The discovery of antibiotics and their subsequent application to clinical medicine is one of the outstanding scientific achievements of the twentieth century. The tale of how antibiotics were discovered is one of scientific legend: Sir Alexander Fleming astutely recognized that a contaminated Petri dish actually contained a bacteria-killing mold. For his discovery of penicillin, Fleming shared the 1945 Nobel Prize in physiology/medicine with Sir Howard Florey and Ernst B. Chain.

The unique feature of penicillin (and other antibiotics) is not merely that it kills bacteria—there are many compounds that have such a capability—but that it specifically affects bacteria. This key feature is absolutely critical for the medical application of antibiotic therapy. Antibiotics administered to humans are lethal to disease-causing bacteria but do not impact the patient. This is possible because antibiotics act on features of the bacterial cell that are absent in humans. For example, penicillin prevents the formation of new bacterial wall materials; human cells do not even contain a cell wall.

During the last half-century, antibiotics have become pervasive in human medicine. Since the discovery of penicillin, a plethora of new antibiotics, semi-synthetic antibiotics, and synthetic antibiotics (antibacterials) have been discovered or developed (Table 1). These new drugs target different features of bacterial physiology, thus expanding the range of bacterial species that can be successfully treated with antibiotics. Antibiotics are also used extensively in agriculture and for other non-medical purposes. Low doses of antibiotics are often included in animal feed to promote growth and increase weight gain, as well as prevent the onset of
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The pioneering work of Stuart Levy
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antibiotic-resistant bacteria in wastewater.
hospital. "It is not difficult to make microbes resistant to penicillin in the laboratory by exposing them to concentrations not sufficient to kill them, and the same thing has occasionally happened in the body. Moral: If you use penicillin, use enough."

Table 1. Major Classes of Antibiotics and Antibacterials, and Representative Drugs in Each Class

<table>
<thead>
<tr>
<th>Class</th>
<th>Representative Drug(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-lactams</td>
<td>Penicillin, Amoxicillin, Methicillin</td>
</tr>
<tr>
<td>Aminoglycosides</td>
<td>Streptomycin, Neomycin, Kanamycin, Gentamicin</td>
</tr>
<tr>
<td>Macrolides</td>
<td>Tylosin, Erythromycin</td>
</tr>
<tr>
<td>Ketolides</td>
<td>Telithromycin</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>Tetracycline, Oxytetracycline</td>
</tr>
<tr>
<td>Lincosamides</td>
<td>Clindamycin</td>
</tr>
<tr>
<td>Ansamycins</td>
<td>Rifampin</td>
</tr>
<tr>
<td>Glycopeptides</td>
<td>Vancomycin</td>
</tr>
<tr>
<td>Quinolones/fluoroquinolones</td>
<td>Nalidixic acid, Ciprofloxacin</td>
</tr>
<tr>
<td>Sulfonamides</td>
<td>Sulfamethoxazole</td>
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</tbody>
</table>

disease. Although reliable estimates are
difficult to obtain, most scientists believe
that approximately 70% of all antibi-
otics are used for agricultural purposes.
In this article, we report on a
research project that investigated the
role of municipal wastewater treat-
ment facilities in the spread or control
of antibiotic-resistant bacteria. The
project was supported by a grant from
CURA’s Faculty Interactive Research
Program, as well as grants from the
Undergraduate Research Opportunity
Program at the University of Minnesota.
We hypothesized that the disinfection
processes most treatment facilities use
would adequately inactivate antibi-
otic-resistant bacteria in wastewater.
However, our research suggests that
treatment facilities, which are primarily
designed to protect water quality,
do not adequately prevent resistant
bacteria from being released into the
environment. We conclude that rela-
tively simple changes in the design,
operation, and regulation of municipal
wastewater treatment facilities could
substantially reduce the release of these
bacteria and, we hope, slow the prolif-
eration of antibiotic resistance among
bacteria appearing in clinical patients.

A Brief History of Antibiotic Resistance
Antibiotic-resistant bacteria were
discovered soon after the medical use
of penicillin began. At the time, the
development of resistant bacteria was
largely viewed as inconsequential. If a
patient had an infection that a resistant
bacterium caused, then an alternative
antibiotic was always available for effec-
tive treatment. However, some fore-
sighted scientists warned of the pending
problem of antibiotic resistance. In his
Nobel acceptance speech, Alexander
Fleming himself cautioned doctors about
the danger of giving an “underdosage”
of penicillin, noting: “It is not difficult
to make microbes resistant to penicillin in the laboratory by exposing them to
concentrations not sufficient to kill them, and the same thing has occasionally
happened in the body. . . . Moral:
If you use penicillin, use enough.”

Ultimately, the proliferation of
antibiotic resistance is caused by the
propagation of specific genes that allow
bacteria to defy the lethal effects of
antibiotics. These antibiotic resistance
genes are probably not new, but likely
result from millions of years of evolu-
tion, during which time bacteria have
developed many mechanisms to survive
the dangers that the world thrusts upon
them. Certainly, many of these genes
were specifically developed to coun-
teract antibiotics, which are, after all,
naturally occurring compounds. Many
antibiotic resistance genes, however,
likely are subtle adaptations of genes
that provide protection against other
toxic compounds. For example, there
is a strong correlation between genes
that encode for resistance to heavy
metals and antibiotic resistance genes.

The Development of Antibiotic Resistance in Bacteria
The simplest method by which bacteria
become resistant to antibiotics is via a
point mutation of the deoxyribonucleic
acid (DNA) within their genome. Point
mutations are typically lethal to the
bacterium or have no effect, but on rare
occasions these mutations are beneficial
(from the bacterium’s perspective) and
allow the organism to become resistant
to antibiotics. Point mutations, however,
are not the major concern with respect
to antibiotic resistance. This form of
bacterial evolution is slow and random,
and it is unlikely that bacteria could
rapidly achieve resistance to multiple
antibiotics via point mutations alone.

The existence of antibiotic resistance,
however, is insufficient to explain the
global proliferation of resis-
tance. Bacteria also harbor other
genes that are specifically designed to
help bacteria rapidly evolve—genes desig-
nated as evolution genes. Some of
these genes enable bacteria to rapidly
develop new genes that are not the major concern with respect
to antibiotic resistance. This form of
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throughout the bacteria population. The evolution genes that allow lateral gene transfer are perhaps the most important class of evolution genes with respect to antibiotic resistance. Lateral gene transfer is the exchange of genetic material between different bacteria; it allows bacteria to share their abilities to resist antibiotics. This is believed to be the principal mechanism by which similar resistance genes are found throughout the world among many different species of bacteria.

During the last 20 years, scientists have also recognized the importance of integrons, another type of evolution gene. Integrons are responsible for integrating resistance genes into the genomes of bacteria, and then controlling the expression of these resistance genes. Because of this unique ability, integrons can be viewed as a genetic “luggage rack” in which different genes can be kept until they are needed. Integrons are a key component in the development of multiple-antibiotic-resistant bacteria because they allow bacteria to easily accumulate numerous genes.

Responding to Antibiotic Resistance

Although scientists have known about antibiotic-resistant bacteria for almost as long as they have known about antibiotics, the assumption was that new antibiotics would be discovered or developed faster than bacteria could become resistant. The discovery of new antibiotics, however, has slowed substantially since the 1960s. In fact, most “new” antibiotics are merely subtle modifications of previously existing ones and have little impact on bacteria that are already resistant.

During the last decade, therefore, there has been a considerable effort to restrict antibiotic use to only those applications where antibiotics are appropriate. Physicians are now reminded to avoid prescribing antibiotics for viral infections such as influenza and the common cold. Likewise, patients are carefully instructed to follow prescription guidelines so that enough of the drug is administered to limit the development of resistant bacteria. There is also increasing pressure to limit or eliminate non-medical use of antibiotics and antibacterials. As noted above, a substantial fraction of all antibiotics are used in agriculture at subtherapeutic concentrations. Although the United States appears to be far from prohibiting this practice, the European Union is banning subtherapeutic antibiotic use in agriculture in 2006. Although more controversial, many scientists—led by the Alliance for the Prudent Use of Antibiotics—are recommending the elimination of triclosan and other antibacterials from liquid hand soap, toothpaste, and other common household items.

A New Paradigm: Resistance Control?

The current situation with respect to antibiotic resistance is bad and the future is bleak. The discovery of new drugs has slowed to a trickle—a problem that will only worsen as pharmaceutical companies devote a greater fraction of their research and development budgets to less essential drugs (e.g., Botox, Viagra). Simultaneously, the ever-increasing use and misuse of antibacterials in common household products can only exacerbate the problem.

From our perspective, current efforts to reduce the spread of antibiotic resistance are an excellent first step. Certainly, our historically indiscriminate use of antibiotics needs to end. The more important issue is to identify novel approaches to limit the spread of antibiotic resistance. Our intention in undertaking this research, therefore, was to take a different approach to solving the problem of antibiotic resistance. We started by asking some simple yet fundamental questions about the proliferation of antibiotic-resistant bacteria.

First, where do the majority of antibiotic-resistant bacteria originate? Certainly, many bacteria are naturally resistant, but the majority of antibiotic-resistant bacteria result from antibiotic use. Therefore, people and animals taking antibiotics are most likely the primary source of antibiotic-resistant bacteria.

Second, how do resistant bacteria spread throughout the world after they originate inside a person? Humans actually contain about 10 times more bacterial cells in their bodies than they do human cells. The overwhelming majority of these bacterial cells reside in our gastrointestinal tracts, and most are released from the body during defecation.

Having asked and answered these two simple questions, we then inferred that municipal wastewater treatment plants, which handle virtually all human toilet waste in large municipalities (in rural areas, septic systems are more commonly used), would be critical in reducing the spread of antibiotic resistance. We hypothesized that municipal wastewater treatment facilities could adequately control the release of antibiotic-resistant bacteria to the world.

Municipal Wastewater Treatment Facilities: How Do They Work?

Municipal wastewater treatment facilities are primarily designed and operated to protect the environment. Municipal wastewater treatment facilities remove readily biodegradable compounds from sewage. Although there is relatively little in human sewage that is toxic, these biodegradable compounds are of environmental concern because if they were released untreated, they would biodegrade in the environment, resulting in oxygen depletion leading to septic conditions. Municipal wastewater treatment facilities, therefore, allow surface waters to maintain high dissolved oxygen levels, improving their aesthetic and recreational use value, as well as their ability to support healthy populations of fish and other aquatic fauna.

Although all municipal wastewater treatment facilities are unique, most are similar in design and involve a common series of unit operations (Figure 1). The first few unit operations, called primary treatment, are designed to remove particles from the wastewater. The bar rack removes large particles (greater than 1 inch), whereas the grit chamber removes sand and other dense, rapid-settling particles. The primary clarifier is a quiescent settling zone that allows organic particles to settle or float so that they can be removed. These primary treatment operations account for about 50% of the treatment that occurs.

The next unit operation, the aeration tank, is designed to remove dissolved organic compounds (which are readily biodegradable) from the wastewater by creating conditions favorable for the growth of bacteria. The tank works by bubbling air through the wastewater, allowing bacteria to metabolize pollutants that are present. Because these bacteria grow in excessive quantities, they must be removed from the wastewater. This is accomplished by the next unit operation, which is a quiescent settling chamber called the secondary clarifier. The combination of the aeration tank and the secondary clarifier is called the activated sludge process, which is the most common technology for the secondary treatment of wastewater.

Following primary and secondary treatment, the quality of municipal wastewater is quite good—not yet potable (i.e., safe to drink), but often.

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as good as or better than the quality of many lakes and rivers. This treated wastewater, however, still contains pathogenic bacteria that could make people sick if they accidentally ingested the water. Municipal wastewater treatment facilities, therefore, perform a final treatment step in which the treated wastewater is disinfected to help reduce the number of disease-causing microbes. Disinfection is required only when recreational use of the receiving stream is a reasonable expectation. In Minnesota, for example, wastewater treatment facilities usually disinfect their wastewater only from April to November.

In addition to treating the wastewater, municipal wastewater treatment facilities must deal with the solid residues that the primary and secondary clarifiers collect. These solid residues are readily biodegradable organic materials that are most commonly treated by a process called anaerobic digestion. The conventional anaerobic digestion process, which largely mimics our gastrointestinal tracts (hence the “digestion” nomenclature), is kept free of oxygen and operated at 98.6°F. Following digestion,
the treated wastewater solids are either applied to farmland as a fertilizer and soil conditioner, or sent to a landfill for disposal. The former alternative is preferred as a “sustainable” practice, whereas landfill space is finite.

Because anaerobic digestors operate at conditions similar to the human body, they are not particularly good at eliminating human pathogens. Numerous alternative treatment technologies, therefore, have been developed to better treat wastewater solids. All of these alternative treatment technologies are more expensive, however, and thus municipalities do not frequently use them. Perhaps the most attractive treatment alternative is thermophilic anaerobic digestion, which operates almost identically to conventional anaerobic digestion, except that it operates at sufficiently high temperatures (greater than 110°F) to kill most human pathogens.

**Methodology and Analysis**

The first goal of our project was to determine the extent to which municipal wastewater treatment facilities prevent the release of antibiotic-resistant bacteria. There are two potential paths by which antibiotic-resistant bacteria can escape a municipal wastewater treatment facility. The most obvious is in the treated wastewater. Our research, therefore, investigated the importance of secondary clarification and disinfection in preventing the release of antibiotic-resistant bacteria from the aeration tank. Resistant bacteria could also be released in the solids collected during primary treatment and from the secondary clarifier. Our research, therefore, compared the effectiveness of two variations of conventional anaerobic digestion and thermophilic anaerobic digestion at destroying resistant bacteria.

We investigated the efficacy of wastewater disinfection at the Metropolitan Wastewater Treatment Facility in St. Paul. This facility is very large, treating an average of 180 million gallons of sewage each day. Typically, the quality of treatment from the Metropolitan plant is top-notch, and the facility regularly wins state and national awards for operational excellence. Throughout the year, we quantified about 100,000 (10^5) tetracycline-resistant bacteria per milliliter of water in the aeration tanks at the Metropolitan plant. From the treated wastewater, we quantified about 300 tetracycline-resistant bacteria per milliliter in the winter (i.e., when disinfection was not performed) and about 30 tetracycline-resistant bacteria per milliliter during the summer (i.e., during the disinfection period). That is, about 99.6% and 99.97% of the resistant bacteria in the aeration tanks are removed in the winter and summer, respectively. Although this removal efficiency might seem sufficient, 30 bacteria per milliliter translates to more than 10 trillion (10^{13}) tetracycline-resistant bacteria released each day from this treatment facility into our waterways.

We also investigated the efficacy of anaerobic digestion at the Western Lake Superior Sanitary District (thermophilic process) and the Empire Wastewater Treatment Facility (conventional process), which are located in Duluth and Farmington, respectively. Both of these plants have also earned awards for operational excellence. We again detected about 100,000 (10^5) tetracycline-resistant bacteria per milliliter in the waste stream entering the anaerobic digestors at each of these treatment facilities. However, we were unable to detect any tetracycline-resistant bacteria in the waste stream leaving the anaerobic digestors at these two treatment facilities, in part because the research method we used is unable to detect levels of tetracycline-resistant bacteria below 1,000 (10^3) per milliliter of sludge solids. However, this suggests that both anaerobic digestion processes were able to inactivate at least 99% of antibiotic-resistant bacteria. We are currently attempting to develop an alternative technique to measure the efficiencies by which these anaerobic digestors inactivate antibiotic-resistant bacteria.

The second goal of our research was to characterize the antibiotic-resistant bacteria in sewage. From the three treatment facilities, we isolated and identified 173 bacterial strains that were resistant to tetracycline. All of these bacterial strains were pathogenic (e.g., *Shigella* or *Klebsiella* spp.), possibly pathogenic (e.g., *Escherichia coli*), or non-pathogenic but related to pathogens (e.g., *Citrobacter* spp.). In more than 50% of these bacteria, we also detected at least one gene encoding for tetracycline resistance.

Based on these initial data, we then studied 14 different tetracycline-resistant bacterial strains in more detail. All 14 of these strains contained an integron and were resistant to at least three different antibiotics (we tested resistance to amoxicillin, ampicillin, chlorotetracycline, enrofloxacin, erythromycin, sulfamethoxazole, trimethoprim, and tylosin). We also tested these bacteria for lateral gene transfer. Although this work is still ongoing, many of these bacterial strains are capable of
encouraging, 1% of a very large number that although a 99% inactivation looks round disinfection. Instead, we learned encourage the implementation of year-
municipal wastewater, and that an antibiotic-resistant bacteria in treated 
fection would adequately inactivate Our original hypothesis was that disin-
resistant bacteria are released from extremely high numbers of antibiotic-
Our research has demonstrated that these strains contained an integron or a gene encoding for resistance to tetracycline. Although this work is also ongoing, our analysis revealed that several of these strains were capable of laterally exchanging genes encoding for resistance to ciprofloxacin.

Conclusion and Policy Recommendations

Our research has demonstrated that extremely high numbers of antibiotic-resistant bacteria are released from municipal wastewater treatment plants, even when disinfection is performed. Our original hypothesis was that disinfection would adequately inactivate antibiotic-resistant bacteria in treated municipal wastewater, and that an outcome of our work would be to encourage the implementation of year-round disinfection. Instead, we learned that although a 99% inactivation looks encouraging, 1% of a very large number (10^{13}, or 1 quadrillion) still represents a very large number (10^{10}, or 10 trillion) of antibiotic-resistant bacteria that are released from the Metropolitan Wastewater Treatment Facility each day.

The bacteria that we studied were all pathogens or related to pathogens and all were resistant to multiple antibiotics. A substantial fraction of these bacteria (greater than 50%) harbored genes encoding for tetracycline resistance. These bacteria frequently harbored integrons (genes that allow bacteria to accumulate multiple genes for antibiotic resistance) and some of them were capable of transferring their resistance to other bacteria. The frequency of lateral gene transfer of ciprofloxacin resistance, which occurred in more than 40% of the strains we studied, is particularly worrisome because this trait is typically very rare (less than 1%) among clinical strains of ciprofloxacin-resistant E. coli. Simply put, the bacteria that we detected in municipal wastewater are some of the most resistant bacteria ever studied. There is a substantial need, therefore, to prevent these organisms from reaching the environment.

At first glance, the most obvious solution to the problem of antibiotic-resistant bacteria in treated municipal wastewater would be to require more stringent disinfection. The majority of municipal wastewater is disinfected using chlorine, which poses a security risk (chlorine gas is very dangerous) and generates disinfection by-products that are known or suspected carcinogens. Although we recommend a policy shift to include year-round wastewater disinfection, we do not recommend that more stringent disinfection regulations be imposed because of these unwanted consequences.

Instead, we recommend that wastewater effluents be passed through a sand filter prior to disinfection. Sand filters can physically remove antibiotic-resistant bacteria from treated wastewater, but without the use of potentially dangerous chemicals. At the present time, sand filters are rarely used in wastewater treatment, but they are commonly used at drinking water treatment facilities, so the technology is well-developed and well-understood. Additional research is needed, however, to optimize the removal/inactivation of antibiotic-resistant bacteria by our proposed combination of sand filtration and effluent disinfection.

Although our research on the fate of antibiotic-resistant bacteria in anaerobic digestors was inconclusive due to the limitations of our research method, we suspect that our ongoing research will demonstrate that thermophilic anaerobic digesters achieve substantially better inactivation efficiencies than conventional technologies. This ongoing research is particularly pertinent because of a recent shift in policy that emphasizes the application of treated wastewater solids to land rather than putting these residues into landfills—that is, the “environmentally friendly” practice of applying wastewater solids to land may have unexpected and undesirable consequences in terms of the proliferation of antibiotic-resistant bacteria.

Timothy M. LaPara is associate professor in the Department of Civil Engineering at the University of Minnesota. His research focuses on the microbial ecology of wastewater treatment. Sara J. Firl was a graduate student in the Department of Civil Engineering at the University of Minnesota during this study. She currently works for Barr Engineering. Leslie J. Onan was an undergraduate student in the College of Biological Sciences at the University of Minnesota during this study. She is currently attending law school at the University of Michigan. Sudeshna Ghosh is a doctoral candidate in the Department of Civil Engineering at the University of Minnesota. Tao Yan is a post-doctoral research associate in the Biotechnology Institute at the University of Minnesota. Michael J. Sadowsky is Distinguished McKnight Professor in the Department of Soil, Water, and Climate and the Biotechnology Institute at the University of Minnesota. His research focuses on the genetics, genomics, and biochemistry of bacteria of environmental importance.

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