton to various concentrations of Cd, Ni, ampicillin, and tetracycline and observing changes in the frequency of resistance to both metals (Cd and Ni) and three antibiotics (ampicillin, gentamicin and tetracycline). Cadmium (representing class B metals) and nickel (representing transition metals) are discharged to aquatic environments by a variety of industries (Nriagu, 1996). Ampicillin (β-lactams), gentamicin (aminoglycosides) and tetracycline (tetracyclines) represent three of the most widely used classes of antibiotics in medicine and agriculture (Kolpin et al., 2002).
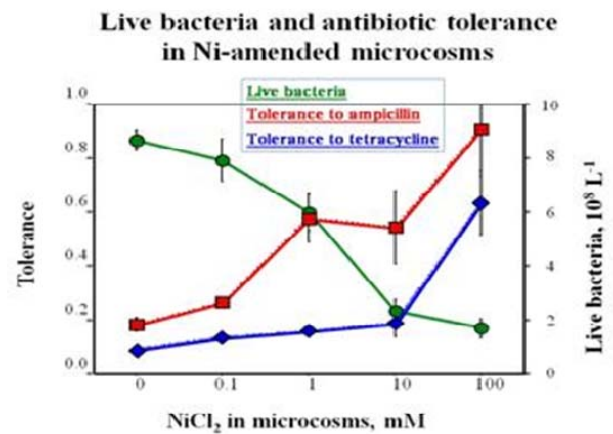

Figure 5. Abundance of total live (green), ampicillin tolerant (red) and tetraclycline tolerant (blue) bacteria as a Function of nickel concentration in mesocosms. (Stepanauskas et al. 2006).
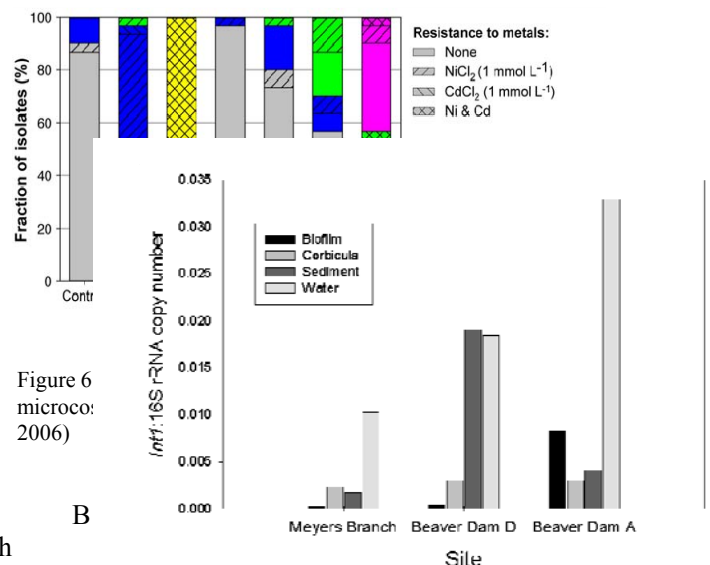

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Figure 7. Abundance of *Int*1 in four microhabitats: biofilm, bivalve digestive tract, sediment and water at three stream sites in Beaver Dam Creek. Site A is nearest the ash settling basin. Meyers Branch is a reference stream.

metals and antibiotics had significant effects on the fraction of cultivable bacteria in the microcosms (Fig. 5 only Ni shown). Fewer than 20% of isolates from the control microcosms were resistant to any of the tested antibiotics, while 50–100% of isolates from the microcosms amended with 0.01–0.1 mM Cd (Fig. 6) and 1 mM Ni (not shown) were resistant to at least one antibiotic. Similarly, while only 3% of isolates obtained from the control microcosms were resistant to Cd or Ni, 50–60% of isolates obtained from the microcosms amended with 30 mg l^{-1} tetracycline and 10 mg l^{-1} ampicillin were resistant to at least one of the two metals. Furthermore, when microbial assemblages were individually exposed to each of the four toxicants, the frequency of microorganisms with multiple resistances increased (Fig. 6). This study provided the first experimental evidence that the exposure of freshwater microbial assemblages to individual metals and antibiotics selects for multiresistant microorganisms (Stepanauskas et al., 2006).

Integrons in the Environment. Class 1 integrons were first discovered in multiple AR pathogenic bacteria isolated from patients with nosocomial infections (Stokes and Hall 1989, Hall et al., 1999, Gillings et al., 2008). Clinical class 1 integrons have been recovered from human commensals (Labbate et al., 2008) and from livestock (Goldstein et al., 2001) and these integrons are very similar, carrying transposition genes that are often found in transposon Tn*402* (Rosewarne et al., 2010). Class 1 integrons have been found in many different and varied ecosystems (Rosenwarne et al., 2010) but those found in ecosystems not impacted by human activities are generally different from the Tn*402*-like transposition genes. This suggests that integrons are older than the use of antibiotics and that they are important in bacterial adaptation.

Rosenwarne et al., (2010) suggest that bacteria possessing class 1 integrons may have an increased selective advantage because of their ability to take up elements from the environmental gene cassette metagenome. This metagenome includes a vast array of bacterial genetic diversity (Stokes et al., 2001, Gillings et al., 2008) conferring, among other things, the ability to harvest metals, pump out metals (efflux pumps), synthesize polysaccharides and bind sulfates (Rosenwarne et al., 2010); traits which can be clearly adaptive under certain environmental conditions.

One key in estimating gene transfer potential and its role in bacterial evolution is to first estimate how abundant MGE's are in bacterial communities under varying degrees of selective pressures. To estimate the potential role of integrons in the evolution of environmental bacteria, we compared the relative abundance of class 1 integrons and quantitatively examined the structure and function of gene cassettes under varying degrees of bacte-

rial stress using real-time combined with conventional PCR. We compared the abundance of the class 1 integron specific gene *Int1* in bacterial communities from industrially contaminated (Beaver Dam Creek, SRS) and reference riverine (Meyers Branch, SRS). We predicted that integrons would be more abundant in the industrially impacted sites, while the diversity and structure of the cassette gene pool would reflect site-specific selective pressures and encode for functions other than that of AR (Wright et al., 2006; Wright *et al.,* 2008).

Our data demonstrated that integron abundance was highest in the water samples from the site nearest the point discharge (Fig. 7). However, highest abundance was found in the sediment samples at the lowest site in the contaminated stream. Abundance of integrons was relatively low in the reference stream for all habitat types. Community comparisons among the three study sites based on tRFLP analyses and the diversity and abundance of gene cassettes using multidimensional scaling showed that while all sites differed with respect to gene cassettes, the two sites on the contaminated stream were more similar than either was to the reference stream.

That class-1 integrons were more abundant in sites with contamination and across a variety of habitats suggests that bacteria at these sites have a higher potential for gene exchange, whether it is for the acquisition of AR gene cassettes or cassettes of unknown function (Wright *et al.,* 2008). Such a finding is of significant relevance to the role of abiotic selective pressures in shaping and influencing the adaptive responses of microbial systems.

CONCLUSIONS

From these studies we have learned that exposing microbial communities to heavy metals results in:

1) Simultaneous selection of both metal and AR traits that were not present in unexposed communities,

2) Significant positive correlation in the levels of AR with increasing concentrations of some metals,

3) Spatial patterns (upstream-downstream) within the microbial community in the prevalence of AR traits as a function of changing metal concentration

4) Increased levels (concentration tolerance) of multiple AR, and

5) The abundance and distribution of class 1 integrons is significantly affected by exposure to heavy metals.

As our studies have moved from the discovery of generalized AR patterns (without quantification of specific genes) to examining the distribution and abundance of integrons, several unanswered questions remain. However these data demonstrate that industrial pollution can and does select

for antibiotic resistance traits and genes and that these traits are preserved under continuous selection. Thus industrially polluted streams and rivers become reservoirs of antibiotic resistance genes that can, under natural conditions, be transferred to water-borne pathogens. Multiple antibiotic resistant water-borne pathogens should be of great concern to public health officials and environmental regulators.

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