12/4/07 Bd. Mtg.
Water Recycling Policy
Deadline: 10/26/07 by Noon

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Date: Mon, Sep 17, 2007 8:15 AM
Subject: Water Recycling Policy

To: State Water Resources Control Board
Fm: Dr Edo McGowan
Re: Comments related to---http://www.swrcb.ca.gov/comments/docs/water_recycling_policy_notice.pdf

http://www.waterboards.ca.gov/comments/docs/water_recycling_policy.pdf

The document and its underpinnings are so weak on pathogens, transfer of antimicrobial resistance, lateral transfer of genetic information to soil and aquatic microbes and environmental niches, all potentially impacting public health as to be blatantly dangerous. Because of the potential impact on public health, an EIR warrants preparation under CEQA to fully discuss the human and environmental health implications and alternatives. The reference resolutions noted within the document are so old with respect to issues and impacts on public health, that they are useless. There are provisions within the resolution (resolved 7 (b) relating to Title 22, Resolve 10 requiring CDPH to have developed MCL, but nothing on pathogens, antibiotic resistant genetic material, antibiotic resistance and the build up of antibiotics such as macrolides that can maintain vancomycin resistance. Thus for constituents that CDPH has not established MCLs, the regional boards may establish such---but where is the expertise to do so?

Therefore let us take some examples for illustrative purposes---------

Let us first discuss Erythromycin, which is a bioaccumulating macrolide that has been shown to maintain or cause cross resistance with vancomycin. Hence, since this can bioaccumulate in soils, and where there are soil microbes, these can develop resistance to both of these antibiotics. There are soil microbes everywhere in soils. Thus one may well see the build up of resistance or similar cellular and molecular machinery established that can supply cross resistance. Additionally, the entrained antibiotic resistant genetic material as discussed by Pruden et al is found in Title 22 water and the pathogens entrained in this water, as discussed by Rose et al, (WERF #00-PUM-2T)will be delivered with the Title 22 water. Thus, we have, under each micro-emitter of a drip system a small biological factory for producing antibiotic resistant pathogens. I have had this Title 22 water run in the lab and it contained multi-drug resistant bacteria. In viewing the Mueller-Hinton plates two days later, we noticed secondary growth within formerly clear areas. This may have represented bacteria in the viable but non-culturable (VBNC) state that resuscitated. These also were resistant. Title 22 does not, as far as I can ascertain, look for either resistance or VBNC. Rose in her paper as published by WERF comments on the fact that the indicators used in ascertaining health risks from recycled water have long been known to be non-reflective of public health risks.

The state's public health agencies appear to have little or no information on these phenomena. I asked the various agencies that are presumed to have some control over public health as to who among them was dealing with antibiotic resistance in water. Each, in response assured me that, now that they understood the situation [many had not even thought about it], it was not within their jurisdiction, but if I would just call this number, that agency over there would be the one that could help me. I received the same or similar reply, went full circle and never found any state agency that was dealing with this issue. The U.S EPA has just started to look at this, but only as relates to sewage sludge (biosolids). EPA does have standards for

sewage solids but has not entered the arena of reclaimed/recycled water; that is an area left to the states. The U.S. EPA has not done a human health risk assessment on pathogens contained within biosolids, and none on reclaimed/recycled water. In discussing this with both U.S. EPA and CDC&P, both admit that there had been no coordinated or focused effort in looking at antibiotic resistance in sewage, hence its risks to man. I am on a newly established U.S. EPA/WERF scientific panel that has been developed to look at antibiotic resistance within biosolids. Some of that work will, of course, translate to reclaimed/recycled water questions, but as yet even the RFP for these studies in the area of biosolids are still in the developmental state. Thus, it seems that to assume that somehow all is well within Title 22 is a major jump and is in fact a fiction as relates to public health. Thus at a minimum, the resolution's actions fall to CEQA and warrant an EIR to explore the issues of public health.

In large irrigation systems such as public parks, we have large pop-up sprinklers that are designed to produce a spray pattern that will water the entire lawn's area. As a second example then, let us assume a family at a picnic on the grass of the public park irrigated with Title 22 water. Did not little Susie, just drop her lollypop on the grass, and then pick it up to suck on it again? Or little Jim, skid across the grass attempting to catch a ball and open his forearm with a skid-rash? Thus the skin's protective barrier is broken.

Now we have the opportunity for entry of antibiotic resistant microbes found within Title 22. In the case of little Susie, she may not get sick, but what is the chance that her gut bacteria will pick up that genetic information, multiply it out. Now three weeks later she is crying and you can't calm her down, her eardrum is infected, and she has a bladder infection, not necessarily related to the lollipop, but from something entirely unrelated. But the pathogens just happened to have met with and exchanged genetic information within her own internal flora that now contain the genetic information from what was on the lollipop. Also one must consider transfer of genetic information from these organisms to more robust organisms as highlighted by Sjolund et al. (2005) [1] indicating that resistance in the normal flora, which may last up to four-years, might contribute to increased resistance in higher-grade pathogens through interspecies transfer.

These bacteria that were on the lollipop were from the Title 22 water. They were able to colonize her gut bacteria through ingestion. Once ingested, the genetic information was transferred to normal flora, and subsequently to the pathogenic bacteria, making later treatment with particular antibiotics ineffective. Little Susie is now in the ICU on vancomycin and that drug is tearing up her vascular system and they had to discontinue it and attempt to find another drug. She is on a respirator, things don't look good.

Sjolund et al go on to note that since populations of the normal biota are large, this affords the chance for multiple and different resistant variants to develop. This thus enhances the risk for spread to populations of pathogens. Furthermore, there is crossed resistance. For example, vancomycin resistance may be maintained by using macrolides.

So, just how fast can a lethal level of antibiotic resistance develop? It does not take long as the example below will illustrate. Schentag, et al. (2003), followed surgical patients with the subsequent results. Pre-op nasal cultures found Staphylococcus aureus 100% antibiotic susceptible. Pre-op prophylactic antibiotics were administered. Following surgery, cephalosporin was administered. Ninety percent of the patients went home at post-op day 2 without infectious complications. Nasal bacteria counts on these patients had dropped from 10/5 to 10/3, but were now a mix of sensitive, borderline, and resistant Staphylococcus sp. By comparison, prior to surgery, all of the patients' Staphylococcus samples had been susceptible to antibiotics. For the patients remaining in the hospital and who were switched on post-op day 5 to a second generation cephalosporin (ceftazidine), showed bacterial counts up 1000-fold when assayed on post-op day 7 and most of these were methicillin resistant Staphylococcus aureus (MRSA). These patients were switched to a 2-week course of vancomycin. Cultures from those remaining in the hospital on day 21, revealed vancomycin resistant enterococcus (VRE) and candida. Vancomycin resistant enterococci infections can produce mortality rates of between 42 and 81%.

Note in the above, that on entry to the hospital none of these patients harbored resistant bacteria in their nasal cavities. But what would be the result if there had been inadvertent acquisition of resistance from

environmental contamination such as through Title 22 water? Gerba and Rusin conducted research on the passage from finger to mouth of pathogens found on typical household objects. But we are not at home, but on a sunny day in the park, innocently having a picnic, on grass irrigated with Title 22 water that carries antibiotic resistant bacteria and other pathogens.

Cottage Hospital in Santa Barbara is a teaching hospital and I occasionally attend grand rounds and other functions to maintain my continuing medical education requirements. Cottage has been, since about 2003, been giving vancomycin as a pre-op prophylactic for many surgical procedures. Vancomycin is not a benign drug. But, now because of the levels of background and community acquired antibiotic resistance, it would be unconscionable not to consider this drug. It was once held in reserve as the drug of last resort. Now from hospitals and nursing homes across California, it is used to quell resistant bacteria. As a pre-op prophylactic, it is also found in the human waste which is going to the sewer, then back into the reclaimed water along with resistant pathogens and their genetic material, and back onto the lawns and parks---a revolving door. Sewer plants have a very hard time with pharmaceuticals, but that connection to Title 22 seems to be seldom discussed.

Because resistance can be and is transferred to the flora of the human gut (skin and mucosal flora as well) the infective dose that has classically been used to estimate public health impacts is confounded. This then brings into question the current paradigm on infection and its dose response to a certain load of a particular pathogen, i.e., ID and LD 50s. Lateral transfer of mobile genetic elements conferring resistance is not considered in this old paradigm. With the prodigious capacity for the gut bacteria to multiply, once the lateral transfer has taken place, very small original numbers---well below the old paradigms can be multiplied into impressive numbers. Since viruses and phages are also involved, their capacity to multiply, which dwarfs that of bacteria, must also be included. Thus there is a need for a new paradigm; unfortunately, the regulatory community seems not to recognize this. When one considers the multiplication within sewer plants and also within their byproducts, disbursement into the environment, the transfer to background organisms, hence to man and his animals, then the remultiplication within commensals of the gut, the emerging picture is worrisome.

Further, there are opportunities and interrelationships between microbes that can degrade antibiotics, eg. antibiotic resistant bacteria, and those that can degrade heavy metals and pharmaceuticals brought in by the Title 22 water as well as well as pesticides and fertilizer chemicals that are already found in soils of the park. In many cases, the involved cellular machinery is the same or similar, i.e., a duality (see for example papers by Schlüter).

Third example-------Your favorite uncle, Uncle Albert who was the youngest of the brothers, was also at the above picnic. He is about 48, has diabetes, a toe has been removed and he has a non-healing open ulcer on the bottom of the same foot. He is a devil-may-care fellow who still thinks he is 26, bullet-proof, and thus does not watch his blood sugar. A week after taking his shoes off and playing ball in the park with the family, he is in the clinic because his foot is infected. He is given a course of gorilla-cillin and it does no good, but in the interim his foot and lower leg are all red and swollen. It is difficult to walk and now he is taking a day off work. He really does not appreciate his situation because he can't feel too much due to diabetic neuropathy in his lower limbs, so he just soldiers on. Thus in a few days leg is much worse, but because of neuropathy he just does not feel things. The gorella-cillin is not working but Albert doesn't know that. His wife and kids tell him to go to the doctor and finally he decides to do just that. His swollen leg is starting to turn a dusky color.

On examination, he is immediately put into the hospital and put on IV antibiotics while lab tests are run to determine the sensitivity of the antibiotics. His foot is badly damaged already but again, Albert really can't feel the pain. Try as they might, because of his poor circulation, the antibiotic can not reach the infection; the infection turns to gangrene and the foot must come off.

Let's now move five years out and revisit Albert. He is now 53, depressed and when he attends picnics he laments the fact that he can't run and play catch. His depression over his state has him on antidepressants. He already had erectile dysfunction, but now because of the anti depressants he is not really interested. His wife 15 years his junior still has a normally functioning libido and marital discord is

showing up.
Albert's other leg is starting to break down due to the added stress.

Move out 3 more years. Albert has just come back from the hospital, for treatment of an ulcer that developed on the stump of the other leg which was removed. He is just like a lot of diabetics that lose a leg. They will lose the other in about 5 years from the added stress. His numerous bouts with infection, the diabetes, his impacted immune system, and the numerous experiences with antibiotics find that his system is easily colonized with antibiotic resistant bacteria. He is relegated to a wheel chair, on heavy meds for depression and has not worked full-time for two years. He lives in a small subsidized housing system. His wife left him and he sees the children on alternate weekends. He is now seeing a cardiologist because his cardiovascular tree is coming apart. In losing his legs he also lost the calf pumps. The calf pumps, contraction of the calf and foot muscles, move the blood back to the heart through a series of check valves in the veins and thus pre-load the heart for normal circulation to occur. Thus Albert's heart is not preloaded and this causes deterioration of the entire cardiovascular tree.

Do you think the Water Quality Board staff will come to visit him in his room at the dingy public assistance rooming-house and cheer him up or lay flowers on Susie's grave?

[1] Emerging Infectious Diseases (Vol. 11, # 9, Sept 2005 @ p. 1389 et seq),

Fm: Dr Edo McGowan, Medical Geohydrology
Re Policy issues involving antimicrobial resistance
ABSTRACT This brief presents an argument that accelerating risks from both antimicrobial resistance and pandemic, especially as now found emerging in the world community, may be related to the disposal of inadequately treated sewage. On most continents, the practice of land applied sewer sludge or reclaimed water have gained unprecedented acceptance based on the need to rapidly rid ourselves of our waste and salvage otherwise discarded water. Recent papers have noted the mixing of genetic material between various organisms provides for new types of pathogens and our immune systems may thus be faced with an unknown foe. Interestingly, much of this mixing goes on every day in sewage treatment plants in almost every city. Tons of poorly treated sewage wastes and reclaimed water are discharged to the environment and the solids, now termed biosolids are spread across thousands of square miles. There, this material is open to background organisms, thus allowing the intermixing with numerous species at the micro and macro biological levels.

My contention is that while over-use of antibiotics may play an important role in the advancement of resistance, other causes of resistance to antibiotics are overlooked, and perhaps even purposefully ignored. A critical but less well understood mechanism for the transfer of multi-drug resistant pathogens is found at the local sewer-treatment plant [1]. As bacteria, other pathogens, and common background-organisms wind their way through municipal sewage treatment processes there is the intermixing of vast quantities of organisms that might otherwise never come together. In the process of sewage treatment, the selective pressures against these organisms is increased. In consequence, there is a greater effort by these organisms to up-regulate or acquire numerous other survival mechanisms to assure that they and their genetic material persist to pass on genetic information. Additionally, as the environmental crowding and stresses increase, these organisms can acquire and pass on antibiotic resistance, virulence, as well as resistance to heavy metals and chlorine. In many cases, these changes to the cellular and metabolic machinery afford the ability to deal with numerous insults, hence development of cross-resistance mechanisms.
Many antimicrobials or their metabolites pass through the body essentially unchanged. Thus feces and urine do contain some impressive levels. As later noted, Kümmerer and others (1999, 2000, 2003, 2004) [2], have followed this and noted levels of antibiotics in sewage that are able to induce or maintain resistance. Added to this are the other materials dumped into the toilet or down the drain that confer resistance. This includes discarded antibiotics and disinfectants such as Triclosan [3] a ubiquitous biocide has been suspected of inducing resistance.

Based on wastewater industry and governmental dogma and standards, released effluent and the land application of sewage sludge are benign and beneficial activities. If however, one reviews the current medical and scientific literature, a different picture emerges, one that raises serious questions about the benevolence of this activity and efficacy of the underlying standards. Thus, the issue takes on aspects of a political and not a scientific argument. In the interim, most regulatory agencies have backed off . This leaves the citizens and patient base standing naked.

In one of several major studies looking at sewage treatment plants, the scientists followed bacteria through a sewer treatment works using fecal coliforms as the test organism [4]. Coliform bacteria were isolated at various locations in the plant, specifically a) the inlet, b) the primary sedimentation tank, c) the activated sludge digestion tank, d) the final settling tank, e) the outlet and f) the return activated sludge drain. They were then examined the presence of drug resistant plasmids. Using this approach, resistant bacteria and those that were still sensitive to antibiotics were detected [5].

Several drugs were tested and included tetracycline, kanamycin, chloramphenicol and streptomycin, ampicillin, nalidixic acid, rifampicin, and sulfisoxazole. A total of 900 separate tests were conducted, of which more than half contained multi-drug resistant plasmids. While this is interesting, there was a new finding that raised considerable concern. The further along that the wastewater had progressed through the treatment process, the greater the tendency was to encounter strains that had developed multiresistance to antibiotics. Additionally, the study demonstrated that these multi-resistant bacteria also simultaneously carried, and then passed around their multiple transferable drug-resistance plasmids. Thus, the development of drug resistance and the transfer of multi-drug resistance are enhanced in sewage wastewater treatment plants [5]. These findings have been documented for more than a decade. They were a harbinger, yet little impact from such studies has been noted. Under the current practices, sewage treatment practices allow the survival of up to 2-million viable coliform per gram of sludge at the point of land application to farmlands [6].

This paper was originally developed a few years ago to discuss the land application of sewage sludge (biosolids) The use of reclaimed water has been much in the news of late and there is much interest in salvaging this water for useful purposes. That stance makes sense only if that use is well controlled, controls that include pathogens, their genetic material and materials that can promote antibiotic resistance. Reclaimed water was reviewed by Rose, et al and these authors found that none of the sewer plants they studied were able to meet even the most minimal standards a significant portion of the time. The level of pathogens as well as their types were not well considered by the standards and the public health was not protected. That report came out in 2004, but where in the import of that report reflected in this resolution? If you look at the number of respondents during the March meeting of this year, many are recipients of WERF documents. Where was the work of Rose, et all discussed within this body? The single comment from Lawyers for Clean Water suggesting further analysis in comparison to that of the water agencies seems to indicate that decisions may rest on political rather that a transparent scientific analysis. This while not surprising, would, considering the cost of health care, be a mistake. Thus an EIR on this resolution seems warranted prior to your consideration for approval. If for some reason, this escapes the rigors of CEQA, a thorough scientific study should be conducted and that should be done by those outside of the industry to avoid bias. Such a study should be vetted by such groups as the American Society of Microbiology or the Canadian Infectious Disease Society.

The use of low-level indicator bacteria, along with the apparent lack in understanding of antibiotic resistance within federal and state regulators should alert anyone that the issue is anything but closed. If the state has not adequately presented the necessary analyses in this area, it not only manufactures uncertainty, but also potentially increased the risk of human disease, disease from some serious pathogens that may not respond to current antibiotics. Sewage technologies that are normally used in the U.S. such as anaerobic digestion and aerobic digestion and heating at these levels as well as composting and land stabilization do not effectively destroy critical pathogens [7]. These practices also do not destroy the genetic material and this and its lack of acknowledgement is a critical shortcoming within the state regulatory systems. Thus if there is antibiotic resistance within sewage, it may be passed through these processes to background organisms including man [8]. Actually several studies have documented the horizontal transfer of genetic information to background environmental systems and such systems can act as lending libraries for this genetic information. Man and animals are exposed daily to such backgrounds [9].

What are the chances for inadvertent acquisition of resistance from environmental contamination such as through sewage sludge? Gerba and Rusin [10] conducted research about the passage from finger to mouth of pathogens found on typical household objects. Others have documented drift as a mechanism for dispersal of pathogens. Thus what of the dwellings down wind from sprinkler irrigation? Tyhere are numerous papers discussing movement via this mode. Further, there are concerns about wash-off from rains of irrigated areas and return flows. Gerba and others have written extensively about the survival of pathogens and their viable infectivity once they are adsorbed onto sediments [11]. These sedimants can be dried and then subjected to wind drift as dust. The USGS has written extensively on the movement of dust from Africa, across the Atlantic and carrying with it viable pathogens thus causing respiratory disease in the Caribbean [12].

The indicator organisms commonly include Escherichia coli and sometimes Salmonella. These are the organisms that are normally killed by low-level disinfection. They are vegetative bacteria that are highly susceptible to both chemical disinfection and heat disinfection. However, sewage contains a large range of organisms besides E. coli, Salmonella, and Staphylococcus. Also highly susceptible and easily inactivated are the enveloped viruses such as Hepatitis B., HIV, and influenza. While these organisms are fairly easily destroyed. This raises the logical question of survival for the more robust organisms.

These bacteria are thus able to colonize environmental niches, and animals, including humans, through ingestion. Once ingested, the plasmids may be transferred to normal flora, and subsequently to pathogenic bacteria found in humans or animals, making later treatment with particular antibiotics ineffective. Also one must consider transfer of genetic information from these organisms to more robust organisms as highlighted by Sjolund et al. (2005) [13] indicating that resistance in the normal flora, which may last up to four-years, might contribute to increased resistance in higher-grade pathogens through interspecies transfer.

These authors go on to note that since populations of the normal biota are large, this affords the chance for multiple and different resistant variants to develop. This thus enhances the risk for spread to populations of pathogens. Furthermore, there is crossed resistance. For example, vancomycin resistance may be maintained by using macrolides [14].

This then brings into question the current paradigm on infection and its dose response to a certain load of a particular pathogen, i.e., ID and LD 50s. Lateral transfer of mobile genetic elements conferring resistance is not considered in this old paradigm. With the prodigious capacity for the gut bacteria to multiply, once the lateral transfer has taken place, very small original numbers---well below the old paradigms can be multiplied into impressive numbers. Since viruses and phages are also involved, their capacity to multiply, which dwarfs that of bacteria, must also be included. Thus there is a need for a new paradigm; unfortunately, the regulatory community seems not to recognize this. When one considers the multiplication within sewer plants and also within their byproducts, disbursement into the environment, the transfer to background organisms, hence to man and his animals, then the remultiplication within commensals, the emerging picture is worrisome.

Further, there are opportunities and interrelationships between microbes that can degrade antibiotics, eg. antibiotic resistant bacteria, and those that can degrade metals as well as pesticides and farm chemicals that are already found in agricultural soils. In many cases, the involved cellular machinery is the same or similar, i.e., a duality (see Schlüter and abstracts of others below).

This duality may have some interesting synergistic survival advantages for the microbes, but bad-for-human-health effects when considering sewer sludge as applied to heavily farmed lands.

The current standards controlling sewer plant operations consider none of these issues. Another hole in the system is that there is no state requirement for sewer plant operators to have this background. Thus in dealing with lay administrators, they can not assure these decision-makers that what is going on within a sewer plant is in fact protective of public health.

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My group had requested, via Freedom of Information Act, certain data from the U.S. EPA on their progress dealing with biosolids and resistance. In providing us answers to this request, EPA delayed its response for about 6 months and then merely directed us to a section of the NERL's website, which contained no usable information. This site was (www.epa.gov/nerlesd1/chemistry/pharma/fq.htm#disposal), as evidenced by the following search results. Similar results were found for other EPA web addresses.
Results of Searching the "Environmental Sciences" Area of EPA's Web Site
No matches found for transposon; 1402 files searched
No matches found for antibiotic resistance + biosolids; 1402 files searched.
No matches found for antimicrobial resistance + biosolids; 1402 files searched
No matches found for virulent pathogens + biosolids; 1402 files searched.
No matches found for plasmids + biosolids; 1402 files searched.
No matches found for mobile genetic elements; 1402 files searched.
No matches found for high level disinfection + biosolids; 1402 files searched.
Results of Searching EPA's Entire Web Site
We have searched the entire EPA site and found the following results. You may also return to searching for the same terms within Environmental Sciences.
No matches found for high level disinfection + biosolids; 494732 files searched.
No matches found for plasmids + biosolids; 494732 files searched.
No matches found for transposons + biosolids; 494732 files searched.
No matches found for mobile genetic elements + biosolids; 494732 files searched.
No matches found for virulent pathogens + biosolids; 494732 files searched.
No matches found for antibiotic resistance + biosolids; 494732 files searched.
No matches found for antimicrobial resistance + biosolids; 494732 files searched.
Results of Searching the "Exposure Research" Area of EPA's Web Site
We have searched the area of EPA's site related to Exposure Research and found the following results. You may also search for the same terms across EPA's entire site.
No matches found for prions + biosolids; 3352 files searched.
Results of Searching EPA's Entire Web Site
We have searched the entire EPA site and found the following results. You may also return to searching for the same terms within Exposure Research.
No matches found for prions + biosolids; 530969 files searched.
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The 64 508 bp IncP-1 antibiotic multiresistance plasmid pB10 isolated from a waste-water treatment plant provides evidence for recombination between members of different branches of the IncP-1 group
A. Schlüter, et al
The complete 64 508 bp nucleotide sequence of the IncP-1 antibiotic-resistance plasmid pB10, which was

isolated from a waste-water treatment plant in Germany and mediates resistance against the antimicrobial agents amoxicillin, streptomycin, sulfonamides and tetracycline and against mercury ions, was determined and analysed. A typical class 1 integron with completely conserved 5' and 3' segments is inserted between the tra and trb regions. The two mobile gene cassettes of this integron encode a -lactamase of the oxacillin-hydrolysing type (Oxa-2) and a gene product of unknown function (OrfE-like), respectively. The pB10-specific gene load present between the replication module (trfA1) and the origin of vegetative replication (oriV) is composed of four class II (Tn3 family) transposable elements: (i) a Tn501-like mercury-resistance (mer) transposon downstream of the trfA1 gene, (ii) a truncated derivative of the widespread streptomycin-resistance transposon Tn5393c, (iii) the insertion sequence element IS1071 and (iv) a Tn1721-like transposon that contains the tetracycline-resistance genes tetA and tetR. A very similar Tn501-like mer transposon is present in the same target site of the IncP-1 degradative plasmid pJP4 and the IncP-1 resistance plasmid R906, suggesting that pB10, R906 and pJP4 are derivatives of a common ancestor. Interestingly, large parts of the predicted pB10 restriction map, except for the tetracycline-resistance determinant, are identical to that of R906. It thus appears that plasmid pB10 acquired as many as five resistance genes via three transposons and one integron, which it may rapidly spread among bacterial populations given its high promiscuity...".

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Risk Analysis
Volume 24 Page 221 - February 2004
doi:10.1111/j.0272-4332.2004.00425.x Volume 24 Issue 1
A Dynamic Model to Assess Microbial Health Risks Associated with Beneficial Uses of Biosolids Joseph N. S. Eisenberg1* Jeffrey A. Soller2, James Scott1, Don M. Eisenberg2, and John M. Colford, Jr.1
There is increasing interest in the development of a microbial risk assessment methodology for regulatory and operational decision making. This document presents a methodology for assessing risks to human health from pathogen exposure using a population-based model that explicitly accounts for properties unique to an infectious disease process, specifically secondary transmission and immunity. To demonstrate the applicability of this risk-based method, numerical simulations were carried out for a case study example in which the route of exposure was direct consumption of biosolids-amended soil and the pathogen present in the soil was enterovirus. The output from the case study yielded a decision tree that differentiates between conditions in which the relative risk from biosolids exposure is high and those conditions in which the relative risk from biosolids is low. This decision tree illustrates the interaction among the important factors in quantifying risk. For the case study example, these factors include biosolids treatment processes, the pathogen shedding rate of infectious individuals, secondary transmission, and immunity. Further refinement in methods for determining biosolids exposures under field conditions would certainly increase the utility of these approaches.
McGowan's comments on the Risk Analysis paper------
A brief read of this paper produced the following comments. Principal amongst my thoughts is the paper's limit to pathogens that would not likely multiply outside the host---i.e., viruses. Thus, the model is quite limited from this important perspective. Secondly, there is no consideration of transfer of mobile genetic elements (MGEs) to terrestrial reservoirs, the potential for shifts in genetic information passing through multiple species, and thus the potential for newly emerging diseases. Consequently the issue of transferred antibiotic resistance and similar molecular and cellular machinery is missed. They also do not discuss colonization or later acquiring of resistance, the fecal veneer and thus movement into other organ systems or orifices.

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The state falls into a problem with its own laws. H&SC 5410 relating to contaminants followed by 5410 (c) would apply to the discharge to areas that might be populated, thus limiting the use of reclaimed water, unless such water was essentially sterile. This is especially where there is contact with the public, such as to new homes and residential areas (WC 13552.4). This also goes along with H&SC 5410(d) and 5411, and if a large area 5410(f) where contaminants, including pathogens, their genetic material, accompanying pharmaceuticals and industrial pollutants, none of which are now absent from Title 22, are released with reclaimed/recycled water.

What is interesting about Amy's work (see below) is that the ARGs are not impacted by chlorine. They sail right through sewer plants and their chlorine treatment, thence into the environment, are picked up as

fresh water raw stock, transit completely through drinking water plants and their chlorine and wind up in the potable water supply with its standard residual chlorine levels----all unaffected. This is because although they are viable bits of genetic information that can be absorbed by soil bacteria or the flora of the human gut, ARGs are not "alive". Chlorine generally is used for things that are "alive"---the vegetative bacteria. This "alive" thing thus also impacts the sewer system's capacity to deal with viable but non-cultutable (VBNC), cysts, spores, and persisters. Thus while vegetative bacteria that require only low-level decontamination may be knocked back 3 or 4 logs, i.e., the indicators, the pathogens that require high-level disinfection will survive as will several forms that are not amenable to the standard disinfection levels used by Title 22. The tests used by Title 22 will not see these and they are not designed to do so. Nonetheless, there is really not much new here about conditions such as VBNC, and it is well known that the standard MPN lab tests don't see these things. Thus merely complying with the current Title 22 standards does not protect public health.

Environ. Sci. Technol., 40 (23), 7445 -7450, 2006. 10.1021/es060413l S0013-936X(06)00413-5
Web Release Date: August 15, 2006

Antibiotic Resistance Genes as Emerging Contaminants: Studies in Northern Colorado
Amy Pruden,* Ruoting Pei, Heather Storteboom, and Kenneth H. Carlson
Department of Civil and Environmental Engineering, Colorado State University, Fort Collins, Colorado 80523
Received for review February 20, 2006
Revised manuscript received July 10, 2006
Accepted July 17, 2006
Abstract:
This study explores antibiotic resistance genes (ARGs) as emerging environmental contaminants. The purpose of this study was to investigate the occurrence of ARGs in various environmental compartments in northern Colorado, including Cache La Poudre (Poudre) River sediments, irrigation ditches, dairy lagoons, and the effluents of wastewater recycling and drinking water treatment plants. Additionally, ARG concentrations in the Poudre River sediments were analyzed at three time points at five sites with varying levels of urban/agricultural impact and compared with two previously published time points. It was expected that ARG concentrations would be significantly higher in environments directly impacted by urban/agricultural activity than in pristine and lesser-impacted environments. Polymerase chain reaction (PCR) detection assays were applied to detect the presence/absence of several tetracycline and sulfonamide ARGs. Quantitative real-time PCR was used to further quantify two tetracycline ARGs (tet(W) and tet(O)) and two sulfonamide ARGs (sul(I) and sul(II)). The following trend was observed with respect to ARG concentrations (normalized to eubacterial 16S rRNA genes): dairy lagoon water> irrigation ditch water> urban/agriculturally impacted river sediments ($p < 0.0001$), except for sul(II), which was absent in ditch water. It was noted that tet(W) and tet(O) were also present in treated drinking water and recycled wastewater, suggesting that these are potential pathways for the spread of ARGs to and from humans. On the basis of this study, there is a need for environmental scientists and engineers to help address the issue of the spread of ARGs in the environment.

COMMENTS as printed in ES&T.

These comments are merely qualifications, not criticisms of Dr. Pruden's fine paper [1]. Resistance has been attributed to drug over-use. Pruden notes a less well-understood mechanism for the amplification of multi-drug resistance, sewage. The local sewer-treatment plant releases pathogens and resistance to the environment and agriculture[2]. Wastewater treatment intermixes organisms otherwise seldom coming together. Selective pressures increase survival mechanisms [3].

Defense strategies include going dormant, entering the viable but non-culturable (VBNC) state. These VBNC organisms are essentially invisible to laboratory tests used in the wastewater industry. Higgins & Murthy recently reconfirmed this [4] in a paper that raises some serious questions about the efficacy of current standards. Those authors noted that during centrifuged dewatering of sewer sludge, indicators in a

VBNC state were resuscitated. The results were several magnitudes greater than standard plate counts had indicated [4]. Such findings raise logical questions. If dewatering by centrifuge brought out the essence of VBNC, would other products of sewage that had not been subjected to the centrifuge also in the VBNC state? If so would they revive in the field following agricultural application of sludge or irrigation with reclaimed wastewater? This seems plausible but needs further study.

Additionally, as stresses increase organisms can acquire genes from or transfer genes to non-related organisms, organisms even within completely different kingdoms [5,6]. There are other materials dumped into the drain that confer resistance. This includes industrial chemicals, heavy metals, and disinfectants. Triclosan a ubiquitous biocide is suspected of inducing resistance, as are many other industrial materials found in sewage [7,8]. Changes to the cellular machinery afford the ability to deal with numerous insults, hence cross-resistance [9].

Many antimicrobials including metabolites enter sewage essentially unchanged to induce resistance in the environment [10]. Kummerer [11,12,13,14,15] and others [16] note levels of antibiotics/pharmaceuticals in sewage able to induce or maintain resistance, hence adding to the risks in crop production through irrigation.

Based on wastewater (sewage) industry and regulatory opinion, the standards, the released effluent, and its use for crop irrigation or the land application of sewage sludge are benign and beneficial activities [17]. If however, one reviews the current medical and scientific literature, a different picture emerges, one that raises serious questions about the benevolence of this activity and efficacy of the underlying standards [18]. Thus, the issue takes on aspects of a political and not a scientific argument [18,19]. In the interim, most regulatory agencies have backed off [20]. This leaves the citizens and patient base essentially standing naked.

In 2002 the NAS/NRC [21] called into question the U.S. EPA Part 503 guidelines for land application of sewage sludge (biosolids) and specifically EPA's failure to consider antibiotic resistance. As of writing this comment, EPA has shown little if any progress in investigating resistance. A Freedom of Information Act request to EPA on this subject was submitted in February 2005. The agency has not answered that request [20]. Additionally, the agency has not done health hazards risk analyses for pathogens. Notwithstanding these shortcomings, the agency and the wastewater industry continue to promote the use of sewage byproducts in crop production. Salinas Valley is an example.

Citations

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The Importance of Municipal Sewage Treatment in the Spread of Antibiotic Resistance
106th General Meeting of the American Society for Microbiology
May 21-25, 2006, Orlando, Florida
For more information on any presentation at the 106th General Meeting of the ASM contact Jim Sliwa, ASM Office of Communications at jsliwa@asmusa.org
EMBARGOED UNTIL: Monday, May 22, 9:00 a.m. EDT
(Session 041/Q, Paper Q-032)
Sara Firl
University of Minnesota
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Our study determined that substantial numbers of antibiotic-resistant bacteria were present in municipal wastewater, and that the existing treatment infrastructure did not adequately prevent release of antibiotic-resistant bacteria into the environment. Many of the bacteria found in the wastewater treatment plant and in the plant effluent were tentatively identified as potential pathogens and were also resistant to multiple antibiotics, raising public health concerns. We believe that wastewater treatment plants could be modified to further prevent the release of resistant bacteria to the environment.
Sara Firl and Leslie Onan performed this study under the supervision of principal investigator Dr. Timothy LaPara at the University of Minnesota, Department of Civil Engineering. Funding was provided by the Center for Urban and Regional Affairs at the University of Minnesota and Geomatrix Consultants, Inc. The work is being presented as a poster at the 106th General Meeting of the American Society for Microbiology in Orlando on May 22.
The spread of antibiotic-resistant bacteria is a major public health concern. Infections previously treatable are increasingly resistant to antibiotics. Scientists believe that the spread of antibiotic resistance results from both misuse of antibiotics and transfer of resistance between bacteria. A potentially large reservoir for antibiotic-resistant bacteria is municipal wastewater. People release resistant bacteria with fecal matter into the wastewater stream, which is collected and treated at municipal treatment facilities before release

to the environment. The objective of this study was to investigate how many resistant bacteria were present at municipal wastewater plants and if the existing infrastructure of waste treatment was adequate to remove resistant bacteria before discharge.
In our study, the effect of effluent treatment (clarification and disinfection) and biosolids treatment (sludge digestion) on the removal of antibiotic-resistant bacteria was investigated at three wastewater treatment facilities. We found substantial numbers of resistant bacteria at the wastewater treatment facilities and that, although effluent treatment reduced the numbers of bacteria, large quantities of resistant bacteria were discharged. Numerous bacteria isolated from the effluent stream were resistant to multiple antibiotics and closely related to potentially pathogenic bacteria. Our research suggests that the existing wastewater treatment infrastructure should be modified to better prevent release of these potentially dangerous bacteria to the environment.

To: Jeff Stone, DHS
Cc: LA RWQCB
Cc: Malzachers
Fm: Dr. Edward McGowan
Re: Comments and issues related to the City of Santa Paula proposal and the Malzacher ranch.

Jeff, hope this finds you well. I would like to discuss the following with you and at some point to include the staff of the LA RWQCB.

http://www.dhs.ca.gov/ps/ddwem/waterrecycling/PDFs/rechargeregulationsdraft-01-04-2007.pdf

In the above document, the strikeout of Section 60320 also removes some critical language--------in essence that the **water shall at all times a quality that is fully protective of public health**. Where is this language reinserted into the changes? Its loss will be a major blow to the protection of public health. This is grave since I am unable to ascertain that discriminate issues related to antibiotic resistance and transfer of genetic information conferring resistance or virulence were considered. Do the criteria of 60320.010, .230, .320 include such a discussion? We know from Amy Pruden's work that the antibiotic resistant genes are considered as contaminants, that they slide right through treatment, are essentially unaffected by current treatment processes including levels of chlorine and will transfer to the human gut flora as noted by Sjolund, et al where they can thus persist for a long time. This then allows for the shift within higher level pathogens, hence the augmentation of community acquired antibiotic resistance. Accordingly, the words highlighted above need to be reinserted.

Additionally, per 60320.010 (c), it appears that a separation of 500 feet is warranted between spreading and uptake of water for drinking (domestic wells included?). Additionally the water must remain within the ground for 6 months. This raises a curious issue with the City of Santa Paula's proposal to use perc ponds for spreading. The issue may be somewhat different as it is not considered recharge and this is a critical point because the heading of the above section is pathogen control.

The Malzacher's property seems to be well within the 500 foot limit from the perc ponds proposed by the City of Santa Paula. Thus, if the 500 feet is a general rule relating to public health, this then puts a serious question before the RWQCB. Further, the proposed loading into the perc ponds is likely to see substantial mounding and thus potentially rapid lateral flow that may cause the water to move to their domestic well considerably in advance of the 6-month limit---especially if a mound is next to a cone of depression. That notwithstanding, there is apparently nothing within the entire document that mentions antibiotic resistance, the movement of genetic materials that confer resistance or virulence, or the interaction of pharmaceuticals or materials that are known to enhance resistance. We have tested the uniformly established Title 22 water and found multi-drug resistant bacteria. Additionally, although 60320.047 (a) (3) mentions pharmaceuticals, it does not correlate the levels with levels that might augment resistance although there is an abundance of literature on the subject----see for example the writings of Klaus Kummerer.

Thus in monitoring efforts there needs to be some further consideration for tracking biotic shifts in the entrained microbial population and that with potential impacts on public health. The pharmaceuticals are not just inert, they are specifically designed to have impacts on microbial systems and cause shifts to internal cellular machinery, to which the microbes respond. Microbes have been noted at 2.8 kilometers below the surface. Entrained microbes and genetic material may be carried to considerable depths within aquifers, again the literature is not silent on these things. I would be most happy to supply you and your agency some of this material to assist you in your efforts at writing the draft regs.
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Characterization of attached bacterial populations in deep granitic groundwater from the Stripa research mine by 16S rRNA gene sequencing and scanning electron microscopy

S Ekendahl, J Arlinger, F Stahl and K Pedersen
Department of General and Marine Microbiology, University of Goteborg, Sweden.
This paper presents the molecular characterization of attached bacterial populations growing in slowly flowing artesian groundwater from deep crystalline bed-rock of the Stripa mine, south central Sweden. Bacteria grew on glass slides in laminar flow reactors connected to the anoxic groundwater flowing up through tubing from two levels of a borehole, 812-820 m and 970-1240 m. The glass slides were collected, the bacterial DNA was extracted and the 16S rRNA genes were amplified by PCR using primers matching universally conserved positions 519-536 and 1392-1405. The resulting PCR fragments were subsequently cloned and sequenced. The sequences were compared with each other and with 16S rRNA gene sequences in the EMBL database. Three major groups of bacteria were found. Signature bases placed the clones in the appropriate systematic groups. All belonged to the proteobacterial groups beta and gamma. One group was found only at the 812-820 m level, where it constituted 63% of the sequenced clones, whereas the second group existed almost exclusively at the 970-1240 m level, where it constituted 83% of the sequenced clones. The third group was equally distributed between the levels. A few other bacteria were also found. None of the 16S rRNA genes from the dominant bacteria showed more than 88% similarity to any of the others, and none of them resembled anything in the database by more than 96%. Temperature did not seem to have any effect on species composition at the deeper level. SEM images showed rods appearing in microcolonies.(ABSTRACT TRUNCATED AT 250 WORDS)
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Microbial contamination of two urban sandstone aquifers in the UK.

Powell KL, **Taylor RG**, **Cronin AA**, **Barrett MH**, **Pedley S**, **Sellwood J**, **Trowsdale SA**, **Lerner DN**.

Robens Centre for Public and Environmental Health, University of Surrey, Guildford, Surrey, GU2 7XH, UK.

Development of urban groundwater has historically been constrained by concerns about its quality. Rising urban water tables and overabstraction from rural aquifers in the UK have led to a renewed interest in urban groundwater, particularly the possibility of finding water

of acceptable quality at depth. This study assessed the microbial quality of groundwater collected from depth-specific intervals over a 15-month period within the Permo-Triassic Sherwood Sandstone aquifers underlying the cities of Nottingham and Birmingham. Sewage-derived bacteria (thermotolerant coliforms, faecal streptococci and sulphite-reducing clostridia) and viruses (enteroviruses, Norwalk-like viruses, coliphage) were regularly detected to depths of 60 m in the unconfined sandstone and to a depth of 91 m in the confined sandstone. Microbial concentrations varied temporally and spatially but increased frequency of contamination with depth coincided with geological heterogeneities such as fissures and mudstone bands. Significantly, detection of Norwalk-like viruses and Coxsackievirus B4 in groundwater corresponded with seasonal variations in virus discharge to the sewer system. The observation of low levels of sewage-derived microbial contaminants at depth in the Triassic Sandstone aquifer is explained by the movement of infinitesimal proportions of bulk (macroscopic) groundwater flow along preferential pathways (e.g., fissures, bedding planes). The existence of very high microbial populations at source (raw sewage) and their extremely low detection limits at the receptor (multilevel piezometer) enable these statistically extreme (microscopic) flows to be traced. Rapid penetration of microbial contaminants into sandstone aquifers, not previously reported, highlights the vulnerability of sandstone aquifers to microbial contamination.
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Filtration and transport of Bacillus subtilis spores and the F-RNA phage MS2 in a coarse alluvial gravel aquifer: implications in the estimation of setback distances.

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Filtration of Bacillus subtilis spores and the F-RNA phage MS2 (MS2) on a field scale in a coarse alluvial gravel aquifer was evaluated from the authors' previously published data. An advection-dispersion model that is coupled with first-order attachment kinetics was used in this study to interpret microbial concentration vs. time breakthrough curves (BTC) at sampling wells. Based on attachment rates (katt) that were determined by applying the model to the breakthrough data, filter factors (f) were calculated and compared with f values estimated from the slopes of log (cmax/co) vs. distance plots. These two independent approaches resulted in nearly identical filter factors, suggesting that both approaches are useful in determining reductions in microbial concentrations over transport distance. Applying the graphic approach to analyse spatial data, we have also estimated the f values for different aquifers using information provided by some other published field studies. The results show that values of f, in units of log (cmax/co) m(-1), are consistently in the order of 10(-2) for clean coarse gravel aquifers, 10(-3) for contaminated coarse gravel aquifers, and generally 10(-1) for sandy fine gravel aquifers and river and coastal sand aquifers. For each aquifer category, the f values for bacteriophages and bacteria are in the same order-of-magnitude. The f values estimated in this study indicate that for every one-log reduction in microbial concentration in groundwater, it requires a few tens of meters of travel in clean coarse gravel aquifers, but a few hundreds of meters in contaminated coarse gravel aquifers. In contrast, a one-log reduction generally only requires a few meters of travel in sandy fine gravel aquifers and sand aquifers.

Considering the highest concentration in human effluent is in the order of 10(4) pfu/l for enteroviruses and 10(6) cfu/100 ml for faecal coliform bacteria, a 7-log reduction in microbial concentration would comply with the drinking water standards for the downgradient wells under natural gradient conditions. Based on the results of this study, a 7-log reduction would require 125-280 m travel in clean coarse gravel aquifers, 1.7-3.9 km travel in contaminated coarse gravel aquifers, 33-61 m travel in clean sandy fine gravel aquifers, 33-129 m travel in contaminated sandy fine gravel aquifers, and 37-44 m travel in contaminated river and coastal sand aquifers. These recommended setback distances are for a worst-case scenario, assuming direct discharge of raw effluent into the saturated zone of an aquifer. Filtration theory was applied to calculate collision efficiency (alpha) from model-derived attachment rates (katt), and the results are compared with those reported in the literature. The calculated alpha values vary by two orders-of-magnitude, depending on whether collision efficiency is estimated from the effective particle size (d10) or the mean particle size (d50). Collision efficiency values for MS-2 are similar to those previously reported in the literature (e.g.) [DeBorde, D.C., Woessner, W.W., Kiley, QT., Ball, P., 1999. Rapid transport of viruses in a floodplain aquifer. Water Res. 33 (10), 2229-2238]. However, the collision efficiency values calculated for Bacillus subtilis spores were unrealistic, suggesting that filtration theory is not appropriate for theoretically estimating filtration capacity for poorly sorted coarse gravel aquifer media. This is not surprising, as filtration theory was developed for uniform sand filters and does not consider particle size distribution. Thus, we do not recommend the use of filtration theory to estimate the filter factor or setback distances. Either of the methods applied in this work (BTC or concentration vs. distance analyses), which takes into account aquifer heterogeneities and site-specific conditions, appear to be most useful in determining filter factors and setback distances.
PMID: 15763354 [PubMed - indexed for MEDLINE]
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Distance and flow effects on microsphere transport in a large gravel column.

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Consumption of microbially contaminated ground water can cause adverse health effects and the processes involved in pathogen transport in aquifers need to be understood. The influences of distance, flow velocity, and colloid size on colloid transport were examined in homogenous pea-gravel media using an 8-m column and three sizes (1, 5, and 10 microm) of microspheres. Experiments were conducted at three flow rates by simultaneously injecting microspheres with a conservative tracer, bromide. Observed concentrations were simulated with CXTFIT and analyzed with filtration theory. The results demonstrate that colloid concentration is strongly log-linearly related to transport distance (as suggested by filtration theory) in coarse gravels, similar to our previous field studies. In contrast, the log-linear relationship is often reported to be invalid in fine porous media. The observed log-linear relationship is possibly because straining is negligible in the coarse gravels investigated. This has implications in predicting setback distances for land disposal of effluent, and suggests that setback distances in gravel aquifers can be estimated using constant spatial removal rates (f). There was an inverse relationship between transport

distance and colloidal concentration, but not with temporal attachment rate (katt) and collision coefficient (alpha). Increases in flow velocity result in increasing colloidal recovery, katt and alpha but decreasing f. Increases in sphere size result in decreasing colloidal recovery with increasing katt, f, alpha, and velocity enhancement. Diffusion is the dominant collision mechanism for 1-microm spheres (81-88%), while settling dominates for 5- and 10-microm spheres (> 87%), and interception is very small for all spheres investigated.
PMID: 16825440 [PubMed - indexed for MEDLINE]

Transport of MS2 phage, Escherichia coli, Clostridium perfringens, Cryptosporidium parvum, and Giardia intestinalis in a gravel and a sandy soil.

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To define protection zones around groundwater abstraction wells and safe setback distances for artificial recharge systems in watertreatment, quantitative information is needed about the removal of microorganisms during soil passage. Column experiments were conducted using natural soil and water from an infiltration site with fine sandy soil and a river bank infiltration site with gravel soil. The removal of phages, bacteria, bacterial spores, and protozoan (oo)-cysts was determined at two velocities and compared with field data from the same sites. The microbial elimination rate (MER) in both soils was generally >2 log, but MER in the gravel soil was higher than that in the fine sandy soil. This was attributed to enhanced attachment, related to higher metal-hydroxides content. From the high sticking efficiencies (>1) and the low influence of flow rate on MER it was deduced that straining played a significant role in the removal of Escherichia coli and Cryptosporidium parvum oocysts in the gravel soil. Lower removal of oocysts than the 4-5 times smaller E. coli and spores in the fine sand indicates that the contribution of straining is variable and needs further attention in transport models. Thus, simple extrapolation of grain size and particle size to the extent of microbial transport underground is inappropriate. Finally, the low MER of indigenous E. coli and Clostridium perfringens observed in the soil columns as well as under field conditions and the second breakthrough peak found for Cryptosporidium and spores in the fine sandy soil upon a change in the feedwater pH indicate a significant role of detachment and retardation to microbial transport and the difficulty of extrapolation of quantitative column test results to field conditions.
PMID: 16295848 [PubMed - indexed for MEDLINE]

To: California DHS
Cc: California WRCB
Fm: Dr. Edward McGowan
Re: Title 22, the need to revisit the scientific underpinnings and medical implications of this area of law

- Hospitals may represent epicenters for the formation of drug resistance.
- Sewer works may multiply this situation.
- Wastewater products, including Title 22 water, may lead to pathogen and genetic transfer into environmental reservoirs which then act as lending libraries for pathogens impacting public health.

As part of a typical local community and also based on my experience as a public member of a county-wide multi-jurisdictional task group dealing with wastewater and human waste, and a national committee reviewing antibiotic resistance related to sewage and its byproducts, I wish to bring to your attention items warranting further consideration. The subject below deals with water and pathogens and, within that macrocosm of inquiry, the more important issue is one of multi-drug resistant bacteria (MDRB). I am assigned to these groups based on my background. I was the Regional Environmental Officer with USAID to a 22-nation section of Africa where water and health problems abounded. I also have a degree in medicine and a PhD in water quality.

It is my contention that Title 22, as currently established, is not protective of public health. First, Title 22 seems not to consider the passage of pathogens and antibiotic resistance from reclaimed/recycled water to the public and to environmental niches. This lack of consideration then ignores public health risks and costs associated with the rising levels of community acquired antimicrobial resistance.

The federal government under EPA has the lead on sewage, but the states are responsible for reclaimed/recycled water.

Since the release of the 2002 NAS/NRC report on land applied biosolids, EPA has been charged with the task of reviewing the status of pathogens and resistance in the solids portion of sewage. I am on that team charged with this undertaking. The area of reclaimed/recycled water, however, is left to the states. Accordingly, the states can not merely defer to EPA but must take a proactive role.

The issue is critical. As will be noted below and through the attached studies and abstracts, the issue of transferred antibiotic resistance to the environment and background organisms, thence to humans is a serious concern and can not be ignored. The WHO has raised the issue of antibiotic resistance to a global crisis.

A response on the following would be appreciated. I may be reached by mail at 3152 Via Real, Carpinteria, CA 93013 or by email edomcgowan@earthlink.net.

Hospitals, Sewer Plants, and Multi Drug Resistant Bacteria---the impacts on Title 22.

In California, the state requires seismic retrofit of hospitals. In consequence, many hospitals will be rebuilt from the ground up, others will be razed. The result will see a concentration of this industry as smaller less viable units fall out. In the revamping of any hospital, serious thought should be applied to the make-up of sewer effluents. In many industrial settings, there is a requirement for pre-treatment. There are extant, package plants and systems that can be plugged into laterals coming from hospitals and their surrounding medical communities---all are major contributors of resistant pathogens as well as materials that contribute to resistance. This is needed to protect down-gradient receiving waters, hence the health of humans and the environment. Accordingly, hospitals should be considered within the category of industrial wastewater generators. The other option is to substantially upgrade sewer plant treatment processes, thus eliminating a need for individual systems.

Amongst the community at large, including staff operating sewer treatment works, there is a distinct lack of recognition for issues relating to the connections between sewage and its byproducts, infectious disease, and MDRB. Nonetheless, staffs at sewer works hold an important position as guardians of sanitation and water quality. The state, however, requires no training in areas relating to transfer of infectious disease and resistant pathogens for obtaining an operator's license. I suspect that this flaw also falls to drinking water.

The issue is that pathogenic organisms carrying resistance pass from sink or toilet through sewer treatment to the environment at large. Although current water quality standards are silent on such issues of MDRB, there is a pressing need for recognition. Thus hospitals and sewer districts, as major members of a community, and for the ultimate needs of their communities, need to go beyond current standards. Unfortunately, there is no real pressure to move these groups beyond current practices.

The state's water supply and quality are diminishing from external competition and water trades, consumption, and pollution. At the same time the population is increasing and demands on this resource will increase its value. Thus a critical aspect is reclaimed/recycled water which will become more prevalent in potable and non-potable supply. Unless we reevaluate Title 22, the rapidly expanding demand and its accompanying politics will see externality costs such as public health both ignored and climbing.

Contrary to popular myth, many pathogens survive their passage through a sewer treatment plant thus remaining to constitute an increased public health risk. Additional inputs to sewer plants such as heavy metals, personal care products, disinfectants, and discarded antibiotics as well as antibiotics passing through the gut or renal system, may augment resistance. These conditions are contrary to directives found in statute, directives that charge both DHS and the state and regional boards with protection of public health (see Water Code 13550(a)(3), et seq and 13521 and 13522 in relationship to health and safety Code sections 5410, et seq. Additionally, see Pruden, et al---Antibiotic resistant Genes as Emerging Contaminants: Studies in Northern Colorado)

That this situation has continued for some time may be attributed, in part, to economics, politics, and the antiquated water quality standards. Nonetheless, readily available scientific and medical literature are, and have been for some time, replete with data demonstrating and confirming this fact—the public health is adversely impacted by the existing standards. Studies reported in the scientific and medical literature dating back to at least the 1970s show failure of treatment. Thus, this is hardly new knowledge. [Fontaine, et al, (1976); Grabow, et al. , (1973); Linton, et al., (1974); Walter et al,. (1985)].

Previous studies have shown that waste effluents from hospitals contain higher levels of antibiotic-resistant enteric bacteria than waste effluents derived from other sources [1,2,3,4,5,6].

In a 2003 meeting of our task-group, one of the members, a well respected wastewater engineer, raised an interesting question relating the survival of pathogens once the material had left the sewer treatment works. The essence of the question is related to the survival of genetic material. Hence, analyses on the underlying issue of surviving MDRB. The question went something like this---"If *Staphyloccus aureus* were found dead, did that mean that the problem was solved?" The corollary--- was it dead or merely in the viable but non-culturable (VBNC) state, was it a persister, was it in another starvation arrested state, or was it killed from a starvation but otherwise recoverable state by sudden nutrient excess in the culture? Did it, while within the persister state, involve an interaction with a phage, and if so, how might that impact continued pathogenesis ? Additionally, there are issues of the re-uptake of naked DNA.

Recently, in discussing mobile genetic elements (MGE), Nielsen, et. al. [7,8], demonstrated that DNA was well protected in dead cells and that transforming activity remained. The survival of such material was found to be up to two years [9]. Other papers have noted survival of genetic information in desiccated soils for centuries. There are interesting situations of "long-lived" bacteria. Vreeland, Rosenzweig, and Lowenstein obtained samples from a deep excavation for a waste disposal site in a southeastern New Mexico rock formation called the Permian Salado Formation. There they found salt crystals with tiny fluid inclusions that emtombed still viable saltloving bacteria. According to the researchers, the now living bacteria spent the last 250 million years in suspended animation. The bacteria "revived" by the scientists

are salttolerant species of the genus Bacillus. Bacillus, incidentally is a bacterium that is not easily disinfected by sewage treatment.

Additionally, there are papers demonstrate that growing plants, via their roots, could transfer MGEs to bacteria. The reverse has also been widely demonstrated. Thus, non-pathogens and non-bacteria can serve as reservoirs for maintaining resistance. Pneumococci, for example, can take up naked DNA from the environment (natural transformation from lysed bacteria). Thus merely finding "dead" bacteria may be no assurance that risk has reached acceptable levels.

There is nothing particularly new about these situations. From the work by Fred Griffith in 1928 on bacteria, we know that pathogens can regain virulence from dead bacteria. This was followed by Berry and Dedrick and Hurst in the 1930s working on virus. Heat inactivated myxoma virus were combined with a more virulent fibroma virus to produce mixoma lesions. These examples, were confirmed by Gardner and Hyde in the 1940s, Shope in 1950, Smith in 1952, Farmer and Marshall in 1959. All these examples generally show how genetic material can be passed between organisms whether bacteria or virus. Unfortunately, in developing Title 22, those doing so seem to have missed these historic lessons and thus, fall prey to what George Santayana noted: "Those who cannot remember the past are condemned to repeat it". Lamentably, it is not the regulatory community that suffers but the public's health.

Additionally, during the above noted meeting, I had mentioned some notes taken during a medical grand rounds. The speaker, an expert on infectious disease from UCLA, indicated that there is strong medical evidence that about one-half of the general, non-hospital community acquired skin infections in the Greater Los Angeles area are now MRSA. The April 2003 issue of *Skin & Allergy News* also had a front-page article on this since dermatologists often stand on the front lines. More recent issues have carried similar articles.

Prior to 1985, vancomycin resistance in human pathogens had not been described in the literature. A decade later, more than one-half of the hospitals in New Jersey contained strains of vancomycin resistant bacteria. By the end of 1998, one quarter of enterococci isolated from intensive care units across the U.S. expressed resistance to vancomycin.

Recent publications in the medical literature discuss the cost of drug resistant bacteria. The annual cost in the U.S. was estimated to be upwards of $30 billion annually (Dominguez EA, et al. Infection Control & Hospital *Epidemiology*, vol 21,#1, supp, Jan 2000, p S4).

It was assumed for a long time that gene transfer between different species of microorganisms is a very rare event at best; that view has changed. The available evidence suggests that interspecific transfer of genes has occurred between the three major groups of organisms: archaebacteria, eubacteria and eukaryotes. There is very strong evidence that gene transfer easily occurs between distantly related bacteria. Marcinek, et al [10] estimated that under the natural conditions of a sewer treatment works, between 10^6 to 10^9 gene transfer events between different *E. faecalis* strains should take place per day. The maximum number of transfer events for the sex pheromone plasmids between different strains of *E. faecalis* in the municipal sewage water treatment plant was found to range from 10^5 to 10^8 events per 4 hour period. This work also indicated that gene transfer should take place under natural conditions following release of sewer effluent.

Iversen, et al, [11] isolated VRE in 21 of 35 untreated sewage samples (60%), from 5 of 14 hospital sewage samples (36%), from 6 of 32 treated sewage samples (19%), and from 1 of 37 surface water samples. It was speculated that antimicrobial drugs or chemicals released into the sewage system sustained VRE in the system. Others [5] have demonstrated direct evidence that related tetracycline resistance-encoding plasmids have disseminated between different *Aeromonas spp.* and *E. coli* and between the human and aquaculture environments in distinct geographical locations. Collectively, these findings provide evidence to support the hypothesis that the aquaculture and human compartments of the environment behave as a single interactive niche.

Ribeiro [12] and others [13] have found that as these organisms progress further through sewer treatment, the level of resistance and number of transferred plasmids increases. Reinthaler et al [14] found that the

highest resistance rates were found in E. coli strains of a sewage treatment plant which treats not only municipal sewage but also sewage from a hospital. **While these papers have been out for a while, it appears that their import has been missed on the regulatory community.** More recent work by Pruden, et al, Firl, Kinney reconfirm these findings. The work by Pruden discusses antibiotic resistant genes (ARGs) which are essentially immune to the effects of chlorine at levels and contact times currently used. This allows ARGs to transit through a POTW and into the Title 22 water. If the works of Kinney are reviewed, it becomes obvious that both pathogens and materials that may augment or sustain resistance can and do build up in soils. Thus, these authors concluded that sewage treatment processes contribute to the dissemination of resistant bacteria in the environment.

Rose, et al (2004) looked at reclaimed water and concluded (she and her team looked at Title 22) that pathogens were getting through and that the plants were not attaining standards. The report by Rose, et al (2004) also concluded that "it has long been known that indicator coliform bacteria alone are inadequate measures for determining microbial water quality and safety." These authors note that Giardia were detected in 84% of the final disinfected samples, enteric viruses in 31% and Cryptosporidum in 71% of the final treated water. Of the plants reviewed across the nation, the California plant producing Title 22 water had the poorest water quality.

This is not a surprising finding. Title 22 uses the MPN and these processes are not able to see microbes in the VBNC and similar states. Higgins and Murthy looked at resuscitation of indicators and noted that within a few minutes, the indicators that had been pronounced within limits of the standards were now at least three magnitudes above that. That finding tends to make fiction of the standards. To that extent, the Pomona Virus Study (PVS) by Parkhurst, upon which Title 22 is in part based, found similar inconsistencies. Coliform of 2.2 MPN/100ml was met at chlorine residual s of 10 mg/L but at lower chlorine levels the standard was not met. Rose et al note that for their study the Title 22 plant surveyed used the longest contact time of all plants reviewed yet had the poorest control over pathogens. The PVS notes that while 5.1—5.5 log virus removal was met with 10 mg/L of ozone, the coliform of 2.2 was not met. This is interesting because Polio Type I was used and it is considerably more resistant to chlorine than coliform, about 5-times more tolerant to chlorine. Thus more needs to be brought into this picture. When, however, ozone levels were pushed up to 50 mg/L, standards for both virus and coliform were met. Nonetheless, viruses were reported to have been recovered from tertiary effluents when coliform counts were below 2.2 MPN/100 ml. Thus low coliform does not equate with low virus (PVS, p. 95). In ozone tests when viruses were not detected, coliform were in compliance only 35% of the time. In chlorine experiments where viruses were not detected, coliform were not in compliance 40% of the time in the tertiary treated water (PVS, p. 95). Further, it was noted that with respect to natural viruses, the variability of data gathered in the natural virus experiments made meaningful calculations of removal efficiency by treatment systems virtually meaningless (PVS, p. 95).

Part of the apparent background for PVS was to find if there might be a technology based correlation between coliform and virus numbers. The time and expense of running viral lab tests might be obviated if one were able to find a reliable correlation between coliform numbers, turbidity and virus numbers. But because the PVS used Polio I, more robust viruses might not give the same results. This was commented upon within the memo between Neil Dunham and Henry Ongerth (Dept of Health April 28, 1978). "Although considering PVS results, there are no data to indicate that the same relationships will hold for other types of viruses. Previous research on relative resistance to of different viruses to chlorine strongly suggests that this relationship would vary significantly with virus type. The Pomona study demonstrated the difficulty that exists when attempting to equate virus removals with coliform concentrations."

This thought is amplified because the 2.2 MPN/100ml did not consider VBNC or similar states. Additionally as noted the DHS paper---Drinking Water & Environmental Management Treatment technology for Recycled Water, Jan 2007, "there has yet to be a demonstrated correlation between turbidity and pathogen concentration. The current turbidity performance standards for media and membrane filtration are based on achievable turbidity performance and do not assure any specific minimum level of pathogen removal. This is a recognized issue. Since the Pomona Virus Study, biological treatment has introduced additional variables into the picture, as the type of biological treatment can impact the particle

size distribution and downstream filter and disinfection performance. However, integration of these processes into a process-train are not well understood at this time.""

One of the issues being studied locally is the leaking of sewer system trunk mains that underlie many American cities. There has been a sufficiency of studies to raise questions about exfiltration—the loss of sewer effluent from these sewer mains. That rising ground water actually enters these older mains and systems is now well beyond question. The issue is one of logic. If rising ground water gets in through failing joints, cracks, and breaks, what keeps sewer water from utilizing these same portals when the surrounding ground water falls below them?

Cenci, et al [15] reviewed the incidence and the patterns of the antibiotic and metal resistance in 106 strains of *Escherichia coli* isolated from ground waters, used also as drinking water supply. These organisms were studied in comparison with the resistance behavior in the 104 strains of the same microorganism isolated from non hospitalized patients. When, however, these were compared to hospitalized patients, the patterns of the antibiotic multiresistances and the strains isolated from patients and from ground waters did not differ greatly. The authors concluded that their findings strengthened the hypothesis that resistance to antibiotics had been acquired by *Escherichia coli* strains before reaching the ground waters.

If the above has any validity, then what of the possible effects of different pharmaceutical groups such as anti-tumour drugs, antibiotics and contrast media as well as Absorbable organically bound halogens (AOX) resulting from hospitals effluent input into sewage? Recently, the occurrence and fate of pharmaceutically active compounds (PhACs) in the aquatic environment was recognized as one of the emerging issues in environmental chemistry and as a matter of public concern [16]. Residues of PhACs have been found as contaminants in sewage, surface, and ground- and drinking water samples. Again this begs the issue of leaking sewer mains and a need for pretreatment.

Most antibiotics and their metabolites are excreted by humans after administration and therefore reach the municipal sewage with the excretions. Kummerer, et al [17] looked at a worst case scenario on found concentrations of the antibiotics in hospital effluents. These concentrations were estimated and compared with minimum inhibitory concentrations for susceptible pathogenic bacteria and with the genotoxic potency. Both the concentrations calculated for hospital effluents and the adverse effects in bacteria were in the same order of magnitude.

Absorbable organically bound halogens (AOX) are mostly persistent in the environment, and accumulate in the food web. One important source of AOX in hospital effluents may be x-ray contrast media containing an iodine carbon bond. These materials may also add to selection pressures and development of resistant strains.

Others [18] have noted that the mere process of chlorinating effluent tends not only to increase resistance, but also increase the competitive edge of these survivors. Thus, we are now seeing developing resistance to chlorine, other antiseptics, and disinfectants. This raises some interesting academic as well as practical questions at the cellular and molecular level. For example, would developing resistance to chlorine also affect the efficacy of hypochlorite released within lysosomes, there by reducing effectiveness of leukocytes? How then might this affect the immune response?

The workers at sewer plants are also at risk. Several papers [19,20,21] have reported on transfer of viral particles and bacteria in aerosols that are generated by and surround many of these plants. In addition, there are studies on wind drift of these plumes into the surrounding neighborhoods.

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Decay of *E. coli* in soil following the application of biosolids

Investigators: **N.L. Lang, S.R. Smith, M. Bellet-Travers, E.B. Pike**
Funding: Environment Agency, Severn Trent Water, Thames Waterm Yorkshore Water, Scottish Water Authorities EPSRC

Introduction

The survival of pathogenic microorganisms in soil is important for assessing the potential microbiological risk to human and animal health associated with the use of biosolids as a soil amendment. Soil is a complex heterogeneous environment and enteric pathogens introduced to soil in sewage sludge are influenced by both climatic and agronomic variables. For example, the application of biosolids to soil may itself alter the soils physical and chemical properties with implications for pathogen survival.

The fate of *Escherichia coli* was assessed over time from soil amended with enhanced and conventionally treated biosolids

Field experiment

Biosolids were applied to the soil at a rate equivalent to 10 t ds/ha and were incorporated immediately using rotary cultivator. The range of biosolids evaluated in the trial included: (1) anaerobically digested cake (DMAD), (2) composted sludge with green waste (CPT) and (3) a thermally dried digested product (TDD). Unamended control soil was also monitored to assess the background status of enteric microorgansims in the trial plots.

A series of plots was covered with perforated polythene film to manipulate soil environmental conditions. A series of plots was covered with perforated polythene film to manipulate soil environmental conditions.

Meteorological data were collected by an automatic weather station. Soil temperature was recorded continuously at depths of 0-5 cm and 5-10 cm and soil moisture was also monitored using an automated time domain reflectomatory system.

Enhanced treated biosolids

Large increases in numbers of *E. coli* in the surface 0-5 cm were observed on day 14 and 42 and this coincided with periods of high mean rainfall and increased soil moisture content.

Significant regrowth of *E. coli* also occurred under the perforated plastic film and the unamended control treatment (0-5 cm) showed the same characteristic decay pattern as in the uncovered condition.

It is well documented that increasing soil moisture may encourage re-growth of enteric bacteria, particularly after a period of soil drying. Other possible explanations include recontamination by animal faeces and transformation of viable non-culturable bacteria to a culturable condition triggered by a change in environmental conditions.

Conventionally treated biosolids

Numbers of E. coli increased by approximately 1-2 $\log_{10}$/g after day 14, following a rainfall event in a similar manner to soil amended with enhanced-treated biosolids. However, no further evidence for regrowth was observed during the subsequent monitoring of *E. coli*. After day 14, *E. coli* populations declined in DMAD amended soil in both the covered and uncovered conditions and the patterns of decay followed the same general trend at both monitoring depths.

Conclusions

Enhanced biosolids do not contribute *E. coli* to soil, but they may influence soil conditions and the patterns of regrowth of indigenous populations of *E. coli* in soil. Survival of *E. coli* in soil following spring applications is limited typically to between 2-3 months and is well within sowing/cropping restrictions permitted in legislation controlling agricultural use of sewage sludge.

McGowan's comments---It would appear that there is ample opportunity for the transfer of antimicrobial resistance from sewer sludge organisms to soil bacteria. This warrants considerably more study because of the worrisome issues of accelerating resistance. Sewer sludge collected from major municipal centers with dischargers such as hospitals and the mixing of genetic material in sewer plants as well as concentrating of organisms in sludge offers an opportunity for amplifying resistance. If this sludge is then applied to thousands of acres, the opportunity fro transfer of resistance to environmental sinks is amplified.

Antibiotic resistance of E. coli in sewage and sludge.

Reinthaler FF, Posch J, Feierl G, Wust G, Haas D, Ruckenbauer G, Mascher F, Marth E.

Institute of Hygiene, University of Graz, Universitatsplatz 4, Austria. franz.reinthaler@uni-graz.at

The aim of the study is the evaluation of resistance patterns of E. coli in wastewater treatment plants without an evaluation of basic antibiotic resistance mechanisms.Investigations have been done in sewage, sludge and receiving waters from three different sewage treatment plants in southern Austria. A total of 767 E. coli isolates were tested regarding their resistance to 24 different antibiotics. The highest resistance rates were found in E. coli strains of a sewage treatment plant which treats not only municipal sewage but also sewage from a hospital.Among the antimicrobial agents tested, the highest resistance rates in the penicillin group were found for Ampicillin (AM) (up to 18%) and Piperacillin (PIP) (up to 12%); in the cephalosporin group for Cefalothin (CF) (up to 35%) and Cefuroxime-Axetil (CXMAX) (up to 11%); in the group of quinolones for Nalidixic acid (NA) (up to 15%); and for Trimethoprime/Sulfamethoxazole (SXT) (up to 13%) and for Tetracycline (TE) (57%).Median values for E. coli in the inflow (crude sewage) of the plants were between 2.0 x 10(4) and 6.1 x 10(4)CFU/ml (Coli ID-agar, BioMerieux 42017) but showed a 200-fold reduction in all three plants in the effluent. **Nevertheless, more than 10(2)CFU E. coli/ml reached the receiving water and thus sewage treatment processes contribute to the dissemination of resistant bacteria in the environment**. Water Res. 2003 Apr;37(8):1685-90. PMID: 12697213 [PubMed - indexed for MEDLINE]

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[Behavior of drug resistant fecal coliforms and R plasmids in a wastewater treatment plant]

[Article in Japanese]

Nakamura S, Shirota H.

Department of Food and Nutrition, Ube College.

Fecal coliforms were isolated from the inlet, the primary sedimentation tank, the activated sludge digestion tank, the final settling tank, the outlet and the return activated sludge drain at the municipal wastewater plant in Ube City, and examined for drug resistance and presence of R plasmids. Drug concentrations employed to distinguish resistant isolates from sensitive isolates were 25 micrograms/ml for tetracycline, kanamycin, chloramphenicol and streptomycin, 50 micrograms/ml for ampicillin, nalidixic acid and rifampicin, and 200 micrograms/ml for sulfisoxazole, respectively. Of a total of 900 isolates, 45.7% were drug resistant and 51.1% of them carried R plasmids. The further along that wastewater had progressed through the treatment process the greater the tendency was for appearance of the multiresistant isolates.

These isolates also were shown to simultaneously carry transferable R plasmids. Observed resistant patterns of R plasmids were mainly multiple and encoded to resistance to tetracycline, chloramphenicol, streptomycin and sulfisoxazole. It became clear that multiplication of R plasmids took place in the activated sludge digestion tank. This study show that drug resistance transfer mediated by these R plasmids may occur in actual wastewater treatment plants. Nippon Koshu Eisei Zasshi. 1990 Feb;37(2):83-90.
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Transferable resistance to gentamicin and other antibiotics in Enterobacteriaceae isolates from municipal wastewater.

Kralikova K, Krcmery V, Krcmery V Jr.

In two sets of Enterobacteriaceae and Pseudomonas bacteria resistant to at least two antibiotics a distinctly upward trend was found in the incidence of strains resistant to gentamicin. The strains examined were either routine isolates from three municipal wastewater treatment facilities or from the Danube river samples collected near the outlet of municipal sewerage. The resistance to gentamicin points to the representation of strains originating from hospitalized patients and its incidence among wastewater strains is recordable since the summer of 1981. Gentamicin resistance transfer could be demonstrated in a sewage sludge strain of Klebsiella pneumoniae resistant to seven antibiotics and in two multiresistant isolates from the river Danube. Resistance transfers in the case of other antibiotics, especially those susceptible to beta-lactamase (ampicillin, carbenicillin), were demonstrated in 10 out of the 24 di- and multiresistant strains tested. These findings show that both municipal wastewater and water in streams may function as the reservoirs of strains bearing the determinants of transferable resistance. Such strains may play an important role not only in the ecology and epidemiology of R plasmids, but also in the accidental spread of the so-called DNA recombinants that might escape during gene manipulations. J Hyg Epidemiol Microbiol Immunol. 1984;28(2):161-6.
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R-plasmid transfer in a wastewater treatment plant.

Mach PA, Grimes DJ.

Enteric bacteria have been examined for their ability to transfer antibiotic resistance in a wastewater treatment plant. Resistant Salmonella enteritidis, Proteus mirabilis, and Escherichia coli were isolated from clinical specimens and primary sewage effluent. Resistance to ampicillin, chloramphenicol, streptomycin, sulfadiazine, and tetracycline was demonstrated by spread plate and tube dilution techniques. Plasmid mediation of resistance was shown by ethidium bromide curing, agarose gel electrophoresis, and direct cell transfer. Each donor was mated with susceptible E. coli and Shigella sonnei. Mating pairs (and recipient controls) were suspended in unchlorinated primary effluent that had been filtered and autoclaved. Suspensions were added to membrane diffusion chambers which were then placed in the primary and secondary setting tanks of the wastewater treatment plant. Resistant recombinants were detected by replica plating nutrient agar master plates onto xylose lysine desoxycholate agar plates that contained per milliliter of medium 10 micrograms of ampicillin, 30 micrograms of chloramphenicol, 10 micrograms of streptomycin, 100 micrograms of sulfadiazine, or 30 micrograms of tetracycline. Mean transfer frequencies for laboratory matings were 2.1 X 10(-3). In situ matings for primary and secondary settling resulted in frequencies of 4.9 X 10(-5) and 7.5 X 10(-5), respectively. These values suggest that a significant level of resistance transfer occurs in wastewater treatment plants in the absence of antibiotics as selective agents. Appl Environ Microbiol. 1982 Dec;44(6):1395-403.
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R factors in coliform-fecal coliform sewage flora of the prairies and Northwest Territories of Canada.

Bell JB, Macrae WR, Elliott GE.

Coliform and fecal coliform populations found in the raw sewages and final sewage effluents of the prairie provinces and the Northwest Territories were examined for antibiotic resistance and the possession of R

factors. It was determined that 8.91% of the total coliform and 10.80% of the fecal coliform populations carried R factors. The following numbers of combinations of R determinants were found: 39 in the Escherichia coli population, 6 in the Citrobacter population, 20 in the Enterobacter populations, 10 in the Klebsiella populations, and 11 in the Aeromonas populations. The maximum number of R determinants transferable simultaneously was seven; organisms with R factors containing determinants for chloramphenicol usually contained determinants for ampicillin. Of the coliform and fecal coliform populations, 2 to 4% were resistant to chloramphenicol in some provinces, and from 17 to 30% of the populations were resistant to three or more antibiotics. It was calculated that coliforms containing R factors in the raw sewage reached population levels of 1.5 X 10(7)/100 ml, and fecal coliforms containing R factors reached population levels of 8.6 X 10(5) ml. Final effluent discharges to the receiving environment contained R factor-containing coliform and fecal coliform populations of 3.1 X 10(4)/100 ml and 5.8 X 10(2)/100 ml, respectively. The incidence of bacteria containing R factors in sewage appears to be increasing with time, and their removal from sewage before discharge to the receiving environment is desirable. Consideration of data on bacteria with R factors should be made in future water quality deliberations and in discharge regulations. Appl Environ Microbiol. 1981 Aug;42(2):204-10

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Elimination of enteric bacteria in biological-chemical wastewater treatment and tertiary filtration units.

Koivunen J, Siitonen A, Heinonen-Tanski H.

Department of Environmental Sciences, University of Kuopio, POB 1627, FIN-70211 Kuopio, Finland. jari.koivunen@uku.fi

The occurrence and removal of salmonellae and faecal indicators in four conventional municipal wastewater treatment plants (MWTP) were investigated. In addition, we tested the efficiency of a semi-technical scale biological nutrient removal process and three pilot-scale tertiary filtration units in microbial removal. All influent samples collected from MWTPs contained salmonellae from 93 to 11,000 MPN/100 ml and indicator bacteria from about 10(7) to 10(8) CFU/100 ml. The reductions in salmonella numbers achieved in full-scale biological-chemical wastewater treatment and semi-technical scale biological nutrient removal processes were usually between 94% and virtually 100% (99.9%) and indicator bacteria reductions between 2 and 3 log units. Microbial numbers in MWTP effluents could be modelled as a function of effluent residual organic matter, suspended solids and total phosphorus concentrations. Pilot-scale tertiary treatment by rapid sand contact filter, chemical contact filter and biological-chemical contact filter reduced salmonella numbers below the detection limit and faecal coliform numbers on average by 99%, 39% and 71%, respectively. A total of 32 Salmonella serovars were identified among 197 Salmonella isolates from municipal wastewaters. Of the isolates, 32% were resistant to nalidixic acid, indicating reduced sensitivity to ciprofloxacin, the drug of choice in the treatment of salmonellosis. In addition, 18% of the isolates were multiresistant. Our results, especially antibiotic resistant Salmonella strains, indicate that **conventional municipal wastewater treatment without efficient tertiary treatment, like filtration or disinfection, may constitute a risk for public health.** Water Res. 2003 Feb;37(3):690-8.

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Antibiotic resistance among different species of fecal coliforms isolated from water samples.

Niemi M, Sibakov M, Niemela S.

The distribution of resistance to ampicillin, chloramphenicol, sulfonamides, tetracycline, and streptomycin among fecal coliforms in sewage, surface waters, and sea water was investigated. The incidence of resistant strains among isolates varied significantly among the water samples, without obvious connection with the water source or the level of pollution. The average frequency of multiple resistance was not always high in the same samples in which the overall resistance was high. The species composition varied considerably in different water samples. A significant correlation was observed between the relative frequency of Klebsiella species and the incidence of ampicillin resistance in water samples. The importance of species

composition of fecal coliforms, affected by their source and by the aquatic environment, on the resistance pattern is noted. Appl Environ Microbiol. 1983 Jan;45(1):79-83.

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[Quantitative studies of the elimination of coliphages and other fecal indicators during wastewater treatment]

[Article in German]

Zaiss U, Hennies HH.

Labor fur Mikrobiologie und Hygiene, Fachrichtung Versorgungstechnik, Fachhochschule Braunschweig-Wolfenbutte.

Concentrations of coliphages, coliforms, enterococci and fluorescent Pseudomonas were monitored in several wastewater purification steps of the treatment plant Wolfenbuttel during one year. Their number varied widely during the investigation period, but was independent of seasons. In the course of sewage treatment, including primary settling, activated sludge purification, simultaneous precipitation, trickling filters and oxidation pond, the concentration of indicators decreased gradually. The coliphages were most resistant, exhibiting only a decimal elimination value of 1.7 log10 units as compared to the bacterial indicators with elimination values ranging between 2.4 and 2.8 log10 units in the whole process. The most efficient purification step revealed to be the activated sludge procedure including simultaneous phosphate precipitation with iron hydroxides and sedimentation. On an average 1.7% of the coliphages present in raw sewage or 9.8.10.11 phages were discharged into the river Oker everyday, 0.64% remained in the sludge. Numbers of indicators in the water of the oxidation pond and those seeded into river water were continuously reduced during 3 days. Also in these laboratory experiments, the coliphages were more resistant than the bacteria, but no evidence was found to support the view that coliphages play a role in the reduction of the number of coliform bacteria. Even after addition of peptone which stimulated growth of E. coli the coliphages were inactivated more rapidly. The behaviour of coliphages during the purification process is compared with literature data about enteroviruses. Zentralbl Bakteriol Mikrobiol Hyg [B]. 1988 Aug;186(5-6):512-25.

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Antibiotic resistance patterns of fecal coliforms isolated from domestic sewage before and after treatment in an aerobic lagoon.

Bell RB.

The resistance of 260 strains of fecal coliforms, isolated from raw domestic sewage and aerobic lagoon effluent, to ampicillin, aureomycin, chloromycetin, gentamicin, streptomycin, sulfadiazine, and tetracycline, was determined. Aerobic lagoon treatment produced a 20-fold reduction in the fecal coliform numbers. No statistically significant difference in antibiotic resistance was observed between the fecal coliforms found in raw sewage and in lagoon effluent despite a trend towards the loss of resistance in the latter. Antibiotic resistance, either single or multiple, did not contribute to, or detract from, bacterial survival in the aerobic lagoon. Of the isolates, 15% showed resistance to two or more antiobiotics. Can J Microbiol. 1978 Jul;24(7):886-8.

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[Development of antibiotic resistance in purified sewage effluents subjected to chlorination]

[Article in Italian]

Morozzi G, Cenci G, Caldini G, Sportolari R, Bahojbi AG.

Antibiotic-resistance is widely spread phenomenon in the environment because of uncontrolled discharge

of urban and animal wastewaters. Sewage treatment can significantly reduce the number of both sensitive and resistant bacteria. A reduction of about 1.5 logarithmic units in faecal coliforms was observed during biological treatment (3, 7), but a simultaneous increase in the percentage of resistant strains occurred because of not well understood selection phenomena. The above reported bacterial reduction is not always sufficient to meet the quality standards of Italian legislation required to discharge the treated effluents into surface waters, and so, chlorination become a compulsory additional treatment whose impact on both sensitive and resistant microflora must be evaluated. The results obtained in the present research have demonstrated that chlorine concentrations in the range of 0.5-2 ppm are able to reduce significantly the faecal coliforms concentrations and, in particular, treatment with 1 ppm of chlorine for 1 hour reduces the concentration of the above reported bacteria to the extent of 2 logarithmic units, so that their final concentration are of the about 10(2)/100 ml. The surviving chlorine tolerant bacteria seem to be antibiotic resistant in higher percentage than the chlorine sensitive ones and so, as a consequence, a significant increase in the antibiotic resistance and multiresistance was observed in the chlorinated effluents. In this context it is interesting to underline the larger variety of resistance patterns observed in the chlorine-resistant bacteria in comparison with the uniformity in the resistance patterns observed in isolated from unchlorinated effluents. The selected chlorine-tolerant strains seem to be less able to transfer their resistances under laboratory conditions, not because of curing effect of chlorine on the plasmids but, probably, because of the damage to cellular cell envelopes. Ann Ig. 1989 Jan-Apr;1(1-2):351-62.

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Fecal coliforms of wastewater treatment plants: antibiotic resistance, survival on surfaces and inhibition by sodium chloride and ascorbic acid.

Abu-Ghazaleh BM.

Department of Biology, Hashemite University, Zarqa, Jordan.

Three hundred and sixteen fecal coliform strains isolated from raw sewage and final effluents of two representative wastewater treatment plants were examined for antibiotic resistance. For plant A (which was disinfecting its effluents before discharge), 55% and 41% of the strains isolated from the influent and the effluent, respectively were resistant to one or more antibiotics. For plant B (which was not disinfecting its effluents before discharge), 33% and 58% of the strains isolated from the influent and the effluent, respectively were resistant to one or more antibiotics. A considerable proportion of these bacteria were resistant to three or more antibiotics. NaCl (3%) and ascorbic acid (0.1%) reduced the growth rate of both sensitive and resistant strains, and more inhibitory effects on the sensitive strains than on the resistant strains were observed. All resistant strains tested survived on various types of surfaces (e.g. glass, stainless steel, agar, cabbage, parsley and sand) significantly better than sensitive strains. New Microbiol. 2001 Oct;24(4):379-87.

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Effect of chlorination on antibiotic resistance profiles of sewage-related bacteria.

Murray GE, Tobin RS, Junkins B, Kushner DJ.

A total of 1,900 lactose-fermenting bacteria were isolated from raw sewage influent and chlorinated sewage effluent from a sewage treatment plant, as well as from chlorinated and neutralized dilute sewage, before and after a 24-h regrowth period in the laboratory. Of these isolates, 84% were resistant to one or more antibiotics. Chlorination of influent resulted in an increase in the proportion of bacteria resistant to ampicillin and cephalothin, the increase being most marked after regrowth occurred following chlorination. Of the other nine antibiotics tested, chlorination resulted in an increased proportion of bacteria resistant to some, but a decrease in the proportion resistant to the remainder. Multiple resistance was found for up to nine antibiotics, especially in regrowth populations. Identification of about 5% of the isolates showed that the highest proportion of Escherichia coli fell in untreated sewage. Some rare and potentially pathogenic species were isolated from chlorinated and regrowth samples, including Yersinia enterocolitica, Yersinia pestis, Pasteurella multocida, and Hafnia alvei. Our results indicate that chlorination, while initially

lowering the total number of bacteria in sewage, may substantially increase the proportions of antibiotic-resistant, potentially pathogenic organisms. Appl Environ Microbiol. 1984 Jul;48(1):73-7.
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[The role of mechanically purified city sewers in the spread of antibiotic-resistant bacteria of the Enterobacteriaceae family]

[Article in Polish]

Grol A, Szymanska B, Wejner H, Kazanowski A, Wlodarczyk K.

The aim of this study was to evaluate a degree of contribution of mechanically cleansed municipal sewage in a spread in on environment of bacteria of Enterobacteriaceae family with special regard to antibiotic resistant strains. High number of bacteria of Enterobacteriaceae family was found in 1 ml of sewage and the number of antibiotic-resistant bacteria was 0.5-50 X 10(3)/ml. Among the strains tested the resistance to more than one antibiotics was encountered. 78.3% of strains transferred antibiotic resistance to E. coli recipient strain, what indicate a participation of potentially pathogenic bacteria from Enterobacteriaceae family in a spread of antibiotic resistance in a environment. Med Dosw Mikrobiol. 1989;41(2):100-5.
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Fecal coliforms of wastewater treatment plants: antibiotic resistance, survival on surfaces and inhibition by sodium chloride and ascorbic acid.

Abu-Ghazaleh BM.

Department of Biology, Hashemite University, Zarqa, Jordan.

Three hundred and sixteen fecal coliform strains isolated from raw sewage and final effluents of two representative wastewater treatment plants were examined for antibiotic resistance. For plant A (which was disinfecting its effluents before discharge), 55% and 41% of the strains isolated from the influent and the effluent, respectively were resistant to one or more antibiotics. For plant B (which was not disinfecting its effluents before discharge), 33% and 58% of the strains isolated from the influent and the effluent, respectively were resistant to one or more antibiotics. A considerable proportion of these bacteria were resistant to three or more antibiotics. NaCl (3%) and ascorbic acid (0.1%) reduced the growth rate of both sensitive and resistant strains, and more inhibitory effects on the sensitive strains than on the resistant strains were observed. All resistant strains tested survived on various types of surfaces (e.g. glass, stainless steel, agar, cabbage, parsley and sand) significantly better than sensitive strains. New Microbiol. 2001 Oct;24(4):379-87.
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Influence of sewage treatment and urbanization on selection of multiple resistance in fecal coliform populations.

Bell JB, Elliott GE, Smith DW.

The fecal coliform populations found in the raw sewages and final sewage effluents of mechanical treatment plants, a long-term retention lagoon, shorter-term retention lagoons, a remote northern Canada river, and a heavily urbanized prairie river were examined for antibiotic resistance and the possession of R factors. It was determined that there was a decrease in the percentage of multiresistant fecal coliform populations in the mechanical sewage treatment plants and shorter-term retention lagoons; however, there was an increase in populations from the long-term retention lagoon. The percentage of the populations possessing transmissible R factors was constant in the mechanical treatment and shorter-term retention facilities; however, the ability to transmit was lost in 50% of the infective population of the long-term retention facility. A striking contrast was found between the populations of the remote northern Slave River and those of the urbanized Red River. Of the fecal coliforms in the Slave River, 7.1% were multiresistant, and only 0.79% possessed transmissible R factors. The Red River fecal coliform populations were 52.9% multiresistant, and 18.77% of the total population possessed transmissible R factors. The influence of

urbanization and the type of sewage treatment have been shown to affect the selection and survival of multiresistant fecal coliforms and R+ fecal coliforms. Determination of other factors influencing the development and the survival of these populations is needed for rational wastewater management and water quality consideration. Appl Environ Microbiol. 1983 Jul;46(1):227-32.

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Transferable resistance to gentamicin and other antibiotics in Enterobacteriaceae isolates from municipal wastewater.

Kralikova K, Krcmery V, Krcmery V Jr.

In two sets of Enterobacteriaceae and Pseudomonas bacteria resistant to at least two antibiotics a distinctly upward trend was found in the incidence of strains resistant to gentamicin. The strains examined were either routine isolates from three municipal wastewater treatment facilities or from the Danube river samples collected near the outlet of municipal sewerage. The resistance to gentamicin points to the representation of strains originating from hospitalized patients and its incidence among wastewater strains is recordable since the summer of 1981. Gentamicin resistance transfer could be demonstrated in a sewage sludge strain of Klebsiella pneumoniae resistant to seven antibiotics and in two multiresistant isolates from the river Danube. Resistance transfers in the case of other antibiotics, especially those susceptible to beta-lactamase (ampicillin, carbenicillin), were demonstrated in 10 out of the 24 di- and multiresistant strains tested. These findings show that both municipal wastewater and water in streams may function as the reservoirs of strains bearing the determinants of transferable resistance. Such strains may play an important role not only in the ecology and epidemiology of R plasmids, but also in the accidental spread of the so-called DNA recombinants that might escape during gene manipulations. J Hyg Epidemiol Microbiol Immunol. 1984;28(2):161-6

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[Fecal coliforms in sewage waters. I. Resistance to antibiotics, heavy metals and colicinogeny]

[Article in Portuguese]

Ribeiro Dias JC, Vicente AC, Hofer E.

Instituto Oswaldo Cruz, Departamento de Bacteriologia, Rio de Janeiro, Brasil.

Qualitative bacteriological analysis was carried out in two sewage treatment plants in the city of Rio de Janeiro during the period 1984-1985. Specific points of the plants were selected for the collection of affluent and effluent samples. The study involved the isolation and the identification of 540 cultures of Escherichia coli that were analyzed for their resistance to eight antibiotics (sulfadiazine, streptomycin, tetracycline, chloramphenicol, kanamycin, ampicillin, nalidixic acid and gentamycin), and three heavy metals (copper sulphate, mercuric chloride and zinc sulphate) as well as colicinogeny. About 95% of the isolated cultures from the effluents had genetic markers while the samples originated from the affluents showed 70%.Mem Inst Oswaldo Cruz. 1987 Jul-Sep;82(3):335-43.

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Antibiotic resistance and R-factors in the fecal coliform flora of urban and rural dogs.

Monaghan C, Tierney U, Colleran E.

The incidence of antibiotic-resistant fecal coliforms in the rectal flora of 106 healthy dogs in the Galway area was investigated. As far as could be determined, none of the dogs had received antimicrobial drugs. Half of the dogs sampled were from homes within the city boundaries, whereas the remainder were from farms within a 40-mile (24.8-km) radius of the city. Of the dogs sampled, 47 had a highly susceptible fecal coliform flora, with less than 1% of the coliform population resistant to any of the four test antibiotics. Fecal coliforms resistant to one or more of the test drugs comprised between 40 and 100% of the total fecal

coliform population of 36% of the rural dogs and 13% of the urban dogs sampled. Of the 473 resistant Escherichia coli isolates studied, the highest number of associated resistance determinants encountered was 5, with a medium number of 2.5. Of the E. coli isolates from rural dogs, 52% were resistant to three or more antibiotics compared with 37% of the isolates from urban dogs. A total of 64% of the isolates were shown to transfer some or all of their resistance determinants by conjugation. The transferability of ampicillin (77%) and chloramphenicol (70%) resistance determinants was higher than that of streptomycin (40%) or tetracycline (44%).Antimicrob Agents Chemother. 1981 Feb;19(2):266-70.
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Incidence of R-plasmids in fecal flora of healthy household dogs.

Hirsh DC, Ling GV, Ruby AL.

Rectal swabs were taken from healthy household dogs that, insofar as could be determined, had not received antimicrobial drugs. Tetracycline-resistant coliforms comprised 80 to 100% of the total number of coliforms in 61 (65%) of the 94 dogs sampled. The median number of other resistance determinants possessed by these tetracycline-resistant coliforms was 5.1. Of the tetracycline-resistant strains studied, 97% were resistant to streptomycin; 76% were resistant to sulfonamides; 59% were resistant to ampicillin; 59% were resistant to kanamycin/neomycin; and 40% were resistant to chloramphenicol. A total of 64% of the strains was shown to transfer resistance by conjugation or by the aid of the sex factor F. Of the strains transferring resistance, 33% were found to transfer all of their resistance determinants. Antimicrob Agents Chemother. 1980 Mar;17(3):313-5.
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Occurrence of multiple-antibiotic-resistant enteric bacteria in domestic sewage and oxidation lagoons.

Walter MV, Vennes JW.

The coliform bacterial population in the Grand Forks, N.Dak. sewage system was examined for multiple-antibiotic-resistant organisms over a 1-year period. Multiple-antibiotic-resistant coliforms were found to be common in the sewage, and their numbers remained fairly constant relative to the total coliform population throughout the year. Resistance to kanamycin, tetracycline, and ampicillin was found to be transferable at variable rates. Transfer rates were found to be temperature sensitive and were optimal at 35 degrees C. Although 75% of the multiple-antibiotic-resistant coliforms were capable of transferring resistance at some level, only 25% were capable of transferring resistance at rates greater than 10(-3) transconjugants per initial donor. Appl Environ Microbiol. 1985 Oct;50(4):930-3.
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The effect of anaerobic and aerobic wastewater treatment on faecal coliforms and antibiotic-resistant faecal coliforms.

Morozzi G, Sportolari R, Caldini G, Cenci G, Morosi A.

Istituto di Igiene, Universita degli Studi, Perugia, Italy.

The efficiency of anaerobic treatment of animal wastes and aerobic treatment of urban sewage in removing faecal coliforms and their effect on the antibiotic-resistant coliforms was evaluated in this study. A two reactor anaerobic digester and six activated sludge plants were studied. The concentrations of both faecal coliforms in sampling from influents and treated effluents were calculated to determine efficiency of plants during depuration treatments. Anaerobic and aerobic treatments resulted in 90% and 97% (arithmetic mean values) efficiency in removing faecal coliforms. Although neither anaerobic nor aerobic treatment seems to significantly increase the percentage of antibiotic-resistant bacteria, during the aerobic treatment of urban sewage there was a tendency for the percent of antibiotic-resistant bacteria to increase. Zentralbl Bakteriol Mikrobiol Hyg [B]. 1988 Jan;185(4-5):340-9.
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Elimination of enteric bacteria in biological-chemical wastewater treatment and tertiary filtration units.

Koivunen J, Siitonen A, Heinonen-Tanski H.

Department of Environmental Sciences, University of Kuopio, POB 1627, FIN-70211 Kuopio, Finland. jari.koivunen@uku.fi

The occurrence and removal of salmonellae and faecal indicators in four conventional municipal wastewater treatment plants (MWTP) were investigated. In addition, we tested the efficiency of a semi-technical scale biological nutrient removal process and three pilot-scale tertiary filtration units in microbial removal. All influent samples collected from MWTPs contained salmonellae from 93 to 11,000 MPN/100 ml and indicator bacteria from about 10(7) to 10(8) CFU/100 ml. The reductions in salmonella numbers achieved in full-scale biological-chemical wastewater treatment and semi-technical scale biological nutrient removal processes were usually between 94% and virtually 100% (99.9%) and indicator bacteria reductions between 2 and 3 log units. Microbial numbers in MWTP effluents could be modelled as a function of effluent residual organic matter, suspended solids and total phosphorus concentrations. Pilot-scale tertiary treatment by rapid sand contact filter, chemical contact filter and biological-chemical contact filter reduced salmonella numbers below the detection limit and faecal coliform numbers on average by 99%, 39% and 71%, respectively. A total of 32 Salmonella serovars were identified among 197 Salmonella isolates from municipal wastewaters. Of the isolates, 32% were resistant to nalidixic acid, indicating reduced sensitivity to ciprofloxacin, the drug of choice in the treatment of salmonellosis. In addition, 18% of the isolates were multiresistant. Our results, especially antibiotic resistant Salmonella strains, indicate that conventional municipal wastewater treatment without efficient tertiary treatment, like filtration or disinfection, may constitute a risk for public health. Water Res. 2003 Feb;37(3):690-8.

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[Quantitative studies of the elimination of coliphages and other fecal indicators during wastewater treatment]

[Article in German]

Zaiss U, Hennies HH.

Labor fur Mikrobiologie und Hygiene, Fachrichtung Versorgungstechnik, Fachhochschule Braunschweig-Wolfenbutte.

Concentrations of coliphages, coliforms, enterococci and fluorescent Pseudomonas were monitored in several wastewater purification steps of the treatment plant Wolfenbuttel during one year. Their number varied widely during the investigation period, but was independent of seasons. In the course of sewage treatment, including primary settling, activated sludge purification, simultaneous precipitation, trickling filters and oxidation pond, the concentration of indicators decreased gradually. The coliphages were most resistant, exhibiting only a decimal elimination value of 1.7 log10 units as compared to the bacterial indicators with elimination values ranging between 2.4 and 2.8 log10 units in the whole process. The most efficient purification step revealed to be the activated sludge procedure including simultaneous phosphate precipitation with iron hydroxides and sedimentation. On an average 1.7% of the coliphages present in raw sewage or 9.8.10.11 phages were discharged into the river Oker everyday, 0.64% remained in the sludge. Numbers of indicators in the water of the oxidation pond and those seeded into river water were continuously reduced during 3 days. Also in these laboratory experiments, the coliphages were more resistant than the bacteria, but no evidence was found to support the view that coliphages play a role in the reduction of the number of coliform bacteria. Even after addition of peptone which stimulated growth of E. coli the coliphages were inactivated more rapidly. The behaviour of coliphages during the purification process is compared with literature data about enteroviruses. Zentralbl Bakteriol Mikrobiol Hyg [B]. 1988 Aug;186(5-6):512-25.

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Fecal coliforms of wastewater treatment plants: antibiotic resistance, survival on surfaces and inhibition by sodium chloride and ascorbic acid.

Abu-Ghazaleh BM.

Department of Biology, Hashemite University, Zarqa, Jordan.

Three hundred and sixteen fecal coliform strains isolated from raw sewage and final effluents of two representative wastewater treatment plants were examined for antibiotic resistance. For plant A (which was disinfecting its effluents before discharge), 55% and 41% of the strains isolated from the influent and the effluent, respectively were resistant to one or more antibiotics. For plant B (which was not disinfecting its effluents before discharge), 33% and 58% of the strains isolated from the influent and the effluent, respectively were resistant to one or more antibiotics. A considerable proportion of these bacteria were resistant to three or more antibiotics. NaCl (3%) and ascorbic acid (0.1%) reduced the growth rate of both sensitive and resistant strains, and more inhibitory effects on the sensitive strains than on the resistant strains were observed. All resistant strains tested survived on various types of surfaces (e.g. glass, stainless steel, agar, cabbage, parsley and sand) significantly better than sensitive strains. New Microbiol. 2001 Oct;24(4):379-87.

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Antibiotic resistance of E. coli in sewage and sludge.

Reinthaler FF, Posch J, Feierl G, Wust G, Haas D, Ruckenbauer G, Mascher F, Marth E.

Institute of Hygiene, University of Graz, Universitatsplatz 4, Austria. franz.reinthaler@uni-graz.at

The aim of the study is the evaluation of resistance patterns of E. coli in wastewater treatment plants without an evaluation of basic antibiotic resistance mechanisms.Investigations have been done in sewage, sludge and receiving waters from three different sewage treatment plants in southern Austria. A total of 767 E. coli isolates were tested regarding their resistance to 24 different antibiotics. The highest resistance rates were found in E. coli strains of a sewage treatment plant which treats not only municipal sewage but also sewage from a hospital.Among the antimicrobial agents tested, the highest resistance rates in the penicillin group were found for Ampicillin (AM) (up to 18%) and Piperacillin (PIP) (up to 12%); in the cephalosporin group for Cefalothin (CF) (up to 35%) and Cefuroxime-Axetil (CXMAX) (up to 11%); in the group of quinolones for Nalidixic acid (NA) (up to 15%); and for Trimethoprime/Sulfamethoxazole (SXT) (up to 13%) and for Tetracycline (TE) (57%).Median values for E. coli in the inflow (crude sewage) of the plants were between 2.0 x 10(4) and 6.1 x 10(4)CFU/ml (Coli ID-agar, BioMerieux 42017) but showed a 200-fold reduction in all three plants in the effluent. Nevertheless, more than 10(2)CFU E. coli/ml reached the receiving water and thus sewage treatment processes contribute to the dissemination of resistant bacteria in the environment. Water Res. 2003 Apr;37(8):1685-90.

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Influence of sewage treatment and urbanization on selection of multiple resistance in fecal coliform populations.

Bell JB, Elliott GE, Smith DW.

The fecal coliform populations found in the raw sewages and final sewage effluents of mechanical treatment plants, a long-term retention lagoon, shorter-term retention lagoons, a remote northern Canada river, and a heavily urbanized prairie river were examined for antibiotic resistance and the possession of R factors. It was determined that there was a decrease in the percentage of multiresistant fecal coliform populations in the mechanical sewage treatment plants and shorter-term retention lagoons; however, there was an increase in populations from the long-term retention lagoon. The percentage of the populations possessing transmissible R factors was constant in the mechanical treatment and shorter-term retention facilities; however, the ability to transmit was lost in 50% of the infective population of the long-term retention facility. A striking contrast was found between the populations of the remote northern Slave River

and those of the urbanized Red River. Of the fecal coliforms in the Slave River, 7.1% were multiresistant, and only 0.79% possessed transmissible R factors. The Red River fecal coliform populations were 52.9% multiresistant, and 18.77% of the total population possessed transmissible R factors. The influence of urbanization and the type of sewage treatment have been shown to affect the selection and survival of multiresistant fecal coliforms and R+ fecal coliforms. Determination of other factors influencing the development and the survival of these populations is needed for rational wastewater management and water quality consideration. Appl Environ Microbiol. 1983 Jul;46(1):227-32.

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Transferable resistance to gentamicin and other antibiotics in Enterobacteriaceae isolates from municipal wastewater.

Kralikova K, Krcmery V, Krcmery V Jr.

In two sets of Enterobacteriaceae and Pseudomonas bacteria resistant to at least two antibiotics a distinctly upward trend was found in the incidence of strains resistant to gentamicin. The strains examined were either routine isolates from three municipal wastewater treatment facilities or from the Danube river samples collected near the outlet of municipal sewerage. The resistance to gentamicin points to the representation of strains originating from hospitalized patients and its incidence among wastewater strains is recordable since the summer of 1981. Gentamicin resistance transfer could be demonstrated in a sewage sludge strain of Klebsiella pneumoniae resistant to seven antibiotics and in two multiresistant isolates from the river Danube. Resistance transfers in the case of other antibiotics, especially those susceptible to beta-lactamase (ampicillin, carbenicillin), were demonstrated in 10 out of the 24 di- and multiresistant strains tested. These findings show that both municipal wastewater and water in streams may function as the reservoirs of strains bearing the determinants of transferable resistance. Such strains may play an important role not only in the ecology and epidemiology of R plasmids, but also in the accidental spread of the so-called DNA recombinants that might escape during gene manipulations. J Hyg Epidemiol Microbiol Immunol. 1984;28(2):161-6.

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R-plasmid transfer in a wastewater treatment plant.

Mach PA, Grimes DJ.

Enteric bacteria have been examined for their ability to transfer antibiotic resistance in a wastewater treatment plant. Resistant Salmonella enteritidis, Proteus mirabilis, and Escherichia coli were isolated from clinical specimens and primary sewage effluent. Resistance to ampicillin, chloramphenicol, streptomycin, sulfadiazine, and tetracycline was demonstrated by spread plate and tube dilution techniques. Plasmid mediation of resistance was shown by ethidium bromide curing, agarose gel electrophoresis, and direct cell transfer. Each donor was mated with susceptible E. coli and Shigella sonnei. Mating pairs (and recipient controls) were suspended in unchlorinated primary effluent that had been filtered and autoclaved. Suspensions were added to membrane diffusion chambers which were then placed in the primary and secondary setting tanks of the wastewater treatment plant. Resistant recombinants were detected by replica plating nutrient agar master plates onto xylose lysine desoxycholate agar plates that contained per milliliter of medium 10 micrograms of ampicillin, 30 micrograms of chloramphenicol, 10 micrograms of streptomycin, 100 micrograms of sulfadiazine, or 30 micrograms of tetracycline. Mean transfer frequencies for laboratory matings were 2.1 X 10(-3). In situ matings for primary and secondary settling resulted in frequencies of 4.9 X 10(-5) and 7.5 X 10(-5), respectively. These values suggest that a significant level of resistance transfer occurs in wastewater treatment plants in the absence of antibiotics as selective agents. Appl Environ Microbiol. 1982 Dec;44(6):1395-403.

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The composition and persistence of faecal coliforms and enterococcal populations in sewage treatment plants.

Vilanova X, Manero A, Cerda-Cuellar M, Blanch AR.

Departament de Microbiologia, Universitat de Barcelona, Barcelona, Spain.

AIMS: The changes in structure and composition of faecal coliforms and enterococcal populations in sewage from different treatment plants, and the elimination of vancomycin- and erythromycin-resistant enterococci (VRE and ERE, respectively) in these treatment plants was analysed to determine any selective reduction. METHODS AND RESULTS: Faecal coliforms, enterococci, VRE, ERE and spores of sulphite-reducing bacteria were enumerated using standard methods. Samples were enriched where necessary in order to isolate antibiotic resistant strains. The structure and composition of these bacterial populations were determined by biochemical fingerprinting and clustering analysis. High diversity and similarity indexes were detected among all the bacterial populations in raw and treated sewage, independently of their origin and the treatment processes employed. Antibiotic resistant strains were detected in all sewage tested and no selective reduction was observed. CONCLUSIONS: The faecal coliforms and enterococci populations did not differ in the sewage samples studied. The vancomycin and erythromycin resistances of the enterococcal populations were similar in the sewage samples. Resistance to both antibiotics persisted after the treatment process independently of raw sewage flow, faecal origin or size of the human population contributing to sewage. However, sewage of mixed origin (human and animal) presented a lower similarity index for the two bacterial populations compared with that of the other human sewage analysed. SIGNIFICANCE AND IMPACT OF THE STUDY: Although a significant reduction in bacterial populations was observed, the persistence of VRE and ERE strains in the same proportions in sewage suggests that there is no selective elimination of bacterial populations during the treatment processes. The ability of antibiotic resistance strains to survive sewage treatment systems should be considered in certain water reuse programmes. J Appl Microbiol. 2004;96(2):279-88.
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Effect of chlorination on antibiotic resistance profiles of sewage-related bacteria.

Murray GE, Tobin RS, Junkins B, Kushner DJ.

A total of 1,900 lactose-fermenting bacteria were isolated from raw sewage influent and chlorinated sewage effluent from a sewage treatment plant, as well as from chlorinated and neutralized dilute sewage, before and after a 24-h regrowth period in the laboratory. Of these isolates, 84% were resistant to one or more antibiotics. Chlorination of influent resulted in an increase in the proportion of bacteria resistant to ampicillin and cephalothin, the increase being most marked after regrowth occurred following chlorination. Of the other nine antibiotics tested, chlorination resulted in an increased proportion of bacteria resistant to some, but a decrease in the proportion resistant to the remainder. Multiple resistance was found for up to nine antibiotics, especially in regrowth populations. Identification of about 5% of the isolates showed that the highest proportion of Escherichia coli fell in untreated sewage. Some rare and potentially pathogenic species were isolated from chlorinated and regrowth samples, including Yersinia enterocolitica, Yersinia pestis, Pasteurella multocida, and Hafnia alvei. Our results indicate that chlorination, while initially lowering the total number of bacteria in sewage, may substantially increase the proportions of antibiotic-resistant, potentially pathogenic organisms. Appl Environ Microbiol. 1984 Jul;48(1):73-7.
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R-plasmid transfer in Salmonella spp. isolated from wastewater and sewage-contaminated surface waters.

Alcaide E, Garay E.

A total of 865 Salmonella isolates from wastewaters and sewage-contaminated natural waters were tested for antimicrobial resistance by using NR10 medium and incubation at 43 degrees C. Of the strains, 12.7% were resistant to one or more of the compounds tested, and 30% transferred resistance to an Escherichia coli recipient. The highest minimal inhibitory concentrations were ca. 1,000 micrograms/ml. Transfer frequencies ranged from 10(-3) to 10(-7). Appl Environ Microbiol. 1984 Aug;48(2):435-8.
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Survival of fecal microorganisms in marine and freshwater sediments.

Davies CM, Long JA, Donald M, Ashbolt NJ.

Australian Water Technologies, EnSight, West Ryde, New South Wales.

The survival of culturable fecal coliforms, fecal streptococci, and Clostridium perfringens spores in freshwater and marine sediments from sites near sewage outfalls was studied. In laboratory studies, the inhibition of protozoan predators with cycloheximide allowed the fecal coliforms to grow in the sediment whereas the presence of predators resulted in a net die-off. C. perfringens spores did not appear either to be affected by predators or to die off throughout the duration of the experiments (28 days). Studies using in situ membrane diffusion chambers showed that, with the exception of C. perfringens, die-off of the test organisms to 10% of their initial numbers occurred in both marine and freshwater sediments within 85 days. The usual exponential decay model could not be applied to the sediment survival data, with the exception of the data for fecal streptococci. It was concluded that application of the usual decay model to the fecal coliform data was confounded by the complex relationship between growth and predation. The survival of seeded Escherichia coli in marine sediment was studied by using an enumeration method which detected viable but nonculturable bacteria. Throughout the duration of the experiment (68 days), the same proportion of E. coli organisms remained culturable, suggesting that sediment provides a favorable, nonstarvation environment for the bacteria. Appl Environ Microbiol. 1995 May;61(5):1888-96.

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Sampling strategy for detecting viruses in a sewage treatment plant.

Rolland D, Joret JC, Villeval F, Block JC, Hartemann P.

A study of pollutant flows was carried out at a wastewater treatment plant in Nancy, France, which used activated-sludge treatment. To carry out observation of hourly flow variation, a sampling strategy needs to be defined. A comparison between two methods of sampling was conducted: dip samples every 2 h over a period of 24 h and one 24-h composite sample were taken from raw and treated wastewater and then analyzed for enteroviruses, fecal coliforms, chemical oxygen demand, biochemical oxygen demand, and suspended solids. The results showed that the hourly variations of these pollutants in the effluents are in good agreement with expectations based upon the customers' usage and the characteristics of the wastewater network. Significant correlations were found between all tested parameters and enteroviruses in raw wastewater. After biological treatment, no correlation remained in treated wastewater between viruses and other parameters. As for the two sampling methods, a rather good representation of the daily load was given by the composite mode of sampling as concerns physicochemical and microbiological parameters. **Biological treatment removed an average of 83% of viruses.** Appl Environ Microbiol. 1983 Jun;45(6):1767-74.

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Incidence of lysogeny, colicinogeny, and drug resistance in enterobacteria isolated from sewage and from rectum of humans and some domesticated species.

Dhillon TS, Dhillon EK.

Enterobacteria were isolated by streaking swabs of sewage and rectal swabs from human volunteers from domesticated animals. Thirty strains of human origin were identified as Escherichia coli. Out of 1,367 rectal isolates of animal origin, 21% were lysogenic (phi+), 29% were colicinogenic (col+), and 7% were col+ phi+. Out of 85 rectal samples more than 60% harbored variable numbers of col+ or phi+ bacteria. Lysogens harboring homoimmune prophages were detectable in six out of eight human subjects in sequential samples taken at weekly intervals. Chickens in Hong Kong are fed on antibiotic-containing feeds; the avian isolates contained the highest frequency (98%) of drug-resistant bacteria, whereas only 39% of the bovine and 61% of the human isolates were drug resistant. Transmissible drug resistance was demonstrable in sewage isolates and those from animal sources; the highest frequency (58%) of resistance

donors was shown by the avian isolates, and the lowest (9%) was shown by the bovine isolates. Unselected marker analysis has shown that a vast majority of multiply resistant donors of diverse origins are able to transmit multiple resistance. Appl Environ Microbiol. 1981 Apr;41(4):894-902.
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Antibiotic resistance of E. coli in sewage and sludge.

Reinthaler FF, Posch J, Feierl G, Wust G, Haas D, Ruckenbauer G, Mascher F, Marth E.

Institute of Hygiene, University of Graz, Universitatsplatz 4, Austria. franz.reinthaler@uni-graz.at

The aim of the study is the evaluation of resistance patterns of E. coli in wastewater treatment plants without an evaluation of basic antibiotic resistance mechanisms.Investigations have been done in sewage, sludge and receiving waters from three different sewage treatment plants in southern Austria. A total of 767 E. coli isolates were tested regarding their resistance to 24 different antibiotics. The highest resistance rates were found in E. coli strains of a sewage treatment plant which treats not only municipal sewage but also sewage from a hospital.Among the antimicrobial agents tested, the highest resistance rates in the penicillin group were found for Ampicillin (AM) (up to 18%) and Piperacillin (PIP) (up to 12%); in the cephalosporin group for Cefalothin (CF) (up to 35%) and Cefuroxime-Axetil (CXMAX) (up to 11%); in the group of quinolones for Nalidixic acid (NA) (up to 15%); and for Trimethoprime/Sulfamethoxazole (SXT) (up to 13%) and for Tetracycline (TE) (57%).Median values for E. coli in the inflow (crude sewage) of the plants were between 2.0 x 10(4) and 6.1 x 10(4)CFU/ml (Coli ID-agar, BioMerieux 42017) but showed a 200-fold reduction in all three plants in the effluent. Nevertheless, more than 10(2)CFU E. coli/ml reached the receiving water and thus sewage treatment processes contribute to the dissemination of resistant bacteria in the environment. Water Res. 2003 Apr;37(8):1685-90.
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The resistance to chemotherapeutic agents of Escherichia coli from domestic dogs and cats.

Moss S, Frost AJ.

The prevalence of antibiotic resistant Escherichia coli in the rectal flora of 168 healthy dogs and 93 cats in the Brisbane area was investigated. Rectal swabs were plated on MacConkey agar with and without antibiotics, and 690 isolates confirmed as faecal E. coli were tested for resistance to tetracycline, streptomycin, chloramphenicol, ampicillin, neomycin, furazolidone and sulphanilamide. Resistant isolates were obtained from 101 (60%) of the dogs and 24 (26%) of the cats sampled. A high percentage of the isolates was resistant to tetracycline, streptomycin, ampicillin and sulphanilamide. Multiple resistance to 3 or more of the drugs was exhibited by the majority of isolates and a total of 31 different multiple resistance patterns was demonstrated. Of the 50 strains tested for transfer of resistance, 30 (60%) transferred some or all of their resistance determinants to an E. coli K12F - recipient. Aust Vet J. 1984 Mar;61(3):82-4.
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Transferable resistance to gentamicin and other antibiotics in Enterobacteriaceae isolates from municipal wastewater.

Kralikova K, Krcmery V, Krcmery V Jr.

In two sets of Enterobacteriaceae and Pseudomonas bacteria resistant to at least two antibiotics a distinctly upward trend was found in the incidence of strains resistant to gentamicin. The strains examined were either routine isolates from three municipal wastewater treatment facilities or from the Danube river samples collected near the outlet of municipal sewerage. The resistance to gentamicin points to the representation of strains originating from hospitalized patients and its incidence among wastewater strains is recordable since the summer of 1981. Gentamicin resistance transfer could be demonstrated in a sewage sludge strain of Klebsiella pneumoniae resistant to seven antibiotics and in two multiresistant isolates from the river Danube. Resistance transfers in the case of other antibiotics, especially those susceptible to beta-lactamase (ampicillin, carbenicillin), were demonstrated in 10 out of the 24 di- and multiresistant strains

tested. These findings show that both municipal wastewater and water in streams may function as the reservoirs of strains bearing the determinants of transferable resistance. Such strains may play an important role not only in the ecology and epidemiology of R plasmids, but also in the accidental spread of the so-called DNA recombinants that might escape during gene manipulations. J Hyg Epidemiol Microbiol Immunol. 1984;28(2):161-6.

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Carriage of antimicrobial resistant Escherichia coli in adult intestinal flora.

Newman MJ, Seidu A.

Dept of Microbiology, University of Ghana Medical School, Accra.

Knowledge of antibiotic resistance in bacteria strains colonizing healthy people is important for several reasons, one of which is that; these organisms form one of the largest reservoirs of resistant genes. Frequency of resistance to eleven different antimicrobial agents was examined in faecal flora of adults with no history of recent antimicrobial treatment. Using the disc diffusion sensitivity test, 106 strains of Escherichia coli were examined, 68% of these were resistant to tetracycline, and 57% were resistant to ampicillin and cotrimoxazole respectively. There was no resistance to cefuroxime but resistance to ceftazidime was 13%. Fifty six out of the eighty eight (64%) isolates, which showed any resistance, were resistant to three or more antimicrobials. The most common resistant pattern was to three drugs tetracycline, ampicillin and cotrimoxazole. Six strains were susceptible to all antibiotics. One strain of Escherichia coli was resistant to eight antimicrobials. Thirty per cent of the Escherichia coli were resistant to gentamicin. This study reveals a high prevalence of resistant bacteria in faecal flora of healthy adults.
West Afr J Med. 2002 Jan-Mar;21(1):48-50.

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carriage of Escherichia coli resistant to antibiotics in healthy populations in Shanghai.

Zhang XL, Wang F, Zhu DM, Wu S, Wu PC, Chen YD, Wang YQ, Zhou L.

Institute of Antibiotics, Huashan Hospital, Shanghai Medical University, China.

Healthy populations represent the largest reservoir of bacteria resistant to antibiotics. We investigated the resistance of Escherichia coli to 12 antibiotics in fecal samples from untreated healthy populations in Shanghai, China by using Kirby-Bauer (K-B) method. The results showed that: (i) All subjects carried resistant strains of Escherichia coli. (ii) The carriage rates of Escherichia coli resistant to various antibiotics were different, less than 10% to amikacin and 30% to 100% to others. (iii) In the elder children group aged 10-11 years, the percentages of strains resistant to gentamicin, streptomycin, chloramphenicol, tetracycline, trimethoprim, and sulfamethoxazole were significantly lower than those in the younger group aged 5-6 years. In the adult group, the percentages of strains resistant to ampicillin, piperacillin, amikacin, streptomycin, chloramphenicol, tetracycline, trimethoprim, and sulfamethoxazole were significantly lower than those in the elder children group. (iv) The number of strains resistant to five or more antibiotics accounted for 31.8% in the younger children group, 23.7% in the elder children group, and 12.1% in the adult group. These findings suggest that all healthy people in Shanghai carry resistant strains of Escherichia coli in the intestine. The younger the populations, the higher the level of resistance of fecal Escherichia coli to antibiotics. Improvement of health behaviors and environmental sanitation and rational use of antibiotics could remarkedly decrease the resistant level of bacteria. Biomed Environ Sci. 1998 Dec;11(4):314-20.

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Patterns of antimicrobial use and antimicrobial resistance among healthy children in Bolivia.

Bartoloni A, Cutts F, Leoni S, Austin CC, Mantella A, Guglielmetti P, Roselli M, Salazar E, Paradisi F.

Clinica Malattie Infettive, Universita di Firenze, Italy. infdis@cesit1.unifi.it

OBJECTIVE: To determine the incidence of antimicrobial-resistant, nonpathogenic Escherichia coli among healthy children aged 6-72 months in Camiri town and a rural village, Javillo, in south-eastern Bolivia. METHOD: A community-based survey: stool samples were obtained from 296 healthy children selected by modified cluster sampling in Camiri and all 25 eligible children in Javillo. E. coli isolates were tested for antimicrobial susceptibility according to the standard disc diffusion method. By a questionnaire survey of 12 pharmacies and by using simulated patients, we investigated the antimicrobial availability and the usage patterns in Camiri town. RESULTS: In Camiri, over 90%, and in Javillo over 70% of children carried E. coli resistant to ampicillin, trimethoprim-sulphamethoxazole (TMP/SMX) or tetracycline. Overall, 63% of children carried E. coli with multiple resistance to ampicillin, TMP/SMX, tetracycline and chloramphenicol. In the simulated patients study, antimicrobials were dispensed inappropriately for 92% of adults and 40% of children with watery diarrhoea, and were under-prescribed for males with urethral discharge (67%) or females with fever and dysuria (58%). The dose and/or duration of antimicrobials dispensed was almost always too low. CONCLUSION: Our study showed a disturbingly high prevalence of carriage of nonpathogenic E. coli resistant to antimicrobials. The prevalence of resistance to ampicillin and TMP/SMX was higher than that previously reported in developing countries. The existence of a large reservoir of resistance genes in healthy individuals in developing countries represents a threat to the success of antimicrobial therapy throughout the world. Programmes to improve rational and effective drug use in developing countries are urgently needed. Trop Med Int Health. 1998 Feb;3(2):116-23

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Carriage of antibiotic-resistant bacteria by healthy children.

Millar MR, Walsh TR, Linton CJ, Zhang S, Leeming JP, Bennett PM; ALSPAC Study Team. Avon Longitudinal Study of Pregnancy and Childhood.

Department of Microbiology, The Royal London Hospital, Barts and The London NHS Trust, Whitechapel Road, London E1 1BB, UK. M.R.Millar@mds.qmw.ac.uk

The frequency of carriage of antibiotic-resistant bacteria in healthy 7- and 8-year-old children in Bristol was studied. Children born in Avon between 1 April 1991 and 31 December 1992, attending the Avon Longitudinal Study of Pregnancy and Childhood (ALSPAC) 7 year follow-up clinic, formed the study population. Carriage was estimated using mouth and stool samples. None of 105 children on whom information was available had received tetracycline, chloramphenicol, ciprofloxacin or an extended-spectrum cephalosporin in the previous year. Staphylococcus aureus was isolated from mouthwashes from 200 (37.1%) of 539 children sampled. Six (3%) of the isolates were resistant to chloramphenicol or tetracycline and four (2%) were methicillin resistant. Haemophilus spp. were isolated from 369 (72%) of 513 samples and 63 (17%) were ampicillin resistant, 49 (13.3%) were erythromycin resistant and seven (1.9%) were tetracycline resistant. Branhamella catarrhalis was isolated from 333 (74%) of 450 samples. Twenty-eight (8.4%) were erythromycin resistant and 14 (4.2%) strains were tetracycline resistant. Group A beta-haemolytic streptococci were isolated from 17 of 507 children sampled. One (5.9%) was tetracycline resistant. Stool samples were returned from 335 (62%) of 539 children from whom they were requested. Eleven per cent of samples yielded Gram-negative bacilli with high-level resistance to chloramphenicol, which was frequently linked to resistance to ampicillin, spectinomycin and streptomycin. Isolates demonstrating resistance to the third-generation cephalosporin ceftazidime were recovered from 17 subjects (3.2%). Six (35%) of 17 isolates possessed extended-spectrum beta-lactamases. Healthy children carry bacteria resistant to antibiotics to which children are not usually exposed. Resistance to ceftazidime, chloramphenicol and tetracycline may be co-selected by exposure to other antibiotics used in children or may be acquired from family members, pets, other children or food. These results suggest that antibiotic-resistant bacteria are widely disseminated and may be acquired by children before exposure to specific selection pressure. J Antimicrob Chemother. 2001 May;47(5):605-10.

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ESTROGENIC ACTIVITY AND VOLUME FRACTION OF WASTEWATER ORIGIN IN MONITORING WELLS ALONG THE SANTA CRUZ RIVER, ARIZONA[1]

David M. Quanrud[2], Konrad Quast[3], Otakuye Conroy[4], Martin M. Karpiscak[2], Charles P. Gerba[5], Kevin .E. Lansey[6], Wendell P. Ela[4], and Robert G. Arnold[4]

The fate of estrogenic activity in wastewater effluent was examined during surface transport and incidental recharge along the Santa Cruz River in Pima County, Arizona. Based on measurement of boron isotopes, the fractional contribution of reclaimed water in surface waters and groundwater wells proximate to the river was determined for a contemporary sample set. Estrogenic activity decreased monotonically by 75 percent over the 38-km length of the river below effluent discharge points in Tucson. In groundwater samples obtained from monitoring wells that are proximate to the Santa Cruz River, both DOC ($p = 0.0003$) and estrogenic activity ($p = 3 \times 10^{-6}$) were highly correlated to fractional wastewater content. Results indicate that local groundwater quality is sensitive to incidental recharge of reclaimed water in the Santa Cruz River bed. In a few locations little attenuation of estrogenic activity was apparent during percolation of effluent in the river channel to well withdrawal points.

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Development of antibiotic resistance in purified sewage effluents subjected to chlorination]

[Article in Italian]

Morozzi G, Cenci G, Caldini G, Sportolari R, Bahojbi AG.

Antibiotic-resistance is widely spread phenomenon in the environment because of uncontrolled discharge of urban and animal wastewaters. Sewage treatment can significantly reduce the number of both sensitive and resistant bacteria. A reduction of about 1.5 logarithmic units in faecal coliforms was observed during biological treatment (3, 7), but a simultaneous increase in the percentage of resistant strains occurred because of not well understood selection phenomena. The above reported bacterial reduction is not always sufficient to meet the quality standards of Italian legislation required to discharge the treated effluents into surface waters, and so, chlorination become a compulsory additional treatment whose impact on both sensitive and resistant microflora must be evaluated. The results obtained in the present research have demonstrated that chlorine concentrations in the range of 0.5-2 ppm are able to reduce significantly the faecal coliforms concentrations and, in particular, treatment with 1 ppm of chlorine for 1 hour reduces the concentration of the above reported bacteria to the extent of 2 logarithmic units, so that their final concentration are of the about 10(2)/100 ml. The surviving chlorine tolerant bacteria seem to be antibiotic resistant in higher percentage than the chlorine sensitive ones and so, as a consequence, a significant increase in the antibiotic resistance and multiresistance was observed in the chlorinated effluents. In this context it is interesting to underline the larger variety of resistance patterns observed in the chlorine-resistant bacteria in comparison with the uniformity in the resistance patterns observed in isolated from unchlorinated effluents. The selected chlorine-tolerant strains seem to be less able to transfer their resistances under laboratory conditions, not because of curing effect of chlorine on the plasmids but, probably, because of the damage to cellular cell envelopes. Ann Ig. 1989 Jan-Apr;1(1-2):351-62.

PMID: 2483077 [PubMed - indexed for MEDLINE]

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Transferable drug resistance associated with coliforms isolated from hospital and domestic sewage.

Fontaine TD 3rd, Hoadley AW.

The incidence of antibiotic-resistant fecal coliforms in raw and treated hospital and municipal wastes was investigated to determine whether such wastes may serve as reservoirs for the spread of resistant bacteria and resistance transfer factors. Multiple resistance occurred in 87.8% of isolates from hospital and 42.6% of isolates from municipal wastes. Antibiotic resistance was transferable to Escherichia coli and Salmonella cholerae-suis recipient strains from 62.3% of resistant isolates from hospital and 90.9% of resistant isolates from municipal wastes, and from 56.2% of all isolates from hospital and 45.9% of all isolates from municipal wastes. Numbers of multiply-resistant fecal coliforms decreased during passage through a sewage treatment plant, but their proportion did not change appreciably, although proportions exhibiting resistance to 3, 4, 5, 6, and 7 drugs decreased. A study of transfer in sewage indicated that transfer of resistance from donors present in sewage to pathogenic Salmonella strains can occur under appropriate conditions. The data suggest that both raw and treated wastes, and especially those from hospitals, may serve as reservoirs for the spread of antibiotic-resistant bacteria and transferable resistance in the environment. Health Lab Sci. 1976 Oct;13(4):238-45

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Survival of coliforms and bacterial pathogens within protozoa during chlorination.

King CH, Shotts EB Jr, Wooley RE, Porter KG.

Department of Medical Microbiology, College of Veterinary Medicine, Athens, Georgia.

The susceptibility of coliform bacteria and bacterial pathogens to free chlorine residuals was determined before and after incubation with amoebae and ciliate protozoa. Viability of bacteria was quantified to determine their resistance to free chlorine residuals when ingested by laboratory strains of Acanthamoeba castellanii and Tetrahymena pyriformis. Cocultures of bacteria and protozoa were incubated to facilitate ingestion of the bacteria and then were chlorinated, neutralized, and sonicated to release intracellular bacteria. Qualitative susceptibility of protozoan strains to free chlorine was also assessed. Protozoa were shown to survive and grow after exposure to levels of free chlorine residuals that killed free-living bacteria. Ingested coliforms Escherichia coli, Citrobacter freundii, Enterobacter agglomerans, Enterobacter cloacae, Klebsiella pneumoniae, and Klebsiella oxytoca and bacterial pathogens Salmonella typhimurium, Yersinia enterocolitica, Shigella sonnei, Legionella gormanii, and Campylobacter jejuni had increased resistance to free chlorine residuals. Bacteria could be cultured from within treated protozoans well after the time required for 99% inactivation of free-living cells. All bacterial pathogens were greater than 50-fold more resistant to free chlorine when ingested by T. pyriformis. Escherichia coli ingested by a Cyclidium sp., a ciliate isolated from a drinking water reservoir, were also shown to be more resistant to free chlorine. The mechanism that increased resistance appeared to be survival within protozoan cells. This study indicates that bacteria can survive ingestion by protozoa. This bacterium-protozoan association provides bacteria with increased resistance to free chlorine residuals which can lead to persistence of bacteria in chlorine-treated water. We propose that resistance to digestion by predatory protozoa was an evolutionary precursor of pathogenicity in bacteria and that today it is a mechanism for survival of fastidious bacteria in dilute and inhospitable aquatic environments. Appl Environ Microbiol. 1988 Dec;54(12):3023-33.

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Environmental antimicrobial contamination from terraccumulation and diffuse pollution pathways.

Rooklidge SJ.

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The fate of antimicrobial pharmaceuticals entering the aquatic environment has become an increasing concern for researchers and regulators in the past decade, and recent research has focused on antimicrobial contamination from point sources, such as wastewater treatment facility outfalls. Terraccumulation is the concentration of pollutants in soils from land application of contaminated biosolids generated by agricultural practices and water and wastewater facilities. The terraccumulation of antimicrobials and mobility in diffuse pollution pathways should not be overlooked as a contributor to the spread of bacterial resistance and the resulting threat to human drug therapy. This review critically examines recent global trends of bacterial resistance, antimicrobial contaminant pathways from agriculture and water treatment processes, and the need to incorporate diffuse pathways into risk assessment and treatment system design. Alignment of environmental scientific and engineering research with strategies applied in clinical situations could contribute to continued efficacy of antimicrobial therapies necessary for human health and welfare. Copyright 2003 Elsevier B.V. Sci Total Environ. 2004 Jun 5;325(1-3):1-13

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Detection of clinically relevant antibiotic-resistance genes in municipal wastewater using real-time PCR (TaqMan).

Volkmann H, Schwartz T, Bischoff P, Kirchen S, Obst U.

Forschungszentrum Karlsruhe GmbH, Institute for Technical Chemistry, Watertechnology and Geotechnology Division, Department of Environmental Microbiology, P.O. Box 3640, D-76021, Karlsruhe, Germany.

Real-time PCR assays were developed for the quantifiable detection of the antibiotic-resistance genes vanA of enterococci, ampC of Enterobacteriaceae, and mecA of staphylococci in different municipal wastewater samples. Primer and probe designs for these resistance genes were constructed and optimised for application in standardised TaqMan PCR assays. Using reference strains, the linear measurement ranges of the assays were defined and covered concentration ranges of five to seven exponential values. Wastewater isolates of vancomycin-resistant enterococci (VRE) and beta-lactam-resistant Enterobacteriaceae were cultivated from municipal wastewaters in order to verify the specificity and sensitivity of the primer-probe systems. Additionally, clinical strains of staphylococci resistant to methicillin (MRSA) confirmed the applicability of the mecA-specific detection system. Total DNAs were extracted from five different wastewater treatment plants and used for direct TaqMan PCR detection of the resistance genes without prior cultivation. In municipal wastewater, the resistance gene vanA was detected in 21% of the samples, and ampC in 78%. The gene mecA was not found in municipal wastewater, but in two clinical wastewater samples. J Microbiol Methods. 2004 Feb;56(2):277-86

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Bacterial population changes in hospital effluent treatment plant in central India.

Chitnis V, Chitnis S, Vaidya K, Ravikant S, Patil S, Chitnis DS.

Department of Microbiology and Immunology, Choithram Hospital and Research Centre, Indore 452001, India. vikrant_chitnis@hotmail.com

Hospital effluent with its high content of multidrug resistant (MDR) enterobacteria and the presence of enteric pathogens could pose a grave problem for the community. It was planned at our tertiary care hospital in central India to study the population changes at various steps of effluent treatment plant (ETP) like collection, aeration, clarification, liquid sludge, dried sludge, high-pressure filter and treated wastewater. The study included viable bacterial counts, coliform counts, staphylococcal, enterococcal, Pseudomonas and multiple drug resistant (MDR) gram negative bacterial counts in the different stages of ETP. In order to study the distribution of bacteria as free floating in liquid and adherent to suspended particles, enumeration of the bacteria in the filterate and the sediment was also carried out. The effluent input showed 55% of the 8.6 x 10(6)/ml bacteria as coliforms and E. coli which was a typical of fecal flora. The prevalence of MDR coliforms was 0.26%. The substantial reduction (> 3log) was seen for the effluent coming from the clarifier. The bulk of the bacteria in the hospital effluent remains firmly adhered to solid particles; aeration and clarification removes bulk of the bacteria by physical processes like flocculation. The treated liquid effluent still contains sizeable loads of MDR bacteria and inactivation by procedure such as chlorination is required. The bacteria get concentrated in sludge and a greater concentration of chlorine is required for decontamination. Water Res. 2004 Jan;38(2):441-7.

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Comparison of enterococcal populations related to urban and hospital wastewater in various climatic and geographic European regions.

Blanch AR, Caplin JL, Iversen A, Kuhn I, Manero A, Taylor HD, Vilanova X.

Department of Microbiology, University of Barcelona, Barcelona, Catalonia, Spain. ablanch@ub.edu

AIMS: Scarce knowledge about the distribution of enterococci species in wastewaters limits any statement on their reliability as faecal indicators or the implications of antibiotic resistance transmission by these organisms through the water cycle. Enterococci have been involved in nosocomial infections and the spreading of antibiotic resistance through the food chain. The species distribution of enterococci and the presence of resistant strains to vancomycin and erythromycin were analysed in more than 400 raw and treated urban wastewaters, surface waters receiving these treated wastewaters and hospital wastewaters from three European countries. METHODS AND RESULTS: A total of 9296 strains were isolated and biochemically phenotyped. The species identification was based on the comparison of biochemical profiles with those of

more than 20000 enterococci isolates from an international study. The prevalence of enterococcal isolates resistant to erythromycin (ERE) and vancomycin (VRE) was also analysed. ERE strains were present in a high proportion in all the studied samples. VRE strains were also isolated in all studied countries despite the time elapsed since the use of antimicrobial glycopeptides in animal production was banned in the European Union. CONCLUSIONS: Enterococcus faecalis and Ent. faecium were the most abundant species in all the studied wastewaters. All the studied wastewaters demonstrated high diversity and similar population structure and composition. ERE and VRE isolates were detected in most of the wastewaters. SIGNIFICANCE AND IMPACT OF THE STUDY: Urban and hospital wastewaters are useful targets for the evaluation of the prevalence of ERE and VRE isolates in the environment. It appears that these bacteria could pass through wastewater treatment plants and be transferred to surface waters. J Appl Microbiol. 2003;94(6):994-1002.
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Antibiotic resistance of E. coli in sewage and sludge.

Reinthaler FF, Posch J, Feierl G, Wust G, Haas D, Ruckenbauer G, Mascher F, Marth E.

Institute of Hygiene, University of Graz, Universitatsplatz 4, Austria.
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The aim of the study is the evaluation of resistance patterns of E. coli in wastewater treatment plants without an evaluation of basic antibiotic resistance mechanisms.Investigations have been done in sewage, sludge and receiving waters from three different sewage treatment plants in southern Austria. A total of 767 E. coli isolates were tested regarding their resistance to 24 different antibiotics. The highest resistance rates were found in E. coli strains of a sewage treatment plant which treats not only municipal sewage but also sewage from a hospital.Among the antimicrobial agents tested, the highest resistance rates in the penicillin group were found for Ampicillin (AM) (up to 18%) and Piperacillin (PIP) (up to 12%); in the cephalosporin group for Cefalothin (CF) (up to 35%) and Cefuroxime-Axetil (CXMAX) (up to 11%); in the group of quinolones for Nalidixic acid (NA) (up to 15%); and for Trimethoprime/Sulfamethoxazole (SXT) (up to 13%) and for Tetracycline (TE) (57%).Median values for E. coli in the inflow (crude sewage) of the plants were between 2.0 x 10(4) and 6.1 x 10(4)CFU/ml (Coli ID-agar, BioMerieux 42017) but showed a 200-fold reduction in all three plants in the effluent. Nevertheless, more than 10(2)CFU E. coli/ml reached the receiving water and thus sewage treatment processes contribute to the dissemination of resistant bacteria in the environment. Water Res. 2003 Apr;37(8):1685-90.
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Elimination of enteric bacteria in biological-chemical wastewater treatment and tertiary filtration units.

Koivunen J, Siitonen A, Heinonen-Tanski H.

Department of Environmental Sciences, University of Kuopio, POB 1627, FIN-70211 Kuopio, Finland. jari.koivunen@uku.fi

The occurrence and removal of salmonellae and faecal indicators in four conventional municipal wastewater treatment plants (MWTP) were investigated. In addition, we tested the efficiency of a semi-technical scale biological nutrient removal process and three pilot-scale tertiary filtration units in microbial removal. All influent samples collected from MWTPs contained salmonellae from 93 to 11,000 MPN/100 ml and indicator bacteria from about 10(7) to 10(8) CFU/100 ml. The reductions in salmonella numbers achieved in full-scale biological-chemical wastewater treatment and semi-technical scale biological nutrient removal processes were usually between 94% and virtually 100% (99.9%) and indicator bacteria reductions between 2 and 3 log units. Microbial numbers in MWTP effluents could be modelled as a function of effluent residual organic matter, suspended solids and total phosphorus concentrations. Pilot-scale tertiary treatment by rapid sand contact filter, chemical contact filter and biological-chemical contact filter reduced salmonella numbers below the detection limit and faecal coliform numbers on average by 99%, 39% and 71%, respectively. A total of 32 Salmonella serovars were identified among 197 Salmonella isolates from municipal wastewaters. Of the isolates, 32% were resistant to nalidixic acid, indicating reduced sensitivity to ciprofloxacin, the drug of choice in the treatment of salmonellosis. In addition, 18% of the isolates were multiresistant. Our results, especially antibiotic resistant Salmonella strains, indicate that conventional municipal wastewater treatment without efficient tertiary treatment, like filtration or disinfection, may constitute a risk for public health. Water Res. 2003 Feb;37(3):690-8.

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Impact of long-term application of industrial wastewater on the emergence of resistance traits in Azotobacter chroococcum isolated from rhizospheric soil.

Aleem A, Isar J, Malik A.

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A total of 57 (36 and 21) Azotobacter chroococcum were isolated from wheat (Triticum aestivum) rhizospheric soil irrigated with industrial wastewater (about a decade) and ground water (uncontaminated) and characterized on the basis of morphological, cultural and biochemical characteristics. Rhizospheric soils were analyzed for metal concentrations by atomic absorption spectrophotometery and the test soil samples were contaminated with Fe, Zn, Cu, Cr, Ni and Pb. All the isolates of A. chroococcum were tested for their resistance against Hg2+, Cd2+,

Cu2+, Cr3+, Cr6+, Zn2+, Ni2+ and Pb2+. Among 36 isolates of Azotobacter from soil irrigated with industrial wastewater, 94.4% were resistant to Pb2+ and Hg2+ and 86.1%, 77.5% and 63.8% were resistant to Zn2+, Cr6+ and Cr3+ respectively. The highest minimum inhibitory concentration of 200 microg/ml for Hg2+ and 1600 microg/ml for other metals were observed against these bacteria from soil. The incidences of metal resistance and MICs of metals for A. chroococcum from wastewater irrigated soil were significantly different to those of uncontaminated soil. All A. chroococcum isolates were tested for their resistance against 11 commonly used antibiotics/drugs. 91.6% were found to be resistant against nitrofurantoin while 86.4% and 80.5% were found to be resistant against polymyxin-B and co-trimoxazole respectively. Agarose gel electrophoresis using the miniprep method for plasmid isolation revealed that these isolates harboured plasmids of molecular weights 58.8 and 64.5 kb using EcoRI and HindIII digests of X DNA and undigested X DNA as standard markers. Bioresour Technol. 2003 Jan;86(1):7-13.

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Fecal coliforms of wastewater treatment plants: antibiotic resistance, survival on surfaces and inhibition by sodium chloride and ascorbic acid.

Abu-Ghazaleh BM.

Department of Biology, Hashemite University, Zarqa, Jordan.

Three hundred and sixteen fecal coliform strains isolated from raw sewage and final effluents of two representative wastewater treatment plants were examined for antibiotic resistance. For plant A (which was disinfecting its effluents before discharge), 55% and 41% of the strains isolated from the influent and the effluent, respectively were resistant to one or more antibiotics. For plant B (which was not disinfecting its effluents before discharge), 33% and 58% of the strains isolated from the influent and the effluent, respectively were resistant to one or more antibiotics. A considerable proportion of these bacteria were resistant to three or more antibiotics. NaCl (3%) and ascorbic acid (0.1%) reduced the growth rate of both sensitive and resistant strains, and more inhibitory effects on the sensitive strains than on the resistant strains were observed. All resistant strains tested survived on various types of surfaces (e.g. glass, stainless steel, agar, cabbage, parsley and sand) significantly better than sensitive strains. New Microbiol. 2001 Oct;24(4):379-87.

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These search terms have been highlighted: hospital sewer effluent

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RSO Magazine6, No. 1 13Concentrations of Iodine-131 Released from a Hospital Into a Municipal Sewer by Ingvar L. Larsen, E. A. Stetar, B. G. Giles, and B. Garrison

ABSTRACT--- Samples of wastewater effluent from a local hospital were collected at the point of entry into a municipal sewer system and analyzed for 131I. The effluent contained high concentrations of 131I as a result of excretions from a patient treated with an ablation dose of 131I. External dose rate measurements of the patient over time allowed the calculation of 131I released into the sewer system. Measured concentrations of the effluent corresponded to estimated values based on typical hospital water usage flow releases. These concentrations were used to assess radiation levels and potential contamination of 131I to sewer maintenance crews performing emergency repairs in the sewer downstream from the hospital effluent. Key words Radioactive 131I Ablation dose Whole body dose readings Wastewater concentrations Effluent release

INTRODUCTION--- Municipal sewer systems receive a wide variety of radionuclides from both natural and anthropogenic sources. Natural sources include contributions from local water supplies, storm runoff, industrial disposal of chemical waste products, and digested food products in excreta.[1]These radionuclides include 40K, 226/228Ra, 7Be, and uranium. Anthropogenic sources include fallout radionuclides such as 137Cs; industrial processed contaminated reactor materials such as 60Co and 137Cs; depleted uranium from uranium processing facilities, and medical radionuclides from hospitals.[2,3]Industrial releases into municipal sewer systems of anthropogenic generated radionuclides are regulated through state or federal licensing procedures. Medical radionuclides, however, in the excreta of patients, are exempt from licensing regulations.[1]Iodine-131 (8.04 d half-life) is one medical radionuclide used for both diagnostic and therapeutic applications. Diagnostic applications typically use ~0.4GBq (10 mCi) while thyroid ablation doses are much higher, typically in the 3-5 Gbq (~100 mCi) range.

RELEASES OF 131I---- Patients when treated with ablation doses of 131I are confined in the hospital for a few days to allow external radiation levels to decline to acceptable levels before release. The relatively rapid decline in activity is due primarily to the biological elimination of 131I from the body. As a result of this elimination, considerable amounts of radioactive 131I are discharged within the patient excreta into the local municipal sewer. Elevated concentrations of 131I have been observed in municipal wastewater treatment

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plants following the application of ablation doses.[1,4] In Oak Ridge, Tennessee, reported concentrations in grab samples following ablation doses have ranged from 1100 Bq/L (~30,000 pCi/L) in the wastewater treatment plant influent (raw sewage) to 1700 Bq/L (46,000 pCi/L) in the plant's secondary treatment system (aerobic) sludge.[2] Based on hospital estimates, roughly 18.5 GBq (500 mCi) of 131I is released to the Oak Ridge sewer each year. Although the 131I concentrations observed within the Oak Ridge treatment plant were not considered a significant source of radiation exposure to plant operators, they did raise concerns about potential exposures to individuals performing sewer-line maintenance downstream of the hospital. During transit through the sewer system, the discharged 131I undergoes significant dilution and is further reduced by decay and physical losses. Therefore, the 131I concentrations in the sewer line at and near the point of discharge were expected to be much higher than those observed entering the plant. To reduce the potential for exposures to workers, a cooperative agreement between the City of Oak Ridge and the local hospital was established to notify sewer-line maintenance crews before the administration of ablation doses of 131I. This agreement in keeping with the practice of ALARA (as low as reasonably achievable), allows routine maintenance activities near the hospital to be rescheduled until 131I activity

levels diminish to near background levels. However, under some circumstances, such as line blockages, delaying sewer line repairs may not be possible. Therefore, evaluating the external exposure rates and 131I concentrations to which sewer-line personnel could be exposed during emergency repairs was necessary. To determine the 131I concentrations released via patient excreta from the hospital into the municipal sewer system following an ablation dose, samples of the local hospital's wastewater effluent into the municipal sewer system were collected and analyzed. On 4 August 1997, at 5pm, a patient was administered an oral dose of 3.8 GBq (102.6 mCi) of 131I (as sodium iodide). During the hospital internment, exposure rate measurements were taken on the patient until the measurements diminished to acceptable levels before the patient's release. The measurements were performed using a beta-gamma ionization survey instrument (Keithley model 36150) at the surface of the neck, stomach, thighs and also at Table 1. Exposure rate measurements on a 20-year-old patient after receiving 3.8gbq (102.6 mCi) of ingested (sodium iodide) 131I ~17:00 4 August 1997. Units are shown in mR/hr. Neck Stomach Date Time Surface @1 m Surface @1 m Thigh Surface 4 August 17:00 110 21.9 275 21.9 40 5 August 17:30 54 5.1 65 5.2 24 6 August 09:00 10 1.1 14 1.3 5 Table 2. Estimated amount of 131I released from the patient into the sewer after correcting for physical decay Date (1997) 131I mCi(Gbq) From To Released 4 Aug 5pm 5 Aug 17:30 70 (2.6) 5 Aug 5:30pm 6 Aug 09:00 17 (0.6) Estimated total released to sewer: 87 (3.2)

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one meter distance (Table 1). On 6 August 1997, the patient was released from the hospital in the early afternoon. Since the patient's residency was outside the Oak Ridge area, any additional contributions of 131I to the local sewer from this source would not be anticipated. However, this does not exclude the possibility of other local residents being treated at other hospitals in the Knoxville, Tennessee, area, and contributing an additional source of 131I to the Oak Ridge municipal sewer system. In addition, diagnostic treatments consisting of 0.37 GBq (~10 mCi) of 131I are occasionally administered and the patient allowed to return home without hospital internment. One Oak Ridge resident did receive such a diagnostic dose on 20 August 1997.

LOSSES OF 131I FROM THE PATIENT The amount of radioactive 131I discharged into the sewer was estimated from exposure readings taken at a distance of 1 meter from the neck and stomach of the patient and corrected for physical decay (Table 2). Based on these calculations some 2.6 Gbq (~70 mCi) of 131I (after correcting for physical decay losses) were released from the hospital into the sewer within approximately a 24-hour period.

SAMPLE COLLECTION On 4 & 5 August 1997, wastewater containing pulses of 131I were discharged from the hospital into the local sewer system. Composited samples of hospital waste-water entering the sewer were collected from a manhole near the entry point using an automated ISCO water sampler. Grab samples were collected before the release and intermittently during the first few hours following the release. Grab sample collection times were based on qualitative measurements of increased radiation levels using an end-window G-M probe within the manhole.

SAMPLE ANALYSES Samples of the collected hospital wastewater and plant influent were transferred to half or one liter Marinelli beakers for 131I analyses. Samples were counted on a high purity germanium detector (HPGe) having a cobalt relative efficiency of 25% and a resolution at 1332 keV of 2.0 keV. The detector, shielded with 10cm of low background lead, is coupled to a Nuclear Data 6700 microprocessor system. Software using a peak search routine, ambient peak background subtraction, nuclide identification, and quantification, was that of the vendor. Calibration of the detector followed the methodology described by Larsen and Cutshall.[5]High activity samples were either diluted with distilled water, or allowed to decay before final analysis. Samples were decay-corrected back to the time of sampling. Corrections for decay-during-sampling compositing were also made similarly to decay-during-counting. That is, a 24-hour composite sample would have an additional 131I decay correction factor of 1.044 to correct for the decay from the beginning of sampling to completion of sampling, in addition for decay corrections to the time of analysis. Table 3 summarizes the 131I concentrations in the grab samples and composite samples collected at the hospital's discharge point into the city's sewer line. Replicate samples of composited wastewater from the hospital were collected and analyzed. Quantifying the actual effluent volume flow associated with the

131I release from the hospital during the sample collection period was not done. However, typical flow releases from previous determinations were available (Table 4) and show the order of magnitude of flow volumes anticipated.

RESULTS Based on grab sampling, the peak 131I concentrations present in the manhole ranged from 0.14 to 2MBq/L (4 to 50 uCi/L). The maximum radiation exposure rates associated with these peak concentrations were approximately 0.02mSv/hour (2 mrem/hour). These radiation levels were transient, lasting only during the passage of the pulse. Between pulses, the radiation levels within the manhole were at background levels. Using the estimated amount of 131I

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released from the patient into the sewer system over the first 24 hours (~70 mCi or 2.6 G Bq), and an estimated mean flow of 3E05 L/d, an overall 131I concentration of 8.7E03 Bq/L (2.6E09Bq/3E05 L) or 2.3E05 pCi/L would be anticipated. This estimated value based on an overall assumed mean flow for the discharge day is within the range of the observed composited samples. Typically, higher concentrations would be anticipated with lower volume discharges, and conversely, lower concentrations with higher volume discharges during this period.

CONCLUSIONS--- An ablation treatment dose of ~3.8 Gbq (102.6 mCi) of 131I in a patient at a local hospital, resulted in 131I releases of approximately 3.2 Gbq (87 mCi) entering the sewer system over a 40-hour period. Measured aqueous concentrations of 131I in grab samples were taken at the point of the hospital's discharge into the local sewer system. These samples revealed concentrations as high as 2MBq/L (5.4E07 pCi/L). Time integrated composited samples ranged from ~2E02 to ~7E04 Bq/L (~5E03 to ~2E06 pci/L), depending on liquid flow discharges coupled with patient excreta releases. These released concentrations diminish with time and dilution and eventually enter the wastewater treatment plant. Table 3.131I Concentrations in wastewater discharges from the hospital into the municipal sanitation sewer line Grab Samples Date/time 131IBq/L +/- sigma 01 pre dose 4 August noon Not Detected 02 Grab 4 August 18:45 1.44E05+/-3.30E02 03 Grab 4 August 19:15 8.51E05+/-1.24E03 04 Grab 4 August 20:15 2.01E06 +/-4.11E03 05 Grab 4 August 21:25 5.92E05+/-1.21E03 Distilled water blank 7 August 7:00 Not Detected Composite samples 01A 4 August 5-8 pm 2.35E04+/-8.29E01 01B (replicate) 2.32E04+/-4.74E01 02A 4 August 8-11 pm 6.72E04+/-2.08E02 02B (replicate) 6.59E04+/-2.12E02 02C (replicate) 6.40E04+/-1.97E02 03A 4/5 August 11pm-6am 1.96E02+/-4.14E00 03B (replicate) 2.11E02+/-3.77E00 04A 5 August 6-9 am 2.36E02+/-3.85E00 04B (replicate) 2.03E02+/-3.49E00 05A 5 August 9am-12 noon 7.73E04+/-1.89E02 05B (replicate) 8.07E04+/-2.01E02 06A - 5-6 August 12 noon 3.77E03+/-2.60E01 06B(replicate) 12 noon 3.88E03+/-3.21E01

Flow-proportional, time-integrated influent 131I water concentrations collected at the treatment plant are listed in Table 5. A complete continuous record was not available, but the influent concentrations correspond to the treatment period and illustrates the decrease over approximately a 5-day period. The purpose of the sampling effort was to assess the radiation levels and concentrations of 131I to which sewer crew workers performing emergency repairs following an ablation dose might be exposed. The maximum radiation levels observed, which were associated with the peaks of the I-131 pulses entering the system, were 0.02 mSv/hour (2 mrem/hour). This exposure rate is assumed to be the "worst-case" level to which sewer workers would be exposed while performing repairs immediately down-stream from the hospital. Based on the concentrations of 131I measured in the sewer line (up to 120,000 dpm/ml), **the potential for contamination of worker clothing, tools, equipment, and the soils surrounding the sewer line is high.** Therefore, in the event repairs must be done downstream from the hospital following an ablation dose, the City of Oak Ridge will ensure that a trained health physicist is present to monitor radiation contamination levels and assist in decontamination as needed. References 1. Larsen, I.L., E.A. Stetar, and K.D. Glass, "In-House Screening for Radioactive Sludge at a Municipal Wastewater Treatment Plant," Radiation Protection Management, 12(4):29-38, 1995. 2. Stetar, E.A., Task 7 Report: "Evaluation of Radionuclide Implications

for Treatment Options for the Oak Ridge Wastewater Treatment Plant Sludge," Technical Memorandum to the City of Oak Ridge, TN., November 22, 1993. 3. Larsen, I.L., S.Y. Lee, H.L. Boston, and E.A. Stetar, "Discovery of Cs-137 Hot Particle in Table 4. Typical effluent flow volumes from the hospital wing into the sewer Liter/Day x 103Liters/Minute Minimum Flow 76 53 Mean Flow 300 220 Normal Peak Flow 490 340 Maximum Peak Flow *600 420 *Maximum peak flow duration typically ranges from 1 to 2 hours near noon and normal peak flow occurs during early morning through late afternoon. Table 5. Wastewater treatment plant flow proportional time integrated volume influent and 131I concentrations Date 6am-6am Influent Volume, Liter/d (x 106) Influent 131I Bq/Liter*4-5Aug 18.9 0.36+/-0.04 5-6 15.9 NS* 6-7 17.4 17.50+/-0.36 7-8 17.0 3.58+/-0.18 8-9 17.8 1.25+/-0.16 9-10 15.9 1.16+/-0.08 10-11 16.3 0.10+/-0.06 11-12 16.3 1.00+/-0.10 12-13 17.4 NS* 13-14 16.3 NS* 14-15 18.2 NS* * NS No Sample

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Municipal Wastewater Treatment Sludge," Health Physics, 62(3):235-238, 1992. 4. Fenner, F.D., and J.E. Martin, "Behavior of Na I-131 and Meta (I-131) Iodobenzylguanidine (mibg) in Municipal Sewerage," Health Physics, 73 (No.2):333-339, 1997. 5. Larsen, I.L., and N.H. Cutshall, "Direct Determination of Be-7 in Sediments," Earth and Planetary Science Letters, 54:379-384, 1981.

ACKNOWLEDGMENTs --- Research performed under subcontract ERD-88-793 with The City of Oak Ridge, Tennessee under Lockheed Martin Energy Research. Contract DE-AC05-96OR22464 with the U. S. Dept of Energy. Accordingly, the U.S. Government retains a nonexclusive, royalty-free license to publish or reproduce the published form of this work, or allow others to do so, for U.S. Government purposes. About the Authors Ingvar L. Larsen has over 35 years of experience in the use of radionuclides with regard to understanding and quantifying environmental processes—the determination of contaminant pathways, transport rates, and accumulation areas. In addition, he has developed instrumental methods for quantifying radionuclide concentrations in various matrices. I.L. Larsen Teledyne-Brown Engineering, Inc. 2508 Quality Lane Knoxveille, TN 37931-3133 Phone: 865/690-6819 E-mail: Lauren.Larsen@tbe.com Elisabeth Stetar is a certified health physicist and consultant in the areas of regulatory compliance, environmental monitoring, and dose assessment. She has extensive experience in decontamination applications and the evaluation of the impact of radionuclides discharged into sanitary sewers. Bruce G. Giles holds a BS degree in environmental health and is an employee of the City of Oak Ridge, Tennessee, serving as the environmental and regulatory compliance coordinator. Beverly Garrison has served as a medical technician in the area of radiology.

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Pharmaceutically Active Compounds in Residential and Hospital Effluent, Municipal Wastewater, and the Rio Grande in Albuquerque, New Mexico by Kathryn D. Brown Water Resources Program The University of New Mexico Albuquerque, New Mexico www.unm.edu/~wrp/Publication No. WRP-9 January 2004

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iiNote: This report is the Professional Project report of Kathryn D. Brown, submitted in partial fulfillment of the requirements for the Master of Water Resources degree at the University of New Mexico. The project was supervised and approved by the following committee: Dr. Bruce M. Thomson, Department of Civil Engineering, UNM (Chair); Dr. Michael E. Campana, Water Resources Program and Department of Earth and Planetary Sciences, UNM; and Mr. Jerzy Kulis, M.S., State of New Mexico Environment Department.

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iiiACKNOWLEDGEMENTS I would like to thank … My committee chairman Bruce Thomson for his knowledge and interest in this project, Michael Campana for his support and encouragement on this project and his tireless dedication to the Water Resources Program, and Jerzy Kulis, from NMED for his willingness to collaborate with me and share his experience. Tim Chapman, Dr. Doug Mawhinney, and Rick Meyerhein from SLD for their impressive technical expertise and willingness to perform the dirty (read "raw sewage") work involved in analyzing my samples. The Graduate and Professional Student Association of the University of New Mexico for the $4,950 high-priority Graduate Research Development grant that covered analytical costs and allowed me to undertake this project; and the Student Research Allocations Committee grant of $376 that helped support travel costs to attend an important PhAC conference. Earl Browning, Tim Mellady, Bobby Kenton, Danny Gonzales, and Michael Garcia for granting me access to the bowels of the city and assisting me with effluent sampling. Dave Kearsey, Hema and Lauren at the City of Albuquerque Lab for their help with and careful handling of my samples. Carolyn Piro at Ted Miller and Associates for loaning me the ISCO sampler. Dr. Laura Crossey and John Craig at the Biology Annex Lab for sharing their precious equipment to allow for general chemistry measurements. My employer, UNM Hospitals, for their flexibility while pursing this Master's degree.

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ivLouis Achusim, Director of Pharmacy at UNM Hospital for his assistance with clinical PhAC issues. The late Marc Reisner and Rachel Carson for their vision and for inspiring me to seek a degree in Water Resources. My parents for nurturing my confidence, teaching me perseverance, and always encouraging me to pursue my dreams. Ona Porter and Miriam Rand for opening up their office space for my use in writing this document…it was a good idea to be without a phone or internet access at a time like this. My friends who have stuck with me despite my busy schedule and distracted behavior…now, I promise, I really will call you back. My dog, Utah, who also stuck by my side and is in desperate need of a good long hike. Jami, my constant source of joy, for her love, encouragement, and shared passion for rivers.

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1**ABSTRACT**--- This project investigated: 1) the contribution of pharmaceutically active compounds (PhACs) from residential and hospital effluent sources, 2) resultant concentrations of PhACs in the Albuquerque Southside Water Reclamation Plant (SWRP) raw influent and treated effluent, and 3) concentrations of PhACs in the Rio Grande, which receives SWRP effluent. PhACs present in surface waters have been shown to adversely impact organisms (Jobling et al., 1998) and, in the case of antibiotics, perhaps increase resistance to these drugs (Ash, 1999; Eichorst, 1999; Guardabassi et al, 1998; Sternes, 1999). In this study, ten sample sites were identified and samples collected and analyzed for the presence of 39 PhACs, consisting of 29 non-antibiotic PhACs and 10 antibiotics. The Scientific Laboratory Division of the New Mexico Department of Health (SLD) conducted all analyses. Antibiotic analyses involved solid phase extraction, high performance liquid chromatography, and tandem mass spectrometry while the non-antibiotic PhACs were analyzed using liquid-liquid extraction, gas chromatography, and tandem mass spectrometry. Six antibiotics (sulfamethoxazole, trimethoprim, ciprofloxacin, ofloxacin, lincomycin, and penicillin G) and caffeine were detected in hospital wastewater (300-35,000 ng/l), while only one antibiotic, ofloxacin, was detected in wastewater from one of the two residential sites (1,300 ng/l). Three antibiotics: sulfamethoxazole, trimethoprim, and ofloxacin were present in both SWRP influent and treated effluent in concentrations ranging from 110 ng/l to 470 ng/l. However, concentrations in the treated effluent were

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reduced 20 to 77 percent. No PhACs were detected in the Rio Grande sample upstream of the SWRP discharge, and only one antibiotic, sulfamethoxazole, was detected in the two Rio Grande samples below SWRP. These results reveal that most of the PhACs analyzed for were absent or at undetectable concentrations in wastewater. However, antibiotics, particularly some sulfonamides and fluoroquinolones, were found at relatively high concentrations in hospital wastewater and were not completely removed by

wastewater treatment. In particular, the sulfonamide antibiotic, sufamethoxazole, displayed high persistence and was detected at concentrations of 300 ng/l in the Rio Grande.

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31.0 **INTRODUCTION---** Pharmaceutically active compounds (PhACs) such as analgesics, anti-convulsants, anti-depressants, anti-inflammatories, hormones, and antibiotics can enter municipal and natural water systems via residential or commercial discharges, including hospital effluent. Although PhACs are intended to be utilized by the human body, in some instances as much as 50 to 90 percent of an administered drug may be excreted by the body in a biologically active form (Raloff, 1998). Wastewater treatment facilities vary in their ability to remove PhACs. Consequently, PhACs are released into surface waters where they may adversely impact aquatic organisms (Jobling et al., 1998) and, in the case of antibiotics, perhaps increase resistance to these drugs (Ash et al., 1999; Eichorst et al., 1999; Guardabassi et al., 1998; Sternes, 1999). In 2000, the New Mexico Environment Department (NMED) and the Scientific Laboratory Division of the New Mexico Department of Health (SLD) initiated a study of PhACs in New Mexico waters. NMED detected a variety of drug residues in 11 of 15 sewage effluent samples and in 4 of 23 surface water samples (McQuillan et al., 2000, 2001, and 2002). Estrogenic hormones were detected in trout and silvery minnow habitats in the San Juan and Rio Grande rivers respectively (McQuillan et al., 2002), at levels that have been shown to cause sexual disruption of wild fish in Europe (Jobling et al., 1998). Antibiotics like those found by NMED in New Mexico sewage effluents (McQuillan, 2002), and in streams worldwide (Heberer et al., 2001; Sedlak and Pinkston, 2001) are of concern due their possible connection to the development of antibiotic-resistant

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organisms, the potential for disruption of microbial ecology, complications surrounding development of water reuse technologies, and even increased human health risks (Daughton and Ternes, 1999; Guardabassi et al., 1998; Huang et al., 2001). The development of antibiotic-resistant bacteria is an increasing concern. Recent studies have found widespread antibiotic-resistant bacteria in the Rio Grande (Sternes, 1999), in several major U.S. rivers (Eichorst et al., 1999), and in wild Canada geese (Ash et al., 1999). The widespread and often inappropriate administration of antibiotics in livestock, pets, and humans has been shown to result in the development of antibiotic-resistant bacteria and is generally accepted to be the primary pathway for proliferation of antibiotic-resistant bacteria in the environment (Shagum, 2003, personal communication, unreferenced). However, there is concern that long-term, low dose concentrations (ng/l-µg/l) of antibiotics, such as those present in wastewater and surface water, could also result in the development of antibiotic-resistant organisms. Although there is a paucity of literature addressing this potential pathway, one study has shown increased prevalence of antibiotic-resistant Acinetobacter spp. in sewers receiving wastewater effluent from a hospital and a pharmaceutical plant (Guardabassi et al., 1998). Specifically, sewers downstream from the hospital displayed an increased prevalence of bacteria resistant to oxytetracycline, while sewers downstream from the pharmaceutical plant showed an increased prevalence of bacteria resistant to multiple drugs, including sulfamethoxazole. The results of this study and in particular, the findings at the pharmaceutical plant, seem

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to lend credence to the concern that antibiotic-resistant bacteria could develop from environmental exposure to pharmaceuticals. However, although concerning, this study alone is not sufficient to determine the relative risk. Consequently, while the presence of antibiotics in wastewater and surface water is discussed widely in the literature as an area of concern it is also identified as a topic in much need of further investigation. Additionally, in hospital effluent, ciprofloxacin was detected at levels from 3 µg/l to 87 µg /l (Hartmann et al., 1998). Ciprofloxacin, a fluoroquinolone antibiotic, was shown to display high genotoxicity at these concentrations. Genotoxic substances are often also mutagenic and carcinogenic and are therefore especially concerning. Furthermore, the presence of genotoxic antibiotics in hospital effluent is of particular concern for its possible connection to proliferation of antibiotic-resistant organisms. Although several studies have detected the occurrence of antibiotics in hospital effluent (Alder et al., 2003; Feldmann et al., 2003; Hartmann et al. 1998), little is known about their fate or effects in the environment (Guardabassi et al, 1998; Hartmann et al. 1998) This study investigated hospital and residential effluents for their potentially significant contribution of PhACs to wastewater systems, such as the Albuquerque Southside Water Reclamation Plant (SWRP). Wastewater effluent has been shown to be a primary

contributor of PhACs to surface water (Daughton and Ternes, 1999). Surface run-off, mainly from confined animal feed operations, is also a significant contributor of PhACs to surface water, but is not specifically addressed in this study (Daughton and Ternes, 1999).

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This professional project was conducted in collaboration with NMED and SLD to investigate: 1) the contribution of PhACs from residential and hospital effluent sources and, 2) the resultant PhAC concentrations in Albuquerque's SWRP raw influent, treated effluent, and in the Rio Grande,which receives SWRP effluent. While it is generally accepted that hospitals are a primary point source for PhACs in water, there is little literature documenting the quantities contributed (Hartmann et al., 1998). In fact, the EPA website identifies the issue of hospital vs. residential contributions of PhACs as one of its top research needs (Daughton, 2002). Additionally, research indicates that sunlight can degrade some PhACs, notably fluoroquinolone and tetracycline antibiotics (Buser et al., 1998; Huang et al., 2001). Given New Mexico's prevalent sunlight, and wide and shallow river morphology, this degradation process is of particular significance. In prior studies, concentrations of PhACs ranging from 1 ng/l to 100 ng/l seemed to correlate with a region's population density (Raloff, 1998). Similarly, the highest concentrations tended to show up in the smallest rivers, where 50 percent of the water could be sewage treatment effluent (Raloff, 1998). The SWRP effluent is a major contributor of flow in the Rio Grande and is considered the fifth largest tributary to the Rio Grande (Stomp, 2003, personal communication, unreferenced). The City of Albuquerque plans to divert additional Rio Grande water as part of the San Juan-Chama Diversion Project and Albuquerque Drinking Water Program (City of Albuquerque, 2003). The diversion of approximately 94,0000 acre-feet/year (af/y) will occur in Albuquerque north of Paseo del Norte Blvd. and the return flow of

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approximately 47,000 af/y will occur in the Albuquerque South Valley via the SWRP effluent. Operation of the diversion is only planned for conditions when the river flow is large, hence although 15 miles of the Rio Grande will have a diminished flow, this reduction will be small. Predicting the actual reduction in Rio Grande flow attributed to this change from ground to surface water diversion is a complicated hydrologic process involving connections between the river and groundwater aquifers. However, the City of Albuquerque predicts the effective loss of water in this stretch of the Rio Grande to be only 34,000 af/y, not the full 94,000 af/y. This prediction is based on the expected contribution of additional water to the Rio Grande from the surrounding aquifer once groundwater pumping is reduced. Ultimately, however, flow in the Rio Grande through Albuquerque will be diminished to some extent while the quantity of SWRP effluent remains the same. Consequently, the flow contribution from the SWRP effluent will be a higher percentage of total river flow, resulting in a greater impact to the water quality of the river. (See Appendix A for calculations of SWRP effluent and Rio Grande flow rates and dilution) As part of the Albuquerque Drinking Water Program, surface water will be used for drinking. This raises the questions of whether PhACs might be present in the surface water to be used and, if present, will treatment techniques be capable of removing them? Because very little is known about safe allowable limits for drinking water or about the temporal and spatial fluctuations of PhACs in surface waters, this is a significant concern.

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While previous studies have found no detectable concentrations of PhACs in drinking water samples in New Mexico (McQuillan, 2000), PhAC have been detected in U.S. municipal drinking water revealing that at least some conventional treatment processes are not fully effective in removing all PhACs (Stackelberg et al., 2003). Additionally, the combined effects of drought and increased diversions could push concentrations of PhACs to levels of concern.

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2.0 **METHODS**--- 2.1 Selection of PhACs A total of 10 antibiotics and 29 other non-antibiotic PhACs were selected for testing (Table 1). Selection of PhACs was based on five factors: 1) analytical capabilities of SLD, 2) data identifying the most commonly prescribed drugs in the US (Table 2) and at UNM Hospital in Albuquerque, NM (Achusim, 2003, personal communication; unreferenced), 3) classes of drugs with known and suspected environmental and species impact (Ash et al., 1999; Eichorst et al., 1999; Jobling et al., 1998; McQuillan et al., 2002), 4) classes of drugs that persist in aqueous environments and have previously been detected in wastewater and natural waters (Huang et al., 2001), and 5) PhACs included in

previous NMED studies that will offer a comparison group (McQuillan et al., 2001). Table 1: PhACs investigated in this study Drug Class Non-antibiotic PhACs (29 Total) Analgesics propoxyphene (Darvon) Anti-Convulsants phenytoin (Dilantin) Anti-Depressants fluoxetine (Prozac), sertraline (Zoloft), amitriptyline, protriptyline, trimipramine maleate, nortriptyline, desipramine, imipramine, doxepin, nordoxepin, paroxetine Anti-Inflammatory methyprednisolone, prednisone Hormones equilin, 17β-estradiol, estrone, 17α-ethynyl estradiol, medroxyprogesterone, megestrol acetate, mestranol, progesterone, norethindrone, norethynodrel, norgestrel, cholesterol Other caffeine, tamoxifen Antibiotics (10 Total) Antibiotics norfloxacin, lincomycin, oxytetracycline HCl, ciprofloxacin, ofloxacin, trimethoprim, penicillin G. 1/2 – benzathine salt, sulfamethoxazole, penicillin V potassium salt, tylosin tartrate

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Table 2: Most commonly prescribed drugs in the United States (McQuillan et al., 2001; RxList, 2003). Drug Class Specific DrugsAnalgesics hydrocodone, ibuprofen, propoxyphene (Darvon), acetaminophen Antibiotics amoxicillin, azithromycin, cephalexin, ciprofloxacin, clarithromycin, penicillin VK Anti-Convulsants diazepam, phenytoin (Dilantin) Anti-Depressants fluoxetine (Prozac), paroxetine (Paxil), sertraline (Zoloft), amitriptyline Cardiovascular amlodipine, digoxin, enalapril, lisinopril, furosemide, diltiazem Hormones thyroxine, estrogen hormones Lipid Lowering Agents atorvastatin, lovastatin, simvastatin

Selection of Sampling Sites Locations of sampling sites are presented in Table 3. Sampling sites were selected to address three primary objectives: 1) investigate point source contributions of PhACs from hospital and residential sources, 2) determine removal of PhACs by a well run treatment plant and 3) investigate the occurrence and fate of PhACs in the Rio Grande, upstream and downstream of SWRP. Contributions of PhACs from hospital and residential sources to wastewater have not been well documented (Daughton, 2002). In this study, sample sites 1-5 were selected to address this issue (Table 3). Hospitals were selected because, while it is generally accepted that they are a significant point source contributor of PhACs, there is little literature documenting the quantities contributed (Hartmann et al., 1998). Three hospitals were selected based on their patient population profiles, ease of accessibility to effluent pipes, and willingness to participate in study. Two residential sites were selected to

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Table 3: Locations of sampling sites Site No. Sample Site Name Details of Site Location 1 Presbyterian Hospital Hospital effluent at NE corner of Silver and Oak St. (manhole) 2 University Hospital Hospital effluent at NE corner of Lomas Blvd and hospital entrance road (sewer access) 3 VA Hospital Hospital effluent under overhang at main entrance to hospital (manhole) 4 UNM Alvarado DormitoryDormitory effluent south of Campus Dr. and west of loading ramp (manhole) 5 Vista del Rio Assisted Living/Retirement CommunityFacility effluent via cleanout pipe located off north side of building at the edge of the NW parking lot (clean-out) 6 SWRP influent City of Albuquerque laboratory official daily influent sample (called T.P. 2.3 by city) 7 SWRP effluentCity of Albuquerque laboratory official daily effluent sample (called T.P. 2.7 by city) 8 Rio Grande 1 At Los Calabacitas Arroyo, north of Paseo del Norte Bridge; upstream of SWRP discharge 9 Rio Grande 2 Approximately 1.0 mile downstream from SWRP discharge 10 Rio Grande 3 Approximately 1.5 miles north of I-25 interstate bridge; approximately 4.0 miles downstream from SWRP represent potential contributions from the population at large. The UNM Alvarado dormitory was selected to represent a relatively young population, while Vista del Rio Assisted Living/Retirement Community was selected to represent a more elderly population. The sample collection times for hospitals were selected based on prior research showing that concentrations of PhACs in hospital wastewater vary throughout the day with peaks between 6 a.m. and 10 a.m., and between 6 p.m. and 8 p.m. (Guiliani et al., 1996; Feldmann, 2003, personal communication; unreferenced). By selecting collection times during the potentially peak hours of 6 a.m. to 10 a.m., samples were more likely to contain PhACs.

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The SWRP influent and effluent were selected to allow comparison of PhAC concentrations before and after wastewater treatment. Rio Grande 1 sampling site was selected to measure the occurrence of PhACs in the river upstream from the SWRP effluent location. Rio Grande 1 is also near the proposed intake for the City of Albuquerque Drinking Water Program (City of Albuquerque, 2003). The Rio Grande 2

sampling site was located downstream of SWRP effluent, and intended to be just far enough below the discharge point to allow mixing of effluent with river water. The comparison of Rio Grande 1 with Rio Grande 2 allows comparison of Rio Grande waters before and after the addition of SWRP discharge. Rio Grande 3 was selected to offer insight into the fate and persistence of PhACs in the Rio Grande.

SAMPLING PROTOCOL ---This study was conducted in accordance with the EPA-approved Quality Assurance Project Plan (QAPP) for the NMED (New Mexico Environment Department, 2003). Samples were collected between March 30, 2003 and May 7, 2003. The sampling at sites 1-5 was performed using an ISCO GLS automated composite sampler (Table 3). At these five sites, forty-eight 125 ml samples were collected in 5-minute increments between 6 a.m. and 10 a.m., resulting in 6-liter composite samples. Each collection was compiled in a 2.5 gallon glass bottle inside the ISCO sampler, surrounded by ice and protected from sunlight. After collection, the composite samples

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were transferred to six 1-liter brown glass bottles, stored on ice, and delivered to SLD within 24 hours. For sampling sites 6 and 7, samples were collected by City of Albuquerque as part of the official sampling events at SWRP (Table 3). Each of these approximate 6-liter composite samples was comprised of approximate 1-liter samples that were collected every four hours and compiled over two consecutive 24-hour periods. The City of Albuquerque lab used three of six liters from each 24-hour sample and donated the remaining three liters for use in this study. Since approximately six liters were required for this study, three liters from the first 24 hour sample remained refrigerated at 4° C and out of sunlight at the City of Albuquerque lab while awaiting the second 24-hour sample. The two 3-liter 24-hour samples were composited and mixed in a large glass jar and then redistributed into six 1-liter brown glass bottles, stored on ice, and delivered to SLD within 24 hours. Although the collection times were dictated by the City of Albuquerque's established protocol, the 48–hour composite sample was ideal as it allowed for capture of a representative sample that accounted for high and low flow periods within a day (Kearsey, 2003, personal communication; unreferenced). For sampling sites 8-10, six 1-liter grab samples were collected in brown glass bottles, composited and mixed in a large glass jar, and then redistributed into six 1-liter brown glass bottles (Table 3). Samples were collected across the channel and at variable depth profiles (shallow to deep) at each river sampling site based on accepted USGS technique (Kolpin et al., 2002). Samples were stored on ice and delivered to SLD within 24 hours.

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Attempts were made by SLD to extract all samples within 48 hours of collection; however, the Rio Grande samples remained refrigerated at 4° C for four days before being extracted. Equipment and field blanks were collected and analyzed based on the NMED QAPP (New Mexico Environment Department, 2003). The equipment blank involved sampling of three liters of de-ionized water from a glass jar using the ISCO composite sampler. The collected sample was then redistributed into three 1-liter brown glass bottles, and immediately stored at 4° C at SLD. A field blank was collected at the VA hospital site by placing three 1-liter brown glass bottles of de-ionized water (open to the environment) next to the ISCO sampler for the duration of the sampling event. The three 1-liter equipment blank samples were stored on ice and delivered to SLD along with the VA sample. Sample temperature, pH, specific/electrical conductance, and total dissolved solids concentrations were collected and are presented along with other sample site collection details in Appendix B. Rio Grande flow rates were obtained from USGS gage data (USGS, 2003). 2.4 Analytical Methods In the process of developing the analytical techniques used in this study, SLD encountered difficulties associated with analyses of very low concentrations of PhACs in raw wastewater. Several PhACs originally intended for analysis had to be eliminated due to difficulties associated with extraction and recovery. For instance, erythromycin

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appeared to dehydrate resulting in poor recovery due to multiple product formation during MS/MS analyses. Tetracyclines tended to complex with metals making them difficult to extract with Solid Phase Extraction (SPE). Additionally, many of the PhACs were very sensitive to pH such that two different pH extractions had to be conducted for optimum recovery to occur. Although clogging of SPE cartridges was anticipated for the raw sewage samples, no centrifuging was necessary to achieve optimal extraction. Similar difficulties arose with analyses of non-antibiotic PhACs; however, these issues were resolved with

prior NMED/SLD studies (Chapman, 2003, personal communication, unreferenced). Antibiotic samples were extracted using Solid Phase Extraction (SPE) at two pHs. The first set was brought to a pH of 9.5 using 2M ammonium hydroxide, while a pH of 3.5 was achieved for the second set using formic acid. All samples were extracted at both pHs to determine optimum extraction. Extracted samples were concentrated to 1ml. and analyzed by high performance liquid chromatography (HPLC) and tandem mass spectrometry (MS/MS) using Agilent 1100 liquid chromatograph interfaced to Applied Biosystems API 4000 mass spectrometer. (Chapman and Mawhinney, 2003, manuscript in preparation; unreferenced). The non-antibiotic PhACs were analyzed using techniques developed in previous NMED/SLD studies (McQuillan, 2001). Samples were extracted with a dichloromethane liquid-liquid extraction (LLE) and then concentrated down to 1 ml. Sampling was performed using a Varian 8200 automatic sampler. Samples were analyzed by gas

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chromatography (GC) and MS/MS using a Varian 3800 gas chromatograph coupled to Saturn 2000 mass spectrometer (Chapman and Mawhinney, 2003, manuscript in preparation; unreferenced). Each sample batch was analyzed along with lab reagent blanks, lab fortified blanks, and lab fortified matrices as controls. All positive results were quantified using freshly prepared chemical standards. The sample detection limit (SDL) for all antibiotics and non-antibiotic PhACs was10 ng/l. Recoveries ranged from 80 to120 percent. Conjugate forms of the PhACs, such as glucuronides and sulfates, were treated as transformation products and are not accounted for in the concentrations detected. Since some conjugates can be converted back into the original PhAC form before or during wastewater treatment processes, this may result in an underestimation of the concentration of PhACs present in samples (Huang et al., 2001). Chemical characteristics and pharmacokinetics for several of the detected antibiotics are presented in Appendix C.

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RESULTS AND DISCUSSION ---First, it is important to note that this study does not quantify the total load of PhACs contributed from any sample source because flow volume during sample collection was not known. Instead, what can be determined is a concentration of parent compound present in the sample at the time of collection. Secondly, the study design did not allow PhACs to be tracked temporally (i.e. from hospital to SWRP to river). Consequently, while results do reflect occurrence concentrations at time of collection, it is not feasible to definitively claim that differences in concentrations detected from source to river actually reflect removal of the PhACs within the system. Finally, only the parent compounds of the 39 PhACs were investigated. Conjugates and metabolites of the parent compounds, while sometimes pharmaceutically active, were not included in analytical testing. **Consequently, by tracking only parent compounds, these results likely underestimated the concentration of PhACs present in the samples**. Ten sampling sites were investigated for the presence of thirty-nine PhACs comprised of 29 non-antibiotic PhACs and 10 antibiotics. Analytical results of all PhACs detected are presented in Table 4. Of the 29 non-antibiotic PhACs tested, only caffeine was found and only at the Presbyterian Hospital site (3000 ng/l). However, a number of antibiotics were detected, with six of the ten antibiotics found (Figure 1). Each of the six antibiotics detected were found at two or more sites (Figure 1). Additionally, of the ten sampling sites investigated, eight sites had at least one of the 39 PhACs present while five sites had three or more PhACs present (Figure 2).

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Table 4: PhACs detected at sampling sites (ng/l). Blank boxes indicate no detection.
7402620020012345678SulfamethoxazoleTrimethoprimNorflaxacinCiprofloxacinOfloxacinLincomycinTylosinOxytetracyclinePenicillinGPenicillinVAntibiotic

Number of Sites Figure 1: Number of sites where a particular antibiotic was detected. This graph also shows that six of the ten antibiotics were detected while four were absent from all sampling sites. PhAC drug classPresbyterian Hospital University Hospital VA Hospital UNM Dormitory Vista del Rio Assisted LivingSWRPInfluentSWRPEffluent Rio Grande 1 Rio Grande 2 Rio Grande 3 sulfamethoxazoleantibiotic-sulfonamide800 2100400NDND390310ND 300 300trimethoprimantibiotic-other5000 2900NDNDND590180ND ND NDciprofloxacinantibiotic-fluoroquinolone2000 ND850NDNDNDNDND ND NDofloxacinantibiotic-fluoroquinolone25500 34500 35500ND1300470110ND ND

NDlincomycinantibiotic-lincosamine2000 300NDNDNDNDNDND ND NDpenicillin Gantibiotic- β-lactamND 5200850NDNDNDNDND ND NDcaffeine other-stimulant3000 NDNDNDNDNDNDND ND ND

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654013301101234567Presbyterian HospitalUniversityHospital VA Hospital UNM Dormitory Vista del Rio Assisted Living SWRP Influent SWRP Effluent Rio Grande 1 Rio Grande 2Rio Grande3

Sample Sites Number of Pharmaceuticals Detected Figure 2: Number of PhACs detected at each sampling site. As expected, hospitals had the most PhACs detected and the river the least. Also, eight of the ten sampling sites had at least one PhAC present and two sites had none.

3.1 Fate and Persistence of PhACs in the Environment Once a PhAC enters wastewater or natural waters, several processes affect its fate and transport in the environment. These processes include 1) sorption, 2) biotic transformation, and 3) abiotic transformation (Huang et al., 2001). The fate and persistence of PhACs in the environment is affected by their sensitivity to these processes. Research based on the chemical properties and structures of PhACs has improved our ability to predict the sensitivity of PhACs to these processes and hence, their expected fate and persistence (Huang et al., 2001). Furthermore, it is now understood that classes of drugs that have similar chemical properties and characteristics tend to behave similarly in the environment. See Appendix D for details regarding

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specific chemical properties and pharmacokinetics of the three antibiotics detected in SWRP influent and effluent. The likelihood of detecting specific drugs can be predicted by combining knowledge regarding the concentrations and fate of PhACs within the same class (Huang et al., 2001). Regarding antibiotics, their persistence and transport in the environment has been predicted by Huang et al. (2001), as follows: sulfonamides and fluoroquinolones are the most persistent followed by macrolides; tetracyclines can persist for relatively long periods if sunlight is not present, but tend to be less mobile, and aminoglycosides and β-lactam antibiotics show the least persistence. However, it is important to realize that it is not essential for a PhAC to be persistent in the environment in order for it to have significant impact. Instead, the PhAC could be present at concentrations of concern simply by continual infusion into the environment (Daughton and Ternes, 1999). With regard to antibiotics in wastewater and surface water, previous studies have shown tendencies for some classes of antibiotics to be detected while others are not. In wastewater and surface waters, tetracycline and β-lactam antibiotics have been found rarely, trimethoprim occasionally, and sulfonamide, fluoroquinolone, and macrolide antibiotics frequently (Huang et. al, 2001). Research by Huang et al. (2001), identified antibiotics that were most likely to be found in wastewater sources by combining information concerning environmental fate with predicted concentration levels of different antibiotics. From their respective classes, sulfamethoxazole, ciprofloxacin, and azithromycin were predicted to be the leading wastewater effluent antibiotics (Huang et

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al., 2001). This predictability of detection is largely related to stability of these compounds in the environment. As such, the sulfonamides and fluoroquinolones, followed by macrolides, are the least susceptible to transformation and more likely to persist and transport in aqueous environments (Huang et al., 2001). Additionally, the fluoroquinolones and tetracyclines degrade very slowly as long as sunlight is limited (Huang et al., 2001). Tetracyclines adsorb to soils and sediments most readily, fluoroquinolones and macrolides moderately, sulfonamides moderately to weakly, and aminoglycosides and β-lactams weakly (Huang et al., 2001). In addition to predictions regarding fate and persistence, Huang et al. (2001) also estimated antibiotic concentrations in untreated wastewater to range from 3.9 ng/l to approximately 27,000 ng/l. Interestingly, these predictions regarding fate, persistence, and concentrations are similar to the results obtained in this project (Table 4). See Appendix D for additional fate, transport and persistence characteristics for common antibiotic classes.

3.2 Detection of Antibiotics vs. Other PhACs ---While antibiotics were detected in all hospital samples, it is surprising and not well understood why none of the non-antibiotic PhACs were detected, or why caffeine was detected at only one site. Although beyond the scope of this study, the absence of these non-antibiotic

PhACs from all samples may be due to 1) lower prescribed use, 2) differences in excretion and metabolism of parent compound, 3) lower persistence and transport due to differences in chemical properties and structures of non-antibiotic drugs, and/or 4) analytical error/inaccuracies associated with the analytical techniques used for the non-antibiotic drugs compared with that used for antibiotics.

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3.3 Occurrence of PhACs in Hospital and Residential Effluent ---The first objective of this study was to investigate the occurrence of PhACs in hospital and residential wastewater and, when present, to document their concentrations. In this regard, data reveals that all three hospitals are in fact significant source contributors of several antibiotics but not of non-antibiotic PhACs (Figure 3). In addition, one hospital was also a source contributor of the PhAC, caffeine. Six of the ten antibiotics investigated were detected at the hospital sites (Figure 3). As predicted by Huang et al., 2001, the drug classes of fluoroquinolones and sulfonamides are well represented. This is reflected by the presence of ofloxacin and sulfamethoxazole at all three hospital sites, and ciprofloxacin at two hospital sites. Ofloxacin was found at particularly high levels in all three hospital's wastewaters.
0500010000150002000025000300003500040000PresbyterianHospitalUniversityHospitalVA HospitalUNM DormVista del Riong/l
(ppt)SulfamethoxazoleTrimethoprimCiprofloxacinOfloxacinLincomycinPenicillin Gcaffeine Figure 3:

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PhACs detected in effluent from hospital and residential sites--- In contrast to hospital effluents, residential point source contributions were minimal as indicated by the absence of PhACs at the UNM Alvarado Dormitory, and the detection of only one antibiotic, ofloxacin, at Vista del Rio Assisted Living (Figure 2). Also, in comparison to the concentrations found at hospital sites, the concentration contributed from Vista del Rio is nominal.

3.4 Genotoxicity in Hospital Effluen --- Genotoxicity refers to the amount of damage a toxin can do to DNA molecules. Genotoxic substances are also often mutagens and carcinogens (Hartmann et al., 1998). Fluoroquinolone antibiotics, particularly ciprofloxacin, have been shown to display genotoxic effects in hospital effluent where concentrations were in the 3000ng/l to 87,000 ng/l range (Hartmann et al., 1998). While ciprofloxacin was only found at a maximum concentration of 2000 ng/l in this study, temporal and spatial variability in effluent concentrations are likely to exist and could result in concentrations within the genotoxic range at times. Additionally, ofloxacin, which is also a fluoroquinolone but was not specifically addressed in the Hartmann et al. study, was found at very high concentrations in all three hospital samples and is therefore also of concern for its potential contribution to genotoxic effects. At concentrations found in hospital effluent, genotoxic effects from ciprofloxacin most significantly impair prokaryotic rather than eukaryotic organisms and do not appear to pose an acute human genotoxic risk (Hartmann et al., 1998). Still, prokaryotic organisms can be found in the activated sludge of sewage treatment plants where they could come

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into contact with significant concentrations of fluoroquinolone antibiotics (Hartmann et al., 1998). While not well understood, there is concern that this type of exposure could result in the disruption of microbial ecology or perhaps facilitate the proliferation of antibiotic-resistant organisms.

3.5 Differences in Occurrence and Concentration of PhACs from Source to SWRP Influent--- While hospital effluent samples contained six different antibiotics and caffeine, the wastewater sample collected at the SWRP influent site contained only three antibiotics (Figure 4). Four antibiotics and caffeine dropped below detection levels between the primary source and SWRP. This difference in concentrations of antibiotics between the source samples 1-5 (Table 3) and the SWRP influent can likely be attributed to: 1) dilution by other wastewater sources that do not contain PhACs, and /or 2) processes affecting the fate and transport of the PhAC such as sorption, biotic, and abiotic transformations (Huang et al., 2001). However, since the study design did not allow for hospital and residential effluent to be tracked temporally from source to SWRP influent, it is possible that the sample of influent collected at SWRP did not contain any of the originally sampled hospital or residential effluent but instead contained effluent that never had detectable concentrations of the PhAC. While it is likely the case that the drop in concentrations of PhACs

in wastewater is primarily due to dilution and/or one of the processes affecting fate and transport, it is important to understand that temporal variations in concentration of PhACs in hospital or residential discharges may also

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contribute. Determination of exact processes affecting concentrations and fate of PhACs from source to river is an important area for further research.

0500010000150002000025000300003500040000SulfamethoxazoleTrimethoprimCiprofloxacinOfloxacinLincomycinPenicillin GCaffeineng/l (ppt)PresbyterianHospitalUniversityHospitalVA HospitalUNM DormVista del RioSWRPInfluent

Figure 4: Differences in concentrations of PhACs between their sources and the SWRP influent. The reduction in concentrations of PhACs between their various point sources and the SWRP influent ranges from 2-81% for sulfamethoxazole, 80-88% for trimethoprim, and 64-99% for ofloxacin. 3.6 Concentrations of PhACs Before and After Wastewater Treatment. The second objective of this study was to assess removal of PhACsby the SWRP. Three antibiotics (sulfamethoxazole, trimethoprim, and ofloxacin) were present both in the SWRP influent and effluent samples. Interestingly, these PhACs appear to have experienced between 20 and 77 percent removal (Figure 5). While the experimental design of this study makes it imprudent to definitively claim that SWRP removed these

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PhACs, the fact that SWRP influent and effluent samples were 48-hour composites does lend some confidence to the results. Consequently, it is likely that one of the SWRP 0100200300400500600700 SulfamethoxazoleTrimethoprim Ofloxacin Antibiotics ng/l (ppt)SWRP Influent SWRP Effluent 20% reduction 69% reduction 77% reduction

Figure 5: Removal efficiency of SWRP for the three antibiotics detected in the SWRP influent treatment processes (activated sludge or chlorination) was responsible for the observed reductions. It is also notable that the removal efficiency by SWRP varies for the three antibiotics. This variability is likely due to differences in chemical properties and structure of the PhACs that make them more or less sensitive to SWRP treatment processes and consequently result in different removal efficiencies. Interestingly, all three PhACs present in SWRP samples were from different drug classes and therefore, as predicted by Huang et al. (2001), were expected to behave differently, and in fact, did. Sulfamethoxazole, (a sulfonamide) demonstrated poor removal, whereas, trimethoprim

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(classified as 'other') and ofloxacin (a fluoroquinolone) were both more efficiently removed, though to differing degrees (Huang et al., 2001). Again, the exact processes (sorption, biotic or abiotic transformation) responsible for the removal are not known and are beyond the scope of this study. However, it would be interesting to collect samples between different treatment phases within SWRP to determine which phase and processes are responsible for the removal or transformation of each PhAC. Following treatment at the SWRP, three antibiotics were detected in the SWRP effluent. This effluent is thus continually infusing antibiotics into the Rio Grande, though at relatively low concentrations. The effects of this discharge are not known. In fact, little is known at all about the acute or long-term effects to aquatic species or, more generally, about safe allowable limits of PhACs in the environment. Consequently, the inability of SWRP to fully remove PhACs is disconcerting.

[redacted]

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3.7 Occurrence and Fate of PhACs in the Rio Grande— The final objective of this study was to investigate the occurrence and fate of PhACs in the Rio Grande by collecting samples both upstream and downstream of SWRP. With regard to occurrence, no PhACs were detected at Rio Grande 1, upstream of SWRP, and only one antibiotic, sulfamethoxazole, was detected at the two sampling sites below SWRP (Figure 6). The lack of detection of PhACs at Rio Grande 1 is consistent with two prior NMED studies in which PhACs were undetected in samples from this location (McQuillan, 2001, 2002). This is good news since this is near the planned diversion site for the City of Albuquerque's Drinking Water Program.
47039059018031011030030001002003004005006007 00SulfamethoxazoleTrimethoprimOfloxacinAntibioticsng/l (ppt)SWRPInfluentSWRPEffluentRioGrande 2RioGrande 3Figure 6: Concentration of antibiotics at SWRP and in the Rio Grande

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Although three PhACs were detected in the SWRP effluent, the trimethoprim and ofloxacin were present at very low concentrations. It is reasonable to assume that dilution by the river caused these two antibiotics to drop below detection limits in the Rio Grande since the Rio Grande flow rate was 5.5 times that of the SWRP effluent (See Appendix A). However, it is possible that photolysis or some other transformative process might also have played a role. Fluoroquinolones are especially susceptible to photodegradation (Huang et al., 2001) for which the wide and shallow river morphology of the Rio Grande offers ample opportunity. Consequently, photodegradation must be considered a possible explanation for the absence of ofloxacin from the Rio Grande samples. Similarly, the fact that the sulfamethoxazole concentration remains relatively stable in the SWRP effluent and in Rio Grande samples 2 and 3, seems to support the predictions made by Huang et al., (2001), that sulfamethoxazole is not particularly sensitive to photolysis or other transformation processes and tends to persist and transport readily in the environment. Alternatively, it is unclear why the concentrations of sulfamethoxazole in the SWRP effluent and Rio Grande samples 2 and 3 remain virtually unchanged when dilution alone should have resulted in a 5.5 fold reduction (Appendix A). Possible explanations for this result might include: 1) the SWRP effluent contained conjugates or metabolites of sulfamethoxazole that were not accounted for in analysis and were not in pharmaceutically-active forms in the SWRP effluent but were later converted back to the detectable parent form of the drug after reaching the river, or 2) temporal variations in sulfamethoxazole concentrations exist in the SWRP effluent from day to day.

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Since study design did not temporally track samples from the SWRP into the Rio Grande, temporal variations could potentially explain this result. In fact, the Rio Grande samples were collected about a week before the SWRP effluent samples. Consequently, if the SWRP effluent entering the Rio Grande on the day of collection of the Rio Grande 2 and 3 samples had concentrations of sulfamethoxazole 5.5 times greater than those detected in the SWRP effluent in this study, the concentration of sulfamethoxazole found in this study in Rio Grande 2 and 3 samples would be consistent with dilution effects. However, since temporal fluctuations of this magnitude are unlikely, it is possible that some combination of factors was responsible for the results obtained. Maintaining adequate flow in the Rio Grande is important for the preservation of water quality because it allows for the dilution of contaminant loads entering the river. With the City of Albuquerque Drinking Water Program, additional water will be diverted from the Rio Grande. The City of Albuquerque will be diverting 94,000 af/y but predicts the effective loss of flow through Albuquerque to be minimal, at 34,000 af/y. At present, on the collection date of 3/31/03, the SWRP effluent was 15.4% of the Rio Grande flow. This would increase to 16.9% if 34,000 af/y were effectively lost as predicted (Appendix A). While not a significant change, this could potentially raise PhAC concentrations as well as other chemical pollutant concentrations to levels of concern. 3.8 Comparisons with Prior Studies The finding of sulfamethoxazole in the Rio Grande is consistent with results obtained by the USGS in their surveillance of US streams in 1999 and 2000 where sulfamethoxazole and trimethoprim were both detected in 12.5 percent of 104 streams with a median

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concentration of 150 ng/l (Kolpin et al., 2002). It is curious, however, that trimethoprim was not detected in the Rio Grande since these two drugs frequently appeared to be detected together and in similar concentrations by the USGS (Kolpin et al., 2002) (Table 5). Perhaps the answer lies in the differences of

removal by SWRP where trimethoprim is reduced by approximately 69 percent and sulfamethoxazole by only 20 percent (Figure 5). This may indicate that treatment processes within SWRP are affecting the trimethoprim more readily than the sulfamethoxazole. Table 5 is included to allow for further comparison of results from USGS, NMED, and this study (Kolpin et al., 2002). Similarly, it is also interesting that in previous NMED studies involving Rio Grande samples, other PhACs, such as estrone, amitriptyline, and caffeine were detected. In light of these findings, it is somewhat surprising that none of these PhACs were detected in this study, particularly since the Rio Grande sample sites in this study focused on Albuquerque, the most populated region in New Mexico. Analyses were performed by SLD for both this study and the prior NMED studies when these other PhACs were detected (McQuillan, 2000, 2001). However, new instrumentation not previously used by SLD was utilized for this study and therefore, might explain the different findings. However, a recent study conducted by the U. S. Fish and Wildlife Service and SLD (using the older instrumentation) investigated the same 29 non-antibioitic PhACs tested for in this study, and detected only 17β-estradiol at the analytical detection limit of 10 communication, unreferenced). None of the 29 non-antibiotic PhACs were detected in

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Table 5: Comparison of PhACs detected in three different studies: 1) U.S. Streams by the USGS (Kolpin et al., 2002), 2) the Rio Grande in prior NMED studies (McQuillan, 2001), 3) the Rio Grande in this study. PhAC USGS* frequency of detection (%)USGS median concentration (ng/l) Concentrations found in Prior NMED studies (ng/l) Concentration detected in this study (ng/l) sulfamethoxazole 12.51500300trimethoprim 12.515000norfloxacin 0.912000lincomycin 19.26000oxytetracycline 1.23400Not testedsulfamethazine 4.82000sulfamethizole 113000tylosin 13.54000fluoxetine 1.212000caffeine 61.981200 and 15000cholesterol 84.383000equiline 1.41470017α-ethynl estradiol 15.7730017β-Estradiol 10.616000mestranol 107400estrone 7.1271400amitriptyline Not testedNot tested300*

The USGS study detected additional compounds but only those also tested for in the other studies are included here for comparison Albuquerque reaches of the Rio Grande despite use of the older analytical instrumentation. This indicates that the difference in instrumentation is unlikely to be responsible for the differences in results. A second possible reason that NMED may have detected PhACs that were not detected by this study or the U. S. Fish and Wildlife Service study, might include differences in dilution of wastewater effluents due to differences in Rio Grande flow rates at time of collection. However, it is unlikely that flow was less during the NMED study than during

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this study as collections for this study were performed during a very low flow period (446 cfs). Alternatively, it is possible that differences are due to spatial variability between the Rio Grande sample locations selected in each study despite relatively close proximity. Specifically, in the NMED study, estrone and caffeine were found "in the South Valley". However, the exact location as it compares to the Rio Grande sample locations from this study is not known but could be miles away. A final possible explanation might be differences in photodegradation effects due to differences in sunlight during or preceding collection of Rio Grande samples, although, it is known that both the NMED and this study collected Rio Grande samples on sunny days. The differences found between all of these studies and the proposed explanations clearly reflect the inherent difficulties in studying PhACs and offer a glimpse of the myriad variables involved in fully understanding this issue.

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CONCLUSION--- This study establishes a preliminary inventory of PhACs in effluent from hospital and residential sources, raw influent and treated effluent at the City of Albuquerque municipal wastewater treatment plant, and the Rio Grande in Albuquerque, NM. As anticipated, hospitals were found to be a significant source of antibiotics with concentrations ranging from 300 ng/l to 35,000 ng/l. On the other hand, sampling of residential wastewater resulted in detection of only one antibiotic. However, it is important to remember that while concentrations from individual residential sources may be low or below detection levels, they can be numerous and, when combined, may contribute a relatively significant load of PhACs to wastewater. While antibiotics were detected in all hospital samples, it is surprising and not well understood why none of the non-antibiotic PhACs were detected, or why caffeine was only detected at one

site. One explanation might be due to differences in chemical properties/structures that limit the persistence and transport of the non-antibiotic PhACs more readily than the antibiotics. It is also possible that interferences associated with the high concentrations of solids and organics in raw wastewater limited the ability to detect these compounds at trace levels. Three antibiotics (sulfamethoxazole, trimethoprim, and ofloxacin) were detected in the SWRP influent and effluent at levels ranging from 470 ng/l in the influent to 110 ng/l in the effluent. Specifically, concentrations of these PhACs in the effluent sample are

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reduced between 20 and 77 percent by the SWRP treatment processes. This difference in response to the treatment processes at SWRP is believed to be attributable to differences in the chemical properties and structures affecting their fate and persistence in the treatment environment. With regard to occurrence of PhACs in the Rio Grande, none were detected at the sampling site upstream of the SWRP, and only one antibiotic, sulfamethoxazole, was detected at the two sampling sites below the SWRP (300 ng/l). The trimethoprim and ofloxacin, present at relatively low concentrations in the SWRP effluent, but absent in the river, are assumed to have dropped below detection limits due to dilution by the river. However, the possibility that photolysis or some other transformative process might also have played a role cannot be ruled out. With respect to the sulfamethoxazole, it appears to be resistant to transformation and persists in the river over a distance of approximately five miles with no change in concentration. Overall, the results of this study are not alarming. Despite prior NMED detection in the Rio Grande, no estrogenic hormones were found. This data may ease the concern regarding possible environmental problems due to the presence of estrogenic hormones. In addition, although antibiotic concentrations in hospital effluent were relatively high, detection and concentration in the Rio Grande was minimal with only sulfamethoxazole being detected.

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[redacted] Presently, little is known regarding environmentally safe levels for antibiotics in wastewater or surface waters. Clearly more research is needed to quantify the risk such that appropriate action can be taken to mitigate harmful effects or alternately, redirect efforts and limited resources. However, complicating factors such as the temporal and spatial variability in PhAC detection make it difficult to compare studies, assess risk, or institute policy. While the relatively low concentrations of sulfamethoxazole detected in the Rio Grande are not known to cause any human or ecological health risks, it is still wise to employ the precautionary principle and focus on reducing or eliminating the occurrence of PhACs whenever possible. To this end, the quantification of PhAC contributions from hospitals provides valuable information that should be used to educate and motivate the medical community to improve clinical practice standards regarding the dispensing and disposal of medications as discussed in the USGS concept of "Green Pharmacy" (Daughton, 2003 [redacted] Ultimately, it is my hope that the results of this study can be used as a foundation for future management decisions affecting water quality and its consequences for aquatic and human species.

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SUGGESTIONS FOR FUTURE WORK In the past decade, concern regarding chemical pollution has expanded beyond the traditional priority pollutants to include micro-contaminants such as PhACs. Research on this topic has been increasing worldwide, particularly in Europe and in the United States, where several instrumental studies have been conducted by U.S. governmental agencies. Although significant advances have been made, many questions remain. More research is needed in all areas; but, areas of particular

importance might include identification of: 1) source contributions, 2) fate and transport characteristics, 3) wastewater treatment removal efficiencies, 4) effects on aquatic and other species, and 5) optimization of analytical techniques. While future research opportunities are seemingly limitless, several topics have been identified during the course of this project. Furthermore, many of the suggestions stated here would specifically benefit New Mexico and could potentially be conducted on relatively limited budgets. Generally speaking, investigation into issues of temporal variability is important. Clarification or illumination of temporal trends might explain some of the observed differences between studies and help to direct future sampling protocols. For instance, results might help illuminate the relative importance of composite vs. grab sampling techniques or help determine appropriate composite collection schedules. Investigation of temporal variability could be addressed for source contributions, wastewaters, surface waters or any sample of concern. Specifically, this might include sampling hourly over a 24-hour period to determine how hospital effluent, wastewater, or surface water concentrations vary. Temporal sampling could also be done daily at the same time of day

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to determine changes not associated with diurnal patterns but rather fluctuations from day to day. Seasonal collections might also be of interest due to potential seasonal variability in PhAC usage. Another general consideration in study design might involve evaluation of analytical techniques. Samples could be sent to two or more labs to evaluate the reliability and validity of results. For instance, results could be compared between labs analyzing the same sample with different techniques or between labs utilizing different techniques to analyze the same sample. With regard to source contributions, it is worth considering determination of the total load of PhACs contributed by a given source. To do this, it is necessary to know the flow rate during sample collection. Total load is important when trying to perform a mass balance approach to tracking PhACs throughout their course to determine fate and transport in the environment. While this is an increasingly popular approach, it does add another level of complexity to the study design. Additionally, it is not necessarily an essential component for inventory studies since the concentration of PhAC is generally the issue of concern in regard to effects on aquatic species. [redacted] As an employee of University hospital it is the author's intention to encourage and participate in the implementation of this process. Additionally, however, further research is needed to assess the environmental risks associated with these genotoxic substances. In general, the potential for antibiotic resistance to develop in organisms exposed to very low concentrations of antibiotics in the aquatic environment needs to be addressed. Although this pathway for the development of antibiotic resistance is widely discussed in the literature as an emerging threat, little documentation is available to validate the concern (Guardabassi et al., 1998). Further research is urgently needed to quantify the risk such that appropriate action can be taken to mitigate harmful effects or alternately, redirect efforts and limited resources. Another major topic for further investigation involves wastewater treatment techniques and their PhAC removal efficiencies. There are numerous studies that have documented the removal of PhACs by various wastewater treatment facilities. However, more clarity is needed in determining the exact processes responsible for the removal. To assess this, samples could be collected at the SWRP influent, between each distinct treatment process, and at the effluent. Another interesting project might be to compare removal efficiencies at SWRP with those at the Santa Fe wastewater treatment plant since these facilities utilize different techniques (UV radiation vs. chlorination specifically). Also, with the planned implementation of the Albuquerque Drinking Water Program and its associated additional Rio Grande diversion, the SWRP will likely become a slightly greater percentage of the Rio Grande flow, consequently minimizing dilution by the river. If the impact on flow is significant, it might be interesting to resample the Rio Grande

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sites investigated in this study to see if additional PhACs are detected or if concentrations are elevated. Since some PhACs tend to adsorb to soils and clay, it would be interesting to investigate the presence of PhACs in riverbed sediments in the Rio Grande, downstream of the SWRP. Similarly, it might be interesting to sample soils near landfills, particularly those that receive wastewater treatment sludge. Soils

from city parks or golf courses that are irrigated with surface water might also be of interest. Furthermore, groundwater associated with each of these soil samples could be sampled to assess for leaching of PhACs into groundwater. Recent literature reflects a trend away from simply quantifying aqueous concentrations and instead, is moving toward the tracking of PhACs utilizing a mass balance approach that addresses the ultimate fate of the PhAC. To address these issues of fate, it is important to determine the transformation and degradation processes involved [redacted] others may biodegrade or form transformation products due to alterations in their chemical structures. A clearer understanding of the fate of PhACs in wastewater treatment plants could be achieved by analyzing the mass of PhACs in the aqueous phase and in wastewater sludge (to account for sorption processes). Additionally, attempts could be made to track other transformation/degradation products when the chemical structures of the products are known.

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In addition to these specific areas of focus, there are many other research opportunities that might best be addressed in a laboratory. For instance, the effects of photodegradation could be evaluated for different PhACs and drug classes to help determine sensitivity to this degradation process. Another project would be to investigate tendencies of PhACs to adsorb to sludge, soils, clay, or minerals. This project would help to advance our understanding of the fate and persistence of PhACs in the environment. Research pertaining to effects on aquatic species is another area where laboratory research is indicated and more research is essential. This is particularly true in regard to estrogenic compounds and as noted previously, antibiotics. Research investigating long-term low dose exposure to PhACs is an area of particular concern. Similarly, there is much interest in the potential risk to aquatic organisms associated with concurrent exposure to combinations of PhACs, particularly when the drugs in combination are from the same drug class or tend to act similarly on the target organism. In conclusion, this issue of PhACs in the environment is an area of much concern for a wide variety of environmental disciplines. Input from diverse fields of study is essential to gain a clear and thorough understanding of this complex topic. While a few suggestions have been made here, there are myriad other opportunities available for further research in this fast-growing and interesting area of study. "Not everything that can be counted counts, and not everything that counts can be counted" - Albert Einstein

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GLOSSARY OF TERMS Abiotic transformation: A non-biologically induced change in the PhAC. Hydrolysis and photolysis are common degradation pathways for PhACs whereas little is know about other types of abiotic transformation such as oxidation and reduction processes as they apply to PhACs in the environment (Huang et al., 2001) Antibiotic: A drug class. Antibiotics are a special class of drug. Antibiotics are drugs that are used to fight infections. In this study they were categorized separately from the other PhAC because they required application of a different analytical detection technique and because as a class they are of particular concern in the environment due to their potential to facilitate the proliferation of antibiotic-resistant organisms. Antibiotic resistance: When bacteria develop this, antibiotics are no longer effective at stopping their growth and infections can flourish. Illnesses caused by such bacteria are consequently difficult to control and can spread rapidly. Strains of bacteria that display antibiotic resistance seem to be on the rise. Anti-convulsants: A drug class; a drug used to treat or control convulsions such as in epilepsy. Anti-depressants: A drug class; a drug used to treat depression (these often have undesirable side effects Anti-inflammatories: A drug class; a drug used to treat or control inflammation Analgesic: A drug class; a drug used to decrease pain. Biochemical processes: Reactions of chemical compounds such as sorption, biotic transformation, and abiotic transformation (Huang et al., 2001) Biotic transformation: Changes in PhACs due to biological processes; biodegradation Class: See Drug class Drug: The active ingredient in a PhAC; a substance other than food intended to affect the structure or function of the body (http://www.m-w.com/cgi-bin/dictionary); In this study drug and PhAC are interchangeable Drug class: A categorization or grouping of drugs based on commonalities regarding their effects on target organs or organisms. Drugs in the same class tend to behave similarly and often have similar chemical and physical properties. Effluent: Flowing out Fate: What happens to a PhAC throughout its existence in the environment; i.e. is it in the aqueous phase, adsorbed to a solid, or transformed or degraded into a non-PhAC. Fluoroquinolone: A type or sub-classification of antibiotics

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Genotoxicity: The amount of damage a genotoxic substance can cause to a DNA molecule. Genotoxicity also relates to mutagenicity and carcinogenicity. Genotoxic effects: DNA damage Hormones: A drug class; a chemical substances created by the body or synthetically produced that control numerous body functions; examples include birth control pills and hormones used for hormone replacement therapy in menopausal women.Hospital source: A location that contributes PhACs from a hospital to the wastewater system. In this study there are three sampling sites that are hospital sources: Presbyterian, UNM, and the VA Hospitals.Influent: Flowing in Lab reagent blank: Whatever solvent is used for analysis. In this study it is de-ionized water for the antibiotics and dichloromethane for the non-antibiotic PhACs. Lab fortified blank: The analytical solvent (de-ionized water or dichloromethane) spiked with standards of all the PhACs to be investigated. Lab fortified matrixes: An actual field sample spiked with standards of all the PhACs to be investigated. Macrolide: A type or sub-classification of antibiotics Microgram/liter (µg/l): A concentration measurement. A microgram is 10-6grams. Although not technically correct for fluid measurements, this is sometimes referred to as ppb (parts per billion) Nanogram/liter (ng/l): A concentration measurement. A nanogram is 10 -9 grams. Although not technically correct for fluid measurements, this is sometimes referred to as ppt (parts per trillion). NMED: New Mexico Environment Department, collaborating agency for this study Persistence: A PhACs ability to remain in a detectable pharmaceutically active form in the environment; a PhAC has high persistence in the aquatic environment if it remains pharmaceutically active over for a long period of time or through a long course of travel. PhAC: Pharmaceutically active compound. A compound with pharmaceutical properties such that it behaves and acts upon target organisms in a manner similar to a pharmaceutical. Pharmaceutical: A medicinal drug (http://www.m-w.com/cgi-bin/dictionary) Photodegradation: See photolysis Photolysis: The chemical breakdown of a compound or in this case, a PhAC, caused by sunlight; photodegradation.

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Point source: Sources of PhAC contributions to wastewater or surface water that can be localized as a specific point. In this study there are five investigated potential point sources that could contribute PhACs into the wastewater system: the three hospitals and the two residential sites. The only point source contributor to the Rio Grande is the SWRP effluent. Non-point source contributors include sources that cannot be localized such as runoff from an agricultural field. Non-point sources were not addressed by this study. Precautionary principle: This is based on the idea that people "must acknowledgeuncertainty is inherent in managing natural resources, recognize it is usually easier to prevent environmental damage than to repair it later, and shift the burden of proof away from those advocating protection toward those proposing an action that may be harmful." (http://www.biotech-info.net/ctw_quote.html). QAPP: Quality Assurance Project Plan drafted by the NMED for EPA-funded projects that involve sample collection and analyses. It requires approval by the EPA. Residential source: A location that contributes PhACs from domestic locations (where people live, not from industrial, commercial or hospital locations) to wastewater system. In this study there are two sampling sites that are residential sources: UNM Alvarado dormitory and Vista del Rio Assisted Living/Retirement Community. Sexual disruption: In the case of wild fish exposed to PhACs, this refers to fish developing characteristics/morphology of the opposite sex (male to female and female to male). In addition to physical characteristics, studies have shown changes in sexual function such as an inability to reproduce. SLD: Scientific Laboratory Division, New Mexico Department of Health. Contract agency for PhAC laboratory analysis.Sorption: A binding of one compound to another. In the case of PhACs this would be a PhAC binding to another compound such as clay material or minerals, soil, or activated sludge. (Huang et al., 2001) Sulfonamide: A type or sub-classification of antibiotics Tetracycline: A type or sub-classification of antibiotics. Transformation: A breakdown of the PhAC structure such that it is no longer pharmaceutically active. Transport: the act of remaining mobile within the environment; a PhAC has high mobility if it remains mobile and is able to move from one environment to another or along a course of travel (i.e. from hospital effluent through the wastewater treatment plant and into the river)

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APPENDIX A: FLOW RATE AND DILUTION CALCULATIONS FOR THE RIO GRANDE AND SWRP EFFLUENT AT PRESENT AND AFTER CITY OF ALBUQUERQUE SAN JUAN-CHAMA DIVERSION Present Flow Rates and Dilution Percentages for SWRP Effluent and the Rio Grande in Albuquerque, NM SWRP effluent average flow rate for collection dates 4/8/03-4/10/03 = 52.5 mg/d (52.5 mg/d x 1 cfs / .6464 mg/d) = 81 cfs Rio Grande at Albuquerque flow rate for collection date 3/31/03 (USGS, 2003) (USGS Albuquerque gage is upstream of SWRP) = 446 cfs Flow rate of the Rio Grande after the SWRP effluent addition (446 cfs + 81 cfs) = 527 cfs Percentage of flow rate contributed by SWRP effluent (81 cfs /527 cfs x 100) = 15.4% Dilution of SWRP effluent by the Rio Grande (81 cfs:446 cfs) = 1:5.5 Predicted Flow Rates and Dilution Percentages for SWRP Effluent and the Rio Grande in Albuquerque, NM After City of Albuquerque San Juan-Chama Diversion The City of Albuquerque is planning to switch from a dependence on ground water to the predominant use of surface water. To do this, the City of Albuquerque will decrease ground water pumping and divert 94,000 af/y from the Rio Grande, north of Paseo del Norte Blvd. in Albuquerque. Although the diversion from the river will be 94,000 af/y, the City has predicted that, due to hydrologic connections between groundwater and the Rio Grande, the end result of this 94,000 af/y diversion will actually be a loss of only

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34,000 af/yr in the Rio Grande through the City of Albuquerque. (City of Albuquerque, 2003). Calculations below show 1) the effect of the full 94,000 af/y to show the maximum potential effect of the diversion if no replacement was gained through aquifer-enhanced flow and, 2) the effective diversion of 34,000 af/y as predicted by the City, based on aquifer-enhanced flow in the Rio Grande due to reduced groundwater pumping. Calculations are based on the 3/31/03 Rio Grande at Albuquerque flow rate of 446 cfs (USGS, 2003) and the 4/8/03-4/10/03 average SWRP effluent flow rate of 81 cfs. Maximum Effect of City of Albuquerque Diversion = 94,000 af/y (94,000 af/y x 43,560 cf/af) = 4.1x109 cf/y (4.1x109 cf/y x 1 yr. / 31,536,000 sec) = 128 cfs Percentage of Rio Grande flow that will be diverted (128 / 446 x 100) = 28.9% Rio Grande at Albuquerque post diversion flow rate (446 cfs – 128 cfs) = 318 cfs Flow rate of the post-diversion Rio Grande after addition of SWRP effluent (318 cfs + 81 cfs) = 399 cfs Percentage of post-diversion flow rate contributed by SWRP effluent (81 cfs / 399 cfs) = 20.3% Maximum dilution of SWRP effluent by the Rio Grande, post diversion = 1:3.9 (81 cfs : 318 cfs) City Predicted Effect of City of Albuquerque Diversion = 34,000 af/y (34,000 af/y x 43,560 cf/af) =1.5 x109 cf/y (1.5 x109 cf/y x 1 yr. / 31,536,000 sec) = 47 cfs Percentage of Rio Grande flow that will be diverted (47/446 x 100) = 10.5%

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Rio Grande at Albuquerque post diversion flow rate (446 cfs – 47 cfs) = 399 cfs Flow rate of the post-diversion Rio Grande after addition of SWRP effluent (399 cfs + 81.2 cfs) = 480 cfs Percentage of post-diversion flow rate contributed by SWRP effluent (81.2 cfs / 480 cfs) = 16.9% Maximum dilution of SWRP effluent by the Rio Grande, post diversion = 1:4.9 (81cfs:399 cfs) It should be noted, that these calculations are based on the 3/31/03 flow of 446 cfs. This is a relatively low flow. The calculations of percent of flow contribution by SWRP of 15.4%, 20.3 % and 16.9% are only accurate for the Rio Grande flow of 446 cfs. Much of the year, the flow is greater than this and the percent contribution of the SWRP would be less. At times, however, the Rio Grande does have even lower flows with a mean low flow or 4Q3 of approximately 250 cfs. This level was reached several times in 2003. To address this, during periods of extremely low flow, the City of Albuquerque plans to stop diversions from the Rio Grande to keep water in

the river to maximize dilution of the SWRP effluent. None the less, the change from ground water pumping to Rio Grande diversion may reduce Rio Grande flow and consequently, the ability of the Rio Grande to dilute the SWRP effluent. This could result in the increase of concentrations of PhACs in the Rio Grande downstream of the SWRP to levels of concern.

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APPENDIX B: SAMPLE SITE COLLECTION DETAILS AND GENERAL CHEMICAL MEASUREMENTS Sample Site Location Collection Date 2003 Timeof Collection Weather Sample Temp. (Celsius) cElectrical Conductance (µS/cm)pHTotal Dissolved Solids (ppt). aTime of ChemicalMeasurements Presbyterian Hospital 04/30 6:00a -10:00a sunny clear 9.5° 725 8.6 11:15a University Hospital 03/31 6:00a -10:00a sunny clear 21.1°b850 7.38 0.43 10:00a VA Hospital 05/07 6:00a -10:00a sunny clear 9.2° 709 7.7 2:30p UNM Dorm 05/01 6:00a -10:00a sunny clear 13.0° 878 12:00p Vista Del Rio 04/07 6:00a -10:00a cloudy 535 7.6 8:30a on 4/8dSWRP Influent 04/08 – 04/10 6:00a – 6:00a (48 hr) varied 3.0° - 4.0° 1127 and 1076 6.96-7.28 eSWRP Effluent 04/08 – 04/10 6:00a – 6:00a (48 hr) varied 813 and 835 7.61-7.83 e Rio Grande 1f03/30 11:50a-12:35psunny clear 5.6° 330 8.6 .17 12:00p on 3/31Rio Grande 2f 03/30 2:15p-2:45psunny clear 3.0° 360 8.41 .18 12:00p on 3/31 Rio Grande 3f 03/30 4:30p-5:00psunny clear 2.7° 570 8.42 .29 12:00p on 3/31 awhen this box is blank = instrumentation not available on this date b temperature collected at sample site at 10 am along with chemical measurements using a portable instrument that was lost and inaccessible for later testing. No ice in ISCO sampler during collection of this sample. c when this box is blank = not collected due to investigator/equipment error. Here, ppt is parts per thousand d these chemical measurements were taken at SLD the day after the sample had been delivered, not by the portable unit, as with the UNM Hospital and not at the Biology Annex Lab as is the case with the other samples e The City of Albuquerque Lab collected the pH and EC measurements around 10 am each day fRio Grande flow as of 4:30 pm on 3/30/03 was 446 cfs (USGS, 2003)

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APPENDIX C: CHEMICAL PROPERTIES AND PHARMACOKINETICS FOR COMMONLY DETECTED ANTIBIOTICS Trimethoprim/Sulfamethoxazole http://www.aegis.com/factshts/network/access/drugs/sulfame.htmlhttp://www.aegis.com/factshts/network/access/drugs/tmp.htmlBrand Names: Bactrim, Septra. When administered alone, sulfamethoxazole brand names include Gantanol and Urobak Excretion Percentage: The free forms of sulfamethoxazole/trimethoprim are considered to be the therapeutically active forms. The average percentage of the dose recovered in urine from 0 to 72 hours after a single oral dose of sulfamethoxazole/trimethoprim is 84.5% for total sulfonamide and 66.8% for free trimethoprim. Thirty percent of the total sulfonamide is excreted as free sulfamethoxazole, with the remaining as N4-acetylated metabolite. When administered together as sulfamethoxazole/trimethoprim, neither sulfamethoxazole nor trimethoprim affects the urinary excretion pattern of the other. Applications: To treat common respiratory infections, and is also prescribed to people who have sinusitis. Bactrim is used for prevention and treatment of PCP pneumonia, particularly in patients with HIV. As a single drug product, sulfamethoxazole is most commonly used to treat urinary tract infections. Pharmacokinetics: Both sulfamethoxazole and trimethoprim exist in the blood as unbound, protein-bound and metabolized forms; sulfamethoxazole also exists as the conjugated form. The metabolism of sulfamethoxazole occurs predominately by N4-acetylation, although the glucuronide conjugate has been identified. The principal metabolites of trimethoprim are the 1- and 3-oxides and the 3'- and 4'-hydroxy derivatives. The free forms of sulfamethoxazole/trimethoprim are considered to be the therapeutically active forms.

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Ciprofloxacin http://www.rxlist.com/cgi/generic/cipro_cp.htmExcretion Percentage: Approximately 40 to 50% of an orally administered dose is excreted in the urine as unchanged drug. Approximately 20 to 35% of an oral dose is recovered from the feces within 5 days after dosing. Four metabolites have been identified in human urine which together account for approximately 15% of an oral dose. The metabolites have antimicrobial activity, but are less active than unchanged. After intravenous administration, approximately 50% to 70% of the dose is excreted in the urine as unchanged drug. Molecular Weight: 331.4 Chemical Formula: C(17)H(18)FN(3)O(3) Ciprofloxacin is 1-cyclopropyl-6-fluoro-1, 4-drhydro-4-oxo-7-(1-piperazinyl)-3-quinolinecarboxylic acid Ciprofloxacin differs from other quinolones in that it has

a fluorine atom at the 6-position, a piperazine moiety at the 7-position, and a cyclopropyl ring at the 1-position. Pharmacokinetics: It is soluble in dilute (0 1N) hydrochloric acid and is practically insoluble in water and ethanol. The serum elimination half-life in subjects with normal renal function is approximately 4 hours. After a 250-mg oral dose, urine concentrations of ciprofloxacin usually exceed 200 mg/ml during the first two hours and are approximately 30 mg/ml at 8 to 12 hours after dosing. The urinary excretion of ciprofloxacin is virtually complete within 24 hours after dosing. Concurrent administration of antacids containing magnesium hydroxide or aluminum hydroxide may reduce the bioavailability of ciprofloxacin by as much as 90%. Following a 200-mg I.V. dose, concentrations in the urine usually exceed 200 mcg/ml 0-2 hours after dosing and are generally greater than 16 mcg/ml 8-12 hours after dosing. Following a 400-mg I.V. dose, urine concentrations generally exceed 400 mcg/ml 0-2 hours after dosing and are usually greater than 30 mcg/ml 8-12 hours after dosing. The renal clearance is approximately 22 L/hr. The urinary excretion of ciprofloxacin is virtually complete by 24 hours after dosing. After I.V. administration, three metabolites of ciprofloxacin have been identified in human urine which together account for approximately 10% of the intravenous dose. The bactericidal action of ciprofloxacin results from interference with the enzyme DNA gyrase which is needed for the synthesis of bacterial DNA.

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Ofloxacin

http://www.rxlist.com/cgi/generic/oflox.htmhttp://www.rxlist.com/cgi/generic/oflox_cp.htmBrand Names: Floxin Excretion Percentages: Ofloxacin has a pyridobenzoxazine ring that appears to decrease the extent of parent compound metabolism. Between 65% and 80% of an administered oral dose of ofloxacin is excreted unchanged via the kidneys within 48 hours of dosing. Studies indicate that <5% of an administered dose is recovered in the urine as the desmethyl or N- oxide metabolites. Four to eight percent of an ofloxacin dose is excreted in the feces. Molecular Weight: 361.4. Chemical Formula: C(18)H(20)FN(3)O(4) Clinical Pharmacology: Chemically, ofloxacin, a fluorinated carboxyquinolone, is the racemate, (±)-9- fluoro-2,3-dihydro-3-methyl-10- (4-methyl-1-piperazinyl)-7-oxo-7H- pyrido[1,2,3-de]-1,4-benzoxazine -6- carboxylic acid. Ofloxacin is an off-white to pale yellow crystalline powder. The molecule exists as a zwitterion at the pH conditions in the small intestine. The relative solubility characteristics of ofloxacin at room temperature, as defined by USP nomenclature, indicate that ofloxacin is considered to be soluble in aqueous solutions with pH between 2 and 5. It is sparingly to slightly soluble in aqueous solutions with pH 7 and freely soluble in aqueous solutions with pH above 9. Ofloxacin has the potential to form stable coordination compounds with many metal ions. This in vitro chelation potential has the following formation order: Fe+3 > Al+3 > Cu+2 > Ni+2> Pb+2 > Zn+2 > Mg+2> Ca+2 > Ba+2. Applications: Floxin Tablets and IV are synthetic broad-spectrum antimicrobial agents for oral or intravenous administration. Pharmacokinetics: Following oral administration, the bioavailability of ofloxacin in the tablet formulation is approximately 98%. Maximum serum concentrations are achieved one to two hours after an oral dose. Absorption of ofloxacin after single or multiple doses of 200 to 400 mg is predictable, and the amount of drug absorbed increases proportionately with the dose. Ofloxacin has biphasic elimination. Following multiple oral doses at steady-state administration, the half-lives are approximately 4-5 hours and 20-25 hours. However, the longer half-life represents less than 5% of the total. Accumulation at steady-state can be estimated using a half-life of 9 hours. The total clearance and volume of distribution are approximately similar after single or multiple doses. Elimination is mainly by renal excretion.

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APPENDIX D: FATE, TRANSPORT, AND PERSISTENCE OF PHARMACEUTICALLY ACTIVE COMPOUNDS Antibiotic Class Persistence Factors Affecting Fate and Transport Sulfonamide High 1Moderate to weak adsorption to soils 1Fluoroquinolone High if sunlight absent 1Moderate adsorption to soils 1Substantial adsorption to sewage sludge 2 With 15-20 hours residence time in river, concentrations of ciprofloxacin and norfloxacin were reduced by 66% and 28% respectively 2Macrolide Moderate 1Moderate adsorption to soils 1Tetracycline High if sunlight absent 1High adsorption to soils; generally low transport 1 Oxytetracycline strongly interacts with clay affecting its mobility and bioavailability; However, when competing solutes are present, this binding will be reduced and the bioavailability and mobility of oxytetracycline will be affected 3Sensitive to transformation via photolysis 1Resists degradation via conventional wastewater treatment 4Complexes with metals making Solid Phase Extraction (SPE) difficult 5B-Lactam Low 1Weak adsorption to soils 1Trimethoprim-other Trimethoprim reduced to

below detection limits by conventional drinking water treatment plant 6Penicillin Penicillin G requires acidic condition for optimum SPE recovery 5Easily degrade in the environment 21 = Huang et al., 2003 2 = Alder et al., 2003 3 = McKay et al., 2003 4 = Kulis, personal communication, unreferenced, 2003 5 = Chapman, personal communication, unreferenced, 2003 6 = Stackelberg et al., 2003
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Occurrence of antibiotic resistances within the water cycle: an emerging problem of the use of environmental resources Thomas Schwartz, Holger Volkmann, Silke Kirchen, Ursula Obst ITC-WGT, Department of Environmental Microbiology Introduction The occurrence of antibiotic-resistant bacteria in aquatic environments has increased dramatically as a consequence of the wide spreaduse of antibiotics in medicine and in intensive animal husbandry[1, 2]. In order to study the fate of these bacteria in environmenta lcompartments, the paths of entry, accumulation and dissemination of resistant bacteria in biofilms of aquatic environments were examined. Both cultivation and molecular biological methods were applied for a detection of resistant enterococci, staphylococci and Enterobacteriaceae and their resistance genes in wastewater, surface water and drinking water. In order to assess the risk potential of antibiotics and the respective bacterial resistances in the environment, two strategies are proposed: the determination of bioeffectivity thresholds of low antibiotic concentrations and the design of an indicator system to characterise the status of bacterial resistance in aquatic compartments. Table 1: Resistant bacteria and their resistance genes in biofilms. Biofilm sample Total genomic DNA Resistant Bacteria Vancomycin resistance gene vanA (enterococci) Hospital wastewater Municipal sewage Surface water Drinking water positive positive positive! positive !positive positive positive! negative ! Methicill in resistance gene mecA (staphylococci) Hospital wastewater Municipal sewage Drinking water positive negative negative positive negative negativeb -lactam resistance gene ampC (Enterobac.) Hospital wastewater Municipal sewage Surface water Drinking water positivepositive not tested! positive !positive positive positive! negative ! Biofilm sample Total genomic DNA Resistant Bacteria Vancomycin resistance gene vanA (enterococci) Hospital wastewater Municipal sewage Surface water Drinking water positive positive positive! positive !positive positive positive! negative ! Methicillin resistance gene mecA (staphylococci)Hospital wastewater Municipal sewage Drinking water positive negative negative positive negative negative Biofilm sample Total genomic DNA Resistant Bacteria Vancomycin resistance gene vanA (enterococci) Hospital wastewater Municipal sewage Surface water Drinking water positive positive positive! positive !positive positive positive! negative ! Methicilllin resistance gene mecA (staphylococci) Hospital wastewater Municipal sewage Drinking water positive negative negative positive negative negativeb- lactam resistance gene ampC (Enterobac .)Hospital wastewater Municipal sewage Surface water Drinking water positive positivenot tested! positive !positive positive positive! negative ! vancomycin E. E. . . faeciumfaeciumvan A gene expression mRNA mRNA mRNAvanA gene induction induction Threshold value for induction of gene expression: 0,032 mg/lFig. 1: Induction of the vanA expression in the presence of vancomycin. antibiotic1234567 amoxicillin++gentamicin
(high)+++amoxicillin/++synercid(+)linezolidteicoplanin+vancomycin+ciprofloxacin++ (+) (+) (+)+imipenem++++cotrimoxazol+ythromycin++++++mpicillin(+)++++acyclin++++eratetrFig. 2: Antibiograms of different indicator organisms in different environmental compartments.

????antibioticantibioticindicatorindicator--organismorganismresistance genresistance genimipenemimipenemPseudomonas Pseudomonas aeruginosaaeruginosablablaVIMVIMimipenemimipenemPseudomonas Pseudomonas aeruginosaaeruginosablablaIMPIMPampicillinampicillinEnterobacter Enterobacter cloacaecloacaeampCampCmethicillinmethicillinCNSCNS, , Staphylococcus Staphylococcus aureus aureus (MRSA)(MRSA)mecAmecAvancomycinvancomycinEnterococcus Enterococcus faeciumfaeciumvanvanAARealReal--TimeTime--PCRPCR((TaqManTaqMan))RealReal--TimeTime--PCRPCR((TaqManTaqMan))primerprimer--extensionextension--reactionreactionprimerprimer--extensionextension--reactionreactionDNADNA--chipchipaim:DNADNA--chipchipaim:cipcip rofloxacinrofloxacinPseudomonas Pseudomonas aeruginosaaeruginosapoint mutationpoint mutationgyrAgyrA,, gyrBgyrB,, parCparCFig. 3: Development of an indicator system to screen the state of resistance in different aquatic environments.Antibiogram assays of pathogens isolatedfrom clinical wastewater reveal typical resistance patterns, that can be ascribedto the application of antibiotics (Fig. 2). Based on such patterns an indicator system for assessing the state ofresistance in aquatic environments can be derived. Molecular biological methodswere used to detect their resistancegenes mecA, blaIMP, blaVIM, ampC, vanA, and point mutations of topoisomerasecoding genes and their propagationpathways beyond the habitat of theirorigin. For their genotypic detection Real-Time-PCR-assays and primer-extension-assays, capable to be transferred to DNA-Chip-technology, were designed (Fig 3).Such microarrays enable in parallel the taxonomical and genotypical surveillanceof aquatic habitats based on such indicator bacteria. Bioeffectivity Indicator system Dissemination of resistance and entry pathways [redacted]. Expectedly enterococci and Enterobacteriaceae were absent in drinking water. Nevertheless their resistance mediating genes, the gene vanA as well as the gene ampC, could be amplified and subsequently sequenced from these drinking water biofilms(Tab. 1)[3]. [redacted]. Further investigations showed, that these resistance genes were carried in viable but not culturable bacteria. The expression of the resistance mediating gene vanA is inducible in the presence of vancomycin and teicoplanin. The bioeffectivity of low concentrated vancomycin in envi-ronmental matrices was evaluated using a bioassay based on the detection of specific mRNA for the vanA gene after induction with different vancomycin concentrations (Fig. 1). A correlation between drug concentration in environmental samples and molecular mechanisms of the bacteria could be demonstrated. The threshold value concerning the induction of biological effects were deter-mined with 0.032 mg/L of glycopeptid antibiotics. The impact of further drugs on bacterial biocoenosis can be described with threshold values determined with such bioassays. Conclusions and call for action Using the introduced approaches a screening of aquatic compartments can reveal hot spots for the spread of antimicrobial resistances. [redacted] References: [1] DAVIES, J.E. (1997): Ciba Foundation Symposium 207, John Wiley & Sons. 15-27 [2] FEUERPFEIL et al.(1999): Bundesgesundheitsblatt42(1): 37-50. [3] SCHWARTZ et al.(2003): FEMS Microbiology Ecology 43: 325-335. This project is funded by the BMBF 02WU013602WU0225

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Disinfection of hospital wastewater by continuous ozonization.

Chiang CF, Tsai CT, Lin ST, Huo CP, Lo KV.

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The disinfection of hospital wastewaters using the ozonization process was studied. The concentrations of ozone required to reach a sudden drop of coliform and Pseudomonas aeruginosa in the wastewater are 4.0-7.0 and 3.0-5.0 mg L(-1), respectively. For the hospital wastewater, the disinfection efficiencies were 0.518S(-1.1) for coliforms, 0.509S(-1.06) for Pseudomonas aeruginosa and 0.254S-(1.54) for total count,

respectively. As to the effects of ozone input methods on the disinfection efficiency, the continuous ozonization process was ten times higher than the batch input process. The low COD removal rate was obtained at 25.0 mgL(-1) of ozone concentration for hospital wastewater. However, more biodegradable compounds resulted in the treated mixture. J Environ Sci Health Part A Tox Hazard Subst Environ Eng. 2003;38(12):2895-908.

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Ozonation: a tool for removal of pharmaceuticals, contrast media and musk fragrances from wastewater?

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A pilot plant for ozonation and UV-disinfection received effluent from a German municipal sewage treatment plant (STP) to test the removal of pharmaceuticals, iodinated X-ray contrast media (ICM) and musk fragrances from municipal wastewater. In the original STP effluent, 5 antibiotics (0.34-0.63 microgl(-1)), 5 betablockers (0.18-1.7 microgl(-1)), 4 antiphlogistics (0.10-1.3 microgl(-1)), 2 lipid regulator metabolites (0.12-0.13 microgl(-1)), the antiepileptic drug carbamazepine (2.1 microgl(-1)), 4 ICM (1.1-5.2 microgl(-1)), the natural estrogen estrone (0.015 microgl(-1)) and 2 musk fragrances (0.1-0.73 microgl(-1)) were detected by LC-electrospray tandem MS and/or GC/MS/MS. ICM, derived from radiological examinations, were present with the highest concentrations (diatrizoate: 5.7 microgl(-1), iopromide: 5.2 microgl(-1)). By applying 10-15 mgl(-1) ozone (contact time: 18 min), all the pharmaceuticals investigated as well as musk fragrances (HHCB, AHTN) and estrone were no longer detected. However, ICM (diatrizoate, iopamidol, iopromide and iomeprol) were still detected in appreciable concentrations. Even with a 15 mgl(-1) ozone dose, the ionic diatrizoate only exhibited removal efficiencies of not higher than 14%, while the non-ionic ICM were removed to a degree of higher than 80%. Advanced oxidation processes (O(3)/UV-low pressure mercury arc, O(3)/H(2)O(2)), which were non-optimized for wastewater treatment, did not lead significantly to a higher removal efficiency for the ICM than ozone alone. Water Res. 2003 Apr;37(8):1976-82.

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Hybrid cavitation methods for water disinfection: simultaneous use of chemicals with cavitation.

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This study brings out the potential efficacy of hybrid techniques for water disinfection. The techniques studied include, hydrodynamic cavitation, acoustic cavitation and treatment with chemicals such as ozone and hydrogen peroxide. The phenomena of cavitation which involves formation, growth and violent collapse of vapor bubbles in a liquid media is known to generate a high intensity pressure which affects the cell and microorganism viability. The hybrid technique which combines hydrodynamic cavitation, acoustic cavitation, hydrogen peroxide and/or ozone appears to be an attractive alternative to a single technique for the reduction in the heterotropic plate count bacteria as well as indicator microorganisms like the Total coliforms, Fecal coliforms and Fecal streptococci. Ultrason Sonochem. 2003 Jul;10(4-5):255-64

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Effect of cavitation on chemical disinfection efficiency.

Jyoti KK, Pandit AB.

Chemical Engineering Division, Institute of Chemical Technology, University of Mumbai (MUICT), Matunga, Mumbai 400019, India.

This study brings out the potential efficacy of hybrid techniques for water disinfection. The techniques studied include hydrodynamic cavitation, acoustic cavitation and treatment with chemicals like hydrogen peroxide and ozone. The hybrid techniques which combine hydrodynamic cavitation, acoustic cavitation and hydrogen peroxide appear to be an attractive alternative to any one technique on its own for the reduction in the heterotropic plate count bacteria as well as indicator microorganisms like the total coliforms, fecal coliforms and fecal streptococci. **Effect of cavitation on chemical disinfection efficiency.**

Jyoti KK, Pandit AB.

Chemical Engineering Division, Institute of Chemical Technology, University of Mumbai (MUICT), Matunga, Mumbai 400019, India.

This study brings out the potential efficacy of hybrid techniques for water disinfection. The techniques studied include hydrodynamic cavitation, acoustic cavitation and treatment with chemicals like hydrogen peroxide and ozone. The hybrid techniques which combine hydrodynamic cavitation, acoustic cavitation and hydrogen peroxide appear to be an attractive alternative to any one technique on its own for the reduction in the heterotropic plate count bacteria as well as indicator microorganisms like the total coliforms, fecal coliforms and fecal streptococci. Water Res. 2004 May;38(9):2248-57.

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Septage treatments to reduce the numbers of bacteria and polioviruses.

Stramer SL, Cliver DO.

Disposal of the pumped contents of septic tanks (septage) represents a possible means of dissemination of enteric pathogens including viruses, **since persistence of enteroviruses in septic tank sludge for greater than 100 days has been demonstrated.** The risk of exposure to potentially infectious agents can be reduced by disinfecting septages before their disposal. Of the septage disinfectants examined (technical and analytical grade glutaraldehyde, hydrogen peroxide, heat treatments, and a combination of heat and hydrogen peroxide), the treatment including hydrogen peroxide (5 mg, plus 0.33 mg of trichloroacetic acid, per ml of septage) and 55 degrees C killed virtually all the bacteria in septage within 1 h, whereas 55 degrees C alone inactivated inoculated polioviruses within 30 min. Virus was the most sensitive to heat, whereas fecal coliforms appeared to be the most sensitive to all chemical treatments. The responses of fecal streptococci and virus to both grades of glutaraldehyde (each at 1 mg/ml) were similar. Virus was more resistant than either fecal streptococci or total bacteria to low concentrations of hydrogen peroxide (1 to 5 mg/ml); however, virus and fecal streptococci were more labile than total bacteria to the highest peroxide concentration (10 mg/ml) examined. It is possible that the treatment combining heat and hydrogen peroxide was the most effective in reducing the concentrations of all bacteria, because catalase and peroxidases as well as other enzymes were heat inactivated, although catalase seems the most likely cause of damage. However, this most effective treatment does not appear to be practical for on-site use as performed, so further work on septage disinfection is recommended. Appl Environ Microbiol. 1984 Sep;48(3):566-72.

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Water disinfection with the hydrogen peroxide-ascorbic acid-copper (II) system.

Ragab-Depre NJ.

Treatment of secondary effluents with hydrogen peroxide (10 mg/liter)-ascorbic acid (10 mg/liter)-Cu2+ (0.5 mg/liter) for 60 min resulted in around 99% reduction of the initial plate count. Hydrogen peroxide could be replaced by other peroxygen compounds; ascorbic acid could be replaced by other reducing agents, of which sodium sulfite and ethanol were the most effective. Cu2+, however, could not be replaced by other metal ions without loss of bactericidal efficiency of the ternary combination. Enterobacteriaceae, total and fecal coliforms, staphylococci, and micrococci were reduced by 99.0 to 99.9%. Group D streptococci aerobic spores were reduced by 80 and 15%, respectively. **Clostridium perfringens, yeasts,**

and molds *were not* killed by the disinfectant combinations. The effect of pH was only minor in the range from 6 to 7.5. At a higher pH value the bactericidal effects tended to decrease. The hydrogen peroxide-ascorbic acid-Cu2+ combination made it possible to obtain 99% reduction within 30 min. When using the hydrogen peroxide-sodium sulfite-Cu2+ or the hydrogen peroxide-ethanol-Cu2+ combinations, 60 min of contact time was necessary to obtain 99% reduction of the initial plate count. Cu2+ combined to an intermediate product of the ascorbic acid autoxidation is the toxic agent, and its penetration into the cell is promoted by hydrogen peroxide. Appl Environ Microbiol. 1982 Sep;44(3):555-60.

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The development and evaluation of electrolysis in conjunction with power ultrasound for the disinfection of bacterial suspensions.

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There is an increasing incidence in health problems related to environmental issues that originate from inadequate treatment of potable waters. This has compelled scientists and engineers to engage in innovative technologies to achieve a maximum disinfection at affordable costs. Some species of bacteria produce colonies and spores that can agglomerate in spherical clusters and thus protect organisms on the inside of the cluster against biocidal attack. Flocs of fine particles (e.g., clay) can entrap bacteria and this can also protect them against the biocides. Other bacteria have the ability to mutate, thus building up resistance to conventional biocides (e.g., chlorine).Ultrasound has been shown to be effective in improving the effectiveness of biocides such as chlorine. The aim of this present study was to investigate the effect of electrolysis and power ultrasound as a disinfection treatment and to provide a greater knowledge of the fundamentals of disinfection through the production of hypochlorite in situ from saline solution via electrolysis. The electrode materials investigated were, carbon (felt and graphite), copper and stainless steel rods. The results show that sonication appears to amplify the effect of electrolysis. A combination of both treatments is significantly better than sonication or electrolysis alone. Ultrason Sonochem. 2003 Jul;10(4-5):231-4

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Fecal coliform removal in wastewater treatment plants studied by plate counts and enzymatic methods.

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Twelve wastewater treatment plants (WWTPs) were sampled in France and Belgium in 1999 and 2000 in order to estimate the fecal coliform (FC) removal efficiency of various types of treatment. **Only one of these WWTPs was equipped with a specific step to eliminate microorganisms (UV disinfection preceded by sand filtration)**. FC abundance was measured in raw and treated sewage by plate counts on selective medium and rapid beta-D-glucuronidase (GLUase)-based assays. Removal of culturable FC was the most efficient in treatments with high retention time (activated sludge process with nitrification and denitrification, lagooning), in biofiltration and in the treatment with a tertiary disinfection step. GLUase activity measurements showed the same removal pattern as plate counts except for UV disinfection, where no reduction of GLUase activity was measured. Specific loads of culturable FC and GLUase activity, i.e. daily amounts of culturable FC or GLUase activity in sewage per inhabitant-equivalent, were calculated in raw and treated wastewater for the different WWTPs. Water Res. 2002 May;36(10):2607-17.

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The cavitation induced Becquerel effect and the hot spot theory of sonoluminescence.

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Over 150 years ago, Becquerel discovered the ultraviolet illumination of one of a pair of identical electrodes in liquid water produced an electric current, the phenomenon called the Becquerel effect. Recently, a similar effect was observed if the water surrounding one electrode is made to cavitate by focused acoustic radiation, which by similarity is referred to as the cavitation induced Becquerel effect. The current in the cavitation induced Becquerel effect was found to be semi-logarithmic with the standard electrode potential that is consistent with the oxidation of the electrode surface by the photo-decomposition theory of photoelectrochemistry. But oxidation of the electrode surface usually requires high temperatures, say as in cavitation. Absent high bubble temperatures, cavitation may produce vacuum ultraviolet (VUV) light that excites water molecules in the electrode film to higher H(2)O(*) energy states, the excited states oxidizing the electrode surface by chemical reaction. Solutions of the Rayleigh-Plesset equation during bubble collapse that include the condensation of water vapor show any increase in temperature or pressure of the water vapor by compression heating is compensated by the condensation of vapor to the bubble wall, the bubbles collapsing almost isothermally. Hence, the cavitation induced Becquerel effect is likely caused by cavitation induced VUV light at ambient temperature. Ultrasonics. 2003 Jun;41(4):313-7.
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Single-Cell Protein Profiling of Wastewater Enterobacterial Communities Predicts Disinfection Efficiency

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ABSTRACT

The efficiency of enterobacterial disinfection is dependent largely on enterobacterial community physiology. However, the relationship between enterobacterial community physiology and wastewater processing is unclear. The purpose of this study was to investigate this relationship. The influence of wastewater treatment processes on enterobacterial community physiology was examined at the single-cell level by using culture-independent methods. Intracellular concentrations of two conserved proteins, the growth-related protein Fis and the stationary-phase protein Dps, were analyzed by epifluoresence microscopy of uncultivated cells by using enterobacterial group-specific polyclonal fluorochrome-coupled antibodies. Enterobacterial single-cell community protein profiles were distinct for different types of biological treatment. The differences were not apparent when bulk methods of protein analysis were used. Trickling filter wastewater yielded Fis-enriched communities compared to the communities in submerged aeration basin wastewater. Community differences in Fis and Dps contents were used to predict disinfection efficiency. Disinfection of community samples by heat exposure combined with cultivation in selective media confirmed that enterobacterial communities exhibited significant differences in sensitivity to disinfection. These findings provide strategies that can be used to increase treatment plant performance, reduce the enterobacterial content in municipal wastewater, and minimize the release of disinfection by-products into receiving water.

INTRODUCTION

The growing demand for water resources is increasing interest in reclamation of municipal wastewater for potable and nonpotable applications (30, 53). Primary wastewater treatment removes solid materials. In secondary treatment a range of strategies collectively based on biological treatment, including activated sludge and biofilms, is used to reduce the organic carbon content and thereby reduce the biological oxygen

demand (37). Despite their differences, secondary treatment strategies overcome variations in microbial community structure, water chemistry, and substrate composition to effect water purification.
Wastewater quality is judged by the content of a subgroup of the *Enterobacteriaceae* termed fecal enterobacteria (fecal coliforms) that provide a biological indicator of fecal pollution (10). In most forms of tertiary wastewater treatment chemical disinfection with chlorine or chloramines is used to inactivate resident microbes. When the receiving water is classified as recreational, residual chlorine must be neutralized by sulfur dioxide treatment (46). The Environmental Protection Agency also mandates that discharged municipal effluent can contain no more than 4,000 fecal coliforms per liter to reduce the risk of fecal contamination in the receiving water. Many treatment plants, however, achieve acceptable microbial contents at the expense of releasing considerable amounts of disinfection by-products (5). Chlorination generates trihalomethane and other by-products with mutagenic and carcinogenic properties (47). Wastewater reuse must therefore balance removing pathogenic microbes with minimizing formation and release of disinfection by-products.
In laboratory experiments the efficiency of disinfection of pure microbial cultures and of mixed-taxon microcosms is dependent upon a range of physiologic characteristics. These include the microbial growth state (25, 31), the availability of nutrients (18, 43), the induction of starvation proteins (4, 19, 24, 25, 36, 43), and alterations in membrane structure (23, 25). Despite the availability of information, the use of culture-based microbiological methods imposes limits on a complete understanding of the relationship between the treatment process and enterobacterial disinfection in environmental samples.
Culture-independent methods provide a direct approach for obtaining information about endogenous microbes in environmental samples. In situ hybridization for detection of individual taxa (32) was initially performed with radiolabeled oligonucleotides (12), and the procedure was quickly refined to incorporate fluorescently labeled oligonucleotide probes. Detection of individual cells and identification of specific taxa in activated sludge also were accomplished by using immunofluorescence-based techniques (13, 29, 48). However, such approaches have been replaced by nucleic acid-based strategies applied to activated sludge (6, 7, 8, 11, 21, 41, 49, 50, 51) and biofilm (22, 35, 44) processes. The studies have focused primarily on the occurrence and distribution of a variety of taxa, such as *Gordonia*, *Acinetobacter*, and *Nitrosococcus*, to understand how these processes influence microbial community composition (15, 52). Although such studies provide important information concerning the taxonomic compositions of communities, the relationship between community physiology and water processing remains obscure (33). Because wastewater release depends on enterobacterial content, particularly the content of feces-associated taxa, it is important to clarify how the treatment process and facility management influence these organisms. To address this question, we developed a culture-independent single-cell method to determine enterobacterial physiological status based on the growth-state-specific protein content (40).
In *Escherichia coli*, Fis is an 11-kDa DNA binding protein (14, 16) which plays a critical role in coordinating rRNA synthesis with growth (27). Fis is present in replicating cells, and its abundance is directly correlated with the growth rate (3, 45). Fis abundance in a cell varies more than 500-fold between the extremes of rapid growth and the stationary phase, and Fis is conserved in the *Enterobacteriaceae* (2, 40). Therefore, we considered Fis abundance a positive indicator of cells in a balanced growth (2, 40). Dps is a highly conserved 19-kDa DNA binding protein (1, 20) that is important in stationary-phase stress physiology (1, 20, 39). Dps abundance is inversely correlated with the growth rate, and the cellular concentration of Dps varies more than 100-fold between the extremes of the stationary phase and rapid growth (1, 20, 28, 39). Dps abundance was therefore used as a positive indicator of cells that had entered the stationary phase (2, 40). In the present investigation, the impact of primary and secondary wastewater treatment on enterobacterial community physiology was determined by measuring single-cell levels of Fis and Dps with previously characterized enterobacterium-specific fluorochrome-conjugated polyclonal antibodies (40). Protein profiling was then combined with direct measurement of disinfection efficiency. The results provide new process strategies for improving enterobacterial disinfection in municipal wastewater.

METHODS

Cloning, purification, and production of antibodies for Fis and Dps.

The *fis* gene was amplified by PCR by using 5' TTGAATTCATGTTCGAACAACGCG 3' (forward primer) and 5' TTCTTAAGAGCATTTAGCTAACC 3' (reverse primer) from *E. coli* strain PBL500 (39). The resulting PCR product was cloned into the *Nco*I and *Xho*I sites of pET28b (Novagen), creating plasmid

pPB916, placing *fis* under control of the T7*lac* promoter, and adding a hexahistidine N-terminal fusion peptide. For induction of the Fis protein we employed *E. coli* strain BL21 DE3 (Novagen). Fis was purified by nickel affinity chromatography by using a His•Bind Quick column (Novagen) as described by the manufacturer. Preparation and purification of recombinant Dps were performed as described previously (39, 40). Rabbit anti-Fis and anti-Dps antibodies were prepared as described previously (39, 40). An *E. coli fis* mutant strain (PB918) was reconstructed by homologous recombination by using a phage-encoded mutant allele of *fis* as described previously (40). Recombinants devoid of integrated phage were verified by PCR after successive cycles of purification for single colonies on Luria-Bertani agar plates containing 0.5% (wt/vol) deoxycholic acid and then Luria-Bertani agar plates containing tetracycline (25 μg/ml).

Antibody probes.

Rabbit sera containing anti-Fis and anti-Dps antibodies were processed with acetone powders derived from homologous mutant strains PB918 and PB664 and then fractionated by affinity chromatography with protein A-Sepharose as described previously (17, 40). Anti-Fis antibodies were coupled to Alexa Fluor 488 (Molecular Probes), and anti-Dps antibodies were coupled to Alexa Fluor 568 (Molecular Probes). Coupling procedures and the extent of antibody labeling were determined as described by the manufacturer. The optimal coupling ratios were 4 to 9 mol of dye per mol of Fis antibodies and 2 to 6 mol of dye per mol of Dps antibodies. The conjugated antibodies were stored at 4°C in light-impermeable containers and were stable for several months. The degree of labeling was determined periodically to ensure that probe quality was maintained. Sample aliquots were used for long-term storage at -20°C to minimize freeze-thaw cycles and light exposure.

Oligonucleotide probe.

The 16S rRNA probe ENT specific for the *Enterobacteriaceae* described previously (26) (sequence, 5'-CATGAATCACAAAGTGGTAAGCGCC-3') was purchased prelabeled with flourescein by the manufacturer (Gibco-BRL). Single-cell 16S rRNA analysis with the fluorescein-labeled oligonucleotide was performed as previously described (12). The fixed cells were counterstained with 4',6'-diamidino-2-phenylindole (DAPI) (0.1 μg/ml) as described previously (34).

Wastewater and sample analysis.

Wastewater was obtained from a local municipal facility that serves a population of 200,000 individuals and treats 18 million gallons of water daily. Grab samples were recovered in 125-ml sterile bottles and processed immediately for protein profiling or placed on ice for transport to the laboratory. Total carbon levels were determined by the carbon combustion method (Leco Corp.). Dissolved oxygen concentrations were determined by using a dissolved oxygen meter (Sper Scientific) as described previously (10). Grab samples were analyzed by plating on selective media, including Endo agar (Becton Dickinson) and A-1 media (Difco).

Fluorescence microscopy and sample preparation.

Grab samples were fixed by addition of paraformaldehyde to a final concentration of 4% (vol/vol) by using a freshly prepared 40% (wt/vol) stock solution prepared in phosphate-buffered saline (PBS) (10 mM NaH_2PO_4, 150 mM NaCl; pH 7.2) and maintained at room temperature protected from light in brown glass bottles. Before fixation, samples were filtered with a 10-μm-pore-size filter paper disk (diameter, 5.5 cm; Fisher) to remove autofluorescent particulate material, fixed with paraformaldehyde, and shaken gently for 2 h at 25°C. Cells were recovered by filtration on 0.2-μm-pore-size membrane filters (Millipore), resuspended in PBS containing 4% (vol/vol) paraformaldehyde, and incubated at 4°C for 1 h with mixing. The cells were then resuspended in 4°C PBS by centrifugation for 5 min at 3,000 x *g*. Washed cells were then resuspended in PBS-100% ethanol (1:1, vol/vol) and stored at -20°C until they were used. Gelatin-subbed slides were prepared by dipping new slides into an aqueous solution containing 0.1% (wt/vol) type A gelatin (approximately 175 bloom; Sigma) and 0.01% (wt/vol) chromium potassium sulfate dodecahydrate [$CrK(SO_4)_2 \cdot 12H_2O$] and then dried for 10 min at 25°C. Fixed cells were applied to treated slides and air dried at 37°C, and then they were dehydrated by successive rinses in 50, 80, and 98% ethanol solutions. Cell permeabilization was accomplished by a lysozyme-EDTA treatment (54) with lysozyme (5 mg/ml) in 100 mM Tris-HCl-50 mM EDTA (pH 8.0). Rinsed and dried slides were then simultaneously treated with two fluorochrome-coupled antibody probes in the dark. Antibody probes in probing buffer (4% [wt/vol] bovine serum albumin, 150 mM NaCl, 100 mM Tris-HCl) were applied to the permeabilized cells on a slide and incubated for 2 h in a humidified chamber at 25°C. After incubation, the slides were washed in a buffer containing 150 mM NaCl, 100 mM Tris-HCl (pH 7.5), and 0.1% (wt/vol) sodium dodecyl sulfate for 20 min, washed in water for another 20 min, and air dried at 37°C. Fluorochrome bleaching was minimized by application of an aqueous solution of phenylenediamine (1 mg/ml) to probed cells, followed

by sealing of the coverslip with clear nail polish. Fluorescence emission from specifically bound fluorochrome-labeled antibody probes was detected with an epifluorescence microscope (Olympus AX 70) fitted with an LEI-750 charge-coupled device camera (DE1 750 Optronic) and light filter sets for fluorescein and rhodamine (Olympus). Images were captured, cells were counted, and fluorescence was quantified by using the Image-1 image analysis software (version 4.0; Universal Imaging). The relationship of fluorescence to the number of cells or to different amounts of Fis or Dps has been described previously (40), and the experiments included mixing experiments in which known numbers of cells having or lacking one of the target proteins (Fis or Dps) were analyzed by fluorescence and bright-field microscopy.

Image analysis.

Single-cell fluorescence intensity was determined by converting wavelength-specific cell brightness into a grey scale pixel value. The total pixel values ranged from 0 to 255 per cell. A midrange pixel value of 100 to 200 per cell was selected for data analysis. The midrange fluorescence data set excluded very faint and very bright fluorescent cells. At least some portion of the weakly fluorescent cells resulted from nonspecific probe binding that reflected insufficient target blocking as a compromise strategy to avoid signal quenching. Also excluded were rare unusually bright cells that fluoresced strongly in both emission ranges. For image analysis we employed automatic fluorescence measurements. The reliability of the automatic mode was validated by comparing automatic fluorescence measurements to manual measurements for identical samples containing a total of 500 cells. Noncellular fluorescent debris and particles were excluded from the analysis by using standard shapes, spheres and rods, for recognition of stationary and growing enterobacteria, respectively. Single-cell fluorescence was converted to a percentage of the maximum fluorescence observed for all cells in each sample for each of the two target proteins. Fluorescent cells were then divided into groups based on 10% incremental relative fluorescence values. The percentage of cells in each incremental group was then determined by determining the fraction of the total number of cells in all incremental groups. The average of the percentages for each incremental group for two replicate water samples was then plotted. The experimental data points included drop lines and a range of data point ball sizes. Ball sizes were proportional to the abundance of the fluorescent cell groups, and the following five ball sizes were used: largest, 100 to 20%; large, 20 to 10%; medium, 10 to 5%; small, 5 to 1.0%; and smallest, 1.0% to undetectable. Since most fluorescent cells fell within the 20 to 1.0% range, a nonlinear division was selected for graph construction.

Heat killing.

To assess the thermal tolerance of enterobacterial community samples, grab samples were collected in 125-ml sterile bottles, placed on ice, and then transported to the laboratory. The time between collection and processing in the laboratory was less than 1 h. To determine the impact of chilling on the community physiology in wastewater samples, *E. coli* K-12 cells (10^6 CFU/ml) were exposed to ice for 60 min and subjected to heat killing at 51°C. The percentages of killed cells before and after exposure to ice were 99.70 and 99.85%, respectively. Since there was no significant difference between the percentages of killed cells, sample chilling appeared to have little impact on subsequent viability determinations. Prior to heating, samples were filtered by using a 10-μm-pore-size filter paper disk (diameter, 12.5 cm; VWR) to remove large particulate material, which recovered more than 99% of the cells. Two-milliliter portions of the filtered samples were subjected to heating at 51°C in a prewarmed heating block for different periods of time. Heated samples were then diluted in sterile distilled water, spread onto duplicate Endo agar (Becton Dickinson) plates, and incubated overnight at 37°C. The surviving population was counted, and the percentage of the unheated population was calculated.

Statistical analysis.

The statistical significance of differences in enterobacterial physiology was investigated for different wastewater processing steps during the 5 weeks of successive sampling. Since there were two variables, week and treatment process, that could affect the dependent variable (growth state physiology), a two-way mixed-design analysis of variance (ANOVA) was used for data analysis. For the clusters of cells with fluorescent Fis and Dps grouped in 10% increments based on individual fluorescence, the fluorescent cell types with 80, 90, and 100% of the total cellular fluorescence were included for statistical analysis. The cells were selected because they exhibited maximum expression of growth-state-specific proteins and thus correlated most closely with the extremes of growth and the stationary phase. Since each week's sample data had been normalized previously to 100%, a portion of the data was included for statistical analysis to compare the variables based on the mean data. The SAS program (version 8.0) was used for ANOVA. Correlation analysis and a Student's *t* test were performed by using MS Excel.

RESULTS

Single-cell enterobacterial protein profiling during wastewater treatment.
The main components of the wastewater treatment plant and the locations of sampling sites are indicated in Fig. 1. Incoming wastewater (influent wastewater) was aerated and diverted into three primary clarifiers after an aerated grit removal process. The three primary clarifiers received approximately equal volumes of wastewater, and the wastewater from these clarifiers had completed primary treatment. Secondary treatment (biological treatment and clarification) followed this initial process and involved the use of trickling filters or activated sludge by surface aeration or submerged aeration methods. Each type of biological treatment was distinguished by the mode and extent of aeration. Wastewater from each biological treatment process was then individually clarified and pooled for tertiary treatment (chlorination). Residual chlorine was neutralized prior to entry into the receiving water.

The specificity and suitability of immunofluorescence techniques for the environmental samples were tested to assess detection of organisms other than enterobacteria present in the wastewater. Fixed cells from each sampling location were probed first with ENT, an oligodeoxynucleotide probe specific for the 16S rRNA of members of the *Enterobacteriaceae*, and counterstained with DAPI. Fixed cells also were probed with both anti-Fis or anti-Dps antibodies and then counterstained with DAPI. There was no statistically significant difference between the percentages of cells detected with the ENT probe and the percentages of cells detected with the anti-Fis and anti-Dps probes (Table 1). This indicated that the antibody probes were specific for enterobacteria and did not detect other organisms.

Single-cell levels of Fis and Dps were determined over a 5-week sampling period for uncultivated enterobacterial cells present in wastewater treatment samples. Samples were collected from the outlet of a treatment process, and cells were fixed immediately after sample collection to prevent variation in the Fis and Dps contents during sample transport. Protein profiles were determined by simultaneous application of enterobacterium-specific fluorochrome-labeled antibody probes. A representative micrograph showing the appearance of these probed cells is shown in Fig. 2. Fis and Dps levels were determined by measuring the fluorescence intensities of the corresponding fluorochrome-labeled antibodies for approximately 1,000 cells per time point over a 5-week period at selected sampling locations (Fig. 3). All samples contained cells with either Dps or Fis (Fig. 3), but the relative proportions varied considerably. In addition, the relative levels of fluorescence of cells within samples varied widely for both proteins at all sampling locations. In wastewater treatment plant influent samples, there were few cells with an abundance of Fis. However, more cells of this type appeared following primary treatment (Fig. 3A to C). The trickling filter process resulted in an increase in the number of cells with an abundance of Fis (Fig. 3D) compared to the number of such cells in primary clarifier wastewater (Fig. 3C). Cells containing large amounts of Fis also were rare in samples after the submerged aeration process (Fig. 3E). In most samples there were few cells with large amounts of Dps. However, the levels observed varied significantly between locations. In most cases, the cellular levels of Dps were low. Cells with relatively high levels of Dps were most evident in primary clarifier wastewater (Fig. 3C) and in submerged aeration basin wastewater (Fig. 3E).

Secondary wastewater from the biological treatment processes was pooled before chlorination (Fig. 1). Protein profiles of this pooled material revealed an abundance of cells with high levels of Fis (Fig. 3G). These cells could have resulted from the mixing of the distinct communities created during biological treatment. Such cells were present in trickling filter wastewater and absent in submerged aeration basin wastewater.
Average values for Fis and Dps cell contents also were determined for each of the treatment plant sampling locations (Table 2). These values were determined by adding all of the fluorescence values for Fis or Dps and normalizing the sum to the total number of cells examined. The resulting data reflected enterobacterial community averages. The values for sampling locations, however, were not statistically significantly different.

Efficiency of disinfection of community samples by heat inactivation.
The protein profile study revealed the presence of an enterobacterial community in submerged aeration basin wastewater containing the smallest number of cells with an abundance of Fis. In contrast, the enterobacterial community present in the trickling filter wastewater contained the largest number of cells

with an abundance of Fis. In general, the pattern for the number of cells with an abundance of Dps was the inverse of the pattern observed with Fis. However, the range of variation between samples was more constrained. To test the hypothesis that the protein profiles were indicative of growing and nongrowing enterobacterial communities, differences in the efficiency of disinfection were examined by using samples from the trickling filter (the location where the Fis cellular fluorescence was highest) and the submerged aeration basin (the location where the Fis cellular fluorescence was lowest). New samples obtained from the treatment plant were placed on ice and transported to the laboratory. Reconstruction experiments demonstrated that short periods of chilling did not alter the survival kinetics. Attempts to use chlorination were confounded by the presence of variable amounts of total carbon in the wastewater samples that titrated added chlorine and interfered with cell killing. Since the general pattern for growth state and resistance to chlorination also applied to killing by heat exposure (9, 38, 39), heating was used as an alternative means of disinfection.

The ambient temperature varied considerably in different seasons in the region occupied by the target wastewater treatment facility. Therefore, analysis of the additional samples also addressed the temporal stability of enterobacterial community protein profiles. Replicate samples of cells from trickling filter and submerged aeration wastewater were subjected either to protein profiling or thermal killing. These samples were obtained approximately 6 months after those described above were obtained (Fig. 3), and they were obtained during the summer months. This study again revealed the presence of both Fis-containing and Dps-containing cells in the water from the two processes, and the distributions resembled the distributions obtained in the experiment described above (Fig. 4). The number of cells with high levels of Fis was large in trickling filter wastewater and small in submerged aeration wastewater. The inverse pattern for Dps was also detected, as observed previously. In thermal killing studies we employed a heat treatment regimen consisting of exposure to 51°C for 50 min. Viability was assessed by plating samples at various intervals onto Endo agar plates to detect the enterobacterial segment of the community (Fig. 5). The thermal tolerance of enterobacteria derived from the submerged aeration wastewater was significantly different from the thermal tolerance of enterobacteria derived from the trickling filter wastewater. Enterobacteria in the trickling filter wastewater were highly sensitive to heat killing, and significant reductions in population sizes were apparent after 10 s of exposure. In contrast, no alteration in the population size was apparent for enterobacteria from the submerged aeration wastewater even after 30 s of exposure (Fig. 5, inset). Most of the enterobacteria in the trickling filter wastewater were killed after 2 min of exposure, compared with only one-half of the enterobacteria from the submerged aeration basin wastewater. More than 1% of enterobacteria from the submerged aeration wastewater were heat resistant even after 50 min of exposure. Similarly, less than 0.01% of enterobacteria in the trickling filter wastewater were resistant to heat killing.

These data reflected the physiological response of the total enterobacterial community to thermal killing because they were obtained by using Endo agar as the plating medium. Since wastewater release into receiving water is regulated by the content of fecal enterobacteria, additional efforts were made to examine this subset of the enterobacterial group. The heat resistance of fecal enterobacteria derived from the wastewater after both forms of biological treatment was examined by using the most-probable-number method of analysis and A-1 medium (42). The sensitivity to heat killing when this medium was used resembled that observed with Endo agar. Fecal enterobacteria derived from trickling filter wastewater were more sensitive to killing than fecal enterobacteria derived from submerged aeration basin wastewater (Fig. 6). After 2 min of exposure, there was no reduction in the number of fecal enterobacteria in water from the submerged aeration process, but after 1 min of exposure approximately 70% of the organisms from the trickling filter water were killed. While there were no surviving fecal enterobacteria after 50 min of exposure in trickling filter wastewater, a significant fraction remained culturable after 50 min of exposure in submerged aeration basin wastewater. These two studies confirmed that the physiology of both total enterobacteria and fecal enterobacteria in submerged aeration basin wastewater was significantly different from the physiology of enterobacteria in trickling filter wastewater.

To test whether the thermal killing profiles observed for the enterobacterial communities from biological treatment wastewater remained apparent after secondary clarification and before pooling and chlorine disinfection, additional samples were subjected to thermal killing. Survival again was measured after plating on a selective medium (Fig. 7). A distinctive pattern was observed, in which the thermal resistance was significantly greater in wastewater from the submerged aeration basin clarifier than in wastewater from either the trickling filter or surface aeration basin secondary clarifier. The dissolved oxygen and total

carbon contents were studied by using three duplicate samples collected simultaneously from the individual and pooled secondary clarifier wastewaters (Table 3). Both the dissolved oxygen and total carbon contents were found to be highest in the trickling filter secondary clarifier. The submerged aeration secondary clarifier wastewater also differed from the trickling filter secondary clarifier wastewater ($P = 0.002$) and the surface aeration basin secondary clarifier wastewater ($P = 0.05$), while the dissolved oxygen content was lowest in samples from the submerged aeration basin secondary clarifier. However, the oxygen levels in the three secondary clarifier wastewaters did not differ significantly from those in the final pooled secondary clarifier wastewater. There was no statistically significant difference among the total carbon levels in these secondary clarifier wastewaters. The variation in sample replicates, however, precluded assessment of the significance of these parameters for establishing or maintaining enterobacterial physiological status during wastewater treatment.

DISCUSSION

Our results reveal that there are distinct enterobacterial communities with characteristic protein profiles that reflect discrete phases of wastewater treatment. Protein profiles were used to predict physiological parameters, including sensitivity to disinfection. Single-cell analysis provided information that was essential for physiological predictions since bulk measurements of Fis and Dps contents that reflected community averages were nearly identical for different sample locations and thus failed to provide information that could be used to predict physiological differences between communities. Single-cell analysis of cellular Fis and Dps contents allowed detection of predictive trends in subsegments of the community that were otherwise obscured by invariant community components.

Two-way ANOVA revealed that there was a significant difference ($P = 0.0001$) in the number of cells with fluorescent Fis between weekly samples, but the difference between locations was not significant. The data for the combined variables (week and location), however, did reveal that there was a significant difference ($P = 0.0039$). Consequently, to understand the difference among the numbers of cells with fluorescent Fis in the processes, a contrast study was performed for the processes, especially the three different types of biological processing. The contrast study revealed that the number of cells with fluorescent Fis in the trickling filter wastewater differed significantly from the number in the submerged aeration basin wastewater ($P = 0.0345$) and the number in the surface aeration basin wastewater ($P = 0.0362$) at the 95% confidence level. Similarly, the cell types with fluorescent Dps present in the five weekly samples were examined by ANOVA. There were significant differences among the cells with fluorescent Dps when different sample weeks were compared, but the difference between locations was not significant. The combined interaction of week and location was also not significant. A contrast study of the processes, especially the three different types of biological processing, revealed that there was not a significant difference between locations for Dps.

If a treatment process favors exponential-phase cells (cells with fluorescent Fis) at one location, there must be a corresponding reduction in the abundance of stationary-phase cells (cells with fluorescent Dps). To confirm the existence of treatment selection in the trickling filter and the submerged aeration basin, a correlation study was performed by using cells with fluorescent Fis and Dps present in the trickling filter and submerged aeration basin wastewaters. The mean values for the cells with fluorescent Fis or Dps (averages of the 5-week sample set) were used for the correlation study. In this study we found that there was a significant negative correlation (correlation coefficient, -760) between cells with fluorescent Fis and cells with fluorescent Dps at a 99% confidence level in trickling filter wastewater. There was not a significant correlation between cells with fluorescent Fis and cells with fluorescent Dps in submerged aeration basin wastewater. The existence of the opposite trend for the types of exponential-phase and stationary-phase cells in **the trickling filter wastewater indicated that physiological selection during the trickling filter process promoted the growth of enterobacteria.**

The main finding of this study was that enterobacteria and fecal enterobacteria in trickling filter wastewater are much easier to kill than enterobacteria in submerged aeration basin wastewater. Protein profile and disinfection analyses conducted for later stages of the treatment process suggested that the distinctive enterobacterial communities were stable and contributed significantly to the community that underwent tertiary treatment (chlorination). **Consequently, inefficient tertiary treatment may result**

from treatment processes that produce disinfection-resistant enterobacterial communities. In addition, improved tertiary treatment may be obtained by directed manipulation of enterobacterial physiology, which has the potential for reducing chlorine use and minimizing release of disinfection by-products into receiving water.

Cells with an abundance of Dps occurred rarely in our studies. This suggests that the enterobacterial communities were metabolically active and capable of proliferating during wastewater treatment. However, the occurrence of cells with variable (although small) amounts of Dps indicated that these cells were in different physiological states in the early stages of the stationary phase. Even though oxygen and nutrients are generally available during wastewater treatment, the presence of Dps indicated that the bacteria were unable to grow well. This condition could reflect the presence of growth-inhibiting compounds or physiological stress.

In influent samples, only a few (<1%) of the cells exhibited high levels of Fis. However, a higher percentage of such cells appeared during subsequent treatment steps. **This indicates that there was** a metabolic shift or **growth transition precipitated by the treatment process**. Such differences were particularly apparent when modes of biological treatment were compared. The differences prompted the use of heat killing to measure the efficiency of disinfection. Since the enterobacterial community present in the submerged aeration basin wastewater was notably more resistant than the enterobacterial community present in trickling filter wastewater, the subfraction comprising fecal enterobacteria was examined. The efficiencies of disinfection of this subfraction exhibited similar process-dependent differences, providing results that are relevant to wastewater tertiary treatment and subsequent water release. **Prolonged exposure to heat resulted in the presence of a small percentage of enterobacterial cells that exhibited extreme resistance to killing**. Such cells are likely to have entered the stationary phase accumulating high levels of Dps and exhibiting concomitant resistance to killing. These findings suggest that most cells in the wastewater enterobacterial community occur in a growing state and are not starving.

It is important to note that the methods employed in this study to examine uncultivated cell physiology failed to distinguish between enterobacterial taxa. Much of the variation observed could reflect species-specific physiological differences. Methods that combine obtaining growth state information with determining taxonomic identity are currently being developed. Together with current strategies, such methods may increase treatment plant performance, reduce wastewater enterobacterial content, and minimize the release of disinfection by-products into receiving water.

Chlorine Dioxide Inactivation of *Cryptosporidium parvum* Oocysts and Bacterial Spore Indicators

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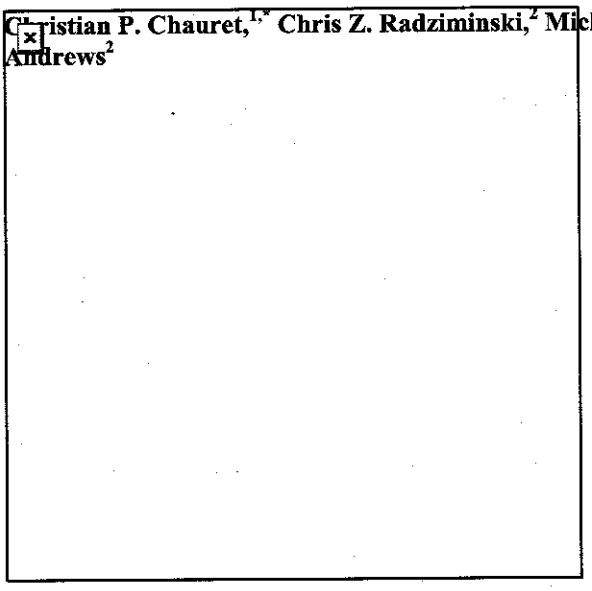

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Cryptosporidium parvum, which is resistant to chlorine concentrations typically used in water treatment, is recognized as a significant waterborne pathogen. Recent studies have demonstrated that chlorine dioxide is a more efficient disinfectant than free chlorine against *Cryptosporidium* oocysts. It is not known, however, if oocysts from different suppliers are equally sensitive to chlorine dioxide. This study used both a most-probable-number-cell culture infectivity assay and in vitro excystation to evaluate chlorine dioxide inactivation kinetics in laboratory water at pH 8 and 21°C. The two viability methods produced significantly different results ($P < 0.05$). Products of disinfectant concentration and contact time (*Ct* values) of 1,000 mg · min/liter were needed to inactivate approximately 0.5 $\log_{10}$ and 2.0 $\log_{10}$ units (99% inactivation) of *C. parvum* as measured by in vitro excystation and cell infectivity, respectively, suggesting that excystation is not an adequate viability assay. Purified oocysts originating from three different suppliers were evaluated and showed marked differences with respect to their resistance to inactivation when using chlorine dioxide. *Ct* values of 75, 550, and 1,000 mg · min/liter were required to achieve approximately 2.0 $\log_{10}$ units of inactivation with oocysts from different sources. Finally, the study compared the relationship between easily measured indicators, including *Bacillus subtilis* (aerobic) spores and *Clostridium sporogenes* (anaerobic) spores, and *C. parvum* oocysts. The bacterial spores were found to be more sensitive to chlorine dioxide than *C. parvum* oocysts and therefore could not be used as direct indicators of *C. parvum* inactivation for this disinfectant. In conclusion, it is suggested that future studies address issues such as oocyst purification protocols and the genetic diversity of *C. parvum*, since these factors might affect oocyst disinfection sensitivity. Applied and Environmental Microbiology, July 2001, p. 2993-3001, Vol. 67, No. 7
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Factors promoting survival of bacteria in chlorinated water supplies.

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Results of our experiments showed that the attachment of bacteria to surfaces provided the greatest increase in disinfection resistance. Attachment of unencapsulated Klebsiella pneumoniae grown in medium with high levels of nutrients to glass microscope slides afforded the microorganisms as much as a 150-fold increase in disinfection resistance. Other mechanisms which increased disinfection resistance included the age of the biofilm, bacterial encapsulation, and previous growth conditions (e.g., growth medium and growth temperature). These factors increased resistance to chlorine from 2- to 10-fold. The choice of disinfectant residual was shown to influence the type of resistance mechanism observed. Disinfection by free chlorine was affected by surfaces, age of the biofilm, encapsulation, and nutrient effects. Disinfection by monochloramine, however, was only affected by surfaces. Importantly, results showed that these resistance mechanisms were multiplicative (i.e., the resistance provided by one mechanism could be multiplied by the resistance provided by a second mechanism).
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