
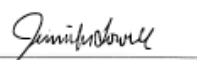


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Title: Arsenic and Tetracycline Resistant Microbial Diversity in High Arsenic Concentration Sediments.

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Abstract:

The Upper Tenmile Creek, which gives Helena most of its public water supply, is contaminated by historic mining activity. This mining adit drains into the creek creating elevated arsenic concentrations in the stream. These elevated arsenic levels can cause co-selection for arsenic and antibiotic resistance in microbial communities. The presence of these resistance genes was assessed through PCR near the Susie Lode and Lee Mountain mines in the Upper Tenmile Creek Superfund Site. Then isolated strains of bacteria were grown on selective media and their 16S genes were assessed to find the identity of each bacterial colony. There was detection of the tetW gene and amplification of the 16S gene, however none of the sequences were of sufficient quality to confirm the identity of bacteria growing on the selective media.

Introduction:

Nearly 2 million people acquire an antibiotic resistant infection annually in the United States (CDC 2013). Although this number is high, antibiotic resistant infections have not always been as much of a concern as they are today. A study conducted in the Netherlands by Knapp et al. (2010) showed that from five archival soil samples spanning from 1940 to 2008, all increased in abundance of the antibiotic resistance genes (ARGs). Knapp et al. (2010) tested the abundance of eighteen different ARGs in the soil samples, which all showed a range of two to fifteen times greater abundance of ARGs, with tetracycline resistance being the most common. Research links this increase with the increase of industrialization of antibiotics after WWII (Finley et al. 2013). Moreover, the use of antibiotics in agriculture has been detected as a source of selection for antibiotic resistant microbes, such as in fish aquacultures (Seiler & Berendok 2012). Seiler and Berendok (2012) also suggest that metals in microbial environments may cause cross-resistance for antibiotics.

At the local level, acid mine drainage causes selection for microorganisms adapted to the contaminated and acidic stream sediments (Delavat et al. 2012). One of the most abundant metals

in the Upper Tenmile Creek is arsenic. Delavat et al. (2012) said that arsenic contaminated sediments can cause selection for certain communities of bacteria that can live in these acidic and arsenic rich environments by using arsenate oxidation mechanisms. The ability to oxidize arsenic may come from the development of various arsenic resistance genes such as *aioA*, *arsC-Trx1a*, and *arrA* (Escudero et al. 2013). These metal resistance genes can also be linked with genes that allow for antibiotic resistance (Baker-Austin et al. 2006). This is possible through co-selective methods by transposable elements that link metal resistance and antibiotic resistance genes (Baker-Austin et al. 2006). Two examples of these genes are drug and metal efflux protein genes that remove arsenic and tetracycline from the bacterial cell, in addition to ribosomal protection proteins that block arsenic and tetracycline (Aminov et al. 2001; Baker-Austin et al. 2006). Guo et al. (2013) also show this co-selective relationship in mice gut microbiota. Increasing exposure to metals, such as arsenic, caused an increase in the abundance of tetracycline resistance genes *tetO*, *tetW*, and *tetQ* (Guo et al. 2013). The same may be true for some efflux mediated genes including *tetA* and *tetB* (Poole, 2005). Examples of genes that encode for tetracycline resistance through ribosomal protection protein mechanisms and are selected for include *tetM*, *tetO*, and *tetW* (Zhang et al. 2008).

Selective media has been used to isolate arsenic resistant strains of bacteria from heavily contaminated communities (Delevat et al. 2012). Strains isolated from acid mine drainages of Carnoules, an ancient mining site in Gard, France were *Paenibacillus sp.*, *Bacillus sp.*, *Streptomyces sp.*, *Rhodococcus sp.*, and *Pseudomonas sp.* (Delevat et al. 2012). Additional studies have worked to identify bacteria that allow for tetracycline resistance through phylogenetic analysis (Aminov et al. 2001). Some of these strains include *Streptomyces lividans* and *Enterococcus faecalis*, which have the *tetM* gene (Aminov et al. 2001). Additionally, there has been isolation of *Enterococcus faecalis* and the detection of both tetracycline and arsenic resistance in the Mololoa River in Tepic, Nayarit, Mexico (Mondragon et al. 2011). These studies of which species have developed resistance and which genes are being passed on that allow for

this resistance have allowed the detection of transferal and biodiversity of antibiotic and arsenic resistance genes, which can illustrate which types of strains more commonly have these genes. Some genes that encode tetracycline ribosomal protection proteins have an evolutionary history in supporting metabolic functions (Aminov et al. 2001). Even though these genes may have evolved earlier, they may have been present in pathogenic bacteria because these genes are located on transposable elements within the bacterial genome (Aminov et al. 2001). Therefore, this body of literature suggests that there could be certain organisms that have acquired resistance and also that there are certain genes that are present within these organisms that are allowing for tetracycline and arsenic resistance.

Water from abandoned mine adits high in metals such as arsenic, flows into the Upper Tenmile Creek, which supplies Helena, Montana with over 50% of its public water supply (U.S. Environmental Protection Agency, 2008). These metal rich environments may cause co-selection in bacteria for heavy metal and antibiotic resistance genes (ARGs) through transposable gene elements usually coding for efflux pump resistance mechanisms (Aminov et al. 2001; Baker-Austin et al. 2006). These genes can be transferred laterally in the bacterial communities located in these highly contaminated environments and may even be transferred to locations further downstream of the mine drainage sources (Baker-Austin et al. 2006). Because of these selective pressures, certain species of bacteria may be selected for in these contaminated sites, causing less diversity in bacterial communities than those that are not exposed to high levels of heavy metals. In the present study, the bacteria in the sediments from these sites were grown on selective media containing arsenic and tetracycline to select for tetracycline and arsenic resistant strains. The 16S gene was sequenced from these isolates to identify each resistant strain. I hypothesize that if there is selection occurring in arsenic contaminated sites and antibiotic resistance in bacterial communities from sediments, then there will be more species of bacteria displaying antibiotic resistance and arsenic resistance phenotypes in Susie Lode and Lee Mountain, Montana than sites

downstream from Susie Lode and Lee Mountain. Susie Lode and Lee Mountain are direct drainages from abandoned mine adits.

Materials and Methods:

Sediment samples were collected at seven sites along the Upper Tenmile Creek along with three negative control sites in the Little Blackfoot River. Sites one and two were taken at the Kading Campground and site three was taken approximately a half-mile downstream from the campground. For the sites in the Upper Tenmile Creek, site four was at the northernmost edge of Moose Creek Campground, site five was the southernmost edge of the Moose Creek Campground, site six was the halfway point along the creek between Rimini and Moose Creek Campground, site seven was right above the point where the mine adit discharge flows just south of the town of Rimini, site eight was where the drainage from a mining adit flows into the Upper Tenmile Creek, site nine was the Lee Mountain superfund site, and site ten was the Susie Lode superfund site (Figure 1).

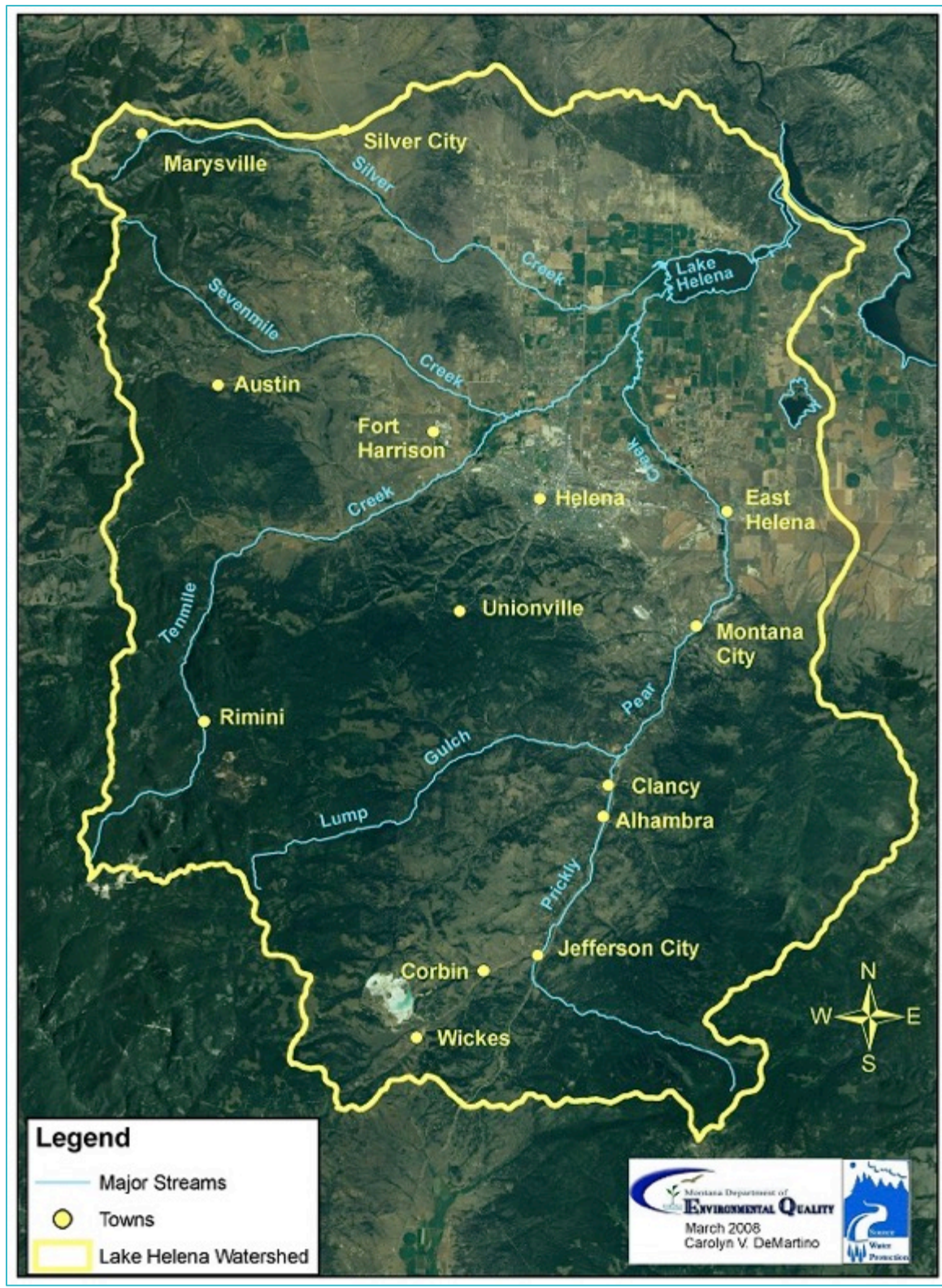


Figure 1. Tenmile Creek where samples were taken.

Each site was tested for specific conductivity, pH, temperature in °C , and dissolved oxygen. All samples were then taken to Energy Laboratories (Helena, MT) to test for arsenic levels in mg/kg of sediment.

Bacterial DNA was extracted from 0.25 grams of soil from each site using MoBio PowerSoil® DNA extraction kits (Carlsbad, CA). The DNA was tested for the presence of arsenic resistance genes including arsenate reductase genes *arrA 1*, *arsC-Grx-Sun mix*, and *arsC-Trx1a* through PCR (Escudero et al. 2013). The ARGs, tetracycline efflux protein genes, *tetA* and *tetB*, and ribosomal protection protein genes, *tetM*, *tetO*, and *tetW*, were also tested from the same samples (Aminov et al. 2001). The PCR protocols for each tetracycline resistance gene were derived from the PCR protocol in Guo et al. 2013. These PCR products were electrophoresed on 1% agarose gels to detect any gene amplification. Any amplified genes were purified using Qiagen purification kits and sent for DNA sequencing at Macrogen USA, Rockville, MD

Bacteria from each site were isolated from the remaining sediment samples after DNA extraction by dislodging the bacteria from 0.5 grams of sediments in four milliliters of PBS, phosphate buffered saline, solution. The supernatant from each site was then individually plated on selective media. The recipe for the nonselective reagents for 500 mL of the selective media is shown in Table 1. In addition to one set of LB agar plates, six different kinds of media were made with differing concentrations of casaminoacids and an alternating pH from 3.5 to 5.5 every other type of media (Table 2). Each media mixture contained 100 µg/mL of tetracycline and 0.1 % arsenic (arsenate and arsenite) to isolate resistant strains of bacteria (Delavat et al. 2012).

Table 1. Recipe for Nonselective Reagents in Media (Delevat et al. 2012).

Reagent	Molarity	Amount/500mL
Calcium Chloride	3.15	5 mL
Magnesium Sulfate	1.66	5 mL
Amonium Sulfate	2.27	5 mL
Trace Solution (Copper Sulfate, Zinc Sulfate, and Magnesium Sulfate)	-	5 mL
Vitamin Solution	-	5 mL
Gellan Gum	-	7.5 g

The bacteria were then incubated at 25°C for three weeks. If there was no observable growth on the plates, they were left in the incubator until there was observable growth. Representative bacterial colonies were picked as templates for amplification of the 16S gene using autoclaved toothpicks, using the 27f and 1492r primers, through PCR. As each colony was picked, it was streaked onto the corresponding media and labeled to have a library of each type of isolated bacteria. These gene products were sequenced at Macrogen USA (Rockville, MD). This allowed for a comparison of bacterial species living in each different site and allowed for observation of the presence of tetracycline and arsenic resistant bacterial strains. This information will then help to determine if antibiotic resistant bacteria live in acid mine drainage, and if certain species of bacteria are being selected for in these contaminated sites.

Table 2. Selective Reagents in Selective Media

Selective Media	Casaminoacids %	pH	Group With Arsenic III	Group With Arsenic V
FD1	.01	3.5	X	X
FD2	.01	5.5	X	X
FD3	.1	3.5	X	X
FD4	.1	5.5	X	X
FD5	1	3.5	X	X
FD6	1	5.5	X	X
LB	---	---	X	X

Results

In order to determine if there is a correlation between arsenic resistance and the genes in the bacteria, the levels of arsenic were tested to determine if there was a significant increase of heavy metals in the Upper Tenmile Creek when compared to the Little Blackfoot River. The Upper Tenmile Creek showed a higher arsenic concentration than the Little Blackfoot River, for example, the highest arsenic concentration in the Little Blackfoot River was 10 milligrams per kilogram of sediment, while the highest arsenic concentration in the Upper Tenmile Creek was 152 milligrams of arsenic per kilogram of sediment (Table 3). Additional measurements taken include: pH, temperature, specific conductivity, dissolved oxygen percentage, dissolved oxygen milligrams/liter and arsenic concentration of the stream sediments in milligrams/kilogram (Table 3).

None of the tetracycline primers from the literature showed amplification except for tetW at 168 bp (Guo et al. 2013). The tetW genes amplified were from sites 9 and 10 (Figure 2). When each site was plated on selective media, observable growth was shown in sites 9 and 10 (Figure 3). Two colonies from site 9 and two from site 10 were used as templates for a 16S PCR, and the amplicons were sent for sequencing. The results of the gene sequence were not of sufficient quality to determine the identity of the colonies.

Table 3. Site Data of Overall Soil Conditions.

Site Data						
	pH	Temp. °C	SPC	D.O. %	D.O. mg/L	As Conc. mg/kg
Site 1: Kading C.G.	8.85	7.6	0.08	114	13.6	10
Site 2: Wildflower	8.39	8.1	0.099	113	13.5	7
Site 3: Kitty Pool	8.46	8.6	0.101	104	12.3	10
Site 4: Moose Creek C.G.	7.84	8.6	0.0077	94.8	11.08	107
Site 5: Big Rock	7.9	9.3	0.08	104	12.05	71
Site 6: Upstream	7.85	9.9	0.073	83.2	9.39	152
Site 7: Red Cave	7.3	9.6	0.436	75.5	8.54	809
Site 8: Red Waters	7.09	9.7	0.439	98.2	11.17	248

Site 9: Pool of Colors	2.93	15.7	2.53	10.4	1.02	6510
Site 10: Susie Lode	3.36	11.9	1.78	86.5	9.3	459

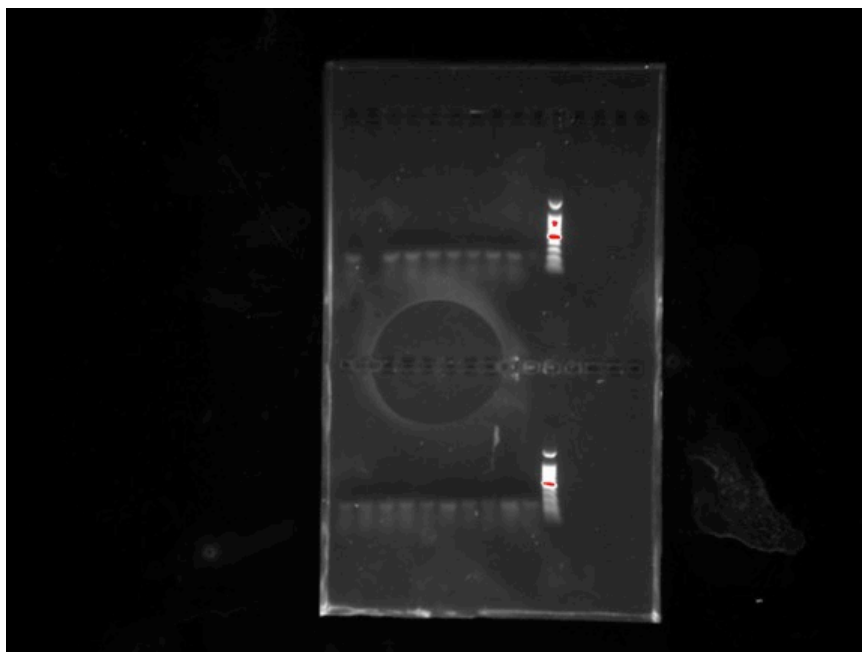


Figure 2. tetW gene amplification.

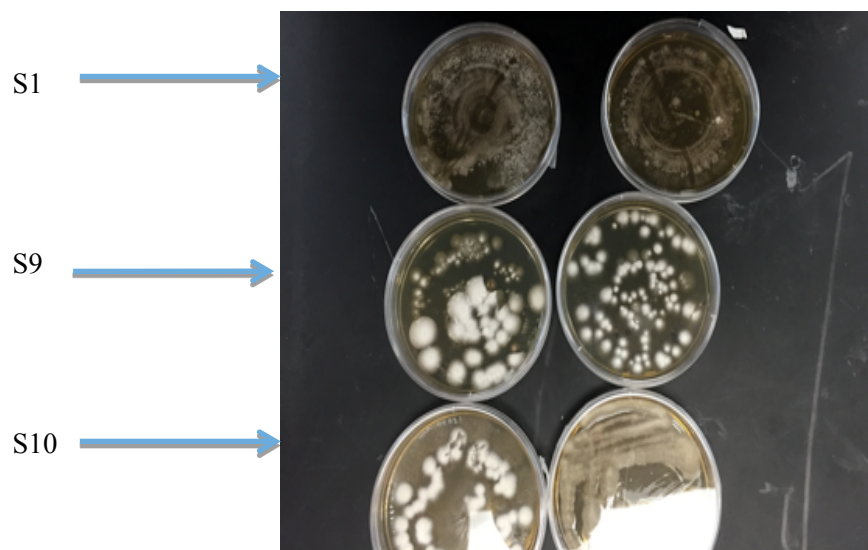


Figure 3. Samples plated on selective media for growth of tetracycline and arsenic resistant bacteria.

Discussion:

The objective of this research was to determine if tetracycline resistance bacteria are found in arsenic contaminated sites in the Upper Tenmile Creek Superfund site, and then to sequence the 16S genes of the bacteria that had these genes. The results show that there are much larger arsenic concentrations in the sites along the Upper Tenmile Creek (Table 3). These concentrations surpass the calculated noncancer guideline for arsenic concentration of 24 mg/kg (Teaf et al., 2010). Escudero et al. (2013) reported selection for arsenic resistance at concentrations of 340 to 9440 mg/ Kg of soil. Additionally, the only tetracycline gene that amplified was tetW in the Lee Mountain and Susie Lode fund sites. Lastly, there was observable growth on the selective media, amplification of the 16S gene from the isolates, but poor quality of sequence.

A study on pig microbiota by Aminov et al. (2001) showed amplification for tetW, tetO, and tetM genes. Similar to their study, we were able to amplify tetW but did not get amplification of tetO and tetM genes. Moreover, amplification of the tetW gene does not correspond with the study on mice gut microbiota conducted by Guo et al. (2013), which found that the most commonly amplified genes in the microbial communities were tetQ, tetO, and tetM.

A problem was encountered with fungus growing on the selective media plates and not allowing for efficient and accurate collection of isolated bacterial colonies. This problem was also encountered in a study on antibiotic resistance in a metal contaminated stream conducted by Tuckfield & McArthur (2006). Their protocol for adding cycloheximide at 100 µg /mL was then used to cut down fungal growth on selective media (Tuckfield & McArthur, 2006).

Lastly, although there was amplification of the 16S gene, none of the sequences was of sufficient quality to identify the bacteria. However, isolates were taken from the selective media and grown in 50 mL screw-cap tubes with their respective media for future use. A future direction is to heat lyse these cells and sequence the genome of each isolate.

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