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An Assessment of Antibiotic Resistant Bacteria in Biosolid used as Garden Fertilizer

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A Masters Thesis

Presented to

Eastern Washington University

Cheney, Washington

In Partial Fulfillment of the Requirements

for the Degree

of Master of Science in Biology

By

Gwendolyn Lenore Hartman

Spring 2012

MASTERS THESIS OF **GWENDOLYN L**. **HARTMAN**
APPROVED BY:

Chairman, Graduate Study Committee Date
Dr. **Prakash H. Bhuta**

Graduate Study Committee Date
Dr. **Sidney K. Kasuga**

Graduate Study Committee Date
Ms. **Doris Munson**

MASTER'S THESIS

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Abstract

Fertilizer grade biosolid is sold to the general public by the Cheney Wastewater Treatment Plant for use as garden fertilizer and as a soil conditioner. Moist biosolid samples were obtained from the Cheney Wastewater Treatment Plant and antibiotic resistant gram negative bacteria were isolated from it. Bacteria isolated from these samples were resistant to tetracycline, ampicillin, streptomycin, kanamycin and chloramphenicol. Tetracycline resistant bacteria were isolated on MacConkey's agar supplemented with tetracycline and their resistance to ampicillin, streptomycin, chloramphenicol and kanamycin were also determined. A total of 48 isolates cultivated to determine the presence of plasmids which often contain antibiotic resistance genes. These isolates were identified using the Vitek2 Identification System (BioMerieux, Durham N.C., U.S.A.). Sixty-seven percent of isolates were discovered to have plasmids and 54% had two or more plasmids. Plasmid size ranged from 2 to 10Kb.

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1.0 Introduction

1.1 Microbes and their Role in Human Health

It is difficult to estimate or even comprehend the impact the smallest of earth's creatures have on almost every aspect of life on this planet. Microbes, from all taxonomic Kingdoms, are often overlooked as factors in larger systems, or have been in the past. Hungarian physician Ignaz Semmelweis proposed that childbirth fever could be prevented with hand washing by attending medics. Prior to this time, microbes were either ignored or regarded as curiosities having no impact on humans (Prescott, 2005). Modern scientists now know that microbes have a profound impact on every ecosystem studied. The study of bacteria is especially important to human health. Although microscopic, bacteria are responsible for essential processes such as fermentation, nitrogen fixation, elemental cycling and the breakdown of many substances that would otherwise remain unusable by other taxa, in addition to causing disease and illness all over the globe (Lipscomb, 1996).

It is well known that bacteria are a major cause of human suffering and disease worldwide; disease causing bacteria are still the leading cause of death, even with the advent of antibiotics. The global mortality rate associated with gram negative bacteria is estimated to be between 20% and 40%; that is mortality not caused by old age, which is the same as in the pre-antibiotic era (Bengmark, 1998). In addition, bacteria are becoming resistant to many of the known antibiotics while the development of new

antibiotics has slowed considerably over the past sixty years, leading to public health concerns (Coates and Yu, 2007; www.cdc.gov/tb/publications\factsheet, last accessed on 02-13-12).

Most bacteria, however, are non-pathogenic and humans have evolved to rely on these bacteria to metabolize dietary polysaccharides and dairy products, and some even provide vitamins and prevent invasion by pathogens. Without such association with them, humans would be unable to survive. (Sonnenburg, et al., 2006; Aminov et al., 2006). These bacteria are considered to be normal flora. This commensal association is not unique to humans or even primates. All herbivorous vertebrates must rely on the fermentative characteristics of bacteria in order to digest cellulose rich diets (Flint et al., 2008). Indeed, eukaryotic organisms rely on prokaryotes for survival, even down to the cellular level in the form of mitochondria and chloroplasts, which many scientists now believe to be the descendants of symbiotic prokaryotes (Slomovic et al., 2005).

1.2 The Spread of Antibiotic Resistance Among Pathogens

Antibiotic resistance has been on the rise almost from the moment antibiotics were put into use (Amyes, 2000). While this trend would occur naturally as a function of the mutation in bacteria, several human practices have compounded the problem. While resistance can be a function of mutation in single bacterial genes, horizontal gene transfer (transfer of antibiotic resistance through plasmid sharing), have become additional means

by which resistance is acquired. This is further complicated by the presence of high levels of antibiotics in the environment creating selective pressure for maintenance of these genes (Alonso et al., 2002; McAdams et al., 2004). Two examples are prescribing antibiotics where an infection would clear up without treatment and prescribing antibiotics for viral infections (Levy, 2000). In many cases, the patients themselves request that the physician prescribe antibiotics and exaggerate symptoms in order to get a prescription in the false belief that the illness will clear up more quickly (Pechere, 2001). Diligence on the part of physicians and public education has helped to alleviate this problem in recent years and the overuse of antibiotics by health organizations has declined in the United States (Finkelstein et al., 2001). For example, the Spokane Regional Health District distributes fliers to physicians for display in waiting areas requesting that patients do not ask for unnecessary antibiotic prescriptions (www.srhd.org).

Another source of selective environmental pressure is the addition of antibiotics to household cleaners and sanitizers. This practice has become quite widespread and a study at the University of Michigan discovered that two common chemical antimicrobials, triclosan and triclocarbon, used in cleaning products could be detected in 60% of waterways in the United States (Kreisberg, 2009).

Similarly, prophylactic treatment of crops and livestock with antibiotics to prevent infection and promote growth introduces significant amounts of antibiotics into

water and surrounding soils. It is estimated that at least 90% of antibiotics used in agriculture are to promote growth and as a preventative measure (Khachatourians, 1998).

Bacteria become resistant to antibiotics through several mechanisms. One mechanism is mutation of the bacterial genome. In the presence of antibiotics, bacteria that contain intrinsic resistance will proliferate or out compete those bacteria that do not have resistance. Mutational acquisition of antibiotic resistance is described as the frequency of detectable mutations which arise in a bacterial population in a given concentration of antibiotic. This rate can be difficult to accurately predict due to the variability in mutation rate, which is affected by physiology of the cell, genetics and the properties of the selective medium, but is a motivating force behind antibiotic resistance (Martinez and Baquero, 2000). Another mechanism is acquired resistance via horizontal gene transfer of plasmid derived resistance genes. This mechanism is one on which this work is focused.

Bacteria need a certain level of antibiotic resistance as protection from other microbes, either bacteria or fungi, but studies have shown that in areas where antibiotics are used frequently, such as hospitals or in countries where they are not regulated, antibiotic resistance amongst bacteria is much higher (Saunders, 1984). While bacterial antibiotic resistance has been widely publicized and studied (eg. MERSA) and has seeped into the public consciousness, the cause or mechanisms behind the rapid spread of antibiotic resistance in a bacterial population is less well understood by that same public (Siegel R.E., 2008; Kim et al., 2006).

1.3 Horizontal Gene Transfer in vivo

Antibiotic resistance can be passed on through horizontal gene transfer between unrelated species of bacteria by transformation, transduction or conjugation. Plasmids are extrachromosomal, circular double-stranded DNA molecules that can be passed to other bacteria via one of the processes previously mentioned (Chen and Dubnau, 2004; Lindsey et al., 2009). It is thought that bacteria do not retain plasmid borne resistance for long without some type of environmental pressure to do so, such as the presence of antibiotics. Some studies have indicated that long term exposure to antibiotics in the environment may slow the recession of resistance genes after antibiotic concentrations in the environment lessens (Diaz-Mejia et al., 2008).

Transfer of genetic information between different species of bacteria has been observed since Frederick Griffith's famous experiment in 1928 in which he demonstrated (and coined the term) transformation of a non-virulent strain of *Streptococcus pneumoniae* to a virulent strain (Griffiths et al., 2000). Since then, many other experiments have been performed that demonstrate the transfer of genes between unrelated species of bacteria (Trieu-Cuot et al., 1985; Salyers et al., 2004).

Antibiotics ingested by humans to treat infection can select for resistant strains of the target organisms in the gut by killing the bacteria without resistance, which allows the proliferation of resistant strains; this resistance can then be transferred, via plasmid, to the natural enteric microbial community of the host (Nijsten et al., 1996; Klimuszko et al., 1989; Akortha and Filgona, 2009; Kurokawa et al., 2007). This exchange of genetic

material in the gut is considered to be a driving force behind the bacterial evolution of resistance, also resulting in "pathogenicity islands" and, therefore, a force behind acquisition and sharing of antibiotic resistance genes (Hacker and Carniel, 2001). Sharing of genetic information does not only occur in the human gut, but also in many other environments as well, such as between Prokaryotes and Eukaryotes. For example, gene transfer between prokaryotic and eukaryotic microbiota in cattle (Ricard et al., 2006) and in marine environments (Stewart and Siniggalliano, 1990) has been demonstrated. The resistant bacteria in the human gut subsequently enter the sewage treatment system after being flushed down the toilet and can act as a reservoir of resistance genes for other microorganisms present in the environment.

During sewage treatment, human bacteria have ample opportunity to mingle with other bacteria. The purpose of sewage treatment facilities is to remove solids, some organics and pathogens. This is accomplished in several stages: primary, secondary and tertiary. During the primary stage, large particulates such as rocks and sticks are removed, as well as oils and fats that are skimmed off the surface in a clarification step.

The secondary stage of sewage treatment includes aeration, and microorganisms are used to degrade organic molecules too small to be removed during the primary stage. Many small wastewater facilities, including Cheney Wastewater Treatment Plant, use Surface Aerated Basins (lagoons) for the secondary step. The microorganisms involved in this secondary stage are indigenous water borne bacteria and protozoans. These microorganisms break down the organic solids in the wastewater through normal

metabolic processes and are provided with plenty of oxygen by use of mechanical aerators. The microbial communities of these lagoons have been analyzed by researchers around the world, are complex and varied, and include, but are not limited to, *Proteobacteria, Bacteroides, Acinetobacter* and *Firmicute*s, as well as nitrogen fixing bacteria such as *Azotobacter* and *Acidobacterium* (Wagner and Loy, 2002; Sanapareddy et al., 2009).

Further processing is accomplished during the tertiary stage. The purpose of the tertiary stage is to remove excess phosphorous and nitrogen and to control odor. This is also when disinfection, to remove excess microbial load, is performed. Cheney Wastewater Treatment Plant performs chlorination and dechlorination during the tertiary step for microorganism removal to produce Class A Biosolid, which is mixed with yard waste and wood chips and sold under the brand name EcoGreen. Biosolid, or sludge, is treated after the secondary step by anaerobic microbial activity and dewatering (Okoh et al., 2007; CityofCheny.org). The purpose of wastewater treatment is not to sterilize the resulting biosolid, but to remove sediment, heavy metals, oil and grease as well as to ensure that numbers of potentially pathogenic bacteria are under acceptable levels. Sterilized biosolid would not be useful as a garden fertilizer as the process would also degrade the desired organic matter and kill bacteria that will enrich the soil. Bacteria are desired for a healthy garden in that they can further break down soil to enhance mineral extraction and also play a crucial role in nitrogen fixation (Kuske et al., 2002; Chu et al., 2007). In addition to the DNA contained in viable cells, cell-free DNA can also be

present in the environment, which can also be picked up by other bacteria (Nielsen et al., 2000).

Class A Biosolid is sold for use as fertilizer for gardens and farms growing crops for human consumption. The samples taken for this study were of Class A biosolid before combination with mulch. The resistance genes of bacteria can be ingested by humans via produce grown in these gardens, either through ingestion of soil or by uptake of resistance genes by the plants themselves (Conte et al., 2009). Once in the gastrointestinal tract these genes may potentially be transferred to other pathogenic or non-pathogenic bacteria in the gut of the host. This would give potential pathogens resistance against antibiotic treatment (Arthurson, 2008).

1.4 Sewage Treatment Facility as a Reservoir for Antibiotic Resistance Genes

Cheney Wastewater Treatment Facility is located south of Cheney and treats between 1.5 and 2.7 million gallons of wastewater per day. Wastewater is reclaimed through the collection system where debris and large objects are removed via passage through a screen to prevent damage to the plant. Wastewater is then processed by aerobic and anaerobic digestion (www.cityofcheney.org). The bacterial genera involved with wastewater treatment is not known, however several studies have been carried out to determine a few of the major metabolic groups of bacteria. The most common genera in sewer sludge from wastewater treatment facilities reported from various locations are

Acidovorax, Delftia, Leptothrix, Methylibium, Polaromonas, Verminephrobacter, Rhodoforax, Azoarcus, Rhodocyclaceae, and *Pseudomonas* (Sanapareddy et al 2009, Gunther et al. 2009).

One byproduct of water treatment is biosolid (or sludge). After the wastewater is treated by microbial remediation, it is processed further to reclaim much of the water. Once much of the water has been reclaimed, the biosolid product is composted, mixed with mulch, which is mostly wood, and is rated for use as a soil supplement and can be used as fertilizer (EPA, 1993). The Biosolid rating depends upon the manner in which the fertilized crops will be consumed. For example, crops that will be eaten raw require a Class A Biosolid rating. A Class A rating ensures that bacteria considered to be human or zoonotic pathogens (ex. *Salmonella*) are below detectable levels of less than 3 to 4 g of dry weight/solid. Class B biosolids, those approved for use on crops that will be cooked before consumption, have higher limits, but must still demonstrate a reduction in microbial load (Arthurson, 2008). Sewage sludge, and subsequently biosolid treatment, is regulated and rated by the U.S. Environmental Protection Agency, which sets out guidelines for toxin levels and testing for known microbial pathogens (EPA, 1993).

Studies have demonstrated the incidence of horizontal gene transfer in wastewater treatment facilities (Marcinek et al., 1998; Auerbach et al. 2006; Tennstedt et al., 2006). In addition, the use of antibiotics in the Cheney area was characterized in a study done by M. Marshall (2007) as a graduate research project at Eastern Washington University. The study demonstrated that antibiotics used in a community are reflected in the type of

antibiotic resistance expressed in the bacterial population. The selection of antibiotics for use in this study was partly based on the survey of antibiotics used in Cheney, Washington, as reported in Ms. Marshall's study.

1.5 Biosolid as a Valuable Resource

The benefits of using biosolid as fertilizer are well documented and include increased phosphorus sorption and increased nitrogen concentrations (Garling and Boehm, 2000; Lu and O'Connor, 2000). In addition, biosolid is readily available, relatively inexpensive and can be used to revitalize areas that have lost topsoil due to erosion or construction (Meyer et al., 2000).

1.6 Project Goals

The purpose of this project was to detect organisms that harbor antibiotic resistance genes in biosolid which is routinely used as fertilizer for crops and home gardens. To do this, samples of biosolid were collected and plated on selective media to isolate bacteria. Gram negative organisms were selected for this study as they are more likely to be enteric and, therefore, of human or animal origin. Organisms resistant to Tetracycline were selected first. To test for multiple antibiotic resistance, the resulting isolates was be screened for resistance to four more antibiotics: Ampicillin, Streptomycin, Chloramphenicol and Kanamycin. The isolates were identified using the

Vitek 2 Identification System. In addition, the presence or absence of plasmids in these isolates was confirmed by plasmid isolation and gel electrophoresis. Such plasmids, if they contain antibiotic resistance genes, could confer antibiotic resistance to other microbes present in the biosolid.

Materials and Methods

2.1 Sample Site and Collection

Approximately 1000g of biosolid was collected from the Cheney Wastewater Treatment Plant in sterilized jars. Samples were collected at the output site after water removal, but before the addition of mulch. The samples were stored at 2-8C and used with 48 hours of collection.

2.2 Isolation of Bacteria

One gram samples of biosolid were added to 100mL sterile phosphate buffer (1.7mM KH_2PO_4, 5.4mM Na_2HPO_4, 0.15mM NaCl, 2.7mM KCl, pH 7.2). Samples were vortexed for one minute to break up clumps and then filtered using sterile gauze to remove most of the solid particles. The filtrate was diluted by serial dilution up to 1:1000 and aliquots of 1:10, 1:100 and 1:1000 were inoculated on MacConkey agar containing 20µg/mL Tetracycline to select for resistant gram negative bacteria. The plates were incubated at 37˚C for 18-24 hours.

2.3 Processing of the Isolates

Forty-eight morphologically distinct colonies from the MacConkey agar containing Tetracycline were selected to determine their resistance to other antibiotics. The isolates were arbitrarily assigned numbers 1-48. Each isolate was screened for resistance to five additional antibiotics on MacConkey agar for a total of twelve plates per antiobiotic type. Plates were prepared with MacConkey agar infused with each antibiotic type separately. Each plate type was inoculated with isolates one through forty-eight. Growth on these plates was indicative of their resistance to the antibiotics present in the growth media. The antibiotics, and their concentrations, used were Ampicillin 50µg/mL, Streptomycin 50µg/mL, Chloramphenicol 30µg/mL and Kanamycin 50µg/mL. The plates were incubated at 37ºC for 24 hours.

2.4 Identification of Isolates

Isolates from each resistance group were identified using the Vitek2 Automated Identification system. The Vitek2 Automated Identification System performs bacterial and yeast identification by analysis of their metabolic properties (Figure 1). Isolates were subcultured onto Columbia Blood agar (BioMerieux) and incubated overnight at 30-35C. A 0.5 to 0.63 McFarland standard solution was prepared by picking a colony off of a culture plate with a sterile loop and placing it in 0.45% sterile saline. The resulting

suspensions were placed onto a cassette that held the tubes and test cards in preparation for the inoculation step. Vitek2 Gram Negative cards (Figure 2) were matched with the samples and inoculum tubes that protrude from the cards were placed into the suspensions. The isolate numbers were entered into the computer for data tracking and matched with barcodes printed on the cards. The cassettes containing the suspension tubes and gram negative Vitek cards with inoculum tubes in the solution were placed into a vacuum chamber. The application of vacuum and then pressure was used to force the solution through the inoculum tube and into the wells of each card. The cards were incubated and read by the Vitek2 at 15 minute intervals until identification was achieved. The Vitek2 monitored the color changes in the reaction chambers from each card and used the color changes to create a distinct biopattern. The biopattern was compared to biopatterns kept in the Vitek2 database and identification was achieved when a match occurred. Upon identification, a confidence level was assigned (Acceptable, Good, Very Good or Excellent) if the probability percentage was over 85% (BioMerieux.com, 2011). Test substrates are listed in Table 1.

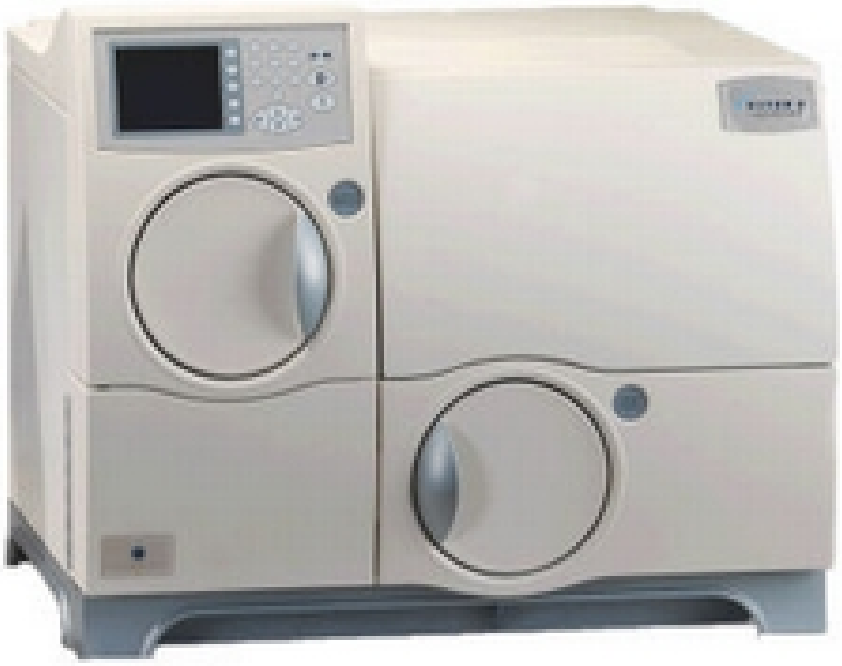

Figure 1. Vitek2 Compact

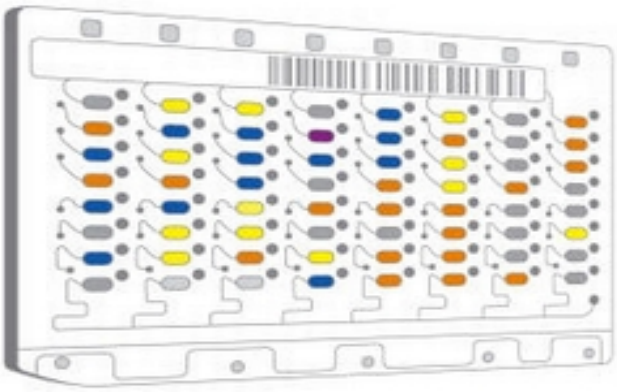

Figure 2. Vitek2 Gram Negative Card

Test	Abbreviation (Abbv)	Test	Abbv
Ala-Phe-Pro-ARYLAMIDASE	APPA	D-SORBITOL	dSOR
ADONITOL	ADO	SACCHAROSE/SUCROSE	SAC
L-Pyrrolydonly-ARYLAMDASE	PyrA	D-TAGATOSE	dTAG
L-ARABITOL	lARL	D-TREHALOSE	dTRE
D-CELLOBIOSE	dCEL	CITRATE (SODIUM)	CIT
BETA-GALACTOSIDASE	BGAL	MALONATE	MNT
H2S PRODUCTION	H2S	5-KETO-D-GLUCONATE	5KG
BETA-N-ACETYL-GLUCOSAMINIDASE	BNAG	L-LACTATE alkalinisation	lLATk
Glutamyl Arylamidase pNA	AGLTp	ALPHA-GLUCOSIDASE	AGLU
D-GLUCOSE	dGLU	SUCCINATE alkalinisation	SUCT
GAMMA-GLUTAMYL-TRANSFERASE	GGT	Beta-N-ACETYL-GALACTOSAMINIDASE	NAGA
FERMENTATION/GLUCOSE	OFF	ALPHA-GALACTOSIDASE	AGAL
BETA-GLUCOSIDASE	BGLU	POSPHATASE	PHOS
D-MALTOSE	dMAL	Glycine ARYLAMIDASE	GlyA
D-MANNOSE	dMAN	ORNITHINE DECARBOXYLASE	ODC
BETA-XYLOSIDASE	BXYL	LYSINE DECARBOXYLASE	LDC
BETA-alanine arylamidase pNA	BAlap	DECARBOXYLASE BASE	0DEC
L-Proline ARYLAMIDASE	ProA	L-HISTIDINE assimilation	lHlSa
LIPASE	LIP	COUMARATE	CMT
PALATINOSE	PLE	BETA-GLUCORONIDASE	BGUR
Tyrosine ARYLAMIDASE	TyrA	O/129 RESISTANCE (Comp. vibrio.)	O129R
UREASE	URE	Glu-Gly-Arg-ARYLAMIDASE	GGAA

Table 1. Test Substrates on the Vitek2 GN Card

2.5 Plasmid Isolation

All isolates were screened for the presence or absence of plasmids. This was carried out using the Fermentas GeneJet Plasmid Miniprep Kit (#K0502).

Samples from each plate were inoculated into 5mL LB broth (Lysogeny broth), placed on a shaker and incubated overnight at 37ºC. The resulting cell cultures were harvested by centrifugation. The cells in the pellet were then lysed, and the Lysate cleared by centrifugation (12000 rpm for 10 minutes). The rinsed Lysate was applied to a silica spin column to selectively bind DNA molecules at a high salt concentration. The adsorbed DNA was washed to remove contaminants, and the pure plasmid DNA was eluted in elution buffer.

2.6 Gel Electrophoresis

The resulting DNA samples were separated on a 0.85% (w/v) agarose gel electrophoresis. The agarose gels were prepared with 1X TAE (Tris-acetate-EDTA) Buffer (0.04mM Tris-acetate, pH 8.0 and 0.001M EDTA) and low EEO Agarose. The gels were prepared by dissolving 0.85 g Agarose powder in 100mL of TAE at 95ºC. A 100 mL volume was poured into the gel apparatus and allowed to solidify at room temperature. The wells were formed by placement of a comb while the gel was still in liquid form. Upon solidification, the comb was removed carefully. The wells were loaded

with 10µL of the plasmid preparations and 4µL of 5x SYBR Gold (Invitrogen, Carlsbad, CA) mixed with loading dye. The electrophoresis chamber was then filled with enough 1X TAE Buffer to cover the gel. The electrodes were connected to an electric power unit. The electrophoresis was carried out at 71 milliamps. A supercoiled DNA ladder was added to the first well of each row to determine the plasmid size (New England Biolabs, #N0472S). The gel was photographed using a closed chamber and UV light with a digital camera.

3.0 Results

3.1 Processing of Isolates

Five of the recovered isolates were resistant to all of the antibiotics tested (isolates 4,19,29,36 and 46) (Table 2). All of the remaining isolates were resistant to at least two of the challenge antibiotics as indicated in Table 2. Some of the isolates were identified as having the same genus and species. This can be expected due to relatively small variations in colony morphology during the visual macroscopic selection process. All isolates were gram negative organisms, but only some are considered enteric and thus could be of human origin. *Aeromonas* was the most prevalent genus followed by *Raoultella*.

Recovered Isolates						
Resistance Pattern		Tetracycline 20µg/mL	Ampicillin 50µg/mL	Streptomycin 50µg/mL	Chloramphenicol 30µg/mL	Kanamycin 50µg/mL
Group 1						
Isolates resistant to five antibiotics	4	+	+	+	+	+
	19	+	+	+	+	+
	29	+	+	+	+	+
	36	+	+	+	+	+
	46	+	+	+	+	+
Group 2						
Isolates resistant to four antibiotics	3	+	+	-	+	+
	8	+	+	-	+	+
	13	+	+	-	+	+
	38	+	+	+	-	+
	44	+	+	+	+	-
Group 3						
Isolates resistant to three antibiotics	1	+	+	-	-	+
	12	+	+	-	-	+
	20	+	+	+	-	-
	32	+	+	+	-	-
	48	+	+	+	-	-
Group 4						
Isolates resistant to two antibiotics	2	+	+	-	-	-
	18	+	+	-	-	-
	23	+	+	-	-	-

Table 2. Multidrug resistance patterns for recovered isolates.

3.2 Identification of Isolates

Preliminary screening of the isolates employed several standard biochemical tests (Table 3). These were performed to confirm the information inferred from observation of colony growth on MacConkey agar plates. These rapid tests were used to determine if the organisms were truly members of the Enterobacteriaceae family before the automatic identification by use of the Vitek2**.**

Biochemical Tests						Biochemical Tests					
Isolate Number	Gram reaction	Lactose Fermentatio	Glucose Fermentatio n	OXI	Indole	**Isolate Number**	Gram reaction	Lactose Fermentatio	Glucose Fermentatio n	OXI	Indole
1	-	+	+	-	+	**25**	-	-	+ Gas	-	-
2	-	-	+	-	+	**26**	-	-	+ Gas	+	+
3	-	+	+ Gas	-	+	**27**	-	-	-	-	+
4	-	+	+ Gas	-	+	**28**	-	+	+ Gas	-	-
5	-	-	+	-	-	**29**	-	-	+ Gas	-	-
6	-	+	+	-	-	**30**	-	-	+ Gas	+	-
7	-	-	+	-	-	**31**	-	-	-	-	+
8	-	+	+ Gas	-	-	**32**	-	+	+ Gas	-	-
9	-	+	+ Gas	+	-	**33**	-	-	+ Gas	-	-
10	-	-	-	+	-	**34**	-	-	+ Gas	+	-
11	-	-	+	-	-	**35**	-	-	-	-	-
12	-	-	+	-	-	**36**	-	+	+ Gas	-	-
13	-	-	+ Gas	-	-	**37**	-	-	+ Gas	-	-
14	-	+	+ Gas	-	-	**38**	-	-	+ Gas	+	-
15	-	-	+	+	+	**39**	-	-	-	-	-
16	-	+	+ Gas	-	-	**40**	-	+	+ Gas	-	-
17	-	-	+ Gas	+	-	**41**	-	-	+ Gas	-	-
18	-	+	+	-	+	**42**	-	-	+ Gas	+	-
19	-	-	+	+	+	**43**	-	-	-	-	-
20	-	+	+	-	+	**44**	-	+	+ Gas	-	-
21	-	+	+	-	-	**45**	-	-	+ Gas	-	-
22	-	-	+	-	-	**46**	-	-	+ Gas	+	+
23	-	+	+	-	-	**47**	-	-	-	-	-
24	-	+	+	-	+	**48**	-	+	+ Gas	-	+

Table 3. Preliminary Biochemical Tests

Identification of the isolates was performed using the BioMerieuxVitek2 Automated Identification System to determine the bacterial Genera that survive through the biosolid processing stream. Identification results are shown in Table 4.

Isolate Identification			
Isolate Number	**Identity**	**Isolate Number**	Identity
1	*Raoultella planticola*	**25**	*Aeromonas salmonicida*
2	*Aeromonas hyrophila/caviae*	**26**	Unidentified Organism
3	*Raoultella ornitholytica*	**27**	Not Identified
4	*Raoultella ornitholytica*	**28**	*Pseudomonas fluorescens*
5	*Serratia liquefaciens group*	**29**	*Aeromonas sobria*
6	Not Identified	**30**	*Acinetobacter lwoffii*
7	Unidentified Organism	**31**	Not Identified
8	*Raoultella planticola*	**32**	*Aeromonas hydrophila/caviae*
9	*Ochrobactrum anthropi*	**33**	Not Identified
10	*Cupriavidus pauculus*	**34**	Not Identified
11	Not Identified	**35**	*Acinetobacter ursingii*
12	*Serratia marcescens*	**36**	*Pantoea spp.*
13	*Citrobacter freundii*	**37**	Not Identified
14	Not Identified	**38**	*Aeromonas hydrophila/caviae*
15	*Aeromonas sobria*	**39**	*Acinetobacter ursingii*
16	Not Identified	**40**	*Acinetobacter ursingii*
17	Not Identified	**41**	*Serratia liquefaciens group*
18	*Raoultella ornitholytica*	**42**	*Comomonas testosteroni*
19	*Aeromonas sobria*	**43**	Not Identified
20	*Raoultella ornitholytica*	**44**	*Aeromonas hydrophila/caviae*
21	Not Identified	**45**	Not Identified
22	*Serratia liquefaciens group*	**46**	*Aeromonas sobria*
23	*Raoultella planticola*	**47**	*Acinetobacter lwoffii*
24	Unidentified Organism	**48**	*Citrobacter freundii*

Table 4. Isolate Identification

3.3 Plasmid Isolation and Gel Electrophoresis

The numbers of plasmids from each isolate and their sizes were determined by gel electrophoresis. Supercoiled DNA was used in lanes 1 and 17 for size determination (New England Biolabs, #N0472S). Thirty-four out of 48 isolates showed DNA bands varying in size from 2 to 10kb. Gel electrophoresis results are shown in Figures 3 and 4 and approximate plasmid numbers and sizes are in Tables 5 and 6.

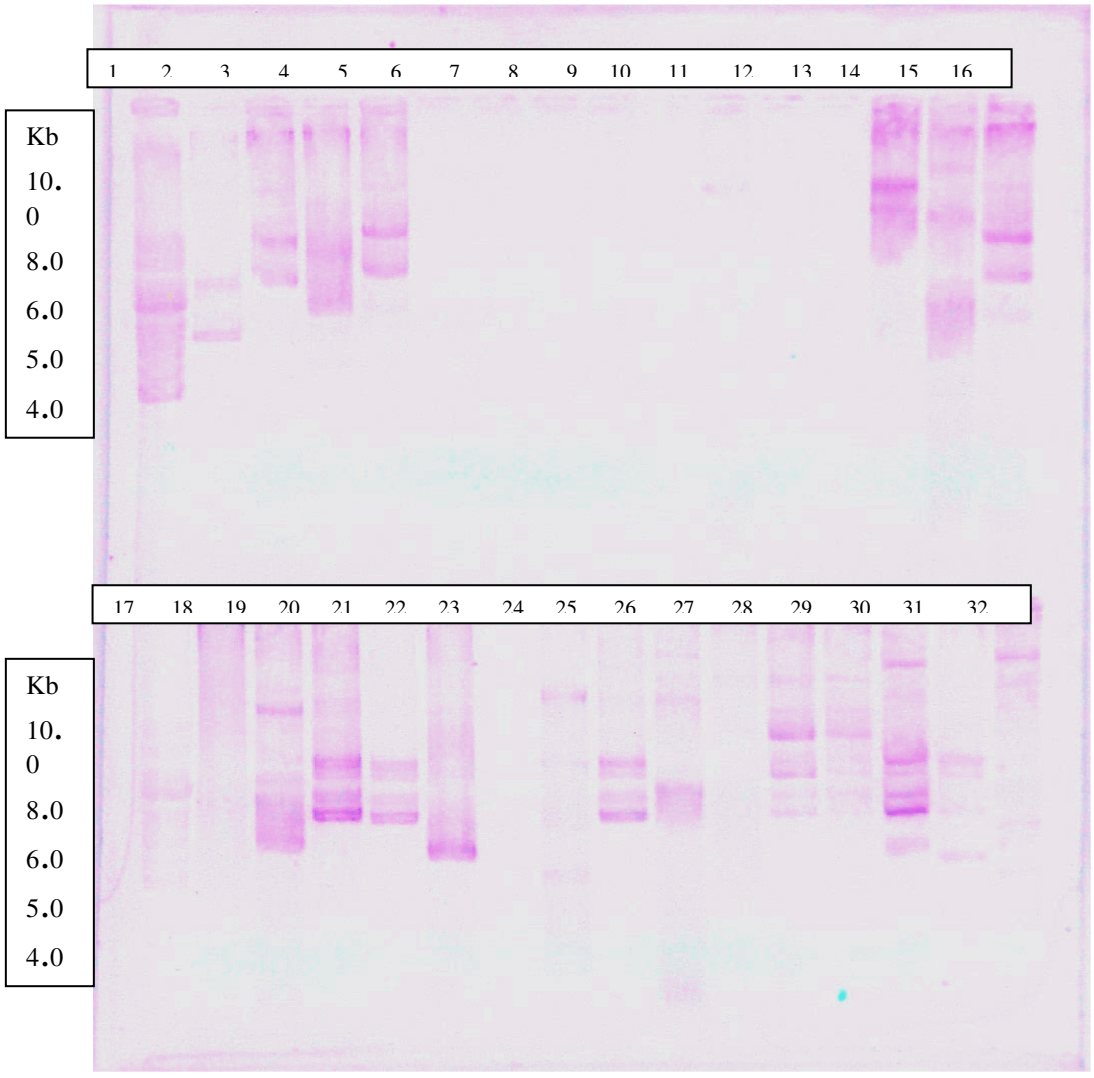

Figure 3. Isolates 1-30 with supercoiled DNA ladder in lanes 1 and 17.

Isolate Number	Lane	Number of Plasmids	Plasmid Size (Kb)	Isolate Number	Lane	Number of Plasmids	Plasmid Size (Kb)
1	2	2	5,6	**16**	18	0	
2	3	4	6,8,9 10	**17**	19	2	6,9
3	4	3	5,7,10	**18**	20	3	5,6,7
4	5	3	6,8.10	**19**	21	3	5-6
5	6	0		**20**	22	1	5
6	7	0		**21**	23	0	
7	8	0		**22**	24	1	9
8	9	0		**23**	25	3	5-6
9	10	0		**24**	26	2	5,9
10	11	0		**25**	27	0	
11	12	0		**26**	28	4	5-8
12	13	0		**27**	29	1	8
13	14	3	8,9,10	**28**	30	5	6-9
14	15	3	5,8,10	**29**	31	1	6
15	16	4	6,7,10	**30**	32	1	10

Table 5. Approximate plasmid number and size for isolates 1-30.

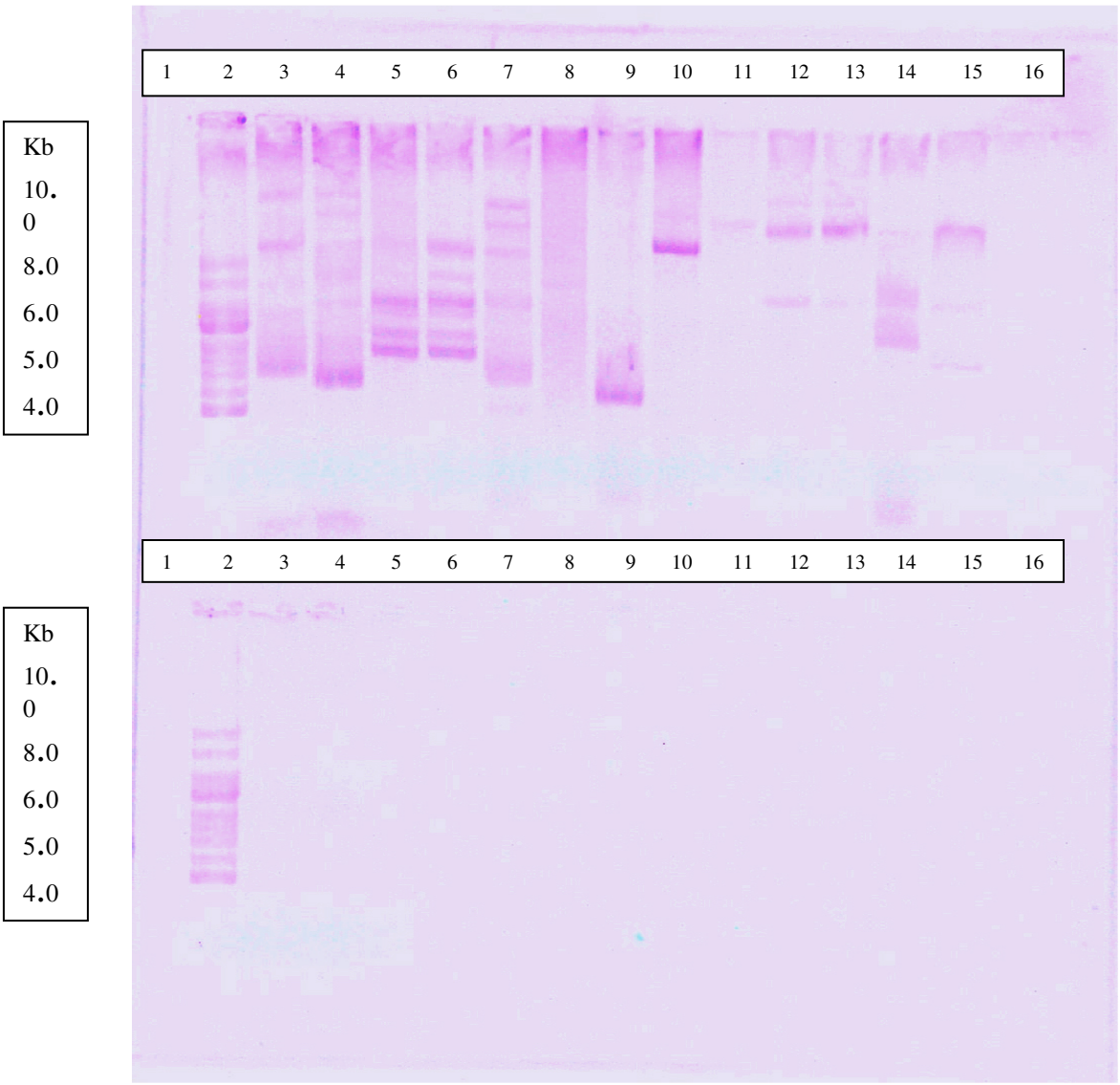

Figure 4. Isolates 31-48 with supercoiled DNA ladder in lanes 1 and 17.

Isolate Number	Lane	Number of Plasmids	Plasmid Size (Kb)	Isolate Number	Lane	Number of Plasmids	Plasmid Size (Kb)
31	2	3	6,8,9	**40**	11	1	9
32	3	3	6,8,9 10	**41**	12	1	9
33	4	4	4,8,9,10	**42**	13	3	5,6,8
34	5	5	5-8	**43**	14	1	9
35	6	4	4-9	**44**	15	0	
36	7	0		**45**	16	0	
37	8	1	4	**46**	18	0	
38	9	1	8	**47**	19	0	
39	10	1	9	**48**	20	0	

Table 6. Approximate plasmid number and size for isolates 31-48.

4.0 Discussion

The development of microbial antibiotic resistance from a human source is fairly straightforward and begins with the fecal shedding of enteric gram negative antibiotic resistant bacteria into the sewage system. Once in the sewage treatment facility, the bacteria freely mix with other genera of bacteria present. Genetic exchanges are possible under such conditions and are well documented (Bale et al., 1987). The cycle continues back to human hosts via garden products, which can have far reaching consequences for all members of a community. In addition to direct uptake of antibiotic resistance genes by ingestion of viable cells containing antibiotic resistance genes or by these genes spilled into the sewage effluent by dead cells, studies have demonstrated that these genes can also seep into surface water in areas surrounding the crops (Auerbach et al., 2006). *Enterobacteriaceae* are a normal part of the normal human gastrointestinal system and normally cause no disease. For an individual suffering from bacterial gastrointestinal

disease, the usual treatment is a course of antibiotics. This allows for selection of antibiotic resistant bacteria in the gut. When the antibiotic resistance is transferred in the gut from non-pathogenic bacteria to infection causing strains, then there is no treatment other than hydration to prevent dehydration in the patient (Rath et al., 2001; Thoren et al., 1980). Already, many deaths occur every year due to non-treatable bacterial infections and this rate is increasing (Blot et al., 2002).

The transfer of antibiotic resistance can occur in the gut or outside the gut when two bacteria are in close proximity to one another. Alternately, the antibiotic resistant bacteria, upon death, may spill their cell contents into the environment, whether water, soil or intestinal tract, releasing plasmids (Maruyama et al., 2006).

All of the isolates recovered from the Cheney Wastewater Treatment Plant biosolids were determined to be resistant to at least two commonly used antibiotics. Antibiotic resistance genes are known to pass between even unrelated strains of bacteria. Enteric bacteria which have a high level of antibiotic resistance are washed into the water treatment system where they mingle with many other bacteria. Sewage treatment plants are ideal environments for genetic transfer due to high nutrient levels and a high bacterial population (Silva et al., 2006). The presence of bacteria with antibiotic resistance genes in a treatment product intended for use on food crops can be problematic as these bacteria or their genes may end up in humans.

There is a cost to the bacterial cell in retaining large numbers of plasmids, and research has shown that antibiotic resistant strains of some bacteria grow more slowly than wild type strains (Saunders, 1984). In addition, studies have shown that antibiotics are persistent in treated and drinking water and can be correlated to human use (Karthikeyan and Bleam, 2003).

Studies to measure the scope of the antibiotic resistance problem have shown that unrestrained use of antibiotics has a definite, measurable effect on the environment and the organisms living in those environments. The human overuse of the very weapons manufactured by microbes to fight other microbes is the cause of the rise in antibiotic resistance and the key to stemming the problem is to simply reduce their use (www.nih.gov).

5.0 Conclusion

Samples of biosolid from Cheney Wastewater Facility showed ample bacterial growth. The high number of organisms recovered, and their diversity, is not surprising because wastewater treatment is not a sterilization process. What is surprising, however, is the incidence of antibiotic resistance. The biosolid samples were taken before the composting step, which may further reduce the bacterial load before the addition of mulch.

Ten genera of gram negative bacteria were isolated from the biosolid sample obtained from Cheney Wastewater facility. They were *Acinetobacter, Aeromonas*,

Citrobacter, Comomonas, Cupriavidus, Ochrobactrum, Pantoea, Pseudomonas, Raoultella and *Serratia*.

The genera *Acinetobacter, Pseudomonas and Aeromonas* belong to the Class Gammaproteobacteria, which is a diverse Class divided into 14 Orders and 25 Families (Prescott et al., 2005). *Acinetobacter* are aerobic inhabitants of water and soil. *Aeromonas* are facultative anaerobes whose natural niche is brackish water. Four out of the five isolates resistant to all five antibiotics were from the genus *Aeromonas*. A proposed reason for this is that *Aeromonas* is not of human origin. *Aeromonas* may be a continual part of the sewage treatment process and constantly exposed to antiobiotics in the system. Aeromonas, being adapted to and aquatic environment, may not survive in a terrestrial setting, such as a garden. Further studies could be done to assess the actual bacterial load after composting and the addition of mulch.

Comomonas and *Cupriavidus* are included in the Class Betaproteobacteria, Order *Burkholderiales*. *Ochrobactrum* are included in the Order Alphaproteobacteria and are organisms normally found in soil. These organisms are not considered to be part of normal human flora although they can be opportunistic human pathogens.

Of the ten genera isolated, only *Raoultella*, *Serratia, Citrobacter* and *Pantoea*, are members of the family *Enterobacteriaceae* and are therefore considered to be a normal part of the human intestinal microbial community. Horizontal gene transfer between these species of *Enterobacteriaceae* and other unrelated bacterial genera has been well documented (Doi et al., 2004; Lawley et al., 2003).

The perceived safety of biosolid application for crop fertilization only reflects the monitoring of viable pathogenic bacteria such as *Escherichia coli* and *Salmonella* (Arthurson, 2008). The potential pathogenicity of these organisms, however, does not address the effect of antibiotic resistance genes in the various bacteria that are not monitored. These organisms are themselves harmless, but carry a very real potential threat in the form of transferrable antibiotic resistance which directly correlates to antibiotic consumption in a community.

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VITA

Author: Gwendolyn Lenore Hartman

Place of Birth: Fort Ord, Monterey, California

Undergraduate Schools Attended: Craven Community College
North Idaho College
Eastern Washington University

Degrees Awarded: Associate of Science, 1999, North Idaho College
Bachelor of Science, 2003, Eastern Washington University

Honors and Awards: Graduate Assistantship, Biology Department, 2005-2007, Eastern Washington University

J. Herman Swartz and Jean Swartz Bennett and Frances and William P. Werschler Graduate Fellowship, 2006-2007, Eastern Washington University

Graduated Cum Laude, Eastern Washington University, 2003

Professional Experience: Teaching Assistant for Microbiology, Medical Bacteriology, Hematology, Microbial Physiology Labs, Eastern Washington University, 2005-2007

Summer Employment at Eastern Washington University, 2006
Preparation of Biochemical tests and media

Spore Strip testing for Eastern Washington University Dental Hygiene Department, 2006-2007

Quality Control Microbiologist I, HollisterStier Laboratories, Spokane Washington, 2007-Present