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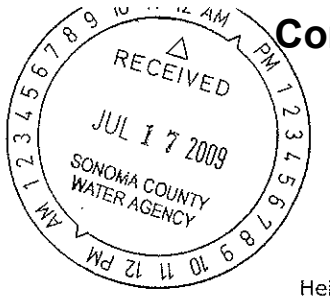

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July 13, 2009

Mr. Marc Bautista,
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P.O. Box 11628
Santa Rosa, CA 95406-1628

Via email: info@NBWRP.org.

CF/70-0-14 North San Pablo Bay Restoration and Reuse Project - EIR

COMMENTS ON THE NORTH BAY WATER REUSE PROJECT DEIR-EIS

Dear Mr. Bautista;

Thank you for this opportunity to comment on the North Bay Water Reuse Project ("NBWRP") DEIR-EIS ("project" or "document"). Having examined the document we are concerned that a number of harmful pollutants known to exist in tertiary-treated sewage effluent have been overlooked and that plans to use treated effluent do not account for significant, scientific, peer-reviewed research. L-1

Many of these contaminants by themselves, not to mention others that result from the combination, reaction or other transformation of two or more of these compounds, are considered toxic and therefore fall under the purview of existing legislative and regulatory stipulations discussed below.

Emerging Contaminants

We are particularly concerned that the distribution of what is casually referred to as "tertiary-treated" sewage would constitute a danger to public health. There is no agreed upon standard that defines "tertiary" treated sewage exactly. In practice, the purity of products from waste water treatment plants claiming "tertiary" treatment can vary wildly. Considerable evidence has accumulated over the past 20 years demonstrating: 1) the inadequacy of sewage treatment, including so-called "tertiary" treated sewage; 2) the ability of sewage treatment plants to actually produce new toxicants from the ingredients contained in raw sewage; and 3) the role that sewage treatment plays in increasing and spreading antibiotic resistance. L-2

The rise of antibiotic resistance in sewage plants was once believed to be a passive process of simply killing off vulnerable pathogens and leaving only a miniscule number of hardy pathogens. No doubt this process continues apace (see below). But as early as 1990, Nakamura and Shirota[1] discovered that multi-drug resistant ("MDR") pathogens do not just survive treatment, they can actually increase as treatment progresses. Additionally, a disturbing number of the survivors detected by these researchers carried extra packets of DNA coded for multi-drug resistance called "R plasmids."

> "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 [sic] that drug resistance transfer mediated by these R plasmids may occur in actual wastewater treatment plants." [emphasis added]

Observations of increased resistance after treatment have become common worldwide. For example, da Silva[2], et. al. observed rather dramatic increases of MDR *E. faecium* compared to levels detected earlier in raw sewage. In other words, antibiotic resistant pathogens actually increased from the amounts detected in the raw state because of treatment. Such examples can be multiplied many fold[3].

Antibiotic Resistance, a Rising Tide

It is difficult to exaggerate the danger of antibiotic resistance. Without antibiotic drugs, modern medicine would revert to a level of care not seen since World War I. In addition to curing a host of often lethal bacterial infections, virtually every major surgical procedure performed today is done using prophylactic antibiotic drugs. Developing resistance to antibiotics eventually will render these drugs obsolete—unless something is done to curb the spread of resistance.

Many factors contribute to antibiotic resistance but it has been well established that sewage treatment plays an integral role in reducing the efficacy of these so-called

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[1] Behavior of drug resistant fecal coliforms and R plasmids in a wastewater treatment plant, Nakamura S, Shirota H., Department of Food and Nutrition, Ube College, Japan, Nippon Koshu Eisei Zasshi. 1990 Feb;37(2):83-90

[2] Antibiotic resistance of enterococci and related bacteria in an urban wastewater treatment plant, Miguel Ferreira da Silva, Igor Tiago, Antonio Verissimo, Rui A. R. Boaventura, Olga C. Nunes & Célia Manaia, Federation of European Microbiological Societies FEMS Microbiol Ecol 55 (2006) 322–329, Published by Blackwell Publishing Ltd., 7 August 2005

[3] Occurrence and fate of antibiotic resistant bacteria in sewage, Luca Guardabassi, Anders Dalsgaard, The Royal Veterinary and Agricultural University, Department of Veterinary Microbiology, Environmental Project No. 722 2002, Miljøprojekt, Danish Environmental Protection Agency, etc.

"miracle drugs"[4]. If the project allows antibiotic-resistant pathogens and antibiotic-resistant genes to be spread via open dumping, and to travel to surface waters, the dramatic increase in antibiotic resistance will continue until we no longer have any "miracles" left. L-3

It is much cheaper to stop the contaminated material in the sewer plant than it is to fight bugs that have become resistant.

> "The cost of treating one person with multidrug-resistant TB is a hundred times greater than the cost of treating non-resistant cases. New York City needed to spend nearly US$1 billion to control an outbreak of multi-drug resistant TB in the early 1990s; a cost beyond the reach of most of the world's cities.[5]"

Sonoma County, Marin County and Napa County have already experienced a frightening rise in antibiotic-resistant pathogens. Methicillin-resistant *Staphylococcus aureus* ("MRSA") is a "fairly significant" problem in homeless shelters in Petaluma[6]. MRSA is not at all uncommon anymore in nursing homes in all three counties.

MRSA now exists in at least five varieties[7] of varying virulence some of which are exceedingly difficult if not impossible to cure, *e.g.* USA300-MRSA. USA300 is well established next door in San Francisco and it is only a matter of time before we see cases appear in the North Bay. Patients infected with USA300 are almost certainly traveling through the North Bay and using sanitary facilities making these pathogens available to waste water treatment plants. New drug resistant pathogens are being discovered with disturbing regularity, including strains that have developed resistance to Vancomycin, once regarded as the antibiotic of last resort.

The danger from antibiotic-resistant pathogens and genes qualifies as a serious pollutant under existing California law and sewage treatment plays an important role in amplifying this danger. Please bear in mind that all infectious pathogens, and the genes coded for antibiotic resistance that they may carry, wind up in sewage treatment plants. The project is obliged to discuss possible methods of either curtailing or eliminating the spread of antibiotic resistance. L-4

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[4] The Importance of Municipal Sewage Treatment in the Spread of Antibiotic Resistance, Sara Firl, University of Minnesota, 106th General Meeting of the American Society for Microbiology, Orlando FL, Session 041/Q, Paper Q-032, 2006

[5] DRUG RESISTANCE THREATENS TO REVERSE MEDICAL PROGRESS, Press Release WHO/41, 12 June 2000

[6] Homeless People at Higher Risk for CA-MRSA, HIV and TB, Healing Hands, HCH Clinician's Network, Vol. 10, No. 5 n December, 2006

[7] Understanding The Impact Of MRSA On Limb Preservation, Loan Lam, DPM, Peter Blume, DPM, FACFAS, and Michael Palladino, DPM, FACFAS, Podiatry Today, Issue Number: 7, VOLUME: 20, Jul 01 2007

Antibiotic-Resistant Genes

A study by Pruden [8], et. al., describes antibiotic-resistant genes ("ARG") as emerging contaminants in treated sewage. Pruden showed that ARGs not only survive sewage treatment they can be detected in drinking water supplies after treated effluent is discharged into surface waters. Since the project proposes that tertiary-treated sewage effluent be used as a substitute for fresh water where potable water is not necessarily required, this material should be thoroughly examined for antibiotic-resistant genes and antibiotic-resistant pathogens. An alternative to rigorous testing would be to employ a treatment process that guarantees ARG removal or their utter destruction. However, none of the wastewater treatment plants ("WWTP") that supply water to the project guarantee this. Removal or complete destruction of ARGs would necessarily require the destruction of all gene fragments as well as all cassettes and plasmids. L-5

Wastewater treatment plants are unique environments that collect a multitude of pathogens from entire sanitary districts—pathogens that would not ordinarily find themselves in close proximity. In addition to this unique population of pathogens is a concomitant collection of antibiotic drugs. Both humans and livestock excrete up to 95% of the antibiotic drugs they ingest[9]. Antibiotic drugs tend to be stable compounds, making the presence of pure, not metabolized, antibiotic pharmaceuticals in the sewage matrix significant.

This unique matrix of pathogens and antibiotics initiates a process where weak, susceptible pathogens die off and ever stronger, resistant pathogens are selected. In a very real sense, sewage treatment facilities are evolution accelerators creating antibiotic resistance on an industrial scale.

A March 24, 2009 study of antibiotic-resistance in WWTP flatly concluded:

> "These results suggest that [the] wastewater treatment process contributes to the selective increase of antibiotic resistant bacteria and the occurrence of multi-drug resistant bacteria in aquatic environments.[10]"

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[8] Antibiotic Resistance Genes as Emerging Contaminants: Studies in Northern Colorado, Amy Pruden, RuoTing Pei, Heather Storteboom, and Kenneth H. Carlson, Environ. Sci. Technol. 2006, 40, 7445-7450

- Report on Antibiotic Resistance and Recycled Water to Marty Blum, Mayor of Santa Barbara, California by Edo McGowan, Ph.D., May 8, 2009

[9] Pruden, et. al. *supra*

[10] Wastewater treatment contributes to selective increase of antibiotic resistance among Acinetobacter spp., Zhang Y, Marrs CF, Simon C, Xi C., Department of Environmental Health Sciences, University of Michigan, Ann Arbor, USA., Sci Total Environ. 2009 Jun 1;407(12):3702-6.

-Sewage Plants May Be Creating "Super" Bacteria, Andrew McGlashen and Environmental Health News, Scientific American, April 16, 2009

Deaths from MDR Bacteria

Antibiotic-resistant bacteria are already a serious public health threat. An American Medical Association study determined that, in 2005, 19,000 Americans died from Methicillin Resistant *Staphylococcus aureus* ("MRSA")[11]. This death toll is greater than the number of Americans who died from complications resulting from HIV-AIDS.

The rise of community-associated MRSA ("CA-MRSA") appears to coincide with the EPA easing Clean Water Act restrictions on sewage sludge and allowing open dumping[12]. The suspicion that hospital-acquired MRSA ("HA-MRSA") escaped the hospital setting because of open dumping of sewage sludge is compelling. More research is needed to confirm these suspicions but it is clear that "treated" sewage plays a not-insignificant role in spreading antibiotic-resistance and WWTP operators should be taking pro-active steps to curtail the spread of ARGs, MRSA, or any other material contributing to the antibiotic-resistant epidemic.

Even if MDR pathogens are destroyed during treatment, the genes these pathogens once carried, encoded for antibiotic-resistance, are deposited into the sewage matrix making them available to other pathogens to incorporate and become resistant to antibiotic drugs. If ARGs enter the body, they can exchange genetic information with gut flora and transfer antibiotic resistance to persons unlucky enough to ingest them. Theoretically, a single bacterium measuring a mere .5 µm ($1x\ 10^{-16}$) is capable of multiplying antibiotic-resistant genes to human gut flora in the billions within the short space of 24 to 36 hours. Bacteria are ferociously promiscuous and are known to exchange genetic material at astounding rates and efficiencies.

Treated effluent discharged from waste water treatment plants ("WWTP") should not be considered free of ARGs or MDR pathogens.

The DEIR-EIS does not discuss ARGs, antibiotic-resistant pathogens or the means by which the project intends to reduce or eliminate their pernicious effects. Also missing are estimates of effective levels of ARG inoculates required to transfer antibiotic-resistance to people who come in contact with treated effluents. Since the project intends to spread treated sewage effluent which may very well contain these contaminants, the DEIR-EIS is obliged to account for these risks in some detail and offer means to mitigate or eliminate the potential threats they pose to public health. L-6

Treated Sewage Can Systemically Contaminate Plants

The project proposes wide distribution of treated sewage effluent on agricultural crops and references "Efficacy of Pathogen Removal During Full-Scale Operation of Water Reuse Facilities In Monterey, California" to justify this practice. This study characterizes irrigation with treated sewage as benign but makes this claim at the expense of a L-7

[11] Infection Killed 19,000 in 2005, Study Says, New York Times, October 16, 2007, Kevin Sack

[12] cf. 40 CFR Part 503, promulgated on February 19, 1993.

considerable body of evidence that has found otherwise. In fact, several studies have found that vegetation, including agricultural crops, readily uptake pharmaceuticals, pathogens, antibiotic-resistant pathogens and other micro pollutants from treated sewage effluent and sludge, sometimes with lethal effect. L-7 cont.

The U.S. Environmental Agency ("EPA") presented data [13] at the recent Micropol & Ecohazard 2009 conference in San Francisco that demonstrates the uptake of antibiotics and illegal drugs in various plants watered with treated effluent or fertilized with treated sewage solids. Yates [14] similarly demonstrated plant uptake of both bacterial and viral pathogens as well as parasites. All these contaminants entered the plants as a result of using treated—and declared safe—but still contaminated, sewage.

The EPA authors note that they were able to detect:

> " . . Azithromycin and Methamphetamine in Bermuda roots sampled from a field that had been treated for several years with biosolids . . . There were traces of uptake of clindamycin into spinach leaves and possibly lettuce root . . . Trace amounts of roxithromycin were detected in lettuce roots. Carrots showed the greatest amount of uptake of roxithromycin, 110 ng/g, from 1000 ng/L of roxithromycin watered into the carrot plots. All of the plants, except the carrots, from the field crops watered with Tucson wastewater effluent showed uptake of n,n'-dimethylphenethylamine, an industrial chemical used in manufacturing, food industry, etc."

The mechanism of vegetative uptake of pollutants is so well established that some alternative sewage treatment technologies actually rely on doing exactly this to "trap" pollutants in trees or other plants [15].

The DEIR-EIS suggests that watering vineyard grapes, for example, with treated sewage would be safe because the edible part of the plant does not come into contact with contaminated waters. There is no mention, let alone estimates, of what types of contaminants, and in what quantities, would migrate into the grapes. Unfortunately, there is nothing in the wine-making process that would remove, sanitize, disinfect or otherwise render harmless the host of possible contaminants known to exist in treated L-8

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[13] A Case Study: Crop (Lettuce, Spinach, and Carrots) Uptake of Three Macrolide Antibiotics (Azithromycin, Clindamycin and Roxithromycin) and Other Drugs, Tammy L. Jones-Lepp, Charles A. Sanchez, Research Chemist U.S. EPA ORD, NERL, Environmental Sciences Division, Las Vegas, NV and University of Arizona Department of Soil, Water, and Environmental Sciences, Yuma Agricultural Center, Yuma AZ, respectively.

[14] PATHOGENS IN RECLAIMED WATER, M.V. Yates, P.h.D., Professor of Environmental Microbiology College of Natural and Agricultural Sciences, University of California Riverside, Informational handout at lecture, 1989.

[15] Wastewater Management Using Hybrid Poplar, Agroforestry Notes, USDA Forest Service, USDA Natural Resources Conservation Service, April 2000

sewage[16]. Exposing grape stock to treated sewage effluent risks polluting both grape and wine.

The risk of contaminating grapes used by the North Bay wine industry could set in motion incalculable economic repercussions. This scenario is particularly credible since every alcoholic beverage business in the world ultimately relies on the perception of pristine water as the foundation for the product. This is true whether the product is beer, wine or whiskey. Tertiary-treated sewage effluent is by definition anathema to this universal principle and very far from the perception of pristine water.

L-8 cont.

Plant uptake of pollutants in crops eaten raw, e.g. strawberries, lettuce, carrots, etc., require extra careful laboratory analysis to guarantee that these food crops are contaminant free. However, discussions, studies and recommendations pertaining to these well-known problems are absent in the DEIR-EIS.

Deaths from Contaminated Plants

In 2008, several hundred dairy cattle in the State of Georgia died from eating hay that had been grown on land fertilized with sewage sludge. The court trials that resulted from this case of mass poisoning documented a clear instance where toxic materials, in this case heavy metals, passed from treated sewage into plants making the feed lethal to consume[17]. Worse, even the milk was contaminated. The Augusta Chronicle, a local newspaper, noted: "In one case, according to test results provided to the AP, the level of thallium—an element once used as rat poison—found in the milk was 120 times the concentration allowed in drinking water by the Environmental Protection Agency."[18]

Phthalate Toxicity and Dosage

Researchers, water suppliers and others can be misled by terms like "trace" or "insignificant" when used to quantify amounts of pollutants that remain after sewage treatment. Increasingly, researchers are discovering appreciable effects from pollutant levels previously believed to be below safe thresholds[19]. Additionally, other chemicals

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[16] Validity of the Indicator Organism Paradigm for Pathogen Reduction in Reclaimed Water and Public Health Protection, Valerie J. Harwood, Audrey D. Levine, Troy M. Scott, Vasanta Chivukula, Jerzy Lukasik, Samuel R. Farrah, and Joan B. Rose, APPLIED AND ENVIRONMENTAL MICROBIOLOGY, June 2005, p. 3163–3170, Vol. 71, No. 6

[17] R.A. McELMURRAY, III, R.A. McELMURRAY, JR ., RICHARD P . McELMURRAY, and EARL D . McELMURRAY, *V*. UNITED STATES DEPARTMENT OF AGRICULTURE, NO . CV105-15 9, Feb 25, 2008

-UNITED STATES SENATE COMMITTEE ON ENVIRONMENT AND PUBLIC WORKS Briefing on "Oversight on the State of Science and Potential Issues Associated with EPA's Sewage Sludge Program" September 11, 2008 TESTIMONY OF ROBERT A. (ANDY) MCELMURRAY, III

-Researchers Link Increased Risk Of Illness To Sewage Sludge Used As Fertilizer, Science Daily, July 30, 2002

[18] "National policy brought sludge to Augusta farms: Ruling for farmer disputes government data" , Augusta Chronicle, Sunday, March 09, 2008

[19] Counterintuitive toxicity: increasingly, scientists are finding that they can't predict a poison's low-dose effects, Raloff, Janet, Jan 20, 2007, Science News, ISSN: 0036-8423

known to survive the treatment process, for example phthalates, behave as endocrine disruptors and therefore mimic hormones.

Hormones are some of the most potent chemicals known to science; vanishingly small doses can provoke impressive, often harmful, biological reactions.[20] In the past, agencies, municipalities, boards and other custodians of water quality, supply and safety have been able to discount very small amounts of contaminants and believe them to be safe. Nevertheless, a growing body of research warns us that ignoring contaminants like phthalates, even in "miniscule" amounts, contradicts prudent scientific discipline.

Hormones, and the chemicals that mimic them, can be biologically active in parts per trillion[21] (*i.e.*, 1 part per 10^{12}).

The DEIR-EIS does not discuss this threat to public health nor does it present the results of studies to determine the extent of damage that the project would contribute to endocrine disruption in human and animal populations. The document offers no means to ameliorate or eliminate this threat. L-9

Chlorine and Residual Pollutants

The DEIR-EIS makes no mention of chemical transformations occasioned by chlorine and the various contaminants known to remain in processed sewage products.

For example, on page 3.4-3, of the NORTH SAN PABLO BAY RESTORATION AND REUSE PROJECT (NORTH BAY WATER RECYCLING PROGRAM), we find: "Free chlorine residual at concentrations of less than 1 milligram per liter (mg/L) usually poses no problem to plants." Chlorine may or may not pose a problem to plants at this level, but chlorine is known to trigger several reactions that are very much a problem to human health. L-10

Chlorinated Triclosan Derivative Products

Triclosan (5-chloro-2-(2,4-dichlorophenoxy)phenol) is a ubiquitous antimicrobial found in soaps, shampoos, toothpastes, and other products, and routinely detected in WWTP sludge and effluent. The sewage treatment plants that supply the NBWRP with effluent do not remove all of the triclosan they receive in raw sewage. Triclosan and chlorine are known to react and create chlorinated triclosan derivative ("CTD") products. When exposed to sunlight, CTDs will photolyse in water and form polychlorodibenzo-*p*-dioxins, dioxin is a potent toxicant and a threat to public health. "It is important to determine the L-11

[20] Effects of relatively low levels of mono-(2-ethylhexyl) phthalate on cocultured Sertoli cells and gonocytes from neonatal rats, Li LH, Jester WF Jr, Orth JM., Department of Anatomy and Cell Biology, Temple University School of Medicine, Philadelphia, Pennsylvania, 19140, USA. Toxicol Appl Pharmacol. 1998 Dec;153(2):258-65.

[21] DETECTION OF HORMONE MIMICS IN WATER USING A MINITURISED SPR SENSOR, ADAMA M. SESAY and DAVID C. CULLEN, Cranfield Biotechnology Centre, Institute of BioScience and Technology, Cranfield University at Silsoe, Silsoe, Bedfordshire, U.K., Environmental Monitoring and Assessment 70: 83–92, 2001

amount of CTDs formed from triclosan during wastewater disinfection, because they may give rise to more highly toxic dioxins."[22]

Yet there is no mention of this toxicant or the "more highly toxic dioxins" it may form; no mention of any studies performed by the project to determine polychlorodibenzo-*p*-dioxins load destined for public waters or suggested methods to eliminate it.[23]

L-11 cont.

Chlorine and MRSA

Exposure to chlorine has been demonstrated to exacerbate virulence in Methicilin-resistant *Staphylococcus aureus* by inducing amino acid synthesis genes as well as enhancing exotoxins, hemolysins, leukocidins, coagulases, and surface adhesion proteins—the very mechanisms that make MRSA so dangerous[24]. Since the sewage treatment facilities for the project do not guarantee the removal of all *Staphylococcus aureus*, we may assume that a certain number will exist in the "recycled" water[25] and that some of these may have been made more virulent through chlorine exposure.

However, the DEIR-EIS makes no mention of most probable number ("MPN") estimates of MRSA in treated effluent nor are there any studies referenced that gauge the potential risk to public health by spraying MRSA-containing water on schoolyards, golf courses, domestic lawns, parks and the other public areas that the project plans to irrigate. Also missing are studies demonstrating increased virulence of MRSA, if any, as a result of exposure to chlorine in WWTP and the fate of any enhanced pathogens once released in public.

L-12

Chlorine and the Immune System

When chlorine is used as a disinfectant, weak bacteria die and strong bacteria survive. This process has gone on long enough for microbiology to recognize many chlorine-

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[22] Formation and Occurrence of Chlorinated Triclosan Derivatives (CTDs) and their Dioxin Photoproducts, Jeffery M. Buth, William A. Arnold, Kristopher McNeill, University of Minnesota, Department of Chemistry, buthx007@umn.edu

[23] *Nota Bene*: Only manufacturers of dioxin products (American Chemical Council members) have attempted to depreciate the CTD study. However, the nexus of profit motive versus negative publicity render these deprecations suspicious to dubious.

[24] Toxicogenomic Response to Chlorination Includes Induction of Major Virulence Genes in Staphylococcus aureus, Matthew Wook Chang,, Freshteh Toghrol, and, William E. Bentley, Environmental Science & Technology 2007 41 (21), 7570-7575

[25] A seasonal study of the mecA gene and Staphylococcus aureus including methicillin-resistant S. aureus in a municipal wastewater treatment plant. Börjesson S, Melin S, Matussek A, Lindgren PE. Department of Clinical and Experimental Medicine, Division of Medical Microbiology, Linköping University, SE-581 85 Linköping, Sweden, stefan.borjesson@liu.se

-Antibiotic Resistance in Wastewater: Methicillin-resistant Staphylococcus aureus (MRSA)and antibiotic resistance genes, Börjesson, Stefan, Linköping University, Medical Microbiology, Doctoral thesis, 2009.

-Harwood, et. al. *supra*

resistant bacteria[26]. Chlorine-resistant bacteria present a serious health challenge because the body's leucocytes destroy pathogens by injecting them with hypochlorite. If, however, disease-causing bacteria are already immune to chlorine, then the unfortunate person infected with such pathogens has, in effect, no working immune system.

The DEIR-EIS contains no discussion of chlorine-resistant bacteria, their affect on the human immune system or the estimated number of such bacteria found in sewage effluent that is intended for public distribution. The DEIR-EIS is missing estimates regarding the project's contribution to chlorine-resistant pathogen populations in general and the overall effect, if any, the project will have on public health as a result. L-13

Chlorine and Acetaminophen

Regardless of the efficacy that chlorination may have in reducing or destroying pathogens, chlorine has been demonstrated to transform certain common chemicals with significant health risks into vastly more potent chemicals with much greater health risks. Chlorine is known to transform acetaminophen (Tylenol®) into two separate toxicants neither of which were introduced to the waste stream[27].

The DEIR-EIS does not account for the potential dangers occasioned by chlorine reactions with acetaminophen, or with any other materials, during the treatment process nor on the fate of such substances once they are released into the environment. L-14

This single study by Bender, et. al. is a cautionary tale. It demonstrates that no one could possibly know that any particular batch of "tertiary" treated effluent is safe because it is impossible to predict all of the potential transformations and reactions that take place in such a rich confusion of chemical compounds.

What You Don't Know Can Hurt You

Numerous reports[28] attest to the persistence of a wide variety of pharmaceutical compounds in treated sewage and treated wastewater that migrate to surface waters.

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[26] Phenotypic and Genetic Diversity of Chlorine-Resistant Methylobacterium Strains Isolated from Various Environments, AKIRA HIRAISHI, KATSUNORI FURUHATA, ATSUHIKO MATSUMOTO, KAZUKO A. KOIKE, MASAFUMI FUKUYAMA, AND KIYOSHI TABUCHI, APPLIED AND ENVIRONMENTAL MICROBIOLOGY, June 1995, p. 2099–2107 Vol. 61, No. 60099-2240/95 Copyright 1995, American Society for Microbiology

[27] Transformation of Acetaminophen by Chlorination Produces the Toxicants 1,4-Benzoquinone and N-Acetyl-p-benzoquinone Imine, Mary Bender, William A. McCrehan, Analytical Chemistry Division, National Institute of Standards and Technology, ENVIRON. SCI. & TECHNOL.,

[28] AP: Drugs found in drinking water, Jeff Donn, Martha Mendoza and Justin Pritchard, Associated Press, USA Today, 2008-03-10;

-Where rivers run high on cocaine, NIGEL HAWKES, Times (UK) Online, August 05, 2005;

-PRESENCE OF PHARMACEUTICALS IN WASTEWATER EFFLUENT AND DRINKING WATER, METROPOLITAN ATLANTA, GEORGIA, JULY–SEPTEMBER 1999, Elizabeth A. Frick, Alden K. Henderson, Ph.D., M.P.H., Deborah M. Moll, Ph.D, Edward T. Furlong, Ph.D., and Michael T. Meyer, Ph.D., Proceedings of the 2001 Georgia Water Resources Conference , held March 26-27, 2001

These discoveries sometimes note that the amounts of drugs detected are below therapeutic dosages and therefore—incorrectly—consider them to be harmless.

Low dosage notwithstanding, endocrine disruptors can be biologically active in parts per trillion, as noted *supra*. Bioaccumulation can create serious health problems as "miniscule" amounts of certain chemicals accrete to biologically toxic levels. Also, thresholds gauging toxicity are constantly being reevaluated, usually downward.

The DEIR-EIS does not take into account the possible chemical reactions amongst the unusually large numbers of pathogens, pharmaceuticals, illegal drugs, industrial chemicals, endocrine disruptors, ARGs, antimicrobial products, bacteria, viruses, prions and other material found in treated sewage and then spread on land or discharged into surface waters. Since these toxicants will be placed in close proximity to the public the DEIR-EIS is obliged to measure the risk to public health from the variety of contaminants intended for distribution. L-15

Ultra-Violet Light Disinfection

The DEIR-EIS notes that some WWTP use Ultra-Violet ("UV") light as a disinfectant instead of chlorine. UV disinfection is often considered more efficient than chlorine and also avoids some of the problems associated with chlorine.

However, UV disinfection has no effect on endosymbiont bacteria nor on the genetic material they contain. Endosymbionts, especially those that have developed antibiotic-resistance, because ARGs stand an excellent chance of surviving disinfection, whether by UV light or chlorination or both. The project does not discuss the problem of endosymbionts nor does it offer a solution. L-16

Resurrection

Even when exposed to UV disinfection, micro organisms are notorious for being able to repair UV damage, either through Photo-Reactivation, if sunlight is available to provide energy, or, if sunlight is denied them, through a complex enzyme chemistry known as Dark Repair. Either way, malignant pathogens can and do resuscitate if not completely annihilated. The DEIR-EIS does not discuss the phenomenon of reactivated pathogens in the sewage effluent that the project plans to use from those plants employing UV disinfection. L-17

Two other means by which pathogens may suddenly seem as if they have come back to life after being pronounced "dead" are Sudden Increase ("SI"), when bacterial counts taken shortly after dewatering can dramatically balloon, and Regrowth, when bacterial counts appreciably increase during storage of dry cake sludge prior to land application.

Dewatering, especially with a centrifuge, can physically rupture some pathogen cell walls allowing the nutrients inside the cells to disgorge into the sludge mixture. The bacteria in the sludge have exhausted their food source due to long time exposure in

the digesters. Essentially, hunger has made them unresponsive and they appear dead because they do not culture. These seemingly dead bacteria are called viable non-culturable ("VBNC"). VBNC bacteria can suddenly come "back to life" when exposed to the rich nutrients released from ruptured bacteria and they feed again. SI readings can be orders of magnitudes higher than measurements taken after anaerobically digested sludge material is tested[29].

When methods of dewatering other than a centrifuge is used, for example a belt press, the seemingly "dead" pathogens can also spark back to life in the presence of nutrients found in soil. The application of sludge on agricultural land will place VBNC pathogens in close proximity with manure, fertilizers and endemic nutrients that can revivify them.

Similarly, processed sludge that simply sits for extended periods prior to land distribution has been shown to "re-grow" pathogens once believed to be "dead".

Sewage sludge is a necessary by-product of the so-called "recycled" water that the project specifies in the DEIR-EIS. If this sludge is not sequestered in a hazardous landfill and if it is destined for land application, as the projects suggests, then storm runoff from sludge will make significant contributions of pollutants to the project's overall water design. The project is obliged to discuss both SI and Regrowth as defined above to account for the potential dangers of infection to the public. L-18

Regulatory Compliance

The project DEIR-EIS makes repeated mention that the sewage plants involved in the project produce reclaimed or recycled water that meets standards established by the USEPA. This may be so but even given the small sample of scientific, peer-reviewed literature referenced in this short letter, there is considerable reason to doubt that the project would be able to comply with the California Health and Safety Code ("CHSC") §§ 5410-5416 inclusive. For example: L-19

> § 5410(d): "Contamination" means an impairment of the quality of the waters of the state by waste to a degree which creates a hazard to the public health through poisoning or through the spread of disease. "Contamination" shall include any equivalent effect resulting from the disposal of waste, whether or not waters of the state are affected.
>
> § 5410(f): "Nuisance" means anything which: (1) is injurious to health, or is indecent or offensive to the senses, or an obstruction to the free use of property, so as to interfere with the comfortable enjoyment of life or property, and (2) affects at the same time an entire community or neighborhood, or any considerable number of persons, although the extent of the annoyance or damage inflicted upon individuals may be

[29] Reactivation of Fecal Coliforms after Anaerobic Digestion and Dewatering, Hendrickson, Donald A.; Denard, Dave; Farrell, Joseph, Proceedings of the Water Environment Federation, Residuals and Biosolids Management 2004 , pp. 1018-1026(9), Water Environment Federation

unequal, and (3) occurs during, or as a result of, the treatment or disposal of wastes.

§ 5411: No person shall discharge sewage or other waste, or the effluent of treated sewage or other waste, in any manner which will result in contamination, pollution or a nuisance.

L-19 cont.

Similarly, the heart of the California Environmental Quality Act ("CEQA"), an act to which the DEIR is specifically subject, requires strict protection of California's environment. In pursuit of this goal public agencies "shall mitigate or avoid the significant effects on the environment . . . *whenever it is feasible to do so.*" (Pub. Resources Code § 21002.1, italics added.) Thoroughly cleaning sewage waste is eminently feasible and many sanitary districts around the country and around the world accomplish this feat on a routine basis.

Modern sewage treatment employs multi membrane reverse-osmosis filtration, ultra violet disinfection; nano-filtration, ozone exposure and other modern technologies all carefully monitored with rigorous, on-going analysis of the resulting product.

CEQA's substantive mandate requires that projects with significant impacts be denied if feasible alternatives accomplish most project objectives. The mandate does not include as a caveat " . . . unless a public agency does not want feasible alternatives to be imposed."

Must a community pay the environmental price when public agencies, local governments and developers determine to build a project simply because it generates the highest possible profits and is the most expedient way to dump sewage? Simply stated, may profit-driven and cost-cutting goals hold CEQA hostage? The law says "no."

L-20

CEQA's definition of "feasible" turns on practicality, not on maximum profits or cost-cutting—for example, by expanding water availability for development, expansion and other growth-inducing mechanisms. (Pub. Resources Code §21061.1.) In failing to understand and properly interpret the law regarding feasible alternatives, and in failing to comply with other mandates of CEQA that require an EIR to adequately study water supply, contamination of public waterways, and other potential public health hazards, the NBWRP DEIR-EIS appears not to comply with CEQA.

Exacerbating community acquired antibiotic-resistance; the spread and even creation of antibiotic-resistant pathogens; the creation and spread of chlorine-resistant pathogens; contamination of waterways with endocrine-disrupting phthalates; and threatening both the wine and agricultural produce industries with deep, systemic pollution, would each appear to contravene both the spirit and letter of these regulatory stipulations.

It appears unclear to us how the DEIR-EIS would satisfy these legal hurdles in its present state.

L-21

Historical Perspective

In the past, in fact in the very recent past, the use of so-called "recycled" water seemed reasonable and safe to both scientists and environmentalists. However, in light of the scientific investigations herein submitted, so-called "recycled" water now occupies an historical moment analogous to that of cigarettes in the 1950's or DDT in the 1970s.

In 1957, most people did not take seriously the warnings of Surgeon General Leroy Burney, M.D., when he declared cigarette smoke injurious to health. Indeed, it took decades of scientific evidence and a slow but inexorable gathering of social opprobrium before Americans fully realized the danger and stopped smoking on a large scale. The number of smokers today is miniscule compared to people who smoked in 1957.

In 1948, the Swiss chemist Paul Müller actually received the Nobel Prize in Physiology or Medicine for his discovery that DDT was an effective contact poison for certain insects. Initially, DDT seemed to be a boon for public health and comfort. But by 1972, the United States had banned DDT completely after discovering that it is a carcinogen and that it posed a serious and targeted threat to avian life.

The widespread use of partially-cleaned sewage effluent, "reclaimed" or "recycled" water, appears to be following a similar trajectory from acceptance to rejection. In the end, we must recycle water, not only to comply with regulations but to survive. "Recycle", however, means to remove all contaminants, not just some of them. The goal is to return, or recycle, contaminated water back into fresh water. L-22

Sincerely,

H.R. Downs
President
O.W.L. Foundation

1: Nippon Koshu Eisei Zasshi. 1990 Feb;37(2):83-90. Links

[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.

PMID: 2131972 [PubMed - indexed for MEDLINE]

Comment Letter L

FEMS

Antibiotic resistance of enterococci and related bacteria in an urban wastewater treatment plant

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Keywords
enterococci; ecology; antibiotic resistance; wastewater treatment.

Abstract

The main objective of this work was to study the ecology of enterococci and related bacteria in raw and treated wastewater from a treatment plant receiving domestic and pretreated industrial effluents in order to assess the influence of treatment on the prevalence of antibiotic resistance phenotypes among this group of bacteria. The predominant species found in the raw wastewater were *Enterococcus hirae*, *Enterococcus faecium* and *Enterococcus faecalis*. Wastewater treatment led to a reduction in *E. hirae* ($\alpha < 0.1$) and an increase in *E. faecium* ($\alpha < 0.1$); the relative proportions of *E. faecalis* remained the same in the raw and in the treated wastewater. Among the isolates tested, no vancomycin resistance was observed among the enterococci. *Enterococcus faecium* and *E. faecalis* showed resistance prevalence values reaching 33%, 40% and 57% for the antibiotics ciprofloxacin, erythromycin and tetracycline, respectively. Antibiotic-resistant strains of enterococci were not eliminated by wastewater treatment. A positive selection of ciprofloxacin-resistant enterococci was indicated by a significant increase in resistance prevalence ($\alpha < 0.02$) in treated wastewater compared with the raw wastewater.

Introduction

Enterococci are important commensal members of the intestinal microbiota of humans and animals. Despite their widespread distribution in food products, enterococci may be opportunistic pathogens and are frequently associated with nosocomial infections (Devriese *et al.*, 1992; Klein, 2003). As a result of the high prevalence of acquired antibiotic resistance, enterococci are recognized as important active spreading agents of this type of resistance both at intra- and at interspecific levels (Klare *et al.*, 2003). Recent studies, focusing on habitats related to human activity such as processing of food products, animal production or wastewater treatment, have provided new insights on the epidemiology and ecology of antibiotic-resistant bacteria (Kuhn *et al.*, 2000; Blanch *et al.*, 2003; Peters *et al.*, 2003; Hayes *et al.*, 2004). Despite the ubiquitous character of enterococci, their distribution in wastewaters and their fate during water treatment have been poorly characterized. This strongly limits our understanding of their role as indicators of faecal pollution, of the ecology of pathogenic strains or of their involvement in antibiotic resistance transmission.

Domestic wastewater treatment plants are important links in the water cycle in urban areas, and study of their microbial ecology may bring valuable insights for two main reasons. The microbial flora mirrors the commensal microorganisms of the human population of a particular area, and such studies provide evidence for the impact of human activity on water microbial ecology.

Here we examined the diversity of culturable entercocci and related bacteria present in raw and treated wastewater of a wastewater treatment plant receiving mainly domestic effluents, and evaluated the resistance prevalence of these microorganisms to six antibiotics.

Materials and methods

Wastewater treatment plant and sampling

Raw and treated wastewater samples were collected at four different times of the year (January, March, July and November 2004) from a wastewater treatment plant in northern Portugal. The treatment plant receives around 75% of the sewage drainage from a municipal area of more then 100 000 inhabitants and a population density above 1000 km^{-2}.

In the plant, raw wastewater consists of domestic sewage (around 70%) and pretreated industrial effluents (about 30%). Occasionally, storm water may enter the sewage network. Raw wastewater undergoes a preliminary treatment to remove voluminous solids, storage in a primary settling tank to remove settleable solids and a secondary biological treatment (activated sludge process). The treated wastewater from the secondary settling tank is discharged without any further treatment into a natural water stream. The total hydraulic retention time of the wastewater is approximately 12 h. One litre of each sample (effluents from the primary and secondary clarifiers) was collected in a sterile container, transported to the laboratory and analysed within a maximum period of 2 h.

Wastewater analysis

Water samples were analysed for determination of chemical oxygen demand (COD) and biochemical oxygen demand (BOD$_5$) according to standard methods (APHA, 1995). Microbiological analyses were performed using the membrane filtration method. Briefly, 1 mL serial dilutions of water samples were filtered and the membranes (cellulose nitrate, 0.45 μm pore size, 47 mm diameter, Albet, Barcelona, Spain) were placed onto four different media: plate count agar (PCA, Merck, New Jersey) for total heterotrophs, m-FC Agar (m-FC, Difco, Chicago, IL) for faecal coliforms, m-Endo-Agar-LES (m-Endo-LES, Difco) for total coliforms and m-Enterococcus Agar (m-Enterococcus, Difco) for total streptococci. After an incubation period of 24 h at 30, 35 and 44.5 °C or 48 h at 35 °C (for enterococci), the number of CFU in each culture condition was registered in the filtering membranes presenting between 20 and 80 typical colonies. Typical colonies on m-Endo LES were confirmed in Lauryl Tryptose Broth (LTB, Difco) and Brilliant Green Bile 2% (BGB, Difco), whereas isolates from m-FC and m-Enterococcus media were confirmed in EC medium (EC, Difco) and on Bile Esculin Agar (BEA, Merck), respectively.

Removal efficiencies of the wastewater treatment were calculated from: % removal = $(X_{raw}-X_{treated})/X_{raw} \times 100$; log removal = log X_{raw}–log $X_{treated}$ where X_{raw} and $X_{treated}$ were for total heterotrophs, total coliforms, faecal coliforms, total enterococci, COD or BOD in raw and treated wastewater, respectively (George *et al.*, 2002).

Isolation of bacteria and preliminary characterization

Bacteria were isolated from the membrane filters containing countable CFU. Twenty-five to 100% of the colonies formed on m-Enterococcus agar were isolated and purified on PCA or, when necessary, on MRS agar (Man, Rogosa and Sharp, Amersham). Purified cultures were characterized by Gram staining, presence of catalase, growth and aesculin degradation on BEA medium, and growth at 45 °C in Tryptone Soy Broth (TSB, Amersham) or in the same medium supplemented with 6.5% (weight in volume, w/v) NaCl. Isolates were maintained at −80 °C in TSB supplemented with 15% (volume in volume, v/v) glycerol.

Antibiotic resistance

Antibiotic resistance phenotypes were tested based on the Kirby–Bauer method according to standard recommendations (National Committee for Clinical Laboratory Standards, 2003). Briefly, bacterial suspensions with an OD of 0.2, at 620 nm, were spread on Mueller Hinton agar (Oxoid) using a sterile cotton swab. Antibiotic discs of amoxicillin (AML, 25 μg), gentamicin (GEN, 10 μg), ciprofloxacin (CIP, 5 μg), sulphamethoxazole/trimethoprim (SXT, 23.75/1.25 μg), tetracycline (TET, 30 μg) and erythromycin (ERY, 15 μg) (all Oxoid) were placed on the surface of each inoculated plate. Additionally, resistance to vancomycin (VAN, 30 μg) and GEN (120 μg) was assayed for some of the strains. After 24 h of incubation at 35 °C, the diameters of antibiotic inhibition of growth were measured and recorded as susceptible (S), intermediary (I) or resistant (R). The criteria used for these designations, based on inhibition zone diameters, were as follows (mm): AML 25: $R < 14$, $I = 20$–14, $S \geq 21$; GEN 10: $R < 14$, $I = 15$–20, $S \geq 21$; GEN 120: $R < 7$, $S \geq 10$; CIP 5: $R < 19$, $I = 19$–21, $S \geq 22$; ERY 15: $R < 17$, $I = 21$–17, $S \geq 22$; TET 30: $R < 17$, $S \geq 19$; SXT 25: $R < 10$, $I = 9$–15, $S \geq 16$; VAN 30: $S > 17$. *Escherichia coli* ATCC 25922, *Enterococcus faecalis* DSM 2570, *Pseudomonas aeruginosa* DSM 1117 and *Staphylococcus aureus* DSM 1104 were used as controls in each experimental set. The inhibition zones observed for these organisms were compared with expected values. The average deviation of the diameters of inhibition zones measured for the control strains ranged between 0.1 and 0.2 mm.

Species distribution and the prevalence of antibiotic resistance or intermediary phenotype to each antibiotic among enterococci isolates from raw and treated wastewater were compared using the chi-squared test.

RAPD typing

Random amplified polymorphic DNA (RAPD) analysis was used to compare and cluster enterococci and related bacteria. The method used was based on that described by Tiago *et al.* (2004). Crude cell lysates were used as DNA templates for genotyping. Amplification reactions were performed with a total volume of 25 μL containing: 0.75 U *Taq*

Comment Letter L

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polymerase, 1.5 mM $MgCl_2$ (Pharmacia Biotech), 0.2 mM of each dNTP, 1.0 μM primer M13 (5′GAGGGTGGCGG TTCT3′) and 0.5 μL of crude cell lysates. After 5 min at 94 °C, samples were subjected to 45 cycles of amplification (Biometra), as follows: 1 min at 94 °C, 1 min at 34 °C and 2 min at 72 °C, and a final extension step of 10 min at 72 °C. Polymorphic DNA fragments were analysed by electrophoresis in a 2% agarose gel in Tris-acetate-EDTA buffer. To allow alignment and comparison of profiles in different gels, the RAPD profile of *E. coli* ATCC 25922 generated under the same conditions was included in the extremities and in the centre of each gel.

The RAPD profiles were visually analysed and the presence or absence of each band was noted for each isolate. The resultant matrix was interpreted using a hierarchical clustering method, based on the Dice coefficient (SPSS 13.0 for Windows), in which isolates were grouped according to resemblance of their RAPD patterns. Clusters were defined for relative rescaled distances below 20%. As a quality control of the RAPD analysis and data interpretation, three isolates randomly selected among the test strains were successively processed together with the remaining strains. The rescaled distances obtained for each of these control strains was below 8%. Identification of those members comprising each cluster and validation of the typing method as discriminating different species were made through analysis of partial 16S rRNA gene sequences. Thus, 10–25% of the isolates of each cluster as well as others that could not be typed by RAPD analysis were selected for 16S rRNA gene sequence analysis.

rRNA gene sequence analysis

The extraction of genomic DNA, PCR amplification and sequencing of the 16S rRNA gene were carried out as described previously (Rainey *et al.*, 1996). DNA sequences were determined using a model 310 Genetic Analyzer (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Primer 519R (5′-GWATTACCGCGGCKGCTG-3′) was used to generate partial 16S rRNA gene sequences comprising 420–450 nucleotides. The quality of the 16S rRNA gene sequences was checked manually using the Bioedit editor (Hall, 1999) and compared with sequences available in the EMBL/GenBank database using Blast network services and also with sequences in the Ribosomal Database Project II (RDP) (Cole *et al.*, 2005).

Results and discussion

Wastewater characterization

Microbiological analyses of the raw wastewater indicated that levels of total heterotrophs and total coliforms were about 10^7–10^8 CFU, and that levels of faecal coliforms and enterococci were about 10^6 CFU per 100 mL (Table 1). The number of organisms in each of these groups decreased following treatment, with reductions of 80–90% for total heterotrophs and total coliforms (i.e. in a range of 0.69–1.02 $\log_{10}$) and of 84–96% for faecal coliforms and enterococci (i.e. in a range of 0.80–1.39 $\log_{10}$). These microbial reduction values are in agreement with previous reports for similar wastewater treatment systems (Rose *et al.*, 1996; George *et al.*, 2002; Blanch *et al.*, 2003).

Relative decreases of COD and BOD observed in March and July ranged, respectively, from 76 to 88% and from 93 to 94%. Lower values were observed in November because of maintenance to the treatment plant: 42 and 47% of COD and BOD, respectively. This maintenance operation was responsible for the release of treated wastewater with COD and BOD values slightly higher than those legally established (125 and 25 mg O_2 L^{-1}, respectively) (Council Directive 91/271/EEC, 1991). Interestingly, the removal of microorganisms was not related to the wastewater COD or BOD reduction, given that in November the decrease in bacterial counts of the treated wastewater was similar to those recorded in January, March and July. These results suggest that the relative bacterial counts in treated and in raw wastewater may not be related to the metabolic activity of microbiota constituting the activated sludge.

Table 1. Chemical and microbiological quality parameters of raw and treated water sampled at four different times of the year

	Raw water				Treated water			
	January	March	July	November	January	March	July	November
	CFU × 10^6 100 mL^{-1}							
Total heterotrophs	52.0	200.0	230.0	250.0	6.5	37.5	29.0	24.0
Total coliforms	ND	139.0	55.0	27.0	3.3	26.0	9.0	5.5
Faecal coliforms	ND	2.5	3.4	3.6	0.6	0.4	0.5	0.2
Faecal streptococci	4.4	3.8	7.4	2.6	0.5	0.5	0.3	0.3
	Oxygen demand (mg O_2 L^{-1})							
Chemical demand	ND	291.0	724.8	398.5	ND	71.4	90.9	231.8
Biochemical demand	ND	233.0	400.0	245.0	ND	16.0	22.7	130.0

ND, not determined.

Isolation and characterization of enterococci and related bacteria

During one year, a total of 167 Gram-positive, catalase-negative cocci were isolated from raw and treated wastewater. Among these isolates, 148 were confirmed as *Enterococcus* spp. based on their ability to grow and produce blackening on BEA, and to grow at 45 °C and in the presence of 6.5% NaCl, after 48 h of incubation. Nineteen isolates produced negative reactions to at least one of these tests and therefore were considered to represent nonenterococci.

Genotyping of the isolates led to the establishment of seven clusters (Figs 1 and 2), from which representative isolates were identified by analysis of their partial 16S rRNA gene sequences, comprising ~450 nucleotides. *Enterococcus hirae* isolates were included in a single cluster, comprising 65 members, defined at approximately 15% rescaled distance. *Enterococcus faecium* isolates were separated into two clusters formed, respectively, at about 12.5 and 15% rescaled distance. Within this latter cluster it was possible to distinguish a subcluster composed of three *E. avium* isolates. The isolates belonging to *E. faecalis* and *E. durans* formed two distinct clusters at 15% rescaled distance. Thirteen of the nonenterococcal isolates clustered together at about 20% rescaled distance and were identified as representing *Lactococcus lactis*. Six isolates could not be typed by RAPD, but 16S rRNA gene sequence analysis demonstrated that they were related to *Lactococcus raffinolactis* (~96%) (two isolates), *Streptococcus bovis* (~99%), *E. pseudoavium* (~100%) and *E. saccharolyticus* (~96%) (two isolates). Whereas the *Lactococcus* spp. strains were isolated from raw wastewater, the others were detected in treated wastewater.

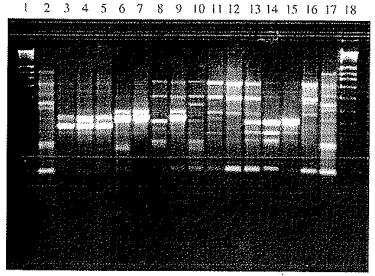

Fig. 1. RAPD profiles of representative isolates integrated in each of the seven clusters. Lanes 1, 18: molecular weight marker; 2, 17: *Escherichia coli* ATCC 25922; 3, 4, 5 *Enterococcus faecalis*; 6, 7: *Enterococcus faecium* (first cluster); 8, 9, 10: *Enterococcus faecium* (second cluster); 11: *Enterococcus avium*; 12: *Enterococcus durans*; 13, 14, 15: *Enterococcus hirae*; 16: *Lactococcus lactis*. Molecular weight marker bands: 23583; 19329; 7743; 5526; 4254; 3281; 2690; 2322; 1882; 1489.

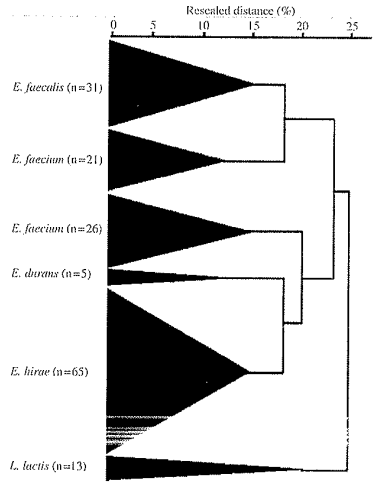

Fig. 2. Dendrogram expressing the RAPD-based similarity between isolates calculated through a Dice coefficient correlation represented using an average linkage method.

Enterococcal species distribution

The most abundant species detected in raw wastewater was *E. hirae*, whereas *E. faecium* and *E. faecalis* were found in lower but similar proportions. The relative abundance of *E. faecalis* was similar in treated and in raw wastewater; it was the least abundant enterococcal species found in the treated wastewater (Fig. 3). Wastewater treatment led to a reduction in *E. hirae* ($\alpha < 0.1$) but an increase in *E. faecium* ($\alpha < 0.1$), with the two species reaching equivalent frequencies in treated wastewater. This result may reflect a higher intrinsic resistance to environmental stress of *E. faecium* in comparison with *E. hirae* (Renner & Peters, 1999).

The predominance of *E. hirae* and the increase in *E. faecium* during wastewater treatment were not observed by (Blanch *et al.*, 2003) in a study involving a large number of enterococci isolated from urban wastewater treatment

3.L-17

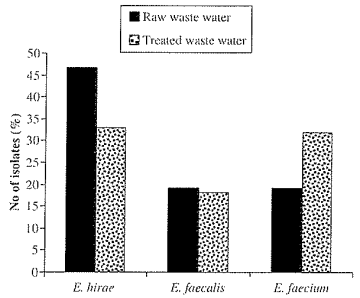

Fig. 3. Relative proportions of enterococcal species in raw and treated wastewater.

plants in Sweden, the UK and Spain. Blanch *et al.* also used m-Enterococcus agar as the isolation medium but pre-enrichment in Brain Heart Infusion was made in order to promote recovery of stressed cells. This latter procedure may explain the discrepancies observed between the results from the two studies

In a comparison of enterococcal populations from human, animal and environmental origin in the UK, Spain, Denmark and Sweden, *E. faecalis* was found to be the most prevalent species, both in urban and in hospital sewage in Sweden, whereas in the same sample types, *E. faecium* was predominant in Spain and in the UK (Kuhn *et al.*, 2003). The authors found a similar distribution of enterococci in surface water as in the treated sewage. According to Kuhn *et al.* (2003), *E. hirae* is predominant in environments associated with cattle and pig effluent. This may suggest that the high percentages of *E. hirae* found in the present study were due to contamination of agricultural land runoff and/or nonauthorized discharges from cattle and pig farms into the sewage system.

Antibiotic resistance phenotyping

A total of 133 enterococcal strains were characterized for their phenotype of antibiotic resistance/susceptibility (Table 2). Within the isolates tested, a low prevalence of resistance was observed for amoxicillin and sulfamethoxazole/trimethoprim. By contrast, in the isolates identified as *E. faecalis* and *E. faecium*, the prevalence of resistance observed to erythromycin, ciprofloxacin and tetracycline ranged between 23 and 57%. *E. hirae* clearly had the lowest prevalence of antibiotic resistance, a fact that may be due to its comparatively low resistance in water. Indeed, with such a short life span in water, these organisms have reduced ability to act as receptors in processes of horizontal gene transfer, and consequently will not have major relevance as disseminators of antibiotic resistance. The high levels of resistance to gentamicin (10 μg) were expected given that enterococci are intrinsically resistant to low concentrations of this antibiotic, although a few susceptible isolates were found. Twenty-seven enterococci isolates chosen at random were tested for resistance to a higher concentration of gentamicin (120 μg); only two isolates belonging to *E. faecium* were found to be resistant. Moreover, among the 65 isolates tested for resistance to vancomycin (30 μg), none was resistant.

Among the 12 isolates of *Lactococcus lactis* (one could not grow on Mueller Hinton agar), seven were resistant to ciprofloxacin and three presented with an intermediary phenotype. Considering that *L. lactis* is typically associated with food products and is used in food production, this result may indicate that regular and extensive antibiotic resistance screening may be desirable in strains of this and other genera used in similar procedures.

Effect of wastewater treatment on antibiotic resistance phenotype

Except for ciprofloxacin, the wastewater treatment did not select positively or negatively for antibiotic resistance phenotypes. Similarly, the treatment process was not related to any variation in the prevalence of isolates exhibiting resistance to more than one antibiotic. Indeed, for ciprofloxacin, wastewater treatment led to an increase in the prevalence of resistant enterococci. Moreover, the phenotype to ciprofloxacin resistance was three times higher in the treated than in the raw wastewater ($\alpha < 0.02$). Despite the fact that horizontal gene transfer between enterococci has been described as a general mechanism of antibiotic resistance acquisition (Klare *et al.*, 2003), namely in activated sludge (Marcinek *et al.*, 1998), this may not be the only explanation for the results observed in the present study. The results indicate that the significant increase in ciprofloxacin resistance may be due, at least in part, to the increase in the proportion of *E. faecium*, with high prevalence of resistance to ciprofloxacin, and the simultaneous decrease of *E. hirae* populations in the treated wastewater. Given the character of opportunistic pathogens together with their high survival rate during wastewater treatment and the high prevalence of the antibiotic resistance phenotype, monitoring levels of *E. faecium* and of *E. faecalis* is strongly recommended.

Our results clearly showed that in the plant studied, treatment is not efficient in eliminating commensal antibiotic-resistant enterococci from wastewater. According to these results, similar treatment plants may act as permanent suppliers of antibiotic-resistant bacteria to the environment, leading to a continuous dissemination and accumulation of resistant organisms in environmental water.

Table 2. Prevalence (%) of enterococci with resistance (or intermediary) phenotype for the antibiotics tested*

	No. of isolates†	AML	GEN	ERY	CIP	SXT	TET	$R > 1$‡
Enterococcus faecalis	30	0	100	35 (35)	23 (60)	0	57	30
Enterococcus faecium	43	2 (2)	39 (61)	40 (40)	33 (26)	0	38	30
Enterococcus hirae	52	0	41 (46)	7 (2)	2	0	13	4
Other§	8	13	0 (100)	71	25	14	29	25
Raw water	56	0	50 (41)	33 (7)	9 (20)	0	31	18
Treated wastewater	77	3 (1)	50 (46)	23 (30)	25 (23)	1	33	21
Total	133	2 (1)	50 (44)	27 (21)	18 (22)	1	32	20

*None of the 65 isolates tested was resistant to vancomycin.
†Twelve isolates of *Enterococcus hirae*, one of *Enterococcus faecalis* and one of *Enterococcus faecium* were not able to grow on Mueller Hinton agar and were not tested for antibiotic resistance phenotype.
‡$R > 1$. Resistant to more than one antibiotic; as all *Enterococcus faecalis* strains were resistant to GEN, this antibiotic was not considered for this analysis.
§Other refers to *Enterococcus avium* and *Enterococcus durans* isolates.
AML, amoxicillin; GEN, gentamicin; ERY, erythromycin; CIP, ciprofloxacin; SXT, sulfamethoxazole/trimethoprim; TET, tetracycline.

Ecology of antibiotic-resistant enterococci

The increased use of antimicrobials useful for treatment of infections is seen as an important factor contributing to the selection of bacteria with natural resistance or only weakly susceptible to target antibiotics. This explains the evident enhancement of infections caused by enterococci resistant to antibio-therapy (Klare *et al.*, 2003). In a Portuguese medical study involving 12184 prescriptions of antibiotics corresponding to 11982 disease episodes, an annual antibiotic prescription frequency of 9.3% (prescriptions per 100 individuals) was reported (Observatório Nacional de Saúde. Médicos Sentinela (ONSA), 2002). In that study the most frequently prescribed antibiotics were penicillins (47%, of which 11% was amoxicillin), macrolides (16%, of which 2% was erythromycin), quinolones (15%, of which 7% was ciprofloxacin), cephalosporines (12%), sulfonamides (5%) and tetracyclines (2%). Comparing these data with the resistance prevalence values observed in the present study, it may be hypothesized that the clinical use of macrolides and quinolones has influenced the positive selection for resistant strains. By contrast, it seems that the clinical use of penicillins has not led to an increase in the prevalence of enterococci resistant to these antibiotics in wastewater.

Assessing the mechanisms of antibiotic resistance dissemination requires an integrated approach, including comparisons of the resistance prevalence among clinical, food and environmental isolates. In a study with *E. faecium* and *E. faecalis* isolated from animal and food products, Peters *et al.* (2003) observed that all of the 118 isolates were sensitive to ampicillin and to amoxicillin/clavulanic acid. In the present study, two strains of *E. faecium* and *E. avium* were found to be resistant to amoxicillin, confirming that environmental enterococci maintain high levels of sensitivity to beta-lactamic antibiotics. These observations are consistent with those obtained with clinical isolates (e.g. Fluit *et al.*, 2000; Mutnick *et al.*, 2003).

The observed levels of resistance to other antibiotics among nonclinical isolates, namely to tetracycline, erythromycin and ciprofloxacin, were higher both in the present study and in previous works. For tetracycline, resistance prevalence values of about 30% are reported for enterococci isolated from animal and food products (Peters *et al.*, 2003; Hayes *et al.*, 2004; Johnston & Jaykus, 2004), whereas among clinical and human isolates this prevalence is above 50% (Fluit *et al.*, 2000; Mondino *et al.*, 2003; Mutnick *et al.*, 2003; Udo *et al.*, 2003). The tetracycline resistance prevalence observed in the present study, mainly for *E. faecalis*, was closer to that observed for clinical isolates. The resistance of enterococci to macrolide antibiotics, namely erythromycin, is of concern because of the high prevalence of intermediary or resistance phenotypes observed in animal or food products or in wastewaters (Blanch *et al.*, 2003; Peters *et al.*, 2003; Hayes *et al.*, 2004; Johnston & Jaykus, 2004; the present study). Intermediary levels of resistance to erythromycin (26–82%) in *E. faecium* and *E. faecalis* are reported by these authors. Moreover, Blanch *et al.* (2003) report prevalence values of erythromycin-resistant enterococci above 60% in raw, treated and hospital wastewaters, as well as in surface waters receiving the treated effluents, suggesting their environmental dissemination.

It was concluded in the present study that wastewater treatment may lead to the positive selection of enterococci showing a resistance phenotype to ciprofloxacin. Previous reports, focused both on animal or food products and on clinical isolates, have evidenced a widespread distribution of enterococci with intermediary or resistant phenotype to quinolone antibiotics, mainly in *E. faecium*, in which resistance prevalence may vary between 28% (Johnston & Jaykus, 2004) and 65% (Peters *et al.*, 2003). In a study with enterococci isolated in medical centres from different European countries, Mutnick *et al.* (2003) reported a ciprofloxacin resistance prevalence of about 35%. Similar findings were reported by Fluit *et al.* (2000) in a European

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surveillance study with isolates from urinary tract infections, one of the most frequent infections caused by enterococci; only 64% of the strains were found to be susceptible to ciprofloxacin. These reports, together with the present study, suggest that ciprofloxacin resistance prevalence in enterococci of food, animal and environmental origin is at similar levels to those observed in clinical isolates.

Enterococcus spp. resistant to vancomycin have been identified recently as a major issue of concern, as they have been found associated with nosocomial infections, in food products and even in sewage (Iversen *et al.*, 2002; Blanch *et al.*, 2003; Klare *et al.*, 2003; Klein, 2003; Witte, 2004). The absence of vancomycin resistance among the enterococcal strains isolated during the present study may be due to the methodology used. According to Nayak *et al.* (2002), the disc diffusion assay may provide unreliable results for vancomycin resistance testing in enterococci, because the results do not correlate with those based on determination of resistance genes.

The underestimation of antibiotic resistance prevalence, using exclusively culturable organisms and phenotyping methods, has been highlighted recently (Schwartz *et al.*, 2004; Volkmann *et al.*, 2004). According to the evidence given in these studies, screening of total DNA for resistance genes provides additional insights into antibiotic resistance prevalence and dissemination. Such an approach represents an important complement to cultivation-dependent methods, allowing the detection of resistance genes in nonculturable bacteria and inferences to be made regarding the processes of horizontal gene transfer.

The present study provides evidence that antibiotic-resistant enterococci are not eliminated during wastewater treatment consisting of primary and secondary activated sludge processes. Moreover, a positive selection for antibiotic-resistant bacteria may occur during the overall treatment process. It was also observed that *E. faecium*, an opportunistic pathogen, was positively selected during wastewater treatment, with an increase in its relative proportion in the treated wastewater. Given that treated wastewater is usually released to rivers or coastal waters, a progressive change to the microbial ecosystem, namely in the antibiotic-resistant enterococci populations, may occur. This kind of change was evidenced recently by Novais *et al.* (2005). And events of this kind may explain the similar prevalence of resistance to some antibiotics found in food products, wastewater, or among human and clinical isolates.

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Environmental Project No. 722 2002
Miljøprojekt

Occurrence and fate of antibiotic resistant bacteria in sewage

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Comment Letter L

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Preface

This report contains the results of a project conducted at the Department of Veterinary Microbiology of The Royal Veterinary and Agricultural University (RVAU). The project is composed of three parts (I, II and III). After an introductory chapter on antibiotics, antibiotic resistance and project structure (chapter 1), the methodology used is described in chapter 2. The outcomes of each part of the project are then reported and discussed separately in chapters 3, 4 and 5.

The following people have taken part in the project:

Luca Guardabassi, Assistant Professor, Department of Veterinary Microbiology, RVAU.

Anders Dalsgaard, Associate Professor, Department of Veterinary Microbiology, RVAU.

The project steering group consisted of:

Linda Bagge, the Danish Environmental Protection Agency.

Eva Vestergård, the Danish Environmental Protection Agency.

Bo Neegard Jacobsen, Spildevandscenter Avedøre I/S, Denmark.

Kim Rindel, Lynettefællesskabet I/S, Denmark.

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The results of the project have been published in national and international journals and presented at scientific meetings as listed in Annex 1.

Summary and conclusions

In this project, we investigated different aspects concerning the occurrence and fate of antibiotic resistant bacteria in sewage:

- the effects of waste effluent from a hospital and a pharmaceutical plant on the prevalence of resistant *Acinetobacter* in the recipient sewers (Part I)
- the effects of tertiary sewage treatment on total numbers and percentages of resistant bacteria (Part II)
- the survival in natural aquatic habitats of multiple-resistant bacteria originating from municipal sewage effluents, and more in general the impact of such effluents on the spread of antibiotic resistance (Part III).

Part I
In Part I of the project, *Acinetobacter* was used as a bacterial indicator for monitoring antibiotic resistance in the sewers receiving waste effluent from two potential sources of resistant bacteria and/or antibiotic residues: a hospital and a pharmaceutical plant manufacturing antibiotics. The choice of *Acinetobacter* was prompted by the normal occurrence of these organisms in water and their remarkable ability to develop antibiotic resistance.

The levels of susceptibility to six antibiotics were determined by the disc-diffusion method in 385 *Acinetobacter* isolates from sewage collected upstream and downstream from the discharge points of the hospital (n=180) and the pharmaceutical plant (n=205). Isolates from the sewers at the pharmaceutical plant were further analysed by plasmid DNA profiling and phenotypic tests to detect any changes in the distribution of *Acinetobacter* species/strains associated with the discharge of waste effluent from this source.

Statistical analysis of the data from antibiotic susceptibility testing showed that the discharge of waste effluent from the pharmaceutical plant was associated with a statistically significant increase in the prevalence of both single and multiple antibiotic resistance profiles amongst *Acinetobacter* isolates from the recipient sewers (logistic regression $P < 0.01$). This increase of antibiotic resistance was observed throughout the period of study and was evident up to 250 m downstream from the discharge point. Strains isolated downstream from the pharmaceutical plant discharge point also demonstrated different plasmid profiles and phenotypic traits compared with those isolated upstream.

In contrast, the hospital waste effluent increased only the prevalence of resistance to one antibiotic, namely oxytetracycline, amongst *Acinetobacter* isolates from the recipient sewers. Furthermore, the increase in tetracycline resistance observed immediately downstream from the hospital discharge point was significantly reduced 500 m further downstream from the discharge point.

Based on this evidence, it was concluded that the discharge of waste effluent from the pharmaceutical plant caused an increase in the prevalence of both single and multiple antibiotic resistance amongst *Acinetobacter* spp. in the recipient sewers and had a higher impact on antibiotic resistance compared with the discharge of hospital waste effluent. Furthermore, waste effluent from the pharmaceutical plant determined a change in the distribution of *Acinetobacter* species/strains in the sewers, probably due to introduction of either antibiotic residues or resistant strains.

Part II
Part II of the project dealt with the effects of tertiary sewage treatment on the prevalence of resistant bacteria in two large-scale municipal treatment plants during a period of six months. Total and relative numbers of resistant bacteria in raw sewage, treated sewage and anaerobically digested sludge were determined by bacteriological counts on agar media with and without inclusion of ampicillin, tetracycline, gentamicin or all three antibiotics. Two different agar media, one selective for coliforms (MacConkey agar) and one selective for *Acinetobacter* (Baumann agar), were used in order to study the effect of treatment on different bacterial populations. In addition, the levels of susceptibility to 14 antibiotics were determined by the disc-diffusion method in 442 *Acinetobacter* isolates identified by colony hybridisation with a genus-specific DNA probe.

Depending on the different antibiotics and media used for bacteriological counts, the total numbers of resistant bacteria ranged between 10 to 1000-fold lower in treated sewage than in raw sewage. For both bacterial populations under study, the prevalence of resistant bacteria in treated sewage and digested sludge were not significantly higher than in raw sewage. On the contrary, the prevalence of ampicillin-resistant acinetobacters (i.e. presumptive *Acinetobacter* not identified at the genus level) was significantly reduced by sewage treatment at one plant (linear regression $P < 0.05$). Similarly, sludge treatment determined a reduction in the prevalence of ampicillin-resistant acinetobacters, as well as ampicillin- and gentamicin-resistant putative coliforms (linear regression $P < 0.05$).

The results obtained by bacteriological counts were confirmed by antibiotic susceptibility testing of *Acinetobacter* isolates. Based on logistic regression analysis, the frequencies of antibiotic resistance in isolates from treated sewage and digested sludge were not significantly higher in comparison with those in isolates from raw sewage. Comparison of the levels of resistance to 14 antimicrobial agents between isolates obtained from raw and treated sewage allowed detection of a statistically significant increase in the prevalence of antibiotic resistance to only one antibiotic (nalidixic acid) and restricted to one plant. It can therefore be concluded that tertiary sewage treatment did not determine a selection for resistant bacteria. In accordance, sewage treatment appears to reduce numbers of bacteria irrespective of their susceptibility to antibiotics.

Although the results of the study clearly indicated that the overall prevalence of antimicrobial resistant bacteria was not increased by sewage treatment, the final effluent of one plant was found to contain low numbers (10 to 10^2 CFU/ml) of bacteria resistant to ampicillin, gentamicin and tetracycline, collectively. This multiple resistance phenotype is not likely to occur naturally in aquatic bacteria, as suggested by the absence of bacterial growth following inoculation of freshwater and seawater samples on agar plates containing these

three antibiotics. Consequently, it was decided to investigate the ability of such multiple-resistant bacteria to survive in natural aquatic environments (Part III).

Part III
In the final part of the project, three multiple-resistant strains isolated from treated sewage were investigated for their ability to survive in natural waters and retain antibiotic resistance. In parallel, survival experiments in laboratory seawater microcosms and membrane-filter chambers immersed in a freshwater pond were also carried out. The three strains were representative of three different bacterial species: *Acinetobacter johnsonii*, *Escherichia coli* and *Citrobacter freundii*. The multiple resistance patterns of these strains were used as selective markers for their detection among the indigenous bacteria. The experiments were performed using low bacterial inoculums (10^3 to 10^4 CFU ml^{-1}) appropriate to reproduce the actual conditions occurring when treated sewage is discharged into natural aquatic recipients.

Two of the three multiple-resistant strains (*Escherichia coli* and *Citrobacter freundii*) survived for at least one month in the seawater microcosms and the freshwater pond, whereas, the *Acinetobacter johnsonii* strain survived for shorter times in both settings. The results demonstrated that some multiple-resistant strains occurring in municipal sewage effluents were able to survive for relatively long periods following their release into natural aquatic habitats. The strains survived longer in autoclaved water better than in untreated water, suggesting that the presence of the indigenous microflora affected survival, presumably due to antagonism or predation. However, the strains maintained their multiple resistance properties following one month of incubation under natural conditions, indicating that stress and nutrient depletion did not affect the stability of their resistance phenotypes.

An aspect of the study in Part III focused on the possibility that antibiotic resistance genes occurring in sewage could be transferred to bacteria living in natural aquatic environments. This was studied by laboratory mating experiments. Ten unrelated tetracycline-resistant *Acinetobacter* strains isolated from sewage (n=10) were mated with a tetracycline-sensitive *Acinetobacter* strain isolated from an unpolluted stream. Only two out of ten donor strains were able to transfer tetracycline resistance under laboratory conditions (in vitro). In one instance, transfer of tetracycline resistance was associated with relocation of multiple small plasmids from the donor to the recipient strain, whereas, in another, transfer was apparently not mediated by plasmid conjugation. These results confirmed that sewage is a possible vehicle for the dissemination of antibiotic resistance genes in the indigenous microflora of aquatic habitats. However, the limited number of strains used in the mating experiments did not allow conclusions to be drawn on the occurrence of this phenomenon in nature.

The impact of municipal sewage effluents on the spread of antibiotic resistance was further investigated by comparing the occurrence of resistant bacteria in blue mussels exposed to sewage effluents and in blue mussels originating from unpolluted sites. Blue mussels were selected as a biological niche due to their ability to harbour high numbers of bacteria through daily filtration of large volumes of water. Higher percentages of ampicillin-resistant bacteria were found in mussels exposed to treated sewage (12.9 to 95.5%) in comparison with mussels not exposed to treated sewage (1.5 to 5.4%), whereas, the percentages of gentamicin- and tetracycline-resistant bacteria

were low (<3%) independent of the origin of the mussels. Small traces (#0.1%) of multiple-resistant bacteria were found only in mussels exposed to treated sewage, suggesting that municipal sewage effluents are potentially a source for the spread of these bacteria in the aquatic environment. The isolation of resistant bacteria was generally higher in mussels collected from the immediate proximity of the outlets of sewage effluents compared with mussels collected at 100 m from the outlets, indicating a correlation between prevalence of resistant bacteria and distance from the outlet.

Conclusions

The results of this project indicate that the occurrence of single and multiple-resistant bacteria in sewage can be increased by the discharge of waste effluent from pharmaceutical plants producing or manufacturing antibiotics. To our knowledge, this was the first study demonstrating an impact of antibiotic manufacturing on the occurrence of resistant bacteria in sewage. The observed increase in the prevalence of resistant *Acinetobacter* could have been due to the presence in the effluent of resistant bacteria selected inside the plant by the presence of high antibiotic concentrations. Alternatively, antibiotic residues may have caused a selection of resistant bacteria in the recipient sewers, or a combination of both. These results indicate that waste effluents from pharmaceutical plants manufacturing antibiotics are an important source for the occurrence and selection of resistant bacteria in sewage. As this study was conducted on a single pharmaceutical plant, further investigation is needed to assess the role of antibiotic manufacturing on selection and/or introduction of resistant bacteria in sewage.

The occurrence and fate of resistant bacteria in sewage should be given carefully consideration due to the ubiquitous nature of bacteria. Sewage is a convenient and suitable vehicle for the dissemination of resistant bacteria, in that it connects antibiotic selective environments, such as hospitals, chemical industries, farms and slaughterhouses to natural environments. Risk assessment was not included as an objective in this study. However, risks for human health may result from the dissemination in the environment of resistant bacteria occurring in sewage and the possible contamination of bathing and drinking water with these organisms.

Our investigation indicates that sewage treatment causes a reduction in the total numbers of resistant bacteria without increasing their percentage in treated sewage compared with raw sewage. Therefore, it appears that treatment of sewage has a positive effect in limiting the dissemination of resistant bacteria.

However, the investigation also demonstrates that:

- Multiple-resistant bacteria occurring in raw sewage can survive treatment and reach natural aquatic environments by municipal sewage effluents.
- Multiple-resistant bacteria occurring in municipal sewage effluents can survive for relatively long periods and maintain their resistance properties following introduction into natural aquatic habitats.
- Bacteria resistant to three or four different classes of antibiotics were found in shellfish exposed to municipal sewage effluents but appear to

be absent in shellfish and water originating from unpolluted aquatic environments.

- Resistant bacteria originating from sewage are able to transfer genes encoding antibiotic resistance to susceptible bacteria living in unpolluted aquatic habitats.

The results from our investigations underline the need to assess the impact of municipal sewage effluents on dissemination of multiple-resistant bacteria. Future studies monitoring the environmental impact of municipal sewage effluents on the spread of antibiotic resistance should focus attention on multiple-resistant bacteria rather than bacteria resistant to single antibiotics. Furthermore, multiple-resistant strains occurring in municipal sewage effluents should be investigated for their ability to transfer resistance genes to aquatic microbial communities under *in vivo* conditions. Similarly, the fate of resistant bacteria and resistance genes occurring in sewage sludge intended for agricultural use should be studied following their introduction into natural soil habitats. Finally, the relative importance and contribution of resistant bacteria in the aquatic environment and the consequent risk for resistance problems in veterinary and human medicine should be assessed.

Sammendrag og konklusioner

I dette projekt har vi undersøgt forskellige aspekter vedrørende forekomst og overlevelse af antibiotika-resistente bakterier i spildevand, herunder:

- hvilken effekt udledning af spildevand fra et hospital og en farmaceutisk virksomhed havde på prævalensen af antibiotika-resistente *Acinetobacter* i kloaksystemet (Del I)
- effekten af tertiær spildevandsbehandling på det totale og procentvise antal resistente bakterier (Del II)
- overlevelsen i naturlige akvatiske miljøer af multi-resistente bakterier fra byspildevand og indflydelsen af byspildevand på spredningen af antibiotika-resistente bakterier (Del III).

Del I
I Del I af projektet blev *Acinetobacter* anvendt som bakteriel indikator til overvågning af antibiotikaresistens i kloakker. Kloakkerne modtog spildevand fra et hospital og en farmaceutisk virksomhed, som forarbejdede antibiotika-holdige produkter. Begge er således potentielle kilder til resistente bakterier og/eller antibiotika-holdigt spildevand:. Baggrunden for valget af *Acinetobacter* som indikator var bakterieslægtens normale forekomst i vandmiljøer og dens udtalte evne til at udvikle antibiotikaresistens.

Resistens overfor seks antibiotika blev bestemt ved tabletdiffusionsmetode af 385 *Acinetobacter* bakterier isoleret opstrøms og nedstrøms for udledning af spildevand fra hospitalet (n=180) og den farmaceutiske virksomhed (n=205). Bakterieisolater i spildevandet fra kloakken ved den farmaceutiske virksomhed blev endvidere karakteriseret ved plasmid analyse og fænotypiske tests til påvisning af ændringer i fordelingen af *Acinetobacter* populationer som følge af udledning af spildevand fra virksomheden.

Statistisk analyse af resultaterne fra antibiotikaresistens undersøgelserne viste, at udledning af spildevand fra den farmaceutiske virksomhed medførte en stigning i prævalensen af såvel enkelt som multi-resistente *Acinetobacter* i kloakspildevandet nedstrøms for virksomheden (logistisk regression $P < 0,01$). Denne stigning i antibiotikaresistens blev observeret gennem hele undersøgelsesperioden og persisterede 250 m nedstrøms for stedet for virksomhedens spildevandsudledning.

Dette var i modsætning til udledning af hospitalsspildevand, som kun medførte en stigning i prævalensen af oxytetracyklin-resistente *Acinetobacter* i kloakspildevandet. Den observerede stigning i tetracyklinresistens umiddelbart nedstrøms for spildevandsudledningen blev dog signifikant reduceret 500 m nedstrøms for udledningsstedet. Bakterier isoleret nedstrøms for den farmaceutiske virksomhed havde forskellige plasmidprofiler og fænotypiske egenskaber sammenlignet med *Acinetobacter* bakterier isoleret opstrøms.

Det kunne således konkluderes på baggrund af undersøgelsens resultater, at udledningen af spildevand fra den farmaceutiske virksomhed medførte en

stigning i forekomsten af antibiotika-resistente bakterier på grund af tilførsel af antibiotika-holdigt spildevand og/eller tilførsel af antibiotika-resistente bakterier. Endvidere medførte spildevandsudledningen også ændringer i forekomsten og fordelingen af forskellige *Acinetobacter* fænotyper.

Del II
I projektets Del II undersøgte vi effekten af tertiær spildevandsbehandling på prævalensen af antibiotika-resistente bakterier i to store spildevandsrensningsanlæg gennem en 6 måneders periode. Det totale og relative antal resistente bakterier i ubehandlet spildevand, behandlet spildevand og anaerob-behandlet slam blev bestemt ved kimtællinger på agarmedier med og uden følgende antibiotika: ampicillin, tetracyklin, gentamicin, samt agarmedier med alle tre antibiotika inkluderet samtidigt. MacConkey agar blev anvendt til påvisning af koliforme bakterier og Baumann agar til påvisning af *Acinetobacter*. Anvendelse af de to agarmedier muliggjorde undersøgelse af effekten af spildevand-og slambehandling på forskellige bakteriepopulationer. Desforuden, blev følsomheden hos 442 *Acinetobacter* isolater overfor 14 antibiotika bestemt ved tabletdiffusionsmetode. Identiteten af de testede *Acinetobacter* isolater var forinden blevet fastlagt ved kolonihybridisering med en genus-specifik DNA probe.

Det totale antal resistente bakterier var mellem 10 og 1.000 gange lavere i behandlet end i ubehandlet spildevand afhængig af de anvendte antibiotika og påvist bakteriepopulationer. Prævalensen af resistente koliforme bakterier og *Acinetobacter* i behandlet spildevand og behandlet slam var ikke signifikant højere end i ubehandlet spildevand. Faktisk blev prævalensen af ampicillin-resistente acinetobacter (suspekte *Acinetobacter*, som ikke er identificeret på slægts niveau) signifikant reduceret ved spildevandsbehandling i et af de to undersøgte anlæg (lineær regression $P<0,05$). Slambehandling medførte også en reduktion i prævalensen af ampicillin-resistente acinetobacter, samt en reduktion i ampicillin- og gentamicin-resistente suspekte koliforme bakterier (lineær regression $P<0,05$).

Resultaterne fra kimtællinger på agarmedier med antibiotika blev underbygget af resistensbestemmelserne af *Acinetobacter* isolater ved tabletdiffusion. Logistisk regressionsanalyse viste, at frekvensen af antibiotikaresistens i bakterieisolater fra behandlet spildevand og behandlet slam ikke var signifikant højere sammenlignet med isolater fra ubehandlet spildevand. Ved sammenligning af resistensprofilerne hos isolater fra ubehandlet og behandlet spildevand overfor de 14 antimikrobielle stoffer, fandtes der kun for et enkelt antimikrobielt stof, nalidixcan syre, i et af rensningsanlæggene, en signifikant højere prævalens af resistente bakterier i behandlet spildevand sammenlignet med ubehandlet spildevand. Det kan således konkluderes, at tertiær spildevandsrensning ikke medfører en selektion for resistente bakterier. Med andre ord ser spildevandsrensning ud til at reducere bakterieantallet uafhængig af deres følsomhed overfor antibiotika.

Selvom resultaterne klart viser, at den overordnede prævalens af resistente bakterier ikke steg som følge af spildevandsrensning, så indeholdt det udledte behandlede spildevand lave bakterieantal ($10\text{-}10^2$ CFU/ml), som var resistente overfor ampicillin, gentamicin og tetracyklin. Sandsynligheden for at sådanne multi-resistente bakterier forekommer i naturen er meget lille. Dette blev understreget af, at der ikke blev set bakterievækst efter udsæd af ferskvands- og saltvandsprøver på agarmedier med de tre antibiotika. På baggrund af

fundet af disse multi-resistente bakterier, besluttede vi, at undersøge deres overlevelsesevne i naturlige akvatiske miljøer (Del III).

Del III
I projektets Del III, blev tre multi-resistente bakteriestammer undersøgt for deres overlevelse og evne til at bibeholde antibiotikaresistens i saltvand i laboratorieforsøg og i membranfilterkamre nedsænket i en ferskvandsdam. De valgte bakteriestammer repræsenterede tre forskellige bakteriearter: *Acinetobacter johnsonii, Escherichia coli* og *Citrobacter freundii*. Multi-resistensen hos de tre teststammer blev udnyttet i forsøgene ved tilsætning af de tre antibiotika som selektiv markør til påvisning af teststammerne blandt den mindre resistente normale bakterieflora. Ved forsøgene blev der tilsat lave kimtal af teststammerne (10^3- 10^4CFU ml^{-1}) til efterligning af de faktiske forhold når behandlet spildevand med multi-resistente bakterier udledes til akvatiske recipienter.

To ud af de tre multi-resistente teststammer, *Escherichia coli* og *Citrobacter freundii*, overlevede længere end 1 måned i saltvand i laboratorieforsøgene og i ferskvandsdammen. Derimod overlevede *Acinetobacter johnsonii* teststammen kun kort tid i begge forsøgsopstillinger. Resultaterne viste, at multi-resistente bakteriestammer som forekom i byspildevand kunne overleve relative lange perioder efter deres udledning i akvatiske recipienter. De multi-resistente bakterier overlevede bedre i sterilt (autoklaveret) vand, hvilket tyder på, at deres overlevelse blev påvirket af den tilstedeværende naturlige mikroflora. Sandsynligvis på grund af antagonisme eller predation. Ydermere, udviste teststammerne stadig multi-resistens efter 1 måneds ophold i ferskvandsdammen. Dette tydede på, at stress og næringsstof sult ikke påvirkede bakteriernes evne til at udvise antibiotikaresistens.

Muligheden for at overføre antibiotika resistensgener fra spildevand til bakterier i naturlige akvatiske miljøer blev undersøgt ved udspredning på agarmedier i laboratorieforsøg i projektets Del III. I alt 10 forskellige tetracyklin-resistente *Acinetobacter* bakterier isoleret i spildevand (n=10) blev udstreget på vækstmedium sammen med en tetracyklin-følsom *Acinetobacter* bakterie isoleret fra en å, som ikke var forurenet med spildevand. Kun to ud af de 10 donorbakterier var i stand til at overfører tetracyklin-resistens med den anvendte metode. I et af disse tilfælde var der en sammenhæng med overførsel af tetracyklin-resistens og overførsel af små plasmider fra donor til recipientbakterien, hvorimod overførsel af resistens i det andet tilfælde ikke var medieret af plasmid conjugation. Disse resultater indikerer, at antibiotika resistensgener i spildevand kan spredes til den naturlige akvatiske mikroflora. Det begrænsede antal anvendte teststammer, og det forhold at kun to donorbakterier var istand til at overføre resistens, tillader dog ikke en bestemmelse af omfanget af resistensoverførsel i naturlige akvatiske miljøer.

Effekten af udledning af byspildevand på spredning af antibiotika resistens blev yderligere undersøgt ved at sammenligne forekomsten af resistente bakterier i blåmuslinger fra områder hvortil der blev udledt spildevand og fra områder som ikke modtog spildevand. Blåmuslinger blev valgt som indikatorer på grund af deres høje bakterieoptag gennem daglig filtration af store vandmængder. Blåmuslinger eksponeret til behandlet spildevand indeholdt en højere procentdel ampicillin-resistente bakterier (12,9 til 95,5%), sammenlignet med blåmuslinger fra områder som ikke modtog behandlet spildevand (1,5 til 5,4%). Derimod var procentdelen af gentamicin og tetracyklin-resistente bakterier lav (<3%), uafhængig af muslingernes

oprindelse. Multi-resistente bakterier blev kun fundet i blåmuslinger eksponeret til behandlet spildevand og her i lave koncentrationer (#0.1%). Disse fund bekræfter, at behandlet spildevand kan være kilde til spredning af multi-resistente bakterier i akvatiske miljøer. Endvidere var der procentvis flere resistente bakterier i blåmuslinger indsamlet fra områder tæt på spildevandsudløbet sammenlignet med blåmuslinger indsamlet 100 m fra spildevandsudledningen. Dette indikerer en sammenhæng mellem forekomsten af resistente bakterier og afstanden til stedet for spildevandsudledning.

Konklusioner
Undersøgelsernes resultater viser, at forekomsten af enkelt og multi-resistente bakterier kan øges ved udledning af spildevand fra farmaceutiske virksomheder som producerer eller forarbejder antibiotika-holdige produkter. Disse undersøgelser syntes at være de første, som har påvist en sammenhæng med udledning af spildevand fra farmaceutiske virksomheder og forekomsten af resistente bakterier i spildevand. Den fundne stigning i prævalensen af resistente *Acinetobacter* kunne være forårsaget af resistente bakterier i virksomhedens spildevand og/eller udledning af antibiotika-holdigt spildevand som selekterede for resistente bakterier i kloakken. Disse fund viser, at spildevand fra farmaceutiske virksomheder sandsynligvis er vigtige kilder til forekomst og spredning af resistente bakterier i spildevand. Det skal dog bemærkes, at undersøgelserne kun blev udført ved en enkelt farmaceutisk virksomhed. Undersøgelser af yderligere virksomheder er derfor nødvendige til bestemmelse af omfanget af selektion og/eller introduktion af resistente bakterier gennem udledning af spildevand fra sådanne virksomheder.

Forekomst og overlevelse af resistente bakterier i spildevand bør vurderes varsomt på grund af muligheden for overførsel af disse bakterier mellem forskellige miljøer. Spildevand og kloakker er således egnede habitater for forekomst og spredning af resistente bakterier. Endvidere modtager og viderefører de spildevand fra en række kilder, hvorfra der udledes antibiotika og resistente bakterier (eksempelvis hospitaler, farmaceutiske virksomheder, landbrug og slagterier), til naturlige vandmiljøer. En egentlig risikovurdering var ikke opstillet som formål i vores undersøgelser. Men mulige sundhedsrisici for mennesker kan eventuelt være behæftet med spredning af resistente bakterier i spildevand, eksempelvis ved en eventuel forurening af drikkevand og badevand.

Vores undersøgelser viser, at spildevandsrensning medfører en reduktion i det totale antal resistente bakterier. Der fandtes ingen forskel i reduktionen af følsomme og resistente bakterier da den procentvise forekomst af resistente bakterier var ens i behandlet sammenlignet med ubehandlet spildevand. Spildevandsrensning ser således ud til nedsætte spredningen af resistente bakterier i spildevand.

Vores undersøgelser viste dog også at:

- Multi-resistente bakterier i ubehandlet spildevand kan overleve behandling og spredes til naturlige vandmiljøer gennem udledning af byspildevand.

- Multi-resistente bakterier i behandlet spildevand kan overleve i relativt lange perioder og bibeholde deres resistens egenskaber efter udledning til recipienten.

- Bakterier som var resistente overfor tre eller fire forskellige antibiotika blev isoleret i blåmuslinger fra områder som modtog behandlet spildevand. Derimod kunne sådanne multi-resistente bakterier ikke påvises i blåmuslinger og vandprøver fra områder som ikke modtog spildevand.

- Resistente bakterier i spildevand kan overfører antibiotika resistens til følsomme bakterier i ikke-forurenede akvatiske miljøer.

Resultaterne fra vores undersøgelser understreger behovet for at fastlægge i hvilket omfang spildevand spreder multi-resistente bakterier. Fremtidige studier bør undersøge forekomst og spredning af multi-resistente bakterier i byspildevand fremfor bakterier med resistens overfor et enkelt antibiotikum. Der er også behov for yderligere undersøgelser af multi-resistente bakteriers evne til resistensoverførsel til den normale akvatiske mikroflora under naturlige forhold. Endvidere bør spredningen af resistente bakterier og deres resistensgener efter udbringning af spildevandsslam på landbrugsjord undersøges. Endelig bør der foretages en vurdering af i hvilket omfang resistente bakterier i vandmiljøer bidrager til resistensproblemerne indenfor det veterinær og humane område.

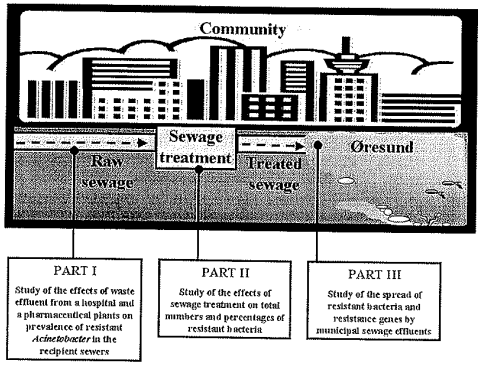
1 Introduction and project background

This chapter describes the structure and objectives of the project, and provides the reader with the basic knowledge necessary to understand the problems addressed in the report. A description of the project is followed by a short introduction on antibiotics and antibiotic resistance, including an explanation of the problems occurring when antibiotic resistance is to be measured in bacterial populations. The public health and ecological concerns associated with the emergence of bacterial resistance are discussed. Finally, considerations are made concerning the importance of sewage in the spread of antibiotic resistance between different bacterial populations and environments.

1.1 Project structure and objectives

The project is composed of three parts, each part focusing on a particular feature concerning the occurrence and fate of resistant bacteria in sewage (Fig. 1). In the Part I, the effects caused by the discharge of waste effluent from a hospital and a pharmaceutical plant manufacturing antibiotics were investigated using *Acinetobacter* as a bacterial indicator. In the Part II, the effects of tertiary sewage treatment on total and relative numbers of resistant bacteria were monitored at two large-scale treatment plants for a period of six months. In the Part III, multiple-resistant strains isolated from treated sewage were analysed for their ability to survive in the aquatic environment. In addition, the impact of municipal sewage effluents on the spread of antibiotic resistance was further evaluated by studying the transfer of tetracycline resistance between *Acinetobacter* strains isolated from sewage and unpolluted freshwater, and by comparing the occurrence of resistant bacteria in shellfish exposed/non-exposed to sewage effluents.

Figure 1.1. Schematic representation of the project



The general aim of the project was to identify factors influencing the occurrence of resistant bacteria in sewage and to study the fate of such bacteria along the sewage system. The assessment of the occupational risks associated with the occurrence of resistant bacteria in sewage treatment plants and the public health risks associated with the spread of multiple-resistant bacteria *via* municipal sewage effluents was not part of this study. The present work, however, represents a good basis for the development of future studies on risk assessment.

The following specific objectives were pursued as part of the project:

- To assess the effects of waste effluent from a hospital and a pharmaceutical plant on the prevalence of resistant *Acinetobacter* in the recipient sewers (Part I).
- To detect changes in the distribution of *Acinetobacter* strains/species associated with the discharge of waste effluent from these sources (Part I).
- To determine to what extent sewage treatment reduces the total numbers of resistant bacteria depending on the antibiotic, the bacterial population and the treatment plant under study (Part II).
- To evaluate the effects of sewage treatment on numbers of single and multiple-resistant bacteria (Part II).
- To determine the ability of multiple-resistant bacteria originating from treated sewage to survive in laboratory marine microcosms and in membrane diffusion chambers immersed in a freshwater pond (Part III).

- To demonstrate *in vitro* transfer of antibiotic resistance from bacteria isolated from sewage to related bacteria occurring in unpolluted aquatic environments (Part III).
- To determine whether differences in the number of resistant bacteria exist in shellfish from sites exposed to treated sewage and in shellfish from unpolluted sites (Part III).

1.2 What are antibiotics?

Antibiotics are substances produced by living organisms, which are able to kill or inhibit the growth of microorganisms. According to the literal sense of the word, substances produced synthetically (e.g. sulfonamides or quinolones) should not be termed antibiotics, and the use of a broader term (i.e. antimicrobial agent) would be more appropriate to indicate the complex of all substances having a harmful effect on microorganisms [1,1]. However, the term antibiotic is used throughout the present report as a synonym of antimicrobial agent.

Antibiotics are selectively toxic substances as they affect pathogenic microorganisms more adversely than the host. The degree of selective toxicity depends on the specific mechanisms of action of the drug. The most selective agents are those affecting structures (e.g. cell wall) or functions (e.g. folic acid synthesis) present only in prokaryotic cells. The less selective antibiotics are those affecting protein (e.g. tetracyclines) or nucleic acid synthesis (e.g. quinolones), which are essential functions for both prokaryotic (bacteria) and eukaryotic cells (the host). Among the antibiotic agents produced synthetically, are some that are toxic for humans and animals, and their use is restricted to inanimate objects (i.e. disinfectants) or the surface of living tissues (i.e. antiseptics). These agents are generally termed biocides.

1.2.1 Classification

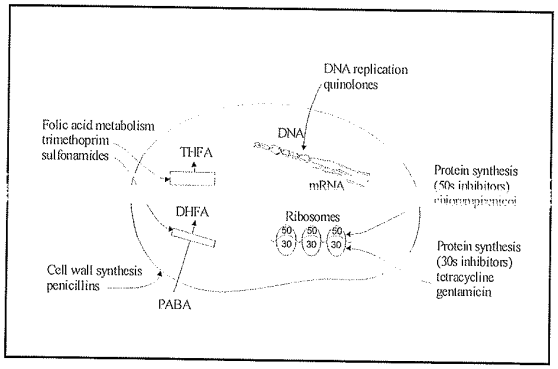
Antibiotics are classified based on their chemical structure. Each class of antibiotics is characterised by a typical core structure and the various members of the class are differentiated by the addition or subtraction of secondary chemical structures from the core structure. The main classes of antibiotics currently used in clinical practice include penicillins, cephalosporins, tetracyclines, aminoglycosides, fluoroquinolones, potentiated sulfonamides, macrolides and glycopeptides.

Antibiotics can also be classified as broad, intermediate or narrow spectrum, depending on the range of bacterial species against which they are active [1]. Broad-spectrum antibiotics include compounds active against both Gram-positive and Gram-negative bacteria like quinolones, tetracyclines, and third generation cephalosporins. Intermediate spectrum antibiotics generally include substances with reduced activity against some Gram-negative bacterial species (e.g. ampicillin, amoxicillin, first and second generation cephalosporins). Narrow spectrum antibiotics are only effective against restricted groups of bacteria. For example, penicillin is only active against Gram-positive bacteria, whereas, aminoglycosides, sulfonamides and trimethoprim are solely active against aerobic bacteria [1].

1.2.2 Mechanisms of action

Antibiotics constitute quite a heterogeneous group of chemicals. Depending on the chemical structure, antibiotics exert an effect on different structures or functions of the bacterial cell (Fig. 1.2). The major mechanisms of action are inhibition of the cell wall synthesis (e.g. penicillins and vancomycin), damage of the cell membrane function (e.g. polymixins), inhibition of protein synthesis (e.g. aminoglycosides, tetracyclines, chloramphenicol, lincosamides and macrolides), inhibition of the nucleic acid synthesis (e.g. quinolones and rifampicin), and metabolic antagonism (e.g. sulfonamides and trimethoprim)[1].

Figure 1.2. Sites of action for selected antibiotics. PABA, para-aminobenzoic acid; DHFA, diydrofolic acid; THFA, tetrahydrofolic acid. Modified from Prescott and Baggot[1].



1.3 What is antibiotic resistance?

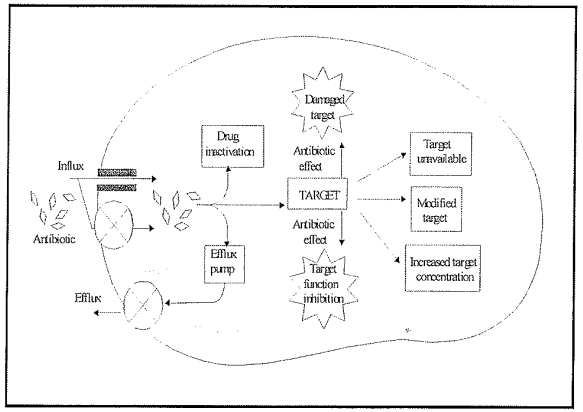
Antibiotic resistance is a relative term. A bacterial strain can be defined resistant if it survives in the presence of higher antibiotic concentrations in comparison with phylogenetically related strains[2]. Thus, antibiotic resistance is not a bacterial property that can be determined by studying a single strain, but only by comparison under identical conditions of two or more strains belonging to the same genus or species.

The above-mentioned definition of antibiotic resistance refers to *in vitro* conditions. Under *in vivo* conditions, antibiotic resistance is a context-dependent term as it depends on the location of the bacterium and the bioavailability of the drug. For example, bacteria are less susceptible to antibiotics when assembled in biofilms (complex communities of microorganisms embedded in a matrix of extracellular material) compared with the same organisms living separately[3]. In aquatic environments, binding of the antibiotic molecule with ions or substances present in sediment strongly reduces both the activity of the drug and its absorption in the fish intestine[4].

1.3.1 Molecular mechanisms

Bacterial resistance to antibiotics can be caused by different molecular mechanisms[5]. The most common mechanisms include: reduced drug uptake (e.g. membrane impermeability to cephalosporins); active drug efflux (e.g. tetracycline efflux pumps); drug deactivation (e.g. hydrolysis of penicillins by beta-lactamases), modification of the drug target (e.g. mutations of the DNA gyrase leading to quinolones resistance); increased concentration of the drug target (e.g. increased folic acid production that counteracts the inhibition of such production by sulfonamides), or alternative pathways to elude the drug effect (e.g. synthesis of folic acid using an enzyme which is not affected by sulfonamides) (Fig. 1.3).

Figure 1.3. Molecular mechanisms of antibiotic resistance. Modified from Hayes and Wolf[5].



1.3.2 Natural and acquired resistance

An important distinction should be made between natural and acquired resistance. Bacteria are termed naturally, intrinsically or constitutively resistant when resistance is due to characteristic features typical of the species. For example, *Pseudomonas aeruginosa* is naturally resistant to penicillins, due partly to the inability of the drug to diffuse through the outer membrane[6] and partly to the deactivation of the drug by chromosomally encoded enzymes (i.e. beta-lactamases)[7].

In contrast, acquired resistance emerges in a bacterial population that was previously susceptible, because of modifications of the bacterial DNA caused by either chromosomal mutation or horizontal gene transfer. Natural resistance results from a long process of genetic evolution, whereas, acquired resistance can arise within a short time (one or few generations)[5].

1.3.3 Acquisition by chromosomal mutations

Mutation is a heritable change in the sequence of the DNA occurring due to errors during DNA replication [8]. The frequencies of chromosomal mutations leading to antibiotic resistance depend on the specific antibiotic. For example, mutation frequencies are high for compounds like nalidixic acid, rifampicin and streptomycin (10^{-8} to 10^{-10} cells per generation), low for erythromycin and are not known to occur for vancomycin and polymixin B [9]. For antibiotics like streptomycin, a single mutation can determine a 1000-fold increase in the resistance levels [9]. In contrast, for other drugs (e.g. quinolones) the acquisition of resistance is a gradual, step-wise process in which different mutations are involved [10].

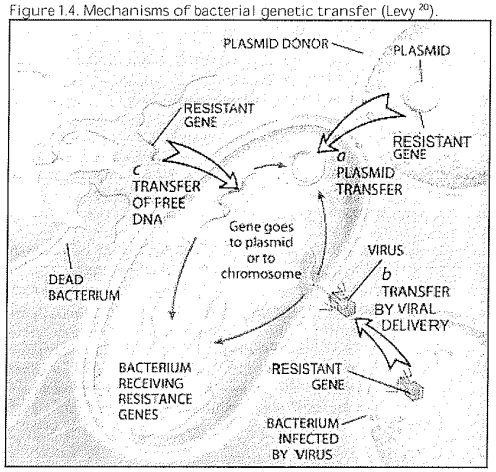
1.3.4 Acquisition by horizontal gene transfer

Horizontal gene transfer is the relocation of genetic material from one bacterial cell (donor) to another (recipient). Such a transfer may occur directly by physical contact (*conjugation*) or indirectly, using the surrounding medium (*transformation*) or bacteriophage (*transduction*) as vectors [11] (Fig. 1.4). Bacterial transfer of antibiotic resistance has been demonstrated to occur in various natural habitats, including water, sediment, soil, plants and animals [12,13]. The DNA transferred from the donor to the recipient may be contained in mobile genetic elements called plasmids, structures of circular DNA that reproduce independently from the chromosome [11]. Unlike chromosomes, plasmids generally do not encode functions essential to bacterial growth, but functions that are of importance under particular conditions, such as antibiotic resistance, heavy metal resistance, metabolic functions, or production of antibiotics, toxins and virulence factors [14].

1.3.5 Intracellular migration of resistance genes

Antibiotic resistance genes can migrate from one site to another on the bacterial genome using small vectors called transposons [15] and integrons [16]. These genetic elements containing antibiotic resistance genes are able to move between different sites of the bacterial genome without any requirement of DNA homology. This process is known as non-homologous recombination (to a site that does not match with the gene) and differs from the normal process of genetic recombination, which requires a high degree of DNA homology (a near perfect match) [11,17]. Both transposons and integrons make it possible for new antibiotic resistance genes to be acquired by plasmids and subsequently spread in the bacterial population by mechanisms of horizontal gene transfer, as suggested by the frequent recovery of these genetic elements as part of broad host plasmids [18,19].

Figure 1.4. Mechanisms of bacterial genetic transfer (Levy [20]).



1.3.6 Measurement of resistance in bacterial populations

The value of the term "measurement of antibiotic resistance" in environmental microbiology generally differs from that in clinical studies. The main concern for environmental microbiologists is to investigate the distribution of antibiotic resistance in bacterial populations rather than the level of resistance in individual strains. Unfortunately, culture methods are not efficient enough to determine the actual prevalence of antibiotic resistance in a bacterial population. In fact, only a small proportion of the aquatic bacterial flora (<1%) can be cultured on laboratory media [21].

The method traditionally used for the measurement of antibiotic resistance at the population level consists in standard bacteriological counts on media containing specific concentrations of antibiotics. The main drawback of this method is the use of a single breakpoint for the determination of antibiotic resistance. In fact, the use of a single breakpoint, corresponding to the amount of antibiotic agent added to the medium, does not take into account the variability in the levels of antibiotic resistance existing among different bacterial species. Consequently, bacteria characterised by intermediate levels of resistance can be classified either as resistant or susceptible depending on the concentration of antibiotic added to the medium.

An alternative approach is to use a group of phylogenetically related organisms as bacterial indicators of antibiotic resistance. This method is based on the principle that spatial and temporal differences observed in the levels of antibiotic resistance of the bacterial indicator are indicative of the selective pressure to which the entire bacterial population is exposed. Thus, this method does not aim to determine the exact prevalence of antibiotic resistance

in the bacterial population under study, but rather to detect the effect of potential sources of antibiotic resistance on the bacterial population.

The use of bacterial indicators offers various advantages compared with bacteriological counts on antibiotic selective media. The isolation and identification of bacteria makes possible the use of standardised methods for antibiotic susceptibility testing, namely the disc-diffusion method and the dilution method. When antibiotic susceptibility testing is performed on a large number of bacterial isolates, results can be used to understand the distribution of antibiotic resistance within the target bacterial population and consequently to define appropriate breakpoints for the classification of resistant and susceptible strains.

Today, the nucleotide sequences of many genes encoding for antibiotic resistance are available. DNA-DNA hybridisation and PCR methods are currently used to investigate the presence of resistance genes in environmental bacteria. In comparison with phenotypic methods, genetic methods offer the great advantage to investigate also non-culturable bacterial species. However, the currently available genetic methods are only able to quantify, to a limited degree, the presence of a gene in a bacterial population. Furthermore, since a number of different genes can encode resistance to the same antibiotic agent, genetic methods cannot be used for quantitative assessment of resistance.

1.4 The microbial threat

In the last decades, bacterial resistance to antibiotics has assumed an increasing importance with regard to its impact on both public health and ecology. Obviously, the primary problem is represented by the emergence of antibiotic resistance among bacteria pathogenic to humans and animals, which makes difficult the treatment of some life-threatening infections. However, independent from the risks for human health, is the spread of antibiotic resistance and the problems raised in ecological nature. In fact, the introduction and selection of resistant bacteria in the environment can lead to structural changes in the composition of microbial communities, with possible deleterious effects on the balance of natural ecosystems.

1.4.1 The emergence of resistance in human pathogenic bacteria

In the past, bacteria were the most important cause of disease and mortality among humans. The introduction of antibiotics in human medicine has markedly reduced the impact of bacterial diseases on human mortality. Nevertheless, the extraordinary capacity for adaptation of bacteria soon allowed these organisms to develop mechanisms of resistance enabling them to overcome the toxic effects of antibiotics.

A survey on enterobacterial isolates collected between 1917 and 1954 has demonstrated that bacteria were generally susceptible to antibiotics before these drugs became commonly available in human medicine [22]. However, other studies indicate that resistant bacteria were present at the time, although they were not prevalent in bacterial populations [23]. Thus, it appears that the indiscriminate use of antibiotics has played a major role in the emergence of antibiotic resistance by exerting a selection in favour of resistant bacteria.

The first case of penicillin resistance in *E. coli* was reported in the 1950's. Since then, things have taken a turn for the worse. Today, antibiotic resistance

represents an important problem in the therapy of various human pathogenic bacteria. Three bacterial species causing life-threatening infections (*Pseudomonas aeruginosa*, *Mycobacterium tubercolosis* and *Enterococcus faecalis)* can demonstrate resistance to any available antibiotic [20]. Vancomycin is the only effective drug for treatment of infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA), but the occurrence of strains with reduced susceptibility to this antibiotic has already been reported [24]. Problems may also occur in the therapy of hospital infections caused by *Acinetobacter baumannii*, *Haemophilus influenzae*, *Klebsiella pneumoniae*, *Neisseria meningitidis* and *Streptococcus pneumoniae* [20].

The problem of antibiotic resistance is of particular concern for immunosuppressed patients, suchas those affected by HIV, cancer or chronic diseases, as antibiotic therapy represents the only way to overcome bacterial infections for these people. Serious problems may also occur in developing countries where the use of new and expensive drugs is limited by their cost and availability. In addition to the risks for human health, this situation incurs a worldwide increase in the cost of hospital care, including the use of new expensive drugs, increased costs for bacteriological analysis and prolonged hospitalisation [25].

1.4.2 The spread of resistance among environmental bacteria

Antibiotic resistance is not only found in pathogenic bacteria but also in environmental organisms inhabiting terrestrial and aquatic habitats. The occurrence of resistant bacteria in nature may have originated from antibiotic-producing organisms, as suggested by the evidence that in some cases the mechanisms and genes protecting these organisms from the antibiotics they produce are similar to those responsible for resistance in clinical isolates [26]. However, higher numbers of resistant bacteria occur in polluted habitats compared with unpolluted habitats [27,28], indicating that humans have contributed substantially to the increased proportion of resistant bacteria occurring in the environment.

Possible mechanisms by which humans enhance the spread of antibiotic resistance among environmental bacteria include the deliberate or accidental introduction of antibiotics, resistant bacteria and resistance genes into the environment. Antibiotics exert a selection in favour of resistant bacteria by killing or inhibiting growth of susceptible bacteria (see section 1.5.1); resistant bacteria can adapt to environmental conditions and serve as vectors for the spread of antibiotic resistance; resistance genes can be taken up by indigenous bacteria and spread by mechanisms of genetic transfer (see section 1.3.4).

The main risk for public health is that resistance genes are transferred from environmental bacteria to human pathogens. The ability of resistant bacteria and resistance genes to move from one ecosystem to another is documented by the various cases in which transmission of resistant bacteria has been demonstrated between animals and humans [29,30]. The inclusion of certain growth promoters in animal feed has been recognised as a cause for the selection of resistance genes in the commensal microflora of animals and their transmission to humans *via* the food chain [29,30]. Similarly, drinking and bathing water could represent a source for the acquisition of resistant bacteria in humans. However, further studies are necessary to validate this hypothesis.

The ecological consequences associated with the dissemination of resistant bacteria in the environment have been scarcely investigated. However, it

appears evident that environmental contamination with antibiotics, resistant bacteria and resistance genes affects the biodiversity of natural ecosystems. Antibiotics are likely to determine a reduction in the levels of microbial diversity by the suppression of susceptible organisms, including bacteria, fungi, protozoa and algae. Resistant bacteria and genetic elements could find favourable conditions to become predominant in habitats contaminated by antibiotics, thereby, altering the original composition (balance) of natural microbial communities.

1.5 Spread of antibiotic resistance in sewage

Sewage is waste matter resulting from the discharge into the sewers of human excreta and wastewater originating from the community and its industries. Sewage contains a high content of both organic and inorganic matter, as well as high densities of living organisms, including pathogenic, commensal and environmental bacteria. This chacteristic composition makes sewage a particularly suitable ecological niche for the growth and spread of antibiotic resistance.

1.5.1 Antibiotic selective pressure

The acquisition of antibiotic resistance genes is generally independent of the presence of antibiotics. However, the exposure of bacteria to antibiotics confers an ecological advantage to resistant strains on susceptible strains, allowing them to become predominant in the bacterial population. This situation is commonly termed as *antibiotic selective pressure* and can occur in either the host *in vivo* (eg. human or animal body) as a consequence of chemotherapy or in the environment, for example when antibiotic residues are introduced in sewage.

Residues of antibiotics administered to humans and animals reach the sewage systems in urine or faeces, in the form of either parent compound or degraded metabolites depending on the pharmacology of the specific antibiotic. Furthermore, an unknown amount of antibiotics enter the sewers by waste derived from antibiotic production and disposal of a surplus of drugs. Indeed, various antibiotics have been found in municipal sewage, including fluoroquinolones, sulfonamides and erythromicin metabolites [31-34]. The antibiotic concentrations found in sewage vary between 1 and 100 µg per liter. Such concentrations are 100- to 1000fold lower compared with those necessary to inhibit resistant bacteria, but are sufficient to affect susceptible bacteria [35,36]. Therefore, the occurrence of such antibiotic concentrations in sewage has the potential to select for antibiotic resistance.

The fate of antibiotics in sewage depends on their chemical properties. Lipophilic and non-readily degradable substances are likely to be retained in the sludge, whereas, hydrophilic substances may be able to pass through treatment plants and end up in the natural recipients receiving treated sewage [37]. It also appears that the solubility in water of drug metabolites is generally higher compared with the parent compounds [37]. Thus, it is likely that a large proportion of the antibiotic residues introduced into the sewage system can reach surface waters through municipal sewage effluents.

1.5.2 Non-antibiotic selective pressure

Among the multitude of substances occurring in sewage, there are some that have the potential to select for antibiotic resistance, even though they are not antibiotics. Heavy metals and biocides are two important groups of non-antibiotic substances showing this property. Heavy metals are widespread in sewage as a consequence of industrial pollution. Biocides are introduced into sewage by hospitals, farms, slaughterhouses and food-processing establishments; where these agents are used for the disinfection of environments and utensils, or by the community, due to the presence of these agents in house-hold products, such as soaps and dishwashing detergents.

There are two possible ways by which heavy metals and biocides can select for antibiotic resistance. The genes encoding resistance to heavy metals and biocides can be located together with antibiotic resistance genes on either the same genetic structure (e.g. plasmid), or different genetic structures within the same bacterial strain. Alternatively, bacteria can have unspecific mechanisms of resistance to different substances, including heavy metals, biocides and antibiotics. In both cases, exposure to one substance results in the selection of bacterial strains able also able to resist the other substance (*co-selection*).

Genes encoding resistance due to heavy metals and antibiotics often co-exist on plasmids [38]. In addition, unspecific mechanisms conferring resistance to both heavy metals and antibiotics are known to exist in some bacterial species (e.g. active pump-efflux system encoded by the *marA* gene in *E. coli*). The co-selective property of heavy metals is confirmed by the indirect evidence that bacteria isolated from heavy metal-polluted marine sediment are significantly more resistant to antibiotics compared with bacteria isolated from unpolluted sites [39].

Although genes encoding resistance to biocides have been found on plasmids and integrons [40], these substances are more likely to select for antibiotic resistance by induction of unspecific mechanisms of multiple resistance. Laboratory experiments have shown that biocides such as triclosan and pine oil can select for resistance to different antibiotics when bacteria are exposed to low concentrations of biocide [41,42]. Accordingly, the co-selective effect of biocides for antibiotic resistance could be particularly marked when these substances are dispersed in the environment, because of dilution and formation of concentration gradients.

1.5.3 Optimal conditions for horizontal gene transfer

Sewage is a suitable habitat for the transfer of resistance genes across different groups of bacteria. In this habitat, environmental bacteria meet resistant bacteria selected by use of antibiotics in human and veterinary medicine. Consequently, resistance genes occurring in bacteria of human and animal origin can be transferred to environmental bacteria, contributing to the formation of an environmental pool of resistant bacteria and resistance genes.

The high concentrations of bacteria, nutrients and suspended solids in sewage are all factors enhancing horizontal gene transfer [43-45]. High bacterial concentrations increase the chance that donor and recipient cells come in contact. Nutrients are more likely to have an indirect influence on the occurrence of gene transfer by increasing the concentration and the metabolic activity of bacteria. Suspended solids provide ideal surfaces on which the

various components contributing to the process of horizontal gene transfer (bacteria, free DNA and bacteriophages) are concentrated.

Plasmids and transposons harbouring antibiotic resistance genes are widespread in the bacterial flora of sewage [46,47]. Multiple-resistant bacteria isolated from sewage can transfer plasmid-mediated antibiotic resistance at high frequencies in the laboratory [48,49]. Experiments performed using membrane chambers immersed in sewage have shown that high frequencies of transfer may also occur under real conditions [50,51].

Comment Letter L

2 Methodology

2.1 Sampling sites, times and methods

As part of the study samples of sewage were collected from sewers and sewage treatment plants. The following two sections detail the sites sampled, sampling times and methods used.

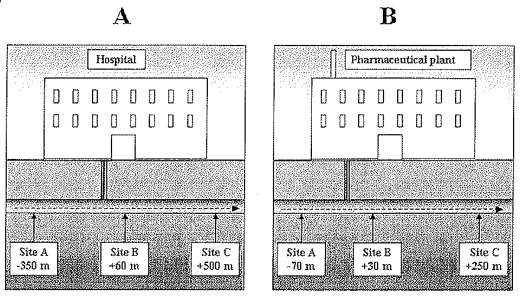
2.1.1 Sampling at sewers

Samples of sewage were collected from two separate sewers receiving waste effluent from a hospital and a pharmaceutical plant manufacturing products containing antibiotics (Paper 1). Sampling sites were situated upstream (site A) and downstream (sites B and C) from the effluent discharge points of the hospital and the pharmaceutical plant (Fig. 2.1). At the hospital, site A was situated 350 m upstream from the discharge point, site B was 60 m downstream and site C was 500 m downstream. At the pharmaceutical plant, site A was located 70 m upstream from the discharge point, site B was 30 m downstream and site C was 250 m downstream.

The selection of the sampling sites was restricted by the access to the sewer system for the collection of samples. No important sources of waste effluent were present between sites A and sites B. Therefore, differences in the occurrence of resistant bacteria between these sites could be used to evaluate the impact associated with the discharge of waste effluent.

Samples were collected between June and September 1997, for a total of four sampling times. Samples of sewage mixed with sediment particles were aspirated from the bottom of sewers using sterile catheters applied to 150 ml sterile syringes. Samples were delivered to the laboratory within one hour after their collection.

Figure 2.1. Schematic representation of the sampling sites at the sewers receiving waste effluent from the hospital (A) and the pharmaceutical plant (B)



2.1.2 Sampling at sewage treatment plants

Samples of raw sewage, treated sewage and anaerobically digested sludge were collected at two large-scale treatment plants in Denmark, Avedøre Spillevandscenter I/S and Lynettefællesskabet I/S (Paper 4). The catchment areas of the two plants have a population of approximately 240,000 and 500,000 inhabitants, respectively. At both plants, sewage undergoes tertiary (advanced) treatment. The treatment process includes: retention of large solids by mechanical screens; separation of sand, grit and grease in aerated chambers; sludge sedimentation in primary settling tanks; biological removal of nitrogen and phosphorus by activated sludge units (supplemented by chemical phosphorus precipitation), and secondary clarifiers.

Sampling was performed monthly from August 1999 to January 2000, for a total of six sampling times at each plant. Twenty-four hour flow-proportional samplers were used to collect raw sewage from the influent, and samples of treated sewage from the final effluent. Grab samples of digested sludge were collected from the digesters following centrifugation. All samples were processed in the laboratory within 6 hours of their collection.

2.2 Measurement of antibiotic resistance

Two different methods were used for measurement of antibiotic resistance of bacteria in sewage samples. *Acinetobacter* isolates were randomly isolated without inclusion of antibiotics in the medium (non-selective method) and the prevalences of antibiotic resistance were evaluated based on antibiotic susceptibility testing of large numbers of isolates. In addition, total and relative numbers of resistant bacteria were calculated based on bacteriological counts on media containing antibiotics. The latter method differs from the former, in that bacteria are selectively isolated with regard to their antibiotic resistance properties (selective method).

2.2.1 Use of Acinetobacter as a bacterial indicator

One of the main innovative aspects of this project was the use of *Acinetobacter* as a bacterial indicator. In fact, most previous studies investigating the occurrence of resistant bacteria in sewage were performed using coliforms as bacterial indicators. The choice of *Acinetobacter* was prompted by the natural occurrence of this organism in the aquatic environment. Based on this characteristic, *Acinetobacter* was considered a more representative indicator of the aquatic bacterial flora in contrast to coliforms, which occur in sewage mainly through human and animal contamination.

In the last decade, *Acinetobacter* has assumed an increasing importance as an opportunistic human pathogen, causing infections often refractory to antibiotic treatment [52]. The particular ability of *Acinetobacter* to develop antibiotic resistance suggested us that this organism that could be used as a sensitive indicator for monitoring antibiotic resistance. Furthermore, *Acinetobacter* represents a good model for studying the ecology of antibiotic resistance genes, as it occurs ubiquitously in a large variety of habitats, including water, soil, food, animals and humans (201).

Several practical features also contributed to the choice of *Acinetobacter* as a bacterial indicator. *Acinetobacter* is a non-fastidious organism in the laboratory

and therefore allows antibiotic susceptibility testing by standardised procedures. Furthermore, the availability of a pre-established selective enrichment medium and a genus-specific DNA probe allowed us to develop a rapid method for isolation and identification (see section 2.3.1). This aspect was of primary importance in the choice of the bacterial indicator, as large numbers of isolates are required for statistically validating data on antibiotic resistance.

2.2.2 Antibiotic susceptibility testing of Acinetobacter isolates

Antibiotic susceptibility testing of *Acinetobacter* isolates was performed by the disc-diffusion method in accordance with the Swedish Reference Group for Antibiotics [53]. All isolates were tested against antimicrobial compounds representative of six antibiotic classes: amoxicillin or ampicillin (penicillins), chloramphenicol (phenicols), ciprofloxacin (quinolones), gentamicin (aminoglycosides), tetracycline or oxytetracycline (tetracyclines), and sulfamethoxazole (sulfonamides) or sulfamethoxazole/trimethoprim (potentiated sulfonamides). *Acinetobacter* isolates from the two sewage treatment plants were additionally tested against aztreonam (monocyclic beta-lactam), cefoxitin (2nd generation cephalosporin), cefotaxime (3rd generation cephalosporin), imipenem (carbapenem), nalidixic acid (older quinolone), piperacillin (piperazine-penicillin), amikacin and tobramycin (aminoglycosides).

The breakpoints for classification of resistant and susceptible isolates were empirically selected based on the distribution of the inhibition zone diameters. The selection of the breakpoints was facilitated by the demonstration of two distinct sub-populations constituting resistance and susceptibility. In most cases, the elected breakpoints did not differ significantly from those used for defining resistance in clinical *Acinetobacter* isolates. In the few instances where they did not correlate, both empirical and clinical breakpoints were used (Paper 4).

2.2.3 Enumeration of resistant coliforms in sewage

Bacteriological counts of total and resistant coliforms were performed by the streak plate method. Total coliforms were enumerated on MacConkey agar following 24 hours incubation at 37°C, without confirmation of presumptive coliforms for gas production. Resistant coliforms were enumerated on the same medium containing ampicillin (16 µg/ml), gentamicin (8 µg/ml), tetracycline (8 µg/ml), or all three antibiotics using the same incubation conditions. The percentages of antibiotic resistance were then calculated for each sample as the number (CFU/ml) of resistant coliforms divided by the number of total coliforms.

Ampicillin, gentamicin and tetracycline were selected as representatives of important classes of antibiotics: beta-lactams, aminoglycosides and tetracyclines, respectively. The antibiotic concentrations added to the medium were in accordance with the minimum inhibitory concentration (MIC) breakpoint values for definition of resistance in clinical practice [53].

2.2.4 Enumeration of resistant acinetobacters in sewage

Total numbers and percentages of resistant acinetobacters (i.e. presumptive *Acinetobacter* spp. not identified by the genus-specific probe) in sewage were

determined as described above, with the exception of the agar (Baumann agar) and the incubation conditions (48 hours at 30°C) used.

2.2.5 Enumeration of total culturable resistant bacteria in blue mussels

Blue mussels were homogenised in a stomacher (5 g blue mussels in 10 ml sterile water for 30 s) and the obtained homogenate serially diluted ten-fold. Total numbers and percentages of culturable antibiotic-resistant bacteria were then determined as described above, with the exception of a different agar (Mueller-Hinton agar) and different incubation conditions (48 hours at 30°C). Mueller-Hinton agar was considered particularly suitable for enumeration of total culturable resistant bacteria, as it does not contain substances that could adversely affect the activity of antibiotics incorporated and permits satisfactory growth of most culturable bacterial species.

2.2.6 Enumeration of resistant *E. coli* in blue mussels

Total numbers and percentages of antibiotic-resistant *E. coli* in blue mussels were determined as detailed above, with the exception of a different agar (tryptone bile agar with X-glucoronide) and different incubation conditions (24 hours at 44°C).

2.2.7 Statistical analysis

Statistical analysis of data on antibiotic resistance was performed using either Statistix (Analytical Software, USA) (Paper 1) or SAS version 6.12 (SAS Institute Inc., USA) (Paper 4). The analyses were used to determine any statistically significant associations between antibiotic resistance (outcome variable) and sampling sites (independent variable), including the variable sampling time to allow for any confounding effect of time.

Data derived from antibiotic susceptibility testing of *Acinetobacter* isolates (dichotomous data) were analysed by logistic regression analysis. When such analysis was not appropriate due to excessive variability of the data, chi-square analysis was performed separately for each sampling time (Paper 1). Data derived from bacteriological counts (continuous data) were analysed by linear regression analysis, after testing whether the residuals were normally distributed.

In the study relating to the effects of waste effluent from the hospital and the pharmaceutical plant, comparisons between sites A and B were carried out to determine whether the discharge of waste effluent was associated with an increase in the occurrence of resistant isolates. Comparisons between sites B and C were carried out to provide information concerning variations in the prevalence of resistant *Acinetobacter* depending on the distance from the discharge point.

In the study concerning the effects of sewage treatment, the statistical analysis was performed separately for each plant. Data pertaining to raw and treated sewage were compared to assess the effect of sewage treatment on the prevalence of resistant bacteria. Data concerning raw sewage and digested sludge were compared to assess the effect of sludge treatment on the prevalence of resistant bacteria.

2.3 Identification and typing of bacteria and resistance genes

During the project, bacteria were characterised at various levels using phenotypic and genotypic methods. *Acinetobacter* isolates were identified at the genus level prior to antibiotic susceptibility testing. A number of isolates was characterised by phenotypically and their plasmid content determined to detect possible effects on strain distribution caused by the discharge of waste effluent from the pharmaceutical plant. Ribotyping was used to confirm the identity of strains introduced into membrane diffusion chambers during the *in situ* experiment on survival of multiple-resistant bacteria. Finally, tetracycline resistance genes were also typed to determine whether the same classes of resistance genes occurred in both clinical and aquatic *Acinetobacter* strains.

2.3.1 Identification of *Acinetobacter* at the genus level

Acinetobacter isolates were identified at the genus level by colony hybridisation using a genus-specific DNA probe. In order to enhance the detection of *Acinetobacter*, colony hybridisation was performed in combination with the use of the Baumann medium, which is a selective medium based on the ability of *Acinetobacter* to grow using acetate as the only carbon source [54]. The protocol used for colony hybridisation is described in Paper 1.

2.3.2 Identification of *Acinetobacter* at the species level

A subset of *Acinetobacter* isolates from the sewers at the hospital and the pharmaceutical plant (n=43) was identified at the species level by phenotypic tests. The following tests were performed: growth at 37EC in Brain Hearth Infusion broth (BHI), acidification of glucose, haemolysis of sheep blood, utilisation of citrate, azelate, glutarate, L-histidine, DL-lactate, L-leucine, L-phenylalanine and L-arginine. The methods used for each test and the criteria used for identification are described in Paper 2.

2.3.3 Plasmid profiles

The same subset of *Acinetobacter* isolates from the sewers at the pharmaceutical plant and the hospital was characterised by plasmid profiling. Plasmids were isolated by a hot alkaline method [55] modified by the addition of lysozyme, and then detected by gel electrophoresis in 0.8% agarose gels.

2.3.4 Ribotyping

Due to its high discriminatory power, ribotyping was considered particularly suitable to confirm the identity of strains inoculated into membrane-filter chambers during the performance of the *in situ* pond experiment (see section 2.5.3).
Ribotyping was performed as previously described [56] using the restriction enzyme *Hin*d III for DNA digestion. The method entails digestion of bacterial DNA by restriction enzymes followed by DNA hybridisation with rRNA-based probes.

2.3.5 Typing of tetracycline resistance genes

Fifty tetracycline-resistant *Acinetobacter* isolates from clinical specimens (n=35), sewage (n=10) and aquaculture habitats (n=5) were analysed by PCR for the occurrence of tetracycline resistance genes of classes A to E, which are the predominant classes among Gram-negative bacteria. The PCR

primers and protocols used for the typing of tetracycline resistance genes are described in Paper 3.

2.4 Experiments on transfer of tetracycline resistance

In vitro experiments on the transfer of tetracycline resistance between *Acinetobacter* strains isolated from different environments and belonging to different species were carried out to assess their ability to transfer tetracycline resistance genes. We decided to focus our attention on tetracycline resistance due to the frequent occurrence of this resistance observed in *Acinetobacter* isolates originating from sewage and aquaculture habitats. Furthermore, tetracycline resistance was considered particularly suitable for studying the ability to transfer antibiotic resistance among aquatic bacteria as it is usually mediated by genetic transfer and only rarely determined by chromosomal mutations.

2.4.1 Bacterial strains

Twenty tetracycline-resistant *Acinetobacter* strains were used as donors in the mating experiments, including 10 strains from sewage, 5 strains from aquacultural habitats and 5 strains resulting from clinical outbreaks. The five clinical strains belonged to the species *A. baumannii* and originated from different European countries. Most aquatic strains (8/15) were identified phenotypically as *A. lwoffii* and *A. johnsonii*, the two species prevalent in the aquatic environment. The remaining strains belonged to *A. junii* (n=3), *A. haemolyticus* (n=1), the unnamed genomic species 16BJ (n=1) or had atypical phenotypic traits precluding speciation (n=2).

Rifampicin-resistant mutants obtained by the gradient plate method [57] were used as recipients. These were strains derived from tetracycline-sensitive *Acinetobacter* strains isolated from an unpolluted stream (recipient A) and sewage (recipients B and C). According to phenotypic identification, recipients A and B belong to unknown *Acinetobacter* species and recipient C belongs to *A. calcoaceticus*.

2.4.2 Mating experiments

Mating experiments were performed on solid media (Luria Bertani agar) as described in Paper 3. The acquisition of tetracycline resistance by recipient strains was confirmed by phenotypic tests differentiating between donors and recipients. Plasmid profiling was used to detect relocation of plasmid DNA from donor to recipient strains. Mating experiments in which transfer of tetracycline resistance was demonstrated, were studied to determine whether transfer was mediated by conjugation or transformation. In order to exclude transfer mediated by transformation, experiments were repeated in the presence of deoxyribonuclease I, an enzyme destroying extracellular DNA. In addition, the ability of the recipient strains to acquire resistance by transformation was tested by experiments in which DNA extracted from the donor strains was used as a source of resistance genes.

2.5 Experiments on survival of multiple-resistant bacteria in natural waters

Three multiple-resistant strains isolated from treated sewage were investigated for their ability to survive in natural waters and retain antibiotic resistance. This was tested using laboratory seawater microcosms and membrane-filter chambers immersed in a pond. The multiple resistance phenotypes characteristic of these strains were used as selective markers for their detection in the presence of indigenous bacteria. The experiments were performed using low bacterial inoculums (10^3 to 10^4 CFU/ml) with the scope to reproduce the actual conditions occurring when treated sewage is released into natural aquatic recipients.

2.5.1 Bacterial strains

Three multiple-resistant strains previously isolated from the final effluent of the Lynetten treatment plant, were used for the survival experiments. The strains were identified phenotypically as *Acinetobacter johnsonii* (strain B1), *Escherichia coli* (strain M1) and *Citrobacter freundiii* (strain M2) by the API identification system (Biomerieux, France). All three strains were resistant to ampicillin, gentamicin and tetracycline. In addition, strain B1 was resistant to tobramycin, nalidixic acid, potentiated sulfonamides, piperacillin and aztreonam. Strain M1 was resistant to nalidixic acid, ciprofloxacin and intermediate resistant to chloramphenicol. Strain M2 was resistant to chloramphenicol, potentiated sulfonamides and cefoxitin.

The strains were detected using Baumann agar (strain B1) and MacConkey agar (strains M1 and M2) containing ampicillin (16 µg/ml), gentamicin (8 µg/ml) and tetracycline (8 µg/ml). No bacterial growth was observed when these media were inoculated with either seawater or pondwater used for the survival experiments, indicating that bacteria with this multiple resistance phenotype were not present in the indigenous microflora.

2.5.2 Laboratory seawater microcosms

The first survival experiment was performed in microcosms containing seawater collected from Øresund. Microcosms (n=7) consisted of 200 ml Erlenmeyer flasks containing 100 ml of either untreated seawater (n=3) or autoclaved seawater (n=3). The three strains were inoculated into separate flasks to reach the final concentration of approximately 5×10^3 CFU/ml. One flask containing untreated seawater was not inoculated with any of the strains and served as a control to observe the behaviour of the indigenous microflora alone.

Flasks were maintained at room temperature under gentle agitation. Samples were collected from each flask immediately after inoculation (day 0) and after 8 h, 24 h (day 1), 48 h (day 2), 1 week (day 7), 2 weeks (day 14), 3 weeks (day 21), and 4 weeks (day 28). For each sampling time, counts of the strains under study were performed with the antibiotic-selective media described above. In addition, numbers of total culturable bacteria were enumerated on Tryptic Soya agar following 48 hours of incubation at 30°C.

2.5.3 *In situ* pond experiment

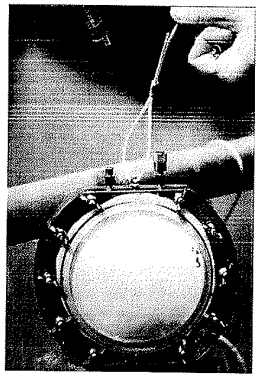
The *in situ* experiment was performed in a freshwater pond using membrane-filter chambers (Technical Services, Montana State University, USA). These

chambers are designed to allow diffusion of water and solutes but without diffusion of the bacteria contained in the chambers (Fig. 2.2). Two bacterial suspensions containing approximately 10^1 CFU/ml of each strain were prepared using water collected from the pond, either without any previous treatment (chamber 1) or autoclaved (sterilised) (chamber 2). Immediately after preparation, 100 ml of the suspensions were inoculated into the chambers, which were then immersed into the pond.

During the experimental period (November-December 2000), the water temperature in the daytime varied from 6 to 9°C, the pH was approximately 7.5 and the concentration of dissolved oxygen was between 11 and 12 mg/L. Samples were collected from the chambers and the pond immediately after inoculation (day 0) and after 4 days (day 4), 1 week (day 7), 2 weeks (day 14), 3 weeks (day 21), and 4 weeks (day 28). Enumeration of the strains under study and total culturable bacteria was performed as described previously. At day 28, representative colonies were isolated from the media used for enumeration of the strains and characterised by phenotypic tests (i.e. colony morphology, cell morphology, glucose O/F and cytochrome oxydase test) and ribotyping to confirm strain identity.

An enrichment procedure was used for detection of stressed cells following 28 days of incubation into the chambers. For this purpose, 1 ml of water was collected from each of the chambers and serial ten-fold dilutions were prepared using peptone buffered water. Dilution tubes were incubated for 48 h at 30°C under gentle agitation and, subsequently analysed for bacterial growth visually.

Figure 2.2. A membrane-filter chamber used in the *in situ* pond experiment.



3 Effects of hospital and pharmaceutical waste effluent on the prevalence of resistant *Acinetobacter* in the recipient sewers

Waste effluent from hospitals contains high numbers of resistant bacteria [48,58,59] and antibiotic residues at concentrations able to inhibit the growth of susceptible bacteria [35]. Accordingly, hospital waste effluent could increase the numbers of resistant bacteria in the recipient sewers by both mechanisms of introduction and selection for resistant bacteria. The effects caused by the introduction of hospital waste effluent into the sewage system were prior to our investigation. Furthermore, no comparison was made with the effects produced by waste effluent from other potential sources of resistant bacteria and antibiotic residues, like pharmaceutical plants producing or manufacturing antibiotics.

In the first part of the project, we investigated the effects of waste effluent from a hospital (section 3.1) and a pharmaceutical plant manufacturing antibiotic products (section 3.2). The levels of susceptibility to six antimicrobial agents were determined in a total of 385 *Acinetobacter* isolates obtained from sites situated upstream (site A) and downstream (sites B and C) from the discharge points of the hospital (n=180) and the pharmaceutical plant (n=205).

In addition, a subset of 43 *Acinetobacter* isolates was characterised by biochemical tests and plasmid profiles to detect possible changes in the composition of the bacterial population associated with the discharge of waste effluent from the pharmaceutical plant (section 3.2). The methods used for sampling, bacterial isolation, antimicrobial susceptibility testing, phenotypic characterisation and plasmid profiling are described in chapter 2.

3.1 Effects of hospital waste effluent

Only minor differences were observed in the levels of antibiotic resistance between *Acinetobacter* isolates from sites situated upstream and downstream from the hospital discharge point (Table 3.1). Oxytetracycline resistance was detected only at sites B and C, and represented 37.2% and 12.5% of the total isolates from those sites, respectively. The occurrence of oxytetracycline-resistant isolates was significantly higher at site B compared with site A ($P<0.01$). However, at site C, 500 m beyond the discharge point, the levels of oxytetracycline resistance were significantly reduced compared with site B ($P<0.01$).

Surprisingly, the occurrence of chloramphenicol-resistant isolates observed at site B was significantly higher than that observed at site A ($P<0.01$). In relation to the other four antibiotics used for susceptibility testing (ampicillin, ciprofloxacin, gentamicin and sulfametoxazole), no significant differences

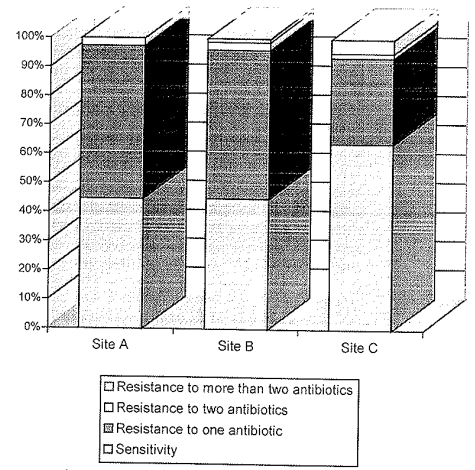
were found between different sites in the occurrence of resistant isolates. Independent of the sampling site, most isolates were either susceptible to all antibiotics tested (51.7%) or resistant to only one compound (43.9%). Isolates resistant to more than two antibiotics were not detected at site A, and were rarely observed at sites B (1.3%) and C (4.7%) (Fig. 3.1).

Table 3.1. Percentages of antibiotic resistance in 180 *Acinetobacter* isolates from the sewers receiving waste effluent from a hospital.

Antibiotic	% of resistant isolates		
	Site A (n=38)	Site B (n=78)	Site C (n=74)
Amoxicillin	2.6	2.6	3.1
Ciprofloxacin	0	2.6	0
Chloramphenicol	55.3[a]	16.7	26.6
Gentamicin	0	0	3.1
Oxytetracycline	0	37.2[b]	12.5[c]
Sulfamethoxazole	0	2.6	4.7

[a] Statistically significant decrease in the numbers of resistant isolates occurring at site B compared with site A.
[b] Statistically significant increase in the numbers of resistant isolates occurring at site B compared with site A.
[c] Statistically significant decrease in the numbers of resistant isolates occurring at site C compared with site B.

Figure 3.1. Percentages of antibiotic sensitivity, resistance to one antibiotic and multiple resistance in 180 *Acinetobacter* isolates from the sewers receiving waste effluent from a hospital.



The reduction in the percentage of chloramphenicol resistance observed at downstream sites is difficult to explain. A possible explanation could be the presence of high numbers of chloramphenicol-sensitive strains in the hospital effluent, as this drug is no longer used in human medicine. Alternatively, substances present in the hospital effluent could affect the survival of chloramphenicol-resistant strains isolated upstream from the discharge point.

Indeed, among the the 21 chloramphenicol-resistant isolates obtained from site A, 20 isolates were susceptible to the other five antibiotics used for susceptibility testing, and only a single isolate was resistant to amoxicillin.

The total numbers of culturable bacteria were markedly higher at the two sites B and C (10^6 to 10^7 CFU/ml) compared to site A (10^5 CFU/ml), indicating that the hospital effluent introduced high numbers of bacteria into the recipient sewers. Since the prevalence of *Acinetobacter* among total culturable bacteria was higher at sites B (23%) and C (9%) than at site A (<1%), it could be deduced that this organism was widely distributed in the hospital effluent. Indeed, it has recently been demonstrated that *Acinetobacter* is the most prevalent bacterial taxon (15% to 56%) among tetracycline-resistant heterotrophic bacteria in hospital waste effluent [60]. These data confirm the suitability of our choice of *Acinetobacter* as a bacterial indicator for monitoring antibiotic resistance in hospital waste effluent.

The results of our study are in accordance with those of a previous study conducted at the same hospital by the former Danish Water Quality Institute (VKI) [61]. Although different methods and target bacterial populations were used for monitoring of antibiotic resistance, both studies indicate a significant increase in the occurrence of tetracycline resistant bacteria associated with the discharge of waste effluent from the hospital. It is difficult to explain these results based on the data on national consumption of antibiotics in hospital care, as tetracyclines account for only a minor component of the antibiotics used in Danish hospitals in recent years [62]. However, resistance to this antibiotic could also be co-selected by exposure to other substances, since tetracycline resistance genes are often located on plasmids together with other resistance genes.

3.2 Effects of pharmaceutical waste effluent

Acinetobacter isolates from the two sites situated downstream from the pharmaceutical plant demonstrated drastically increased levels of antibiotic resistance compared with isolates from the site situated upstream (Table 3.2). Significantly, higher percentages of *Acinetobacter* isolates resistant to amoxicillin, chloramphenicol, gentamicin, oxytetracycline and sulfamethoxazole were obtained from site B in comparison with site A ($P<0.01$). No statistically significant differences were detected between the two sites situated downstream from the sewage discharge point, indicating that the increase in the percentages of resistant isolates persisted at least 250 m from the discharge point.

Table 3.2. Percentages of antibiotic resistance in 205 *Acinetobacteer* isolates from the sewers receiving waste effluent from a pharmaceutical plant.

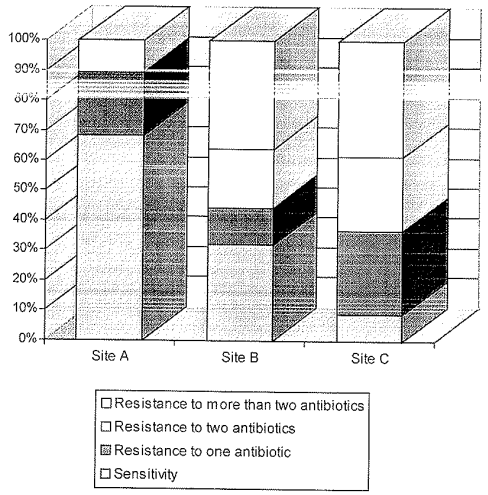
Antibiotic	Antibiotic resistant isolates (%)		
	Site A (n=76)	Site B (n=72)	Site C (n=57)
Amoxicillin	10.5	26.4[a]	31.6
Ciprofloxacin	1.3	0	0
Chloramphenicol	25.0	51.4[a]	61.4
Gentamicin	0	20.8[a]	12.3
Oxytetracycline	5.3	48.6[a]	61.4
Sulfamethoxazole	0	51.4[a]	57.9

[a] Statistically significant increase in the numbers of resistant isolates occurring at site B compared with site A.

High numbers of multiple-resistant *Acinetobacter* isolates were found at the sites situated downstream from the pharmaceutical plant (Fig. 3.2). Multiple resistance to three or more antibiotics was frequently observed among isolates from sites B (36.1%) and C (38.6%), whereas, this was not seen with isolates from site A. A statistically significant increase in the percentage of multiple-resistant isolates was shown for site B in comparison with site A ($P<0.001$), but only at the first three sampling times.

Resistance to sulfamethoxazole and oxytetracycline was often found associated among isolates from sites B (47.2%) and C (54.4%). This resistance pattern was observed in association with chloramphenicol resistance both among isolates from sites B (34.7%) and C (29.8%). Multiple resistance to all antibiotics used, except ciprofloxacin, was observed in 15.3% and 17.5% of the isolates from sites B and C, respectively. None of the above mentioned multiple resistance patterns were detected among isolates from site A.

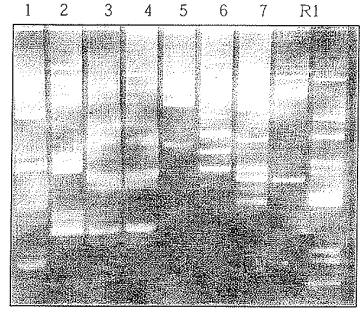
Figure 3.2. Percentages of antibiotic sensitivity, resistance to one antibiotic and multiple resistance in 205 *Acinetobacter* isolates from the sewers receiving waste effluent from a pharmaceutical plant.



The observed increase in the prevalence of antibiotic resistance was associated with differences in the distribution of *Acinetobacter* species/strains between sites situated upstream and downstream from the pharmaceutical plant. *Acinetobacter* isolates from sites B and C showed different phenotypic patterns and plasmid profiles compared with isolates from site A. Seven different clones of *Acinetobacter* were found to be responsible for the high levels of multiple resistance observed at the two sites situated downstream from the pharmaceutical plant (Fig. 3.3).

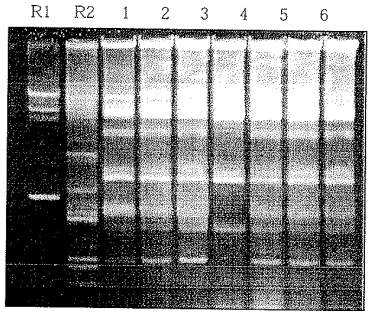
According to phenotypic characterisation, the multiple-resistant strains isolated from the sewers receiving waste effluent from the pharmaceutical plant belonged to at least four different groups of *Acinetobacter* species: *A. lwoffii/A. johnsonii* (n=4), *Acb* complex (n=1), *A. junii* (n=1) and genomic species 16BJ (n=1). Some of the strains contained plasmids of similar size, but no single plasmid appeared to be present in all seven strains (Fig. 3.3). Consequently, the occurrence of multiple antibiotic resistance in the indigenous *Acinetobacter* population was not mediated by horizontal transfer of a unique plasmid structure.

Figure 3.3. Plasmid profiles of seven multiple-resistant *Acinetobacter* strains occurring in the sewers situated downstream from the pharmaceutical plant (lanes 1-7). Lanes R1-R2, reference strains *E. coli* 39R 861 and *E. coli* V517.



The combined use of antibiogram typing and plasmid profiling demonstrated that identical *Acinetobacter* strains were repeatedly isolated throughout the period of study. Isolates showing identical antibiotic resistance patterns, and identical or closely related plasmid profiles, were obtained from samples collected at different times or sites (Fig. 3.4). The clonal relationship of isolates showing identical plasmid profiles was further confirmed by Random Amplified Polymorphic DNA (RAPD) analysis, a DNA fingerprinting method for bacterial typing (data not shown). Based on these results, it could be concluded that multiple-resistant *Acinetobacter* clones, or presumptive clones, were established in the bacterial population of the sewers receiving waste effluent from the pharmaceutical plant.

Figure 3.4. Examples of identical or closely identical plasmid profiles in *Acinetobacter* isolates obtained at different times or from different sites situated downstream from the pharmaceutical plant (lanes 1-7). Lanes R1-R2, reference strains *E. coli* 39R 861 and *E. coli* V517.



Previous studies on the effects of antibiotic resistance caused by the discharge of waste effluent from pharmaceutical plants, have been scarce. A brief report previously conducted at the same pharmaceutical plant showed that the numbers of enterobacteria resistant to chloramphenicol, tetracyclines and aminoglycosides were higher at sites situated downstream from the effluent discharge point [63]. A similar effect on antibiotic resistance was observed in sewers receiving waste effluent from another pharmaceutical plant producing fusidic acid [63]. Therefore, according to the data currently available, it seems that waste effluent from pharmaceutical plants producing or manufacturing antibiotics enhances the occurrence of resistant bacteria in sewage.

The mechanisms by which waste effluent derived from antibiotic production or manufacture increases the numbers of resistant bacteria in sewage has not been investigated. At the pharmaceutical plant under study, products containing penicillins, tetracyclines, tylosin, and to a lesser extent aminoglycosides and sulfonamides, were manufactured during the period of sampling. At the end of each production cycle, the tanks where antibiotic agents had been produced were washed and the residual water, containing antibiotic residues and possibly resistant bacteria, was released directly into the sewage system. Consequently, the increased occurrence of resistant bacteria in the recipient sewers could be due to introduction of either antibiotic residues or resistant bacteria.

3.3 Conclusions

Waste effluent from the pharmaceutical plant was shown to have a higher impact on both single and multiple antibiotic resistance compared with waste effluent from the hospital, which had little effect on occurrence of resistant *Acinetobacter* in the recipient sewers. Therefore, waste effluent from pharmaceutical plants producing or manufacturing antibiotics could represent an important source for the occurrence of resistant bacteria in sewage.

This study indicates that human activities other than the indiscriminate use of antibiotics in human medicine, animal husbandry and agriculture, may disrupt the microbial balance in favour of resistant bacteria. Antibiotic resistance can develop not only in humans and in animals treated with antibiotics, but also in aquatic environments where antibiotics are present as residues derived from industrial production.

Further studies are necessary to assess the actual impact of antibiotic manufacturing on the spread of resistant bacteria in sewage. In particular, more pharmaceutical plants should be investigated, since various factors, such as type and size of production, could influence the occurrence of resistant bacteria and/or antibiotic residues in waste effluent derived from antibiotic production or manufacturing. Furthermore, studies should be implemented to investigate whether antibiotic residues originating from this source may negatively affect the biological treatment process at sewage treatment plants.

4 Effects of sewage treatment on total numbers and percentages of resistant bacteria

The possibility that resistant bacteria occurring in sewage can reach natural aquatic habitats is correlated to their ability to survive sewage treatment. It is generally assumed that sewage treatment determines a marked reduction in the bacterial numbers, including the total numbers of resistant bacteria. However, some studies have documented higher percentages of multiple-resistant bacteria in treated sewage compared with raw sewage [64,65], indicating that resistant and susceptible bacterial populations may not be equally affected by treatment.

In the second part of the project, we investigated the effects of tertiary sewage treatment on total numbers (section 4.1) and percentages (section 4.2) of resistant bacteria. The numbers of resistant bacteria in raw sewage, treated sewage and anaerobically digested sludge from two large-scale treatment plants (Avedøre Spillevandscenter I/S and Lynettefællesskabet I/S) were enumerated on media containing ampicillin, gentamicin, tetracycline or all three antibiotics (antibiotic selective method). Bacteriological counts were determined using media selective for two distinct bacterial populations, i.e. coliforms and acinetobacters. This afforded possible discrimination as to whether the effects of sewage treatment varied among different bacterial populations.

In addition, the levels of susceptibility to 14 antibiotics were determined in 442 *Acinetobacter* isolates obtained from culture on agar media without antibiotics (antibiotic non-selective method) and identified at the genus level. The use of two different methods for quantitative assessment of antibiotic resistance allowed us to compare results obtained by different methods. The description of the sampling sites and the methods used for sampling, bacteriological counts, bacterial isolation, identification, antibiotic susceptibility testing and statistical analysis are described in Chapter 2.

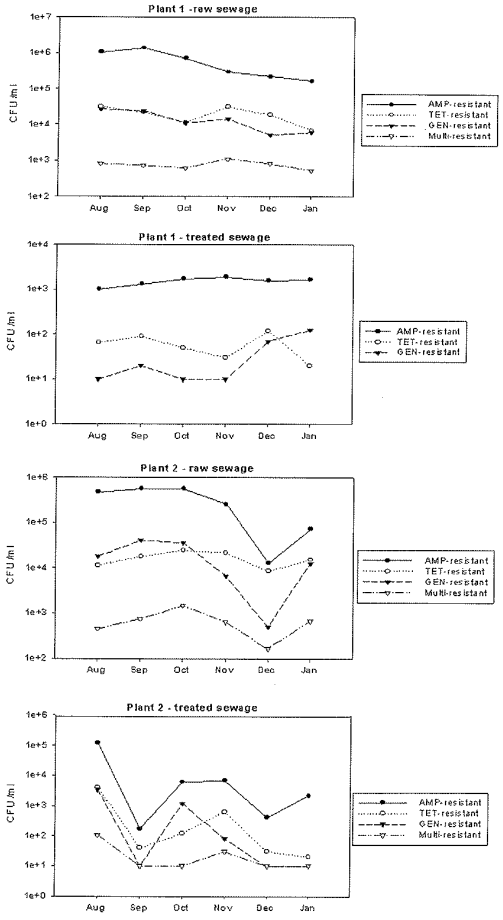
4.1 Effects on total numbers of resistant bacteria

At both plants under study, sewage treatment was associated with a marked reduction in the total numbers of resistant bacteria. The numbers of coliforms resistant to ampicillin, gentamicin and tetracycline were generally 100 to 1000 times lower in treated sewage compared with raw sewage (Figs. 4.1). The only exception was the first sampling time (August) at Lynetten plant, where the numbers of resistant coliforms in treated sewage were about 10 times lower compared with raw sewage. Similar results were seen with acinetobacters (see Paper 4).

Independent of the sample type, coliforms resistant to ampicillin occurred more frequently in comparison with bacteria resistant to gentamicin or tetracycline (Fig. 4.1). Bacteria resistant to all three antibiotics in raw sewage

were relatively frequent with regard to both coliforms (10^2 to 10^3 CFU/ml) and acinetobacters (10^3 to 10^4 CFU/ml). Such multiple-resistant bacteria were not detected in treated sewage from the Avedøre plant, and were seen only at low numbers (#10^2 CFU/ml) in treated sewage from Lynetten.

Figure 4.1. Numbers of coliforms resistant to ampicillin (AMP-resistant), gentamicin (GEN-resistant), tetracycline (TET-resistant) or all three antibiotics (Multi-resistant) in raw and treated sewage from the two treatment plants under study.



Single- and multiple-resistant bacteria occurred in high numbers in digested sludge from both plants (Table 4.1), indicating that anaerobic digestion had little effect on the total numbers of resistant bacteria occurring in sludge. Differences were observed between the two plants, with sludge from Avedøre plant containing lower numbers of resistant bacteria compared with sludge from Lynetten plant. Since the total numbers of resistant bacteria occurring in raw sewage were similar at the two plants (Fig. 4.1), it appeared that the process of sludge treatment at the Avedøre plant reduced the numbers of resistant bacteria more efficiently than at Lynetten plant. However, at both plants digested sludge was further treated by incineration (850°C for 2 sec), which eliminates any form of bacterial life. Furthermore, the resulting ash was not used for agricultural purposes. Therefore, these results do not imply any potential risks for the dissemination of resistant bacteria in the environment through use of sludge in agriculture.

Table 4.1. Average numbers (CFU/g) of coliforms and acinetobacters resistant to antibiotics occurring in anaerobically digested sludge.

Antibiotic	Avedøre plant		Lynetten plant	
	Coliforms	Acinetobacters	Coliforms	Acinetobacters
Ampicillin	4.4×10^{4}	9.3×10^{4}	9.1×10^{5}	1.0×10^{6}
Gentamicin	1.3×10^{3}	3.2×10^{4}	1.9×10^{4}	2.5×10^{5}
Tetracycline	5.3×10^{3}	2.2×10^{4}	4.5×10^{4}	1.5×10^{6}
All 3 antibiotics	5.7×10^{2}	1.3×10^{3}	2.0×10^{3}	7.9×10^{3}

The average percentages of acinetobacters and coliforms among total heterotrophic bacteria for each plant and sample type are shown in Table 4.2. Independent of the sample type, acinetobacters were more prevalent in the total heterotrophic bacterial population compared with coliforms. In the Lynetten plant, the prevalence of coliforms in treated sewage was significantly higher than in raw sewage ($P<0.05$). No other significant differences were observed in the prevalence of the two bacterial populations in different sample types (Table 4.2).

Table 4.2. Average percentages of coliforms and acinetobacters among total culturable bacteria in raw sewage, treated sewage and anaerobically digested sludge.

Bacterial population	Avedøre plant			Lynetten plant		
	Raw sewage	Treated sewage	Digested sludge	Raw sewage	Treated sewage	Digested sludge
Coliforms	12.6	14.8	8.0	5.8	15.1*	7.0
Acinetobacters	19.3	23.6	25.7	26.1	72.9	28.6

*The percentage of coliforms in treated sewage was significantly higher than in raw sewage ($P<0.05$).

4.2 Effects on percentages of resistant bacteria

The relative numbers of antibiotic-resistant coliforms and acinetobacters were not significantly increased by sewage treatment (Table 4.3). On the contrary, at the Avedøre plant the percentage of ampicillin-resistant acinetobacters in treated sewage was significantly lower than in raw sewage ($P<0.05$). At the same plant, digested sludge contained significantly lower percentages of ampicillin-resistant acinetobacters ($P<0.005$), ampicillin-resistant coliforms ($P<0.05$) and gentamicin-resistant coliforms ($P<0.05$) compared with raw sewage, indicating that anaerobic digestion also had a positive effect on percentages of resistant bacteria.

Although these results clearly indicate that the relative numbers of single- and multiple-resistant bacteria were not increased by sewage treatment, it should be noted that strains demonstrating multiple-resistant to ampicillin, gentamicin and tetracycline, such as those detected in treated sewage from the Lynetten plant, are unlikely to occur naturally in the environment. Indeed, the occurrence of such multiple-resistant strains was not detected in seawater collected from Øresund that was analysed using the same media and antibiotic concentrations employed in this study (data not shown).

The average percentages of resistant bacteria occurring in raw sewage, treated sewage and digested sludge are shown in Table 4.3. Ampicillin resistance occurred more frequently than tetracycline and gentamicin resistance in both coliforms and acinetobacters. This could be due to the fact that many bacterial species are intrinsically resistant to penicillins in general but particularly to ampicillin. Coliforms were generally more resistant to ampicillin and less resistant to gentamicin and tetracycline compared with acinetobacters (Table 4.3).

Table 4.3. Average percentages (%) of antibiotic-resistant coliforms and acinetobacters in raw sewage, treated sewage and digested sludge determined by counts on media countaining antibiotics.

Antibiotic[a]	Avedøre plant						Lynetten plant					
	coliforms			acinetobacters			coliforms			acinetobacters		
	Raw sewage	Treated Sewage	Digested Sludge	Raw sewage	Treated sewage	Digested sludge	Raw sewage	Treated sewage	Digested sludge	Raw sewage	Treated sewage	Digested sludge
Ampicillin	51.4	60.3	34.2[b]	47.2	27.0[a]	20.0[b]	47.7	50.5	38.5	18.2	22.3	12.2
Gentamicin	1.4	1.8	1.1[c]	3.9	7.2	5.5	3.3	3.1	1.7	3.4	5.8	3.8
Tetracycline	2.0	2.2	3.7	10.8	11.5	4.3	4.9	2.2	3.2	12.3	12.9	16.7
All antibiotics	0.1	-[c]	0.3	0.1	-[c]	0.3	0.2	0.3	0.3	0.3	0.3	0.1

[a] The percentage of resistant bacteria in treated sewage was significantly lower than in raw sewage (P<0.05).
[b] The percentage of resistant bacteria in digested sludge was significantly lower than in raw sewage (P<0.05).
[c] Multiple resistance to ampicillin, gentamicin and tetracycline was not detected in treated sewage from Avedøre.

The results obtained by bacteriological counts were confirmed by antibiotic susceptibility testing of *Acinetobacter* isolates. Among the 14 antibiotics tested, statistically significant differences were rarely observed in the percentages of resistant isolates originating from different sample types (Table 4.4), indicating a limited effect of sewage treatment on percentages of resistant bacteria. Only the percentage of isolates resistant to nalidixic acid was significantly higher in treated sewage than in raw sewage (P<0.05). Furthermore, the increase in the percentage of nalidixic acid resistance in treated sewage was observed only at the Avedøre plant, indicating that this effect was not generally associated with sewage treatment *per se*, but more specific to the conditions occurring at this particular plant.

The highest overall percentages of *Acinetobacter* isolates resistant to antibiotics were observed for aztreonam (38.0%), cefoxitin (23.8%), chloramphenicol (18.6%), cefotaxime (10.2%), tetracycline (8.4%) and nalidixic acid (6.8%). Resistances to amikacin, imipenem and tobramycin were not detected. For the remaining antibiotics, the percentages of resistant isolates were less than 3% (Table 4.4). The percentage of ampicillin resistance among *Acinetobacter* isolates was very low (0.9%) in comparison with the percentages of ampicillin-resistant acinetobacters obtained by bacteriological counts on Baumann agar containing this antibiotic (see Table 4.3). This is probably because bacterial

species other than *Acinetobacter*, including bacteria intrinsically resistant to penicillins (e.g. *Pseudomonas*), are able to grow on this medium [54].

As described in section 2.2.2, in some cases antibiotic resistance was defined according to two different breakpoints, a breakpoint determined empirically based on the distribution of the inhibition zone diameters and a breakpoint used in clinical practice for definition of antibiotic resistance in clinical *Acinetobacter* isolates. Although the two breakpoints differed only by 1 to 3 mm, such a difference was seen to substantially influence the overall percentages of resistance to aztreonam and chloramphenicol (Table 4.4).

For chloramphenicol and tetracycline, the determination of the breakpoint also influenced the results of the statistical analysis (Table 4.4). According to one breakpoint value, isolates resistant to chloramphenicol occurred more frequently in digested sludge when compared with raw sewage at both the Avedøre plant (P<0.005) and the Lynetten plant (P<0.05), and no statistically significant differences were observed in the occurrence of tetracycline-resistant isolates between different sample types. On the other hand, the use of the second breakpoint value showed that the differences in the occurrence of chloramphenicol-resistant isolates between digested sludge and raw sewage were no longer statistically significant and only tetracycline-resistant isolates from Lynetten occurred more frequently in digested sludge compared with raw sewage (P<0.01).

Table 4.4. Percentages (%) of antibiotic resistance among *Acinetobacter* isolates from raw sewage, treated sewage and digested sludge.

Antibiotic	Total (n=442)	Avedøre plant			Lynetten plant		
		raw sewage (n=65)	treated sewage (n=60)	digested sludge (n=83)	raw sewage (n=63)	treated sewage (n=102)	digested sludge (n=69)
Ampicillin	0.9	0	0	1.2	1.6	0	2.9
Amikacin	0	0	0	0	0	0	0
Aztreonam 1*	38.0	33.8	45.0	33.7	39.7	45.1	29.0
Aztreonam 2*	28.1	26.2	30.0	26.5	25.4	35.3	21.7
Cefotaxime	10.2	16.9	5.0[a]	4.8[b]	11.1	11.7	11.6
Cefoxitin	23.8	27.7	21.7	18.1	22.2	22.3	31.9
Chloramphenicol 1*	18.6	15.4	18.3	34.9[d]	7.9	12.7	20.3[d]
Chloramphenicol 2*	5.7	7.7	6.7	14.5	1.6	0	4.3
Ciprofloxacin	2.7	0	0	1.2	4.8	5.9	2.9
Gentamicin	0.7	0	0	1.2	1.6	0	1.4
Imipenem	0	0	0	0	0	0	0
Nalidixic acid	6.8	1.5	10.0[c]	3.6	11.1	5.9	10.1
Piperacillin	0.7	0	0	0	3.2	1.0	0
Sulf.+ trimethoprim	1.1	4.6	0	0	1.6	0	1.4
Tetracycline 1*	8.4	9.2	13.3	3.6	6.3	6.9	13.0
Tetracycline 2*	7.7	7.7	13.3	3.6	3.2	6.9	13.0[d]
Tobramycin	0	0	0	0	0	0	0

* Resistances to aztreonam, chloramphenicol and tetracycline were defined according to two different breakpoints.
[a] The percentage of resistant isolates in treated sewage was significantly lower than in raw sewage (P<0.05).
[b] The percentage of resistant isolates in digested sludge was significantly lower than in raw sewage (P<0.05).
[c] The percentage of resistant isolates in treated sewage was significantly higher than in raw sewage (P<0.05).
[d] The percentage of resistant isolates in digested sludge was significantly higher than in raw sewage (P<0.05).

The analysis of the distribution of the inhibition zone diameters showed an atypical distribution for aztreonam-resistance and chloramphenicol-resistance among *Acinetobacter* isolates, which made the distinction between resistant and susceptible populations quite arbitrary (Paper 4). This type of distribution indicates the occurrence of multiple and gradual levels of resistance to these compounds. A similar distribution was observed also for cefoxitin and cefotaxime, although changes in the selection of the breakpoint values for definition of resistance to these antibiotics did not significantly affect the results of the study.

It should be noted that *Acinetobacter* isolates obtained from the two plants showed markedly lower percentages of single and multiple antibiotic resistance in comparison with *Acinetobacter* isolates previously obtained from sewers receiving waste effluent from a pharmaceutical plant (see section 3.2). The fact that this pharmaceutical plant was located in the catchment area of the Avedøre plant suggests that the numbers of resistant bacteria were substantially reduced along the sewage system before sewage reached the treatment plant.

The results of our study are in contrast with some previous studies [61,65], which indicated an increase in the percentage of multiple-resistant bacteria following sewage treatment. The divergence could be due to either factors affecting the efficiency of removal of resistant bacteria at different plants (e.g. initial composition of sewage, type of treatment, plant operation, etc.) or differences in the bacterial indicators and methods used for quantitative assessment of antibiotic resistance (e.g. type of medium, definition of the breakpoint value, etc.). Indeed, even in this study, slightly different results were obtained depending on the plant, the target bacterial population and the antibiotic under study, as well as on the method and the breakpoint values used to define antibiotic resistance.

4.3 Conclusions

Based on the analysis of samples obtained from the two large-scale sewage treatment plants during a period of six months, it can be concluded that sewage treatment substantially reduces the total numbers of resistant bacteria without increasing their relative numbers. In some cases, the relative numbers of resistant bacteria in treated sewage appeared even to be reduced in comparison with raw sewage.

Nevertheless, it was shown that in some cases low numbers of multiple-resistant bacteria survived sewage treatment and persisted in treated sewage. Consequently, the release of municipal sewage effluents into natural aquatic habitats appears to contribute to the spread of multiple antibiotic resistance in the indigenous aquatic microflora. Similarly, anaerobically digested sludge could contribute to the dissemination of multiple-resistant bacteria when applied directly to agricultural land without any further treatment. In countries such as Denmark, where further treatment is required for the use of sewage sludge as a fertilizer, it would be relevant to investigate the effects of post-treatment and storage of digested sludge on the occurrence of multiple-resistant bacteria.

The results of this investigation indicate that there is a need to understand how long multiple-resistant bacteria originating from municipal sewage effluents are able to survive after they are introduced in natural aquatic

habitats. Furthermore, their ability to retain their resistance properties and transfer resistance genes to aquatic bacteria should also be elucidated. The results of our studies on survival and *in vitro* transfer of antibiotic resistance by multiple-resistant strains isolated from the municipal sewage effluent of the Lynetten plant are described in Chapter 5.

5 Spread of resistant bacteria and resistance genes by municipal sewage effluents

The results presented in Chapter 4 showed that, although in low numbers, multiple-resistant bacteria are able to survive sewage treatment and reach natural aquatic habitats through their presence in treated sewage. As some types of multiple-resistant bacteria are unlikely to occur naturally in the aquatic environment, a possible risk could be that novel resistance genes are taken up by the indigenous microflora and spread by mechanisms of horizontal gene transfer. The actual environmental impact incurred depends on the ability of such multiple-resistant bacteria to survive in the aquatic environment, retain their antibiotic resistance properties and transfer resistance genes to the indigenous microflora.

In the Part III of the project, we investigated the fate of multiple-resistant bacteria occurring in treated sewage, and more generally, the impact of municipal sewage effluents on the spread of antibiotic resistance. Multiple-resistant strains isolated from treated sewage were studied for their ability to survive and retain antibiotic resistance in natural waters (section 5.1). The transfer of tetracycline resistance genes from bacteria in sewage to bacteria in natural aquatic habitats was investigated by laboratory mating experiments (section 5.2). Finally, the impact of municipal sewage effluents on spread of antibiotic resistance was evaluated by comparing the occurrence of resistant bacteria in blue mussels exposed to treated sewage and blue mussels collected from unpolluted sites (section 5.3).

5.1 Survival in the environment of resistant bacteria originating from sewage

The survival in natural waters of three multiple-resistant strains isolated from the effluent of the Lynetten plant was studied by laboratory seawater microcosms (section 5.1.1) and membrane-filter chambers immersed in a freshwater pond (section 5.1.2). The strains represented three different bacterial species: *Acinetobacter johnsonii*, *Escherichia coli* and *Citrobacter freundii*. Survival experiments were performed both in the presence (i.e. untreated water) and in the absence (i.e. autoclaved water) of the indigenous microflora. A detailed description of the methods used is provided in Chapter 2 (section 2.5).

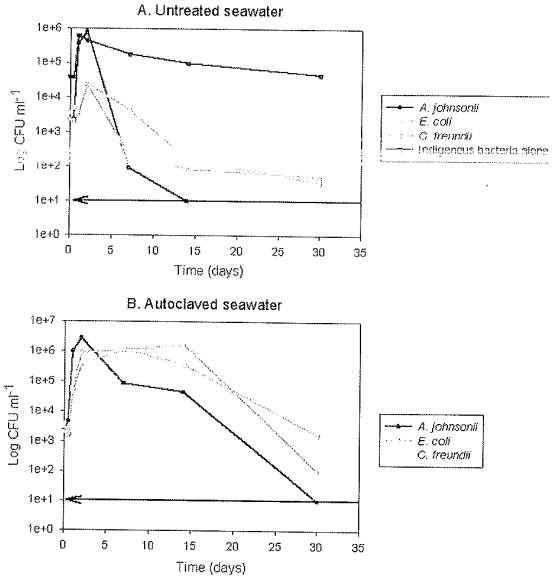
5.1.1 Survival in laboratory seawater microcosms

Growth of the multiple-resistant strains was observed during the 48 hours following the inoculation of the strains into the microcosms (Fig. 5.1). The initial growth of the multiple-resistant strains was likely to be due to the incubation conditions used to maintain the microcosms in the laboratory rather than to a particular ability of the strains to multiply in seawater *per se*,

since similar growth was also observed in the microcosm containing the indigenous microflora alone (Fig. 5.1).

After 48 hours of incubation, the numbers of multiple-resistant strains gradually declined (Fig. 5.1), whereas, the total numbers of culturable bacteria remained stable at approximately 10^5 CFU/ml in all microcosms (Fig. 5.1). All three multiple-resistant strains survived longer in autoclaved seawater (Fig. 5.1B) than in untreated seawater (Fig. 5.1A). A similar finding was seen in a previous study investigating the survival of another *E. coli* strain in seawater from Køge Bugt [16]. The reduced survival of *E. coli* in untreated seawater could be due to both antagonism of the indigenous microflora and predation by protozoa.

Figure 5.1. Survival of multiple-resistant strains isolated from treated sewage in laboratory seawater microcosms. The arrows indicate the detection limit (10 CFU/ml).



Surprisingly, the environmental species *A. johnsonii* showed a more rapid die-off compared with the enteric bacteria *E. coli* and *C. freundii*. In fact, the numbers of *A. johnsonii* strain fell below the detection limit (10 CFU/ml) after 14 days of incubation in untreated water, and after 30 days of incubation in autoclaved water, whereas, the *E. coli* and *C. freundii* strains were still detected after 30 days (Fig. 5.1).

The *E. coli* strain used in this study survived longer in seawater compared with a previously investigated laboratory strain of *E. coli* (i.e. *E. coli* K12), for which a survival of only five days was observed under similar laboratory conditions [66]. Physiological and/or structural changes associated with the exposure to various stressful conditions (e.g. antibiotic selective pressure, survival in sewage, survival of sewage treatment, etc.) could enable multiple-resistant bacteria in sewage to survive environmental stresses compared with the laboratory strains generally used in this kind of experiments.

This study demonstrated that multiple-resistant bacteria occurring in municipal sewage effluents were able to survive in seawater for at least one month following their inoculation into the microcosms. This result is particularly interesting in consideration of the fact that a low bacterial inoclula was used in comparison with previous studies concerning bacterial survival. The temperature in the laboratory microcosms (26°C to 30°C during the day) was higher compared with natural conditions. However, this should not detract from the validity of the result, as previous studies have demonstrated that *E. coli* survive longer in seawater at low temperatures [66].

5.1.2 Survival in a freshwater pond

The multiple-resistant strains showed a more rapid die-off in membrane-chambers immersed in a freshwater pond compared with laboratory seawater microcosms. In the chamber containing untreated pond water (Fig. 5.2A), the numbers of the multiple-resistant strains fell below the detection limit (1 CFU/ml) after either 21 days (*A. johnsonii* and *E. coli* strains) or 28 days (*C. freundii* strain). In the chamber containing autoclaved pond water (Fig. 5.2B), *E. coli* and *C. freundii* strains survived slightly longer compared with the *A. johnsonii* strain. This was also the case in the chamber containing untreated pond water. Only the *C. freundii* strain was recovered after 28 days, although at very low numbers (2 CFU/ml). The numbers of total bacteria were constant in the chamber containing untreated pond water, as well as outside of the chambers.

As for the previous experiment conducted in laboratory seawater microcosms (section 5.1), the *A. johnsonii* strain survived for a shorter period compared with the other two multiple-resistant strains under study. The strain could not be recovered after 28 days of incubation in the pond, even when an enrichment procedure in peptone buffered water was used for detection of damaged and stressed cells. Therefore, it appeared that the *A. johnsonii* strain was no longer present in the chambers.

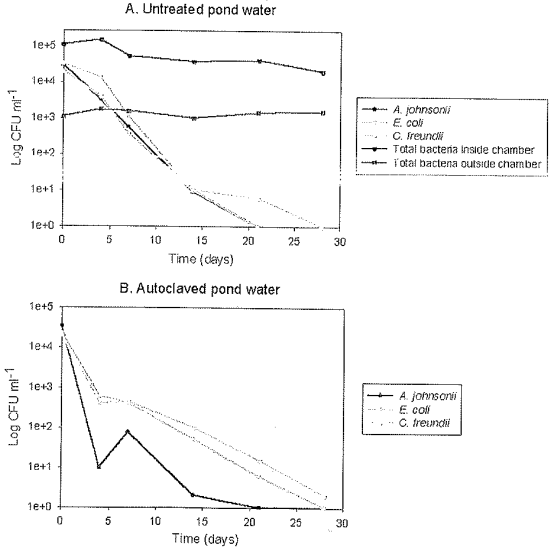
The use of the enrichment procedure in peptone buffered water revealed that the *E. coli* strain and *C. freundii* strains were both present in the two chambers after 28 days. Bacterial isolates (n=15) obtained following inoculation of the enrichment culture on the selective medium for these two strains (i.e. MacConkey agar with added antibiotics) showed the same colony morphology, resistance pattern, plasmid profile and ribotype of the respective *C. freundii* strain (n=14) and *E. coli* strain (n=1). These results confirm that the multiple-resistant isolates obtained after 28 days were identical to the test strains initially inoculated into the chambers.

After 28 days of incubation in the pond, the *E. coli* strain and *C. freundii* strains could be recovered from the first two enrichment dilutions (10^{-1} and 10^{-2}), but not from further dilutions, indicating that the level of the two strains in

the chambers was between 10^2 and 10^3 CFU/ml. A proportion of the two strain populations were probably in a stressed state since lower bacterial densities were detected by direct plating on the selective media (Fig. 5.2).

Characterisation of the bacterial isolates obtained from the chambers after 28 days of incubation in the pond revealed that the strains generally maintained their original plasmid profiles and multiple resistance properties. Only three isolates showed slight variation in the number of plasmid bands compared with the strain originally inoculated into the chambers. Rare differences were observed with regard to the level of susceptibility to one antibiotic (i.e. cefoxitin), with two isolates showing larger inhibition zone diameters (32 mm) compared with the strain originally inoculated into the chamber (10 mm).

Figure 5.2. Survival of multiple-resistant strains isolated from treated sewage in membrane-filter chambers immersed in a pond.



No bacteria showing the same multiple resistance patterns of the test strains were recovered in the pond. Furthermore, bacteria isolated randomly on MacConkey agar without antibiotics showed phenotypic and genotypic traits different from those of the test strains, indicating that the reduction in the numbers of the multiple-resistant strains observed during the experiment was actually due to bacterial die-off and not to loss of their multiple resistance properties.

The results of this experiment showed that two of the three multiple-resistant strains under study were able to survive in the freshwater pond for at least 28 days. Furthermore, the two strains maintained their multiple resistance

properties following one month of incubation under natural conditions. Therefore, it appears that multiple-resistant bacteria occurring in municipal sewage effluents can survive in natural freshwater environments for relatively long periods.

5.2 Transfer of resistance genes from sewage to aquatic bacteria

The possibility that tetracycline resistance is transferred from bacteria in sewage to bacteria in natural aquatic environments was studied under laboratory conditions. Mating experiments were carried out using unrelated tetracycline-resistant *Acinetobacter* strains isolated from sewage (n=10), aquacultural habitats (n=5) and clinical specimens (n=5) as donors and a tetracycline-sensitive *Acinetobacter* strain isolated from an unpolluted stream as a recipient (section 5.2.1). Furthermore, tetracycline-resistant *Acinetobacter* isolates from sewage (n=10), fish farms (n=5) and clinical specimens (n=35) were analysed by PCR for the occurrence of tetracycline resistance genes of the classes Tet A to E, with the aim to determine whether the same genes occur in *Acinetobacter* populations inhabiting different environments (section 5.2.2).

5.2.1 Laboratory mating experiments

Among the 20 *Acinetobacter* strains tested as potential donors of tetracycline resistance, transfer was demonstrated from only three aquatic strains, two from sewage and one from an aquaculture habitat (Paper 3). The two sewage strains capable of transferring tetracycline resistance originated from sewers receiving waste effluent from a hospital (strain LUH 5618) and a pharmaceutical plant (strain LUH 5613) (see Chapter 3). Transfer of tetracycline resistance was not apparent from any of the clinical *A. baumannii* strains to the aquatic recipient strain used in the laboratory matings.

Transfer did not occur when DNA from the donor strains was added to the recipient cultures and was not affected by the presence of deoxyribonuclease I, suggesting a conjugative nature of the transfer. Multiple plasmids of a relatively small size (<36 kb) were transferred from the donor strain LUH 5613 into the recipient strain (Paper 3). In the case of the donor strain LUH 5618, the transfer of tetracycline resistance was apparently not mediated by plasmids, since novel bands were not observed in the plasmid profile of the recipient strain (Paper 3).

This laboratory experiment showed that transfer of tetracycline resistance from sewage bacteria to bacteria living in natural aquatic habitats is possible. However, the limited number of strains used in the mating experiments does not permit broad conclusions on the frequency of such a transfer occurring in nature. Additionally, transfer did not occur between distantly related *Acinetobacter* species, suggesting the existence of physical or physiological barriers limiting the exchange of antibiotic resistance genes between different bacterial species belonging to the same genus.

5.2.2 Distribution of tetracycline resistance genes

Among the 15 aquatic *Acinetobacter* strains tested, three strains contained Tet B (Paper 3). The remaining aquatic strains contained unspecified tetracycline resistance determinants, which did not belong to any of the common classes

occurring in Gram-negative bacteria (Tet A, B, C, D, E, G and M). The three strains containing Tet B had previously been isolated from sewers receiving waste effluent from a pharmaceutical plant (see Chapter 3). The three strains belonged to different species according to both phenotypic and genotypic identification, indicating that Tet B was widespread in the *Acinetobacter* population of this habitat.

A different distribution of tetracycline resistance determinants was observed in clinical strains in comparison with the aquatic strains. Among the 35 clinical strains tested, 33 strains contained either Tet A (n=16) or Tet B (n=17) (Paper 3), indicating that these two classes of tetracycline resistance genes are widely distributed in *Acinetobacter* populations of hospital environments. The different distribution of tetracycline resistance genes in clinical and aquatic strains indirectly provides evidence that the predominant genes occurring in environmental *Acinetobacter* populations do not originate from clinical environments.

5.3 Occurrence of resistant bacteria in blue mussels exposed to treated sewage

The occurrence of resistant bacteria was studied in blue mussels collected from sites exposed to treated sewage (i.e. the outlets of the Avedøre and Lynetten plants) and a control site not exposed to treated sewage (i.e. Limfjorden). Numbers of resistant bacteria were determined for both total culturable bacteria and *E. coli* as previously described (sections 2.2.5 and 2.2.6). The numbers of resistant bacteria in blue mussels either exposed or not exposed to treated sewage were compared to detect possible associations between antibiotic resistance and exposure to treated sewage.

5.3.1 Antibiotic resistance of total culturable bacteria in blue mussels

Occurrence of ampicillin resistance and to a lesser extent nalidixic acid resistance was more frequent than gentamicin and tetracycline resistance in the bacterial flora of mussels exposed to treated sewage (Table 5.1). A different distribution of antibiotic resistance was observed in the bacterial flora of mussels collected from Limfjorden, with nalidixic acid-resistant bacteria being surprisingly more frequent compared with ampicillin-resistant bacteria. Although at very low percentages (#0.1%), multiple-resistant bacteria occurred at the outlets of the two treatment plants. Multiple-resistant bacteria were also isolated from mussels collected at 100 m from the outlet of the Lynetten plant, but not from mussels collected at 100 m from the outlet of the Avedøre plant. This suggests that the presence of multiple-resistant bacteria in mussels living in the proximity of municipal sewage effluents depends on the specific conditions occurring at each treatment plant.

Higher percentages of ampicillin resistance were found in mussels exposed to treated sewage (12.9 to 95.5%) in comparison with mussels not exposed to treated sewage (1.5 to 5.4%). However, interpretation of the data on ampicillin resistance was difficult due to extremely variable percentages of resistant bacteria found in mussels collected from the same sites at different times (Table 5.2). The percentages of gentamicin and tetracycline resistance were low (<3%) independent of the origin of the mussels and the time of sampling.

Table 5.1. Counts of total and resistant bacteria (CFU/g) in blue mussels collected from sites exposed (Avedøre and Lynetten outlets) or not exposed (Lymfjorden) to treated sewage.

Source	Month	Total bacteria	Resistant bacteria					
			AMP	GEN	NAL	TET	AGT	AGNT
Avedøre 1	Aug	$7.2x10^4$	$2.1x10^3$	$5.1x10^2$	$7.4x10^3$	$9.8x10^2$	$2.4x10^2$	$1.2x10^2$
	Nov	$2.2x10^6$	$2.1x10^6$	$1.4x10^4$	$1.6x10^5$	$9.3x10^3$	$1.4x10^3$	$1.0x10^3$
Avedøre 2	Aug	$4.3x10^4$	$1.2x10^4$	N.D.	$1.2x10^3$	N.D.	N.D.	N.D.
	Nov	$2.4x10^6$	$1.7x10^6$	$2.0x10^1$	$1.7x10^5$	N.D.	N.D.	N.D.
Lynetten 1	Aug	$5.0x10^6$	$2.5x10^6$	$4.6x10^4$	$1.2x10^6$	$1.4x10^5$	$1.7x10^3$	$2.2x10^3$
	Nov	$2.9x10^4$	$1.1x10^4$	$6.0x10^1$	$1.5x10^3$	$2.1x10^2$	$6.0x10^1$	$4.0x10^1$
Lynetten 2	Aug	$6.2x10^4$	$8.0x10^3$	$1.6x10^2$	$5.4x10^3$	$1.0x10^3$	$6.0x10^1$	$4.0x10^1$
	Nov	$2.2x10^6$	$2.0x10^6$	$1.8x10^4$	$3.4x10^5$	$7.9x10^2$	$9.2x10^2$	$1.8x10^3$
Limfjorden	Aug	$1.8x10^4$	$9.8x10^2$	$1.0x10^1$	$4.7x10^3$	N.D.	N.D.	N.D.
	Nov	$1.8x10^5$	$2.7x10^3$	$3.9x10^2$	$2.1x10^4$	$1.0x10^2$	N.D.	N.D.

A, ampicillin; G, gentamicin; N, nalidixic acid; T, tetracycline; AGT, ampicillin, getamicin and tetracycline; AGNT, ampicillin, getamicin, nalidixic acid and tetracycline; Avedøre 1, outlet; Avedøre 2, ca. 100 m from the outlet; Lynetten 1, outlet; Lynetten 2, ca. 100 m from the outlet; N.D., not detected.

At sites exposed to treated sewage, a correlation was generally found between the percentages of antibiotic resistance and the distance from the outlets, with higher percentages of resistant bacteria observed in mussels collected from the outlets compared with mussels collected 100 m from the outlets (Table 5.2.). The only exception was the November sampling at the Lynetten plant where an opposite trend was observed (Table 5.2). This was probably because of the presence of strong currents in the direction of the sampling site situated 100 m from the outlet.

Table 5.2. Percentages (%) of resistant bacteria in blue mussels collected from sites exposed (Avedøre and Lynetten plants) or not exposed (Lymfjorden) to treated sewage.

Source	Month	Resistant bacteria					
		AMP	GEN	NAL	TET	AGT	AGNT
Avedøre 1	Aug	29.2	0.7	10.3	1.3	0.3	0.2
	Nov	95.5	0.6	7.3	0.4	0.1	<0.1
Avedøre 2	Aug	27.9	N.D.	2.8	N.D.	N.D.	N.D.
	Nov	70.8	<0.1	7.1	N.D.	N.D.	N.D.
Lynetten 1	Aug	50.0	0.9	24.0	2.8	<0.1	<0.1
	Nov	37.9	0.2	5.2	0.7	0.2	0.1
Lynetten 2	Aug	12.9	0.2	8.7	1.6	0.1%	0.1
	Nov	90.9	0.8	15.5	0.3	<0.1%	0.1
Limfjorden	Aug	5.4	0.1	26.1	N.D.	N.D.	N.D.
	Nov	1.5	0.2	11.6	0.1	N.D.	N.D.

A, ampicillin; G, gentamicin; N, nalidixic acid; T, tetracycline; AGT, ampicillin, getamicin and tetracycline; AGNT, ampicillin, getamicin, nalidixic acid and tetracycline; Avedøre 1, outlet; Avedøre 2, ca. 100 m from the outlet; Lynetten 1, outlet; Lynetten 2, ca. 100 m from the outlet; N.D., not detected.

This study demonstrates that, while resistance to single antibiotics can be found in any environment, including habitats characterised by low levels of pollutants, multiple-resistance to three or four different classes of antibiotics is not likely to occur in natural aquatic bacterial populations. Bacteria demonstrating multiple-resistance to ampicillin, gentamicin and tetracycline were found only in blue mussels exposed to treated sewage, confirming that municipal sewage effluents are likely to represent an important source for the dissemination of these bacteria in the environment.

5.3.2 Antibiotic resistance of *E. coli* in blue mussels

E. coli was only detected in blue mussels exposed to treated sewage, although only sporadically (Table 5.3). This situation did not allow comparison of data on antibiotic resistance between different sampling sites and times. Therefore, *E. coli* could not be used as a bacterial indicator for monitoring antibiotic resistance in blue mussels.

The selective medium used for enumeration of *E. coli* (i.e. TBX agar) could be usefully employed for microbiological analysis of blue mussels. Among 43 representative bacterial isolates tested by the API 20E identification system, 21 isolates (48.8%) were identified as *E. coli* with either a very good or good identification score, 14 isolates (32.6%) were identified as *E. coli* with a low discrimination profile, and 8 isolates (18.6%) showed a doubtful or unacceptable profile. Accordingly, the proportion of verified *E. coli* on the medium varied from 50% to 82% depending on the interpretation of the identification scores obtained by the API 20E identification system.

Table 5.3. Counts of total and resistant *E. coli* (CFU/g) in blue mussels collected from sites exposed (Avedøre and Lynetten plants) or not exposed (Lymfjorden) to treated sewage.

Source	Month	Total *E. coli*	Resistant *E. coli*					
			AMP	GEN	NAL	TET	AGT	AGNT
Avedøre 1	Aug	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
	Nov	$2.1x10^3$	$3.2x10^2$ (15.2%)	$2.0x10^1$ (0.9%)	$6.0x10^1$ (2.9%)	$1.2x10^2$ (5.7%)	$2.0x10^1$ (0.9%)	N.D.
Avedøre 2	Aug	$2.0x10^1$	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
	Nov	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Lynetten 1	Aug	$5.0x10^4$	$1.2x10^4$ (24.0%)	$1.9x10^2$ (0.4%)	$9.8x10^2$ (2.0%)	$4.8x10^3$ (9.6%)	$1.7x10^2$ (0.3%)	$8.0x10^1$ (0.2%)
	Nov	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Lynetten 2	Aug	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
	Nov	$8.5x10^2$	$1.0x10^2$ (11.8%)	N.D.	N.D.	$4.0x10^1$ (4.7%)	N.D.	N.D.
Limfjorden	Aug	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
	Nov	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

A, ampicillin; G, gentamicin; N, nalidixic acid; T, tetracycline; AGT, ampicillin, getamicin and tetracycline; AGNT, ampicillin, getamicin, nalidixic acid and tetracycline; Avedøre 1, outlet; Avedøre 2, ca. 100 m from the outlet; Lynetten 1, outlet; Lynetten 2, ca. 100 m from the outlet; N.D., not detected.

5.4 Conclusions

The results of the survival experiments demonstrate that multiple-resistant strains isolated from treated sewage survived and retained antibiotic resistance for the duration of the study (28 days) in both seawater and freshwater. Above all, the multiple resistance properties of the strains under study remained unchanged after one month of incubation under natural conditions. Therefore, it seems that multiple-resistant bacteria occurring in municipal sewage effluents have sufficient time to transfer resistance genes to indigenous aquatic bacteria once they are released into natural aquatic environments.

Indeed, transfer of tetracycline resistance was shown to occurr under laboratory conditions from *Acinetobacter* strains isolated from sewage to a recipient strain originating from an unpolluted freshwater habitat. However, the actual ability of strains originating from sewage to transfer antibiotic resistance genes to aquatic bacteria under natural conditions needs further evaluation through laboratory experiments using larger numbers of recipient and donor strains resistant to different antibiotics, as well as *in situ* experiments. The lack of transfer of tetracycline resistance from clinical to aquatic *Acinetobacter* strains and the differences observed in the distribution of tetracycline resistance genes between the two bacterial populations, suggest that most tetracycline-resistant bacteria occurring in sewage and aquacultural habitats do not originate from clinical environments.

Bacteria that were multiple-resistant to ampicillin, gentamicin and tetracycline were found in treated sewage and in blue mussels collected at the outlets of municipal sewage effluents, but not in seawater, pond water or blue mussels collected from sites not exposed to treated sewage. This finding substantiates the hypothesis that municipal sewage effluents contribute to the dissemination of multiple-resistant bacteria in the environment. Consequently, future studies investigating the impact of munipal sewage effluents on the spread of

antibiotic resistance should focus on the occurrence of multiple-resistant bacteria rather than on the occurrence of bacteria resistant to single antibiotic compounds.

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Annex 1. Scientific papers and dissemination of results

The results achieved during the three years of this project have been disseminated by publications in international and national scientific journals, as well as by presentations at internationational conferences and national meetings. In all cases, the financial support of the Danish Environmental Protection Agency to the project was acknowleged.

The following is a list of publications in international peer-reviewed journals, which has been referred to throughout the report using the paper number designation:

Paper 1. Guardabassi, L., Petersen, A., Olsen, J. E., Dalsgaard, A. (1998). Antibiotic resistance in *Acinetobacter* spp. isolated from sewers receiving waste effluent from a hospital and a pharmaceutical plant. *Applied and Environmental Microbiology*, 64:3499-3502.

Paper 2. Guardabassi, L., Dalsgaard, A., Olsen, J. E. (1999). Phenotypic characterisation of *Acinetobacter* spp. isolated from aquatic sources. *Journal of Applied Microbiology*, 87:659-667.

Paper 3. Guardabassi, L., Dijkshoorn, L., Olsen, J. E., Dalsgaard, A. (2000). Distribution and *in vitro* transfer of tetracycline resistance determinants in clinical and aquatic *Acinetobacter* strains. *Journal of Medical Microbiology*, 49: 929-936.

Paper 4. Guardabassi, L., Lo Fo Wong, D. M. A., Dalsgaard, D. (2001). The effects of tertiary wastewater treatment on the prevalence of antimicrobial resistant bacteria. *Water Research*, 36: 1955-1964.

This is a list of presentations at conferences or meetings and publications in national journals:

1. Guardabassi, L., Olsen, J. E., and Dalsgaard, A. (1998) The effect on the prevalence of antibiotic resistance of *Acinetobacter* spp. in sewage determined by the discharge of waste effluent from a hospital and a pharmaceutical plant. Poster presentation at the Symposium on Microbial Ecology, Halifax, Canada, 1998.

2. Guardabassi, L. (1998) Sources of antibiotic resistance in the aquatic environment. Oral presentation at the Meeting of the Danish Association of Engineers, Copenhagen, Denmark.

3. Guardabassi, L., Olsen, J. E., and Dalsgaard, A. (1999) Transfer of tetracycline resistance among *Acinetobacter* strains isolated from different habitats. Oral presentation at the 6th Symposium on Bacterial Genetics and Ecology, Florence, Italy.

4. Guardabassi, L. (2000) The use of *Acinetobacter* spp. as bacterial indicators for monitoring antimicrobial resistance in aquatic environments. Ph.D. thesis.

5. Guardabassi, L., and Dalsgaard, A. (2000). Wastewater treatment plants are unlikely to select for antimicrobial resistant bacteria. Poster presentation at the 1st World Congress of the International Water Association (IWA), Paris, France.

6. Guardabassi, L., Olsen, J. E., and Dalsgaard, A. (2000) The use of *Acinetobacter* spp. as bacterial indicators of antimicrobial resistance in aquatic environments. Poster presentation at the 5th Symposium on the Biology of *Acinetobacter*, Noordwijkerhout, The Netherlands.

7. Petersen, A., Guardabassi, L., Olsen, J. E., and Dalsgaard, A. (2000) Selektion of antibiotikaresistens i *Acinetobacter* spp. i spildevand. Dansk Veterinær Tidsskrift, 7: 6-9.

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The listed papers can be found at the Danish National Library of Science and Medicine (http://www.dnlb.dk).

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)

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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.

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Press Release WHO/41
12 June 2000

DRUG RESISTANCE THREATENS TO REVERSE MEDICAL PROGRESS

Curable diseases – from sore throats and ear infections to TB and malaria -- are in danger of becoming incurable New report warns that increasing drug resistance could rob the world of its opportunity to cure illnesses and stop epidemics WHO calls for "Wiser and Wider" effort against infectious diseases.

Increasing levels of drug resistance are threatening to erode the medical advances of recent decades, according to a report released today by the World Health Organization (WHO).

"We currently have effective medicines to cure almost every major infectious disease," said Dr Gro Harlem Brundtland, Director-General of WHO. "But we risk losing these valuable drugs – and our opportunity to eventually control many infectious diseases - because of increasing antimicrobial resistance."

WHO sounded this alarm with the release of its annual Report on Infectious Diseases, titled "Overcoming Antimicrobial Resistance." The report is the first of its kind to present a comprehensive picture of the perilous situation the world is facing as once-effective medicines become increasingly ineffective.

The report describes how almost all major infectious diseases are slowly – but surely – becoming resistant to existing medicines. In Estonia, Latvia, and parts of Russia and China, over 10% of tuberculosis (TB) patients have strains resistant to the two most powerful TB medicines. Because of resistance, Thailand has completely lost the means of using three of the most common anti-malaria drugs. Approximately 30% of patients taking lamivudine – a drug recently developed to treat hepatitis B – show resistance to therapy after the first year of treatment. In India, 60% of all cases of the tropical disease visceral leishmaniasis no longer respond to first-line drugs. A small but growing number of patients are already showing primary resistance to AZT and other new therapies for HIV-infected persons.

In many instances, poorly planned or haphazard use of medicines has caused the world to lose these drugs as quickly as scientists have discovered them.

"It took 20 years to develop penicillin for medical use, and then 20 years for this drug to become virtually useless for treating gonorrhoea in most parts of the world," said Dr David Heymann, Executive Director of WHO's programme on Communicable Diseases. In much of South-East Asia, resistance to penicillin has been reported in 98% of gonorrhoea strains.

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A decade ago in New Delhi, India, typhoid could be cured by three inexpensive drugs. Now, these drugs are largely ineffective in the battle against this life-threatening disease. Likewise, ten years ago, a shigella dysentery epidemic could easily be controlled with cotrimoxazole – a drug cheaply available in generic form. Today, nearly all shigella are non-responsive to the drug.

Those admitted to hospital wards are especially vulnerable. In the United States alone, some 14,000 people are infected and die each year as a result of drug-resistant microbes picked up in hospitals. Around the world, as many as 60% of hospital-acquired infections are caused by drug-resistant microbes.

Antimicrobial resistance is a naturally occurring biological phenomenon amplified many-fold due to human misuse and neglect of antimicrobial drugs. The effect of antimicrobial resistance is that it can reduce the curative power of once life-saving medicines to that of a sugar pill.

The social causes fuelling the spread of antimicrobial resistance are paradoxical. In some settings – especially in poorer countries – the under-use of drugs encourages the development of resistance. For example, where patients are unable to afford the full course of the medicines to be cured of their illnesses, or can only afford to purchase counterfeit drugs on the black market, the weakest microbes in the body may be killed by these insufficient doses while the more resistant microbes are given opportunity to survive and multiply.

In wealthy countries, resistance is emerging for the opposite reason – the overuse of drugs. Unnecessary demands for drugs by patients are often eagerly met by health services prone to over-prescription. Similarly, overuse of antimicrobials in food production in wealthy countries is also contributing to increased drug resistance. Currently, 50% of all antibiotic production is used to treat sick animals, promote livestock and poultry growth, or rid cultivated foods of destructive organisms.

Regardless of where drug resistance originates, globalization, increased travel and trade ensure that these strains quickly travel elsewhere. With new DNA finger-printing technology, scientists have been able to identify drug resistant TB strains originating in Eastern Europe, Asia and Africa and track them as they increasingly reappear in patients in Western Europe and North America.

"The world may only have a decade or two to make optimal use of many of the medicines presently available to stop infectious diseases," said Dr Heymann. "We are literally in a race against time to bring levels of infectious disease down worldwide, before the diseases wear the drugs down first."

The economic consequences of antimicrobial resistance can be staggering. The cost of treating one person with multidrug-resistant TB is a hundred times greater than the cost of treating non-resistant cases. New York City needed to spend nearly US$1 billion to

control an outbreak of multi-drug resistant TB in the early 1990s; a cost beyond the reach of most of the world's cities.

"If we fail to make full and proper use of medicines discovered in our lifetime, many of these drugs will slip through our grasp," said Dr Rosamund Williams, who heads WHO's team working on drug resistance. "Before long, we may have missed our opportunity to control the most dangerous infectious diseases. Indeed, if we fail to make rapid progress during this decade, it may become very difficult and expensive – if not impossible – to do so later."

A common misconception is that the pharmaceutical industry is frequently making new drug discoveries to replace those drugs that become ineffective in fighting the major infectious diseases. In reality, while new versions of older drugs continue to be developed, there is a dearth of new classes of antibacterials. On average, research and development of anti-infective drugs takes 15 to 20 years, and can cost over $500 million, according to pharmaceutical companies.

"Currently, there are no new drugs or vaccines ready to quickly emerge from the research and development pipeline," said Dr Heymann. "We are making a high-risk gamble with the public's health if we are betting on the discovery of new medicines and vaccines, and neglecting our opportunity to make wiser and wider use of the effective medicines we currently have available."

According to the report, the most effective strategy against antimicrobial resistance is to get the job done right the first time – to unequivocally destroy microbes – thereby defeating resistance before it starts. The challenge is to get the right treatment to the patient, each and every time.

"Used wisely and widely, the drugs we have today can be used to prevent the infections of today and the antimicrobial-resistant catastrophes of tomorrow," said Dr Brundtland. "However, if the world fails to mount a more serious effort to fight infectious diseases, antimicrobial resistance will increasingly threaten to send the world back to a pre-antibiotic age. Our grandparents lived during an era without effective antibiotics. We don't want the same situation for our grandchildren."

For further information please contact Gregory Hartl, WHO Spokesperson, WHO, Geneva, telephone: (+41 22) 791 4458, mobile (+41 79) 203 6715, fax: (+41 22) 791 4858. E-mail: hartlg@who.int or Andy Seale, WHO Media Officer for communicable diseases at (+41 22) 791 3670. In the U.S., contact Jim Palmer at (+1 202) 262 9823. In the U.K., contact Janice Muir or Amanda Barnes at (+44 207) 407 3313. There will be an EBU feed on Monday including interview clips from David Heymann and broadcast quality radio interviews will be accessible from the mediacentre reached via http://www.who.int/multimedia. Any enquiries about the EBU feed or broadcast media should be directed to Chris Powell on +41 22 791 2888. All WHO press releases, fact sheets and features as well as other information on this subject can be obtained on internet on the WHO home page at http://www.who.int

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A PUBLICATION OF THE HCH CLINICIANS' NETWORK

HEALING HANDS

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Homeless People at Higher Risk for CA-MRSA, HIV and TB

Homelessness increases one's risk for infectious diseases and complicates access and adherence to treatment. Three infectious agents that disproportionately affect homeless populations — community-associated methicillin-resistant Staphylococcus aureus (CA-MRSA), the human immunodeficiency virus (HIV), and Mycobacterium tuberculosis (TB) — are the focus of this issue of Healing Hands, which highlights recent research on screening, treatment and adherence strategies found to be successful in preventing and arresting these potentially virulent diseases in homeless populations.

CA-MRSA Community-associated (or acquired) methicillin-resistant *Staphylococcus aureus* has recently emerged as a major cause of skin infections in the U.S. At the 2006 Interscience Conference on Antimicrobial Agents and Chemotherapy, MRSA (primarily the USA 300-0114 strain) was identified as the "pathogen *du jour*" because it has "exploded" worldwide over the past 6 years (Bartlett 2006; Moellering 2006). The Association for Professionals in Infection Control recently launched a comprehensive initiative to fight MRSA, calling it the "superbug."

MRSA infections were first described in the early 1980s, but it was not until the mid 1990s that reports of community-associated MRSA infections occurring in patients without identifiable risk factors began to appear in the U.S. (Frazee 2005). The sentinel event was a series of fatal CA-MRSA infections in Native American children in the Midwest, attributed to a MRSA strain known as USA 400.

Since then, studies of urban hospitals and community settings have found that large proportions of reported skin and soft tissue infections (SSTIs) are caused by MRSA, mostly community-associated (Moran et al. 2006; Frazee 2005; Charlebois 2002). One study of SSTIs in 11 city emergency departments across the U.S., for example, found a prevalence of 59 percent MRSA; 74 percent of the MRSA infections were the USA 300-0114 strain (Moran et al. 2006). These authors also found considerable regional variation, with prevalence ranging from 15 to 74 percent overall.

To date, cases of CA-MRSA infection are more common to children and young adults; prevalence is higher among individuals who are members of racial/ethnic minorities and those with low socioeconomic status (Gorwitz, 2006). Research on MRSA in urban populations, though inconsistent on several risk factors, indicates higher risk for individuals with histories of homelessness,

Photo/Toledo-Lucas County Health Department

Pustules resulting from a methicilllin-resistant Staphylococcus aureus skin infection in a tattoo recipient – Ohio, 2005 (Long et al., 2006)

incarceration, frequent emergency department visits, and injection drug use (Frazee 2005; Gilbert 2006; Beam and Buckley 2006; Young et al. 2004; Charlebois 2002).

HCH providers confirm an apparent increase in MRSA over the past few years in homeless clinics and shelters. Higher rates of hospitalization, HIV infection, and injection drug use, as well as crowded living conditions and/or poor hygiene place homeless people at higher risk for acquisition and transmission of CA-MRSA (Charlebois 2002; Pan et al. 2005).

HIV More than one million individuals in the U.S. are living with HIV, and 40,000 new infections are expected to occur this year. About one-quarter of those infected with HIV and as many as half of some subpopulations are undiagnosed and may unknowingly spread HIV to partners. Prevalence of HIV is generally estimated to be at least 3 times higher among people who are homeless than in the general population. In New York the rate of new HIV diagnoses is

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reported to be 16 times higher. One-third to one-half of individuals living with AIDS are estimated to be homeless or at risk of homelessness (Conanan et al. 2003).

TB The incidence of tuberculosis in the U.S. has steadily declined since 1992, the final year of the latest resurgence. The 14,097 cases reported in 2005 represent a decrease of 2.9 percent since 2004 and a 47 percent decrease since 1992. Cases in 4 states (CA, TX, NY, and FL) comprised nearly half (48%) of the 2005 case total (CDC 2005). But because tuberculosis is primarily a disease of poverty and crowding, the prevalence of this disease among urban homeless populations is disproportionately high and emergency shelters remain volatile transmission sites.

Of all TB cases reported in the US between 1994 and 2003, over 6 percent were among persons classified as homeless during the 12 months prior to diagnosis (Haddad et al. 2005; CDC 2005). A recent survey of over 100,000 individuals in New York who had spent at least one night in a homeless shelter found the prevalence of TB infection to be 11 times higher than in the general population (Santora 2006).

COMORBID HIV & TB Of particular concern is the comorbidity of HIV and TB. Because HIV infection severely strains the immune system, people dually infected with HIV and TB have 100 times greater risk of developing active, infectious tuberculosis than do people with inactive ("latent") TB who are not infected with HIV. The CDC estimates that 10 to 15 percent of all TB cases and nearly 30 percent of TB infections among people 25–44 years of age occur in HIV-infected individuals.

As many as one-third (34%) of TB-infected homeless persons nationwide are co-infected with HIV (Haddad et al. 2005). Clinicians are urged to have "a high index of suspicion for TB in a homeless patient who has tested positive for HIV" (Barker et al. 2006). The CDC explicitly notes that any acceleration in the natural elimination of tuberculosis would necessitate prevention efforts targeting at-risk populations, including "persons living with HIV, and persons living in poverty with limited access to medical care and adequate housing and nutrition" (Exec Commentary 2005).

People with HIV/AIDS are also at higher risk of developing multiple drug-resistant TB (MDR TB), which results from inconsistent or partial treatment. In October 2006, the World Health Organization convened a meeting to develop a response to an even more ominous threat—extensively drug-resistant tuberculosis (XDR TB), defined as TB with extensive resistance to second-line drugs. XDR TB has been declared "virtually untreatable." In the U.S., patients with XDR TB are 64 percent more likely to die during treatment than are patients with MDR TB. The emergence of XDR TB reinforces the need for vigilance in screening for TB and HIV.

Preventing Infection & Transmission of Disease

MRSA EDUCATION & TRAINING Acknowledging that MRSA is a "fairly significant" problem in their homeless shelter, **Annie Nicol, FNP, PA** of the Petaluma Health Center in Sonoma County, CA, emphasizes that early identification and prevention are critical to controlling MRSA transmission. Nursing staff provide education about germs, demonstrate sneezing and hand washing techniques, and display visual images of MRSA infections—which is particularly helpful for clients who have difficulty reading or who might otherwise ignore what commonly look like "spider bites."

When a couple of MRSA cases appeared in homeless family shelters this fall, **Elizabeth Browning, RN**, Infectious Control Nurse for the Philadelphia Health Management Corp., said it brought home the need to educate staff and clients about wounds and wound care. She tries to determine how shelter staff can be "practitioners of infection control" because "it takes everybody to do this." "Infection control will become even more important as the prevalence of MRSA increases," warns Ms. Browning. "I can't stress enough the importance of hand washing."

PREVENTING MRSA SKIN INFECTIONS

1. Keep your hands clean by washing thoroughly with soap and water or using an alcohol-based hand sanitizer.
2. Keep cuts and scrapes clean and covered with a bandage until healed.
3. Avoid contact with other people's wounds or bandages.
4. Avoid sharing personal items such as towels or razors.

CDC Guidelines: "Community-Associated MRSA Information for the Public"

http://www.cdc.gov/ncidod/dhqp/ar_mrsa_ca_public.html

The literature confirms the value of these providers' approaches, urging education and attention to basic hygiene and wound care to control the spread of MRSA (Allen 2006; see also Gorwitz 2006 on clinical management of CA-MRSA).

HIV SCREENING & TESTING The CDC has issued revised recommendations for HIV testing in healthcare settings, effective September 2006, in response to concerns that such a large proportion of HIV-infected individuals are unaware of their infection until they have developed symptoms. The recommendations urge HIV screening for all patients ages 13–64 years and testing of high-risk populations at least once a year. Written consent for screening and prevention counseling is no longer required as part of HIV screening programs.

Rapid testing for HIV can facilitate testing of homeless patients in non-clinical settings because results are ready in about 20 minutes. (Positive tests still require confirmation, which can take 1–2 weeks.)

A recent study assessed the use of OraQuick in a mobile health van. The test was chosen for its efficacy in a wider range of settings and temperatures. Of 1,150 tests given, all but 5 results (over 99%) were delivered to the persons tested with OraQuick, while only 50 percent of clients tested using traditional methods returned for results. Authors of adapted clinical guidelines for the care of homeless patients with HIV/AIDS assert the usefulness of rapid testing, but advise that sufficient pre-test counseling be conducted to ensure patient readiness (CN 2003).

Wayne Centrone, MD, Medical Director for Outside In, a program targeting homeless youth and young adults, has used rapid HIV 1 and 2 antibody testing for over two years. Staff provide counseling, advocacy, testing and follow-up referrals for high-risk populations in bath houses, night clubs, sex venues and on the street, as well as in a clinic for high risk MSM (men who have sex with men). "This has been a great way for us to bring a prevention message to a population that might not otherwise be open to such a message, and enables clients to ask questions about creating healthful harm reduction strategies," reports Dr. Centrone.

CDC is currently sponsoring HIV rapid testing by 7 community-based organizations across the country, targeting high-risk populations in a wide variety of non-clinical settings including outreach mobile vans, homeless shelters, and needle exchange programs. New information from these studies will help to determine the appropriateness of this test in homeless populations and settings.

TB CONTROL Tuberculosis control demands vigilance. Ms. Nicol provides TB screening to approximately 800–900 people a year in a new, 130-bed homeless shelter in Sonoma County. She reports an overall decline in TB cases during the past year, just a few converters (from latent to active TB), and only a slight increase in MDR TB. Efforts to maintain a clean environment are partially responsible for these successes, she says. The new facility has a good air filtration system with a structure that is conducive to ventilation. The entire shelter is cleaned twice a day and "double scrubbed" every weekend.

Ms. Browning says Philadelphia shelter providers do their best to keep facilities clean and well-ventilated, but they are often constrained by budget and structural limitations. For example, "if the shelter has few windows, there's very little they can do about ventilation."

These providers also stress the importance of educating shelter staff and clients to dispel common myths about TB—e.g., that a positive test always means someone has infectious tuberculosis.

Published research reinforces the pressing need for sustained, robust TB screening and intervention programs which address multiple risk factors. One study of 415 homeless persons from Skid Row in Los Angeles found deficits in their knowledge about modes of TB transmission and risk factors for infection (Nyamathi 2004; see also Lofy et al. 2006).

Resources developed by HCH providers across the country, including adapted clinical guidelines for homeless patients and practice-based case reports, recommend TB skin tests every 6 months rather than annually for people with HIV infection who are homeless, due to their increased risk for co-infection and unpredictable follow-up (Conanan et al. 2003, Barker et al. 2006).

Studies of TB outbreaks in homeless populations underscore the complexities and challenges of prevention, especially with multiple chains of transmission. A report on a 2003 outbreak in New York recommends use of CDC's universal *Myobacterium tuberculosis* rapid genotyping system (CDC 2005). Authors assessing another outbreak that same year in King County, WA, attributed successful transmission control to genotyping using PCR-based methods that were both rapid and cost-effective (Freeman et al. 2005; Lofy et al. 2006).

Sadly, a conclusion drawn by clinicians seven years ago remains true today: "Despite significant progress in the control of infectious TB … the underlying causes of the resurgence … remain unchanged" (CN, 1999).

Promoting Treatment Adherence & Continuity of Care

Living without stable housing creates a variety of barriers to treatment adherence, such as: limited access to food and water; lack of a safe place to store medications (particularly those requiring refrigeration); managing gastrointestinal side effects when restrooms and bathing facilities are not readily available; and inconsistent shelter and eating arrangements, making it difficult to adhere to a regular schedule. Mental illness and substance abuse are also significantly related to treatment non-adherence (Gordon 2006; Berg et al. 2005; Waldrop-Valverde and Valverde 2006).

The following strategies have proven to be especially effective at promoting treatment adherence and continuity of care, even under difficult circumstances:

CARE COORDINATION Case management throughout treatment, particularly when care is coordinated by an interdisciplinary team, is associated with improved adherence for HIV- or TB-infected homeless people and marginally-housed adults (Kushel et al. 2006; Haddad et al. 2005). "Relationship building, coordination of care by multiple providers, and maintaining contact with homeless clients during and after medical confinement are key to assuring treatment adherence" (Barker et al. 2006). Where possible, homeless clients should be assisted in maintaining a "medical home" for their documentation and treatment records (Conanan et al. 2003).

HCH providers stress the importance of care coordination in their work. Ms. Browning considers collaborations with other homeless agencies and outreach teams to be absolutely essential to locating

homeless clients with active TB who are lost to follow-up. "It becomes like a detective game to figure out where they've gone," she says.

Rod Heilman, MSW, who provides case management to homeless clients for the AIDS Volunteers of Cincinnati, agrees that the most important thing in assuring continuity of care is to be connected to community resources. His clients frequently complain about negative side effects of HIV medications, such as nausea and diarrhea. Regular contact with healthcare professionals is essential to their continuity of care, he says. Although clients may discontinue treatment for reasons unrelated to the treatment itself, medication side effects can be minimized if clients have more personal contact with a healthcare professional, concurs Ms. Browning.

Outreach is a critical component of care coordination for people who are homeless. A recent study in Baltimore, MD, found that homeless HIV-positive individuals with substance use disorders and persistent mental illness were successfully enrolled in integrated treatment services through outreach at shelters, soup kitchens and on the streets. The outreach team consisted of a nurse, a substance abuse counselor, and a formerly homeless person (Tommasello et al. 2006).

HOUSING Supportive housing, including respite care programs that provide interim housing following hospital discharge, are consistently effective in helping clients adhere to treatment for HIV/AIDS and TB (Buchanan et al. 2006; National AIDS Housing Coalition 2005). Research repeatedly confirms that housing is a cost-effective intervention for homeless people, regardless of individual characteristics and behaviors. Permanent supportive housing has a positive impact on prevention of disease, continuity of care, and treatment adherence. A report summarizing the National Housing and HIV/AIDS Research Summit in 2005 is aptly titled, "Housing Is the Foundation of HIV Prevention and Treatment" (National AIDS Housing Organization).

A program in Georgia that provides housing for homeless people with TB demonstrated a 97 percent compliance rate with directly observed therapy and a 91 percent completion of therapy rate among the 538 homeless clients who used their services between July 1, 1996 and June 30, 2004 (Fraser 2005).

INCENTIVES Research has shown that monetary incentives in combination with case management can improve treatment adherence rates for homeless people with latent TB (Hwang et al. 2005; Tulsky et al. 2004). A recent randomized controlled trial of treatment programs for homeless adults with latent tuberculosis infection (LTBI) found that those receiving nurse case management, education, tracking, and incentives had 3 times greater odds of completing a full 6-month course of LTBI treatment than did patients in the control group receiving only standard care plus incentives (Nyamathi et al. 2006).

RECOMMENDATIONS FOR TREATMENT OF CA-MRSA The first challenge for the clinician in treating CA-MRSA infections is "to identify the organisms and recognize that they are resistant to the oral cephalosporins and antistaphylococcal penicillins, which have been the mainstay in treating SSTIs" (Moellering 2006).

TREATMENT OF MRSA: RECOMMENDATIONS FOR CLINICIANS

- Be aware that the prevalence of CA-MRSA is rapidly increasing in the U.S. and there is a low threshold for obtaining material for culture and susceptibility testing from community-associated abscesses and other skin infections, especially those that resemble "spider bites" with areas of necrosis.
- Know when to use drugs with activity against CA-MRSA—if the patient does not respond rapidly to appropriate drainage and standard antimicrobial agent therapy or in settings with a high incidence of CA-MRSA infections.
- In settings of more invasive infections, the clinician should immediately start therapy with vancomycin or linezolid and obtain an infectious disease consultation.

Moellering 2006

Adi Gundlapalli, MD, PhD, infectious disease specialist at the University of Utah and former Medical Director at Wasatch Homeless Health Care in Salt Lake City, advises HCH clinicians to "be aware that MRSA is on the rise." He also notes that oral medications for CA-MRSA (including bactrim, doxycycline and rifampin) can be useful, depending on local resistance patterns. (See box for other recommendations.)

ONCE-A-DAY TREATMENT FOR HIV In July 2006, the Federal Drug Administration approved Atripla, the first once-a-day, fixed-dose combination tablet of three widely used antiretroviral drugs. This is a potential "boon for HIV treatment compliance and adherence," notes Dr. Gundlapalli, but consistent access and availability of the treatment for homeless persons may be a long time in coming.

WHOLE-BLOOD TEST FOR LTBI Use of whole-blood interferon-gamma release assay (IGRA) has been recommended as an alternative to the tuberculin skin test (TST). The fact that whole-blood tests such as QuantiFERON-TB (QFT) and QunatiFERON-TB Gold do not require a return visit makes them especially appealing for homeless

QUANTIFERON TB BLOOD TEST
A whole-blood test for diagnosing latent TB infection (LTBI)

Advantages	Disadvantages
• Single patient visit only – no return visit required	• Additional tests needed to exclude TB disease and confirm LTBI (as with TST)
• No booster phenomenon, which can happen with repeat tuberculin skin tests (TST)	• Blood samples have to be processed within 12 hours of blood draw
• Less subject to reader bias and error than with TST	• Not yet approved for all patients: children <15 years; TB case contacts; pregnant; immunocompromised patients (including known HIV+)
• Assesses responses to multiple antigens simultaneously	

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patients. A recent study found IGRA implementation in a TB control program to be "feasible and acceptable" among homeless persons, injection drug users, and immigrant patients from 6 community clinics in San Francisco. Inclusive of phlebotomy, laboratory, and personnel costs, IGRA cost $33.67 per tested patient (Dewan et al. 2006).

HCH practitioners, however, have mixed attitudes about the test. **Frank Alvarez, MD, MPH**, Deputy Health Officer of the Santa Barbara County Public Health Department's Disease Control & Prevention Division, and Disease Control Manager **Paige Batson, RN, PHN**, recently collaborated with the Santa Barbara HCH clinic to use the QF-TB Gold test on a small group of homeless clients with positive tuberculin skin tests to determine the blood test's efficacy. The QFT test ruled out "nearly all" of those individuals who had recently tested positive with the skin test. For this reason, Ms. Batson says the overall cost savings of using the test are great, even though the test is pricy. She calls the test one of their "greatest resources." Dr. Alvarez concurs that "it's a great test," but adds that there are logistical limitations in the field. For example, "once you draw the serum, you have to get it to a lab within 12 hours or it's worthless."

Dr. Gundlapalli concurs that the logistical challenges in using this test are immense, citing the 12-hour lab restriction, high cost, and limited availability as factors that make the QFT test difficult to use with patients who are homeless. It should also be noted that in those with impaired immune function (including HIV/AIDs), a negative QFT test alone may not be sufficient to rule out *M. tuberculosis* infection. (See box for a summary of advantages and disadvantages of the QFT test on p. 4; CDC Dec 15 05; Barker et al. 2006.)

SHORT-COURSE TREATMENT FOR LTBI A short-course treatment recommended for latent TB infection involves using 60 daily doses of rifampin and pyrazinamide. While this approach seems promising for improving treatment adherence for homeless persons, only one study has assessed this. The study included incarcerated individuals from 5 county jails (n=844) and homeless persons drawn from TB outreach clinics in 3 cities (n=367). While completion rates using the regimen exceeded historical rates using isoniazid, it also found an increased risk of significant hepatoxicity in 6 percent of the patients. The authors conclude that it is important to seek an effective short-course treatment regimen for these populations, but recommend relying upon better known interventions to improve adherence, such as DOT and the use of incentives, until further research has been conducted (Lobato et al. 2005). ■

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STRUCTURAL FACTORS LIMITING TB CONTROL

"Authorities rarely blame the recrudescence of tuberculosis on the inequalities that structure our society. Instead, we hear mostly about biological factors (the advent of HIV, the mutations that lead to drug resistance) or about cultural and psychological barriers that result in "noncompliance." Through these two sets of explanatory mechanisms, one can expediently attribute high rates of treatment failure either to the organism or to uncooperative patients."

"Many would find it scandalous that one of the world's leading causes of preventable adult deaths is a disease that, with the possible exception of emerging resistant strains, is more than 95 percent curable, with inexpensive therapies developed decades ago."

— Paul Farmer, MD, *Pathologies of Power: Health, Human Rights, and the New War on the Poor*

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Other Resources on Infectious Disease Prevention & Control:

1. Association for Professionals in Infection Control and Epidemiology, Inc.: http://www.apic.org
2. Centers for Disease Control and Prevention: http://www.cdc.gov

The HCH Clinicians' Network is operated by the National Health Care for the Homeless Council. For membership information, call 615/ 226-2292.

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Antibiotic Resistance Genes as Emerging Contaminants: Studies in Northern Colorado†

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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.

Introduction

The spread of antibiotic-resistant pathogens is a growing problem in the U. S. and around the world. Recently a 2000 World Health Organization (WHO) report (*1*) focused on antibiotic resistance as one of the most critical human health challenges of the next century and heralded the need for "a global strategy to contain resistance". According to the report, more than two million Americans are infected each year with resistant pathogens and 14 000 die as a result. The rapid growth of the problem emphasizes the need for intervention. For example, vancomycin is currently considered to be the most powerful antibiotic of "last resort", yet within 10 years the incidence of vancomycin-resistant enterococci (VRE) increased in the United States from 0% to 25% (*2*, *3*). Resistance to penicillin, the antibiotic that originally revolutionized human health 50 years ago, is now as high as 79% in *Staphylococcus pneumoniae* isolates in South Africa (*4*, *5*). Alarmingly, diseases that were once considered to be eradicated, such as tuberculosis, are now beginning to make a comeback because of antimicrobial resistance (*1*, *6*, *7*). As with other dangerous pollutants that spread in the environment and threaten human health, there is a need for environmental scientists and engineers to help address the critical problem of microbial resistance to antibiotics.

The rise of antibiotic resistance is considered to be closely linked with the widespread use of antibiotic pharmaceuticals in humans and animals. In particular, more than one-half of the antibiotics used in the U. S. are administered to livestock for purposes of growth promotion or infection treatment (*8*, *9*). In both animals and humans, up to 95% of antibiotics can be excreted in an unaltered state (*10*, *11*). Some removal has been observed in wastewater treatment plants (WWTPs); however, as is true with the larger problem of pharmaceutical compounds, WWTPs are not designed for the removal of micropollutants (*12*–*14*). Residual antibiotics thus are released into the environment where they may exert selection pressure on microorganisms. While overprescribing or other improper use/disposal of antibiotics in humans is generally considered to contribute to the problem, several studies have also linked agricultural antibiotic use with antibiotic-resistant infections in humans (*15*–*23*). For example, avoparcin, an antibiotic growth-promoter used in poultry, was recently banned in Europe because of its association with the development of vancomycin-resistant enterococci (*24*).

Because of the direct selection pressure that antibiotics exert on organisms carrying antibiotic resistance genes (ARGs), the transport pathways of antibiotic-resistant microorganisms and the ARGs that they carry are expected to be similar to the pathways of antibiotic pharmaceuticals. In fact, it is likely that ARGs persist further in the pathway, considering that in many cases they are maintained in the microbial populations even after the antibiotic selection pressure has been removed (*25*–*28*). Also, horizontal gene transfer (HGT) is a major mechanism for sharing ARGs between microbes and has been documented to occur between nonpathogens, pathogens, and even distantly related organisms, such as Gram-positive and Gram-negative bacteria (*25*, *29*–*31*). In many cases, ARGs have been discovered to occur as part of multiple antibiotic resistant (MAR) superintegrons, which may contain over 100 ARG cassettes (*32*). These MAR superintegrons cause multiple-drug resistance in organisms, meaning that even when very different antibiotics are used, one antibiotic may coselect for resistance to other antibiotics (*5*, *33*). MAR gene cassettes and ARGs are notorious for being associated with plasmids and/or transposons that facilitate HGT. Finally, even if cells carrying ARGs have been killed, DNA released to the environment has been observed to persist, to be protected from DNAse, especially by certain soil/clay compositions, and to be eventually transformed into other cells (*34*–*36*). For all of these reasons, ARGs in and of themselves can be considered to be emerging "contaminants" for which mitigation strategies are needed to prevent their widespread dissemination.

The purpose of this study was to document the occurrence of tetracycline and sulfonamide ARGs in various environmental compartments in northern Colorado. These two ARG groups were chosen because sulfonamide and tetracycline antibiotics have been previously characterized in Poudre River sediments and shown to relate to urban/agricultural activity (*37*). The breadth of the study included Cache La Poudre (Poudre) River sediments, dairy lagoon water, irrigation ditch water, a wastewater recycling plant (WRP), and two drinking water treatment plants (DWTPs). The hypothesis was that environmental compartments most directly impacted by urban/agricultural activity would have significantly higher concentrations of ARGs than less impacted and pristine environments. Irrigation ditch waters, which were directly adjacent to farms, were investigated as a potential pathway of ARGs from farms to the Poudre River, while the WRP and the DWTPs were explored as potential routes of human environmental input and consumption. The presence/absence of several ribosomal protection factor tetracycline ARGs and folic acid pathway sulfonamide ARGs was determined using a polymerase chain reaction (PCR) detection assay, and four commonly occurring ARGs were further quantified by quantitative real-time PCR (Q-PCR). Documenting the baseline occurrence of ARGs in a cross-section of environmental compartments will take a step toward understanding and modeling the fate and transport phenomena associated with these emerging contaminants.

Experimental Section

Poudre River Sediment Sampling. Because of its pristine origins and zonation corresponding to land use, the Poudre River has served as a good model for relating human and agricultural activities with the occurrence of antibiotic pharmaceuticals (*37*) and ARGs (*38*). Five sampling sites were the focus of this study, numbered sequentially in the direction of flow from west to east, with the following characteristics: site 1, pristine location at the river origin in the Rocky Mountains; site 2, light-agriculture-influenced area; site 3, urban-influenced area at the outlet of the Fort Collins Drake WWTP; site 4, heavy-agriculture-influenced area between Fort Collins and Greeley; and site 5, heavy-agriculture- and urban-influenced area just east of Greeley, which is a major center for the meat-packing industry. Over 90 confined animal feeding operations (CAFOs), dairies, and ranches are located between sites 3 and 5. Further attributes of the Poudre River watershed that contribute to its suitability for investigating the impacts of urban and agricultural activity on antibiotics and ARGs have been described previously (*37*, *38*).

Sediment samples were collected along the Poudre River at the five sites on August 18, 2005, October 27, 2005, and February 17, 2006. The flow rates on these three dates were 1.04, 14.19, and 0.14 $m^3 s^{-1}$, respectively (U. S. Geological Survey station number 06752260, Fort Collins, CO). Sampling at three points in time provided insight into potential temporal variations in ARG concentrations, and the February 17th date is exactly 1 year later than a previously published sampling date (*38*). The upper sediments (about 5 cm) from the middle and two sides of a cross-section at each site were sampled and composited. Samples were collected using a shovel and mixed well in sterilized centrifuge tubes. Fifty-five grams of mixed sample at each site were stored at −80 °C for subsequent molecular analysis.

Bulk Water Sampling. Irrigation ditch waters were investigated as a potential pathway of ARGs from farms to the Poudre River. Grab samples of bulk water were collected in sterile containers from irrigation ditches on August 18, 2005, corresponding to the August sampling date of the Poudre River sediments. All irrigation ditches were located between site 4 and site 5 on the Poudre River within a 3.5 km × 2 km zone north of the river, and a total of ten locations were sampled. To investigate a potential source of ARGs within this zone, a microaerophillic dairy lagoon (~1 mg/L dissolved oxygen in the upper 1 m) and an anaerobic dairy lagoon (0 mg/L dissolved oxygen) from an anonymous farm located 8 km from site 5 were sampled on October 20, 2005. Finally, source water, and pre-chlorinated, and post-chlorinated bulk water were collected from two anonymous DWTPs and an anonymous WRP in northern Colorado in February, 2005. The DWTP was studied as a potential direct route of ARGs to consumers, and the WRP was considered a potential human input into the environment. To collect fine particulates from the dilute ditch water, DWTP, and WRP samples for subsequent analysis, 500 mL of well-mixed sample was filtered using a 0.45 μm glass fiber filter (Whatman). This concentration step was not required for dairy lagoon samples.

DNA Extraction. DNA was extracted from 0.5 g of composited sediment using the FastDNA Spin Kit for Soil (MP Biomedicals) and from 1.8 mL of dairy lagoon water using the Ultraclean Microbial DNA Kit (MoBio Laboratories, Inc.) according to manufacturer protocol. Both approaches employ a bead-beating procedure. For fine particulates collected on filters from bulk water, the filters were cut into small pieces and added directly to the extraction tubes. Extraction yield and the quality of the DNA were verified by agarose gel electrophoresis and spectrophotometry.

Detection and Quantification of ARGs. Polymerase chain reaction detection assays were used for broad-scale screening of the presence/absence of five ribosomal protection factor tetracycline ARGs (tet(BP), tet(O), tet(S), tet(T), and tet(W)) (*39*) and four folic acid pathway sulfonamide ARGs (sul(I), sul(II), sul(III), and sul(A)). Development and validation of sul primers was described in Pei et al. (*38*). Positive controls consisted of cloned and sequenced PCR amplicons obtained from Poudre River sediments. Both positive and negative controls were included in every run, and negative signals were confirmed by spiking positive control template into the sample to verify a signal. Forty cycles were used to improve chances of product formation from low initial template concentrations. Further details on reaction mixes and temperature programs are available in Pei et al. (*38*); note that annealing temperatures for tet primers vary from Aminov et al. (*39*). Two tetracycline ARGs (tet(W) and tet(O)) and two sulfonamide ARGs (sul(I) and sul(II)) that were commonly occurring according to the PCR presence/absence assays were further quantified by Q-PCR using a SybrGreen approach. For further details on Q-PCR methods, see Pei et al. (*38*). Eubacterial 16S rRNA genes were quantified according to the TaqMan Q-PCR method described by Suzuki et al. (*40*) so that ARGs could be normalized to the total bacterial community. This provided a means to correct for potential variations in extraction efficiencies. By quantification of 16S rRNA genes, it was also possible to compare ARGs proportionally between samples of different overall population sizes. Matrix effects associated with extraction of DNA from environmental samples were corrected for by performing spiked matrix control tests and determining template suppression factors as described in Pei et al. (*38*). All Q-PCR analyses were performed using a Cepheid SmartCycler (Sunnyvale, CA).

Statistics. The influences of the environment (sites, ditch water, and dairy lagoons) on the normalized and non-normalized copies of ARGs were analyzed using the Mixed Procedure, which fits a variety of mixed linear models to data. This provides the flexibility of simultaneously modeling means, variances, and covariances (*41*–*44*). Through the use of this test, it was thus possible to comprehensively compare overall differences between different environmental compartments with respect to ARG concentrations. For comparison of the five Poudre River sites, multiple sampling time points were treated as replicates. Mixed Procedures were conducted using SAS 9.0 (SAS Institute Inc., Cary, NC). A

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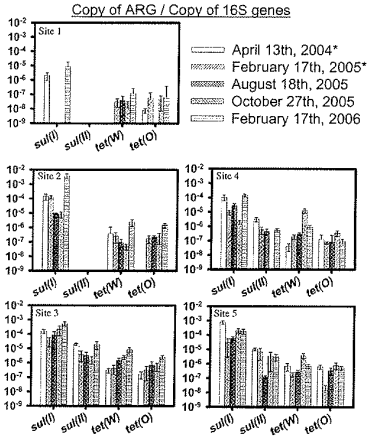

FIGURE 1. Distribution of four ARGs (sul(I), sul(II), tet(O), and tet(W)) in Poudre River sediments on three sampling dates, compared to two previously published sampling dates (April 13, 2004, and February 17, 2005 (*38*)), as determined by Q-PCR: site 1, pristine site; site 2, light agricultural activity; site 3, heavy urban activity; site 4, heavy agricultural activity; site 5, heavy urban and agricultural activity. Error bars represent the standard deviation of six measurements from three independent Q-PCR runs analyzing DNA extract from composite samples.

p-value <0.05 was considered to indicate significance. Averages and standard deviations of all data were determined using Microsoft Excel, 2003.

Results and Discussion

Occurrence of ARGs in Northern Colorado. Figure 1 summarizes the Q-PCR data obtained for the four ARGs at the five Poudre River sites, while Figure 2 summarizes the same analyses for the ditch waters and dairy lagoon water. When August 2005 data for the Poudre River sediments are compared with the dairy lagoon and ditch water, the following trend is observed with respect to ARG concentrations: dairy lagoon water > ditch water > river sediments ($p < 0.0001$), for all ARGs except sul(II), which was absent from the ditch waters. This is based on pooling of all 10 ditch water sites, the two dairy lagoons, and sites 4 and 5, which were directly adjacent to the ditch water sampling locations. Within each of these three pools, there was no statistical difference observed among the samples. Therefore, it was observed as expected that environmental compartments most directly impacted by human/agricultural activity showed higher concentrations of ARGs. This trend is even stronger in considering absolute quantities of ARGs (not normalized to 16S rRNA genes), because the concentration of cells in the dairy lagoon water was orders of magnitude higher than that of the ditch water or the sediments.

In developing a hypothetical pathway for ARGs, a trend is not as clear. The overall trend in terms of ARG concentrations of dairy lagoon water > ditch water > river sediments suggests that on-farm compartments, such as lagoons may be the source of ARGs, which are subsequently attenuated in ditch water before reaching Poudre River sediments.

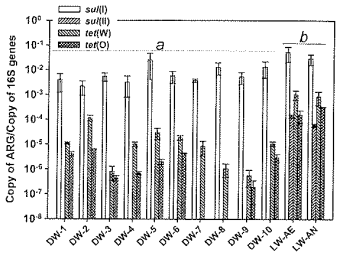

FIGURE 2. Distribution of four ARGs (sul(I), sul(II), tet(O), and tet(W)) at 10 sampling points of irrigation ditch water (DW-1—DW-10) located between site 4 and site 5 compared with that of a microaerophillic dairy lagoon (LW-AE) and an anaerobic dairy lagoon (LW-AN). DW samples were concentrated from 500 mL, and LW samples were extracted directly from 1.8 mL. All samples were normalized to the total 16S rRNA genes. Error bars represent three independent Q-PCR runs in duplicate. The labels a and b indicate that the data sets fell into two statistically different groups, according to the Mixed Procedure.

However, this trend is not supported in terms of sul(II), which is entirely absent from the ditch water and therefore cannot be the source of what is observed in the Poudre River sediments. An alternative source of the sul(II) that appears at sites 4 and 5 could instead be human inputs. This is supported by the data presented in Figure 1, in which it is observed that sul(II) is consistently present at high levels on average at site 3, which is at the point of discharge of the Drake WWTP, while consistently lower (comparing each date sampled) at site 4 (entirely absent for the October event) and equivalent or lower at site 5, which has mixed human/agricultural inputs. Because sul(II) is present in the dairy lagoon waters, it must also have agricultural sources, but it may attenuate too quickly to be transported to the ditches and subsequently to the river sediments. On the basis of this study and a previous study (*36*), it is appears that of the four ARGs quantified sul(II) is the most sensitive indicator of human/agricultural impact, and thus it is suggested that it attenuates quickly in the absence of direct inputs. The other ARGs in the Poudre River sediments at sites 4 and 5 may be of either/both human and agricultural origin, since they followed a decreasing trend from the dairy lagoon through the ditch water but were also present at site 3.

In addition to having higher concentrations of three out of four of the ARGs, the dairy lagoon water was also observed to have more different kinds of ARGs present than the irrigation ditch water according to the PCR assay (Table 1). Together with the Q-PCR results, these data further support the concept that there is some attenuation of ARGs between any linkages that may connect dairy lagoon water and irrigation ditch water. Future work should implement ARG fingerprinting/source tracking to fully characterize the potential pathways.

Temporal Variations of ARG in Poudre River Sediments. As observed in a previous study that compared a high-flow sampling point (6.8 $m^3\ s^{-1}$, April 2004) with a low-flow sampling point (0.6 $m^3\ s^{-1}$, February 2005), the ARG concentrations in the Poudre River sediments are variable with time (*38*). To better understand temporal variations in ARG concentrations, the Poudre River sediments were sampled at three additional time points and compared with the two previously published time points. The February sampling point in this study took place exactly 1 year after

TABLE 1. PCR Presence/Absence Assay of Various ARGs in Ditch (DW)[a] and Dairy Lagoon (LW) Water[b]

ARG	DW-1	DW-2	DW-3	DW-4	DW-5	DW-6	DW-7	DW-8	DW-9	DW-10	LW-AE	LW-AN	+ control
tet(BP)	–	–	–	–	–	–	–	–	–	–	–	–	+
tet(O)	+	+	+	+	+	+	–	–	+	+	+	+	+
tet(S)	–	–	–	–	–	–	–	–	–	–	–	–	+
tet(T)	–	–	–	–	–	–	–	–	–	–	+	+	+
tet(W)	+	+	+	+	+	+	+	+	+	+	+	+	+
sul(I)	+	+	+	+	+	+	+	+	+	+	+	+	+
sul(II)	–	–	–	–	–	–	–	–	–	–	+	+	+
sul(III)	–	–	+	+	+	–	–	–	–	–	+	+	+
sul(A)	–	–	–	–	–	–	–	–	–	–	–	–	+

[a] Collected August 18, 2005. [b] Collected October 20, 2005.

the previous February event. In support of the relationship between ARG concentration and relative environment impact observed above, the pristine site (site 1) consistently had the lowest average concentrations of ARGs with time, with sul(II) completely absent and no individual ARG consistently present at all five sampling times (Figure 1). When presence/absence of ARGs are compared, site 2 appears to be the next lowest in terms of overall impacts. For example, sul(II) is consistently absent at site 2, and tet(O) was absent in one of the five sampling events, whereas these genes were consistently present at sites 3, 4, and 5. In terms of ARG concentrations, tet(W) and tet(O) at site 2 were equal or less than site 3; however, these two genes were sometimes higher and sometimes lower than at sites 4 and 5. On the basis of ARG averages and presence/absence of ARGs, sites 1 and 2 were the least impacted, as expected.

When the Mixed Procedure was applied to the data, in which the time points were pooled as replicates, it was found that there was no statistical difference between the five sites for the 16S normalized data, except in the case of sul(II) ($p = 0.0117$). However, when the same test was performed with non-normalized data, it was found that sites 1 and 2 were statistically lower than sites 3, 4, and 5 in terms of sul(I) ($p = 0.00296$), sul(II) ($p = 0.0199$), and tet(O) ($p = 0.0102$). Though normalizing to 16S genes provides a comparison of ARGs as a proportion of the total population, arguably it may be the absolute quantities of ARGs that are more critical.

While spatial variations in ARGs could be fairly well-characterized, it is difficult to identify clear temporal patterns. Comparison of the two February sampling dates that were exactly a year apart provides some insight. All four genes were either the same on average for both events (tet(O) for sites 1 and 4 and sul(II) for sites 4 and 5) or higher in the 2006 event (all other genes, except sul(II) at sites 1 and 2, where it was not present) (Figure 1). This suggests the possibility that all ARGs are increasing in concentration with time. However, the trends in between these two dates do not support this. Only tet(W) and tet(O) at site 3 increase consistently with time. All remaining ARGs at the five sites either decrease before increasing (e.g., tet(W) at site 2 and sul(II) at site 3), are constant and then increase (e.g., tet(O) at site 2 and tet(W) at site 1), or increase and then decrease (e.g., tet(W) at sites 4 and 5) (Figure 1). Therefore, no clear trend was identified with time.

It was also attempted to analyze trends in the data with respect to river flow rate. This was of interest because flow rate directly relates to runoff and nonpoint source inputs, which were hypothesized in the previous study to play a role in the observed increase in the number of kinds of ARGs detected in Poudre River sediments (*38*). The October 2005 sampling date provided a second sampling date at high flow (14.9 $m^3\ s^{-1}$), compared to the previously published April 2004 high-flow sampling date (6.8 $m^3\ s^{-1}$). (All other dates were at or below 1.0 $m^3\ s^{-1}$.) Interestingly, all four ARGs increased on average at site 5 in comparing the high-flow

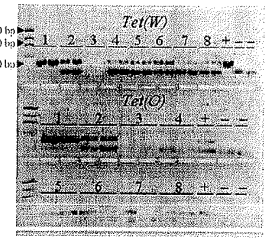

FIGURE 3. Agarose gel analysis of PCR presence/absence (in duplicate) of two ARG families, tet(W) and tet(O): + = positive control; – = negative control. The presence of a band at the same molecular weight as + indicates the presence of an ARG: 1 = WRP effluent; 2 = WRP chlorinated effluent; 3 = DWTP a influent; 4 = DWTP a treated water pre-chlorination; 5 = DWTP a treated water post-chlorination; 6 = DWTP b influent water; 7 = DWTP b treated water pre-chlorination; 8 = DWTP b treated water post-chlorination. The band appearing below 200 bp is consistent with a primer dimer.

October event with the immediately previous low-flow event in August (Figure 1). At site 4, tet(W) and tet(O) increased, but sul(II) stayed the same, and sul(I) decreased. There was no effect at all at site 3, which is affected primarily by point discharge rather than runoff, site 2, or site 1. However, attempts to plot ARG concentrations versus flow rate did not reveal any clear trend. Thus, it is still not possible to make a conclusive judgment on the effect of flow rate on ARG concentrations, though the role of nonpoint source inputs merits further investigation. To accomplish this, it would be necessary to gather more data with time/flow or monitor a much more controlled and smaller-scale system.

Wastewater Recycling Plant and Drinking Water Treatment Plants. A PCR presence/absence assay was conducted on the influent, intermediate effluent, and final effluent of two drinking water treatment plants (DWTP "a" and DWTP "b") and the pre-chlorinated and chlorinated effluent of a WRP. It was observed that both tet(W) and tet(O) were present at detectable levels in all samples except the source water for DWTP "a" (Figure 3). This indicates that the same two genes that were common in various environmental compartments in northern Colorado are also present in treated recycled wastewater and bulk drinking water. These two genes also showed a response to the level of impact; e.g., they were highest in dairy lagoon water and ditch water and lowest on average at the pristine site. On the basis of the intensity of the signal, they were also higher in the recycled wastewater than in the drinking water, as would be expected. Though these two ARGs are not directly associated with any known human pathogens, they may be indicators of links

between human/agricultural activity and ARGs in drinking water. Considering that drinking water is a direct route to human consumers, this emphasizes the need to better understand the pathways by which ARGs are spread in the environment and potential ways that the spread of ARGs may be reduced. For example, vancomycin resistance genes were found in drinking water biofilms in a recent study (*45*). Considering that vancomycin is typically the antibiotic of last resort when all else fails, this underscores the need to address this issue before it is too late. One possibility may be to make simple modifications to wastewater and drinking water treatment plants to reduce the spread of ARGs.

ARGs as Emerging Contaminants. On the basis of this study it is clear that ARGs are present in various environmental compartments, including river sediments, irrigation ditch water, dairy lagoon water, DWTPs, and a WRP. Furthermore, quantitative techniques incorporating Q-PCR provide a means to compare the concentrations of ARGs associated with the known urban and agricultural impacts, which provides a more direct measure than previous culture-based methods. On the basis of this occurrence survey, it is argued that ARGs are emerging contaminants that need to be further studied in the paradigm of environmental science and engineering. The concept of ARGs as "pollutants" has also been suggested by Rysz and Alvarez (*46*).

It should be noted that besides the tetracycline and sulfonamide ARGs that were the focus of this study, there are numerous other ARGs that have been described in the literature and likely even more that have not yet been discovered, each potentially with its own unique properties. Thus, each ARG may have different behaviors with respect to fate and transport and response to physical, chemical, and/or biological treatment. In terms of defining fate and transport characteristics of ARGs in general, it is expected that their behavior will be distinct in comparison to "typical" contaminants. For example, ARGs may be sequestered with bacteria, which are themselves transported, or they may be present as naked DNA bound to clay particles (*47*). Furthermore, ARGs may actually amplify in the environment under some conditions. This is indeed a unique contaminant property. Considering the significance of the problem of the spread of antibiotic resistance, further effort by environmental researchers to better understand these emerging contaminants is well-warranted. This is especially true as the rate of discovery and development of new antibiotics is continually declining (*48*), while the corresponding development and spread of resistance is occurring at a rapid pace. On the basis of this study, understanding ARGs as emerging contaminants can add a new and important angle to helping to approach this important problem.

Acknowledgments

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3.L-61

To: Mayor Marty Blum

Fm: Dr Edo McGowan

Re: Report on Santa Barbara's recycled water

Dt: 5-8-09

Some time ago, you suggested that I compile a report on recycled water that contained scientific citations, and then submit that report to you. Having compiled such a report, I am submitting it at this time in an administrative draft stage. The report is set up initially as a discussion. That is then followed by pertinent question sections, each accompanied by applicable abstracts pulled from the peer reviewed scientific literature. Also included are comments that I make explaining how this information may relate to the issue at hand.

We have now compiled several years of data on the local water as produced by the sewer plant at El Estero. Our original main issue was the exposure of students at SBCC to water that might carry transmissible pathogens. In 2006, we noted multi-drug resistant bacteria at the point of use (SBCC campus). This information was brought to the attention of the City. In prior years, the water had been tested by the students of Medical Microbiology Department and in these tests the water was shown to contain high levels of bacteria. Because of this history and my interest in the subject, I repeated these tests in 2006, finding bacteria coming through that were multi-antibiotic resistant (resistant to 11 of the 12 antibiotics). These tests were again run in 2007/2008 and the findings were consistent with former year's findings and again reported to the City. Thus one might conclude that previous findings were not just rare happen-stance occasions but were the actual background of the recycled water as produced by Santa Barbara. Again, this conclusion was discussed with the City. Our latest work completed in April of this year again showed high counts of bacteria being delivered to the SBCC campus, levels that far exceed safe limits, thus the students, staff, and community may be excessively exposed to some serious pathogens. As previously mentioned, Cottage which is a teaching hospital, went to vancomycin as a pre op prophylactic in about 2003 exactly because the levels of resistance were excessive. Previously, vancomycin had been held by the CDC as the drug of last resort. Thus again we see that our supply of useful tools is being squandered, perhaps through thoughtlessness, but in any event, we are now seeing increasing resistance to vancomycin. Thus it appears that we have a revolving door and the grease on the hinges may well be recycled water.

Today, as you will note we are faced with ever increasing antibiotic (antimicrobial) resistance in an increasing number of pathogens as produced in sewage treatment works and consequently released to the environment. In discussing the concept of antibiotic resistance and the management of municipal wastewater effluent, the concept of antibiotic resistance seemed to be missing. I was unable to find a single instance of the concept within state government agencies dealing with water quality. The question is why?

Comment Letter L

There have been numerous scientific studies that have looked at the standards used to "assure" water quality. Many of these studies have commented on the failure of current standards to be protective of public health. These studies date back decades. The mantra of those within the industry is, however, that they meet the current standards. Thus, while the public is not protected, the industry that is in place to assure protection of public health seems moribund to effect change. In the interim, the rates of community acquired antibiotic resistance continue to escalate.

Since this report is dealing mainly with recycled wastewater as produced under the state's standards (Title 22), the reader may wish to consider some of the uses to which this water is put.

Current uses include sprinkler irrigation of fruit and vegetable crops consumed raw. The uses also include sprinkler irrigation of various greenscapes such as lawns, school playing fields, golf courses, and road mediums. For the most part these are high public-contact areas. In addition, this water is used for cooling towers, including cooling towers on large buildings such as hospitals. Cooling towers are, by their nature, major generators of aerosols. Another use is for fire fighting, again a system where aerosols are generated but often within in confined conditions. Aerosols can initiate surface contamination that can set up colonized niches of pathogens. Once surfaces are colonized, this allows for later transfer to susceptible hosts. Surfaces can remain contaminated with viable pathogens for months (see Ellis below).

Sewer plants can not easily deal with materials in solution, thus these solutions pass through the system and into the environment. The USGS has documented the rising levels of pharmaceuticals in our waterways. Klaus Kummerer in Germany, Kate Brown and Chad Kinney here in the US have looked at this and indicate that the levels found down stream from sewage outfalls are sufficient to either maintain resistance or initiate it. Stu Levy notes that once resistance is gained, the average resistant bug does not revert. Thus terrestrial niches are developed and these are lending libraries for later lateral transfer. Municipal wastewater effluent, especially when receiving effluent from hospitals and other facilities that treat the very sick, is the source of much of this.

Many of these current pathogens were former commensals that, through the transfer of genetic information, have gained the status of pathogen. Sewer plants as currently designed and operated, the standards under which they operate, and the staff that operate them, have been shown to be incapable stemming the tide of released pathogens into the open environment. Part of the problem is that the legislative systems do not recognize this problem and the bureaucratic systems either do not have the capacity to deal with it or are not sufficiently energized or trained to be proactive. This, notwithstanding the fact that development of antibiotic resistance within sewer plants is well known. Amy Pruden's work for which she recently received the highest US civilian national award from the President, demonstrated that the genetic fragments, antibiotic resistant genes (ARGs) which are developed within sewer plants pass to the environment and in spite of chlorination, are subsequently picked up in fresh water intakes, pass

through drinking water treatment (chlorination and filtration) and end in the potable water supply.

Amy Pruden, Joan B Rose, and I were recently on a WERF/US EPA expert team looking at this subject. In my work here in California, we also follow this movement into potable water. In two major pharmacies in our town, water was tested. This was presumed sterile water used to mix prescription drugs. Testing demonstrated that this water had acquired antibiotic resistance but the pharmacies were ignorant of this until it was tested. The bugs were resistant to all challenges in the Kirby Bauer screen except neomycin, i.e., 11 of 12 antibiotics.

Contrary to popular myth sewer plants fail to effectively deal with pathogens, including their genetic material and the transfer of resistance and virulence. In fact sewer plants augment resistance and virulence transfer between organisms. These bugs, absent being commingled within sewer plants, would probably never get together. Sewer plants, although originally designed to only treat human waste (sewage), have been overcome with other materials that they were never designed to treat. The incremental addition to these materials over time and the bracket creep within policies and regulations controlling allowed materials now comprises a daunting list that arrives at sewer plants to confound these treatment designs. Many of the emerging contaminants that one reads about are sewered in abundance by industry.

To deal with this from a political perspective, the regulatory community has ignored these impacts, assuming at first that such incremental additions could be treated by the old adage that "dilution is the solution to pollution". This may have been acceptable as an interim solution during the now by-gone era when rivers and the ocean seemed to have unlimited absorptive capacities. That is physically no longer the case but political inertia is difficult to overcome. Additionally, many of the regulatory agencies that were established to deal with this issue cut their teeth on a chemical pollutant basis and their engineers are steeped within that narrower thought process. Unfortunately, what we are facing is now a major biological, not chemical issue. That issue is antibiotic resistance and increased virulence. Thus, dilution, while it may have momentarily worked for chemical pollutants does not work well for pollutants that have the capacity for vast multiplication and adaptation to environmental niches.

Consequently, in general, the North American Continent's recent crisis in antibiotic resistance may be traced, in part, to improperly treated sewage and its byproducts. The byproducts of municipal sewage are serious potential risks to public health.

So where do we find these sewage byproducts? There are two major byproducts produced from the treatment of sewage. The solids are separated from the wastewater. The solids, called sewage sludge or biosolids, are generally put onto agricultural land or sprayed on irrigated pastureland with the animals being excluded in the US only for 30 days. Many pathogens survive in nature well beyond 30 days, but that is the current US regulatory limit. Dairy pasture is one such use for this "top-dressed" sewage sludge. The wastewater that is separated from the solids is further treated and either released to a river, the ocean, or recycled and put onto crops consumed raw, lawns, school playing fields and golf courses, or used for cooling or fire fighting.

Comment Letter L

We tested some of this recycled water produced in California under state criteria. These criteria are produced by the California Department of Health Services, as found in the California regulations under Title 22. This is recycled water that is tertiary treated and chlorinated prior to release. What we found when we ran tests on this finished water on Mueller-Hinton plates through the Kirby Bauer disc diffusion, was multi-drug resistance, in one case resistance to 11 of the 12 test materials. The tests were preliminary. We noted serratia-like and pseudomonas-like bacteria that were obviously also resistant to chlorine. We tested water from two separate sewer districts. We attempted to test a third district source that uses the water to spray irrigate strawberries and broccoli. When we stated why we wanted to test this water we were promptly refused and immediately handed off to the district's legal counsel.

Since 2003 or so, vancomycin has been used pre-operatively as a routine prophylactic at our local teaching hospital because the background of resistance in the area is so high. The interesting thing here is that one of the test antibiotics used in our study was vancomycin and the bugs were resistant to it.

Now if the recycled water is going out onto public parks and other areas with high human contact, is this an added risk for picking up resistance? Taking a precautionary approach here would seem prudent.

In a recent meeting of our task-group, one of the members, a well-respected wastewater engineer, raised the compelling question relating to land application of sewage byproducts. The essence of the question related to the survival of pathogens, hence the underlying issue of surviving multi-drug resistance. 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 classic persister? In either of these survival states, the standard tests demanded by the state would not pick them up. Further, this says nothing for uptake of released naked DNA (see for example Pruden below). Actually knowledge of the ability to transfer pathogenic traits from dead bacteria to non-pathogens has been known since the work by Griffith in 1928. Griffith took heat-killed bacteria and introduced them into test animals. The animals did not become ill. He then took the same heat killed pathogens and blended them with non pathogens and the genetic information that allowed for pathogenesis was transferred and the animals died. As recently reported by Higgins and Murthy in their study for the wastewater industry (WERF 2006), the issue of VBNC completely misses the bulk of the indicator bacteria. In testing biosolids that were dewatered by centrifuge, material that had passed standard tests just 20 minutes prior to dewatering showed viable bacterial counts that were up several magnitudes.

Biosolids in the US are allowed to legally contain 2-million viable coliform per gram. But these are vegetative bacteria that are easily killed by low-level disinfection. If the 2-million easily killed bacteria per gram are allowed to survive, what of the more robust organisms that require high-level disinfection such as those found on semi-critical medical devices? There is no test for these high-level disinfection-requiring bacteria. Thus, it appears that the standards by ignoring these issues present a fictional picture of safety. The same or similar argument may be made for recycled wastewater. A certain

level of viable indicator is allowed. Again, these indicators are vegetative bacteria requiring only low-level disinfection.

Additionally, during the above noted meeting, I had mentioned a continuing medical education Grand Rounds lecture at the teaching hospital where a speaker from UCLA had noted that that there is now strong medical evidence that about ½ of the non-hospital but community acquired skin infections in the Greater Los Angeles area are now MRSA.

For example, Stuart Levy found that the resistance in gut bacteria of cattle moved to gut bacteria of mice having access to the same area, then from the mice to pigs, to chickens, and finally to flies, then the kitchen. He notes a Dutch study that followed bacteria from animals to the human food chain and entered the consumer's kitchen. In other cited examples, he noted the distinct relationship between multi-drug resistant bacteria in animals and thence to humans attending them, even though the humans used no antibiotics or ate the animals. Levy's work is not new. (Levy SB, MD. *The Antibiotic Paradox*. New York, Plenum Press 1997). Rusin and Gerba have written on the transfer of pathogens from common household surfaces via finger to mouth. Gerba in unpublished work has noted the ease in which contamination can be spread within a home. Others have discussed dust as a carrier of viable pathogens. Gerba has written extensively on the movement of pathogens in sediment, their protection for long periods within sediments and their re-transport as viable pathogens. The NRC in its 2002 report on sewage sludge admonished EPA to look at off-site movement and resistance. There is no evidence that this re-analysis has taken place, yet the World Health Organization has raised the subject of resistance to a Global crisis. The WERF/EPA team that I was on was only recently assigned this task but it has been in excess of five years to get to this point.

THE PROBLEM---DOES RECYCLED EFFLUENT WATER REPRESENT A POTENTIAL PUBLIC HEALTH RISK?

Validity of the Indicator Organism Paradigm for Pathogen Reduction in Reclaimed Water and Public Health Protection†

Valerie J. Harwood,1* Audrey D. Levine,2 Troy M. Scott,3 Vasanta Chivukula,1 Jerzy Lukasik,3 Samuel R. Farrah,4 and Joan B. Rose5

Appl Environ Microbiol. 2005 June; 71(6): 3163–3170.

The validity of using indicator organisms (total and fecal coliforms, enterococci, Clostridium perfringens, and F-specific coliphages) to predict the presence or absence of pathogens (infectious enteric viruses, Cryptosporidium, and Giardia) was tested at six wastewater reclamation facilities. Multiple samplings conducted at each facility over a 1-year period. Larger sample volumes for indicators (0.2 to 0.4 liters) and pathogens (30 to 100 liters) resulted in more sensitive detection limits than are typical of routine monitoring. Microorganisms were detected in disinfected effluent samples at the following frequencies: total coliforms, 63%; fecal coliforms, 27%; enterococci, 27%; C. perfringens, 61%; F-specific coliphages, ~40%; and enteric viruses, 31%. Cryptosporidium oocysts and Giardia cysts were detected in 70% and 80%, respectively, of reclaimed water samples. Viable Cryptosporidium, based on cell culture infectivity assays, was detected in 20% of the reclaimed water samples. No strong correlation was found for any indicator-pathogen combination. When data for all indicators were tested using discriminant analysis, the presence/absence patterns for Giardia cysts, Cryptosporidium oocysts, infectious Cryptosporidium, and infectious enteric viruses were predicted for over 71% of disinfected effluents. The failure of measurements of single indicator organism to correlate with pathogens suggests that public health is not adequately protected by simple monitoring schemes based on detection of a single indicator, particularly at the detection limits routinely employed. Monitoring a suite of indicator organisms in reclaimed effluent is more likely to be predictive of the presence of certain pathogens, and a need for additional pathogen monitoring in reclaimed water in order to protect public health is suggested by this study.

Comment Letter L

PATHOGENS IN RECLAIMED WATER

M. V. Yates

University of California Riverside

The need to conserve water has resulted in an increase in the use of treated sewage effluent, or reclaimed water, for many non-potable purposes. However, reclaimed water may contain potentially harmful contaminants with which the user must be familiar in order to minimize detrimental environmental or human health effects. The focus of this paper is on human pathogenic (disease-causing) microorganisms that may be present in reclaimed water.

Antibiotic-Resistance DNA Showing Up in Drinking Water

Thursday, November 02, 2006

By Charles Q. Choi

DNA that helps make germs resistant to medicines may increasingly be appearing as a pollutant in the water.

This was found "even in treated drinking water," researcher Amy Pruden, an environmental engineer at **Colorado State University** in Fort Collins, told.

The spread of this DNA could exacerbate the already growing problem of drug resistance among potentially infectious microbes.

McGowan's comment---Amy and I are on a WERF team looking at this for US EPA. She indicates that the genetic fragments are not affected by the chlorine treatment nor stopped by the typical filters used by both wastewater and drinking water treatment plants. Thus these fragments (ARGs) make it into the potable water supply. If that is the case there is no reason to assume that they are also not within tertiary treated and chlorinated recycled water. In fact we recently ran this water and found profoundly resistant bacteria from the serratia-like and pseudomonas-like groups. Both of these groups contain some serious pathogens.

QUESTION----ONCE APPLIED TO PUBLIC AREAS SUCH AS PARKS, CAN THE PUBLIC PICK UP THESE MICROBES?

Significance of Fomites in the Spread of Respiratory and Enteric Viral Disease

Stephanie A. Boone* and Charles P. Gerba

Applied and Environmental Microbiology, March 2007, p. 1687-1696, Vol. 73, No. 6

Worldwide annually there are 1.7 million deaths from diarrheal diseases and 1.5 million deaths from respiratory infections (56). Viruses cause an estimated 60% of human infections, and most common illnesses are produced by respiratory and enteric viruses (7, 49). Unlike bacterial disease, viral illness cannot be resolved with the use of antibiotics. Prevention and management of viral disease heavily relies upon vaccines and antiviral medications (49). Both vaccines and antiviral medications are only 60% effective (39, 49). Additionally, to date there are no vaccines or antiviral drugs for most common enteric and respiratory viruses with the exception of influenza virus and hepatitis A virus (HAV). Consequently, viral disease spread is most effectively deterred by preclusion of viral infection.

McGowan's comment here----------These findings are critical to the argument that if the pathogens are getting through in recycled water to human hosts or to vectors that have contact to humans (pets) then merely tracking antibiotic resistance is not enough. As noted, viruses are another issue, the flip side of the same coin.

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Comparative surface-to-hand and fingertip-to-mouth transfer efficiency of gram-positive bacteria, gram-negative bacteria, and phage.

Rusin P, Maxwell S, Gerba C.

University of Arizona, Department of Soil and Water Science, Building 38, Tucson, Arizona 85721 USA. rusin@ag.arizona.edu

AIMS: To determine the transfer efficiency of micro-organisms from fomites to hands and the subsequent transfer from the fingertip to the lip. METHODS AND RESULTS: Volunteers hands were sampled after the normal usage of fomites seeded with a pooled culture of a Gram-positive bacterium (Micrococcus luteus), a Gram-negative bacterium (Serratia rubidea) and phage PRD-1 (Period A). Activities included wringing out a dishcloth/sponge, turning on/off a kitchen faucet, cutting up a carrot, making hamburger patties, holding a phone receiver, and removing laundry from the washing machine. Transfer efficiencies were 38.47% to 65.80% and 27.59% to 40.03% for the phone receiver and faucet, respectively. Transfer efficiencies from porous fomites were <0.01%.

In most cases, M.luteus was transferred most efficiently, followed by phage PRD-1 and S. rubidea. When the volunteers' fingertips were inoculated with the pooled organisms and held to the lip area (Period B), transfer rates of 40.99%, 33.97%, and 33.90% occurred with M. luteus, S. rubidea, and PRD-1, respectively. CONCLUSIONS: The highest bacteral transfer rates from fomites to the hands were seen with the hard, non-porous surfaces. Even with low transfer rates, the numbers of bacteria transferred to the hands were still high (up to 10(6) cells). Transfer of bacteria from the fingertip to the lip is similar to that observed from hard surfaces to hands. SIGNIFICANCE AND IMPACT OF THE STUDY: Infectious doses of pathogens may be transferred to the mouth after handling an everyday contaminated household object.

McGowan's comments---------How does this stack up against something dropped on the grass of a public park that was irrigated with recycled water? Assume that you and your family are sitting on the park's grass and your three year old daughter drops her lollipop. She reaches down and begins to suck on it again. What is the result, her immune system is not yet developed?

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Fomites and Infection Transmission

Kris Ellis 11/07/2006

In a systematic review of the literature, German researchers explored the ability of infectious organisms to survive on inanimate surfaces.1 They found that most gram-positive bacteria, including vancomycin-resistant enterococcus (VRE), methicillin-resistant *Staphylococcus aureus* (MRSA), and *Streptococcus pyogenes* can survive for months on dry surfaces. "In general, there was no obvious difference in survival between multiresistant and susceptible strains of *Staphylococcus aureus* and *Enterococcus spp.*," the authors write. "Only in one study was such a difference suggested, but the susceptible strains revealed a very brief survival as such. Many gram-negative species, such as *Acinetobacter spp.*, *Escherichia coli*, *Klebsiella spp.*, *Pseudomonas aeruginosa*, *Serratia marcescens,* or *Shigella spp.* can survive on inanimate surfaces even for months. These species are found among the most frequent isolates from patients with nosocomial infections. A few others, such as *Bordetella pertussis*, *Haemophilus influenzae*, *Proteus vulgaris*, and *Vibrio cholerae*, however, persist only for days. Mycobacteria — including *Mycobacterium tuberculosis* and spore-forming bacteria, including *Clostridium difficile* — can also survive for many months on surfaces."

Overall, the review noted that gram-negative bacteria persist for longer periods of time than gram-positive bacteria. Climactic factors can play a role in persistence as well — humid conditions were found to increase survival times for most types of bacteria, such as *Salmonella typhimurium*, *Pseudomonas aeruginosa*, and *Escherichia coli*. Only *Staphylococcus aureus* was found to persist longer at lower humidities.

McGowan's comment-----So, the above was mainly a one time application of contaminants to surfaces. But the critical issue is that recycled water is applied, then reapplied in that week followed by continued application on a periodic basis. How does that then play into the risk equation?

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QUESTION---IS ANTIBIOTIC RESISTANCE MERELY A PASSING THING?

The impact of antibiotic use on resistance development and persistence.

Barbosa TM, Levy SB.

the Departments of Molecular Biology, Microbiology, Center for Adaptation Genetics Drug Resistance

The intense use and misuse of antibiotics are undoubtedly the major forces associated with the high numbers of resistant pathogenic and commensal bacteria worldwide. Both the volume and the way antibiotics are applied contributes to the selection of resistant strains. Still, other social, ecological and genetic factors affect a direct relationship between use and frequency of resistance. Resistant bacteria, following their emergence and evolution in the presence of antibiotics, appear to acquire a 'life of their own'. They proliferate and maintain the resistance traits even in the absence of antibiotics, thus jeopardizing the reversal of bacterial resistance by simple reduction in antibiotic use. Reversing resistance requires restoration of the former susceptible flora in people and in the environment. Copyright 2000 Harcourt Publishers Ltd.

PMID: 11498398 [PubMed - as supplied by publisher]

3.1-66

Dissemination of Multidrug-Resistant Bacteria into the Arctic

Maria Sjölund,*

Abstract We show that *Escherichia coli* isolates originating from Arctic birds carry antimicrobial drug resistance determinants. This finding implies that dissemination of drug-resistant bacteria is worldwide. Resistance genes can be found even in a region where no selection pressure for resistance development exists.

Bacteria display a unique ability to adapt to changes in their environment and to develop mechanisms to protect themselves against toxic compounds. Their ability to develop resistance mechanisms to antimicrobial drugs has assumed catastrophic proportions, rendering more and more infections difficult or impossible to treat (*1*). Most reports suggest that the main force behind emergence of drug resistance is the use and misuse of antimicrobial drugs during the past few decades, but there is also evidence for the epidemic spread of drug-resistant bacteria as a contributing factor (*2*). EID Journal Home > Volume 14, Number 1–January 2008 Volume 14, Number 1–January 2008

Persistence of Resistant *Staphylococcus epidermidis* after Single Course of Clarithromycin

Comment Letter L

Maria Sjölund,

> We examined how a common therapy that includes clarithromycin affects normally colonizing *Staphylococcus epidermidis*. Samples from the nostrils of 5 patients receiving therapy were collected before, immediately after, 1 year after, and 4 years after treatment. From each patient and sample, *S. epidermidis* strains were isolated and analyzed for clarithromycin susceptibility and presence of the *erm*(C) gene. We show that macrolide-resistant strains of *S. epidermidis* were selected during therapy and that the same resistant strain may persist for 4 years, in the absence of further antimicrobial treatment.

McGowan's comments------As noted above, once transferred to the human flora, the transfer of genetic information allowing for antibiotic resistance can be maintained for long periods. This then allows later sharing with an incoming pathogen and the result may later be an infection that can not be stopped. It is like running around with tiny time-bombs within one's guts. I am often asked—"where are the dead bodies?" Well, today, antibiotic resistance kills more people that AIDS. Look at those with cystic fibrosis (CF) and an antibiotic resistant infection. What happens when a tourist guest comes to Santa Barbara and that tourist has CF but currently no resistant bacteria in the lung? Remember, in our lab tests of recycled water coming out of El Estero, we did find Pseudomonas-like colonies that were resistant to 11 of the 12 antibiotics.

High Frequency of Hypermutable *Pseudomonas aeruginosa* in Cystic Fibrosis Lung Infection

Antonio Oliver, Rafael Cantón, Pilar Campo, Fernando Baquero, * Jesús Blázquez *

The lungs of cystic fibrosis (CF) patients are chronically infected for years by one or a few lineages of *Pseudomonas aeruginosa.* These bacterial populations adapt to the highly compartmentalized and anatomically deteriorating lung environment of CF patients, as well as to the challenges of the immune defenses and antibiotic therapy. These selective conditions are precisely those that recent theoretical studies predict for the evolution of mechanisms that augment the rate of variation. Determination of spontaneous mutation rates in 128 *P. aeruginosa* isolates from 30 CF patients revealed that 36% of the patients were colonized by a hypermutable (mutator) strain that persisted for years in most patients. Mutator strains were not found in 75 non-CF patients acutely infected with *P. aeruginosa*. This investigation also reveals a link between high mutation rates in vivo and the evolution of antibiotic resistance. *cience* 19 May 2000: Vol. 288. no. 5469, pp. 1251 - 1253

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QUESTION----HOW FAST CAN ANTIBIOTIC RESISTANCE DEVELOP?

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].

Schentag, et al. (2003), in Walsh, 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 10/5TH to 10/3RD, 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 these patients harbored NO resistant bacteria in their nasal cavities upon entry to the hospital. But what would be the result if there had been inadvertent acquisition of resistance from environmental contamination such as through wastewater byproducts? Gerba and Rusin [9] conducted research about the passage from finger to mouth of pathogens found on typical household objects. Others have documented dust as a mechanical vector for pathogens. Thus what of the dwelling down wind sprinkler application of recycled wastewater, from land application of sewer sludge, or from a sewage sludge composting facility? Gerba and others have written extensively about the survival of pathogens and their viable infectivity once they are absorbed onto sediments [10]. Anyone who lives in an agricultural area knows that tillage and wind cause large movements of soil and 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 [11].

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

Comment Letter L

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.

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[10] Rusin P, et al. Comparative surface-to-hand and fingertip-to-mouth transfer

efficiency of gram-positive bacteria, gram-negative bacteria, and phage. *J Appl Microbiol.* 2002;93(4):585-92; See also: Shivi Selvaratnam and J. David Kunberger. Increased frequency of drug-resistant bacteria and fecal coliforms in an Indiana Creek adjacent to farmland amended with treated sludge. Can. J. Microbiol./Rev. can. microbiol. 50(8): 653-656 (2004).

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QUESTION---IS AERIAL DRIFT FROM SEWAGE BYPRODUCTS AND SPRINKLER IRRIGATION OF EFFLUENT LIKELY TO PRESENT A PUBLIC HEALTH RISK?

There are two sources under discussion here. The spray irrigation of treated effluent and the sewer plant it self. Both have been shown to be significant sources of aerosol generation.

Pathogen distribution in waste water sprinkler irrigation]

Zentralbl Bakteriol Mikrobiol Hyg [B]. 1984 May;179(2):151-61.

Ahmed RE, Geuenich HH, Müller HE.

The spray irrigation with pretreated wastewater was investigated on the sewage farm of the Braunschweig Sewage Utilization Association. The emission of airborne bacteria was measured by means of Andersen sampler (AS), Reuter centrifugal sampler (RCS), and sedimentation plates (SP). There was a good correlation of results obtained by parallel measurings of AS and RCS. The RCS was more effective by the factor 11.5 than the AS sampling airborne microorganisms. However, the AS gaves the distribution curve of differently big airborne particulates (Table 1). The medium decrease of bacteria from the sprinkler determined by AS and SP was similar (Fig. 1). With low wind velocity, an aerosol containing enterobacteria was yet detectable at a distance of 60-160 m down-wind from the spray sprinkler. The transport of bacteria as a function of the wind velocity is given in Fig. 2 increasing about 25 m for an increase of the wind velocity of 1 m/sec.

Comment Letter L

During the spray irrigation, the composition of bacteria in the airborne particles is varying continuously. The following order of succession of die-away rates was found: Aeromonas, Plesiomonas, Vibrio greater than Acinetobacter, Pseudomonas greater than Enterobacter greater than Citrobacter greater than E. coli greater than Klebsiella greater than gram-positive bacteria (Table 3). Only seldom and under extreme conditions, gram-negative bacteria were detected in a range between 200-300 m beyond of concentrations as they were found also in controls without irrigation. This result substantiates a minimum protective distance of 300 m between sprinkler and human settlements.

PMID: 6377753 [PubMed - indexed for MEDLINE]

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Comparison of coliphage and bacterial aerosols at a wastewater spray irrigation site.

Bausum HT, Schaub SA, Kenyon KF, Small MJ.

Microbiological aerosols were measured on a spray irrigation site at Fort Huachuca, Ariz. Indigenous bacteria and tracer bacteriophage were sampled from sprays of chlorinated and unchlorinated secondary-treatment wastewaters during day and night periods. Aerosol dispersal and downwind migration were determined. Bacterial and coliphage f2 aerosols were sampled by using Andersen viable type stacked-sieve and high-volume electrostatic precipitator samplers. Bacterial standard plate counts averaged 2.4 x 10(5) colony-forming units per ml in unchlorinated effluents. Bacterial aerosols reached 500 bacteria per m3 at 152 m downwind and 10,500 bacteria per m3 at 46m. Seeded coliphage f2 averaged 4.0 x 10(5) plaque-forming units per ml in the effluent and were detected 563 m downwind. Downwind microbial aerosol levels were somewhat enhanced by nighttime conditions. The median aerodynamic particle size of the microbial aerosols was approximately 5.0 micrometer. Chlorination reduced wastewater bacterial levels 99.97% and reduced aerosol concentrations to near background levels; coliphage f2 was reduced only 95.4% in the chlorinated effluent and was readily measured 137 m downwind. Microbiological source strength an meteorological data were used in conjunction with a dispersion model to generate mathematical predictions of aerosol strength at various sampler locations. The mean calculated survival of aerosolized bacteria (standard plate count) in the range 46 to 76 m downwind was 5.2%, and that of coliphage f2 was 4.3 %.

PMID: 7055376 [PubMed - indexed for MEDLINE]

To get a feeling for the level of data easily available on the web relating aerosols and sewer plants, the following is presented from a Google Scholar key word search:

Results 1 - 10 of about 6,260 for aerosols + sewer plants.

Results 1 - 10 of about 2,090 for aerosols + wastewater + disease.

http://digitalcommons.ilr.cornell.edu/cgi/viewcontent.cgi?article=1001&context=manuals

Health Hazard Manual S WORKERS - digitalcommons.ilr.cornell.edu ... Chicago,

Illinois; and Memphis, Tennessee (Cincinnati group included **sewer** maintenance workers **...** with wastewater and/or sludge and exposure to bacterial **aerosols**. **...** View as HTML - Web Search

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Science 18 September 1970: Vol. 169. no. 3951, pp. 1218 - 1220 DOI: 10.1126/science.169.3951.1218

Coliform Aerosols Emitted by Sewage Treatment Plants

A. Paul Adams 1 and J. Clifton Spendlove 1

1 Deseret Test Center, Fort Douglas, Utah 84113

Development of the science of aerobiology has furnished a tool for the investigation of potential sources of microbial aerosols. An investigation of aerosols emitted by trickling-filter sewage treatment plants revealed that coliforms were indeed emitted and have been sampled to a distance of 0.8 mile (1.2 kilometers) downwind. Factors affecting survival of Escherichia coli *are presented.*

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Health effects among workers in sewage treatment plants

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OBJECTIVES: To further assess the presence of fatigue, symptoms of diarrhoea, and inflammation of airways among people working in sewage plants and the relation to airborne bacterial endotoxin at the workplace. METHODS: 34 Employees in sewage treatment plants and 35 controls were selected. They underwent a questionnaire investigation, and spirometry and airway responsiveness were measured. Measurements were made of airborne endotoxin at different workplaces. RESULTS: The amount of airborne endotoxin varied between 3.8 and 32,170 ng/m3. Workers reported significantly higher nose irritation, tiredness, and diarrhoea. Airway responsiveness was increased among sewage workers, but no differences between the groups were found for spirometry. CONCLUSIONS: The results confirm previous studies on the presence of airways and intestinal inflammation among workers in sewage treatment plants. The most likely causative agent is endotoxin, and at 14 of 23 workplaces, concentrations exceeded recommended guidelines. Occupational and Environmental Medicine, Vol 56, 354-357

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Appl Environ Microbiol. 1985 May; 49(5): 1191-1196

Effect of an activated sludge wastewater treatment plant on ambient air densities of aerosols containing bacteria and viruses.

K F Fannin, S C Vana and W Jakubowski

ABSTRACT

Bacteria- and virus-containing aerosols were studied during the late summer and fall seasons in a midwestern suburb of the United States before and during the start-up and operation of an unenclosed activated sludge wastewater treatment plant. The study showed that the air in this suburban area contained low-level densities of indicator microorganisms. After the plant began operating, the densities of total aerobic bacteria-containing particles, standard plate count bacteria, total coliforms, fecal coliforms, fecal streptococci, and coliphages increased significantly in the air within the perimeter of the plant. Before plant operations, bacteria were detected from five genera, Klebsiella, Enterobacter, Serratia, Salmonella, and Aeromonas. During plant operations, the number of genera identified increased to 11. In addition to those genera found before plant operations, Escherichia, Providencia, Citrobacter, Acinetobacter, Pasteurella, and Proteus, were also identified. Enteric viruses were detected in low densities from the air emissions of this plant. Only standard plate count bacteria remained at significantly higher than base-line densities beyond 250 m downwind from the center of the aeration tanks. Fecal streptococci and coliphages appeared to be more stable in aerosols than the other indicator microorganisms studied. In general, the densities of microorganism-containing aerosols were higher at night than during the day. The techniques used in this study may be employed to establish microorganism-containing aerosol exposure during epidemiological investigations.

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Detection of coliphages and enteroviruses in sewage and aerosol from an activated sludge wastewater treatment plant.**Carducci A**, **Arrighi S**, **Ruschi A**. Lett Appl Microbiol. 1995 Sep;21(3):207-9.

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Health Serv Rep. 1973 Aug–Sep; 88(7): 640–652.

Copyright notice

Emission of microbial aerosols from sewage treatment plants that use trickling filters.

G D Goff, J C Splendlove, A P Adams, and P S Nicholes

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Pontiac fever at a sewage treatment plant in the food industry.

Gregersen P,
Grunnet K,
Uldum SA,
Andersen BH,
Madsen H.

Department of Occupational Medicine, Koge Hospital, Denmark.

BACKGROUND AND OBJECTIVES: During a hot and humid summer period workers became ill with fever and flu-like symptoms after repairing a decanter for sludge concentration at a sewage treatment plant. The work took place over a period of 10 days in a small closed room, while another decanter was in operation and was consequently

emitting aerosol to the environment, to which the workers were exposed. The aim of this study was to determine the cause of this outbreak of febrile illness so that additional cases could be prevented. METHODS: All 5 patients were seen and examined in the Department of Occupational Medicine. Furthermore 2 of the workers had recurrent illness and were examined during hospitalization. As Pontiac fever (nonpneumonic legionellosis) was suspected, antibodies to legionellae were measured in blood samples. After positive antibody titers to Legionella pneumophila were found, samples of the sludge were collected for legionellae culture. RESULTS AND CONCLUSIONS: The clinical picture agreed with that described for Pontiac fever, and positive antibody titers to L. pneumophila serogroup 1 were found in blood from all 5 patients. L. pneumophila serogroup 1 was cultured in high amounts from sludge from the decanter. It was concluded that the fever was caused by L. pneumophila emitted to the environment by the uncovered decanter. Procedures for preventing new cases were established.

PMID: 10450782 [PubMed - indexed for MEDLINE]

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... impact of microbial **aerosols** generated by wastewater treatment **plants** utilizing different aeration ... - all 7 versions » G Brandi, M Sisti, G Amagliani - Journal of Applied Microbiology, 2000 - Blackwell Synergy **...** generated by municipal wastewater treatment **plants** operating with **...** found between the quantity of sewage treated and the entities of microbial **aerosol** dispersion **...** Cited by 11 - Related Articles - Web Search - BL Direct

McGowan's comments---------It should be somewhat clearer now that aerosols are involved with the dissemination of pathogens, that sewer plants may act as generators of aerosols, and that sprinkler irrigation systems may also act as generators of aerosols. Thus, this information should also feed into disaster preparedness thinking, especially if a serious easily distributable pathogen were to hit Santa Barbara. With the swine flu topic currently under consideration, the local decision-making bodies need to be aware of this. As a suggestion, much might be accomplished by merely taking large sheets of plastic and covering open aerosol-generating systems and turning off the recycled water going to the sprinklers. A longer-term approach might be to sub-irrigate areas now irrigated with sprinklers and of course, cleaning up the recycled water prior to its release. Interestingly, Goleta San does a far better job of cleaning up its recycled water. In April of 2009 (this year) I again ran bacterial tests on the recycled water coming out of both Goleta and Santa Barbara. At the plant, samples taken, using a PR lactose/Durham tube most probable number three set, we found the following: Santa Barbara recycled water—-no growth; Goleta water—--no growth. However, at the sprinkler head, for Santa Barbara the result was 3/3/3, which is technically off the chart (the cgart goes up to 3/3/2. The 95% confidence limit for a 3/3/3 shows that bacterial counts could be as high as 4800 cfu/100 ml---way outside of the safe limits.

Goleta on the other hand was producing 0/0/0 at the plant and at the sprinkler head some miles distant, 3/0/0, or a MPN of 28, which would be the upper limit of a one-time

reading. Thus the question that should be ask, why this difference and does the staff of Santa Barbara need to discuss processing with the staff at Goleta? Actually, this is not a new suggestion because in 2006 and again in 2007, our findings were essentially the same and the suggestion that El Estero staff communicate with Goleta was made at that time. In discussions with Goleta, I am informed that Santa Barbara did not contact Goleta. The question might thus be ask, why? It seems that this discussion needs to go on because Santa Barbara is not doing a good job of protecting its citizens.

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QUESTION---DOES TIME OF DAY AFFECT RISK FOR SPRINKLER IRRIGATION?

Airborne enteric bacteria and viruses from spray irrigation with wastewater.

Teltsch B, Katzenelson E.

The relationship between bacterial concentrations in wastewater used for spray irrigation and in the air was examined. Aerosolized coliforms were detected when their concentration was 10(3)/ml or more in the wastewater. Relative humidity and solar irradiation appeared to affect viable bacteria in the air; a positive correlation was found between relative humidity and the number of aerosolized bacteria. The correlation between solar irradiation and bacterial level, on the other hand, was negative. During night irrigation, up to 10 times more aerosolized bacteria were detected than with day irrigation. Wind velocity did not play an important role in the survival of aerosolized bacteria. Echovirus 7 was isolated in 4 out of 12 air samples collected 40 m downwind from the sprinkler.

PMID: 345967 [PubMed - indexed for MEDLINE]

By definition, an aerosol is able to remain in suspension for prolonged periods because of its low settling velocity. The energy supplied by aeration of sewage, especially when the overlying air is cold, may see the mist rise several meters. For spherical particles of unit density the settling time for a 3-M fall is noted in the table below. From this, considering the size of both bacteria and viruses and aerosol generation from large open systems, it will be noted that aerosol movement is considerable. Remember that the average bacteria is 1 uM and a virus about 1/00 of that.

TABLE*

Assumptions: 5 mph average wind speed, laminar flow. The assumptions would be upset within an urban setting with buildings, up-currents, and turbulence from traffic.**

Particle Diameter.....Settling Time.....Distance at wind speed 5 mph

100 uM.....10 sec.....44 ft

20 uM.....4 minutes.....1780 feet

10 uM.....17 minutes.....7480 feet (1.4 miles)

5 uM.....62 minutes.....approx 5 miles

< 3uM.....These essentially will not settle.

* Adapted from Tellier's work

** 5 mph is about as fast as a rapid walk.

The median diameters at which particles exhibit aerosol behavior also corresponds to the size range that will reach the deepest recesses of the respiratory tract.

While keeping in mind the material below in the abstracts discussing drift from sewer plants and then the idea of drift distance for a bacterium or smaller particle, the issue of sewer plant aerosol generation is worrisome. Since sewer works also provide the opportunity for numerous organisms to be brought into close proximity with each-other, there is ample and well documented opportunity for genetic exchange. Thus sewer plants, as currently designed and operated, are generators of disease and not, as myth would lead one to believe, providing treatment that reduces disease.

Since a plant, even with new tertiary upgrades will generate aerosols and allow through-put of pathogens as well as pathogenic laden aerosols the issue is of current importance.

In discussing an epidemic and the non-pharmacologic approaches to infection control and issues of preparedness, please consider the following. There is a need to look at alternatives to current sewer plant technology. As currently designed, sewer plants are major generators of aerosols. These plants receive vast numbers of pathogens and thus during times of an epidemic, the human waste is collected, mixed and then discharged into the environment.

Thus, the average sewer treatment plant represents an increased, but unnecessary risk to both human and environmental health. These increased risks accrue to the discharge of partially treated effluent, the use of recycled wastewater for various sues discussed above. the application of heavily contaminated sludge to land and generation of aerosols, all with an accompanying release of pathogens

Below are some abstracts for your consideration

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Review of Aerosol Transmission of Influenza A Virus

Raymond Tellier*† comment comment *Hospital for Sick Children, Toronto, Ontario, Canada; and †University of Toronto, Toronto, Ontario, Canada

Suggested citation for this article

Abstract In theory, influenza viruses can be transmitted through aerosols, large droplets, or direct contact with secretions (or fomites). These 3 modes are not mutually exclusive. Published findings that support the occurrence of aerosol transmission were reviewed to assess the importance of this mode of transmission. Published evidence indicates that aerosol transmission of influenza can be an important mode of transmission, which has obvious implications for pandemic influenza planning and in particular for recommendations about the use of N95 respirators as part of personal protective equipment.

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Comment Letter L

Comparative investigation of airborne culturable microorganisms in sewage treatment plants.

Haas DU,
Reinthaler FF,
Wust G,
Posch J,
Ruckenbauer G,
Marth E.

The present study investigated emissions and emmissions of airborne microorganisms (mesophilic bacteria, Escherichia coli, molds, Aspergillus fumigatus, thermophilic actinomycetes/bacilli) in sewage treatment plants. For the aerobiological investigations three sewage treatment facilities with an activated-sludge process, capacities between 2000 and 28,000 PE and different cleaning steps were selected. The measurements of microorganism emission were conducted in the area of the intake (screen), in the area of biological treatment (activated sludge tank) and at a distance of 10 m from the activated sludge tanks. In order to determine the emmission, additional measurements were conducted leeward of the plant at a distance of 200 m. Samples were taken using four parallel six-stage Andersen 1 AFCM volumetric samplers. In the area of the intake counts for bacteria were 7.4 x 10(2) CFU/m3 (median), for thermophilic actinomycetes 1.8 x 10(1) CFU/m3, for thermophilic bacilli 7.1 x 10(1) CFU/m3, for molds 2.4 x 10(3) CFU/m3 and for Aspergillus fumigatus 1.8 x 10(1) CFU/m3. Only isolated airborne coliform recoveries, i.e. E. coli, were detected. In the area of the activated sludge tank, in the adjoining area (10 m) and in the vicinity of the plants (200 m), the counts for all microorganism groups investigated corresponded to natural conditions. The results show that the counts of culturable aerogenic microorganisms in and in the immediate surrounding of the sewage plants investigated are low. Although the possibility of an infection through inhalation cannot be ruled out, the direct contact with sewage is much more critical.

PMID: 12096685 [PubMed - indexed for MEDLINE]

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QUESTION---WHAT ARE THE SOURCES OF ANTIBIOTIC RESISTANCE REACHING THE SEWER PLANT?

The treatment of hospital wastewater: an appraisal.

Pauwels B, Verstraete W.

Laboratory of Microbial Ecology and Technology (LabMET), Ghent University, Coupure Links 653, B-9000 Ghent, Belgium.

Hospitals discharge considerable amounts of chemicals and microbial agents in their wastewaters. Problem chemicals present in hospital wastewater belong to different groups, such as antibiotics, X-ray contrast agents, disinfectants and pharmaceuticals. Many of these chemical compounds resist normal wastewater treatment. They end up in

surface waters where they can influence the aquatic ecosystem and interfere with the food chain. Humans are particularly exposed by the drinking water, produced from surface water. Microbial agents of special concern are multiresistant microbial strains. The latter are suspected to contribute to the spread of antibiotic resistance. In this paper, we will discuss the different approaches towards hospital wastewater treatment. The principle of uncoupling hospitals from public sewers warrants indepth evaluation by technologists and ecotoxicologists as well as public health specialists.

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Comparison of the antimicrobial tolerance of oxytetracycline-resistant heterotrophic bacteria isolated from hospital sewage and freshwater fishfarm water in Belgium.

Huys G, Gevers D, Temmerman R, Cnockaert M, Denys R, Rhodes G, Pickup R, McGann P, Hiney M, Smith P, Swings J.

Laboratory of Microbiology, University of Gent, Ghent, Belgium. geert.huys@rug.ac.be

The aim of this study was to investigate the relationship between antimicrobial tolerance and taxonomic diversity among the culturable oxytetracycline-resistant (Ot(r)) heterotrophic bacterial population in two Belgian aquatic sites receiving wastewater either from human medicine or from aquaculture. The study of Ot(r) heterotrophs and mesophilic Aeromonas spp. allowed comparison of tolerance data at the intergenus as well as at the intragenus level. In total, 354 independently obtained Ot(r) isolates were subjected to antimicrobial tolerance testing and identified by GLC analysis of their cellular fatty acid methyl esters (FAMEs), by API 20E profiling and/or by Fluorescent Amplified Fragment Length Polymorphism (FAFLP) DNA fingerprinting. In general, Ot(r) hospital heterotrophs displayed a higher frequency (84%) of ampicillin (Amp) tolerance compared to the Ot(r) heterotrophs from the freshwater fishfarm site (22%). FAME results indicated that this effect was linked to the predominance of intrinsically ampicillin-resistant Ot(r) Aeromonas strains over representatives of Acinetobacter and Escherichia coli within the hospital strain set. Among the Ot(r) mesophilic Aeromonas strain set, the global tolerance profiles of the two sites only differed in a higher number of kanamycin (Kan) -tolerant strains (43%) for hospital aeromonads in comparison with the fishfarm aeromonads (8%). To some extent, this finding was correlated with the specific presence of Aeromonas caviae DNA hybridisation group (HG) 4. Collectively, these results suggest that the profiles for Amp and Kan tolerance observed in both sites arose from taxonomic differences in the culturable Ot(r) bacterial population at the generic or subgeneric level. In addition, our identification data also revealed that Enterobacter sp., Stenotrophomonas maltophilia, and A. veronii biovar sobria HG8 may be considered potential indicator organisms to assess microbial tolerance in various compartments of the aquatic environment.

PMID: 11403391 [PubMed - indexed for MEDLINE]

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Occurrence of antibiotics in hospital, residential, and dairy effluent, municipal wastewater, and the Rio Grande in New Mexico.

Brown KD, Kulis J, Thomson B, Chapman TH, Mawhinney DB.

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This study had three objectives: 1) determine occurrence of antibiotics in effluent from hospitals, residential facilities, and dairies, and in municipal wastewater 2) determine antibiotic removal at a large wastewater treatment plant (WWTP) in Albuquerque, NM, and 3) determine concentrations of antibiotics in the Rio Grande, which receives wastewater from the Albuquerque WWTP. Twenty-three samples of wastewater and 3 samples of Rio Grande water were analyzed for the presence of 11 antibiotics. Fifty-eight percent of samples had at least one antibiotic present while 25% had three or more. Hospital effluent had detections of sulfamethoxazole, trimethoprim, ciprofloxacin, ofloxacin, lincomycin, and penicillin G, with 4 of 5 hospital samples having at least one antibiotic detected and 3 having four or more. At the residential sampling sites, ofloxacin was found in effluent from assisted living and retirement facilities, while the student dormitory had no detects. Only lincomycin was detected in dairy effluent (in 2 of 8 samples, at 700 and 6600 ng/L). Municipal wastewater had detections of sulfamethoxazole, trimethoprim, ciprofloxacin, and ofloxacin, with 4 of 6 samples having at least one antibiotic present and 3 having 3 or more. The relatively high concentrations (up to 35,500 ng/L) of ofloxacin found in hospital and residential effluent may be of concern due to potential genotoxic effects and development of antibiotic resistance. At the Albuquerque WWTP, both raw wastewater and treated effluent had detections of sulfamethoxazole, trimethoprim, and ofloxacin, at concentrations ranging from 110 to 470 ng/L. However, concentrations in treated effluent were reduced by 20% to 77%. No antibiotics were detected in the Rio Grande upstream of the Albuquerque WWTP discharge, and only one antibiotic, sulfamethoxazole, was detected in the Rio Grande (300 ng/L) below the WWTP.

PMID: 16313947 [PubMed - indexed for MEDLINE]

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Promoting resistance by the emission of antibiotics from hospitals and households into effluent.

Kümmerer K, Henninger A.

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OBJECTIVE: There is growing concern about bacterial resistance to antimicrobials. The majority of antibiotics used are only partially metabolized after administration, and are released via patient excreta into the municipal sewage system. Data on the use of antibiotics and their emission into hospital effluent are not available. METHODS: Antibiotic consumption in Germany was calculated on the basis of five hospitals of varying size and medical service spectrum and on prescriptions issued by medical practitioners. The predicted environmental concentration (PEC) was calculated for hospital effluent and for municipal sewage. The PECs were compared both with

published minimum inhibitory concentrations (MIC50) for sensitive pathogenic bacteria and with the predicted no-effect concentrations (PNECs). RESULTS: The amount of antibiotics emitted into hospital effluent may reach and exceed the MIC50 of susceptible pathogenic bacteria. The PEC/PNEC ratio is highest for hospital effluent (in some cases 10-20 times the MIC50) and frequently > 1 for municipal sewage. PECs are high enough for some compounds to have a PEC/PNEC ratio > 1 even in surface water. CONCLUSION: The volume of antibiotics used in hospitals and private households and released into effluent and municipal sewage indicates a selection pressure on bacteria. Steps should be taken to reduce the risk by proper handling of antibiotics and their residues both in hospitals and by private users.

PMID: 14686985 [PubMed - indexed for MEDLINE]

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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.

PMID: 14675656 [PubMed - indexed for MEDLINE]

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Antibiotic resistance in Acinetobacter spp. isolated from sewers receiving waste effluent from a hospital and a pharmaceutical plant.

Guardabassi L, Petersen A, Olsen JE, Dalsgaard A.

Department of Veterinary Microbiology, The Royal Veterinary and Agricultural University, 1870 Frederiksberg C., Denmark. lg@kvl.dk

The possible increase of antibiotic-resistant bacteria in sewage associated with the discharge of wastewater from a hospital and a pharmaceutical plant was investigated by using Acinetobacter species as environmental bacterial indicators. The level of susceptibility to six antimicrobial agents was determined in 385 Acinetobacter strains isolated from samples collected upstream and downstream from the discharge points of the hospital and the pharmaceutical plant. Results indicated that while the hospital waste effluent affected only the prevalence of oxytetracycline resistance, the discharge of wastewater from the pharmaceutical plant was associated with an increase in the prevalence of both single- and multiple-antibiotic resistance among Acinetobacter species in the sewers.

PMID: 9726904 [PubMed - indexed for MEDLINE]

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A REVIEW OF THE EFFECTS OF SEWER LEAKAGE ON GROUNDWATER QUALITY

J. H. Reynolds, BSc. DMS. MICE; M. H. Barrett, BSc. PhD. FGS

Exfiltration (of sewage into the ground from faults in ageing sewerage networks) has been recognised for many years but has never been quantified. It was considered that this leakage did not pose a groundwater-contamination threat because it was assumed that pathogens in sewage would either die off or be attenuated before reaching the groundwater level.

This paper identifies why sewers have faults that could allow sewage exfiltration where the sewer is above a groundwater resource. Because of a lack of UK data on sewage exfiltration, research work in Germany is discussed and analogies are made between the sewerage networks of the two countries.

Until recently, it has not been possible to positively identify sewage contamination of groundwater because of other pollutants and potential sources within an urban environment. The development of sewage-fingerprinting techniques has overcome these difficulties and provides conclusive evidence of sewage contamination of shallow and deep groundwater resources in researched areas. Studies of groundwater recharge and total solute loadings allow quantification of sewage exfiltration reaching the groundwater within the Midlands conurbation. The paper concludes that urban aquifers are potentially more vulnerable to microbiological contamination from leaking sewers than has previously been assumed.

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Microbial contamination of two urban sandstone aquifers in the UK

Karen L. Powell**a**, Richard G. Taylor**b**, Aidan A. Cronin; Mike H. Barrett**a**, Steve Pedley**a**, Jane Sellwood**c**, Sam A. Trowsdale**d** and David N. Lerner**d**

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.

Author Keywords: Groundwater; Urban; Sewage; Viruses; Bacteria; UK

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SUMMARY

Until the government and its regulatory agencies as well as the sewer districts begin to appreciate their involvement in the spread of antibiotic resistance, the problem can not be solved. The health care profession will continue to fight an up hill battle, and one that it is fast losing. We are seeing diminishing capacities of the current stock of pharmaceuticals to effective deal with increasing resistance. Further, the pharmaceutical industry is becoming reluctant to invest in new antimicrobial research. When new drugs are showing resistance within the second level of clinical trials, the handwriting is on the wall. It is better to invest research into drugs that are developed for chronic diseases that must be taken for life rather than ones taken for a mere two weeks duration.

To conclude, the following thought is a statement by the WHO's chief of Communicable Disease, David Heymann, before the US Senate hearing on The Spread of Communicable Disease, in 2001.

Some microbes have accumulated resistant genes to virtually all currently available drugs. Thus, these have the potential to cause untreatable infections. Accordingly, such diseases may have no effective cures over the next 10 years unless there is some uncharacteristic breakthrough in drug therapy. Therefore, if current trends continue, many important medical and surgical procedures, including cancer therapy, bone marrow and organ transplant, hip and knee replacement, and coronary bypass surgery could no longer be undertaken without undue risk of unstoppable infection.

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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

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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.

: Environ Sci Technol. 2007 Apr 1;41(7):2651-2.Links

Comment on:
Environ Sci Technol. 2006 Dec 1;40(23):7445-50.

Comment on "antibiotic resistance genes as emerging contaminants: studies in northern Colorado".