**McGowan E.**

PMID: 17438829 [PubMed - indexed for MEDLINE]

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

Defense strategies include going dormant, entering the viable but non-culturable (VBNC) state. These VBNC organisms are essentially invisible to laboratory tests used in the wastewater industry. Higgins & Murthy recently reconfirmed this [4] in a paper that raises some serious questions about the efficacy of current standards. Those authors noted that during centrifuged dewatering of sewer sludge, indicators in a VBNC state were resuscitated. The results were several magnitudes greater than standard plate counts had indicated [4]. Such findings raise logical questions. If dewatering by centrifuge brought out the essence of VBNC, would other products of sewage that had not been subjected to the centrifuge also in the VBNC state? If so would they revive in the field following agricultural application of sludge or irrigation with reclaimed wastewater? This seems plausible but needs further study.

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

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

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

In 2002 the NAS/NRC [21] called into question the U.S. EPA Part 503 guidelines for



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

**Citations**

[1] Pruden, A.; Pei, R.; Storteboom, H.; Carlson, K. H Antibiotic Resistance Genes as Emerging Contaminants: Studies in Northern Colorado.Some time ago, you suggested that I compile a report that contained scientific citations, and then submit that report to you. Having compiled such a report, I am submitting such at this time. 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 waher that might carry transmissible pathogens. In 2006, we noted multi-drug resistant bacteria at the point of use (SBCC campus). 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. Thus one might conclude that previous findings were not just rare happen-stance occasions but were the actual background of the recycled water as prodced by Santa Barbara. 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 armentarium of useful tools is being squandered, perhaps theough 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. Environ. Sci. Technol.; (Article); 2006; 40(23); 7445-7450.

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The Importance of Municipal Sewage Treatment in the Spread of Antibiotic Resistance

106th General Meeting of the American Society for Microbiology May 21-25, 2006, Orlando, Florida For more information on any presentation at the 106th General Meeting of the ASM contact Jim Sliwa, ASM Office of Communications at *jsliwa@asmusa.org*

EMBARGOED UNTIL: Monday, May 22, 9:00 a.m. EDT (Session 041/Q, Paper Q-032) Sara Firl University of Minnesota Minneapolis, MN, United States Phone: 612 626 8865 **firl0002@umn.edu**

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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1: Sci Total Environ. 2009 Jun 1;407(12):3702-6. Epub 2009 Mar 24.



**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.

The occurrence and spread of multi-drug resistant bacteria is a pressing public health problem. The emergence of bacterial resistance to antibiotics is common in areas where antibiotics are heavily used, and antibiotic-resistant bacteria also increasingly occur in aquatic environments. The purpose of the present study was to evaluate the impact of the wastewater treatment process on the prevalence of antibiotic resistance in Acinetobacter spp. in the wastewater and its receiving water. During two different events (high-temperature, high-flow, 31 degrees C; and low-temperature, low-flow, 8 degrees C), 366 strains of Acinetobacter spp. were isolated from five different sites, three in a wastewater treatment plant (raw influent, second effluent, and final effluent) and two in the receiving body (upstream and downstream of the treated wastewater discharge point). The antibiotic susceptibility phenotypes were determined by the disc-diffusion method for 8 antibiotics, amoxicillin/clavulanic acid (AMC), chloramphenicol (CHL), ciprofloxacin (CIP), colistin (CL), gentamicin (GM), rifampin (RA), sulfisoxazole (SU), and trimethoprim (TMP). The prevalence of antibiotic resistance in Acinetobacter isolates to AMC, CHL, RA, and multi-drug (three antibiotics or more) significantly increased ($p<0.01$) from the raw influent samples (AMC, 8.7%; CHL, 25.2%;

RA, 63.1%; multi-drug, 33.0%) to the final effluent samples (AMC, 37.9%; CHL, 69.0%; RA, 84.5%; multi-drug, 72.4%), and was significantly higher ($p<0.05$) in the downstream samples (AMC, 25.8%; CHL, 48.4%; RA, 85.5%; multi-drug, 56.5%) than in the upstream samples (AMC, 9.5%; CHL, 27.0%; RA, 65.1%; multi-drug, 28.6%). These results suggest that wastewater treatment process contributes to the selective increase of antibiotic resistant bacteria and the occurrence of multi-drug resistant bacteria in aquatic environments.

PMID: 19321192 [PubMed - indexed for MEDLINE]





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contributes to selective

An Acanthamoeba sp. containing two

Microorganisms Resistant to Free-Living

The New York Times

October 16, 2007

# Infection Killed 19,000 in 2005, Study Says

By KEVIN SACK

ATLANTA, Oct. 16 — Nearly 19,000 people died in the United States in 2005 after being infected with a virulent drug-resistant bacterium that has spread rampantly through hospitals and nursing homes, according to the most thorough study to be conducted of the disease's prevalence.

The study, which was published today in The Journal of the American Medical Association, suggests that invasive infections with methicillin-resistant Staphylococcus aureus, or M.R.S.A., may be twice as common as previously thought, according to its lead author, Dr. R. Monina Klevens. If the mortality estimates are correct, the number of deaths associated with M.R.S.A. each year would exceed those attributed to HIV/AIDS, Parkinson's disease, emphysema or homicide.

By extrapolating data collected in nine locations, the researchers established the first true baseline for M.R.S.A. in the United States, projecting that 94,360 patients developed an invasive infection from the pathogen in 2005 and that nearly one of every five, or 18,650 of them, died.

The authors, who work for the Centers for Disease Control and Prevention, cautioned that their methodology differed significantly from previous studies and that direct comparisons were therefore risky. But they said they were surprised by the prevalence of the serious infections they found, which they calculated as 32 cases per 100,000 people.

In an accompanying editorial in the medical journal, Dr. Elizabeth A. Bancroft, an epidemiologist with the Los Angeles County Department of Public Health, characterized that finding as "astounding." She wrote that the prevalence of invasive M.R.S.A. — when the bacteria has not merely colonized on the skin, but has attacked a normally sterile part of the body, like the organs or bloodstream — is greater than the combined rates for other conditions caused by invasive bacteria, including bloodstream infections, meningitis and flesh-eating disease.

The study also concluded that 85 percent of invasive M.R.S.A. infections are associated with health-care treatment. Previous research had indicated that many hospitals and long-term care centers have become breeding grounds for M.R.S.A. because bacteria may be transported from patient to patient by doctors, nurses and unsterile equipment.

"This confirms in a very rigorous way that this is a huge health problem," said Dr. John A. Jernigan, the deputy chief of prevention and response in the C.D.C.'s Division of Healthcare Quality Promotion. "And it drives home that what we do in health care will have a lot to do with how we control it."

The findings are likely to further stimulate an already active debate about whether hospitals and other medical centers should test all patients for M.R.S.A. upon admission. Some hospitals have had notable success in reducing their infection rates by isolating infected patients and then taking extra precautions, like requiring workers to wear gloves and gowns.

But other research has suggested that such techniques may be excessive, and may have the unintended consequence of diminishing medical care for sequestered patients. The C.D.C., in guidelines released last year, recommended that hospitals attempt to reduce their infection rates by first improving hygiene procedures and that they resort to screening high-risk patients only if other methods fail.

Dr. Lance R. Peterson, an epidemiologist with Evanston Northwestern Healthcare, said the Chicago-area hospital system reduced its rate of invasive M.R.S.A. infections by 60 percent after it began screening all patients

in 2005.

"This study puts more onus on organizations that don't do active surveillance to demonstrate that they're reducing their M.R.S.A. infections," he said. "Other things can work, but nothing else has been demonstrated to have this kind of impact. M.R.S.A. is theoretically a totally preventable disease."

Numerous studies have shown that busy hospital workers disregard basic standards of hand-washing more than half the time. This week, Consumers Union, the nonprofit publisher of Consumer Reports, called for hospitals to begin publishing their hand-washing compliance rates.

"This study just accentuates that the hospital is ground zero, that this is where dangerous infections are occurring that are killing people every day," said Lisa A. McGiffert, manager of the group's "Stop Hospital Infections" campaign.

Though the C.D.C. estimates that M.R.S.A. represents only 10 percent to 20 percent of all infections acquired in health-care settings, the bacterium is feared for its opportunism and deadliness.

First isolated in the United States in 1968, it is resistant to a number of antibiotics and can cause infections of surgical sites, the urinary tract, the bloodstream and the lungs, leading to extensive and expensive hospital stays. The bacteria can be brought unknowingly into hospitals and nursing homes by patients who show no symptoms, and then takes advantage of weakened immune systems, incisions and wounds.

Of the infections studied by Dr. Klevens and her colleagues, 27 percent were considered to have originated during a patient's current hospital stay. Another 58 percent were deemed to be associated with a previous hospitalization, nursing home stay, surgery or dialysis. Only 14 percent were cases without a defined health care risk factor, meaning the infection likely originated in the community.

A major difference with previous analyses is that the new study compiled actual confirmed cases of M.R.S.A. infection, rather than relying on coded patient records that sometimes lack precision. In the new study, higher prevalence rates and death rates were found for the elderly, blacks and men. The figures also varied greatly by geography, with Baltimore's incidence rates far exceeding those of the eight other locations studied.

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Dr. Klevens said further research would be needed to understand the racial and geographic disparities.

The C.D.C.'s latest estimate of all infections associated with health care, also taken from a study by Dr. Klevens, was 1.7 million cases and 99,000 associated deaths in 2002.



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Special Applications — 3

Agroforestry Notes

USDA Forest Service • USDA Natural Resources Conservation Service

AF Note — 17 April, 2000

# Wastewater Management Using Hybrid Poplar

## Introduction

Hybrid poplars are rapidly growing trees that are well suited to use agricultural, industrial, and community wastewater. They are being used as an alternative to expensive wastewater treatment systems, and methods which apply wastewater to annual crops or pasture. The trees serve a dual purpose as a nutrient sink for wastewater use and as a means to produce a short-rotation harvested wood product which helps offset the cost of installation and maintenance.

Planning tree wastewater use systems requires both agroforestry and engineering expertise. Omission of either in the planning process can lead to disappointing results and reduced benefits. This *Agroforestry Note* outlines and discusses the major planning and engineering considerations for a short rotation woody crop (SRWC) wastewater application system.

## Planning Considerations

**Dual-Purpose Aspect.** The dual objective of this technology for wastewater management and wood production will affect site selection, stand spacing, utility function, and marketing/utilization of wood products. Trees may be grown for chips for pulp or oriented strand board, solid wood products (veneer, paneling, molding, etc.), or as biomass for fuel. Product goals will influence initial stand spacing and future management regimes (i.e. pruning, thinning). Harvesting in short rotations (6 to 15 years) will keep the stand from deteriorating and take advantage of the rapid growth of young trees for wastewater uptake.

**Interdisciplinary Team and Commitment.** An interdisciplinary team knowledgeable in forestry/plant science, soil science, environmental and irrigation engineering, farming, and wood product utilization can provide critical input for the planning, design, and management of the project. This will require a series of meetings to discuss the project and to develop an appropriate plan of work. It is essential to determine the local commitment for the project in terms of coordination, technical design assistance, installation, maintenance, wood product marketing, permits, and public information.

**Permits.** Meetings with the appropriate agencies (i.e. U.S. Environmental Protection Agency (EPA), State Department of Environmental Quality (DEQ), USDA Natural Resources Conservation Service (NRCS), etc.) should occur early in the planning effort. Most municipal wastewater reuse systems require a permit. Examples include the National Pollutant Discharge Elimination System (NPDES) permit for industrial and municiple waste, and Concentrated Animal Feeding Operations (CAFO), or a Water Pollution Control Facility (WPCF) permit. Some states have additional state issued animal waste permits for agriculture. Facility plans, engineering reports, and management plans are just some of the possible support documents required during development.

USDA
National Agroforestry Center

**Wastewater Design Components**

**Effluent Quantity and Quality**. A clear understanding of the quantity and quality of the effluent to be managed is needed and will dictate the amount of land to be planted. Quantity is often established by the operations permit for the wastewater treatment plant and will vary depending on the water supply, waste inputs, treatment process, and type of storage/retention facility. These permit conditions may limit effluent discharge to a receiving stream during a specific time period and require a minimum level of treatment. The table to the right provides a general list of effluent quality parameters to consider. *In most cases, wastewater effluent from municipal systems has proven to be an ideal source of irrigation water for SRWC plantations.*

**Key Parameters of Effluent Quality**

| Parameter (mg/L) |
|---|
| Biological Oxygen Demand |
| Suspended Solids |
| Dissolved Solids |
| Nitrogen |
| Total Nitrogen |
| Ammonia |
| Nitrate-nitrite |
| Phosphorus |
| Potassium |
| Chlorides |
| Sodium |
| Heavy metals |
| Fecal coliform (#/100 ml) |

**Irrigation Requirement and Nutrient Loading Rates** Water requirements and nutrient uptake capacity of the trees determines the land area requirements. Water needs for various crops can be found in the NRCS *Guide to Individual State Guidelines on Crop Water Use and Net Irrigation Requirement*, through agricultural extension agents, experiment stations, and/or state universities. The nutrient uptake capacity (fertilizer requirements) for tree crops can also be found through regional extension guides. In addition, general guidelines can be found in the EPA *Guidelines for Water Reuse and Process Design Manual for Land Treatment of Municipal Wastewater.* Several demonstration sites, as well as industrial scale hybrid poplar tree plantations, have been developed in the Pacific Northwest to reuse wastewater effluent and biosolids and have helped to better define the crop water use, irrigation requirement, and nutrient uptake capacity.

**Water Delivery System.** The water delivery system generally includes pumps and pipelines needed to convey the wastewater from the treatment facility to the irrigation system at the SRWC plantation. The sizing and design criteria vary according to: 1) quantity of reuse water and required delivery pressure, 2) peak daily irrigation rate, and 3) distance and elevation between the wastewater treatment facility and the irrigation system.

The material selection for pump, pipeline, and filtration components must involve the consideration of the corrosivity of the effluent and the anticipated sediment load. California and Nevada's *Guidelines for Distribution of Nonpotable Water* provides specific guidelines on pipelines. Filtration after the pump station should be considered to protect the irrigation system from clogging. The mesh size of the filter depends on the type of irrigation application system used. Micro-spray sprinklers should have a minimum of a 50-mesh filtration system and drip lines should have a minimum of 150 mesh. Sand media filtration may be required for drip systems when the effluent has a heavy load of suspended organic particles.

**Irrigation System**. Several planning considerations are needed to identify the proper irrigation system components and layout: 1) tree spacing, 2) infiltration rate of the soil, 3) goals surrounding uniformity of water application, 4) water quality of the effluent and filtration desires, and 5) overall operations and management of the plantation. The two most common irrigation systems used in SRWC plantations are micro-spray sprinklers and drip emitters. Both of these systems generally employ an above ground lateral line that delivers water to the sprinklers or drip emitters spaced down the rows. Any buried flexible piping should be considered with caution due to the potential for crimping by tree roots. Drip systems require one lateral line per tree row, however the spacing between the rows for the micro-spray sprinkler system depends on the tree spacing and application uniformity





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desired. For example, a plantation with a 10 foot X 10 foot tree spacing may have a lateral line every other tree row. The spacing within a tree row depends on the tree spacing down the row, flow rate of the emitters or micro-spray sprinklers, and the desired application rate. Other types of irrigation application systems such as impact sprinklers, ditches, and/or gated pipe have also been successfully used.

**Operation and Maintenance.** Operation and maintenance considerations include: 1) sprinkler and/or emitter performance, 2) filtration cycle times, 3) pump flows and pressures, 4) tracking of total application rates and effluent quality, 5) soil moisture conditions, 6) soil pore water quality, and 7) groundwater quality. A routine schedule should be followed for flushing the system, cleaning and maintaining the filtration system, and repairing and/or replacing the micro-spray sprinklers or drip emitters.

**Plant Design Components**

**Soils/Site Conditions at Proposed Project Location**. Ideally, the site should be level with good drainage and a neutral pH (6 to 7). Loamy soils are best. Soil depth should be at least three feet, with four to six feet preferred. Good root development is needed for effective wastewater uptake and to reduce the potential for wind throw damage. Clayey, poorly drained soils or soils with a high pH (8+) or salts are not recommended for tree/wastewater projects. Extra caution should be taken with very sandy or gravely soils due to the potential for excessive leaching. It is also important to identify adjacent surface waters and communities that could be affected.

**Hybrid Poplar Suitability**. The technology for hybrid poplar varieties is rapidly advancing, so it is important to acquire the most current information on suitable varieties. This information may be available from locations such as university extension offices, hybrid poplar nurseries, USDA Forest Service Research Stations, private timber companies, private forestry consultants, and conservation district offices. Knowledge of the soil attributes, wastewater content, and common local pests and diseases is essential to variety selection. Matching varieties for the product desired as well as their utility function will benefit the overall economic outlook of the project.

**Design – Initial Stand Spacing**. Initial stand spacing is determined by a combination of factors like wood product desired, irrigation system, and weed control methods. Biomass plantings for fuel production will have close spacing (e.g. 3 foot X 5 foot) vs. plantings for chips for paper or strand board with moderate spacing (e.g. 8 foot X 10 foot). Wider spacing, such as 12 foot X 12 foot or wider, may be desired for producing a solid wood product like veneer, however, it may add to the number of years of weed control needed and may reduce the amount of wastewater uptake in the early growth phase of the planting. Weed control methods will influence spacing depending on the types of equipment used for mowing and/or tillage operations, and herbicide applications. The wastewater irrigation system may also influence stand spacing.

**Site Preparation, Planting, and Maintenance**

**Site Preparation**. Existing vegetation is typically killed the year before planting and tilled to mellow over winter. If the soil is compacted and/or there are clay or plow pan layers present in the upper two feet of the soil profile, the site will need to be ripped or subsoiled to allow for root growth. Ripping is usually done along the tree row line and sometimes again perpendicular to the tree row line. In the spring, before planting, the site may be tilled. In some wet climates, trees should be planted in early spring when soil conditions are still too wet for tillage. "Flour" type conditions are created with too much tillage and present major problems for plant establishment and potential soil erosion. Cover crops or orchard grass mixtures are sometimes established between the tree rows but three feet should be left clear on either side of the trees.

**Planting and Maintenance**. Branch or stem cuttings 8 to12 inches in length and

greater than 3/8 inch diameter are planted by inserting them into the soil until only one bud remains above ground. This will generally produce a single dominant stem. Avoid very fine or cloddy soil conditions at the planting site to insure good root development. Very fine or powdery soil conditions cause the soil to settle after irrigation, while cloddy conditions will create air pockets next to the stem. It is extremely important to plan for weed control. Pre-emergent and contact herbicide information can be obtained from extension offices, consultants, nurseries, etc. Usually, pre-emergence herbicides are applied within the tree rows just before or after planting and they are used until the trees have shaded out competing vegetation. Control of weeds between rows may be important for reducing habitat for rodents, such as mice/voles, that can girdle young tree stems. In irrigated plantings, such as wastewater applications, mowing or shallow tillage can accomplish this. Make sure the mowing cycle is diligent and weeds are not allowed to go to seed. Sprinkler lines need to be placed in the tree rows to avoid damage by mowing or tillage equipment for between-row weed control.

**Management**. Management consists of protection from animals, insects, diseases, and stand treatments to produce a harvestable product. Deer browse the terminal bud of stems in the first year causing loss of growth and excessive branch development. This reduces stem wood development and quality. Observations from plantation owners and nurseries suggest that deer tend to browse the outer rows of a planting.

For insect and disease control, the best defense is to plant the most tolerant varieties suitable for a region. Leaf rust, stem canker diseases, leaf beetles, aphids, stem borers, and grasshoppers can be important pests to monitor throughout the growth of the planting.

Stand treatments like thinning and pruning are essential for producing a high-value wood product. Initial stand spacing and growth rates will dictate thinning and pruning operations. Wide spacing will reduce the need for thinning, however, it may increase the need for pruning. To determine the harvesting cycle it is useful to target an average stand diameter; common averages are: biomass - four to five inches; chips - seven to eight inches; and sawlogs (lumer, veneer, moldings) - 12 to 16 inches. The length of time trees are grown can determine whether they fall under agricultural or forestry practice laws. State and local laws should be understood when determining harvest cycles.

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**For More Information**

"Crop Water Use and Net Irrigation Requirements." USDA-NRCS. Individual state guidelines. Available at USDA-NRCS offices.

"Guidelines for Distribution of Nonpotable Water." 1983. American Water Works Association (AWWA) - California-Nevada Section.

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NAC is a partnership of the USDA Forest Service, Research & Development (R&D) (Rocky Mountain Research Station) and State & Private Forestry (S&PF) and the USDA Natural Resources Conservation Service. The Center's purpose is to accelerate the development and application of agroforestry technologies to attain more economically, environmentally, and socially sustainable land-use systems. To accomplish its mission, the Center interacts with a national network of cooperators to conduct research, develop technologies and tools, establish demonstrations, and provide useful information to natural resource professionals.







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

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**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.**

Reclaimed water is derived from treated municipal wastewater. The treatment processes used for production of reclaimed water provide multiple barriers (biological treatment, physical removal, and chemical disinfection) for control of pathogens. Reclaimed water is used for nonpotable applications such as irrigation, cooling water, industrial process water, and environmental enhancement (17). Indirect potable reuse occurs through groundwater recharge or surface water replenishment, and is assuming greater importance with increased production of reclaimed water. As water use in the United States (7) and worldwide increases, the importance of reclaimed water to sustainable water resources will continue to increase (17).

A major goal of wastewater reclamation facilities is to reduce pathogen loads in order to decrease public health risks associated with exposure. The effectiveness of pathogen control is indirectly assessed through routine monitoring of the reclaimed water (final effluent) by using grab samples to detect standard indicator bacteria such as total or fecal coliforms. Treatment practices for production of reclaimed water vary depending on the ultimate intended use(s) of the water and local regulatory requirements. Currently, there are no universal standards governing the production and quality of reclaimed water, although the World Health Organization has developed guidelines for the use of reclaimed water (35) that recommend monitoring fecal coliforms and intestinal nematodes. In the United States, there are no federal standards controlling the quality of reclaimed water, and individual states have developed guidelines or implemented specific treatment and monitoring requirements that are intended to protect the public from exposure to pathogens. Due to the inherent constraints associated with pathogen monitoring, indicator organisms are employed as surrogates for pathogens. In some states, total coliform bacteria are used as the indicator organism (6); however, in the majority of states that have specific regulations, the microbiological safety of reclaimed water is evaluated by daily monitoring of fecal coliform bacteria in the disinfected effluent based on a single, 100-ml grab sample (3). In addition, periodic monitoring of viruses and/or protozoan pathogens has been required by a few states, including Arizona, California, and Florida (3).

It has been widely demonstrated that coliform bacteria do not adequately reflect the occurrence of pathogens in disinfected wastewater effluent due to their relatively high susceptibility to chemical disinfection (18) and failure to correlate

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TABLE 1. Comparison of wastewater reclamation facilities sampled for indicator organisms and pathogens in this study

| Facility | Avg capacity ($m^3 \cdot s^{-1}$) | Biological treatment | Chemical use prior to filtration | Filter composition (depth, m) | Backwash frequency (h) | Type of disinfection |
|---|---|---|---|---|---|---|
| A | 0.04 | Activated sludge | None | Fabric (0.02) | 24 to 72 | Chloramines[a] |
| B | 0.4 | Activated sludge | Chlorine | Sand (0.3) | Automatic (daily) | Chloramines[a] |
| C | 0.4 | Activated sludge | Cationic polyelectrolyte | Anthracite (1.2) | 48 | Chloramines[a] |
| D | 0.7 | Activated sludge | None | Anthracite (0.8), sand (0.25) | 48 to 168 | Chloramines |
| E | 0.08 | Nitrification | None | Sand (1.2), upflow | Continuous | Ultraviolet light |
| F | 0.13 | Biological nutrient removal | None (alum added to secondary clarifier) | Anthracite (0.6), sand (1.2) | 48 to 168 | Chlorine |

[a] Chloramines are formed due to the reaction of chlorine with residual ammonia.

with protozoan parasites such as *Cryptosporidium* (5) and enteric viruses (13). Alternative microbiological indicators have been suggested for evaluation of wastewater, drinking water, and environmental waters, including *Enterococcus* spp. (18), *Clostridium perfringens* (9, 20), and coliphages (8, 10, 20).

To date, there have been only a few studies of reclaimed water in which the levels of indicator organisms have been directly compared to those of viral, bacterial, or protozoan pathogens at each stage of treatment (23, 24). In this work, the validity of using coliform bacteria and alternative microbial indicators to predict the presence or absence of pathogens, and thus to assess the public health risk, was evaluated using disinfected effluent from six wastewater reclamation facilities in the United States. The facilities varied in location (Arizona, California, and Florida), size, and treatment practices and were each sampled at least five times over a 1-year period. Each sample was analyzed for a suite of indicator bacteria, coliphages, enteric viruses, and protozoan pathogens, and predictive relationships among the microbial groups were evaluated by several statistical methods, including binary logistic regression and discriminant analysis (DA).

## MATERIALS AND METHODS

**Facilities.** Six wastewater reclamation facilities in the United States were each sampled at least five times over a 1-year period. A comparison of the treatment characteristics is given in Table 1. The facilities represent a cross-section of typical treatment approaches that are used for production of reclaimed water.

**Sampling.** All samples were aseptically collected in sterile containers (or sterile filters). Samples were immediately placed on ice in coolers and were kept on ice until processed. At each facility, samples were collected from the influent (untreated wastewater), secondary clarifier (biological treatment), filtered effluent, and disinfected effluent (reclaimed water). Samples were collected under peak flow conditions to provide a "worst-case" scenario for each facility. Each facility was sampled approximately bimonthly over a 1-year period, resulting in at least five samplings per facility.

Sample volumes collected for bacterial enumeration were 50 ml of influent, 500 ml from the secondary clarifier, 2 liters of effluent from the filtration stage, and 2 liters of disinfected effluent. Assays were performed in triplicate. Large volumes (up to 53 liters) were filtered for protozoan parasite and virus assays. Detection limits for bacterial indicators in disinfected effluent were 0.2 to 0.6 CFU · 100 $ml^{-1}$, those for coliphages were 10 PFU · 100 $ml^{-1}$, those for enteric viruses were 0.3 to 1.4 most probable number (MPN) · 100 $liters^{-1}$, those for *Cryptosporidium* oocysts were 2.0 to 6.9 oocysts · 100 $liters^{-1}$, those for infectious *Cryptosporidium* were 0.29 to 4.1 MPN · 100 $liters^{-1}$, and those for *Giardia* were 1.8 to 5.2 cysts · 100 $liters^{-1}$.

**Bacterial enumeration.** Indicator bacteria were quantified by membrane filtration using 47-mm cellulose acetate filters with a nominal pore size of 0.45 μm. Total coliform bacteria were cultured on mEndo LES agar (Difco, Sparks, MD) for 24 h at 37°C. Colonies that produced a green sheen were enumerated as total coliforms (2). Fecal coliform bacteria were cultured on mFC agar (Difco, Sparks, MD) for 24 h at 44.5°C in a water bath. Blue colonies were enumerated as fecal coliforms (2). *Escherichia coli* (ATCC 9637) was used as the positive control for all coliform measurements. Enterococci were cultured on mEI agar (31, 32). Plates were incubated at 41°C for 24 h, and colonies with a blue halo were enumerated as enterococci. *Enterococcus faecalis* (ATCC 19433) was used as a positive control. *Clostridium perfringens* was isolated on mCP agar (Acumedia Manufacturers, Inc.) (4). Plates were transferred to gas pack bags (BBL GasPak; Becton Dickinson) and sealed. After 24 h of incubation at 45°C, colonies were exposed to ammonium hydroxide fumes. All of the yellow/straw-colored colonies that turned pink/magenta were counted. *C. perfringens* (ATCC 13124) was used as a positive control.

**Bacteriophage analysis.** Coliphages were analyzed by the agar overlay method of Adams (1). Two *E. coli* host strains were used in separate assays: *E. coli* HS (pFamp) R (ATCC 700891), which infects male-specific ($F^+$) coliphages very efficiently and somatic coliphages poorly (8), and *E. coli* C3000 (ATCC 15597), which should host both somatic and $F^+$ coliphages (14). Serial dilutions of samples were made in phosphate-buffered saline according to expected phage concentrations at each treatment step. Five replicate volumes of 0.1 ml to 2 ml were plated for each dilution, except in the case of the disinfected effluent samples, for which 10 replicates of 2 ml each were plated. PFU · 100 $ml^{-1}$ were calculated after 24 h of incubation (2).

**Enteric viruses.** The U.S. Environmental Protection Agency (EPA) methodology (30) was used for the detection of enteric viruses. Influent sample volumes were based on the amount of water that could be processed without clogging the filter. Typically less than 100 liters was filtered for each influent sample, depending on water quality (i.e., content of suspended solids). Larger sample volumes were used for the other sample locations, i.e., ~190-liter samples from the secondary clarifiers and ~380-liter samples from the filtration and disinfection processes. Water samples were pumped through Virusorb 1MDS filters (Cuno, Inc.), which were eluted with 1 liter of 1.5% beef extract (BBL V) in 0.05 M glycine (pH 9.5, ~25°C) (U.S. EPA/ICR). The eluted sample was concentrated by organic flocculation and assayed for enterovirus by the observation of cytopathic effects (CPE) on recently passed (<4 days) cell lines. Three cell lines, i.e., buffalo green monkey, rhabdosarcoma (ATCC CCL-136), and MA-104 (ATCC CRL-2378.1) cells, were used for this purpose. Positive controls were processed in a separate room, using poliovirus 1. CPE on each cell line were observed, and the most dilute sample showing CPE was recorded. MPN determinations were performed using EPA-released software (Most Probable Number Calculator version 4.04; http://www.epa.gov/microbes/other.htm).

**Protozoa.** For the detection of *Giardia* and *Cryptosporidium*, samples were concentrated by filtration using Gelman Envirochek HV cartridge filters and processed according to the manufacturer's instructions. Following filtration, samples were processed by immunomagnetic separation (Dynal, Inc.) and immunofluorescent antibody detection (Easy Stain; Biotech Frontier, Australia) according to the procedure outlined in U.S. EPA method 1623 (33). Sample volumes varied depending upon the treatment stage and the amount of water that could be filtered, i.e., 0.5 to 1.0 liters influent, ~19 liters secondary effluent, ~38 liters effluent from filters, and up to 53 liters disinfected effluent. Detection limits varied with the total volume of sample filtered and processed. Each concentrated sample was divided into two aliquots: one for cell culture viability testing and the other for microscopic enumeration. Equivalent volumes were calculated, and the results were reported as cysts or oocysts · 100 $liters^{-1}$.

***Cryptosporidium* infectivity.** Concentrates from the immunomagnetic separation procedure were inoculated onto HCT-8 cell monolayers in eight-well chamber glass cell culture slides. The cultures were incubated in a 5% $CO_2$ atmosphere at 37°C for 48 h. Infective *Cryptosporidium* was enumerated by the focus detection method-MPN assay (27). Results were reported as infectious oocysts · 100 $liters^{-1}$.





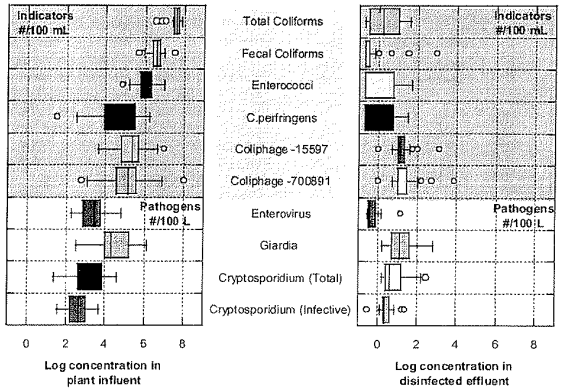


FIG. 1. Mean indicator organism and pathogen concentrations in untreated wastewater and disinfected effluent from six wastewater reclamation facilities ($n = 30$). $Log_{10}$ concentrations of bacterial indicators (CFU · 100 $ml^{-1}$), coliphages on *E. coli* 15597 and *E. coli* 700891 (PFU · 100 $ml^{-1}$), enteric viruses (MPN · 100 $liters^{-1}$), *Giardia* total counts (cysts · 100 $liters^{-1}$), and *Cryptosporidium* total and viable counts (oocysts · 100 $liters^{-1}$) are shown. Detection limits were used as concentrations for parameters that were nondetectable. The boxes represent 50% of the data, the vertical lines represent the mean, the lines extending from the boxes represent the 95% confidence limits, and the individual data points represent outliers.

**Statistical analysis.** Statistical analyses were conducted using SAS software version 8.2 (SAS Institute, Cary, NC) or SPSS version 12.0. Data distributions were evaluated with the Shapiro-Wilk test, which was conducted on the raw data, $log_{10}$-transformed data, and square-root-transformed data. Nonparametric statistical tests were utilized for nonnormally distributed data. Parametric tests were used for analysis of variance, and the Tukey post-hoc test was used to compare treatment means. The Spearman rank correlation was used to test the relationship between indicator organism and pathogen concentrations in the final effluent. A binary logistic regression model (SPSS 12.0) was utilized to determine whether indicator organism concentrations predicted the probability of the occurrence of pathogens in disinfected effluent samples. The dependent variable (pathogen) was treated as a binary variable; that is, a score of 0 was assigned when the organism was not detected, and a score of 1 was assigned when the organism was detected. The independent variables were continuous, and values for samples in which organisms were not detected were reported as 0. True-positive, true-negative, false-positive, and false-negative values were calculated as the number of samples falling into each category divided by the total sample number.

Discriminant analysis was performed on data from effluent samples by using the DISCRIM procedure of SAS (prior probabilities, equal; covariance matrix, pooled). The results of six assays for indicator organisms (total coliform, fecal coliform, *C. perfringens*, enterococci, and F-specific coliphage assays on two hosts) were converted into a string of binary variables representing the presence or absence of each indicator. The ability of the indicator data string to predict the presence or absence of each pathogen (*Giardia*, *Cryptosporidium*, and enteric viruses) was assessed separately. Results are expressed as the percentage of samples correctly classified into the "pathogen present" and "pathogen absent" categories.

## RESULTS

The results presented here represent multiple samplings from six facilities producing reclaimed water and focus on microbial concentrations in the influent and in the reclaimed water (disinfected effluent), which is distributed to end users.

**Microbial concentrations through treatment.** Concentrations of indicator organisms and pathogens before (untreated wastewater) and after (disinfected effluent) treatment are shown in Fig. 1 in a box plot format. The limit of detection (see Materials and Methods) was substituted for measured values for samples in which the organism was not detected, which was rare in influent samples but common in effluent samples. Total coliform concentrations were the highest of the microbial measurements in influent samples (>$10^7$ CFU · 100 $ml^{-1}$), followed by fecal coliforms and enterococci (~$10^6$ CFU · 100 $ml^{-1}$) (Fig. 1). *Clostridium perfringens* values ranged from $10^4$ to >$10^6$ CFU · 100 $ml^{-1}$. Coliphage levels were highly variable, ranging from $10^3$ to $10^8$ PFU · 100 $ml^{-1}$. Pathogen concentrations in the influent (Fig. 1) were 4 to 5 orders of magnitude lower than indicator organism concentrations (note that the unit for pathogen concentrations is 100 $liters^{-1}$). It should be noted that while the enteric virus concentrations represent infectious viruses, *Cryptosporidium* and *Giardia* concentrations reflect the total number of cysts or oocysts (infectious and noninfectious) viewed under immunofluorescence microscopy. In the influent samples, about 40% of the detected *Cryptosporidium* organisms were infective as defined by the focus detection method-MPN cell culture assay. Microbial concentrations in disinfected effluents were much lower, as expected (Fig. 1), and in most cases were near or below the detection limits for each assay.

The percentages of samples from each treatment step that contained detectable levels of each indicator organism and pathogen are summarized in Table 2. Total and fecal coliforms, enterococci, and coliphages were detected in 100% of

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TABLE 2. Percentage of samples with detectable indicator organisms and pathogens

| Indicator or pathogen | % of samples positive in each stage[a] | | | |
|---|---|---|---|---|
| | Influent | Biological treatment | Filter effluent | Disinfected effluent |
| Indicators | | | | |
| Total coliforms | 100 | 100 | 94 | 63 |
| Fecal coliforms | 100 | 97 | 65 | 27 |
| Enterococci | 100 | 94 | 84 | 27 |
| *Clostridium perfringens* | 93 | 86 | 79 | 61 |
| Coliphages on 15597 | 100 | 97 | 83 | 38 |
| Coliphages on 700891 | 100 | 93 | 80 | 45 |
| Pathogens | | | | |
| Enteric viruses | 100 | 73 | 58 | 31 |
| *Giardia* | 100 | 94 | 88 | 80 |
| *Cryptosporidium* | | | | |
| Total oocysts | 74 | 84 | 71 | 70 |
| Infectious oocysts | 32 | 19 | 19 | 20 |

[a] Data from all sampling events at the six facilities were pooled for each treatment step.

influent (untreated) wastewater samples, in which detection limits were generally 33.3 CFU or PFU · 100 ml$^{-1}$. Three of the 30 untreated wastewater samples were below the detection limit for *C. perfringens* (33.3 CFU · 100 ml$^{-1}$). Enteric viruses (detection limit, 100 MPN · 100 liters$^{-1}$) and *Giardia* (detection limit, 500 cysts · 100 liters$^{-1}$) were also found in 100% of untreated wastewater samples. *Cryptosporidium* oocysts were detected in 74% of the untreated wastewater samples; however, infective oocysts were identified in only 32% of these samples. The detection limit for *Cryptosporidium* in the influent samples depended upon the volume that could be filtered and ranged from 300 to 2,100 oocysts · 100 liters$^{-1}$. Following biological treatment, the concentrations of indicators and pathogens were reduced by about 1 to 2 $\log_{10}$, thus decreasing the frequency of detection of most organisms; i.e., enteric viruses were detected in only 73% of the secondary effluent samples, compared to 100% of the influent samples. The frequency of detection of *Cryptosporidium* increased from 75% in the influent samples to 84% in the secondary effluent samples, due to the more sensitive detection limits in secondary effluent (21 to 94 oocysts · 100 liters$^{-1}$); however, the frequency of detection of infectious oocysts decreased from 32% to 19%. Filtration further decreased the frequency of detection of microorganisms, particularly for enterococci, the coliphages, and *Giardia* (Table 2).

In disinfected samples, total coliforms and *C. perfringens* were detected most frequently, and fecal coliforms and enterococci were least frequently detected (Table 2). While the frequencies of detection of fecal coliforms and enterococci in disinfected effluents were similar (27%), they were simultaneously detected in only one sample, whereas either fecal coliforms or enterococci were detected in 50% of the samples. An assessment of the correlation between total residual chlorine and fecal coliform concentrations in treated effluent samples from all the facilities showed no significant relationship between these parameters (data not shown).

Pathogens, measured on the scale of 100 liters$^{-1}$, were detected in 80% (*Giardia*) to 31% (enteric virus) of samples. Both *Giardia* and *Cryptosporidium* were detected by microscopy in 60% of disinfected effluent samples. Unlike the trend noted for the other organisms, the percentage of samples in which *Cryptosporidium* oocysts were detected remained fairly consistent through the treatment stages (71 to 84%); however, detection limits became progressively more sensitive through the treatment stages, reaching 2.2 to 6.9 oocysts · 100 liters$^{-1}$ in the reclaimed water (disinfected effluents). The percentage of samples containing detectable levels of infectious oocysts decreased from 32% in the untreated wastewater samples to 20% in the reclaimed water samples.

The frequency of detection of the various microorganisms in disinfected effluent samples was compared using Fisher's exact test. Total coliforms and *C. perfringens* were detected in significantly more samples (63% and 61%, respectively) than enterococci or fecal coliforms (both 27%). Other proportional comparisons between indicator organism detections were not significantly different. The protozoan parasites were detected in significantly more disinfected effluent samples than enteric viruses, but there was no significant difference in the proportion of samples in which *Giardia* cysts versus *Cryptosporidium* oocysts were detected. Infective *Cryptosporidium* was detected in significantly fewer disinfected effluent samples than total *Giardia* or *Cryptosporidium*.

Of all the indicator organisms, including the coliphages, the fecal coliforms were found at the lowest concentrations in final effluent samples (Fig. 1) and were among the least frequently detected (Table 2). At hypothetical detection limits of 2 CFU · 100 ml$^{-1}$, total coliforms would be detected in 43% of the disinfected effluent samples, whereas fecal coliforms would be detected in only 10% of the samples ($n$ = 30). Reducing the detection limit to 0.2 CFU · 100 ml$^{-1}$ (the actual detection limit) increased the frequency of detection of fecal coliforms and total coliforms to 27% and 63%, respectively. The relationship between hypothetical detection limit and detection frequency was log linear ($r^2$ = 0.96 for total coliforms and 0.94 for fecal coliforms).

**Predictive relationships between microorganisms.** Data from disinfected effluent samples were analyzed separately (by facility) and as a pooled data set (all facilities) to determine if the concentrations of any of the indicators (total coliforms, fecal coliforms, enterococci, *C. perfringens*, or coliphages) were correlated with each other or with concentrations of pathogens (enteric viruses, *Giardia*, or *Cryptosporidium*). Analysis of results by facility did not yield significant correlations (probably due to small sample sizes); however, significant correlations between indicator organism concentrations were observed in the pooled data sets: i.e., for total coliform and fecal coliform (Spearman's $r_s$ = 0.5986; $P$ = 0.0005), *C. perfringens* versus coliphages on host *E. coli* 15597 ($r_s$ = 0.5303; $P$ = 0.0031), *C. perfringens* versus coliphages on host *E. coli* 700891 ($r_s$ = 0.4981; $P$ = 0.0060), and coliphages on the two *E. coli* hosts ($r_s$ = 0.7915; $P$ < 0.0001). No significant correlation between concentrations of any combination of indicator organism and pathogen was observed.

Enteric viruses were above detection limits in 31% of the disinfected effluent samples ($n$ = 30); however, coliphages and enteric viruses co-occurred in only 13% of the disinfected effluent samples. Concentrations of coliphages on both *E. coli* hosts were plotted against enterovirus concentrations using





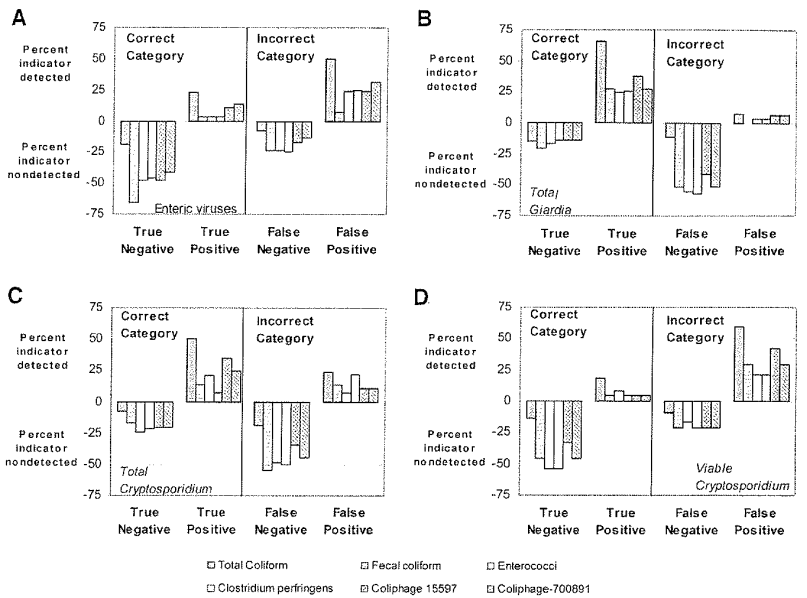


FIG. 2. Relationship between detection of individual indicators and accuracy of pathogen detection in disinfected effluent. All percentages were calculated from the total sample number. Detection limits were 0.2 CFU · 100 ml$^{-1}$ for total and fecal coliforms, enterococci, and *Clostridium perfringens* and 10 PFU · 100 ml$^{-1}$. for coliphages. (A) Enteric viruses; (B) *Giardia* cysts; (C) *Cryptosporidium* oocysts; (D) infectious *Cryptosporidium*.

only samples in which coliphages and enteric viruses were detected, but the slopes of the relationships were not significantly different from 0 (data not shown).

Binary logistic regression was used to test the hypothesis that indicator organism concentrations were predictive of the presence or absence of pathogens in disinfected effluent. Observations of enteric viruses, *Cryptosporidium* oocysts, and *Giardia* cysts were converted to binary data, and the relationship between the concentration of each indicator organism and the presence or absence of each pathogen was assessed, as well as the relationships between the pathogens. Nagelkerke's $R$-square, which can range from 0.0 to 1.0, denotes the strength of the association; stronger associations have values closer to 1.0. Three indicator-pathogen combinations displayed very weak correlations: coliphage concentration (host, *E. coli* 15597) and enteric virus presence/absence ($R$-square = 0.226), fecal coliform concentrations and *Giardia* presence/absence ($R$-square = 0.222), and total coliforms and infectious *Cryptosporidium* presence/absence ($R$-square = 0.241). In each case, the variability in $x$ accounted for only a fraction of the variability in $y$ (odds that a pathogen would be present). A much tighter association was evidenced, for example, between the two coliphage assays on different hosts ($R$-square = 0.762), as would be expected for the two similar assays. No correlations between indicators and pathogens were found using the Spearman correlation; however, this is not unusual as binary logistic regression relies on maximum likelihood, does not require linear relationships between variables (19), and utilizes a binary (0, 1) dependent variable.

The analytical consequences of the failure of indicators to correlate with pathogens are shown in Fig. 2. True negatives are samples in which neither indicators nor pathogens were detected, true positives are samples in which both indicators and pathogens were detected, false negatives are samples in which pathogens were detected when indicators were not detected, and false positives are samples in which indicators were detected when pathogens were not detected. These values add up to 100% for each indicator-pathogen combination. Total coliforms frequently survived the disinfection process; therefore, they tended to be present when pathogens were present,

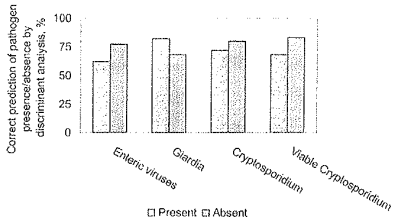


FIG. 3. Discriminant analysis, with results showing the percentage of samples correctly categorized with respect to presence or absence of each pathogen. All of the indicators were used as binary dependent variables. Present, percentage of samples with pathogens actually present and in which pathogen presence was predicted by DA. Absent, percentage of samples in which pathogens were not detected and in which pathogen absence was predicted by DA.

resulting in a relatively high true-positive rate compared to the other indicators (Fig. 2A to D). However, total coliforms also tended to have a low true-negative rate (which would ideally be high) and a relatively high false-positive rate, particularly in the cases of enteric viruses and viable *Cryptosporidium*. In contrast, fecal coliforms, which were relatively infrequently detected in disinfected effluent, tended to have a high true-negative rate but also a low true-positive rate. The percentage of results in the correct categories (true positive and true negative) was not much greater than 50% for any of the indicator-pathogen combinations, although ideally these categories would comprise 100% of observations. Each type of correct and incorrect categorization has distinct implications for public health protection (see Discussion).

DA is a multivariate statistical technique that can be used to classify observations into categories based on a series of independent variables. DA was used to test the hypothesis that the presence or absence of indicator organisms in disinfected effluent samples could predict the presence versus absence of each pathogen (Fig. 3). Indicator organism data for each sample were represented as a string of six binary variables (presence or absence of total coliforms, fecal coliforms, enterococci, *C. perfringens*, coliphages on *E. coli* 15597, and coliphages on *E. coli* 700891). The presence or absence of each of the pathogens was relatively accurately predicted by the suite of indicator organism data for the 29 effluent samples analyzed (Fig. 3). The data are presented as (i) the percentage of samples with pathogens actually present in which pathogen presence was predicted by DA and (ii) the percentage of samples in which pathogens were actually not detected and in which pathogen absence was predicted by DA. When pathogen-positive and pathogen-negative samples were considered together, 72% of enteric virus samples, 79% of *Giardia* samples, 75% of *Cryptosporidium* oocyst samples, and 71% of infectious *Cryptosporidium* samples were placed in the correct category (presence or absence of the pathogen) by discriminant analysis. The absence of all pathogens except *Giardia* was more accurately predicted than pathogen presence. In most cases, removal of one variable (indicator organism) from the data string caused the correct classification rate to decrease by a few percentage points, as one or two additional observations would be misclassified. No single indicator was most highly predictive of membership in the "presence" or "absence" category for pathogens. Interestingly, when coliphage assayed on *E. coli* 700891 was excluded as a variable, it improved the results of the enteric virus analysis by correctly categorizing one additional "presence" sample.

## DISCUSSION

The current monitoring approach to assess the microbial safety of reclaimed water is the measurement of total or fecal coliform concentrations in a single daily grab sample. Utilities and regulatory agencies have assumed a predictive relationship between indicator organism and pathogen levels to protect the public from exposure to pathogens; however, the imperfect relationship between coliform bacteria and pathogens, such as viruses (12, 13, 25) and protozoa (5), through wastewater treatment has been known for some time (see reference 16 for a review). A major goal of this work was to examine monitoring strategies and to determine whether any predictive relationship between conventional and alternative indicator organisms and pathogens in reclaimed water could be discerned among a group of treatment facilities producing reclaimed water.

**Detection of microorganisms.** $\text{Log}_{10}$ reduction of microorganisms through wastewater treatment trains is frequently reported (23, 24) but should not be relied upon as the sole measurement of treatment efficacy. Organisms with very high initial concentrations may experience large log reductions while maintaining detectable levels in disinfected effluents, as illustrated by the total coliforms in this study. Total coliforms experienced an average $\log_{10}$ reduction of >7 from influent to final effluent but were still detected in 67% of disinfected effluent samples.

The linear relationship between hypothetical detection limits and the percentage of samples in which total or fecal coliforms would be detected demonstrates the usefulness of larger sample volumes for detecting indicators, but this ability did not generally translate to a significant predictive relationship between indicators and pathogens. However, if normal volumes (100 ml) had been assayed for fecal coliforms and if we assume that no detection would have occurred in samples in which <1 CFU/100 ml was present, the weak correlations between fecal coliforms versus *Giardia* presence or absence and total coliforms versus infectious *Cryptosporidium* presence or absence would not have been detected (data not shown).

Bacteriophages have been suggested as an alternative indicator for enteric viruses, as their morphology and survival characteristics resemble those of some of the enteric viruses (13, 29). This study found a weak but significant relationship between the presence or absence of enteroviruses and coliphages on *E. coli* 15597 by binary logistic regression. A significant relationship was not found between enteroviruses and coliphages on *E. coli* 700891. This observation, coupled with the improvement in prediction of enterovirus presence or absence by discriminant analysis when coliphage on *E. coli* 700891 was removed as a variable, suggests that the use of other *E. coli* hosts for coliphage assays should be further explored.

The use of U.S. EPA method 1623 for detection of *Cryptosporidium* oocysts does not permit determination of oocyst viability or infectivity, which is crucial information for assessment of the human health risk associated with this parasite. The focus detection method of detecting infectious *Cryptosporidium* (27) has been utilized in a number of studies (11, 15, 21, 22, 26–28, 34), and results coincide well with those of mouse infectivity assays (15). Approximately one-quarter of the disinfected effluent samples with detected *Cryptosporidium* oocysts had detectable levels of infectious *Cryptosporidium*, a disturbing observation in that reclaimed water represents a potential human exposure pathway, depending on how the reclaimed water is used. None of the indicators correlated with *Cryptosporidium* oocysts or infectious *Cryptosporidium*.

Because indicators were not predictive of pathogen presence, the results yielded a high percentage of false-negative or false-positive results for all indicator-pathogen combinations. The relationship of indicators with pathogens that were detected more frequently, such as *Giardia*, tended to show a greater frequency of false negatives (indicators absent but pathogens present). The relationship of indicators with pathogens that were less frequently detected, such as enteric viruses and infectious *Cryptosporidium*, generally showed a higher frequency of false-positives (indicators present but pathogens absent). False-positive results are undesirable because they represent "false alarms." An indicator that is frequently present in the absence of pathogens, such as total coliforms in this study, is not very informative as to the true risk to human health but is relatively conservative in terms of human health protection. False negatives, on the other hand, suggest that probable human health risks are not being detected, which certainly compromises efforts to protect public health. This study suggests that choosing one indicator to predict the survival and/or occurrence of a wide variety of microbial pathogens forces a choice between the two types of error.

Although individual indicator organisms and pathogens were weakly correlated or uncorrelated, the use of discriminant analysis on the composite data set resulted in the relatively accurate prediction of the presence or absence of enteric viruses, *Giardia*, *Cryptosporidium* oocysts, and infectious *Cryptosporidium*. With the exception of *Giardia*, errors tended to be false negatives, as the absence of enteric viruses and *Cryptosporidium* was more accurately predicted than their presence. Further analysis of larger data sets and other indicators, perhaps coupled with measurement of key pathogens, may allow us to refine the predictive capabilities demonstrated by this multivariate analysis. Such a monitoring strategy should protect public health better than the one-indicator system currently used.

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**In the United States District Court**
**for the Southern District of Georgia**
**Augusta Division**

| | | |
|---|---|---|
| R. A. McELMURRAY, III, R. A. McELMURRAY, JR., RICHARD P. McELMURRAY, and EARL D. McELMURRAY, | : | CIVIL ACTION |
| Plaintiffs, | : | |
| v. | : | |
| UNITED STATES DEPARTMENT OF AGRICULTURE, | : | |
| Defendant. | : | NO. CV105-159 |

**O R D E R**

Plaintiffs, R. A. McElmurray, III, R. A. McElmurray, Jr., Richard P. McElmurray, and Earl D. McElmurray (collectively, the "McElmurrays"), filed the above-captioned case against the United States Department of Agriculture ("USDA"), seeking judicial review of an administrative decision, which denied the McElmurrays' application for a "prevented planting" federal farm subsidy.

Presently before the Court are the parties' cross-motions for judgment on the administrative record. Because the agency's decision was arbitrary and capricious, Plaintiffs' motion will be **GRANTED** and Defendant's motion will be **DENIED**.

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BACKGROUND

The City of Augusta operates the Messerly/Butler Creek Wastewater Treatment Plant, which treats industrial and household wastewater. Administrative Record ("AR") 1862.

Before Congress passed the Clean Water Act in 1972, industrial wastewater effluent was dumped into the nation's rivers, oceans, and other waterways, not subject to much, if any, oversight or regulation. See Rapanos v. United States, 165 L. Ed. 2d 159, 168 (2006). One infamous result of this pollution was that the Cuyahoga River, near Lake Erie in Cleveland, Ohio, caught on fire in the 1960s.

After unregulated dumping of industrial pollutants into the nation's rivers was prohibited, effluent from industries began being routed through the municipal wastewater treatment plants across the country, along with household sewage. At municipal treatment plants, wastewater is treated to remove chemicals, pathogens, and toxic metals from the effluent. These materials are concentrated in the byproduct remaining after treatment, sewage sludge. This byproduct also contains beneficial materials like those found in commercial fertilizer. AR 1233-34. Municipalities were left with a









considerable amount of sewage sludge to dispose of in some manner. See Peter Scalamandre & Sons, Inc. v. Kaufman, 113 F.3d 556, 559 (5th Cir. 1997). In the late 1970s, the treated sewage sludge was re-christened "biosolids" and a "land application/recycling" program was started.

The Clean Water Act recognizes that municipal sewage sludge contains toxic pollutants, and it requires that the United States Environmental Protection Agency ("EPA") establish numerical limitations for each such pollutant. 33 U.S.C. § 1345(d)(2)(A)(i) (2001). In 1979, the EPA enacted rules governing the land application of sludge to farmland where crops are grown. 40 C.F.R. § 257.4 (2007). In 1993, the EPA enacted the "Part 503 Sludge Rule," which further regulates the amounts of heavy metals that may be contained in biosolids applications, and reinforced the agency's view that such municipal waste is safe for spreading on farms where crops are grown. 40 C.F.R. Part 503 (2007).

Because the sludge applications that took place in this case ended before Part 503 was enacted, the Part 503 Rules do not supercede the Part 257 regulations in the instant dispute. "Retroactivity is not favored in the law. Thus, congressional enactments and administrative rules will not be construed to have retroactive effect unless their language

requires this result." Bowen v. Georgetown Univ. Hosp., 488 U.S. 204, 208 (1988). The McElmurrays insist that Part 257 governs, and the USDA has never advanced any argument explaining why Part 503 should apply retroactively.

The EPA's Inspector General has criticized the EPA's biosolids program sharply, finding in a 2002 report that the "EPA does not have an effective program for ensuring compliance with land application requirements of Part 503. Accordingly, while EPA promotes land application, EPA cannot assure the public that current land application practices are protective of human health and the environment." AR 1485, 1518.[1]

Since 1938, the McElmurrays have owned and operated a family dairy farm near Hephzibah, Georgia. In the 1970s, Augusta developed a land application program, whereby treated sewage sludge from the Messerly plant was recycled as fertilizer and applied to private farmland, at no cost to the farmers. In 1979, the McElmurrays and Augusta entered into a series of agreements, and the City began applying its sewage sludge at the McElmurrays' farm. Plaintiffs contend

[1] Likewise, the Fifth Circuit has noted that the experts have yet to reach a consensus regarding the safety of land application of sewage sludge generally. Scalamandre & Sons, 113 F.3d at 561-62.









that they were told the fertilizer was safe, and the applications continued on their land through 1990.

According to R. A. McElmurray, III, in November 1990, he was having trouble with his crops. McElmurray described the problem to his brother-in-law, who had a degree in agriculture from the University of Georgia. McElmurray related that his brother-in-law opined that the problem was probably aluminum toxicity. Thereafter, McElmurray asked Augusta's land application supervisor to test for aluminum in the sludge. When the result was high, McElmurray ceased allowing sludge applications on his family's farmland. AR 1743.

McElmurray conceded that he did not quit planting the land involved in this dispute until 1998. The land produced a full crop that year, but planting was ceased due to "[l]iability, and what it was doing to our dairy cows[.]" AR 1777. According to Plaintiffs, only years after the sludge applications took place did they learn the full extent of the damage that the sewage sludge had wrought on their land. The McElmurrays accused the City of withholding pertinent information about the particular locations on their land where the sludge was applied, the volume applied, and the presence and amount of toxic metals contained in the

sludge. The McElmurrays contend that the sludge poisoned plants grown on the land, which were fed to their dairy cattle, causing the cows to become seriously ill and die.

As part of the Farm Bill of 2002, Congress provided certain farmers with subsidies, which were based on historical acreage and yields, not current production choices. Direct and Counter-Cyclical Program, 67 Fed. Reg. 64,748 (Oct. 21, 2002). A farmer could establish his base acres and payment acres by including "any acreage on the farm that the producers were prevented from planting during the 1998 through 2001 crop years to covered commodities because of drought, flood, or other natural disaster, or other conditions beyond the control of the producers. . . ." 7 U.S.C. § 7911(a)(1)(A)(ii) (2007 Supp.)(emphasis added).[2]

> Prevented plant[ing] means, for the purpose of establishing base acres under § 1412.201, the inability to plant a crop with proper equipment during the established planting period for the crop or commodity. A producer must prove that the producer intended to plant the crop and that such crop could not be planted due to a natural disaster rather than managerial decisions. The natural disaster that caused the prevented planting must have occurred during the established planting period for the crop.

7 C.F.R. § 1412.103 (2007).

On January 15, 2003, Plaintiffs submitted a request for acreage/disaster credit to the USDA, listing environmental contamination of the land on their application as the reason for the "prevented planting." The McElmurrays listed the intended crops as 907.1 acres of cotton[3] and 204.8 acres of corn for the years 1999 to 2001. The following day, the McElmurrays submitted additional forms, stating that their request included an additional 559.1 acres of cotton and 59.5 acres of corn for the years 1999 to 2001. The total request was for a prevented planting credit of 1466.2 acres of cotton and 264.3 acres of corn. AR 2134.

At first, Plaintiffs' applications were reviewed by the USDA's Farm Service Agency ("FSA") County Committee. After a preliminary review by the County Committee, the McElmurrays' application was denied because the damage was

[2] While it is not very material, in light of the stipulation made by Deputy Administrator Johnson, discussed below, the Court takes notice of the language used in the statute. The law does not appear to support government counsel's suggestion at oral argument that the Court should view the McElmurrays' claim skeptically because they did not qualify under the law for the credit, but were only able to apply because a special exception was made for them.

[3] While it may seem odd at first blush, the parties agree that cotton is a food-chain crop. It is common for cows to be fed cotton hulls after the cotton lint is removed from the plant (and people consume beef and dairy products), and cottonseed oil is a common ingredient in many snack foods that people eat, like potato chips. AR 1262. Moreover, there is substantial evidence that cotton is not an economically viable crop without considering the marginal value of cottonseed. AR 1049-50 & 1055-56.









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not caused by a natural disaster, as the County Committee believed was required for relief. Yet, a superior FSA official in Washington, D.C., John A. Johnson, reversed the basis for that determination. Johnson, the FSA Deputy Administrator for Farm Programs, stipulated that the McElmurrays could receive the subsidy if their land was contaminated, and the contamination caused the McElmurrays to refrain from planting the intended acreage. On April 22, 2003, the FSA County Committee again denied Plaintiffs' application for payments.

The McElmurrays appealed to the FSA State Committee. This five-member committee of farmers oversees USDA farm programs in Georgia, sets local policies, and settles agriculture-related disputes that involve farmers and public policy. After reviewing the record and conducting multiple hearings, the FSA State Committee voted in favor of Plaintiffs' application, by a vote of three to two. In finding for the McElmurrays, the State Committee discounted the advice of its attorney, Donald Kronenberger, who had opined that the State Committee was bound by certain documents provided to the Committee by the EPA, and had to deny the McElmurrays' application. AR 1988 & 2745.









However, the State Committee's decision was stayed, pending a review by the FSA's Deputy Administrator for Farm Programs, pursuant to 7 C.F.R. § 1412.102(d). Although the entire agency record was forwarded to Johnson, there is no indication that the Deputy Administrator reviewed the file. AR 2134 & 2433. On March 18, 2004, the Deputy Administrator overruled the State Committee and denied Plaintiffs' application. AR 2256-57. In part, Johnson's determination was based on a decision of the Richmond County Superior Court, which had granted summary judgment in favor of Augusta, against the McElmurrays in a related civil lawsuit. AR 2000-01. At the time, that decision was on appeal before the Georgia Court of Appeals. AR 2066. Johnson's decision was made over the State Committee's continuing objection. AR 0002 & 2259-60.

On April 22, 2004, Plaintiffs filed another appeal, this time with the USDA's National Appeals Division ("NAD"). On September 2 and 3, 2004, a final hearing was held before NAD hearing officer James Mark Jones. On December 3, 2004, Jones upheld the denial of the farm credit, finding no error

in the FSA's decision to deny the McElmurrays' application, which was based on certain opinions provided by the EPA.[4]

On January 3, 2005, Plaintiffs brought this action for judicial review of the NAD's final administrative determination in the United States District Court for the Northern District of Georgia, pursuant to 7 U.S.C. § 6999 (1999). On September 12, 2005, the case was transferred to the Southern District of Georgia.

On December 27, 2005, Plaintiffs amended their complaint, and on February 2, 2007, they moved to supplement the administrative record. On March 5, 2007, the USDA moved for judgment on the administrative record. On September 28, 2007, Chief Judge William T. Moore, Jr., denied Plaintiffs' motion to supplement the administrative record. On October

[4] During the NAD appeal process, Jones opined that he did not have the authority to determine whether the land was contaminated, and suggested that the EPA had decided that the land was not polluted. To the contrary, Plaintiffs' counsel, F. Edwin Hallman, Jr., indicated that the EPA had not resolved the issue properly, and argued that the question of contamination was appropriately before Jones. AR 2633-34. Jones also stated that, as far as his review was concerned, "anybody's that's been untruthful, is not going to make any difference." AR 2682 & 2694. Based on these statements, it appears that Jones' view of his authority in deciding the case was unduly narrow, which preordained his conclusion in favor of the agency. To the extent that Jones found the EPA's position questionable or unreliable, either because of the underlying data it was based on, or because the sister agency failed to consider the actual applications presented by the McElmurrays, then Jones should not have relied on, or deferred to, such findings. AR 1495.







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4, 2007, Chief Judge Moore reassigned the case to the undersigned for plenary disposition.

STANDARD OF REVIEW

Judicial review of the USDA's final determination to deny a prevented planting credit is governed by the Administrative Procedures Act ("APA"). 7 U.S.C. § 6999 (1999); 5 U.S.C. § 701-706 (2007). An agency's decision, including its actions, findings, and conclusions, should not be overturned unless the decision is "arbitrary, capricious, an abuse of discretion, or otherwise not in accordance with law" or unless it is "unsupported by substantial evidence." 5 U.S.C. § 706(2)(A) & (E) (2007).

> The scope of review under the "arbitrary and capricious" standard is narrow and a court is not to substitute its judgment for that of the agency. Nevertheless, the agency must examine the relevant data and articulate a satisfactory explanation for its action including a "rational connection between the facts found and the choice made." . . . In reviewing that explanation, we must "consider whether the decision was based on a consideration of the relevant factors and whether there has been a clear error of judgment." . . . Normally, an agency rule would be arbitrary and capricious if the agency has relied on factors which Congress has not intended it to consider, entirely failed to consider an important aspect of the problem, offered an explanation for its decision that runs counter to the evidence before the agency, or is so

> implausible that it could not be ascribed to a difference in view or the product of agency expertise. The reviewing court should not attempt itself to make up for such deficiencies; we may not supply a reasoned basis for the agency's action that the agency itself has not given.

Motor Vehicle Mfrs. Ass'n v. State Farm Mut. Auto. Ins. Co., 463 U.S. 29, 43 (1983)(internal cited and quoted sources omitted)(emphasis added).

> Substantial evidence is more than a scintilla, and must do more than create a suspicion of the existence of the fact to be established. "It means such relevant evidence as a reasonable mind might accept as adequate to support a conclusion." . . . and it must be enough to justify, if the trial were to a jury, a refusal to direct a verdict when the conclusion sought to be drawn from it is one of fact for the jury.

NLRB v. Columbian Enameling & Stamping Co., 306 U.S. 292, 300 (1939)(internal case citation omitted)(emphasis added).

The Eleventh Circuit has explained that "[t]he substantial evidence test is no more than a recitation of the application of the 'arbitrary and capricious' standard to factual findings." Fields v. United States, 173 F.3d 811, 813 (11th Cir. 1999). The agency must give reasons for its findings. When the evidence is in conflict, the agency must explain why it credited some probative evidence but not the conflicting evidence. Vemco, Inc. v. NLRB, 79 F.3d 526, 529 (6th Cir. 1996). The substantial evidence standard does









not excuse the agency from its duty to engage in reasoned decision-making. Haebe v. Dep't of Justice, 288 F.3d 1288, 1301 (Fed. Cir. 2002).

"Except as otherwise provided by statute, the proponent of a rule or order has the burden of proof." 5 U.S.C. § 556(d) (2007); Am. Trucking Ass'ns, Inc. v. United States, 344 U.S. 298, 319-20 (1953); Dir., Office of Workers' Comp. v. Greenwich Collieries, 512 U.S. 267, 272-81 (1994). In this case, the McElmurrays bear the burden of proof because they sought the federal subsidy. AR 2440.

While Daubert does not apply to agency decisions in any formal respect, the principles underlying that decision do apply. Pasha v. Gonzalez, 433 F.3d 530, 535 (7th Cir. 2005). Significantly, the APA demands that agency decisions not be based on unreliable evidence, and an agency must provide a coherent reason for refusing to consider the testimony of expert witnesses. Chao v. Gunite Corp., 442 F.3d 550, 559 (7th Cir. 2006). In other words, "deference has its limits." Id.

Nonetheless, contrary to Plaintiffs' repeated contentions throughout the administrative proceedings, agencies may rely on hearsay in making their determinations. Richardson v. Perales, 402 U.S. 389, 402-04 (1971); AR 1427.

The APA provides that any oral or documentary evidence may be considered, so long as the agency excludes irrelevant and immaterial evidence. 5 U.S.C. § 556(d) (2007).

The Court's consideration of the case is limited to the administrative record before the agency when the USDA made its determination to deny Plaintiff's application for prevented planting credits. Dkt. No. 61; see Alabama-Tombigbee Rivers Coal. v. Kempthorne, 477 F.3d 1250, 1262 (11th Cir. 2007)(court should consider evidence outside the administrative record "only where there is initially 'a strong showing of bad faith or improper behavior' by the agency").

**DISCUSSION**

The issue presented in this case concerns whether the McElmurrays' land was contaminated by sludge applications such that the soil was unsafe for growing food-chain crops. The only dispute presented in this case concerns whether the McElmurrays' land was too polluted to use. The agency has never disputed the question of causation, and the evidence of record supports a finding that causation was established. AR 1777.









To determine whether Plaintiffs have met their burden of proof, the Court will examine the sludge data provided by Augusta, the views of the experts as to contamination, and the EPA's contributions, in turn. Along the way, the Court will examine the proof of contamination, and consider the appropriate remedy in light of the evidence submitted.

**I. Augusta's Data**

Much of the evidence that was considered by the federal agencies in this case, and by Plaintiffs' experts, is based on data collected by the City of Augusta, with respect to its sludge application program from 1979 to 1990. Although there is a broad consensus that Augusta's reports were unreliable, incomplete, and in some cases, fudged, the City's information is an integral part of this case.

According to the deposition testimony of Hugh Avery, Augusta's sewage sludge land application supervisor beginning in 1984, the City's sludge application data going back to 1979 were inaccurate, and the records predating his tenure were "in shambles." AR 2604-05. Specifically, Avery testified that the records were incomplete and missing

critical information about which fields received sludge applications. AR 2604.

Jeff Larson, an official with the Georgia Environmental Protection Division ("EPD"), conducted an audit of the Messerly plant in 1998, and reported in an internal memorandum that problems with the sludge application program persisted, even after the program had been delegated in part to a reputable contractor, AMSCO, Inc. Larson stated that two hundred truckloads of sludge were leaving the facility for land application each day, "much of which may not be meeting requirements[.]" AR 0985 & 1669.

Larson found fault with the City's digestion system and its inappropriate sludge sampling techniques. Larson asserted that the City ignored certain results to make the program look better than it was in fact. AR 1668 & 1670. The plant was in "very poor condition," with major units rusted and out of service. Larson also reported that management at the facility was "literally a joke[,]" and that the "staff is the most demoralized bunch of people I have ever witnessed[.]" AR 0986.

The final EPD report based on Larson's investigation found that "[t]he sludge regulations are based on a well run pretreatment program which is not the case in Augusta. The









sludge is highly corrosive. . . ." AR 1670. The report recommended that the plant be shut down immediately. AR 1671. Neither the USDA nor the EPA asserted that conditions at the Messerly plant had deteriorated since 1990. Indeed, Larson indicated that the plant had "been grossly neglected for years." AR 0986.

Dr. Lewis Goodroad, Plaintiff's expert soil scientist, reported that Augusta manipulated its data by averaging lab results over several months in an attempt to reduce the levels of metals present in the sludge. AR 0681. A former Supervisor of the Messerly Wastewater Treatment Plant, Allen Saxon, confirmed that this was the case. AR 0808. An employee of the USDA, Tommy Weldon, agreed that it "would be hard to come to a conclusion based on [Augusta's] data[,]" because of the City's "sloppy record-keeping and inaccurate data." AR 2758.

There is also evidence that the City fabricated data from its computer records in an attempt to distort its past sewage sludge applications. AR 502-03. In January 1999, the City rehired Saxon to create a record of sludge applications that did not exist previously. Saxon prepared sludge spreadsheets in 1999, which showed cumulative loading calculations for the first time in the twenty-year history

of the City's land application program. AR 0798-818, 844-52, & 659-685.

In other instances, there is evidence that Augusta altered its records to show that the sludge was applied to different, incorrect fields. Handwritten notes on the City's records contradict the number of acres involved, and the volume of sludge applied, as those figures are represented in the 1999 spreadsheet developed by Saxon. AR 2598. Other evidence indicates that City officials altered the spreadsheets in 1999 in an attempt to remove any record of the application of hundreds of thousands of gallons of sludge to hundreds of acres on the McElmurrays' farm. AR 0643-47. Goodroad reported that 18.9 million gallons of sludge had been applied to Plaintiffs' fields but was not recorded by Augusta. AR 0650.

Notwithstanding these facts, USDA employee Ronald Carey testified that evidence that Augusta changed its records years after applications were made, to reflect that the sludge was applied to larger plots of land than was actually the case, would not concern him. AR 2590.

The McElmurrays contend that Augusta's records, under-representative though they are, show that Augusta violated federal law in placing the sludge onto their land, citing,









inter alia, 40 C.F.R. § 257.3-5 (2007). This federal regulation governs allowable cadmium and polychlorinated biphenyl ("PCB") limits. Plaintiffs contend that this violation is plain evidence of contamination of Plaintiffs' land and the unsuitability of the property for the production of food-chain crops. AR 658-685. The Court will explore that evidence and regulation below.

## II. The Experts' Responses: Hall and Haaland Describe the Evidence of Contamination

During the administrative proceeding, Plaintiffs presented credible evidence from qualified experts that supported their contention that their farmland was contaminated. That evidence was not considered by the EPA or the USDA, but the McElmurrays' applications were denied anyway.

William L. Hall is a professional engineer and the CEO of NewFields, Inc., an environmental consulting firm based in Atlanta, Georgia. Plaintiffs retained Hall and NewFields as experts in separate litigation against the City of Augusta relating to the sludge applications to their land. On April 1, 2003, Hall signed an affidavit that was submitted to the FSA and included in the administrative

record. AR 0329-0336. Hall has extensive experience with respect to the impact of heavy metals on the environment, and has been the project manager on seven Superfund sites that reached final closure. AR 0329, 0361-68, & 0691-92.

Hall made extensive findings about Augusta's sludge data and the specific instances of contamination on the McElmurrays' farm. Hall opined that about 2,234 acres of the McElmurrays' farm was unusable, due to contamination from the heavy metals contained in the sewage sludge. AR 0330. Hall noted that high contaminant concentrations were based on the limited sampling that had been completed, and opined that there was a correlation between cow mortality and the consumption of silage, which is animal feed made from forage plants, grown on contaminated fields. AR 0331.

Hall reported that Augusta allowed companies to dump industrial waste into an open pit at the Messerly plant, and that the City failed to monitor the amount and type of waste being dumped into the pit while the McElmurrays were receiving sludge. Hall also faulted the plant's managers for failing to keep records showing when and where dangerous contaminants were placed on the McElmurray land. AR 0332 & 0782. Hall recounted that the sludge applications were unpredictable and variable in terms of the kinds and amounts









of contaminants contained in the sludge. This resulted in "hot zones" of extremely high contaminant ratings on random parts of the McElmurray farm. AR 0333.[5]

Of particular concern, Hall noted that over ten percent of samples showed highly elevated cadmium concentrations, at levels up to seven times the limits that had been established at some Superfund sites, which were being remediated under the Comprehensive Environmental Response, Compensation, and Liability Act ("CERCLA"), 42 U.S.C. § 6901-6992k (2003).

Further, Hall criticized the City's sampling practices, explaining that Augusta took less than five cubic feet of dirt per million cubic feet of soil, and only within the top eight inches of the soil column. According to Hall, this part of the soil is the least likely to retain contaminants over time, due to leaching. Hall points out that the City's data shows that the sludge contaminant concentrations became highly erratic, with extreme metal concentration spikes, beginning in 1986. Hall opined that this time frame coincided with a significant increase in mortality in the

[5] Dr. Goodroad reported that former county agent Bill Craven had agreed that sludge applications on the McElmurrays' land were not uniform. AR 0372.

McElmurrays' dairy herd, when compared with the state average. AR 0335.

In an expert report, Hall reported specific shortcomings in Augusta's field update report data, which purport to record "year to date" ("YTD") and "lifetime total" ("LTT") applications of particular heavy metals on the McElmurrays' land. The reports are inconsistent in that they show YTD figures that match LTT figures and, relatedly, subsequent application data that does not account for prior applications in reckoning the LTT data.

In other instances, the field update report data show cumulative LTT figures that decrease from one application to the next. AR 0342 & 0350. Still, Augusta's data indicated that cadmium and molybdenum levels on the McElmurray farm were above regulatory limits in certain instances, in amounts ranging from 37% to 1400%. AR 0352-53. Hall opined that the high concentration of molybdenum in the McElmurrays' silage was particularly serious, given the time that had elapsed since the sludge was placed on the land. The McElmurray samples were taken in 1998, eight years after Plaintiffs halted the land application program. AR 0356.

Additionally, Hall faulted Augusta's data for lacking information. Complete months and years were missing from









the field update reports, which meant that Augusta's sludge application estimates were under-reporting the toxicity of the soil by a wide margin. Hall also called attention to the City's failure to monitor molybdenum, despite evidence of its presence, given that it is a known hazard on land used by dairy cattle. AR 0343.[6] Hall reported that after the City learned about high concentrations of molybdenum in its sludge, it failed to notify researchers at the University of Georgia about the presence of this heavy metal. Because the University scientists failed to test for molybdenum, the researchers' advice to apply lime to raise the soil's pH level, and thereby limit crop toxicity, was faulty or incomplete. AR 0348.

Dr. Ron Haaland, an Auburn University professor in the School of Agriculture, was hired by Augusta's attorney as an

[6] To the extent it has any relevance, Hall noted that even though 40 C.F.R. Part 503 limits concentrations of molybdenum to 75 parts per million ("ppm"), the sample concentrations on the McElmurrays' land ranged from 25 ppm to almost 140 ppm. AR 0344. Hall drew attention to the fact that the USDA expressed concern about the molybdenum levels permitted in the EPA's Part 503 Rules. The USDA recommended that the EPA reduce the ceiling concentration limit for molybdenum in biosolids to 54 ppm. Even under the EPA's more relaxed limit, 75 ppm, Hall pointed out that Augusta's sludge was applied at about twice that level in some cases. AR 0756. Nonetheless, it is not apparent that this particular test result shows contamination of the soil, in light of the McElmurrays' protestations that Part 503 does not apply in the instant case.

expert witness in the Superior Court litigation. AR 0423. Haaland performed testing at the McElmurrays' farm, and concluded that the soil was not unsafe for growing food-chain crops. Haaland blamed any ill effects from the sludge on the McElmurrays' failure to pay attention to detail and oversee the sludge application program properly. AR 0420 & 1374.

The McElmurrays took issue with Haaland's soil-testing methodology before the State Committee. Plaintiffs asserted that Haaland attempted to find a way to discredit the McElmurrays' samples and show no contamination on their property. The McElmurrays claimed that Haaland set up their property using a nine acre grid system, and pulled one sample from each acre in the nine acre grid. Plaintiffs submit that Haaland then combined the samples together to dilute any results showing the presence of contaminants. AR 1868.

Although Haaland is the only expert that the parties have disclosed that tested the McElmurrays' soil and disagreed with Plaintiffs' experts' conclusions of contamination, the agency never responded to this criticism of Dr. Haaland's methodology. At oral argument, the government's lawyer declined to address this point, leaving









lingering doubt about there being any evidence that supports the government's determination that the land was not contaminated.

Evidence related to the pH level of the soil also supports Plaintiffs' position that the land was too polluted to grow crops for human consumption. Food-chain crops may not be grown when the pH of the sludge and soil mixture is less than 6.5 and the cadmium level therein exceeds 2 ppm. 40 C.F.R. § 257.3-5(a)(1)(i) (2007). Nor may such crops be grown where the annual application of cadmium from solid waste exceeds 0.5 kilograms per hectacre, or .45 pounds per acre. 40 C.F.R. § 257.3-5(a)(1)(ii) (2007).

Plaintiffs' evidence shows that sewage sludge with cadmium concentrations of between 4.2 ppm (January 1980) and 1200 ppm (February 1990) were deposited on Plaintiffs' farmland for ten years. Many fields contained annual cadmium deposits that were two or three times the federal limit. AR 1132-1157. According to the information supplied by Augusta, the pH level of the sludge and soil mixture at the McElmurrays' farm was below the 6.5 minimum consistently. These figures were accepted as credible by Plaintiffs and their experts, and the EPA, which relied on

Augusta's data only in reaching its conclusions in this case. AR 892-913.

Another factor supporting Plaintiffs' argument that the land was contaminated is that certain metals react to the soil's pH level differently. Augusta advised the McElmurrays to keep the pH level of their soil elevated, to attenuate the effect that certain heavy metals would have on their crops. Generally, most metals will accumulate from the soil into the plants grown thereon when the soil has a low pH level. However, molybdenum and arsenic are the exception to this rule. AR 1783. According to experts retained by both parties, molybdenum accumulates in plants more easily and directly when soil pH levels are high. AR 0345 & 0411. As a result, Augusta's suggestion that applying lime to raise the pH level would mollify any contamination concerns was misleading or incomplete. AR 0348.

Other specific evidence showed that heavy metals were found at levels that were above the regulatory limits on the McElmurrays' farm, making the land unfit for food grown for human consumption. On one piece of property alone, antimony levels registered at 96.8 ppm, while the regulatory limit was 4 ppm. Arsenic registered at 44.2 ppm, more than twice









the amount allowed by law. Cadmium was found at a level of 6.41 ppm, which was more than three times the level deemed safe under the law. Selinium registered at 5.4 ppm, although the cleanup standard provided under the law was set at 2 ppm. Thallium was found at 51.6 ppm on that particular piece of property, although the regulatory limit is 2 ppm.[7] AR 1801-03. The levels were similar on other parcels farmed by the McElmurrays. AR 1803-06.[8]

At oral argument, the McElmurrays noted that the administrative record showed that Augusta's lab reports demonstrated that PCBs were placed on their land at a level in excess of 5,000 ppm, even though the allowable limit under EPA standards was 2 ppm. See 40 C.F.R. § 257.3-5

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[7] According to the evidence contained in the administrative record, Thallium is quite dangerous to dairy herds. AR 0916. Plaintiffs maintain that Thallium was used as a catalyst by NutraSweet in making its sweetener, and NutraSweet was the largest user of the Augusta sewer system during the 1980s. AR 1808. The McElmurrays contend that the City did nothing to limit large or illegal dumping, like that by NutraSweet. A 1998 EPD audit provided some support for this contention, finding that "[t]here are no local limits for conventional pollutants" at the Messerly plant. AR 1669.

[8] This portion of the administrative record discusses the limits allowed under Georgia law. At oral argument, Plaintiffs' attorney conceded that federal law controlled, but reported that Georgia law was coextensive with federal requirements in this respect. Although counsel for Defendant expressed no opinion about the applicability or the relevance of state law, the Government's lawyer did not disagree that the relevant state and federal standards were the same.

(2007). The government has not disputed that characterization of the evidence, and it is supported by the administrative record. AR 0535.

Moreover, Plaintiffs submitted evidence that the sludge contained hazardous levels of chlordane, and that it was applied to their land from 1980 to 1985, even though it was banned in 1978. AR 843-883 & 1109-57; Velsicol Chemical Co., et al.: Consolidated Heptachlor/Chlordane Cancellation Proceedings, 43 Fed. Reg. 12,372, 12,373 (March 24, 1978). Plaintiffs cite the following additional sources as evidence that the sludge was applied to their land in violation of federal law: AR 0329-85, 0623-837, 1064-1073; see 40 C.F.R. Part 257, 40 C.F.R. Part 261, 40 C.F.R. Part 258, Appendix I and II.

The evidence in the administrative record shows that the McElmurrays' land is contaminated and unfit for growing food-chain crops. Plaintiffs maintain that they would have violated the law by planting crops, putting human health and welfare at risk. The McElmurrays submit that the high mortality level experienced by their dairy herd is proof of the dangers associated with planting food crops on their land.









The Court concludes that the evidence of contamination on the McElmurrays' land was substantial, and the data provided by Augusta was flawed.

## III. The EPA's Contributions: Mehan, Brobst, Kaufman, and Breen

The USDA submits that applications for prevented planting subsidies, like the one submitted by Plaintiffs, are usually based on the effects of natural disaster to land and crops. Because Plaintiffs' claim had a more unusual basis, alleged contamination of the land, the USDA had to consider the alleged biological effects of sewage sludge on Plaintiffs' land.

Therefore, in evaluating Plaintiffs' application, the USDA sought the opinions of officials at the EPA. The USDA recognized that it possessed limited knowledge regarding the biological effects of sewage sludge on soil, and it sought the advice of the EPA. An FSA handbook allowed it to do so, in certain instances where it lacked the expertise to make proper findings:

> If a reviewing authority receives a request for review involving a technical determination by a Federal Agency other than FSA and NRCS, the reviewing authority shall . . . contact a representative of the applicable Agency to

> discuss and clarify the technical findings, as needed[,] . . . [and] accept as binding, written factual findings or technical determinations of the other Agency.

AR 1495.

The USDA received varying responses from EPA officials about the safety of the sewage sludge land application program and the McElmurrays' applications. Finally, the EPA declared that its official position as to the McElmurrays' petition was set out in a letter written by EPA's Assistant Administrator, G. Tracy Mehan, III. Consequently, the Court will focus on Mehan's letter first.

On December 24, 2003, Mehan wrote a letter responding to a petition from the Center for Food Safety seeking a nationwide moratorium on the land application of sewage sludge. Mehan's letter was broad in scope and only mentioned the McElmurrays' situation in a brief aside. Instead, Mehan considered a number of other issues in rejecting the proposed moratorium, concluding that "[p]etitioners do not present scientifically-based evidence or documentation that links the land application of sewage sludge or chemical pollutants allegedly contained in sewage sludge to human health and environmental impacts that are described in the petition." AR 1521.









Mehan did address Augusta's sludge application program, but all of his specific remarks focus on the Boyce dairy farm's litigation against Augusta, which was a companion case to the Superior Court lawsuit that the McElmurrays had filed against the municipality. For any opinions that Mehan does express about the Messerly treatment plant, Mehan relies on Augusta's sludge data only, which has been called into question by representatives of both parties in this case, as well as disinterested third parties, and Augusta's own representatives. AR 0023, 0332-35, 0342-43, 0350-56, 502-03, 0643-47, 0650, 0681, 0782, 0798-818, 0844-52, 0985-86, 1512-15, 1668-71, 2604-05, 2758, & 2598.

Specifically, Mehan recounts the Center for Food Safety's assertion that, "On June 24, 2003, a court in Georgia ruled that the land application of sewage sludge was the legal cause of the damage to the farmland and the deaths of the farm's prize-winning cattle[.]" AR 1512. Mehan commented that the "EPA understands that the jury awarded $550,000 of the $12.5 million in damages sought by the plaintiffs without any findings of fact." AR 1512.

Mehan quoted from a letter written by Augusta's lawyer, James Ellison, to the EPA about the verdict. According to Ellison,

> [o]ne of the breaches contended by the Boyces was an alleged failure to keep and maintain good records. Unfortunately and regrettably in the early days of Augusta's land application program, record-keeping was a problem, mostly due to programming problems with the biosolids application software used by Augusta. The verdict may well have represented the jury's dissatisfaction with the records maintained by Augusta.

AR 1512.[9]

Plaintiffs argue that Defendant is wrong to rely on Mehan's letter as a factual finding or a technical determination by the EPA that Plaintiffs' land was not contaminated because Mehan's letter was not written in response to Plaintiff's applications. Mehan's letter contains no factual findings regarding Plaintiffs' land, and is not addressed to the USDA. Rather, Mehan wrote in response to a petition from a public interest group seeking

[9] Not surprisingly, Hallman, who also represented the Boyce family in the Superior Court case, takes issue with Ellison's characterization of the verdict. Hallman asserts that, under Georgia law, a general verdict ratifies the claims contained in the operative complaint. AR 1556 (citing Ga. Code Ann. § 9-12-1). What motivates any particular jury verdict (and the amount of damages awarded) is subject to endless speculation, and what happened in the Boyce case is not particularly germane to whether the McElmurrays' land was contaminated. Still, the information is material to the extent that it shows the basis for the EPA's opinion.









a moratorium on the land application of sewage sludge in the United States.

The procedure described in the FSA Handbook for obtaining a technical determination from another agency requires a representative of an agency to "contact a representative of the applicable Agency to discuss and clarify the technical findings, as needed . . . ." AR 1495. Such was not done by the USDA's representatives with Mehan. In addition, Mehan makes clear that the petition relates only to the application of sludge under Part 503. AR 1504. As has been discussed, this law does not apply to the McElmurrays, whose land applications of sludge ceased before the enactment of the regulation in 1993. In short, Mehan's letter is largely irrelevant to the McElmurrays' applications before the USDA.

USDA employees Ronald Carey and Tommy Weldon also asked Robert Brobst, a member of the EPA's Biosolids Incident Response Team ("BIRT"), about the contamination averments made by the McElmurrays. AR 1227-1229. In response, Brobst opined in a letter that the McElmurrays' land was not contaminated. AR 1230-1240.

Because Brobst concluded that Augusta's data sets were the most "complete and reliable," he used its information,

and did not consider (or find any particular fault with) the information provided by the McElmurrays. Brobst's letter focused on cadmium levels at the farm, and at least in his letter, he found that cadmium levels there were within normal national background ranges. Notably, the data, which Brobst claims was obtained in 1999, puts cadmium concentrations on the Plaintiffs' land at .41 mg/kg, which is twice the national average cited by Brobst, .175 mg/kg. AR 1281-1283. Brobst also stated that other metals found in the sludge, or on the land, were within normal background ranges. AR 1238.

On December 11, 2003, Brobst further explained his results to the FSA State Committee. AR 1876-1899. Plaintiffs emphasize that on that day, Brobst made an important qualification to his earlier representation, when he conceded that his original conclusions, which were based on national background concentrations, should not, or need not, be used because those levels are dissimilar to the characteristics present in soil located in Burke County, Georgia. AR 1888, 1477, & 1567-68. Perhaps more importantly, Brobst admitted that one of the McElmurrays' fields contained about forty to fifty times the allowable lifetime loading level of cadmium. AR 2652.









Brobst provides scant support for his determination that the land was not contaminated. Although his letter cites to some data in support of that conclusion, he never explains where such data were found, or how he arrived at such figures. AR 1237-38. It is difficult, if not impossible, to evaluate the trustworthiness of such a conclusion without this information.

As Plaintiffs note, Brobst's letter does not address information contained in Plaintiffs' applications, but exclusively addressed data obtained from the City of Augusta in 1999. Brobst admitted that he did not evaluate the data presented in support of Plaintiffs' applications for prevented planting credit. Because Brobst concedes that his conclusion is based on Augusta's unreliable, and to some extent, invented, data, Brobst's finding has little merit on its own.

On December 31, 2003, Plaintiffs submitted an affidavit from Hugh Kaufman, a senior policy analyst at the EPA, to the State Committee in an effort to rebut Brobst's position. Kaufman explained that he had been involved with testing and evaluating the McElmurrays' land, and opined that the McElmurrays' land was contaminated, and unfit for growing food-chain crops. AR 1478, 1487-1489, & 1548.

On January 28, 2004, Barry Breen, the EPA's Principal Deputy Administrator, wrote a letter to the FSA explaining that Kaufman's affidavit was not the official view of the EPA, and that Mehan's letter was the agency's position. AR 1545. Indeed, the FSA relied on Mehan's letter as the official position of the EPA. AR 2600. Yet, there is no evidence that Mehan ever reviewed the Plaintiffs' applications, other data in the administrative record, or any of the reports detailing the sewage sludge applications on Plaintiffs' land from 1979 to 1990. AR 2663. USDA employee Carey allowed that Mehan made no specific finding that the McElmurrays' land was not contaminated. AR 2664-66.

The EPA's unexplained rejection of Kaufman's position, in favor of the largely irrelevant Mehan letter shows that the decision was not based on substantial evidence. It was arbitrary and capricious for the USDA to defer to Mehan's letter as a technical determination or a written factual finding. Sierra Club v. Martin, 168 F.3d 1, 4-7 (11th Cir. 1999). To the extent that the USDA relied on Brobst's opinions, that was arbitrary and capricious because Brobst did not consider all the relevant data. Motor Vehicle Mfrs. Ass'n, 463 U.S. at 43.









An agency may discredit the uncontradicted witness testimony based on credibility grounds, but only if the agency provides reasons for its credibility determination. Tieniber v. Heckler, 720 F.2d 1251, 1254-55 (11th Cir. 1983); NLRB v. Walton Mfg. Co., 369 U.S. 404, 406-07 (1962). Breen failed to justify why the EPA accepted Mehan's letter over Kaufman's affidavit, or even attempt to explain how Mehan's letter could qualify as a written factual finding or technical determination of the McElmurray matter. Moreover, no one at the EPA ever took the time to evaluate Plaintiffs' applications or their experts' conclusions.

Likewise, Breen failed to investigate the findings made by Kaufman. Carey asked Breen what the basis was for Kaufman's statement that the McElmurrays' land had received sludge applications making the land unsuitable for growing food-chain crops. Breen replied "I do no have information with which to answer this question." AR 1545.

As the Supreme Court has stated, "[t]he substantiality of evidence must take into account whatever in the record fairly detracts from its weight. This is clearly the significance of the requirement . . . that courts consider the whole record." Universal Camera Corp. v. NLRB, 340 U.S. 474, 488 (1951).

Other evidence of record calls into question the fairness and objectivity of the EPA's opinions with respect to the sludge land application program. The administrative record contains evidence that senior EPA officials took extraordinary steps to quash scientific dissent, and any questioning of the EPA's biosolids program.

On February 4, 2004, Dr. David Lewis, a former EPA employee, testified before the House of Representatives' Subcommittee on Energy and Mineral Resources about improper use of the scientific peer review process by senior EPA officials, with respect to a University of Georgia study relating to the Messerly plant, and the deficiencies in the agency's position in support of land application of sewage sludge. AR 1610 & 1616.[10] Lewis criticized the EPA for its handling of the allegations involving the Messerly plant in Augusta, especially its reliance on the dubious data provided by the City. AR 1622-24.

[10] Lewis' work as a microbiologist first drew national and international attention in the early 1990s when six dental patients of the same dentist in Florida contracted HIV. Lewis published a series of articles in the leading British medical journal The Lancet, showing that blood trapped in lubricants inside dental devices can escape disinfection and spread HIV, the virus that causes AIDS. This research prompted new heat sterilization guidelines worldwide. AR 1625.









Lewis explained that he had worked at the EPA for thirty-one years, and was forced to resign in May 2003 because his biosolids research was at odds with official EPA policy. AR 1619. Lewis testified before Congress that the EPA had politicized scientific research at the agency, and utilized unreliable and fraudulent data to support the continuation of the sludge land application program. AR 1619. Lewis recounted to the Committee that he researched adverse health consequences of sewage sludge from 1996 to 2003. Specifically, Lewis wrote a research paper with University of Georgia scientists that linked chemical irritants from airborne dusts, as a result of sewage sludge applications, to children's illnesses. AR 1620.

Lewis reported that a senior EPA official, Dr. John Walker, acted unethically in protecting the Part 503 sludge Rule, which Walker had helped to create. Lewis claimed that Walker had stated that he was qualified to review Lewis' microbiological research, although Walker was untrained in the field. Lewis stated that Walker approached a friend who was a corporate executive at a company involved in the sewage sludge business to help come up with criticisms of Lewis' paper. In addition, according to Lewis' testimony, Walker asked a USDA microbiologist for help with a technical

review, and then plagiarized the USDA official's work as his own. Thereafter, Lewis stated that Walker distributed the critique widely, within the EPA, to trade associations, and among regulated businesses in the industry. AR 1621.

Walker also distributed an anonymous twenty-eight page critique of Lewis' research, which had not been peer reviewed, and contained false scientific arguments aimed at discrediting Lewis. Lewis told the Congressional panel that a colleague at the National Academy of Sciences, Ellen Harrison, testified in a separate proceeding that the paper damaged Lewis' reputation. AR 1621-22. Thereafter, Walker's associates attempted to pressure EPA Administrator Christine Todd Whitman to end Lewis' research immediately. AR 1627. Walker faced no discipline for his actions by the EPA. AR 1620-21.

On May 28, 2003, the EPA forced Lewis to resign for publishing articles in the leading scientific journal Nature, which were critical of the EPA's biosolids policies. During his Congressional testimony, Lewis detailed how EPA administrators attempted to force him out after his article, "EPA Science: Casualty of Election Politics," was published in Nature in 1996. Lewis described how further retaliation in 1999 by senior EPA officials, against him and his









supervisor, Dr. Rosemarie Russo, prompted a separate hearing before Congress and helped spur enactment of the "No Fear" Act, a law protecting federal employees against retaliation. AR 1625-27.

The distribution of the false scientific reports by Walker caused University of Georgia officials to scrap their plans to hire Lewis after he left the EPA. Even letters from United States Senators James Inhofe and Charles Grassley, in an attempt to save Lewis' job at the EPA, were ineffective. AR 1627-28. Lewis reported that he had been blacklisted by Walker, and that he remained unemployed since he left the EPA. Lewis indicated that he had taken up an unrelated area of research without compensation because of the EPA's actions, stating that he was directing research on hepatitis C infections in Egypt. AR 1628.

## IV. Summary Findings and the Appropriate Remedy

Any data that was considered by Mehan and Brobst that related to the McElmurrays' farm was that collected as of 1999. Neither official considered Goodroad's 2001 analysis detailing the deficiencies in the data collected as of 1999. The men did not discuss or acknowledge the serious

limitations and deficiencies of Augusta's data. Neither official considered Plaintiffs' applications or the reports of their experts contained therein. AR 1235.

Neither Mehan nor Brobst made either a written factual finding or a technical determination about Plaintiffs' applications. Mehan, who represented the EPA's official position, did not find any material facts as to the application, and his letter was not a technical determination, but a statement of policy. Brobst may have attempted to produce a technical determination, but he did not consider the McElmurrays' applications, just old data, and he failed to consider anything the McElmurrays or their experts had to say to the contrary. Breen's conclusory rejection of the specific findings contained in Kaufman's affidavit was not binding on the USDA.

The administrative record indicates that the members of the FSA State Committee reviewed the Plaintiffs' applications thoroughly. The members of the State Committee were familiar with Plaintiffs' expert reports, and the import of that evidence. That committee voted in favor of the applications for credit. Likewise, EPA employee Kaufman was familiar with the McElmurrays' applications, expert reports, and the testing on their land. He had conducted an







Comment Letter L

investigation by visiting Augusta and looking into the problems at the Messerly treatment plant. Kaufman's affidavit indicates that the land is unfit for growing crops for human consumption. AR 1487-1489. Hearing Officer Jones also considered the evidence in the case, but his comments indicated that he felt he was bound by EPA opinions to which he ought not have deferred. AR 2144. See infra, note 4.

In short, it appears that the only persons to consider Plaintiffs' applications ended up ruling in their favor, or did not believe they had the authority to rule in the McElmurrays' favor. The USDA's decision to accept a contrary decision, based on no review of the applications by the EPA, was arbitrary and capricious. The conclusions of the EPA were not based on substantial evidence, and the USDA was not compelled by their handbook to rely on the letters presented in this case.[11]

An administrative determination cannot be upheld without an articulated, rational connection between the facts before the agency and the agency's decision. Zahnd v. Sec'y of Dep't of Agric., 479 F.3d 767, 773 (11th Cir. 2007).

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[11] Contrary to the McElmurrays' suggestion, that is not to say that the USDA could not defer to a sister agency if that agency made appropriate findings.



AO 72A
(Rev. 8/82)

Because the record supports Plaintiffs' petition for farm subsidies, the Court reinstates the original decision of the FSA State Committee, and directs the USDA to grant the McElmurrays' application for prevented planting credits. Remand is inappropriate because the record was unevaluated or ignored by agency officials at the USDA and the EPA. In other words, while the record was inadequate to support the agency's decision, it is adequate to support Plaintiffs' applications.

The Court has the obligation under the APA to conduct judicial review of administrative decisions. That statute requires the Court to "hold unlawful and set aside agency action, findings, and conclusions found to be . . . arbitrary and capricious." 5 U.S.C. § 706(2)(A). The agency "is not entitled to a second bite of the apple just because it made a poor decision--if that were the case, administrative law would be a never ending loop from which aggrieved parties would never receive justice." McDonnell Douglas Corp. v. NASA, 895 F. Supp. 316, 319 (D.D.C. 1995); Nelson v. United States, 64 F. Supp. 2d 1318, 1326 (N.D. Ga. 1999); Florida Power & Light Co. v. Lorion, 470 U.S. 729, 744 (1985).









**CONCLUSION**

For the reasons explained above, the USDA's motion for judgment on the administrative record is **DENIED**, and the McElmurrays' cross-motion is **GRANTED**. Dkt. Nos. 54 & 57, respectively. The Court hereby **DIRECTS** the USDA to grant the McElmurrays' application for prevented planting credits.

**SO ORDERED**, this 25th day of February, 2008.

JUDGE, UNITED STATES DISTRICT COURT
SOUTHERN DISTRICT OF GEORGIA

**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[1]

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Chairman Boxer, Ranking Member Inhofe and Honorable Members of the Committee, thank you for the privilege of testifying today about the destruction of our dairy farm business by hazardous wastes in sewage sludge, which was land-applied by the City of Augusta, Georgia.

### Cattle Deaths, Milk Contamination

My name is Andy McElmurray, and with me today is my attorney, Ed Hallman of Decker, Hallman, Barber & Briggs in Atlanta, Georgia. Mr. Hallman has led a team of attorneys and experts for the last 10 years in an effort to recover compensation for the destruction of my family's dairy farm business, which resulted from hazardous wastes in Augusta, Georgia's sewage sludge. My

[1] Andy McElmurray is represented at the Briefing by F. Edwin Hallman, Jr., Esq. Decker, Hallman, Barber & Briggs, Atlanta, Georgia. Mr. McElmurray's testimony draws from several lawsuits filed by Mr. Hallman, including *McElmurray v. United States Department of Agriculture*, United States District Court, Southern District of Georgia, Case No. CV105-159, and two qui tam lawsuits against senior EPA officials and others involved with Augusta's land application program.



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testimony addresses the history of sewage sludge applications to my family's farmlands. The City of Augusta invited us to participate in its land application program and assured us that the sewage sludge was safe for growing forage crops to feed to our dairy cattle.

We began receiving sewage sludge applications in 1979 and continued until 1990. On our farm, we grew forage crops to feed to our dairy cattle, and we grew row crops as well. In 1998, after hundreds of head of cattle sickened and died, we learned that Augusta's sewage sludge contained extremely high levels of hazardous wastes that were toxic to diary cattle.

Another prize-winning dairy farm in the area owned by the family of Bill Boyce was hit even harder, and the owners had to abandon the dairy farm business altogether. Our families, who have farmed our land for three generations, have lost tens of millions of dollars in property value, lost property and agricultural products.

For over two decades, the City of Augusta, Georgia failed to enforce federal and state regulations requiring local industries to treat hazardous wastes before discharging them into the City's sewers. The City also fudged, fabricated and invented data required under the Clean Water Act to make its sewage sludge appear to qualify as "Class B biosolids." The bogus fertilizer ended up sickening and killing hundreds of dairy cows on the two dairy farms.

Milk samples collected from one of our farms still using forage grown on lands which received sewage sludge contained high levels of heavy metals and other sludge contaminants. Additional samples of milk pulled from shelves in grocery stores in Georgia and surrounding states also contained some of the same heavy metals at levels exceeding EPA's safe drinking water standards.[2] Unsafe levels of heavy metals in various samples included thallium, a rat poison toxic to humans in very small doses.

Earlier this year, U.S. District Court Judge Anthony Alaimo rejected Augusta's fabricated data and ruled that the U.S. Department of Agriculture must compensate me and my family for crops that could not be planted, because thousands of acres of land were too contaminated with hazardous chemical wastes from Augusta's sewage sludge.[3] Our dairy, which was once one of Georgia's most productive dairy farms, was destroyed by the heavy metals, PCBs, chlordane, and other hazardous wastes that local industries dumped into Augusta's sewer system.

[2] J. Heilprin and K. S. Vineys. Associated Press. "Sewage-Based Fertilizer Safety Doubted." Mar. 6, 2008.

[3] *McElmurray v. United States Department of Agriculture*, United States District Court, Southern District of Georgia, Case No. CV105-159. Order issued Feb. 25, 2008.

### How It Happened

In 1976, Congress enacted the Resource Conservation and Recovery Act (RCRA) for controlling all solid hazardous wastes from "cradle to grave," *i.e.*, from the time that they are created until the time they are destroyed or safely sealed and permanently buried. "Hazardous wastes" include toxic chemicals, radioactive materials, and biological (infectious) wastes that meet certain criteria for being dangerous or potentially harmful to human health or the environment. They can be liquids, solids, contained gases, or sludges.

EPA regulations established under RCRA specifically exclude mixtures of hazardous wastes and domestic sewage passing through publicly-owned treatment works (POTW), *i.e.*, sewage treatment plants. To qualify under this exclusion: 1) the materials in the sewer line to which hazardous wastes are added must be domestic sewage; 2) the mixture of hazardous wastes and domestic sewage must flow into a POTW; and 3) any hazardous wastes in excess of 33 pounds per month must be "pretreated" before being discharged into sewer lines. Pretreatment standards are designed to protect waste treatment plants from non-domestic wastes that may cause explosion or fire, or interfere with the treatment process. They are also aimed at improving the quality of effluents and sludges so that they can be used as fertilizers and soil amendments (biosolids).

In Augusta, the pretreatment program was so lax that it essentially did not exist. Each industrial discharger applied for a pretreatment permit that limited the number of constituents that were monitored in the discharged effluent. Thousands of pounds of chemicals were dumped into the sewers everyday that were not monitored at all. Each industrial discharger self-reported the contents of the effluent discharged into the sewer lines. Even if there were gross violations of the pretreatment standards, there was not one instance in the history of Augusta where a discharger of hazardous wastes into the sewer lines was shut down or prevented from discharging into Augusta's sewer system.

Local metal plating operations and manufacturers of pharmaceuticals, artificial sweeteners, and other products dumped their wastes into the sewers of Augusta, Georgia. As toxic chemicals made their way to the waste treatment plant, they mixed with the human wastes and concentrated in the sewage sludge in settling tanks.

From there, the sludge was pumped into digesters to reduce levels of disease-causing bacteria and viruses. A small battery of tests developed by the EPA was performed to determine the concentrations of nine heavy metals, a few other chemical parameters including nitrogen, and the levels of at least one "indicator" pathogen.

Employees of the Messerly Wastewater Treatment Plant reported their results to the Georgia Environmental Protection Division (EPD), as required under federal and state environmental laws since Augusta's land application





program began in 1979. They gave the City's processed sewage sludge a passing grade as "Class B biosolids" and had it trucked out to local farmers, including to our farm and the farm owned by the Boyce family. Augusta assured us that the City's sewage sludge was completely safe for fertilizing food-chain crops.

The only problem was that Augusta's digesters and other critical equipment were not working properly – sometimes not at all. The pH of the City's "fertilizer" was so low that it dissolved metal fences and parts of the building where lab tests were performed. Employees tested only one of two waste streams of sewage sludge, and those results showed that the sludge that was tested contained hazardous levels of PCBs, chlordane, heavy metals and other highly toxic wastes.

To appear to be in compliance with the federal Clean Water Act and other environmental laws, City officials routinely altered or outright invented the numbers they reported to the EPD.[4] Records concerning how much sludge was applied per acre were manipulated, and levels of metals in different batches of sludge were averaged to make it appear that annual maximum loading rates for molybdenum and cadmium were not exceeded.

The total amounts of sewage sludge that Augusta applied each year to area farms could not be accurately reconstructed. Different sets of records were kept for amounts of sludge hauled by City and contract employees, and the EPD lost all of Augusta's annual reports showing the combined amounts. The City also lost all of its files showing the amounts of sludge hauled by its contractors. The combined totals reflected in field update reports, the City's only remaining records showing how much sludge was hauled, were inconsistent. Neither EPA, EPD, nor the University of Georgia has ever produced the records EPA and UGA authors used to create summaries of Augusta's historical data, which they published in a scientific journal in 2003.

What is certain is, that had Augusta complied with the law, it would have incinerated or buried its sewage sludge as hazardous wastes. Instead, City workers cooked the books to keep from spending the tens of millions of dollars it

[4] This testimony concerns fraud and scientific misconduct disclosed in *Lewis v. EPA*, U.S. Department of Labor, Office of Administrative Law Judges, Washington, DC. Case Nos. 2003-CAA-00005, 2003-CAA-00006; ARB Case 04-117; *Lewis v. U.S. Dept. of Labor*, United States Court of Appeals for The Eleventh Circuit, Appeal No. 08-12114-HH; *U.S. ex. rel. Lewis, McElmurray and Boyce v. Walker, et al.*, United States District Court, Middle District of Georgia, Civil Action No. 3:06-CV-16; *McElmurray v. United States Department of Agriculture*, United States District Court, Southern District of Georgia, Case No. CV105-159. Order issued Feb. 25, 2008; and *R.A. McElmurray, III, G. William Boyce, and David L. Lewis v. The Consolidated Government of Augusta-Richmond County, Georgia.* United States District Court for the Northern District of Georgia, Civil Action No. 1:05-CV-1575-ODE (2005).

would have taken to upgrade Augusta's dilapidated wastewater treatment system and produce sewage sludge that could legally be land applied.[5]

The case is not that Augusta lacked the funds to make the needed repairs. The Clean Water Act allows municipalities to collect user fees for upgrading treatment systems to meet federal and state environmental standards. City officials, however, diverted these proceeds to the City's general fund. The few repairs and improvements that were made were covered by low-interest government loans. To qualify for these loans, City officials relied on their false and fabricated environmental monitoring data to certify that the wastewater treatment system complied with the Clean Water Act.

As we and the Boyce families used Augusta's sewage sludge to fertilize forage crops, we noticed that our land was becoming more and more acidic. To continue growing crops, we applied large amounts of lime to raise the pH – first on our farm in 1985 and then on the Boyces' farm in 1996. But as soon as we did, the dairy herds developed an odd reddish tinge to their fading coats, a symptom of molybdenum poisoning. Molybdenum, a toxic heavy metal that attacks the liver and kidneys, dissolves at a very high pH, such as when lime is added. Molybdenum was but one of many toxic chemicals in Augusta's sludge that City officials were either underreporting or not reporting at all.[6]

Milk production from both of our dairies plummeted. Within months, many cows looked emaciated and, on our farm, developed *Salmonella* infections. Many of the cattle on both farms developed various infections and looked as if they were suffering through the last stages of AIDS. Veterinarians and other experts tested soil and forage samples as well as liver and kidney tissue samples. They found high levels of cadmium and other sludge-related contaminants. When the experts finally figured out what was happening, they fed one of the herds forage not grown with sewage sludge. Those animals slowly recovered over a period of two years. In the end, both of our family-owned dairy businesses were destroyed.

[5] Classes A and B sewage sludges (biosolids) have the same requirements for levels of chemical pollutants, but different requirements for indicator pathogen levels. Indicator pathogens in Class A material are reduced to undetectable levels; however, traces of indicator pathogens (e.g., *Salmonella*) and other pathogens that escape detection, or are not tested for, may proliferate (re-grow) after the fully processed materials are stored or applied in the field.

[6] Until the mid-1990s, Augusta tested its sewage sludge for priority pollutants and found that it was highly contaminated with chemicals regulated as hazardous wastes under the Resource Conservation and Recovery Act (RCRA), including chlordane, which is banned from use on dairy farms. The City, however, never acted on the information and stopped testing for these pollutants after the 503 sludge rule (40 C.F.R. Part 503) went into effect. This rule, passed in 1993 and modified in 1994, does not require testing for any organic pollutants, and restrictions for certain toxic heavy metals (*e.g.*, thallium, chromium and molybdenum) were reduced or eliminated.





In 1998, my family and the Boyce family sued the City of Augusta over damages caused by hazardous wastes in the City's sewage sludge. EPA dispatched Robert Brobst from Region 8 in Denver, Colorado to investigate. Brobst headed EPA's Biosolids Incident Response Team (BIRT). Brobst had investigated at least one other incident involving cattle, and had ruled that sludge was not the cause.

The EPD responded to our lawsuits by auditing the Messerly Wastewater Treatment Plant in Augusta. They found clear evidence that the City's environmental monitoring reports were being fudged to cover up high levels of contaminants – just as we and our experts discovered Augusta had been doing for decades. One of the reasons Augusta was fabricating data is because the City was not enforcing federal and state pretreatment regulations. The auditors recommended that Augusta's land application program be shut down immediately and the sludge be buried as hazardous wastes.

### The Gatekeepers

You are probably thinking by now that this is a story about corrupt City officials being sent to prison. That is what should have happened. Government forms that were used for reporting their false and fabricated environmental monitoring data included a warning in bold-faced type that it is a criminal violation, punishable by fines and imprisonment, to knowingly report false data under the Clean Water Act. This was a clear case of fraud for EPA's criminal investigation division to refer to the U.S. Department of Justice for prosecution.

But that never happened. Test results from soil and forage samples collected from our farm and the Boyce farm indicated that the dairy cows could have died from ingesting levels of molybdenum that are PERMISSIBLE under EPA's 503 sludge rule. In other words, what happened on our dairy farms suggested that EPA's sludge rule may have a major loophole – one that allows toxic heavy metals and other pollutants to contaminate food chain crops and milk supplies. Federal bureaucrats in the EPA Office of Water, who developed the EPA's sludge regulations, had too much to lose if local Augusta officials were held accountable.

EPA headquarters was not unprepared to deal with the bad news coming from Augusta. The ink on our lawsuits had hardly dried when architects of EPA's 503 rule engaged UGA in a strategy for rebottling the evil genie of Augusta, Georgia.[7] Their plan, which Walker initiated in November of 1998, was to get City officials to provide Robert Brobst with a "scientifically reliable" version of Augusta's historical reports showing that sewage sludge spread on either or both

[7] John Walker's typewritten notes of his telephone calls to Julia Gaskin, Robert Brobst, William Miller and others who Walker involved in the Gaskin study. November 25, 1998; J. Walker's outline of the Gaskin project including input from Rufus Chaney, e-mailed to Nancy Prock, EPD, Dec. 3, 1998; R. Chaney e-mail to Julia Gaskin, Apr. 22, 2005.

of the McElmurray and Boyce farms from the late-1980s through the late-1990s, when the cattle died, was safe for growing forage crops. Prior to 1999, however, the reports were "in shambles" and the data were "sloppy."[8] The reports would require more fudging and inventing.

To get the data into peer-reviewed scientific literature, EPA funded UGA land application specialist, Julia Gaskin, to publish a research study co-authored by Brobst. Brobst provided Gaskin with Augusta's fabricated data upon which to base the report. Later on, the plan included giving the article to the National Academy of Sciences to use in a 2002 report.[9] If all went well, the research article and academy report would be introduced as evidence at our jury trials.

In 1999, when the Gaskin study was conducted, Alan Saxon was rehired by the City and went to work "fudging" and "inventing" a new and improved version of Augusta's data, which Brobst needed to publish in a scientific journal. Brobst summarized and tabulated Saxon's work product for the Gaskin article. When Saxon and Brobst were finished, years of data that were once in "shambles" now fit tidily into a single table complete with what appeared to be statistically valid means, standard deviations, and maximum pollutant values that could pass muster at almost any reputable scientific journal.[10]

Everything actually worked quite well up until the time U.S. District Court Judge Anthony Alaimo did what no one expected. He spent weeks methodically and meticulously combing through court proceedings and mountains of related testimony and exhibits in our cattle cases, and in Dr. David Lewis' Labor Department case as well, until he pieced the puzzle together. Judge Alaimo ruled that Augusta's reports, which Brobst used in the UGA study and the Department of Agriculture case, were "incomplete," "unreliable," "fudged," "fabricated," and, in some cases, "invented."

Using nitrogen data, which Alan Saxon admitted under oath were off by four orders of magnitude, plus sewage sludge application rates, which Judge Alaimo described as "invented," along with metals concentrations, which Judge Alaimo described as "fudged," Gaskin and her co-authors concluded that Augusta's sewage sludge was applied at agronomic (proper nitrogen) rates and generally met federal and state requirements for levels of regulated metals.

[8] Terms used by Augusta employee Hugh Avery and EPA employee Robert Brobst to describe Augusta's data.

[9] *U.S. ex. rel. Lewis, McElmurray and Boyce v. Walker*. United States District Court, Middle District of Georgia, Civil Action No. 3:06-CV-16.

[10] Brobst's data appear on the bottom of page 148 (Table 2) of: Gaskin, Julia W., Robert B. Brobst, William P. Miller, and E. William Tollner, "Long-term Biosolids Application Effects on Metal Concentrations in Soil and Bermudagrass Forage." *J. Environ. Qual.* **32**:146-152 (2003). http://jeq.scijournals.org/cgi/content/full/32/1/146





Another member of EPA's BIRT, Robert Bastian, e-mailed the National Academy of Sciences panel a copy of Gaskin's draft manuscript in 2001.[11] The panel used the manuscript's preliminary, unpublished data to discount our lawsuits and conclude that there was no evidence that sewage sludge applied under EPA's 503 rule has ever harmed public health or the environment. In a national press release issued by UGA when the paper was published in 2003,[12] Julia Gaskin announced:

> "Some individuals have questioned whether the 503 regulations are protective of the public and the environment. This study puts some of those fears to rest."

Finally, Augusta's attorney, James Ellison, turned on the overhead projector and illuminated the courtroom in Atlanta where my case was under appeal. He displayed the Gaskin article page-by-page until he came to the conclusion at the end: "Overall, forage quality from fields with long-term application of biosolids was similar to that having only commercial fertilizer and should not pose a risk to animal health." When all was said and done, a jury awarded the Boyces only $550,000 in damages and our case settled out of court for $1.5 million. These amounts were not even enough to pay our experts, much less make a dent in the tens of millions of dollars that each of our families lost when our dairy farm businesses collapsed.

### The Mehan Letter

Brobst was more successful at using his and Gaskin's article to dismiss a public petition on sewage sludge filed with the EPA in 2003 by 73 farm, health, and environmental organizations. The groups called for a moratorium on land application of sewage sludge until the scientific issues raised by the Boyce verdict and three human deaths linked to sewage sludge could be resolved. EPA Assistant Administrator G. Tracy Mehan, III rejected the petition for the land application moratorium based upon information provided by Bastian and Brobst.

That November, Bastian e-mailed Madolyn Dominy at EPA-Region IV Atlanta a version of the letter he and Brobst were preparing for Mehan to sign.[13] Bastian wrote:

> Madolyn. I have been drafted by OST to develop a write-up on the Augusta, GA, case to include in the petition. [The attached version is] such a write-up developed from various materials that ... have been provided to me by various sources that incorporates some

[11] [E-mail] Robert Bastian, U.S.EPA Office of Wastewater Management. Mar. 13, 2001.

[12] C. Holmes, University of Georgia. "Sludge study relieves environmental fears." Jan. 29, 2003.

[13] [E-mail] Robert Bastian to Madolyn Dominy, Nov. 25, 2003.

suggestions that Bob Brobst and I came up with during a conversation earlier today. Do you know if the City of Augusta has or plans to appeal the jury award? Please let me know what you think of this write-up. Bob Bastian

Assistant Administrator Mehan's final letter issued on Christmas Eve used the Gaskin study to dismiss the jury verdict in favor of the Boyce family.[14] Mehan wrote:

> On February 2, 1999, Region 4 staff and the BIRT met with University of Georgia veterinarian scientists and soil scientists to discuss the livestock deaths and the University's possible participation in assessing soil and forage characteristics in Burke and Richmond Counties. On August 5, 1999, EPA Headquarters issued a grant to the University of Georgia ... This effort resulted in the publication of a paper entitled Long-Term Biosolids Application Effects on Metal Concentrations in Soil and Bermudagrass Forage (Gaskin *et al.*, 2003).
>
> The University of Georgia's findings of their analyses of trace metals levels in soils and feed that were implicated in the Georgia case. The paper indicates 'that toxic levels of metals have not accumulated in the soils due to long-term biosolids application. Overall forage quality from the biosolids-amended fields was similar to that of commercially fertilized fields...'
>
> ...Thus, EPA's investigation of the site and the sewage sludge did not find any substantiation to the allegations that exposure to sewage sludge applied to the pasture land caused illness or death of the dairy cattle that grazed on the pasture.

According to John Walker's typewritten notes of a telephone conversation he had with Dominy in November of 1998, Dominy told Walker that analyses of soil samples from our farms showed that the land was contaminated with 30 ppm (mg/kg) of molybdenum compared with a background concentration of only 0.5 ppm. However, the Gaskin study of other farms reported mean soil molybdenum concentrations of only 0.089 (± 0.041) ppm (Table 3, p. 149). EPA officials involved in drafting the Mehan letter knew that the results in the Gaskin paper grossly misrepresented contaminant levels found on our farm and the Boyce farm.

[14] [Letter] G. Tracy Mehan, III, Assistant Administrator, EPA Office of Water to J. Mendelson, III, December 24, 2003.





**Ruling By Judge Anthony A. Alaimo**

In January of 2003, we filed for economic relief under the federal Farm Bill to cover losses from corn and cotton crops during the 1998-2001 growing seasons. We claimed that our land was too polluted by PCBs, chlordane, heavy metals, and other hazardous wastes in Augusta's sewage sludge to grow food-chain crops. USDA, however, rejected these claims based mainly on information in the Gaskin study supplied by the study's EPA co-author, Robert Brobst.

In February of 2008, Judge Anthony Alaimo ruled that the USDA's conclusions were "arbitrary and capricious." Regarding Brobst's summaries of Augusta's historical data concerning sludge application rates and pollutant concentrations, Alaimo wrote: "Although there is a broad consensus that Augusta's reports were unreliable, incomplete, and in some cases, fudged, the City's information is an integral part of this case."

To support these findings, Judge Alaimo referred to detailed analyses of Augusta's reports performed by our experts, an audit in which the EPD confirmed the conclusions made by these experts, and other key evidence such as sworn testimony taken from employees working for the City of Augsuta. For example, Judge Alaimo found:

> There is also evidence that the City fabricated data from its computer records in an attempt to distort its past sewage sludge applications. ... In January 1999, the City rehired [former City of Augusta supervisor Alan] Saxon to create a record of sludge applications that did not exist previously.

In addition to ruling that environmental data summarized in the Gaskin paper were fudged, fabricated, and invented by the City of Augusta, Judge Alaimo ruled that EPA and the USDA relied on data collected in 1999 (when the Gaskin study was performed) while ignoring ample data collected as much as a decade earlier, at or about the time our cattle were dying. These data proved that our property was highly contaminated. Judge Alaimo wrote:

> Other specific evidence showed that heavy metals were found at levels that were above the regulatory limits on the McElmurrays' farm, making the land unfit for food grown for human consumption. On one piece of property alone, antimony levels registered at 96.8 ppm, while the regulatory limit was 4 ppm. Arsenic registered at 44.2 ppm, more than twice the amount allowed by law. Cadmium was found at a level of 6.41 ppm, which was more than three times the level deemed safe under the law. Selenium registered at 5.4 ppm, although the cleanup standard provided under the law was set at 2 ppm. Thallium was found at 51.6 ppm on that particular piece of property, although the regulatory limit is 2 ppm...

### How Widespread Are The Problems?

My attorney, Mr. Hallman, invited Dr. David Lewis to meet with the experts working on our cases in April of 2003. This is the first time I ever met Dr. Lewis. We were surprised to learn that Dr. Lewis and our veterinarians and other experts had independently come to the same conclusion regarding infections linked to sewage sludge. Dr. Lewis and the scientists working with him concluded that many people living near land application sites, who breathed sewage sludge dusts blowing from the fields, suffered from chemical irritation of the skin, eyes, and respiratory tract. This chemical irritation, Dr. Lewis postulated, lead to a variety of infections.

Our experts had concluded that chemical wastes in Augusta's sewage sludge sickened and killed our cattle in the same way, by attacking internal organs when the contaminated forage was eaten. Once the organs were damaged, the animals started contracting various kinds of infections. My father and I both experienced the same symptoms described in Dr. Lewis' research articles. We stayed on antibiotics. Then, as my father's condition worsened, he had to be kept on massive doses of corticosteroids. He almost died and still suffers serious medical problems from having worked in the sludge-amended fields and from getting steroid treatments. We never made the connection between our illnesses and what was happening to our dairy herds – not until we read the research articles published by Dr. Lewis.

Dr. Lewis provided us with many of the documents that he had collected when he worked on sewage sludge at EPA. These documents filled in many of the gaps in what we knew about what was happening in Augusta. We learned, for example, that EPA set up a cooperative agreement with the Water Environment Federation in 1992 to promote sewage sludge as safe and beneficial. The agreement included studying (and no doubt dismissing) ten "unsubstantiated horror stories." One internal EPA memo discussed the problems on our two dairy farms, mine and the Boyces'. The memo stated: "Biosolids Horror Stories. We asked Bob [Brobst] for real life examples of adverse environmental effects from biosolids. Bob sent us a list of sites with groundwater contamination."

The tables of field data attached to the memo indicated widespread groundwater contamination with nitrates and heavy metals at multiple sites in a study conducted in California, Colorado, Georgia, Illinois, Maine, Minnesota, New Mexico, Nebraska, and South Carolina. I do not believe that Augusta is unique. We have heard from dairy farmers elsewhere in Georgia, and in other states as well, where cattle were sickening and dying after being fed forage crops fertilized with sewage sludge. In one case, autopsies demonstrated that molybdenum poisoning was the likely cause of death.

We also learned from Dr. Lewis' documents that, in 1992, EPA's Office of Research and Development (ORD) identified six major weaknesses in the science





used to support the 503 sludge rule. According to an Inspector General report ten years later, EPA's Office of Water never funded ORD to fix any of these problems.[15] OW claimed that it did not fund ORD because research on sewage sludge became a low priority in 1993 under the Clinton Administration. OW, however, worked with the WEF from 1992-1999 to put tens of millions of dollars in congressional earmarks into funding proponents of land application to publish research supporting the 503 rule. This research did not find any problems with sewage sludge – only benefits.

Some of the weaknesses that ORD identified were the very problems that showed up on our dairy farm and on the Boyce farm as well. For example, ORD wanted to determine the bioavailability of sewage sludge contaminants for uptake by plants and animals. Our cattle were killed when they ingested sludge contaminants taken up by plants. This is also how milk on the Boyce farm became contaminated.

The ORD found weaknesses in the science EPA uses to support land application of sewage sludge, which have existed since the program first began. For example, the ORD pointed out that we need to understand long-term changes at land application sites, including changes in soil pH, land use, and the capacity for sewage sludge to bind chemical contaminants. Again, these kinds of changes are exactly what led to our cattle being poisoned. Our soil pH gradually had dropped over years of sludge applications. Then, when we switched to growing alfalfa – a change in land-use – we had to add lime. The lime caused the soil to lose its ability to bind molybdenum, which had built up to high levels from Augusta's sewage sludge. If the ORD had been able to address the weaknesses its scientist had identified in the sludge rule, and the Office of Water had fixed these problems, then the Boyce family and my family would not have lost our dairy businesses.

Before Dr. Lewis stopped doing the research, UGA approved a grant proposal that he submitted for a Swiss foundation to fund his research. Our farm was going to participate in the study, in which we planned to collect and analyze soil and groundwater samples. We also planned to collect milk samples from dairies using sewage sludge and test them for heavy metals and priority pollutants. This project would have addressed some of the weaknesses the ORD had identified. But, once again, senior EPA officials in the Office of Water stopped the work from being done.[16]

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[15] U.S. EPA. Land Application of Biosolids Status Report. 2002-S-000004. Office of Inspector General. Washington, DC, (2002).

[16] *See* Judge Alaimo's findings concerning the successful efforts by Office of Water officials to end Dr. Lewis' research at the University of Georgia. *R.A. McElmurray III v. United States Department of Agriculture*, United States District Court, Southern District of Georgia, Case No. CV105-159. Order dated Feb. 25, 2008, pp. 38-41.

## Conclusion

In conclusion, ORD clearly identified many of the main weaknesses with the 503 sludge rule when it was first reviewed in 1992. Office of Water has prevented ORD from addressing any of these weaknesses for the past 16 years and tried to cover up any harm to public health or the environment. The same few people have run this program since the 1970s, and the program has only gotten more inept and corrupt with every passing year.

The first step toward fixing problems with land application of sewage sludge, therefore, is to clean up the longstanding corruption associated with this program in EPA's Office of Water, take the millions of dollars the Office of Water is funneling to it supporters with congressional earmarks, and redirect all future funding in this area to ORD.





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# Researchers Link Increased Risk Of Illness To Sewage Sludge Used As Fertilizer

ScienceDaily (Jul. 30, 2002) — Burning eyes, burning lungs, skin rashes and other symptoms of illness have been found in a study of residents living near land fertilized with Class B biosolids, a byproduct of the human waste treatment process.

This study is the first linking adverse health effects in humans to the land application of Class B biosolids to be published in a medical journal. It was co-authored by David Lewis, a UGA research microbiologist also affiliated with the U.S. Environmental Protection Agency (EPA)'s National Exposure Research Laboratory; David Gattie, assistant professor of agricultural engineering at the University of Georgia's College of Agricultural and Environmental Sciences; Marc Novak, a research technician at UGA's School of Marine Sciences; Susan Sanchez, assistant professor of veterinary medicine at UGA; and Charles Pumphrey, a physician from Prime Care of Sun City in Menifee, Calif. The article appeared this month in the British medical journal, BMC Public Health.

Researchers found that affected residents lived within approximately one kilometer (0.6 miles) of land application sites and generally complained of irritation after exposure to winds blowing from treated fields. A prevalence of Staphylococcus aureus infections, a condition commonly accompanying diaper rash, was found in the skin and respiratory tracts of some individuals. Approximately 25 percent of the individuals surveyed were infected, and two died. The 54 individuals surveyed lived near 10 land application sites in Alabama, California, Florida, New Hampshire, Ohio, Ontario, Pennsylvania and Texas. S. aureus is commonly found in the lower human colon and tends to invade irritated or inflamed tissue.

"The EPA did not consider S. aureus to be a significant public health risk even though it is a leading cause of hospital-acquired infections and is commonly found in sewage," said Lewis. "When approving sludge for use as a fertilizer, EPA looked at chemical and pathogen risks separately without considering that certain chemicals could increase the risk of infection."

Chemicals such as lime, which is added during sludge processing, can irritate the skin and respiratory tract and make people more susceptible to infection, according to Lewis. The American Chemical Society recently published another article on pathogen risks from sludge by Lewis and Gattie in their journal Environmental Science & Technology.

Though modern treatment can eliminate more than 95 percent of the pathogens, enough remain in the concentrated Class B sludge leaving treatment plants to pose a health risk, according to Lewis and Gattie.

On July 2, the National Research Council of the National Academy of Sciences (NAS) concluded that there may be public health risks from using processed sewage sludge as a commercial fertilizer. Approximately 60 percent of an estimated 5.6 million tons of dry sludge is used or disposed of annually in the United States.

The NAS report entitled "Biosolids Applied to Land: Advancing Standards and Practices" cites growing allegations that exposure to Class B sludge, the most common form, is causing illnesses and sporadic deaths among residents. The report concludes that certain types of exposure, such as inhalation of sludge particles, "were not adequately evaluated" previously and no work has been done on risks from mixtures of pathogens and chemicals found in sludge. In 1989, an EPA study

found 25 groups of pathogens in sludge, including bacteria such as E. coli and salmonella; viruses, including hepatitis A; intestinal worms; harmful protozoa; and fungus.

Sludge also includes traces of household chemicals poured down drains, detergents from washing machines, heavy metals from industry, synthetic hormones from birth control pills, pesticides, and dioxins, a group of compounds that have been linked to cancer.

Fertilization of land with processed sewage sludge, or "biosolids," has become common practice in Western Europe, the United States and Canada. Local governments, however, are increasingly restricting or banning the practice in response to residents reporting adverse health effects.

"Most people are not aware this is going on in the U.S.," said Gattie. "Most people don't realize that a concentrated sludge of waste products is being processed into a cheap commercial fertilizer and applied to fields near our homes. 'Biosolids' does not connote 'sewage' to most people." He notes this practice has become more common after ocean dumping of sewage was prohibited.

*Adapted from materials provided by University Of Georgia.*

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◉ APA

○ MLA

University Of Georgia (2002, July 30). Researchers Link Increased Risk Of Illness To Sewage Sludge Used As Fertilizer. *ScienceDaily*. Retrieved March 5, 2008, from http://www.sciencedaily.com /releases/2002/07/020730075144.htm

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# National policy brought sludge to Augusta farms

## Ruling for farmer disputes government data

Sunday, March 09, 2008

It was a farm idea with a big payoff and supposedly no downside: ridding lakes and rivers of raw sewage and industrial pollution by converting it all into a free, nutrient-rich fertilizer.

Then two weeks ago, a federal judge ordered the Agriculture Department to compensate a farmer whose land was poisoned by sludge from the waste treatment plant in Augusta. His cows had died by the hundreds.

The Associated Press also has learned that some of the same contaminants showed up in milk that regulators allowed a neighboring dairy farmer to market, even after some officials said they were warned about it.

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.

The contaminated milk and the recent ruling by U.S. District Judge Anthony Alaimo raise new doubts about a 30-year government policy that encourages farmers to spread millions of tons of sewage sludge over thousands of acres each year as an alternative to commercial fertilizers.

The program is still in effect.

Judge Alaimo ordered the government to compensate dairy farmer Andy McElmurray because 1,730 acres he wanted to plant with corn and cotton to feed his herd was poisoned. The sludge contained levels of arsenic, toxic heavy metals and PCBs two to 2,500 times federal health standards.

Also, data endorsed by Agriculture and EPA officials about toxic heavy metals found in the free sludge provided by Augusta's sewage treatment plant was "unreliable, incomplete, and in some cases, fudged," Judge Alaimo wrote.

EPA-commissioned research by the University of Georgia based on the Augusta data was included in a National Academy of Sciences report and served as a linchpin for the government's assertion that sludge didn't pose a health risk.

In his 45-page ruling, Judge Alaimo said that along with using the questionable data, "senior EPA officials took extraordinary steps to quash scientific dissent and any questioning of EPA's biosolids program."

Benjamin H. Grumbles, the EPA's assistant administrator for water programs, said Thursday that the judge's order underscored the significance of what he called strong national standards on sludge rather than undercutting the giveaway program.

"This unfortunate instance of poor recordkeeping and biosolids sampling techniques on the part of one plant reiterates the importance of our national biosolids program," Mr. Grumbles said in a written response to AP questions about the ruling.

About 7 million tons of biosolids are produced each year as a byproduct from 1,650 waste water treatment plants around the nation. Slightly more than half is used as fertilizer; the rest is incinerated or burned in landfills. Giving it away to farmers is cheaper than burning or burying it, and the government's policy has been to encourage the former.

**JUDGE ALAIMO'S DECISION** was a bittersweet victory for Mr. McElmurray, whose silos and dairy barns have sat mostly empty since his herd was wiped out. He contends the cows were done in by grazing on sludge-treated hay for more than a decade, beginning in 1979.

Interviewed before the ruling, Mr. McElmurray scowled at the empty pastures and idle equipment where his prize-winning herds once grazed.

"This farm never would have looked like this if we hadn't used the city's sludge," he said angrily.

The city of Augusta recently settled a lawsuit with him for $1.5 million. Another nearby dairy farmer, Bill Boyce, won a $550,000 court judgment against the city on his claim that sludge was responsible for the deaths of more than 300 of his cows.

The deaths of the farmers' cows in the 1990s and their suits against Augusta raised a red flag with officials at the EPA, which since 1978 has been promoting the use of sludge as a fertilizer.

In 1999, the agency awarded a $12,274 grant to the University of Georgia to study the problem. It resulted in a study published in 2003 in the

Journal of Environmental Quality finding that the city's sludge was safe and that the EPA's regulations were working. Cities' sewage and industrial pollution had spewed untreated into lakes, rivers and oceans until 1972, when Congress passed the Clean Water Act.

Back then, cleaning up waterways was the first target of the newly created EPA. The agency oversaw a multibillion-dollar grant program that Congress set up to help cities and counties build wastewater treatment plants that would filter out pollutants.

Judge Alaimo, citing data from an environmental engineer hired by Mr. McElmurray, found that the Augusta plant was sending out hundreds of truckloads of sludge daily with dangerously high levels of cadmium, molybdenum and chlordane.

The engineer, William Hall of Atlanta, had been a project manager at seven Superfund cleanup sites and had extensive experience with toxic chemicals and heavy metals. His tests found polychlorinated biphenyls, or PCBs, in the Augusta sludge at levels 2,500 times higher than the EPA standard; thallium levels 25 times the legal limit; and arsenic levels twice the government's health standard.

William Miller, a University of Georgia soil scientist who co-wrote the 2003 study commissioned by the EPA, stands by the conclusions it drew on how much sludge had been applied to Mr. McElmurray's and Mr. Boyce's land and the composition of it.

But in a draft of the paper obtained by the AP, he wrote a note by hand saying the authors should "fess up" that they didn't know those things.

"Now, we didn't really know exactly how much sludge, and we didn't know the quality of sludge," Mr. Miller told the AP in an interview. Despite the discrepancies, he maintained the study was valid. "It does not include fake data," he said.

Mr. Boyce told the AP that in January 1999 he informed Georgia dairy regulators and the EPA that tests he had ordered on milk from his cows had come back showing high levels of thallium, molybdenum and cadmium. A top state official alerted the Food and Drug Administration, but Mr. Boyce said no one ever told him to stop selling his milk or mentioned a possible threat to public health.

"We were a little startled," Mr. Boyce recalled. "They concluded that our permit was good, and we could continue to sell milk. So we did."

**THE EPA LISTS THALLIUM** as a toxic heavy metal that can cause gastrointestinal irritation and nerve damage, but the agency has no standard on the metal's presence in milk. Neither does the Agriculture Department, even though it regards thallium as one of the most dangerous agents of potential bioterrorism against the nation's food supply.

State and EPA officials followed up by testing Mr. Boyce's milk, but he said they wouldn't share all their results with him or Mr. McElmurray. There is no evidence those officials took any further action. Mr. Boyce said he decided to reveal the contamination to the AP to illuminate a broader issue.

"The real problem was the state and federal regulatory agencies did not do their jobs," he said, adding that the EPA and Augusta officials "tried to say we were just a disease-infested herd. Well, that's just a bunch of bullhockey."

Charles Murphy, who was then the head of Georgia's dairy program, said he told the FDA's administration office in Atlanta about Mr. Boyce's contaminated samples.

"I don't think they sent anybody out," he said.

Mr. Murphy said he was persuaded by evidence provided by the two farmers to seek broader state testing of dairy cows, but there wasn't enough money. FDA officials in Atlanta and Washington said they had no record of Mr. Murphy's account.

But in 1999, two senior EPA officials, Robert Bastian and Bob Brobst, huddled with the two farmers and their lawyer, Ed Hallman, to talk about sludge.

"They showed us some data," Mr. Bastian recalled. "I don't ever remember seeing any milk data." Mr. Boyce and Mr. McElmurray insist they shared all of their data with the two EPA officials, including separate tests they ran on milk pulled from stores in Charleston, S.C. That milk, which came from other farms in the Southeast, suggested more widespread contamination, they said. It had heavy metals similar to those found in Mr. Boyce's milk.

There are no records that anyone became ill because of milk tainted with heavy metals or other contaminants that could have come from sludge.

## AT A GLANCE

Recent court rulings involving Augusta farmers whose cows died as a result of contaminated sludge raise new doubts about a 30-year





government policy that encourages farmers to spread sewage sludge as an alternative to commercial fertilizers.

**REUSING WASTE:** About 7 million tons of biosolids -- the term waste producers came up with for sludge in 1991 -- are produced each year as a byproduct from 1,650 wastewater treatment plants around the nation. Slightly more than half is used on land as fertilizer; the rest is incinerated or burned in landfills.

**THE AUGUSTA CASE:** An environmental engineer hired by one of the farmers found that the Augusta wastewater treatment plant was sending out hundreds of truckloads of sludge daily with poly-chlorinated biphenyls, or PCBs, at levels 2,500 times higher than the EPA standard; levels of the toxic heavy metal thallium -- an element once used as rat poison -- 25 times the legal limit; and arsenic at levels twice the government's health standard.

**WHAT'S THE HARM:** Contaminated sludge could poison the land it's supposed to be fertilizing. The sludge was to blame in both of the Augusta cases in which hundreds of cows died.

**WHAT HAPPENED:** The city of Augusta recently settled a lawsuit with dairy farmer Andy McElmurray over his dead cows for $1.5 million. Former dairy farmer Bill Boyce won a $550,000 court judgment against the city for the deaths of more than 300 of his cows.

**-- Associated Press**



S.1-19

# Counterintuitive toxicity: increasingly, scientists are finding that they can't predict a poison's low-dose effects.

Print

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For decades, researchers largely assumed that a poison's effects increase as the dose rises and diminish as it falls. However, scientists are increasingly documenting unexpected effects--sometimes disproportionately adverse, sometimes beneficial--at extremely low doses of radiation and toxic chemicals.

Consider the environmentally ubiquitous plastic-softening agent, di-2-ethylhexyl phthalate (DEHP). A German team recently found that in newborn male rats, the lowest DEHP doses tested suppressed the brain activity of an enzyme critical for male development. This was a surprise because higher DEHP doses stimulated that enzyme's action.

Anderson J.M. Andrade and his colleagues at Charite University Medical School in Berlin note that the enzyme's suppressive action would have been missed if they had done what most toxicologists do--project low-dose impacts from high-dose tests. The low dose that suppressed aromatase in the rodents was comparable to exposures occurring in the general human population, Andrade's team reports in the Oct. 29, 2006 Toxicology.

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Other toxic agents have unexpectedly beneficial effects. X-rays and gamma radiation are well-recognized carcinogens. Data collected over decades have shown that exposures to 1 gray (Gy)--the dose from perhaps 100 computerized tomography seans--typically increase an individual's lifetime risk of cancer by 5 percent. However, a growing body' of animal data now indicates that lower radiation exposures can defend against cancer-inducing biological changes.

"The little dose is turning on some kind of protective mechanisms so that when a big dose comes along, it's not as damaging," says radiation biologist J. Leslie Redpath of the University of California, Irvine. Conceptually, it's analogous to a vaccine.

Many such effects have been overlooked because researchers prematurely stopped probing for biological impacts as soon as they identified dosage levels of a poison that appear benign, says toxicologist Edward J. Calabrese of the University of Massachusetts-Amherst. Poisons can have a variety of effects at both high and low doses--whether they trigger release of a hormone, switch a gene on or off, or stimulate cell growth. Indeed, Calabrese told Science News, that he has seen the same low dose of a chemical have beneficial effects on one tissue and detrimental effects on another.

He and others worry that if researchers don't begin regularly probing the effects of these agents at very. low doses, scientists will continue to miss important health impacts--both bad and good--of pollutants, drugs, and other agents.

ANOMALY OR NORM? Regulatory agencies don't require scientists to evaluate a poison at exposures below that at which no harm is apparent. This dose is referred to as the NOAEL, for "no observable adverse-effects level".



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Counterintuitive toxicity: increasingly, scientists are finding that they can't predict a poison's low-dose effects. 6/16/09 12:42 PM

Calabrese has campaigned relentlessly over the past 15 years to draw attention to biological effects that occur below a NOAEL. These include nonlinear effects, such as a toxicity that initially decreases as concentration goes down but eventually increases, producing a U-shaped curve. In a related class of nonlinear effects, called hormesis, a compound at high doses has an inhibitory--and generally toxic--effect on some biological process but the opposite effect at certain low doses. Unlike many other toxicologists, Calabrese uses the term hormesis to cover most nonlinear low-dose effects.

Radiation offers one of the best examples of hormesis in its narrower definition. At the Environmental Mutagen Society meeting last September in Vancouver, British Columbia, Redpath reported that cells exposed to no more than 0.1 Gy of radiation were less likely to spawn tumors than were cells receiving either far higher doses or no radiation.

In another study; Brenda E. Rodgers of Texas Tech University, in Lubbock gave mice a small dose of radiation by caging them in a Ukrainian forest roughly 1.5 kilometers from where the Chernobyl nuclear accident occurred 18 years earlier. Depending on their location, it took between 10 and 45 days for each mouse to receive a dose of 0.1 Gy. A day after an animal had reached that dose, it was moved to a nearby lab and quickly bombarded with 1.5 Gy. Blood tests showed that the lab radiation produced only half as many chromosome breaks--an indicator of damage that could lead to cancer--in these animals as it did in mice without the earlier low-dose exposure.

A low dose of radiation can reduce damage even if it comes after a larger dose, says Tanya K. Day of Flinders University in Bedford Park, Australia. In one study, her team gave mice a 1-Gy dose of radiation. Four hours later, some mice received a second, far smaller dose. Rodents getting both doses developed only half as many DNA inversions--a particular type of cellular damage--as did mice getting just the first dose, and often fewer inversions than did mice receiving no radiation at all.

Day reported her findings in June at the International Hormesis Society meeting in Amherst, Mass.

Calabrese's team reviewed hundreds of toxicology papers that document a biological effect below the NOAEL for chemical poisons. He terms all these effects as instances of hormesis, although only about 5 percent of the articles did. The rest described the results as inexplicable or as evidence of some type of nonlinear toxicity.

To determine how commonly trace exposures trigger unanticipated biological, impacts, Calabrese's team has analyzed databases of biological responses to potentially toxic chemicals, each throughout a broad range of doses. In their most recent study, described in the December 2006 Toxicological Sciences, Calabrese and his colleagues analyzed data showing how cell proliferation in 13 different yeast strains responded to various doses of 2,189 potential anticancer drugs.

Almost 80 percent of the drugs exhibited a NOAEL, the team found. Among these, the group further looked for reports of biological effects triggered by doses even lower than that level. The authors had expected that 25 percent of these drugs, just by chance, would exhibit activity above that seen with no exposure. In fact, 60 percent did.

The effects observed at those low doses were modest, perhaps 60 percent higher or lower than those that occur in the absence of any exposure, Calabrese notes. He acknowledges that such changes might not always have clinical significance.

These findings and earlier analyses by his group, Calabrese says, show that measurable biological effects at low doses appear to be more the norm than an anomaly.

Indeed, even pollutants that don't have a NOAEL may have nonlinear effects at low doses, notes Bernard

Weiss of the University of Rochester (N.Y.) School of Medicine and Dentistry. For example, the drop in a child's IQ for each 1 microgram per deciliter of lead in the blood is much higher at concentrations below 10 [micro]g/dl than at concentrations above that value (SN: 5/5/01, p. 277).

So, Weiss concludes, toxicity estimates based on high-dose measurements greatly underestimate low-dose harm.

HOW DOES IT WORK? Scientists have recently begun to discover mechanisms to explain hormesis and other nonlinear dose responses. For instance, Rodgers has been looking at what genes are preferentially turned on or off in the mice exposed to Chernobyl radiation. Compared with unexposed mice, those caged in the Ukraine forest had 600 to 1,200 genes whose activity had been altered.

"We expected to see an increase in the expression of genes involved in D NA repair," Rodgers says. "What we found instead was an increase in the expression of genes that respond to oxidative stress--such as free radicals."

Another explanation of hormesis was suggested in 2000 by researchers working with human-cancer cells exposed to epigallocatechin gallate (EGCG), the principal cancer-fighting ingredient in green tea. The team showed that although high-dose exposures of EGCG inhibited cell growth, low doses stimulated cell proliferation. D. James Morre of Purdue University in West Lafayette, Ind., says that his team several years ago found a unique enzyme on cell surfaces that appeared to be "a molecular target for chemical hormesis."

The group subsequently determined that this enzyme can bind to various substances, in addition to EGCG, and alter their cellular effects. Those responses disappeared when the enzyme was inactive.

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Some scientists have suggested additional processes that play a role in hormesis. In an upcoming issue of the International Journal of Low Radiation, Bobby R. Scott of the Lovelace Respiratory Research Institute in Albuquerque, N.M., and his colleagues report that low doses of radiation induce mild oxidative stress in cells, activating a high-efficiency form of DNA repair and stimulating the immune system. This stress also "activates a special apoptosis [cell suicide] process"--one that culls genetically unstable cells, he says.

Scott suggests that these same processes probably work to counteract chemical poisons.

IMPLICATIONS Although most toxicologists today agree that hormesis oceurs--a big change from a decade ago--some argue that Calabrese and his team greatly overstate its frequency. A major portion of this controversy hinges on differences in the use of the term hormesis.

"I totally believe that [nonlinear] low-dose responses occur frequently," says Kristina A. Thayer of the National Institute of Environmental Health Sciences in Research Triangle Park, N.C. "In fact, I have no problem accepting that most of the time they might be stimulatory."

However, she says that Calabrese equates stimulatory low-dose effects with benefits when there's no reason to expect that they would necessarily be beneficial. Her research with the plastic-softening agent bisphenol A, a hormone-mimicking agent, illustrates a detrimental effect of low-dose stimulation similar to what Andrade found for DEHP.

Among toxic agents that show positive biologic effects at low doses, Calabrese sees the possibility for better drug design. For example, he says, current treatments for dementia provide tiny doses of drugs that at high doses would be toxic. For instance, he says, "every Alzheimer' s drug on the market today acts via hormetic [low-dose] activities."

Even though a hormetic treatment may show only a small effect, Calabrese proposes that several treatments might be put together to achieve a therapeutic benefit.



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Counterintuitive toxicity: increasingly, scientists are finding that they can't predict a poison's low-dose effects. 6/16/09 12:42 PM

Scott suggests a related therapeutic application of hormesis that uses small doses of radiation to trigger immunological and cell-death processes. However, cancer cells are "reluctant" to undergo programmed death, Scott notes. Because certain compounds--such as resveratrol, a polyphenol in grapes (SN: 11/4/06, 293)--sensitize cancer cells to radiation, Scott envisions pretreating people with such compounds and following this up with a hormetic dose of radiation. "For lung cancer," he says, "perhaps just low-dose diagnostic X rays would do."

Beyond new medical applications, information gleaned from research into low-dose exposures might help fine-tune regulation of chemicals. Scientists may find that many pollutants aren't as toxic at low doses as has been assumed, Calabrese says.

"You can imagine why industry loves hormesis" Weiss says. It suggests pollution may not need to be cleaned up as thoroughly as regulations have been asking for.

Calabrese counters, however, that if traces of certain pollutants are not as dangerous as earlier estimates had suggested, why not investigate whether some regulations are unduly stria?

Indeed, proving that some low-dose exposures are "of no regulatory concern could make a qualitative difference in regulations," observes economist Lester B. Lave of Carnegie Mellon University in Pittsburgh. However, he adds that to justify changing guidelines for regulations, far more research would be needed.

For instance, there has been much discussion suggesting that low doses of chemicals--even pollutants--might rev up immunity in a beneficial way. However, because many people have compromised immune systems, Lave says that before raising the acceptable environmental limits of a pollutant, "I'd want to know if we see a [beneficial] hormetic response in those people, or babies with undeveloped immune systems, or the elderly." Moreover, he says, effects at low doses tend to be subtle, so "I'd want to see them documented in humans, not just animals"--and to know at precisely what dose they turn detrimental.

Jonathan Borak, a toxicologist at the Yale School of Medicine in New Haven, Conn., agrees that it's too early for hormesis or other nonlinear low dose-effects data to be "practically relevant" for altering regulatory or health policy.

Although "I believe hormesis is real, it is fundamentally difficult-and expensive--to demonstrate," Borak says. Looking for relatively small low-dose effects could quadruple the cost of toxicology studies, he estimates, underscoring"practical and economic reasons why today almost nobody looks for them."

1: Toxicol Appl Pharmacol. 1998 Dec;153(2):258-65. Links

## Effects of relatively low levels of mono-(2-ethylhexyl) phthalate on cocultured Sertoli cells and gonocytes from neonatal rats.

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Di-(2-ethylhexyl) phthalate (DEHP), one of the abundant man-made environmental chemicals, induces testicular damage in both developing and adult animals. However, the nature and mechanism underlying the action of phthalates on testicular development remain largely unexplored. In the present study, we used cocultures of neonatal Sertoli cells and gonocytes (precursors of spermatogonia) to characterize in detail the effects of mono-(2-ethylhexyl) phthalate (MEHP; the active metabolite of DEHP) on these cells and to explore the underlying mechanism(s). Sertoli cells and gonocytes were isolated from rat pups on the 2nd day after birth, cocultured, and exposed to MEHP at concentrations of 0.01, 0.1, or 1.0 microM, or to 0.5% DMSO (vehicle control), or 10 microM DEHP (negative control) for a total of 48 h. We found that exposure to MEHP induced gonocyte detachment from the Sertoli cell monolayers in a time- and dose-dependent manner. When exposed to 1.0 microM MEHP, many gonocytes started to detach after 12 h of exposure and most gonocytes were lost during the media change at 24 h. Gonocyte detachment was also observed in cocultures treated with 0.1 microM MEHP for 24 h of exposure, but not in cultures treated with 0.01 microM MEHP for 48 h. Detached gonocytes were viable as indicated by their ability to exclude trypan blue. Furthermore, when proliferation of cultured Sertoli cells was detected by BrdU labeling and subsequently quantified, we found that exposure to 0.1 or 1.0 microM MEHP for 48 h resulted in a decrease in labeling indices of 33.6 and 83.6%, respectively, compared to the vehicle control ($p < 0.01$), while the labeling index was unchanged by treatment with 0.01 microM MEHP. In addition, we also tested the potential effect of MEHP on FSH-stimulated Sertoli cell proliferation by simultaneously treating cultures with 200 ng/ml human FSH and different concentrations of MEHP for 48 h. Exposure to 0.1 or 1.0 microM MEHP resulted in decreases of 24.2 and 74.2%, respectively, in FSH-stimulated Sertoli cell proliferation ($p < 0.01$). Furthermore, MEHP also inhibited dibutyl cAMP-stimulated Sertoli cell proliferation, regardless of whether dibutyl cAMP was added to the cultures before or at the same time as MEHP. Finally, addition of FSH or dibutyl cAMP had no effect on MEHP-induced gonocyte detachment, and none of the observed effects on either Sertoli cells or gonocytes were detected in control cultures treated with 0.5% DMSO only or with 10 microM DEHP. Therefore, short exposure to low levels of MEHP disrupted adhesion of gonocytes to Sertoli cells and inhibited both basal and FSH-stimulated Sertoli cell proliferation in a dose-dependent manner. The lowest effective dose of MEHP in vitro was 0.1 microM, which is about 10- to 1, 000-fold lower than the dose shown to affect Sertoli cells from prepubertal animals. Moreover, our data indicate that MEHP impairs division of neonatal Sertoli cells by acting at a post-cAMP site in the FSH-response pathway or via a mechanism independent of FSH. These data provide direct new evidence that relatively low levels of MEHP disrupt Sertoli cell-gonocyte physical interactions and suppress Sertoli cell proliferation in neonates via mechanisms specific to neonatal testis where the foundations of adult fertility are established. The results also highlight the neonatal period of testicular development as one particularly sensitive to environmental chemicals. Copyright 1998 Academic Press.

PMID: 9878596 [PubMed - indexed for MEDLINE]







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Effects of relatively low levels of mono-(2-ethylh...[Toxicol Appl Pharmacol. 1998] - PubMed Result 6/16/09 2:15 PM

ethylhexyl) phthalate in

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# DETECTION OF HORMONE MIMICS IN WATER USING A MINITURISED SPR SENSOR

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**Abstract.** The ubiquitous presence of chemicals, both natural and synthetic, in the environment with the potential to mimic hormones that may in turn interfere with the endocrine system in both wildlife and humans has in the last decade become a major international concern. Hormone mimics or endocrine disrupting compounds (EDCs) are especially prevalent in surface and waste-waters and therefore, there is a need for an at-source or at-line analytical device for the monitoring of EDC levels. We have incorporated a miniature integrated surface plasmon resonance (SPR) liquid sensor from Texas Instruments into a field analyser and developed a competition/inhibition assay for a model estrogenic compound in aqueous samples. The analyser has the potential for *in situ* and semi-continuous analysis of EDCs. A novel regeneration scheme employing the use of a domestic laundry detergent has been used to remove immobilised assay components between each assay cycle. The resultant re-usable sensor has been demonstrated using estrone-3-glucuronide (E3G) as a model EDC and an anti-E3G antibody producing a current detection range of 10 to 150 ng $mL^{-1}$.

**Keywords:** endocrine disrupting compounds, estrone-3-glucuronide, hormone mimics, miniturised SPR sensor, surface plasmon resonance

## 1. Introduction

Hormones are biologically active substances that are secreted into the blood system via ductless glands of the endocrine system. They are active at very low concentrations (ng $mL^{-1}$ to pg $mL^{-1}$, i.e. ppb or ppt) and bind specifically to target receptor sites on cell surfaces or within the cell nucleus. Once associated with their corresponding target site they exert important regulatory, growth, homeostatic or reproductive effects. The complexity of the endocrine system with cascading loops of hormone signals and responses lends it self to the interference of the system at many points (Arnold and McLachlan, 1996; US EPA, 1997) and hence sensitivity to environmental natural and non-natural hormones and hormone-mimics, i.e. endocrine disrupting compounds.

The purpose of the work reported here is to provide an initial demonstration of the application of a portable biosensor or bioanalyser device that could be implemented near or at source (i.e. wastewater sewage treatment works or surface waters) to determine concentration levels of hormones and their mimics in aqueous environments. It is the aim of this on-going study to achieve this via a simple,

*Environmental Monitoring and Assessment* **70:** 83–92, 2001.

sensitive and stable surface bio/chemistry for the biosensor that can be replaced remotely and automatically by a simple combination of fluidics, non-covalent immobilisation and cleaning steps. Therefore for immobilisation, we have adopted the physical adsorption from aqueous solution to the gold sensor surface of a carrier protein-EDC conjugate to immobilise the specific analyte/hapten for a subsequent competition/inhibition assay. A competition/inhibition assay was chosen to enable the sensitive detection of low molecular weight EDC analytes/haptens in a SPR sensor assay. The assay comprises the pre-incubation of samples with a soluble anti-EDC IgG antibody prior to passing over the SPR sensor with immobilised analyte/hapten. By detecting inhibition of antibody binding due to the presence in a sample of an unknown concentration of an appropriate EDC, a potential 500 fold amplification compared to direct sensing of the low molecular weight EDC analyte/hapten can be expected.

To enable sensor reusability, a novel regeneration step using a domestic liquid laundry detergent was used to achieve reproducible removal of all protein and other molecular assay components from the sensor surface, i.e. regenerating a clean gold surface layer prior to each sensor assay cycle.

## 2. Materials and Methods

### 2.1. Instrumentation – Sensor analyser

The experiments were carried out using the Spreeta™ evaluation Module Kit manufactured by Texas Instruments Inc. (TI) (Texas, U.S.A.) (Woodbury *et al.*, 1998; Elkind *et al.*, 1999; Kukanskis *et al.*, 1999). The commercially available package consists of 50 miniature SPR sensors, associated electronic control box, flow-cell and software. The Spreeta™ sensor is a fully integrated device were all the components required for SPR such as light source and detector are integrated on a small chip and encapsulated in an optical clear epoxy element to enable a standard 'wedge-beam' Kretschmann prism SPR type arrangement that requires no further optical alignment after manufacture (see Figure 1).

The Spreeta™ SPR sensor has been integrated into a self-contained analyser (see Figure 2) and is comprised of a steel housing containing the Spreeta™ SPR device, TI flow-cell, TI control electronics, manual sample loop injection valve and liquid switching valve (Ominfit, Cambridge, U.K.) and reagent reservoir bottles. Reagents and buffers are pulled through the flow-cell using a peristaltic pump (at present using an external pump – Minipuls 3, Gilson, U.K.) at a typical flow rate of 60 $\mu$L min$^{-1}$.



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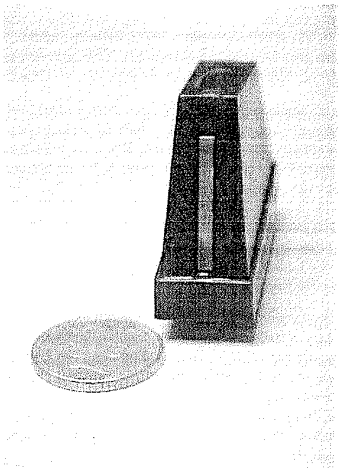

*Figure 1a.* The Texas Instrument Spreeta™ sensor showing gold sensing surface and encapsulation.

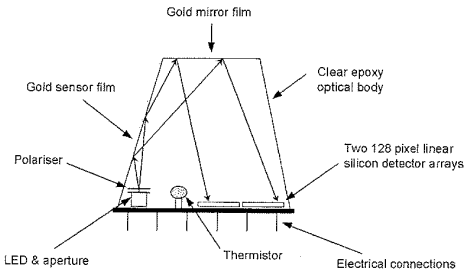


*Figure 1b.* Schematic of internal structure of an early Texas Instrument SPR sensor–sensing region is labelled 'gold sensor film'.

### 2.2. Software setup

The Spreeta™ SPR system is supplied with dedicated control and data handling software and was run on a notebook personal computer associated with the bioanalyser (see Figure 2). Typical software parameters used for experimentation were as follows:

- Number of automated measurements set to 20 (therefore SPR data output over 20 recorded data event values averaged to make 1 datapoint);
- Minimal monitoring interval (time required to drive the sensor and analyse the result) set to 0.25 sec (i.e. $20 \times 0.25 = 5$ sec per recorded datapoint);
- Every other data point event recorded (i.e. 10 sec between each saved data value);
- First Moment used as analysis method to determine SPR minimum position and hence refractive index.

### 2.3. Buffers and reagents

Phosphate buffered saline, pH 7.4 (PBS) (Sigma, U.K.) used as a running buffer to prime the sensor surface and for rinsing and Analar Water (BDH, Poole, U.K.) used for buffers and for refractive index calibration. The domestic laundry detergent used as a surface Regeneration Buffer was 1% (v/v) Persil Biological Liquid (Unilever, U.K.) in water.

Anti-Estrone-3-Gulcronide antibody (monoclonal IgG clone 4155, a kind gift provided by Unilever Research plc. U.K.) was made up to 200 $\mu$g mL$^{-1}$ in PBS and 0.05% Tween 20.

Calibration samples of E3G (Sigma, U.K.) were prepared by dissolving 1 mg of E3G in 1 mL of dimethylformamide (DMF) and then making a stock solution of E3G at 1 $\mu$g mL$^{-1}$ with PBS. Ovalbumin-E3G conjugate was supplied by Unilever Research plc (U.K.) at 150 $\mu$g mL$^{-1}$ made up in PBS. All solutions prior to use were degassed under vacuum at room temperature to minimise bubble formation in the fluidic system.

### 2.4. Procedures

The gold surface of the sensor was cleaned prior to performing an assay with the Regeneration Buffer for 10 min and then washed with water for a further 10 min.

This was important especially when using a new sensor or after extended dry storage. Once washed the sensor was then dried with a nitrogen gas stream prior to performing sensor initialisation. A typical procedure for performing an assay cycle follows:

- Before commencement of an experiment the sensor was initialised in air and then calibrated in water to establish a background reading were all following measurements would be referenced to;



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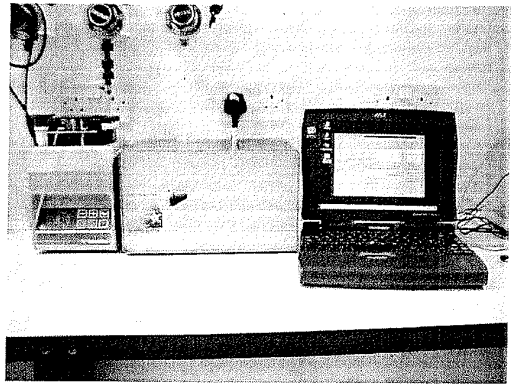

*Figure 2a.* External view of field-anaylser showing external peristaltic pump and control notebook computer.

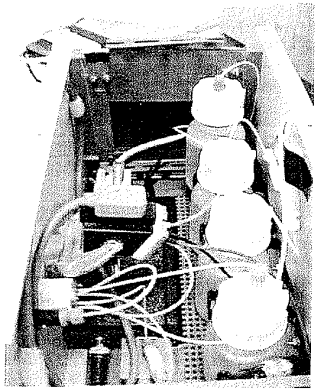

*Figure 2b.* Internal view showing sensor and flow-cell connected to sample valve and reagent bottles.

- PBS running buffer was flown past the surface for 5 min to obtain a baseline;
- Ovalbumin-E3G conjugate (200 $\mu$L) was injected in to the sample loop and then allowed to pass over the sensor surface after a PBS baseline was established;
- PBS was allowed to wash the sensor for 10–15 min to remove any loosely bound protein conjugate and obtain adsorbed conjugate only baseline measurement;
- An equal volume of anti-E3G IgG (100 $\mu$g mL$^{-1}$) was pre-incubated with sample (known/unknown concentrations of E3G) for 30 min;
- 200 $\mu$L of preceding assay mixture was then injected and allowed to flow passed the sensor surface with immobilised protein conjugate;
- PBS again washed over the sensor for 10–15 min to remove any loosely bound antibody and obtain a baseline measurement;
- The sensor surface was then cleaned/regenerated by flowing past the regeneration buffer over the gold surface for 15 min and then rinsing the surface with water for 10 min.

Once the assay procedure was completed the cycle was repeated for next sample measurement. Air initialisation and calibrating in water was not a required step for subsequent measurements.

### 2.5. SPR DETECTION AND DATA ANALYSIS

The automatically recorded data according to the parameters set, were viewed from the data table and transferred directly in to a Microsoft Excel 97 spreadsheet. The time verse refractive index or angle sensorgram scan obtained was then analysed manually to obtain changes in angle over time and relevant information on signal to noise data.

## 3. Results and Discussion

### 3.1. INTERFACE REGENERATION

One of the main aims of this on-going research is to provide a practical and reproducible affinity assay to be used with the Spreeta™ liquid SPR sensor. As we have considered the gold sensing surface to be practically irreplaceable, our main objective was to use the Spreeta™ sensor as a biosensor and attach a biologically specific layer on the sensing surface and in turn find a regeneration protocol that would be able to strip the surface of adsorbed protein thereby enabling the sensor to be reused. Typically 'washing' or 'regeneration' procedures for affinity sensor surfaces commonly involves exposure to single component detergents, variations of pH, variations of ionic strength, etc. We have employed a domestic laundry



| Regeneration with Persil | SPR Resonance Angle (°) |
|---|---|
| Water Baseline | 69.158 ± 0.164 |
| PBS Baseline | 69.340 ± 0.063 |
| Conjugate Baseline (in PBS) | 69.356 ± 0.045 |

*Figure 3.* Demonstration of repeated regeneration of a gold SPR sensor surface after physical adsorption of protein using a commercial laundry detergent (±1 SD, n = 15).

detergent as an alternative that contains a defined complex mixture of surfactants, proteases, cellulases, lipases and bleaching agents compared to simple traditional regeneration approaches.

Figure 3 summarises the effect on the optimum incident angle for SPR excitation for repeated immobilisation of E3G-ovalbumin conjugate and regeneration of the bare gold surface via the domestic laundry detergent. The average SPR angle increases as expected from water to PBS to conjugate as the refractive index experienced by the surface plasmons increases. The small average increase due to the adsorption of the E3G-ovalbumin conjugate is due to the de-naturation of the protein upon adsorption. Since the functionality of the conjugate is due to the availability of the E3G, the loss of the native ovalbumin structure is of no direct consequence. The variation of the SPR angle for the various steps in the 15 repeats included in Figure 3, demonstrate a significant level of variability in the absolute SPR angle value though the relative changes within cycles are not as significant.

### 3.2. SENSOR ASSAY RESULTS

The second aim of this current report was to demonstrate that the Spreeta™ sensor could be used as a biosensor to determine low molecular weight analytes such as EDC's for the eventual purpose of using the instrument as an automated field-analyser. Although the current competition/inhibition assay is not optimised, we have demonstrated a calibration curve for the model EDC, E3G, with good reproducibility for a current working range between 10 and 150 ppb (see Figure 4). This situation compares with published microtitre-plate based receptor assays with lower limits of detection of 0.1 ppb (0.1 $\mu$g L$^{-1}$) for the related 17$\beta$-estradiol (Seifert *et al.*, 1999). For the current study, E3G was chosen as the model EDC due to the ready availability of appropriate antibodies within our laboratories. The SPR data for an example single assay cycle is also shown in Figure 5.

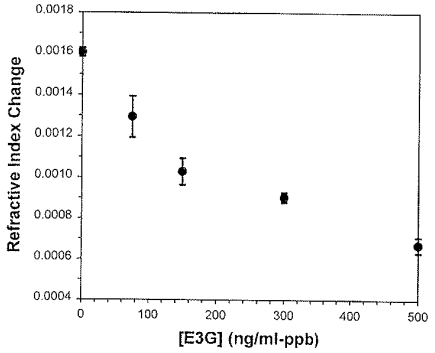


*Figure 4.* Calibration curve for the SPR sensor assay for E3G with the RI difference before and after the flow of the antibody-sample solution over the immobilised E3G layer. From this preliminary data, an approximate working range between 10 and 150 ppb E3G can be estimated (error bars are 1 SD, n = 3).

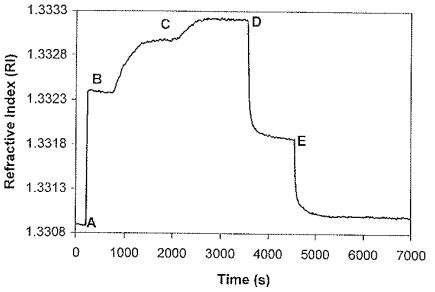


*Figure 5.* A typical assay time trace where A is the baseline of water, B is the baseline of phosphate buffer saline solution 7.4 pH, C is the addition and binding of anti-E3G, in the present of competing E3G, to E3G-ovalbumin conjugate, D is the regeneration of the gold surface using 1% Persil solution and E is the final rinsing with water. The amount of antibody binding is found by determining the refractive index change between conjugate baseline and antibody baseline (difference between immediately prior to point C and prior to point D).



Comment Letter L



The procedure for each assay cycle is as described in the Materials and Methods section. Figure 5 shows a typical SPR output trace of such an assay cycle and shows the various phases of the cycle and including the immobilisation of the ovalbumin-E3G conjugate, the binding of the anti-E3G antibody and the regeneration of gold sensing surface.

### 3.3. Further developments

Further refinement and optimisation of the current assay is required as time-per-assay-cycle, reduction of reagent (i.e. antibody) consumption and detection levels needs to be addressed. The time for a single assay cycle is currently excessive as it takes approximately 70 min for a full cycle to be completed. Significant reductions are expected by modification of the flow-cell fluidics and increased automation. By reducing the flow-cell volume and increasing the flow rate we will be able to improve the kinetics and mass transfer of solutes flowing passed the sensing surface while reducing the amount of reagents used. Additionally, the length of sample-antibody pre-incubation has not been optimised.

The current detection limits demonstrated needs to be lowered if the sensor is to be used in the field as levels of natural oestrogen are usually in the parts-per-trillion range. The use of a pre-concentration step, i.e. solid-phase extraction or an affinity column, will help to address detection limits and to reduce potential matrix effects present in real samples. Furthermore, to broaden the range of EDC's detectable and eventually have a true EDC sensor, the incorporation of recombinant endocrine receptors as affinity molecules can be envisaged.

## 4. Conclusions

There is a growing need for the analysis of potential environmental contaminates to be conducted at or near source and in real-time. Therefore, the need for portable, simple, low cost devices is of great importance. As hormone mimics are especially prevalent in surface and waste-water, our aim was to investigate whether the Spreeta™ SPR sensor was an applicable device to be used as a portable bioanalyser.

The current status of this on-going project provides evidence that the 'off-the-shelf' miniaturised SPR Spreeta™ liquid sensor can be incorporated into a field analyser and can be used with a simply assay protocol for a model EDC analyte (estrone-3-glucuronide) using a complementary antibody for sub-ppm detection. The desired reusability of the sensor was achieved by employing a novel approach using a domestic laundry detergent allowing the regeneration of the bare gold sensing surface and reproducible protein conjugate immobilisation via physical adsorption.

Future developments of this approach will include, (i) optimisation of assay protocols, (ii) improved fluidics, (iii) integration into the analyser of pumps and auto-

mated sampling/fluid switching and (iv) up-stream sample pre-treatment including filtering and solid-phase extraction/pre-concentration.

**Acknowledgements**

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Comment Letter L

Oral Abstract - #5

## Formation and Occurrence of Chlorinated Triclosan Derivatives (CTDs) and their Dioxin Photoproducts

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Triclosan, a widely used antimicrobial, has been frequently detected as a contaminant in waterways throughout the world. The fraction of triclosan that persists through wastewater treatment may be chlorinated during chlorine disinfection, resulting in chlorinated triclosan derivative (CTD) products. Triclosan and CTDs are of concern, because they have the potential to undergo photolysis in aquatic environments to form polychlorodibenzo-*p*-dioxins. While the occurrence of triclosan in wastewater effluent and natural waters has been well-studied, few measurements have been made of CTDs. It is important to determine the amount of CTDs formed from triclosan during wastewater disinfection, because they may give rise to more highly toxic dioxins.

This work has undertaken to develop an analytical method to determine triclosan and CTD concentrations in wastewater effluent before and after chlorine disinfection to assess the formation of CTDs. The method utilizes solid phase extraction (SPE) for pre-concentration and clean-up, followed by capillary liquid chromatography-electrospray ionization-triple quadrupole mass spectrometry (LC-ESI-QqQMS) analysis. Because triclosan and CTDs may be discharged into aquatic systems with wastewater effluent, the photochemistry of triclosan and CTDs has been investigated in natural waters under solar irradiation. The generation of a dioxin photoproduct has been confirmed for each CTD. Photolysis rates were found to be highly dependent on pH, as the phenolate forms degraded one to two orders of magnitude faster than the phenol forms. Photolysis quantum yields were also determined. An understanding of CTD formation during chlorine disinfection of wastewater and the photochemistry of CTDs enables an estimation of the total triclosan-derived dioxin load to the aquatic environment.

**Biography:**

Jeffrey Buth is an analytical chemistry Ph.D. candidate at the University of Minnesota, Twin Cities. He obtained his B.A. in chemistry at Augustana College in Rock Island, Illinois. Advised by Kris McNeill and Bill Arnold, his research focuses on chemical transformations of pharmaceutical and personal care product (PPCP) pollutants and the environmental occurrence of PPCPs and their transformation products.

Environmental Science & Technology

Article

# Toxicogenomic Response to Chlorination Includes Induction of Major Virulence Genes in *Staphylococcus aureus*

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Abstract | Full Text HTML | Hi-Res PDF [138 KB] | Supporting Info

## Abstract

Despite the widespread use of chlorination for microbial control in aqueous environments, cellular response mechanisms of human pathogens, such as *Staphylococcus aureus*, against chlorination remain unknown. In this work, genome-wide transcriptional analysis was performed to elucidate cellular response of *S. aureus* to hypochlorous acid, an active antimicrobial product of chlorination in aqueous solution. Our results suggest that hypochlorous acid repressed transcription of genes involved in cell wall synthesis, membrane transport, protein synthesis, and primary metabolism, while amino acid synthesis genes were induced. Furthermore, hypochlorous acid induced transcription of genes encoding major virulence factors of *S. aureus*, such as exotoxins, hemolysins, leukocidins, coagulases, and surface adhesion proteins, which all play essential roles in staphylococcal virulence. This work implies that chlorination may stimulate production of virulence factors, which provides new insight into host−pathogen interactions and effects of chlorine application for microbial control.

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Toxicogenomic Response of *Staphylococcus aureus* to Peracetic Acid *Environmental Science & Technology*

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Comment Letter L

1: Water Res. 2009 Mar;43(4):925-32. Epub 2008 Dec 6.



**A seasonal study of the mecA gene and Staphylococcus aureus including methicillin-resistant S. aureus in a municipal wastewater treatment plant.**

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Department of Clinical and Experimental Medicine, Division of Medical Microbiology, Linköping University, SE-581 85 Linköping, Sweden. stefan.borjesson@liu.se

The spread of methicillin-resistant Staphylococcus aureus (MRSA), in which the mecA gene mediates resistance, threatens the treatment of staphylococcal diseases. The aims were to determine the effect of wastewater treatment processes on mecA gene concentrations, and the prevalence of S. aureus and MRSA over time. To achieve this a municipal wastewater treatment plant was investigated for the mecA gene, S. aureus and MRSA, using real-time PCR assays. Water samples were collected monthly for one year, at eight sites in the plant, reflecting different aspects of the treatment process. The mecA gene and S. aureus could be detected throughout the year at all sampling sites. MRSA could also be detected, but mainly in the early treatment steps. The presence of MRSA was verified through cultivation from inlet water. The concentration of the mecA gene varied between months and sampling sites, but no obvious seasonal variation could be determined. The wastewater treatment process reduced the mecA gene concentration in most months. Taken together our results show that the mecA gene, S. aureus and MRSA occur over the year at all sites investigated.

PMID: 19084256 [PubMed - indexed for MEDLINE]



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| **Author:** | Börjesson, Stefan (Linköping University, Medical Microbiology )(Linköping University, Faculty of Health Sciences) (Per-Eric Lindgren) |
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| **Alternative title (sv) :** | Resistenta gula stafylokocker (MRSA) och antibiotikaresistensgener förekommer i svenskt kommunalt avloppsvatten |
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| **Abstract(en) :** | A large part of the antibiotics consumed ends up in wastewater, and in the wastewater the antibiotics may exert selective pressure for or maintain resistance among microorganisms. Antibiotic resistant bacteria and genes encoding antibiotic resistance are commonly detected in wastewater, often at higher rates and concentrations compared to surface water. Wastewater can also provide favourable conditions for the growth of a diverse bacterial community, which constitutes a basis for the selection and spread of antibiotic resistance. Therefore, wastewater treatment plants have been suggested to play a role in the dissemination and development of antibiotic resistant bacteria. Methicillin-resistant *Staphylococcus aureus* (MRSA) is a large problem worldwide as a nosocomial pathogen, but knowledge is limited about occurrence in non-clinical environments, such as wastewater, and what role wastewater plays in dissemination and development of MRSA.<br><br>In this thesis we investigated the occurrence of MRSA in a full-scale wastewater treatment plant (WWTP). We also investigated the concentration of genes encoding resistance to aminoglycosides (*aac*(6')-Ie+*aph*(2")), β-lactam |

1: Quantification of genes encoding resistance to

antibiotics (*mec*A) and tetracyclines (*tet*A and *tet*B) in three wastewater-associated environments: (1) soil from an overland flow area treating landfill leachates, (2) biofilm from a municipal wastewater treatment plant, and (3) sludge from a hospital wastewater pipeline. In addition, concentrations of *mec*A, *tet*A and *tet*B were investigated over the treatment process in the WWTP. These investigations were performed to determine how the prevalence and concentration of MRSA and the antibiotic resistence genes are affected in wastewater and wastewater treatment processes over time. The occurrence of MRSA was investigated by cultivation and a commercially available real-time PCR assay. In order to determine concentrations of the genes *aac*(6')-Ie+*aph*(2''), *mec*A, *tet*A and *tet*B in wastewater we developed a LUX™ real-time PCR assay for each gene.

Using cultivation and real-time PCR we could for the first time describe the occurrence of MRSA in wastewater and show that it had a stable occurrence over time in a WWTP. MRSA could mainly be detected in the early treatment steps in the WWTP, and the wastewater treatment process reduced the number and diversity of cultivated MRSA. However, our results also indicate that the treatment process selects for strains with more extensive resistance and possibly higher virulence. The isolated wastewater MRSA strains were shown to have a close genetic relationship to clinical isolates, and no specific wastewater lineages could be detected, indicating that they are a reflection of carriage in the community. Taken together, these data indicate that wastewater may be a potential reservoir for MRSA and that MRSA are more prevalent in wastewater than was previously thought.

The real-time PCR assays, for *aac*(6')-Ie+*aph*(2''), *mec*A, *tet*A, and *tet*B that we developed, were shown to be sensitive, fast, and reproducible methods for detection and quantification of these genes in wastewater environments. The highest concentrations of all genes were observed in the hospital pipeline, and the lowest in the overland flow system, with *tet*A and *aac*(6')-*Ie*+*aph*(2'') detected in all three environments. In the full-scale WWTP, we continuously detected *mec*A, *tet*A and *tet*B over the treatment process and over time. In addition, it was shown that the treatment process reduces concentrations of all three genes. The data presented in this thesis also indicate that the reduction for all three genes may be connected to the removal of biomass, and in the reduction of *tet*A and *tet*B, sedimentation and precipitation appear to play an important role.

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| **Supervisor:** | Lindgren, Per-Eric , Docent (Linköping University, Medical Microbiology )(Linköping University, Faculty of Health Sciences) |
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**LIST OF PAPERS**

aminoglycosides, β-lactams and tetracyclines in wastewater environments by real-time PCR

2:
A seasonal study of the mecA gene and *Staphylococcus aureus* including methicillin-resistant *S. aureus* in a municipal wastewater treatment plant

3:
Methicillin-resistant *Staphylococcus aureus* (MRSA) in municipal wastewater: An uncharted threat?

4:
Genes encoding tetracycline resistance in a full-scale municipal wastewater treatment plant investigated during one year

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, June 1995, p. 2099–2107 Vol. 61, No. 6
0099-2240/95/$04.00+0


# Phenotypic and Genetic Diversity of Chlorine-Resistant *Methylobacterium* Strains Isolated from Various Environments

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**Strains of pink-pigmented facultative methylotrophs which were isolated previously from various environments and assigned tentatively to the genus *Methylobacterium* were characterized in comparison with authentic strains of previously known species of this genus. Most of the isolates derived from chlorinated water supplies exhibited resistance to chlorine, whereas 29 to 40% of the isolates from air, natural aquatic environments, and clinical materials were chlorine resistant. None of the tested authentic strains of *Methylobacterium* species obtained from culture collections exhibited chlorine resistance. Numerical analysis of phenotypic profiles showed that the test organisms could be divided into 19 clusters at a similarity level of 80%, at which all established *Methylobacterium* species tested were separated from each other except *M. organophilum* and *M. rhodesianum*. The chlorine-resistant isolates were randomly distributed among all clusters. The 16S ribosomal DNA (rDNA) sequence-based phylogenetic analyses showed that representatives of the isolates together with known *Methylobacterium* species formed a line of descent distinct from that of members of related genera in the alpha-2 subclass of the *Proteobacteria* and were divided into three subclusters within the *Methylobacterium* group. These results demonstrate that there is phenotypic and genetic diversity among chlorine-resistant *Methylobacterium* strains within the genus.**

The genus *Methylobacterium* (40) is a group of strictly aerobic, facultatively methylotrophic, gram-negative, rod-shaped bacteria that are able to grow on one-carbon compounds more reduced than carbon dioxide as sole carbon and energy sources (for a review, see reference 19). Mass cultures of these facultative methylotrophs are pink to red because of the presence of carotenoids. They also produce bacteriochlorophyll *a* under aerobic growth conditions (39, 45, 54), and some of them have proven to contain a photochemical reaction center similar to the reaction center of purple phototrophic bacteria (38, 50). Because of their production of photopigments with photochemical activity under aerobic conditions, *Methylobacterium* strains are categorized as aerobic phototrophic bacteria (47, 48). The genus *Methylobacterium* now consists of nine species, with *M. organophilum* as the type species (20, 21, 40, 53, 54). They are phenotypically and chemotaxonomically quite similar, and phenotypic differences among the species are found in only limited properties, such as carbon source utilization. In recent years, 16S rRNA sequence information has been used for phylogenetic placement and identification of different physiological groups of methylotrophic proteobacteria, including *Methylobacterium* species (4, 6, 27, 51, 52). The molecular data have shown that the genus *Methylobacterium* represents a line of descent in the alpha-2 subclass of the class *Proteobacteria*.

Members of the genus *Methylobacterium* are distributed in a wide variety of natural habitats, including soil, dust, air, fresh water, and aquatic sediments. These bacteria also occur in human-made environments, including potable water supplies (10–13, 18, 33), bathrooms (14), and washstands (14), where they sometimes produce pink ropy masses of growth. It is important to note that most of the *Methylobacterium* strains isolated from these environments are highly resistant to chlorine (11–13, 33). The capacity of the methylotrophic bacteria for chlorine resistance may explain why these organisms frequently occur in human environments. Moreover, some pink-pigmented bacteria that are now assigned to the genus *Methylobacterium* were isolated as opportunistic pathogens from clinical sources (15, 16, 22, 43), including the blood of a patient with AIDS (31), and from hospital environments (10). In Japan, therefore, the frequent occurrence and colonization of these bacteria in potable water systems have in recent years received much attention as a potential public health hazard. Nevertheless, while many species of acrobic chemoheterotrophic bacteria have been isolated from drinking water systems and other chlorinated environments throughout the world (8, 36, 37, 41, 42), little attention has been paid to the incidence, taxonomic identity, and chlorine resistance capacity of *Methylobacterium* strains possibly predominating in those environments.

We have hitherto isolated large numbers of chlorine-resistant *Methylobacterium* strains from various environments, including potable water supplies, in Japan. However, the classification of our isolates was tentative, and their taxonomic identity at the species level has remained unclear. Detailed systematic studies on these isolates are indispensable for elucidating the ecological and public health significance of the facultative methylotrophs in chlorinated environments. The present study was undertaken to characterize our isolates more

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thoroughly in this respect and to determine whether the capacity for chlorine resistance is widespread among known members of the genus *Methylobacterium* or is characteristic of particular species. Our approaches to this investigation were twofold: one involved physiological and biochemical characterization of the isolates, followed by numerical analysis of phenotypic profiles, and the other involved genetic analysis by PCR sequencing of 16S ribosomal DNA (rDNA). A previous phylogenetic study has shown that some *Methylobacterium* species, including those closely related to *M. extorquens*, are responsible for the formation of pink biofilms in a potable water treatment system (27).

## MATERIALS AND METHODS

**Bacterial strains and cultivation.** A total of 77 strains isolated previously from a variety of environments (10–14, 33) were studied (see Fig. 1). These test strains consisted of 30 strains from potable water tanks (strains F01 to F30), 28 strains from the air (F31 to F58), 10 strains from wells and ponds (F59 to F68), 7 strains from clinical materials (F69 to F75), and 2 strains from a potable water treatment system (GK101 and GK118). All of these isolates were strictly aerobic, facultatively methylotrophic, pink-pigmented, chemoorganotrophic bacteria that had gram-negative or gram-variable, non-spore-forming, motile, rod-shaped cells. They were also positive for catalase and oxidase and produced bacteriochlorophyll *a* under aerobic growth conditions. These phenotypic data led to tentative classification of the isolates as members of the genus *Pentomonas* (now reclassified as *Methylobacterium* [3]) or *Methylobacterium* (10–14, 27, 33). As reference organisms, the following seven strains were also used: *M. extorquens* JCM 2802[T] (the superscript T indicates the type strain), *M. mesophilicum* JCM 2829[T], *M. organophilum* JCM 2833[T], *M. radiotolerans* JCM 2831[T], *M. rhodesianum* JCM 2830, *M. rhodinum* JCM 2831[T], and *M. zatmanii* JCM 2819. All strains with JCM numbers were obtained from the Japan Collection of Microorganisms, Riken, Wako, Japan, and were originally isolated from nonchlorinated environments, including soil, lake, leaf surface, rhizosphere, and fermentors (20, 21, 40, 54). All test organisms were maintained on agar slants at 10°C and subcultured every 3 months. Complex medium PBY (28) or standard agar (SA) medium (Nissui, Tokyo, Japan) was used for cultivation of the organisms. PBY medium was solidified by adding 1.5 and 1.8% agar when it was used for agar slants and plates, respectively.

**Chlorine resistance activity.** Colonies of the test organisms grown on SA medium at 30°C for 5 days were harvested, washed three times with autoclaved distilled water, and resuspended in this water. These cell suspensions were used immediately for tests for chlorine resistance. For these tests, 200-ml portions of a chlorine solution (0.1 mg as free residuals per liter) were prepared in 300-ml Erlenmeyer flasks by diluting and autoclaving a stock 10% aqueous solution of sodium hypochlorite in 10 mM phosphate buffer (pH 7.0). To this solution, the washed cells were resuspended to give a cell density of ca. $10^5$ CFU/ml and incubated at 25°C for 5 min with gentle stirring. Then, 5-ml portions of the cell suspensions were taken and mixed with 0.05 ml of 0.3 M sodium thiosulfate to be neutralized. Viable cells in the suspensions were counted on SA medium which was incubated at 30°C for 7 days. Upon chlorine treatment, strains giving viable cell counts of more than 10% of the initial counts were considered chlorine-resistant strains. In these tests, most of the chlorine-resistant strains had viable-cell counts equivalent to the initial counts, suggesting that the thiosulfate used as a neutralizer had no effect on the viability of these organisms.

**Phenotypic characterization and numerical analysis.** Cells grown aerobically in PBY medium or on SA medium at 30°C for 2 to 3 days were subjected to a set of 88 phenotypic tests. This set of tests consisted of carbon source utilization tests for 49 organic compounds, biochemical reaction tests for 19 enzymes, and susceptibility tests for 20 antibiotics. In all tests, incubation was at 30°C. Tests for carbon source utilization and enzyme activities were performed with the API 50CH and API ZYM systems (BioMerieux, Montalieu-Vercieu, France), respectively; the final reading was made after 3 weeks of incubation for the former and 4 h for the latter. Antibiotic susceptibility was determined with the TRIDISC system (Eiken Chemical Co., Tokyo, Japan) after 1 week of incubation. For numerical analysis, tests for which all test strains were positive or negative were excluded, and the results for the remainder applied to each strain were coded as 0 (negative), 1 (weakly positive), 2 (moderately positive), or 3 (strongly positive). The codes were entered into a data matrix, which was then used to calculate a simple matching coefficient. A cluster analysis for the similarity coefficient was performed by the unweighted pair group mathematical averaging (UPGMA) method (49). Calculation of the similarity values and clustering by the UPGMA method were performed on an NEC personal computer with the Lotus 1-2-3 Multivariate Analysis program (Audemain Co., Tokyo, Japan).

**Analyses of fatty acids and quinones.** Cells grown in PBY medium were harvested by centrifugation from cultures at the early stationary phase of growth, washed, and lyophilized. Fatty acids were extracted as their methyl esters from lyophilized cells and analyzed by gas-liquid chromatography as described previously (29). Quinones were extracted with an organic solvent mixture from freeze-dried cells, purified by thin-layer chromatography, and analyzed by reverse-phase high-performance liquid chromatography (26).

**Amplification and sequencing of 16S rDNA.** Colonies grown on SA medium were harvested, washed with sterilized 1% saline, resuspended in sterilized pure water, and then stored at −20°C. For PCR experiments, crude cell lysates were prepared by treating cells with proteinase K as described previously (30), but the procedure was modified by introducing sonic treatment prior to protease digestion. Stock cell suspensions were thawed, sonicated for 20 s with 2-s intermittent bursts (20 kHz; output power, 50 W), digested with proteinase K in the presence of detergents, and then heated at 95°C for 5 min, followed by centrifugation to remove unbroken cells and large debris. The resultant cell lysates as sources of template DNA were used directly for PCR. The 16S rDNA fragments that corresponded to positions 8 to 1510 of *Escherichia coli* 16S rRNA (5) were amplified by PCR, purified by agarose gel electrophoresis with resin binding, and sequenced directly by the linear PCR sequencing (cycle sequencing) method modified for automated fluorescence detection with a Pharmacia DNA sequencer. The PCR and sequencing procedures used have been described in previous papers (25, 30).

**Phylogenetic analysis.** Sequences were compiled from overlapping sequence data, and binary sequence similarities were calculated on an Apple Macintosh personal computer with the GENETYX-MAC program (Software Development Co., Tokyo, Japan). Multiple alignments of sequence, calculation of nucleotide substitution rates ($K_{nuc}$) (34), and construction of neighbor-joining phylogenetic trees (44) were performed with the CLUSTAL V program (24). Alignment gaps and unidentified base positions were not taken into consideration for the calculations. The topology of the phylogenetic tree was evaluated by bootstrap analysis (9) with 1,000 bootstrapped trials.

**Nucleotide sequence accession numbers.** The sequence determined in this study has been deposited in the DDBJ, GSDB, EMBL, and NCBI nucleotide sequence databases under accession numbers D32224 to D32237. The accession numbers for the sequences used in phylogenetic analysis are as follows: *Acidiphilium cryptum*, D30773; *Afipia felis*, M65248; *Agrobacterium tumefaciens*, M11223; *Azorhizobium caulinodans*, X67221; *Bartonella quintana*, M11927; *Caulobacter crescentus*, M83799; *Erythrobacter longus* M59062; *Hirschia baltica* M59069; *Hyphomicrobium facilis*, D25311; *Methylobacterium* sp. strain GK101, D25305; *Methylobacterium* sp. strain GK118, D25306; *Rhodobacter capsulatus*, D16428; *Rhodobium marinum*, D30790; *Rhodopila globiformis*, M59066; *Rhodoplanes roseus*, D25313; *Rhodopseudomonas palustris*, D25312; *Rhodospirillum rubrum*, D30778; *Rhodospirillum sulfexigens*, M59070; *Rhodovulum sulfidophilum*, D16423; *Roseobacter denitrificans*, M59063; and *Sphingomonas paucimobilis*, D13725.

## RESULTS

**Phenotypic characterization and numerical analysis.** The 77 isolates and 7 authentic strains of *Methylobacterium* species were subjected to a set of 88 phenotypic tests. As a result of these tests, 40 characteristics could be taken into consideration in numerical analysis. A simplified dendrogram constructed on the basis of the results of our phenotypic tests is shown in Fig. 1. At a similarity level of 80%, the test organisms were divided into 19 clusters (designated phena 1 to 19), each of which contained more than two strains. Although phenon 7 included only one strain, we designated it a cluster because the strain was the type strain of *M. rhodinum*. The established species of the genus *Methylobacterium* could be separated from one another at an 80% level of similarity. For example, in addition to *M. rhodinum* in phenon 7, we found *M. zatmanii* in phenon 1, *M. extorquens* in phenon 2, *M. mesophilicum* in phenon 13, and *M. radiotolerans* in phenon 19. The exceptional cases were *M. organophilum* and *M. rhodesianum*, both of which fell into a single cluster (phenon 3) at an 80% level of similarity. Thus, each of the 19 phena which we defined may correspond to a species or a similar taxonomic level within the genus *Methylobacterium*.

The frequencies of positive characteristics in the 19 phena are shown in Table 1. As already described as key characteristics for the identification of *Methylobacterium* species (19), carbon source utilization tests for L-arabinose, D-xylose, D-fucose, fructose, glucose, and some other substrates were found to be useful for the classification of the test strains belonging to different phena. These strains showed resistance to a number of antibiotics tested, but most of them were susceptible to josamycin, tetracycline, and gentamicin. All of

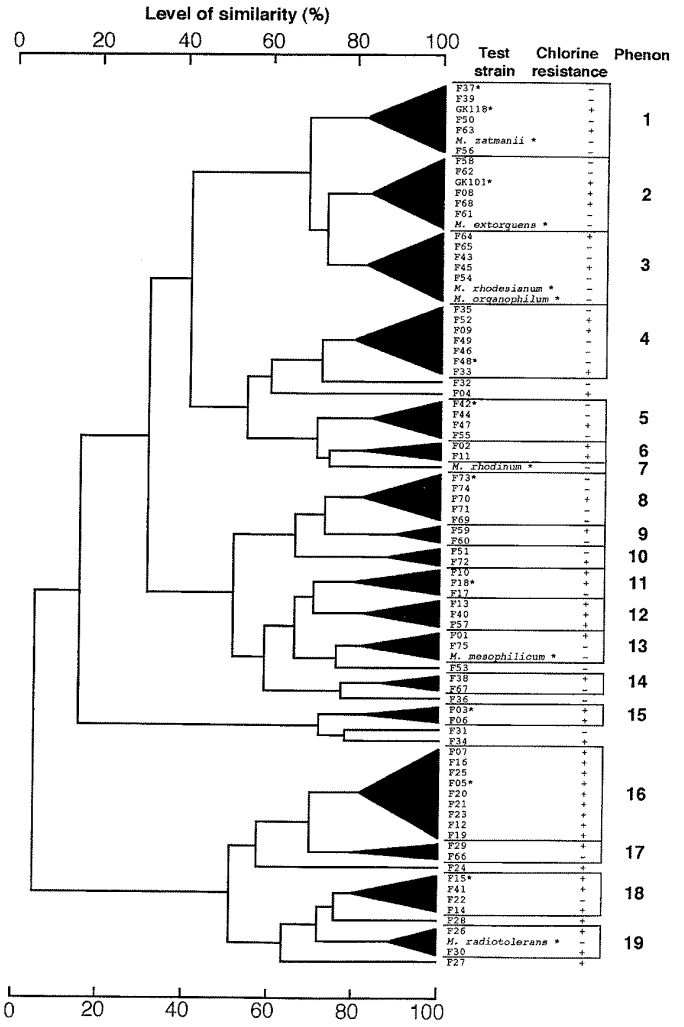


FIG. 1. Simplified dendrogram showing phenotypic clusters of *Methylobacterium* strains obtained by UPGMA linkage of similarity values. Test strains which were used for 16S rDNA analysis are marked with an asterisk.







TABLE 1. Characteristics of *Methylobacterium* strains belonging to different phena

| Test | % of isolates positive in phenon: | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 (7)[a] | 2 (7) | 3 (7) | 4 (7) | 5 (4) | 6 (2) | 7 (1) | 8 (5) | 9 (2) | 10 (2) | 11 (3) | 12 (3) | 13 (3) | 14 (2) | 15 (2) | 16 (9) | 17 (2) | 18 (4) | 19 (3) |
| Carbon sources | | | | | | | | | | | | | | | | | | | |
| Glycerol | 100 | 100 | 100 | 29 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| D-Arabinose | 0 | 0 | 0 | 14 | 0 | 100 | 0 | 0 | 0 | 0 | 0 | 67 | 33 | 0 | 50 | 100 | 50 | 100 | 100 |
| L-Arabinose | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 50 | 100 | 100 | 100 | 100 |
| D-Ribose | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 33 | 0 | 67 | 50 | 0 | 22 | 0 | 25 | 33 |
| D-Xylose | 0 | 0 | 0 | 0 | 0 | 50 | 0 | 0 | 0 | 50 | 100 | 100 | 100 | 100 | 50 | 100 | 100 | 100 | 100 |
| L-Xylose | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 67 | 0 | 0 | 56 | 0 | 0 | 67 |
| D-Galactose | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 100 | 100 | 100 | 33 | 100 | 100 | 50 | 0 | 100 | 100 | 100 | 100 |
| D-Glucose | 0 | 0 | 86 | 29 | 0 | 100 | 100 | 80 | 50 | 0 | 100 | 100 | 0 | 0 | 100 | 89 | 100 | 100 | 100 |
| D-Fructose | 71 | 57 | 100 | 43 | 100 | 50 | 100 | 0 | 0 | 0 | 0 | 33 | 0 | 100 | 0 | 100 | 50 | 25 | 0 |
| D-Turanose | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 50 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| D-Lyxose | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 100 | 33 | 100 | 100 | 0 | 100 | 0 | 100 | 100 |
| D-Fucose | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 0 | 100 | 100 | 100 | 100 |
| L-Fucose | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 11 | 0 | 0 | 33 |
| Gluconate | 0 | 0 | 0 | 14 | 75 | 100 | 0 | 0 | 0 | 0 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| 2-Ketogluconate | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 67 | 0 | 0 | 0 | 0 | 0 | 67 |
| 5-Ketogluconate | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 33 | 0 | 0 | 0 | 0 | 0 | 0 |
| Enzyme activity | | | | | | | | | | | | | | | | | | | |
| α-Glucosidase | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 80 | 0 | 0 | 33 | 0 | 0 | 0 | 0 | 22 | 0 | 25 | 33 |
| Alkaline phosphatase | 14 | 14 | 14 | 14 | 0 | 50 | 100 | 0 | 0 | 0 | 0 | 0 | 0 | 50 | 50 | 0 | 0 | 25 | 33 |
| Acid phosphatase | 29 | 0 | 14 | 14 | 50 | 100 | 0 | 100 | 100 | 50 | 33 | 67 | 67 | 0 | 0 | 56 | 50 | 100 | 33 |
| Phosphoamidase | 14 | 14 | 43 | 14 | 0 | 0 | 100 | 100 | 100 | 50 | 33 | 0 | 67 | 0 | 0 | 22 | 100 | 50 | 0 |
| Esterase (C4) | 71 | 100 | 86 | 71 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 67 | 50 | 100 | 100 | 100 | 100 | 100 |
| [illegible] | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] |
| Leucinearylamidase | 57 | 71 | 71 | 29 | 75 | 0 | 0 | 20 | 0 | 0 | 0 | 33 | 0 | 100 | 0 | 0 | 50 | 25 | 0 |
| Valinearylamidase | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 20 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Trypsin | 14 | 0 | 29 | 0 | 25 | 0 | 0 | 100 | 0 | 0 | 0 | 0 | 0 | 50 | 0 | 11 | 50 | 50 | 0 |
| Antibiotics | | | | | | | | | | | | | | | | | | | |
| Penicillin | 0 | 14 | 0 | 14 | 25 | 0 | 0 | 0 | 0 | 50 | 0 | 0 | 0 | 0 | 100 | 56 | 100 | 50 | 0 |
| Carbenicillin | 0 | 29 | 57 | 57 | 100 | 100 | 100 | 80 | 0 | 100 | 33 | 67 | 67 | 0 | 100 | 100 | 100 | 100 | 100 |
| Amoxicillin | 29 | 57 | 86 | 100 | 100 | 100 | 100 | 100 | 0 | 100 | 67 | 100 | 100 | 50 | 100 | 100 | 50 | 100 | 100 |
| Erythromycin | 43 | 71 | 43 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| Josamycin | 71 | 86 | 86 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| Chloramphenicol | 0 | 14 | 0 | 0 | 75 | 50 | 0 | 0 | 0 | 50 | 0 | 33 | 33 | 0 | 100 | 89 | 100 | 100 | 100 |
| Thiamphenicol | 0 | 14 | 0 | 43 | 100 | 50 | 0 | 60 | 0 | 100 | 0 | 33 | 67 | 0 | 100 | 100 | 100 | 100 | 100 |
| Tetracycline | 86 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| Gentamicin | 57 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| Cephalothin | 0 | 0 | 29 | 43 | 75 | 0 | 0 | 60 | 0 | 100 | 0 | 0 | 33 | 0 | 100 | 100 | 100 | 100 | 67 |
| Cephalexin | 0 | 43 | 29 | 29 | 50 | 100 | 0 | 100 | 0 | 100 | 67 | 0 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| Cefazolin | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 50 | 0 | 50 | 0 | 0 |
| Cephapirin | 0 | 14 | 0 | 43 | 25 | 0 | 0 | 20 | 0 | 50 | 0 | 0 | 0 | 0 | 100 | 78 | 100 | 100 | 0 |

[a] Numbers in parentheses indicate the number of strains isolated and tested.

the test organisms were susceptible to minocyclin and kanamycin (data not shown).

Gas-liquid chromatographic analyses of whole-cell fatty acids showed that all of the test strains contained saturated and monounsaturated straight-chain fatty acids, with $C_{18:1}$ predominating (>80% of the total content). They also contained 3-hydroxy fatty acids, with 3-OH $C_{14:0}$ predominating. Quinone profiling by high-performance liquid chromatography revealed that all test strains contained ubiquinone-10 as the sole quinone component. These findings confirmed the results of previous studies on the chemotaxonomic characteristics of the methylotrophic bacteria (54).

**Relationships of phenotypes, chlorine resistance, and sources.** Figure 1 shows the results of tests for chlorine resistance in addition to the results of numerical analysis of phenotypic profiles. Also, the relationships between the chlorine resistance capacity and the sources of the test organisms are summarized in Table 2. The two isolates obtained from a raw-water treatment system and almost all of the isolates from the potable-water tanks showed chlorine resistance, whereas 29 to 40% of those from the air, wells, ponds, and clinical materials had this property. None of the authentic strains of known *Methylobacterium* species obtained from the culture collections were resistant to chlorine. The isolates from tank

TABLE 2. Capacity for chlorine resistance of isolates from different sources and authentic *Methylobacterium* strains

| Test organism and source | No. of strains tested | No. resistant[a] | % Resistant |
|---|---|---|---|
| Isolates | | | |
| Potable-water tanks | 30 | 28 | 93 |
| Air | 28 | 9 | 32 |
| Wells and ponds | 10 | 4 | 40 |
| Clinical materials | 7 | 2 | 29 |
| Raw-water treatment system | 2 | 2 | 100 |
| Authentic strains from culture collections | 7 | 0 | 0 |

[a] Strains resistant to treatment with chlorine (0.1 mg/liter) for 5 min.

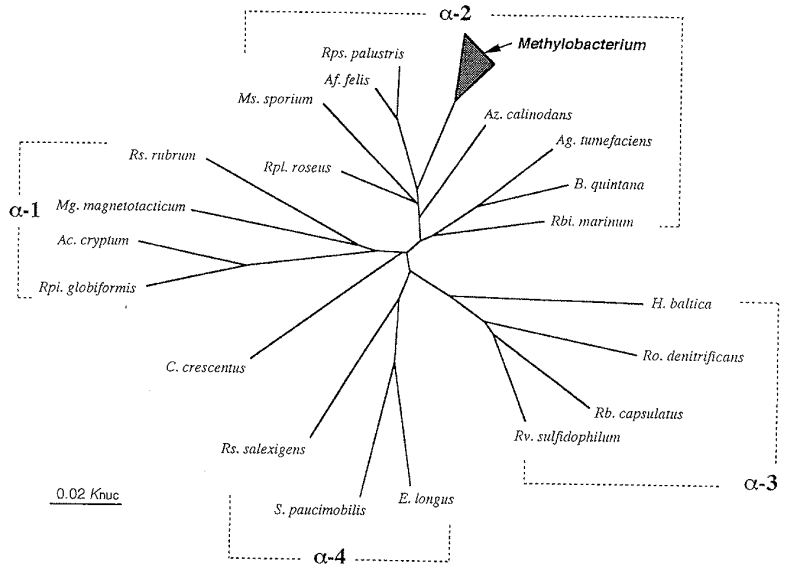


FIG. 2. Distance matrix tree showing phylogenetic positions of *Methylobacterium* strains among members of the alpha subclass of the *Proteobacteria*. The cluster including the *Methylobacterium* strains tested is shown as a shaded triangle. The species given on the tree are as follows: *Ac. cryptum*, *Acidiphilium cryptum*; *Af. felis*, *Afipia felis*; *Ag. tumefaciens*, *Agrobacterium tumefaciens*; *Az. calinodans*, *Azorhizobium calinodans*; *B. quintana*, *Bartonella quintana*; *C. crescentus*, *Caulobacter crescentus*; *E. longus*, *Erythrobacter longus*; *H. baltica*, *Hirschia baltica*; *Mg. magnetotacticum*, *Magnetospirillum magnetotacticum*; *Ms. sporium*, *Methylosinus sporium*; *Rb. capsulatus*, *Rhodobacter capsulatus*; *Rbi. marinum*, *Rhodobium marinum*; *Rpl. roseus*, *Rhodoplanes roseus*; *Rps. palustris*, *Rhodopseudomonas palustris*; *Rs. rubrum*, *Rhodospirillum rubrum*; *Rs. salexigens*, *Rhodaspirillum salexigens*; *Rv. sulfidophilum*, *Rhodovulum sulfidophilum*; *Ro. denitrificans*, *Roseobacter denitrificans*; *S. paucimobilis*, *Sphingomonas paucimobilis*; *Rpi. globiformis*, *Rhodopila globiformis*.

water, most of which were chlorine resistant, were relatively abundant in phenon 16 but also occurred in 10 other phena. The isolates from the other sources were also randomly distributed in different phena. As a result, all recognized phena included at least one chlorine-resistant strain, except phenon 7, which consisted of only one strain, *M. rhodinum* JCM 2811$^T$. These results suggest that the capacity for chlorine resistance of the isolates is related to their source but not to their taxonomic properties.

**16S rDNA sequencing.** For 16S rDNA sequence studies, representative isolates with or without the chlorine resistance capacity were selected from 8 of the 19 clusters, i.e., phena 1, 4, 5, 8, 11, 15, 16, and 18. All the phena from which the test organisms were selected are different from the clusters including the established *Methylobacterium* species except phenon 1 (see Fig. 1). Of the nine species of the genus *Methylobacterium* currently established, five species have been studied for 16S rRNA or rDNA sequences (6). However, we found that all of the published sequence data for the methylotrophs include a number of undetermined positions and possibly erroneous gaps of more than 20 positions in each sequence. Exclusion of these undetermined positions may have serious effects on the elucidation of interspecies relationships, because the sequences of *Methylobacterium* strains have high levels of similarity to one another, as described below. Therefore, we attempted to determine the 16S rDNA sequences not only for our isolates but also for most *Methylobacterium* species, including those species whose sequences are already available, and we used our new versions of the sequences in phylogenetic analyses.

The 16S rRNA genes from crude lysates of the eight isolates and seven established *Methylobacterium* species were amplified successfully by PCR except for one strain (strain F03), which yielded a very small PCR product, not sufficient for full sequencing. The amplified double-stranded 16S rDNAs were sequenced directly by the cycle sequencing method followed by automated fluorescence detection. The sequences which we determined included 1,400 to 1,407 residues of a continuous nucleotide stretch, covering 95% of the entire 16S rRNA gene. The 16S rDNAs of all test strains exhibited large nucleotide deletions at about positions 80, 210, and 470, features placing them in the alpha subclass of the *Proteobacteria*.

**Phylogenetic analysis.** Our sequence data were aligned with a data set consisting of 20 sequences from representative species within the alpha subclass of the *Proteobacteria*, and the evolutionary distance ($K_{nuc}$) values were calculated for the 1,226 positions that could be aligned. On the basis of the $K_{nuc}$ values obtained, a phylogenetic tree was reconstructed by the neighbor-joining method (Fig. 2). All *Methylobacterium* test strains formed a tight cluster (as indicated by the shaded triangle) in the alpha-2 subclass of the *Proteobacteria*.

Since the tested *Methylobacterium* strains showed high levels of binary sequence similarity (93.8 to 99.6%) (see Table 3) and represented a line of descent distinct from that of other members of the alpha-2 subclass, it was necessary to take as many sequence positions as possible into consideration to elucidate interspecies relationships more accurately. Thus, we made another data set consisting of sequences for only the *Methylobacterium* strains and *Rhodospirillum rubrum* as a representative of outgroups and calculated $K_{nuc}$ values for the alignable 1,394 positions of this set. The evolutionary distance ($K_{nuc}$) values thus obtained and the levels of binary sequence similarity are shown in Table 3. A neighbor-joining phylogenetic tree which was reconstructed on the basis of these distance matrix data is shown in Fig. 3. The test organisms could be divided into three major groups that were designated subclusters I, II, and III. The known *Methylobacterium* species fell into two of the subclusters, one of which included *M. extorquens*, *M. organophilum*, *M. rhodesianum*, and *M. zatmanii* (subcluster I), and one of which encompassed *M. mesophilicum* and *M. radiotolerans* (subcluster II). Highly chlorine-resistant strains GK101 and [illegible], both of which were studied previously for 16S rRNA sequences (27), belonged to subcluster I together with *M. extorquens* and related species. Five of our tested isolates were placed in subcluster II, and the remaining two isolates (strains F37 and F48) formed another lineage (subcluster III) distinct from the above two clusters. However, the relationships between subcluster III and the other two clusters appeared to be unstable, as suggested by a low level of bootstrap confidence.





## DISCUSSION

Many species of aerobic chemoheterotrophic bacteria have been isolated from potable water and chlorinated environments. LeChevallier et al. (36, 37) isolated members of the genera *Acinetobacter*, *Arthrobacter*, *Bacillus*, *Corynebacterium*, and *Pseudomonas* as the major constituents of aerobic heterotrophs from chlorinated and raw-water supplies and distribution system biofilms. Ridgway and Olson (42) isolated *Acinetobacter*, *Alcaligenes*, *Flavobacterium*, *Moraxella*, and *Pseudomonas* strains from potable-water distribution systems. Reasoner et al. (41) detected high populations of pigmented bacteria, including members of the genera *Chromobacterium*, *Flavobacterium*, and *Serratia*, and of gram-positive organisms from potable water. Other investigators isolated similar species of aerobic chemoheterotrophs from drinking water (2, 8, 23) and also found a large number of antibiotic-resistant strains among the bacteria detected (2, 8). These observations suggest that a wide variety of bacterial species have the potential to resist chlorine or to survive under chlorinated conditions. Some studies have provided evidence for the persistence in chlorinated water of aerobic heterotrophic bacteria, such as mycobacteria (7, 17), *Legionella pneumophila* (35), and *Pseudomonas aeruginosa* (46).

On the other hand, until recently, there have been only a few reports in the literature concerning the isolation and chlorine resistance of *Methylobacterium* strains from chlorinated environments. One of the reasons for this is that the standard method used previously for heterotrophic plate counts of bacteria involved incubation at 37°C for 48 h on standard agar

TABLE 3. Sequence similarities and evolutionary distances for 16S rDNAs of isolates and authentic *Methylobacterium* strains

| Strain no. | Species or strain no. | Sequence similarity (%) or evolutionary distance ($K_{nuc}$) with strain:[a] | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 |
| 1 | *M. extorquens* | | 99.5 | 99.1 | 98.9 | 98.8 | 97.7 | 96.3 | 95.1 | 95.2 | 94.9 | 95.0 | 95.0 | 94.9 | 94.2 | 95.1 | 95.2 | 86.3 |
| 2 | *M. rhodesianum* | 0.0057 | | 99.1 | 98.7 | 98.5 | 97.3 | 96.1 | 95.6 | 94.9 | 94.7 | 94.7 | 94.7 | 94.6 | 93.9 | 94.8 | 95.5 | 86.1 |
| 3 | GK101 | 0.0086 | 0.0086 | | 98.9 | 99.0 | 97.7 | 96.9 | 95.1 | 95.2 | 94.8 | 94.9 | 94.9 | 94.7 | 94.3 | 95.6 | 95.2 | 86.7 |
| 4 | GK118 | 0.0093 | 0.0122 | 0.0093 | | 98.6 | 97.2 | 96.7 | 95.0 | 94.8 | 94.7 | 94.7 | 94.8 | 94.6 | 94.2 | 95.5 | 95.2 | 86.4 |
| 5 | *M. zatmanii* | 0.0122 | 0.0151 | 0.0100 | 0.0129 | | 97.3 | 96.2 | 94.6 | 94.7 | 94.5 | 94.7 | 94.5 | 94.4 | 93.8 | 95.0 | 95.1 | 86.7 |
| 6 | *M. rhodinum* | 0.0230 | 0.0258 | 0.0230 | 0.0265 | 0.0244 | | 96.4 | 95.6 | 95.8 | 95.7 | 95.8 | 95.3 | 95.7 | 93.8 | 94.9 | 95.2 | 85.9 |
| 7 | *M. organophilum* | 0.0352 | 0.0380 | 0.0301 | 0.0337 | 0.0373 | 0.0373 | | 96.6 | 96.3 | 96.1 | 96.2 | 95.9 | 96.0 | 95.2 | 96.7 | 95.9 | 86.7 |
| 8 | *M. radiotolerans* | 0.0502 | 0.0531 | 0.0509 | 0.0531 | 0.0552 | 0.0445 | 0.0373 | | 99.6 | 99.4 | 99.3 | 98.1 | 97.7 | 96.6 | 96.9 | 95.6 | 87.1 |
| 9 | F73 | 0.0481 | 0.0509 | 0.0488 | 0.0509 | 0.0531 | 0.0423 | 0.0366 | 0.0050 | | 99.4 | 99.3 | 97.9 | 97.9 | 96.7 | 96.7 | 95.3 | 86.7 |
| 10 | F05 | 0.0488 | 0.0516 | 0.0495 | 0.0516 | 0.0531 | 0.0416 | 0.0373 | 0.0057 | 0.0050 | | 99.6 | 97.9 | 97.4 | 96.4 | 96.6 | 95.2 | 86.8 |
| 11 | F15 | 0.0481 | 0.0509 | 0.0488 | 0.0509 | 0.0524 | 0.0409 | 0.0366 | 0.0079 | 0.0057 | 0.0036 | | 97.8 | 97.5 | 96.4 | 96.4 | 95.3 | 86.9 |
| 12 | *M. mesophilicum* | 0.0495 | 0.0524 | 0.0502 | 0.0509 | 0.0545 | 0.0473 | 0.0387 | 0.0187 | 0.0144 | 0.0201 | 0.0222 | | 99.4 | 95.7 | 96.4 | 95.3 | 85.9 |
| 13 | F18 | 0.0509 | 0.0538 | 0.0516 | 0.0524 | 0.0560 | 0.0430 | 0.0402 | 0.0244 | 0.0212 | 0.0244 | 0.0237 | 0.0057 | | 95.6 | 95.9 | 95.2 | 85.9 |
| 14 | F42 | 0.0595 | 0.0610 | 0.0574 | 0.0595 | 0.0624 | 0.0466 | 0.0373 | 0.0344 | 0.0313 | 0.0344 | 0.0337 | 0.0416 | 0.0430 | | 95.1 | 94.8 | 86.5 |
| 15 | F37 | 0.0509 | 0.0538 | 0.0459 | 0.0481 | 0.0531 | 0.0509 | 0.0337 | 0.0344 | 0.0344 | 0.0359 | 0.0352 | 0.0380 | 0.0395 | 0.0502 | | 96.4 | 87.2 |
| 16 | F48 | 0.0473 | 0.0502 | 0.0438 | 0.0459 | 0.0488 | 0.0473 | 0.0409 | 0.0473 | 0.0459 | 0.0459 | 0.0452 | 0.0445 | 0.0452 | 0.0466 | 0.0516 | 0.0337 | 86.8 |
| 17 | *R. rubrum* | 0.1370 | 0.1392 | 0.1334 | 0.1370 | 0.1370 | 0.1413 | 0.1306 | 0.1334 | 0.1317 | 0.1320 | 0.1313 | 0.1399 | 0.1413 | 0.1356 | 0.1313 | 0.1313 | |

[a] The values on the upper right are percent sequence similarity, and the values on the lower left are evolutionary distance ($K_{nuc}$ values).

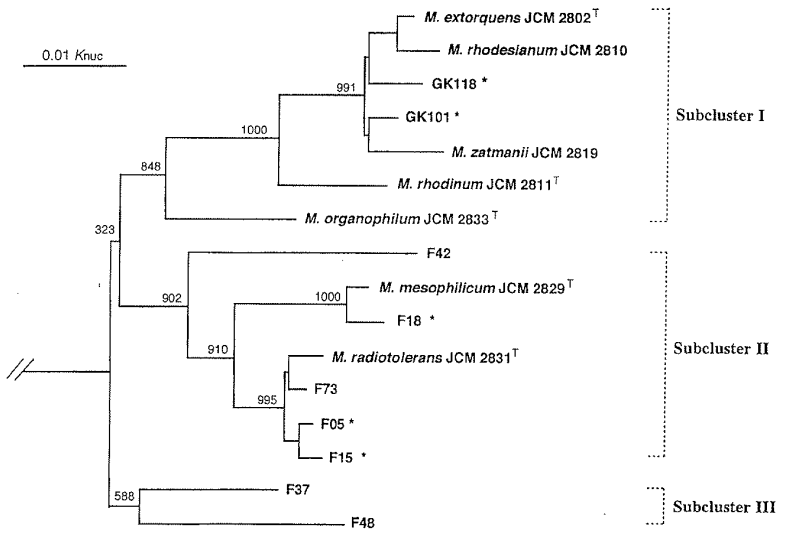


FIG. 3. Distance matrix tree showing phylogenetic relationships among the *Methylobacterium* strains within the genus. *Rhodospirillum rubrum* was used as a member of outgroups. Chlorine-resistant strains are marked with an asterisk. Bootstrap confidence values are shown at branching points of interest.

medium (1), although these culture conditions are unfavorable for the detection of *Methylobacterium* strains. Since the methylotrophic bacteria are relatively slow growers (19, 22), it is necessary to prolong incubation time to recover these bacteria from the environment. Thus, it can be assumed that methylotrophs as major inhabitants of drinking water and chlorinated environments have been overlooked for a long time. In fact, improved isolation techniques with prolonged incubation yielded high numbers of *Methylobacterium* strains from water supplies and other chlorinated environments (11, 14, 33).

One of our approaches to the present study, which was designed to determine the identity of chlorine-resistant *Methylobacterium* strains, was phenotypic characterization with numerical analysis. By this approach, the test organisms, including seven known species of the genus *Methylobacterium*, were found to fall into 19 clusters at an 80% level of similarity, at which the established species could be differentiated from each other with a few exceptions. The chlorine-resistant strains which we isolated were distributed among most of these clusters. These results suggest that chlorine-resistant *Methylobacterium* strains are phenotypically diverse and may be classified into a number of species, including those other than the seven authentic species within the genus.

The other approach, involving 16S rRNA gene sequence comparisons, has shown that the *Methylobacterium* test strains, including seven known species, form a tight cluster at interspecies similarity levels of more than 93% within the alpha-2 subclass of the *Proteobacteria* and that they fall into three major lines of descent, termed here subclusters I, II, and III, within the *Methylobacterium* cluster. Among the established species of *Methylobacterium*, *M. extorquens*, *M. organophilum*, *M. rhodesianum*, *M. rhodinum*, and *M. zatmanii* are positioned in subcluster I, whereas *M. mesophilicum* and *M. radiotolerans* are included in subcluster II. The topology of the 16S rDNA-based phylogenetic tree is similar to the topology of the dendrogram obtained by UPGMA linkage of DNA-DNA hybridization values for the methylotrophs reported previously (32). Our test isolates, including chlorine-resistant strains, were distributed randomly among the three phylogenetic subclusters. These results demonstrate that chlorine-resistant *Methylobacterium* strains are phylogenetically diverse as well as phenotypically heterogeneous.

Why members of the genus *Methylobacterium* occur frequently in and colonize potable water and chlorinated environments has been a subject of major concern. The data presented here and elsewhere (11–13, 33) demonstrate that the capacity for chlorine resistance is one of the most important factors. In view of the results of the present study, this property of *Methylobacterium* strains is independent of their taxonomic and phylogenetic positions but closely related to their sources. Our data show that strains derived from chlorinated water supplies are most remarkable for chlorine resistance. This fact suggests that *Methylobacterium* strains acquire the capacity for chlorine resistance by adapting to chlorinated environments, and therefore they can compete with coexistent chemoheterotrophs, survive, and in some cases exhibit massive growth in





these environments. The mechanism for acquiring chlorine resistance is unknown, and our attempts to demonstrate that this trait is plasmid borne have given negative results so far.

Another important factor may be that *Methylobacterium* strains are able to produce bacteriochlorophyll *a* under aerobic conditions, suggesting their capacity for yielding energy by photophosphorylation. Potable water and chlorinated water systems are oligotrophic environments, in which carbon energy sources for the growth of chemotrophic bacteria are very scant. However, the fact that *Methylobacterium* strains have a cyclic photosynthetic electron transport system, like the phototrophic purple bacteria (50), indicates that their ability to acquire ATP helps them to survive without the need for carbon energy sources.

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Comment Letter L

# Transformation of Acetaminophen by Chlorination Produces the Toxicants 1,4-Benzoquinone and *N*-Acetyl-*p*-benzoquinone Imine

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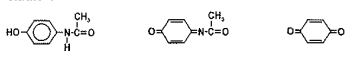
The reaction of the common pain reliever acetaminophen (paracetamol, 4-acetamidophenol) with hypochlorite was investigated over time under conditions that simulate wastewater disinfection. Initially, the reaction was studied in pure water at neutral pH (7.0), a range of reaction times (2–90 min), and a molar excess of hypochlorite (2–57 times) relative to the acetaminophen concentration. The reaction was monitored using reversed-phase liquid chromatography (LC) with ultraviolet absorbance, electrochemical, and mass spectrometric detection. At 1 μmol/L (150 ppb) and 10 μmol/L (1.5 ppm) levels, acetaminophen readily reacted to form at least 11 discernible products, all of which exhibited greater LC retention than the parent. Two of the products were unequivocally identified as the toxic compounds 1,4-benzoquinone and *N*-acetyl-*p*-benzoquinone imine (NAPQI), which is the toxicant associated with lethality in acetaminophen overdoses. With a hypochlorite dose of 57 μmol/L (4 ppm as $Cl_2$), 88% of the acetaminophen (10 μmol/L initial) was transformed in 1 h. The two quinoidal oxidation products 1,4-benzoquinone and NAPQI accounted for 25% and 1.5% of the initial acetaminophen concentration, respectively, at a 1 h reaction time. Other products that were identified included two ring chlorination products, chloro-4-acetamidophenol and dichloro-4-acetamidophenol, which combined were approximately 7% of the initial acetaminophen concentration at 1 h. The reaction was also studied in wastewater, where similar reactivity was noted. These results demonstrate that acetaminophen is likely to be transformed significantly during wastewater chlorination. The reactivity of the chlorine-transformation products was also studied with sulfite to simulate dechlorination, and 1,4-benzoquinone and NAPQI were completely reduced.

## Introduction

Acetaminophen (I) is the most widely used over-the-counter analgesic in the U. S. with production of $3.6 \times 10^9$ g in 2002. It is a safe drug when consumed at therapeutic dosages, where the body metabolizes acetaminophen to labile sulfate and glucuronide conjugates for excretion (*1*). However, overdosage with acetaminophen can be fatal. At high doses, liver microsomes oxidize acetaminophen to *N*-acetyl-*p*-benzoquinone imine (NAPQI; II), a toxic metabolite that results in hepatic necrosis (*1*). NAPQI, however, is known to be fairly unstable and readily hydrolyzes to the toxicant 1,4-benzoquinone (III) in aqueous solution (*2*). The wide use and potentially nefarious chemistry exhibited by acetaminophen render it an important pharmaceutical compound to investigate in the environment. Acetaminophen has already been identified as one of the most frequently detected anthropogenic compounds in a survey of 139 streams in the U.S. by the United States Geological Survey (*3*). In their evaluation, acetaminophen was detected in as many as 24% of the samples with a median concentration of 0.73 nmol/L (0.11 ppb), but concentrations as high as 66 nmol/L (10 ppb) were also reported (*3*). Unlike many other more "traditional" persistent pollutants, little is known about the aquatic ecosystem effects of pharmaceutical compounds, which are less persistent but could pose a threat due to their continuous release into the environment (*4*).

**CHART 1**



Acetaminophen I — *N*-Acetyl-*p*-benzoquinone imine (NAPQI) II — 1,4-Benzoquinone III

Pharmaceutical compounds such as acetaminophen that are detected in the environment are subjected to both waste and drinking water treatment processes. While some researchers have investigated the effect of the individual treatment processes (e.g., biological digestion, chlorination) in reducing the concentrations of parent pharmaceutical compounds (*5–8*), little attention has been placed on their potential transformations during treatment. A study of activated sludge processing suggested that most hydrophilic pharmaceuticals such as acetaminophen exhibit negligible sorption onto sludge and incomplete biological transformation (*8*). Therefore, many pharmaceutical compounds are further subjected to chemical treatment processes. Chlorination is the most widely used chemical process for disinfecting wastewater and drinking water in the U. S. Chlorine is a strong, general oxidant that is capable of rapidly transforming pharmaceutical compounds with reactive functional groups such as the phenol found in the acetaminophen molecule. Understanding the chemical fate of acetaminophen and the toxicological nature of the chlorine-transformation products is an important first step to understanding its environmental significance. To date, limited studies of the transformation rates of parent pharmaceuticals such as acetaminophen and endocrine disruptors by chlorine have been reported (*9–11*). Identification of potential transformation products has been limited to an investigation of the sulfa drugs (*10*). Another significant chemical process that is often employed in wastewater treatment processing is dechlorination with a reducing agent like sulfite, although it is not as universally practiced as chlorination. Further reactions of the chlorine-transformation products of acetaminophen are possible during dechlorination and require consideration.

In this study, we characterize the acetaminophen chlorination products in both pure water and in wastewater under conditions encountered in wastewater treatment processes. The reactivity of acetaminophen and its chlorination products were further characterized by investigating dechlorination with sulfite. This study presents the most comprehensive

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evaluation of the reactivity of acetaminophen in the chemical processes used in wastewater treatment to date. All reactions and products were studied using reversed-phase liquid chromatography (LC) with ultraviolet absorbance (UV), electrochemical (EC), and mass spectrometric (MS) detection.

## Experimental Section

**Reagents.** Acetaminophen (4-acetamidophenol; 98%) was purchased from Aldrich (Milwaukee, WI). Solutions of acetaminophen were prepared in pure water at different concentration levels, including 1, 10, and 337 $\mu$mol/L, and buffered with phosphate buffer, pH 7, to 1, 1, and 10 mmol/L total phosphate, respectively. The solutions also contained traces of methanol (<1% volume fraction), which aided in preparing solutions. Standards for 1,4-benzoquinone (98%) and NAPQI were purchased from Aldrich and Sigma (St. Louis, MO), respectively. Aqueous calibration solutions of 3 $\mu$mol/L 1,4-benzoquinone were freshly prepared and analyzed on days when chlorination experiments were performed. Due to the instability of NAPQI in water (*2*), a 117 $\mu$mol/L solution was prepared in acetonitrile and used for calibration.

For chlorination experiments, a 5% solution (> 5% as Cl) of reagent grade sodium hypochlorite (NaOCl) was obtained from Alfa Aesar (Ward Hill, MA) and diluted to approximately 5 mmol/L. The solution was standardized weekly using an iodometric titration, and the concentration was found to be stable over a period of months. At pH 7, the reactive chlorine species will be distributed between hypochlorite ion and hypochlorous acid based on the $pK_a$ value of 7.5. In this paper we will refer to this equilibrium mixture as "hypochlorite". Subsamples of the acetaminophen solutions (10 mL) were chlorinated in an amber vial by adding an appropriate aliquot of the standardized NaOCl solution and vortex mixing for 10 s. Both the 1 and 10 $\mu$mol/L acetaminophen solutions were chlorinated to 57 $\mu$mol/L with hypochlorite, which is equivalent to 4 ppm as $Cl_2$, a representative disinfection dose used for wastewater treatment. A 337 $\mu$mol/L acetaminophen solution was chlorinated at 674 $\mu$mol/L $OCl^-$ for mass spectrometric determinations. All chlorination experiments were performed at room temperature, 23 ± 1 °C.

For the dechlorination experiment, a solution of sodium sulfite (analytical reagent grade, Mallinckrodt, Paris, KY) was prepared in nitrogen-purged water. An aliquot was added to the 10 $\mu$mol/L acetaminophen chlorination mixture following 1 h of reaction time and vortex-mixed. The concentration of sulfite was made to be 84 $\mu$mol/L (1.5 times the initial hypochlorite dose). Dechlorination was allowed to proceed for 2 min.

Water (OmniSolv, EMD Chemicals, Gibbstown, NJ) and HPLC-grade methanol (Burdick and Jackson, Muskegon, MI) were used for the LC mobile phases. A 1 mol/L buffer solution was prepared from ammonium acetate and acetic acid (Mallinckrodt, Paris, KY) to be pH 5.8 and was diluted to prepare the mobile phases. Trifluoroacetic acid (TFA) was obtained from Fluka (Buchs, Switzerland) and used to prepare a mobile phase for MS with positive mode electrospray ionization, $ESI^+$.

**Wastewater.** Wastewater samples were collected from the Seneca Wastewater Treatment Plant, operated by the Washington Suburban Sanitary Commission in Germantown, MD. This plant utilizes an aerobic, biological activated sludge reactor. Chlorination is achieved with $Cl_2$(g) and a contact time of 1 h. Dechlorination is achieved with $SO_2$(g). The monthly average values during the time of sampling were ammonia-N 0.2 mg/L, organic-N 0.7 mg/L, and pH 7.5. Wastewater for laboratory chlorination was collected at a point downstream from biological digestion, clarification/sand filtration, and just prior to chlorination in the treatment process. The wastewater was transported to the lab and stored in a refrigerator at 4 °C. Before the wastewater was used in experiments, it was allowed to equilibrate to room temperature and centrifuged at 5000 rpm for 5 min to remove any suspended solids that were present. An acetaminophen solution was prepared to be 10 $\mu$mol/L in wastewater with 1 mmol/L pH 7 phosphate buffer. A subsample was chlorinated to 57 $\mu$mol/L as described for the pure water experiments and allowed to react for 1 h.

**Chromatography.** Samples were analyzed using two LC systems, both of which employed UV detection. The LC/UV/EC system consisted of two pumps (model 510, Waters, Milford, MA) and also employed an EC detector (model LC-4A, Bioanalytical Systems, West Lafayette, IN), while the LC/UV/MS system was an Agilent 1100 LC with an SL series mass selective detector (MSD; Palo Alto, CA) that used both ESI and atmospheric pressure chemical ionization (APCI) sources. Both systems utilized a Zorbax C-18 SB RP analytical column (Agilent) that was 3.0 mm × 250 mm and packed with 5 $\mu$m particles and a two-solvent isocratic mobile phase that consisted of 61% "A" and 39% "B." For LC/UV/EC, "A" was 50 mmol/L ammonium acetate/acetic acid buffer in water, pH 5.8. "B" was 99% (volume fraction) methanol, 1% (volume fraction) water, 10 mmol/L ammonium acetate/acetic acid buffer, pH 5.8. For LC/UV/MS, "A" was 10 mmol/L ammonium acetate/acetic acid buffer in water, pH 5.8, and "B" was the same as for LC/UV/EC when APCI was used and 99% (volume fraction) methanol, 1% (volume fraction) water, with TFA added to be 0.025% (volume fraction) when $ESI^+$ was used. The mobile phases were delivered at 0.4 mL/min. Injections of 50 $\mu$L were used for the 1 and 10 $\mu$mol/L acetaminophen solutions, while an injection of 40 $\mu$L was used for the 337 $\mu$mol/L solution. UV detection of acetaminophen and the reaction products was performed at 245 nm, which is the absorbance maximum for acetaminophen. Electrochemical detection on the LC/UV/EC system was performed at −0.1 V, which allowed for the selective detection of easily reducible oxidation products. MS detection was used with both ESI and APCI techniques. $ESI^+$ used $N_2$ drying gas flowing at 9.0 L/min and 350 °C, nebulizer pressure 0.24 MPa, fragmentor 75 V, and a capillary voltage of 3000 V. APCI was performed with negative polarity, $N_2$ drying gas flowing at 4.0 L/min and 350 °C, nebulizer pressure 0.41 MPa, vaporizer temperature of 300 °C, corona current 12 $\mu$A, fragmentor 125 V, and a capillary voltage of 3000 V. The mass spectrometer was scanned over the range from $m/z$ 80 to $m/z$ 380 to obtain spectra.

The linearity of the chromatographic responses was verified for acetaminophen, NAPQI, and 1,4-benzoquinone using at least three points, and $R^2$ values ranging from 0.99 to 1.00 were found. As a result of the remarkable linearity, single-point calibration factors were used and calculated as a ratio of the concentration to the peak area (assuming a zero intercept) using the standard solutions of acetaminophen (10 $\mu$mol/L), NAPQI (117 $\mu$mol/L), and 1,4-benzoquinone (3 $\mu$mol/L) described in the "reagents" section.

## Results

**Chlorination of Acetaminophen.** In our acetaminophen/chlorination experiments, hypochlorite doses were selected to provide a significant excess of chlorine relative to the acetaminophen concentration, reflecting the large excess of chlorine in a typical wastewater treatment process. The reactions were monitored up to 90 min, a reaction time that is representative of the 1−2 h chlorine contact times used in wastewater treatment. Reactions were studied at pH 7 using buffered solutions. The chlorination of acetaminophen was initially evaluated using a liquid chromatographic separation and UV absorbance detection with the LC/UV/EC system. Figure 1 presents chromatograms obtained with UV detection for two different acetaminophen levels at a chlorination time of 10 min. The lower chromatogram reveals a separation of the products formed from reactions of a 10 $\mu$mol/L (1.5 ppm) acetaminophen solution with 57 $\mu$mol/L hypochlorite (left absorbance scale). The first critical observation is the preponderance of separated components in this chromatogram, with at least 13 peaks being discernible. Peak 2 is acetaminophen, and peak 1 corresponds to monochloramine formed from the reaction of the excess hypochlorite with the ammonium ion present in the mobile phase buffer. The remaining 11 peaks therefore are transformation products from the acetaminophen/hypochlorite reaction.

The upper chromatogram in Figure 1 represents chlorination of the 1 $\mu$mol/L acetaminophen level (0.15 ppm) with the same 57 $\mu$mol/L hypochlorite dose (right absorbance scale). This chromatogram is plotted on a scale $^1/_{10}$th that of the 10 $\mu$mol/L solution, so that the relative peak proportions may be compared. All of the products formed at the higher acetaminophen level are formed at the lower level. At both levels, peak 4 is the most prominent of the products at this absorbance wavelength. However, the relative proportions of the peaks are different in the two experiments. At the lower level, acetaminophen (peak 2) has reacted to a greater extent, as shown by the significantly lower relative peak height. Also, the peak heights of the product peaks are relatively greater at the lower level than at the higher level (in particular, peak 13), indicating more efficient conversion to products. The increased reactivity can be attributed to the greater molar excess of hypochlorite relative to acetaminophen (57×) at the lower level.

**Chromatographic Identification of Reaction Products.** To identify the products of the acetaminophen/hypochlorite reaction, we utilized LC with multiple detectors. Because hypochlorite is such a strong oxidant, we investigated whether the products of the reaction retained oxidizing ability. The electrochemical detector in the LC/UV/EC system evaluated the reducibility of the products and was used in tandem with the absorbance detector. The lowest traces in Figures 2A and 2B show chromatographic separations of the acetaminophen reaction products at 21 min obtained by UV and EC detection, respectively. The lowest UV chromatogram shown in Figure 2A is similar to the lower chromatogram in Figure 1, which is expected given the identical concentrations of acetaminophen and hypochlorite. The only difference in Figure 2A is the longer reaction time (21 min vs 10 min). The lowest EC chromatogram shown in Figure 2B reveals that four products are easily reducible. The number labels correspond to the same peaks as in Figure 1.

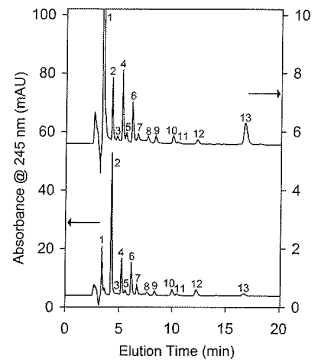


**FIGURE 1. Separation of chlorination products for two acetaminophen concentration levels using the following conditions: reaction time 10 min, hypochlorite dose 57 $\mu$mol/L, 1 mmol/L pH 7 phosphate buffer, and temperature 23 ± 1 °C. Lower chromatogram: 10 $\mu$mol/L acetaminophen (left absorbance scale). Upper chromatogram: 1 $\mu$mol/L acetaminophen (right absorbance scale). Peaks numbered in order of elution. Peak identities: 1, monochloramine; 2, acetaminophen; all others, reaction products.**

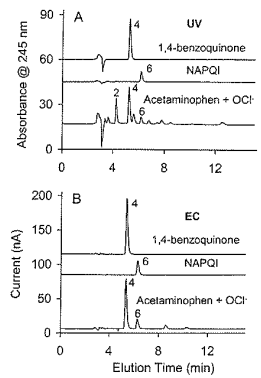


**FIGURE 2. Separations of a chlorinated acetaminophen solution and standard solutions of 1,4-benzoquinone and NAPQI as determined with (A) UV and (B) EC detection. Acetaminophen concentration was 10 $\mu$mol/L with a hypochlorite dose of 57 $\mu$mol/L for a reaction time of 21 min. The concentration of 1,4-benzoquinone was 3.4 $\mu$mol/L, and the responses were divided by 2 to obtain a response similar to that observed in the sample. The concentration of NAPQI was 12 $\mu$mol/L, and the responses were divided by 10 for the same reason. Peak identities: 2, acetaminophen; 4, 1,4-benzoquinone; 6, NAPQI.**

We investigated whether the acetaminophen/hypochlorite reaction might produce NAPQI using a chromatographic evaluation. A dilute aqueous solution of NAPQI was prepared from the calibrant solution in acetonitrile to be 12 $\mu$mol/L and analyzed immediately using the LC/UV/EC system; see middle traces in Figures 2A and 2B. Since NAPQI has a quinoid structure, it is very likely to be an oxidizing agent and therefore readily reducible. The chromatograms of NAPQI obtained by UV (Figure 2A) and EC (Figure 2B) indicate that it is detectable by both methods. More significantly, when the chromatograms for NAPQI are compared to the chlorinated acetaminophen solution in the lowest traces, it appears that peak 6 corresponds to NAPQI. On the basis of the equivalence of the retention times and two detection modes, NAPQI was tentatively identified as one of the oxidation products of acetaminophen. NAPQI is known to be relatively unstable in buffered aqueous solutions, where it is readily hydrolyzed to 1,4-benzoquinone (*2*). It is therefore likely that another of the detected products might be 1,4-benzoquinone. A solution of 1,4-benzoquinone was prepared and analyzed using the LC/UV/EC system, and the UV and EC chromatograms are shown as the uppermost traces in Figures 2A and 2B. As with NAPQI, 1,4-benzoquinone has a quinoid structure and is easily detectable using both detection modes. Also, the retention time for 1,4-benzoquinone in both the UV and the EC chromatograms corresponds with peak 4 in the chlorinated acetaminophen solution in the lowest traces, indicating the likely formation of this product.

We also investigated the potential formation of an N-chlorinated acetaminophen product using EC detection combined with iodide-postcolumn reaction chemistry (see method details in ref *12*). However, no chloramides were detected by this technique, in accord with prior observations that amide nitrogens, such as that contained within the acetaminophen structure, are not favorably N-chlorinated (*13*).

**Mass Spectral Identification of Reaction Products.** Mass spectrometric analysis using the LC/UV/MS system was employed to further elucidate the identities of the acetaminophen/hypochlorite reaction products using sufficiently high concentrations to provide full-scan spectra of the products. ESI was initially investigated because of its general utility for detecting many pharmaceutical compounds (*14, 15*). Both positive and negative polarities were evaluated, but more definitive spectra were obtained using positive mode, $ESI^+$. A mass spectrum was obtained for the NAPQI product. The peak of highest intensity in the mass spectrum occurred at $m/z$ 182.1, corresponding to the methanol adduct ion, $[M + H + methanol]^+$, where M for NAPQI is 149.0. The $[M + H]^+$ was less intense, but was present at $m/z$ 150.1. These spectral characteristics were confirmed by MS analysis of the standard NAPQI solution.

Mass spectra were also obtained for some of the other products using $ESI^+$. The most prominent ion in the spectrum for peak 7 was $m/z$ 186.1, but there was a second ion at $m/z$ 188.1. The relative intensities of these two ions indicated the presence of one chlorine atom (as $^{35}Cl$ or $^{37}Cl$) in the molecule. Given that the nominal molecular weight of acetaminophen is 151, an $[M + H]^+$ at $m/z$ 186 corresponds to a monochlorinated acetaminophen molecule. Since we have evidence that no chloramide is formed in this reaction, we propose that this product derives from monochlorination of the aromatic ring of acetaminophen to form a chloro-4-acetamidophenol. The strongly activating phenol group probably directs the chlorine atom to the ortho position. As ESI is a soft ionization technique, there were no notable fragmentation ions in the mass spectrum to confirm the exact position of the chlorine on the aromatic ring.

A mass spectrum was also obtained for peak 12 in Figure 1. The most prominent ion was $m/z$ 220.0, but ions at $m/z$ 222.0 and $m/z$ 224.1 were also notable. The 2 $m/z$ unit spacing and relative intensities of these ions indicated the compound has two chlorine atoms, and the spectrum could therefore correspond to a dichlorinated acetaminophen. Prominent ions were also found at $m/z$ 237.0 and $m/z$ 239.0, which correspond to ions formed from addition of ammonium, $[M + NH_4]^+$, and sodium, $[M + Na]^+$, respectively. For reasons analogous to peak 7, peak 12 may be tentatively identified as a dichloro-4-acetamidophenol. Formation of mono- and dichloro-4-acetamidophenol was also noted in a study of the reaction of acetaminophen with hypochlorite for 48 h using LC/MS with a particle-beam interface (*16*).

We found it was not possible to obtain good spectra for 1,4-benzoquinone with ESI. However, a mass spectrum of 1,4-benzoquinone was obtained using APCI operated with negative polarity. There was only one prominent ion in the spectrum at $m/z$ 108.1. Interestingly, the nominal molecular weight of 1,4-benzoquinone is 108.0, and the ion can be explained by either electron capture to form a radical anion or by a mass gain that was exactly balanced by a mass loss to make a negative ion. This same spectrum was obtained for a standard solution, indicating it is characteristic of 1,4-benzoquinone.

Some of the additional reaction products (peaks 3, 5, 7, 8, 9, 10, 11, and 13 in Figure 1) provided spectra that were not readily interpretable in either ESI or APCI modes. Several products had prominent ions with $m/z$ of 300 or greater, indicating that coupling reactions such as dimerization of the acetaminophen had occurred. These products remain to be identified.

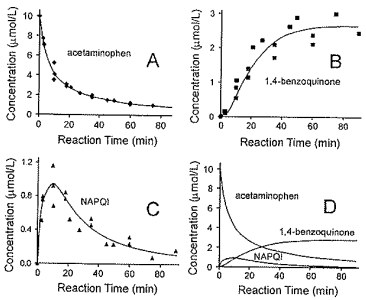


**FIGURE 3. Concentrations of (A) acetaminophen and two of its reaction products (B) 1,4-benzoquinone and (C) NAPQI as a function of chlorine reaction time. Initial conditions: acetaminophen concentration 10 μmol/L; hypochlorite dose 57 μmol/L; 1 mmol/L phosphate buffer, pH 7; temperature 23 ± 1 °C. (D) Concentration trends of acetaminophen, NAPQI, and 1,4-benzoquinone as a function of time plotted on the same scale.**

**Time Course of Reaction.** In addition to the identification of the products of the acetaminophen/hypochlorite reaction, [illegible] varies with reaction time. An initial acetaminophen concentration $[A]_0$ of 10 μmol/L and a hypochlorite concentration of 57 μmol/L were used. The reaction mixture was chromatographically monitored over a period of 90 min by making successively later injections of the acetaminophen/hypochlorite solution. The time of injection was recorded as the reaction time. Upon injection, the chromatographic separation of acetaminophen from hypochlorite, combined with the large dilution factor, quenches further reaction. Peak areas of acetaminophen and its reaction products were then determined and converted to concentration units using response factors obtained from analysis of the standards. The concentrations for each of these compounds as a function of reaction time are shown in Figure 3, which includes all data from three replicate experiments. In the figure, the lines are drawn to help visualize trends and do not represent mathematical models. The loss of acetaminophen over time is shown in Figure 3A. At a reaction time of 60 min, the concentration of acetaminophen remaining is about 1.2 μmol/L (12% of $[A]_0$). The half-life of the reaction was graphically estimated to be 7.2 min under these conditions. The concentrations of the reaction products 1,4-benzoquinone and NAPQI as a function of time are shown in Figures 3B and 3C, respectively. We found that formation of NAPQI is favored initially, reaching a maximum concentration of about 1 μmol/L (10% of $[A]_0$) after 10 min, then decaying to about 0.15 μmol/L (1.5% of $[A]_0$) at 60 min). 1,4-Benzoquinone, however, increases over time, reaching a concentration of about 2.5 μmol/L (25% of $[A]_0$) at 60 min. Figure 3D plots the trend lines for acetaminophen, NAPQI, and 1,4-benzoquinone concentrations over time on the same scale to indicate their relative concentrations.

Pure compounds were not commercially available to quantify the formation of chloro-4-acetamidophenol and dichloro-4-acetamidophenol. Their concentrations were therefore estimated by assuming the molar absorptivity and response factor to be the same as acetaminophen. Consider, for example, the simpler system involving phenol and 2-chlorophenol, where molar absorptivity values of 2190 L $cm^{-1}$ $mol^{-1}$ and 2400 L $cm^{-1}$ $mol^{-1}$, respectively, were found at the phenol absorbance maximum of 272 nm (NIST chemistry webbook, http://webbook.nist.gov/chemistry/). Since the addition of a chlorine atom to a phenol does not drastically change the absorptivity, we approximated the concentrations of chloro-4-acetamidophenol and dichloro-4-acetamidophenol using the molar response factor calculated for acetaminophen. After 60 min of reaction time, chloro-4-acetamidophenol and dichloro-4-acetamidophenol were approximated to be 0.3 μmol/L (3% of $[A]_0$) and 0.4 μmol/L (4% of $[A]_0$), respectively.

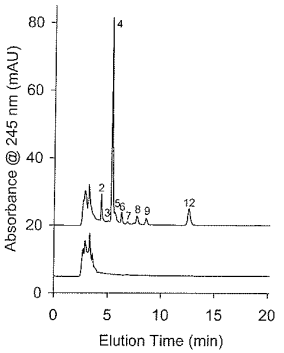


**FIGURE 4. Separations of chlorinated wastewater after 1 h. Upper trace: chlorination of wastewater containing 10 μmol/L acetaminophen. Lower trace: chlorinated wastewater blank. Initial conditions: hypochlorite dose 57 μmol/L; 1 mmol/L phosphate buffer, pH 7; temperature 23 ± 1 °C. Peak identities: 1, monochloramine; 2, acetaminophen; 4, 1,4-benzoquinone; 6, NAPQI; 7, chloro-4-acetamidophenol; 12, dichloro-4-acetamidophenol.**

**Dechlorination of Chlorinated Acetaminophen Mixture.** The reactivity of the acetaminophen chlorination products toward dechlorination processes was evaluated using the common agent sulfite. After 1 h chlorination of the 10 μmol/L acetaminophen solution, sulfite was added in 1.5 times molar excess to the initial hypochlorite dose. After 2 min reaction time, the products were evaluated by LC. The NAPQI and 1,4-benzoquinone formed in the chlorination reaction were quantitatively reduced by the addition of sulfite, whereas the chloro- and dichloro-4-acetamidophenol products appeared to be unaffected. Additionally, the peak area of acetaminophen increased after dechlorination with sulfite.

**Chlorination of Acetaminophen in Wastewater.** The reactivity of acetaminophen with hypochlorite was studied in wastewater collected prior to chlorine disinfection at an operating treatment plant. A solution of 10 μmol/L acetaminophen was prepared in the wastewater and studied using the same chlorine dose, pH, and temperature as those used in the pure water experiments. The chromatographic separation of this reaction mixture after 1 h is shown as the upper trace in Figure 4. Also shown in the lower trace in this figure is a wastewater blank that had been chlorinated for 1 h. This wastewater clearly has components that absorb light at 245 nm as shown by the group of incompletely resolved peaks at the solvent front and the trailing absorbance from about 4 to 6 min. However, this blank does not prohibit the clear identification of acetaminophen (peak 2) and the chlorination products that are numbered in the upper chromatogram. All of the products that were identified in the pure water experiments, particularly 1,4-benzoquinone and NAPQI, are also formed in similar proportions in this wastewater.

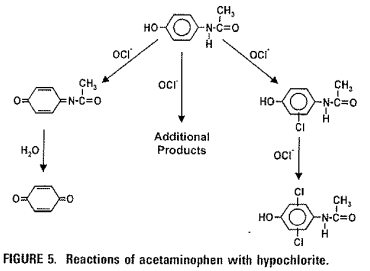


**FIGURE 5. Reactions of acetaminophen with hypochlorite.**

## Discussion

**Transformation of Acetaminophen by Hypochlorite.** Acetaminophen exhibited significant reactivity with hypochlorite in pure water and in wastewater under conditions that closely simulate wastewater treatment: neutral pH, reaction times of approximately 1 h, and molar excess of hypochlorite. In all experiments, acetaminophen was transformed rapidly into multiple products. The reaction proceeded via a number of pathways: general oxidation to quinoidal products, ring chlorination, and possibly free-radical coupling. Because of the *p*-aminophenolic structure of acetaminophen, the formation of quinoidal oxidation products was enabled, leading to the production of NAPQI and 1,4-benzoquinone. Another reaction pathway observed was chlorination of the ring, leading to the products chloro-4-acetamidophenol and dichloro-4-acetamidophenol. Figure 5 summarizes the observed reactions of acetaminophen with hypochlorite. In addition, there was mass spectral evidence of the formation of several products with molecular weights over 300 in the acetaminophen/hypochlorite reaction in pure water. Such products are consistent with free-radical-coupling reactions via a semi-quinone imine or other radicals.

The reactivity of acetaminophen with hypochlorite was similar in pure water and this wastewater sample. However, this single wastewater sample cannot represent all wastewaters where such factors as the ammonia and amine content, pH, etc. are likely to modulate the rate of reaction and proportions of products. To understand the underlying chemistry, the reaction was characterized in detail using pure water at 1 h by determining the concentrations of the identified products relative to the initial acetaminophen concentration $[A]_0$. NAPQI and 1,4-benzoquinone were estimated to be 1.5% and 25% of $[A]_0$, respectively. Although the formation of NAPQI was initially favored (10% of $[A]_0$ at 10 min), 1,4-benzoquinone was a much more significant product as the reaction proceeded. When displayed on the same scale in Figure 3D, it suggests that NAPQI was the initial product of the oxidation reaction, which then quickly hydrolyzed to form 1,4-benzoquinone. The ring chlorination products choro-4-acetamidophenol and dichloro-4-acetamidophenol had a combined contribution estimated to be 7%. Therefore, the estimated concentrations of the known products accounted for 33% of the change in $[A]_0$ at 1 h. However, acetaminophen was not completely consumed in the reaction in 1 h, with 12% of $[A]_0$ remaining. From this semiquantitative evaluation, we have accounted for about 45% of $[A]_0$ at 1 h. We have been unable to conclusively identify products 3, 5, 8, 9, 10, 11, and 13, so determining their relative contributions is not possible. In addition to these UV-

absorbing products, there are also likely to be transformation products formed that have lost the chromophoric structure present in the acetaminophen molecule by such reactions as oxidative opening of the aromatic ring. This has been demonstrated for the reaction of paracetamol (acetaminophen) with ozone, which forms many small molecule products that have lost the aromaticity found in the parent (*17*).

The reactivity of acetaminophen with chlorine observed in our experiments is in general accord with a previous study that predicted the reaction rate of acetaminophen with hypochlorite would be sufficiently fast to be significant in many chlorine disinfection systems (*9*). However, the half-life value determined in this study, 7.2 min, is shorter than would be projected from the estimate provided in the previous investigation (*9*). Assuming a second-order kinetic model and accounting for the differences in reactant concentrations in our experiment from Table 2 of ref *9*, the half-life would be expected to be approximately 26 min. We believe that the use of thiosulfate to reductively quench chlorine in the previous investigation (*9*) concomitantly reduced NAPQI and 1,4-benzoquinone to acetaminophen and 1,4-hydroquinone, respectively. Acetaminophen produced by reduction would be measured as unreacted acetaminophen in their chromatographic separation, leading to an anomalously low determined reaction rate. This is supported by the results from our dechlorination experiment, where the acetaminophen peak area was greater after addition of sulfite and the NAPQI product was completely reduced.

## Environmental Implications

In the wastewater experiments, most of the acetaminophen was transformed into new products via chlorination. It is therefore prudent to consider the toxicity and reactivity of these products. When $LD_{50}$ toxicity values (intraperitoneal injections in mouse) are compared, acetaminophen has a value of 500 mg/kg (*2*), while 1,4-benzoquinone and NAPQI have significantly lower $LD_{50}$ values of 8.5 mg/kg (*18*) and 20 mg/kg (*2*), respectively. 1,4-Benzoquinone and NAPQI are therefore approximately 58 and 25 times more toxic than acetaminophen, respectively. In humans, NAPQI is of particular concern due to its hepatotoxicity in acetaminophen overdoses. 1,4-Benzoquinone is a benzene metabolite implicated with genotoxic and mutagenic effects (*19*). In addition to their toxicity, both NAPQI and 1,4-benzoquinone showed significant reactivity in our experiments. In our LC analysis of buffered aqueous solutions, NAPQI began to produce significant amounts of 1,4-benzoquinone over the period of an hour. Neutrally buffered aqueous 1,4-benzoquinone solutions also demonstrated instability over the period of days, possibly via nucleophilic addition of water to form 2-hydroxyhydroquinone (*20*). In wastewater treatment operations that employ dechlorination with sulfite, it is likely that NAPQI will be reduced to acetaminophen and 1,4-benzoquinone will be converted into 1,4-hydroquinone. Thus, due to these multiple reaction modes, NAPQI and 1,4-benzoquinone are unlikely to persist in the environment. However, their toxicity and mutagenicity are a concern for the immediate environment continuously receiving chlorine-treated wastewater, particularly near plants that do not employ dechlorination. Unfortunately, no toxicity data or reactivity data are available for the chloro- and dichloro-4-acetamidophenol products, although they might be expected to have similar aqueous stability to acetaminophen. In our LC evaluations, unbuffered and neutrally buffered aqueous acetaminophen solutions demonstrated good stability for at least several weeks. Although formed in smaller amounts, chloro- and dichloro-4-acetamidophenol may also have environmental impact due to their potential persistence and increased hydrophobicity relative to acetaminophen.

## Acknowledgments

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**Comment Letter L**



## AP: Drugs found in drinking water

**By Jeff Donn, Martha Mendoza and Justin Pritchard, Associated Press**

A vast array of pharmaceuticals — including antibiotics, anti-convulsants, mood stabilizers and sex hormones — have been found in the drinking water supplies of at least 41 million Americans, an Associated Press investigation shows.

To be sure, the concentrations of these pharmaceuticals are tiny, measured in quantities of parts per billion or trillion, far below the levels of a medical dose. Also, utilities insist their water is safe.

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But the presence of so many prescription drugs — and over-the-counter medicines like acetaminophen and ibuprofen — in so much of our drinking water is heightening worries among scientists of long-term consequences to human health.

In the course of a five-month inquiry, the AP discovered that drugs have been detected in the drinking water supplies of 24 major metropolitan areas — from Southern California to Northern New Jersey, from Detroit to Louisville

Water providers rarely disclose results of pharmaceutical screenings, unless pressed, the AP found. For example, the head of a group representing major California suppliers said the public "doesn't know how to interpret the information" and might be unduly alarmed.

L-3

How do the drugs get into the water?

People take pills. Their bodies absorb some of the medication, but the rest of it passes through and is flushed down the toilet. The wastewater is treated before it is discharged into reservoirs, rivers or lakes. Then, some of the water is cleansed again at drinking water treatment plants and piped to consumers. But most treatments do not remove all drug residue.

And while researchers do not yet understand the exact risks from decades of persistent exposure to random combinations of low levels of pharmaceuticals, recent studies — which have gone virtually unnoticed by the general public — have found alarming effects on human cells and wildlife.

"We recognize it is a growing concern and we're taking it very seriously," said Benjamin H. Grumbles, assistant administrator for water at the U.S. Environmental Protection Agency.

Members of the AP National Investigative Team reviewed hundreds of scientific reports, analyzed federal drinking water databases, visited environmental study sites and treatment plants and interviewed more than 230 officials, academics and scientists. They also surveyed the nation's 50 largest cities and a dozen other major water providers, as well as smaller community water providers in all 50 states.

Here are some of the key test results obtained by the AP:

• Officials in Philadelphia said testing there discovered 56 pharmaceuticals or byproducts in treated drinking water, including medicines for pain, infection, high cholesterol, asthma, epilepsy, mental illness and heart problems. Sixty-three pharmaceuticals or byproducts were found in the city's watersheds.

• Anti-epileptic and anti-anxiety medications were detected in a portion of the treated drinking water for 18.5 million people in Southern California.

• Researchers at the U.S. Geological Survey analyzed a Passaic Valley Water Commission drinking water treatment plant, which serves 850,000 people in Northern New Jersey, and found a metabolized angina medicine and the mood-stabilizing carbamazepine in drinking water.

• A sex hormone was detected in San Francisco's drinking water.

• The drinking water for Washington, D.C., and surrounding areas tested positive for six pharmaceuticals.

• Three medications, including an antibiotic, were found in drinking water supplied to Tucson.

The situation is undoubtedly worse than suggested by the positive test results in the major population centers documented by the AP.

The federal government doesn't require any testing and hasn't set safety limits for drugs in water. Of the 62 major water providers contacted, the drinking water for only 28 was tested. Among the 34 that haven't: Houston, Chicago, Miami, Baltimore, Phoenix, Boston and New York City's Department of Environmental Protection, which delivers water to 9 million people.

Some providers screen only for one or two pharmaceuticals, leaving open the possibility that others are present.

The AP's investigation also indicates that watersheds, the natural sources of most of the nation's water supply, also are contaminated. Tests were conducted in the watersheds of 35 of the 62 major providers surveyed by the AP, and pharmaceuticals were detected in 28.





**Comment Letter L**

Yet officials in six of those 28 metropolitan areas said they did not go on to test their drinking water — Fairfax, Va.; Montgomery County in Maryland; Omaha; Oklahoma City; Santa Clara, Calif., and New York City.

The New York state health department and the USGS tested the source of the city's water, upstate. They found trace concentrations of heart medicine, infection fighters, estrogen, anti-convulsants, a mood stabilizer and a tranquilizer.

City water officials declined repeated requests for an interview. In a statement, they insisted that "New York City's drinking water continues to meet all federal and state regulations regarding drinking water quality in the watershed and the distribution system" — regulations that do not address trace pharmaceuticals.

In several cases, officials at municipal or regional water providers told the AP that pharmaceuticals had not been detected, but the AP obtained the results of tests conducted by independent researchers that showed otherwise. For example, water department officials in New Orleans said their water had not been tested for pharmaceuticals, but a Tulane University researcher and his students have published a study that found the pain reliever naproxen, the sex hormone estrone and the anti-cholesterol drug byproduct clofibric acid in treated drinking water.

Of the 28 major metropolitan areas where tests were performed on drinking water supplies, only Albuquerque; Austin, Texas; and Virginia Beach; said tests were negative. The drinking water in Dallas has been tested, but officials are awaiting results. Arlington, Texas, acknowledged that traces of a pharmaceutical were detected in its drinking water but cited post-9/11 security concerns in refusing to identify the drug.

The AP also contacted 52 small water providers — one in each state, and two each in Missouri and Texas — that serve communities with populations around 25,000. All but one said their drinking water had not been screened for pharmaceuticals; officials in Emporia, Kan., refused to answer AP's questions, also citing post-9/11 issues.

Rural consumers who draw water from their own wells aren't in the clear either, experts say.

The Stroud Water Research Center, in Avondale, Pa., has measured water samples from New York City's upstate watershed for caffeine, a common contaminant that scientists often look for as a possible signal for the presence of other pharmaceuticals. Though more caffeine was detected at suburban sites, researcher Anthony Aufdenkampe was struck by the relatively high levels even in less populated areas.

He suspects it escapes from failed septic tanks, maybe with other drugs. "Septic systems are essentially small treatment plants that are essentially unmanaged and therefore tend to fail," Aufdenkampe said.

Even users of bottled water and home filtration systems don't necessarily avoid exposure. Bottlers, some of which simply repackage tap water, do not typically treat or test for pharmaceuticals, according to the industry's main trade group. The same goes for the makers of home filtration systems.

Contamination is not confined to the United States. More than 100 different pharmaceuticals have been detected in lakes, rivers, reservoirs and streams throughout the world. Studies have detected pharmaceuticals in waters throughout Asia, Australia, Canada and Europe — even in Swiss lakes and the North Sea.

For example, in Canada, a study of 20 Ontario drinking water treatment plants by a national research institute found nine different drugs in water samples. Japanese health officials in December called for human health impact studies after detecting prescription drugs in drinking water at seven different sites.

In the United States, the problem isn't confined to surface waters. Pharmaceuticals also permeate aquifers deep underground, source of 40% of the nation's water supply. Federal scientists who drew water in 24 states from aquifers near contaminant sources such as landfills and animal feed lots found minuscule levels of hormones, antibiotics and other drugs.

Perhaps it's because Americans have been taking drugs — and flushing them unmetabolized or unused — in growing amounts. Over the past five years, the number of U.S. prescriptions rose 12% to a record 3.7 billion, while non-prescription drug purchases held steady around 3.3 billion, according to IMS Health and The Nielsen Co.

"People think that if they take a medication, their body absorbs it and it disappears, but of course that's not the case," said EPA scientist Christian Daughton, one of the first to draw attention to the issue of pharmaceuticals in water in the United States.

Some drugs, including widely used cholesterol fighters, tranquilizers and anti-epileptic medications, resist modern drinking water and wastewater treatment processes. Plus, the EPA says there are no sewage treatment systems specifically engineered to remove pharmaceuticals.

One technology, reverse osmosis, removes virtually all pharmaceutical contaminants but is very expensive for large-scale use and leaves several gallons of polluted water for every one that is made drinkable.

Another issue: There's evidence that adding chlorine, a common process in conventional drinking water treatment plants, makes some pharmaceuticals more toxic.

Human waste isn't the only source of contamination. Cattle, for example, are given ear implants that provide a slow release of trenbolone, an anabolic steroid used by some bodybuilders, which causes cattle to bulk up. But not all the trenbolone circulating in a steer is metabolized. A German study showed 10% of the steroid passed right through the animals.

Water sampled downstream of a Nebraska feedlot had steroid levels four times as high as the water taken upstream. Male fathead minnows living in that downstream area had low testosterone levels and small heads.

Other veterinary drugs also play a role. Pets are now treated for arthritis, cancer, heart disease, diabetes, allergies, dementia, and even obesity — sometimes with the same drugs as humans. The inflation-adjusted value of veterinary drugs rose by 8%, to $5.2 billion, over the past five years, according to an analysis of data from the Animal Health Institute.

Ask the pharmaceutical industry whether the contamination of water supplies is a problem, and officials will tell you no. "Based on what we now know, I would say we find there's little or no risk from pharmaceuticals in the environment to human health," said microbiologist Thomas White, a consultant for the Pharmaceutical Research and Manufacturers of America.

But at a conference last summer, Mary Buzby — director of environmental technology for drug maker Merck & Co. Inc. — said: "There's no doubt about it, pharmaceuticals are being detected in the environment and there is genuine concern that these compounds, in the small concentrations that they're at, could be causing impacts to human health or to aquatic organisms."

Recent laboratory research has found that small amounts of medication have affected human embryonic kidney cells, human blood cells and human breast cancer cells. The cancer cells proliferated too quickly; the kidney cells grew too slowly; and the blood cells showed biological activity associated with inflammation.

Also, pharmaceuticals in waterways are damaging wildlife across the nation and around the globe, research shows. Notably, male fish are being feminized,

creating egg yolk proteins, a process usually restricted to females. Pharmaceuticals also are affecting sentinel species at the foundation of the pyramid of life — such as earth worms in the wild and zooplankton in the laboratory, studies show.

Some scientists stress that the research is extremely limited, and there are too many unknowns. They say, though, that the documented health problems in wildlife are disconcerting.

"It brings a question to people's minds that if the fish were affected ... might there be a potential problem for humans?" EPA research biologist Vickie Wilson told the AP. "It could be that the fish are just exquisitely sensitive because of their physiology or something. We haven't gotten far enough along."

With limited research funds, said Shane Snyder, research and development project manager at the Southern Nevada Water Authority, a greater emphasis should be put on studying the effects of drugs in water.

"I think it's a shame that so much money is going into monitoring to figure out if these things are out there, and so little is being spent on human health," said Snyder. "They need to just accept that these things are everywhere — every chemical and pharmaceutical could be there. It's time for the EPA to step up to the plate and make a statement about the need to study effects, both human and environmental."

To the degree that the EPA is focused on the issue, it appears to be looking at detection. Grumbles acknowledged that just late last year the agency developed three new methods to "detect and quantify pharmaceuticals" in wastewater. "We realize that we have a limited amount of data on the concentrations," he said. "We're going to be able to learn a lot more."

While Grumbles said the EPA had analyzed 287 pharmaceuticals for possible inclusion on a draft list of candidates for regulation under the Safe Drinking Water Act, he said only one, nitroglycerin, was on the list. Nitroglycerin can be used as a drug for heart problems, but the key reason it's being considered is its widespread use in making explosives.

So much is unknown. Many independent scientists are skeptical that trace concentrations will ultimately prove to be harmful to humans. Confidence about human safety is based largely on studies that poison lab animals with much higher amounts.

There's growing concern in the scientific community, meanwhile, that certain drugs — or combinations of drugs — may harm humans over decades because water, unlike most specific foods, is consumed in sizable amounts every day.

Our bodies may shrug off a relatively big one-time dose, yet suffer from a smaller amount delivered continuously over a half century, perhaps subtly stirring allergies or nerve damage. Pregnant women, the elderly and the very ill might be more sensitive.

Many concerns about chronic low-level exposure focus on certain drug classes: chemotherapy that can act as a powerful poison; hormones that can hamper reproduction or development; medicines for depression and epilepsy that can damage the brain or change behavior; antibiotics that can allow human germs to mutate into more dangerous forms; pain relievers and blood-pressure diuretics.

For several decades, federal environmental officials and non-profit watchdog environmental groups have focused on regulated contaminants — pesticides, lead, PCBs — which are present in higher concentrations and clearly pose a health risk.

However, some experts say medications may pose a unique danger because, unlike most pollutants, they were crafted to act on the human body.

"These are chemicals that are designed to have very specific effects at very low concentrations. That's what pharmaceuticals do. So when they get out to the environment, it should not be a shock to people that they have effects," says zoologist John Sumpter at Brunel University in London, who has studied trace hormones, heart medicine and other drugs.

And while drugs are tested to be safe for humans, the timeframe is usually over a matter of months, not a lifetime. Pharmaceuticals also can produce side effects and interact with other drugs at normal medical doses. That's why — aside from therapeutic doses of fluoride injected into potable water supplies — pharmaceuticals are prescribed to people who need them, not delivered to everyone in their drinking water.

"We know we are being exposed to other people's drugs through our drinking water, and that can't be good," says Dr. David Carpenter, who directs the Institute for Health and the Environment of the State University of New York at Albany.



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L-138







Health news

The Times August 05, 2005

# Where rivers run high on cocaine

By Nigel Hawkes

Analysis of waste water in Italy shows a startlingly high level of drug abuse

THE rivers of Italy are flowing with cocaine, say scientists who have adopted a new approach to measuring the extent of drug misuse. The biggest river, the Po, carries the equivalent of about 4kg (8lb 13oz) of the drug a day, with a street value of about £20,000.

Cocaine users among the five million people who live in the Po River basin in northern Italy consume the drug and excrete its metabolic by-product, benzoylecgonine (BE). This goes from sewers into the river. So a team led by Dr Ettore Zuccato, of the Mario Negri Institute for Pharmacological Research in Milan, estimated the use of cocaine by testing the waters of the Po for BE, and for any cocaine that had passed through the body unaltered or reached the sewers in other ways.

What they found surprised them. They calculated that for every 1,000 young adults in the catchment area, about 30 must be taking a daily dose of 100 milligrams of cocaine, which greatly exceeds official national figures for cocaine use.

According to official Italian statistics, 1.1 per cent of people between the ages of 15 and 34 admit to having used cocaine "at least once in the preceding month". Almost all cocaine use occurs in this age group.

Assuming that there are 1.4 million young adults in the Po River basin, the official statistics suggest that there would be 15,000 cocaine-use events per month. But the evidence from the water suggests that the real usage is about 40,000 doses a day, a vastly greater figure.

"The economic impact of trafficking such a large amount of cocaine would be staggering," Dr Zuccato said. "The large amount of cocaine — at least 1,500kg — that our findings suggest is consumed per year in the River Po basin would amount, in fact, to about $150 million in street value, based on an average US street value of $100 per gram."

To confirm their findings, the team also sampled urban waste water from Cagliari in Sardinia, Latina

in central Italy, and from Cuneo and Varese in the north — all medium-sized cities. The values they obtained from the undiluted waste water were far higher than those in the Po, as would be expected. But when translated into likely local use of the drug, they produced very similar figures — which suggests that the Po region is not exceptional in its cocaine consumption. The results cannot be explained by assuming that some drug trafficker was panicked into dumping his stash down the lavatory. If so, much more pure cocaine would have been found, and much less of its human metabolite, BE. In fact, the ratio of cocaine to BE was consistent throughout all the samples.

If anything, Dr Zuccato said, the method would be expected to underestimate rather than to overestimate cocaine use, because some would be lost or absorbed in sediments. So the real consumption may be even higher.

This method has previously been used by the same team to measure the by-products of widely-used prescription drugs, and has produced results consistent with known prescribing patterns. So it seems to work.

The technique has been developed by the Italian team and is complex, as it needs to be to detect such tiny residues — of the order of billionths of a gram per litre of water.

The scientists say that the method needs to be tested further before being brought into general [illegible] and much cheaper way of tracking trends in drug use than by using population surveys.

"The approach tested here, which is in principle adaptable to other illicit drugs, could be refined and validated to become a general, rapid method to help estimate drug abuse at the local level," they report in the journal *Environmental Health*.

"With its unique ability to monitor changing habits in real time, it could be helpful to social scientists and authorities for continuously updating the appraisal of drug abuse."

The levels of the drug and the metabolite found in river water are so low that any effect on natural life is very unlikely. But this is not true of all chemicals. Research indicates that chemicals that mimic natural hormones are having an effect on fish in many rivers, including "feminising" many male fish. The sources of these chemicals include hormones excreted by the human body and industrial chemicals that reach the waterways.

# Reactivation of Fecal Coliforms after Anaerobic Digestion and Dewatering

wef proceedings

**Authors:** Hendrickson, Donald A.; Denard, Dave; Farrell, Joseph
**Source:** Proceedings of the Water Environment Federation, Residuals and Biosolids Management 2004 , pp. 1018-1026(9)
**Publisher:** Water Environment Federation

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**Abstract:**

In August of 2001, the largest known installation of a phased temperature anaerobic process at a 60-MDG wastewater treatment plant was placed into operation. The facility met the time and temperature requirement for Class A biosolids. Testing of the biosolids following thermophillic/anaerobic digestion followed by mesophilic/anaerobic digestion revealed no detectable levels of fecal coliform bacteria in the treated biosolids. However, subsequent testing of the biosolids following dewatering by high solid centrifugation revealed high levels of fecal coliform bacteria. These biosolids, following high solid centrifugation, did not meet Class B requirements.

This study indicated a very serious reactivation of fecal coliform bacteria following high solid centrifugation. Fifty-three percent of the fecal coliforms isolated were identified as Eschericia coli with two of the isolated organisms identified as E.coli 0157:H7. E.coli 0157:H7 has been shown to be capable of formation of an autoinducer in the presence of norephinephrine. The autoinducer triggers the growth of gram-negative bacteria or the conversion of gram-negative bacteria such as fecal coliforms from a non-culturable to culturable state. It is, therefore, hypothesized that the presence of E.coli 0157:H7 may be involved in the reactivation of fecal coliform bacteria.

**Document Type:** Research article
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Comment Letter L

# L. Open Space Water Resource Protection Land Use (O.W.L.) Foundation, H.R. Downs, President, 7/13/2009

L-1 Comment acknowledged. Please refer to **Master Response 2.6, Recycled Water Quality**, in Chapter 2, Master Responses.

L-2 Comment acknowledged. Please refer to **Master Response 2.6, Recycled Water Quality**, in Chapter 2, Master Responses. Tertiary treatment is typically the advanced treatment of wastewater that occurs beyond the secondary or biological treatment phase. According to §60301.230 of Title 22, "disinfected tertiary recycled water" means filtered and subsequently disinfected wastewater that meets specific criteria on the contact time for chlorine disinfection process and concentration of total coliform as noted in the section. As shown in Table 3.4-6 on page 3.4-15 of the Draft EIR/EIS, recycled water treated at different levels is regulated in terms of its allowable end uses.

L-3 Comment acknowledged. The statement noted in the comment, "If the project allows antibiotic-resistant pathogens and antibiotic-resistant genes to be spread via open dumping…any "miracles" left", is speculative. It is noted that the spread of antibiotic-resistant bacteria is a public health concern and, as described in the study referenced in the comment, scientists believe that the spread of antibiotic resistance results from both misuse of antibiotics and transfer of resistance between bacteria. The objective of the study, however, 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. The study suggests that the existing wastewater treatment infrastructure be modified to better prevent release of the potentially dangerous bacteria to the environment. As noted in **Master Response 2.6, Recycled Water Quality**, the proposed action would not alter primary and secondary treatment processes at WWTPs, which are regulated by the RWQCB.

The commenter states that sewage treatment plays an important role in amplifying the danger from the antibiotic-resistant pathogens; however, the role of sewage treatment in the spread of antibiotic-resistant microbes compared to the other pathways (direct ingestion and consumption of chemicals) is yet to be determined and cannot be established except in studies specific to certain WWTPs. With advances in technology and testing techniques, the detection of antibiotic-resistant genes is anticipated to continue and will need further investigation (e.g., relevance to human pathogens) along with any updates on regulations that govern the detection and control of toxic pollutants and pathogens. Please refer to **Master Response 2.6, Recycled Water Quality**, in Chapter 2, Master Responses.

L-4 Comment acknowledged. Please refer to **Master Response 2.6, Recycled Water Quality**, in Chapter 2, Master Responses.

L-5 Comment acknowledged. The study by Pruden et. al., noted in the comment concludes that there is a need for environmental scientists and engineers to help address the issue of the spread of the antibiotic-resistant genes in the environment. The presence of antibiotic-resistant genes in the wastewater at the subject WWTPs however, would need to be established prior to planning for complete destruction or removal of the genes in the wastewater. Additional research (e.g., level of risk with the concentrations present) would be necessary to determine the appropriate best available technology to achieve the desired results. Please refer to **Master Response 2.6, Recycled Water Quality**, in Chapter 2, Master Responses. Based on the current available information for the project and the current regulatory framework discussed in Section 3.4, Water Quality, of the Draft EIR/EIS, the recycled water use under the proposed project would comply with the applicable regulatory requirements.

L-6 Comment acknowledged. Please refer to **Master Response 2.6, Recycled Water Quality**, in Chapter 2, Master Responses. The Draft EIR/EIS is based on best available information and the regulatory standards that form the significance thresholds for the water quality impact analyses. The risk to human and environmental health is one of the critical drivers in establishing the regulatory standards and the compliance schedule through testing and controlling of the constituents of concern in the treated discharges. Under the current regulatory framework, Title 22 requirements would apply to the recycled water quality for the proposed project and are discussed in Section 3.4, Water Quality, of the Draft EIR/EIS.

L-7 Comment acknowledged. Please refer to **Master Response 2.6, Recycled Water Quality**, in Chapter 2, Master Responses. The commenter states that "the project proposes wide distribution of treated sewage effluent of agricultural crops". However, as discussed in Chapter 2, Project Description, of the Draft EIR/EIS, the proposed project would involve use of recycled water, which is tertiary disinfected wastewater treated to comply with specific criteria established under Title 22, for irrigation in the local service areas of the NBWRA Member Agencies. The commenter states that there are several studies that have found uptake of pharmaceuticals and pathogens from treated effluent, "sometimes to lethal effect". It should be noted that a lethal effect depends upon the dose of the chemical and the length of the exposure to the chemical. Detection of a chemical may not necessarily result in a lethal effect. The Draft EIR/EIS presents the impact analysis based on best available information and reflects the current regulatory framework. The proposed project would utilize the treatment technologies that are acceptable to California Department of Public Health (CDPH) under Title 22.

L-8 Comment acknowledged. Please refer to **Master Response 2.6, Recycled Water Quality**, in Chapter 2, Master Responses. The tertiary disinfected recycled water that would be generated and used under the proposed project would not be "contaminated waters" as noted by the commenter. Recycled water is already being used by some vineyards in the North Bay. (Refer to response to comment M-19).The secondary-treated wastewater at the WWTP that would be in compliance with the NPDES permits would be

further treated (tertiary disinfected under Title 22) under the proposed project prior to use. The wine-making process is not part of the proposed project, therefore it is not discussed further in this response.

The study referred to in the comment describes results from the application of biosolids; however, the proposed project involves tertiary-treated and disinfected recycled water for irrigation. Recycled water is used widely in California for agriculture purposes. A five-year pilot project[1] in the vicinity of Castroville demonstrated that irrigation of raw-eaten vegetable crops with recycled water was as safe as irrigation with other sources of water. Also refer to Response L-15.

The comment on incalculable economic repercussions on the North Bay wine industry due to irrigation with recycled water assumes risk of contamination of the grapes from use of recycled water. As noted in **Master Response 2.6, Recycled Water Quality**, while potential human health effects continue to be monitored, there is currently no scientific basis to establish risk factors or set allowable discharge concentrations for microconstituents. Similarly, the availability of research data on the potential uptake of microconstituents by crops irrigated with recycled water, including the fate of the contaminants, does not support conclusive determination of the significance of any potential effect generated at this time.

Additionally, this comment makes several assumptions regarding public perception, changes in market behavior, and subsequent economic effects to the wine industry as a result of recycled water use for irrigation. While it cannot be determined with certainty whether recycled water use would have an effect on public perception of vineyard production within the NBWRA service area, it is important to note that recycled water is currently used on over 4,500 acres of vineyard in Sonoma and Napa Counties (please refer to Response M-19), and is used through the State. Recent market trends identified in a study conducted by the Natural Marketing Institute indicate that 78 percent of table wine consumers are open to, if not actively motivated by, sustainability. These results are based on a Lifestyles of Health and Sustainability (LOHAS) panel used to identify households that buy high end wines ($20+) at lease once a month.[2] However, due to the lack of definitive data or thresholds regarding this issue, further analysis of this issue is speculative and is not required under CEQA or NEPA (Section 15064(f)(5); 40 CFR 1502.22).

L-9 Comment acknowledged. Please refer to **Master Response 2.6, Recycled Water Quality**, in Chapter 2, Master Responses. As described in the master response, while potential human health effects continue to be monitored, there is currently no scientific

1 *Recycled Water Food Safety Study for Monterey County Water Recycling Projects*, Sponsored by Monterey County Water Resources Agency and Monterey Regional Water Pollution Control Agency, August 1998.

2 *Green Movement Sprouts Opportunity for Wine and Spirits.* January 1, 2009, The Free Library. Brian Lechner, Director, Beverage Alcohol, the Nielsen Company, during presentation at Green Wine Summit, Santa Rosa California. Data from The LOHAS Report: Consumers and Sustainability – A Focus on Food and Beverage. Natural Marketing Institute, 2009.

basis to establish risk factors or set allowable discharge concentrations for microconstituents. Similarly, the availability of research data on the potential uptake of microconstituents by crops irrigated with recycled water, including the fate of the contaminants, does not support conclusive determination of the significance of any potential effect generated at this time.

L-10 Comment acknowledged. Free residual chlorine in the treated wastewater discharge at levels stipulated in the NPDES permits is protective of the environment. Further treatment of this water through additional filtration and disinfection would help provide the treatment necessary to destroy the pathogens and break down chemicals that would otherwise have an adverse effect on public health. The statement "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" is unsubstantiated, therefore not discussed further.

L-11 Comment acknowledged. The statement, "the sewage treatment plants that supply the NBWRP with effluent do not remove all of the triclosan they receive in raw sewage" is unsubstantiated. The sanitary districts within the NBWRA test for all required contaminants at the WWTPs, pursuant to conditions specified in their NPDES permits. The issues such as determining the amount of chlorinated triclosan derivative products formed from triclosan – a common ingredient of antimicrobial personal care products[3] – during wastewater disinfection are not required by applicable state and federal regulations and are beyond the scope of this EIR/EIS. Please refer to **Master Response 2.6, Recycled Water Quality**, in Chapter 2, Master Responses, for details.

L-12 Comment acknowledged. The comment assumes presence of Staphlylococcus aureus (S. aureus) in the recycled water used under the proposed project without substantive evidence of the presence as well as the risk involved at the levels present. Testing and treating for pathogens is part of the compliance procedures under the NPDES permits for wastewater discharge. As discussed in the response to comments above, studies demonstrating increased virulence of methycillin-resistant S. aureus as a result of exposure to chlorine in the WWTPs are not required by applicable state and federal regulations and are beyond the scope of this EIR/EIS. Please refer to **Master Response 2.6, Recycled Water Quality**, in Chapter 2, Master Responses, for details.

L-13 Comment acknowledged. As discussed above, constituents of concern identified in the NPDES permits and Title 22 are parts of the compliance monitoring for the proposed project. Studies on estimates of chlorine-resistant bacteria are not required by applicable state and federal regulations and are beyond the scope of this EIR/EIS. Please refer to **Master Response 2.6, Recycled Water Quality**, in Chapter 2, Master Responses.

[3] Halden, R.U. and Katz, J., Occurrence, Fate, and Impact of Triclosan and Other Antimicrobials to Wastewater, The Biodesign Institute at Arizona State University, Microconstituents/Industrial Water Quality 2009.

L-14 Comment acknowledged. Please refer to **Master Response 2.6, Recycled Water Quality**, in Chapter 2, Master Responses. Studies on chlorine reactions with acetaminophen or other materials during the wastewater treatment process are not required by applicable state and federal regulations.

L-15 Comment acknowledged. As noted in response to Comment L-9, a five-year pilot project near Castroville demonstrated that irrigation of raw-eaten vegetable crops with recycled water was as safe as irrigation with other sources of water. The study was designed to determine whether or not pathogenic microorganisms of concern to food safety such as *E. coli*. The sampling did not detect any *Salmonella*, *Cyclospora*, or *E. coli* in any of the recycled water from the Monterey County Water Recycling Projects. The results from samples of recycled water are comparable to those from similar tests at other tertiary recycled water treatments plants and compare favorably with most sources of drinking water supply. Also, please refer to **Master Response 2.6, Recycled Water Quality**, in Chapter 2, Master Responses. Studies on reactions between chemicals and pathogens are not required by applicable state and federal regulations and are beyond the scope of this EIR/EIS.

L-16 Comment acknowledged. Please refer to **Master Response 2.6, Recycled Water Quality**, in Chapter 2, Master Responses. Novato SD and LGVSD WWTPs are considering using chlorine for disinfection processes; SVCSD and Napa SD currently use chlorine, not UV (CDM, 2009). Studies on the effects of UV disinfection on endosymbiont bacteria are not required by applicable state and federal regulations and are beyond the scope of this EIR/EIS.

L-17 Comment acknowledged. The phenomenon of "resurrection" of microorganisms noted in the comment is termed as microbiostatis, where the growth of microorganisms is inhibited, which does not necessarily indicate that the organisms are killed but that they are unable to grow (processes such as refrigeration, desiccation, and use of certain antimicrobial drugs exert a microbiostatic effect). Please refer to **Master Response 2.6, Recycled Water Quality**, in Chapter 2, Master Responses. Studies on microbiostatis and reactivation of pathogens in the wastewater treatment process are not required by applicable state and federal regulations and are beyond the scope of this EIR/EIS.

L-18 Comment acknowledged. Solids separated from the wastewater treatment at the WWTPs form sludge, which, at most WWTPs, is dewatered and treated further prior to discharge to a landfill[4]. Sludge generation and land application of the sludge is not a part of the proposed project. The liquid stream of the treated wastewater would be further treated as described in Chapter 2, Project Description, to produce recycled water. Please refer to **Master Response 2.6, Recycled Water Quality**, in Chapter 2, Master Responses, for details.

[4] Some WWTPs have implemented the practice of using sludge as beneficial reuse through land application.

L-19 Comment acknowledged. Please refer to **Master Response 2.6, Recycled Water Quality**, in Chapter 2, Master Responses. As stated in Section 3.4, Water Quality, of the Draft EIR/EIS, recycled water use under the proposed project would occur in compliance with the standards established by CDPH and San Francisco Bay RWQCB. The California Health and Safety Code Sections 5410 (d) and (f) and Section 5411 noted in the comment are in fact listed under Chapter 6, General Provisions With Respect to Sewers, under Article 2, Sewage and Other Waste. The wastewater discharges from WWTPs in California are subject to the sections noted in the comment. Further, the subject WWTPs discharge treated wastewater into receiving waterways in compliance with their respective NPDES permits that include federal, state, and local standards. The contamination or pollution noted under the Health and Safety Code is governed by the regulatory standards. For example, the RWQCB's 303(d) list consists of impaired water bodies and identifies water quality criteria or maximum daily loads for constituents that may be added by the dischargers without impairing the receiving waters). These standards are intended to be protective of the environment and public health and apply to the treated wastewater discharge from the LGVSD, Novato SD, SVCSD, and Napa SD WWTPs. However, the proposed project does not involve treatment and discharge of wastewater; rather it involves tertiary treatment (i.e., filtration and additional disinfection) and use of treated wastewater that would be otherwise discharged. The project would involve generation and use of recycled water for several purposes as outlined in Chapter 2, Project Description, of the Draft EIR/EIS. The Draft EIR/EIS identifies any significant impacts as part of the impact analysis in Chapters 3, 4, and 5, and recommends mitigation measures to reduce the significant impacts to less-than-significant levels, where applicable.

L-20 Comment acknowledged. The statement "Must a community….highest possible profits and …the expedient way to dump sewage" is noted but irrelevant to the project. As described on page 2-2 of Chapter 2, Project Description, in the Draft EIR/EIS, the purpose of the project is to provide recycled water for agricultural, urban, and environmental uses thereby reducing reliance on local and imported surface and groundwater and reducing the amount of treated effluent releases to tributaries of San Pablo Bay.

Public Resources Code 21061.1, as noted in the comment, states that "Feasible" means capable of being accomplished in a successful manner within a reasonable period of time, taking into account economic, environmental, social, and technological factors. Under CEQA, the Draft EIR/EIS provides the impact analysis for the project proposed by the NBWRA based on a detailed feasibility study conducted prior to the EIR/EIS phase, which is discussed in Section 6.2, Alternatives, of the Draft EIR/EIS. The NBWRP is based on the Feasibility Study, which was prepared as per the Title XVI requirements, and was analyzed as the proposed project in the Draft EIR/EIS. Also as required under CEQA and NEPA, Chapter 6, Alternatives, presents the different alternatives that were studied and not carried forward for the analysis as well those that were considered in terms of the project objectives and environmental impacts. For additional discussion of

the analysis of alternatives, refer to **Master Response 2.2, Alternatives Analysis**, in Chapter 2, Master Responses.

L-21 Comment acknowledged. The commenter states how the Draft EIR/EIS would satisfy "these legal hurdles". Please refer to **Master Response 2.6, Recycled Water Quality**, in Chapter 2, Master Responses. Section 3.4, Water Quality, of the Draft EIR/ES describes the governing regulatory requirements for water quality. Regulatory compliance for the proposed project is mandatory and the Draft EIR/EIS lists the potential permits and approvals and regulatory standards with which the projects would be required to comply.

L-22 Comment acknowledged. The commenter defines "recycle" as "removal of all contaminants, not just some of them, with a goal to return, or recycle, contaminated water back into fresh water". However, the legal definition of "recycled water" under subdivision (n) of Section 13050 of the California Water Code is "water which, as a result of treatment of waste, is suitable for a direct beneficial use or a controlled use that would not otherwise occur and is therefore considered a valuable resource". Please refer to **Master Response 2.6, Recycled Water Quality**, in Chapter 2, Master Responses. The treatment of wastewater to enable water reuse is determined and regulated by CDPH and RWQCB under Title 22 requirements, also described in Section 3.4, Water Quality, of the Draft EIR/EIS.