Water Resources Research Institute
Annual Technical Report
FY 2002

Introduction

Research Program

Microbial source tracking to determine the host origin of fecal contamination in two Puerto Rican watersheds

Basic Information

Title:	Microbial source tracking to determine the host origin of fecal contamination in two Puerto Rican watersheds
Project Number:	2002PR2B
Start Date:	3/1/2002
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Principal Investigators:	Eduardo Schroder

Publication

Title: Microbial source tracking to determine the host origin of fecal contamination in two Puerto Rican watersheds

Statement of critical water problems

In the first year of the proposed research, we will isolate a fecal coliform, *Escherichia coli*, from the feces of a wide variety of warm-blooded animals, including humans, located in two watersheds in Puerto Rico. We will isolate the DNA from these *E. coli* isolates and obtain a DNA "fingerprint" of each isolate. Each "fingerprint" represents the portion of DNA that encodes for ribosomal RNA (rRNA) and is called a ribotype. The method, called ribotyping, shows considerable promise in being able to associate specific *E. coli* ribotypes to specific animal hosts. If one then obtains ribotypes of *E. coli* isolates from waters contaminated with feces, one should be able to identify the sources of fecal contamination by matching the ribotypes from the water against ribotypes contained in a host origin database. This area of research is called microbial source tracking. In the second and third years, we will expand the host origin database and use microbial source tracking to determine the host origin of nonpoint fecal contamination in the Yaguez and Grande de Añasco Rivers.

With regards to the Puerto Rico Water Resources Research Priorities for FY2001, the proposed research responds directly to Priority #1, Watershed and Water Sources Management. Because of ribotyping's discriminatory power, it is possible to identify the contamination source of many *E. coli* isolates from Puerto Rican water sources. With a suitable host origin database, microbial source tracking offers water resource managers the ability to direct their efforts at controlling sources of *E. coli* (as fecal coliforms) where reasonable control is possible (e.g., malfunctioning septic drainfields) and not at sources over which they have limited control (e.g., wildlife defecating in the water). The proposal also responds indirectly to Priority #10, Drinking Water Quality Research, because TMDL implementation plans will require identifying the host origin of nonpoint fecal contamination sources. The current method to identify nonpoint sources of fecal contamination, land use, does not work well. Therefore, microbial source tracking with ribotyping offers a better solution than the current method.

Statement of the results, benefits, and/or information

The main benefit of microbial source tracking, being able to identify the host origin of unknown *E. coli* isolates (as fecal coliforms) from Puerto Rican water sources, is not possible if a host origin database from a wide variety of warm-blooded animals does not exist. The more extensive the host origin database, the greater the likelihood is of obtaining matches. In the first year, we will obtain the ribotype of 400 *E. coli* isolates from a wide variety of

warm-blooded animals located in two watersheds in Puerto Rico. In this manner, the first host origin database for Puerto Rico will be established. In the second year, we will obtain the ribotypes of an additional 400 *E. coli* isolates, of which 150 will be used to expand the host origin database, and the remaining 250 will be used to test Yaguez River, a river that is impaired for fecal contamination. The third and final year will repeat the second year sampling except the Grande de Añasco, another impaired river, will be tested.

The benefit of the proposed research is that if water resource managers are able to direct their efforts at controlling sources of *E. coli* (as fecal coliforms) where reasonable control is possible (e.g., human sewage) and not at sources over which they have limited or no control (e.g., wildlife), then this constructive allocation of Puerto Rican resources would maximize the possibility of improving water quality. With respect to the proposed research, the results will be published in refereed scientific journals, and the host origin database will be placed on the worldwide web so that it is publicly accessible.

Nature, scope, and objectives of the research

A. Nature and scope

Fecal coliforms consist of several bacterial genera from the family Enterobacteriaceae that can grow on a selective medium at 44.5 °C for 24 hours. Fecal coliforms normally inhabit the intestinal tract of warm-blooded animals and their presence in soil or water is a good indicator that the soil or water was contaminated by bacterial pathogens. For example, when numbers of fecal coliforms exceed 2,000 per 100 mL of water, the likelihood of bacterial pathogens in the water is 98.1% (Geldreich, 1970). Fecal coliform counts are typically used to monitor the microbiological quality of Puerto Rico's waters. One of the most vexing problems in isolating fecal coliforms from water samples is not knowing the host origin of these bacteria. In the past, the only way to identify the host origin of a bacterium was to observe the bacterium's various phenotypic markers (i.e., characteristics expressed by the bacterium, like multiple antibiotic resistance). The main problems with using phenotypic markers are their lack of reproducibility and lack of discriminatory ower (ability to distinguish two closely related strains). However, in recent years, it has become possible to identify the host origin of a bacterium based on its DNA. This alternative method, called genotyping, not only has increased reproducibility, but also has increased discriminatory power.

The most common of these genotypic methods include chromosomal DNA restriction analysis, plasmid typing, pulsed field gel electrophoresis, various polymerase chain reaction (PCR) methods, and ribotyping (Farber, 1996).

Each genotypic method has its advantages and disadvantages

with respect to strains that can be typed, reproducibility, discriminatory power, ease of interpretation, and ease of performance. In this proposal, the genotypic method we selected is ribotyping. Ribotyping is based on ribosomal RNA (rRNA). Ribosomal RNA is present in all bacteria, and is composed of three species, 5S, 16S, and 23S. The DNA in the bacterium that codes for these three species of rRNA is usually present in 2 to 11 copies and is highly conserved (Grimont and Grimont, 1986). In ribotyping, the DNA is isolated from the bacterium and cut with a special enzyme that only recognizes certain DNA sequences (i.e., a restriction enzyme). The DNA is electrophoresed in a gel and the DNA transfered to a nylon membrane (this is called Southern blotting). The membrane is probed with a chemiluminescent copy of the 5S, 16S, and 23S portions of the DNA and, when properly treated, the membrane gives a pattern that can be scanned with an imager.

As a method for distinguishing a subspecies of a bacterium, ribotyping is considered to have excellent reproducibility, good discriminatory power, excellent ease of interpretation, and good ease of performance (Farber, 1996).

In this proposal, the fecal coliform we selected for ribotyping is *Escherichia coli*. This bacterium was selected for five reasons. First, as a fecal coliform, *E. coli* is accepted by the American Public Health Association as a good indicator of pathogenic bacteria (Clesceri et al., 1998). Second, most environmental ribotyping has been done with this bacterium (see Related Research). As a result, the methodology for ribotyping this bacterium is established. Third, there is good scientific evidence that specific strains of *E. coli* are associated with different host species (Faith et al., 1996). Fourth, *E. coli* does not exist as a stable population in the environment unless the source of contamination is persistent (Savageau, 1983). Fifth, *E. coli* is easy to isolate and easy to manipulate genetically.

With an extensive host origin database of *E. coli* ribotypes from Puerto Rico, one should be able to isolate *E. coli* from any water source (as well as from other sources, like soil) in Puerto Rico and identify the host origin of that *E. coli* isolate. To develop this database, a large number of *E. coli* isolates must be obtained from warm-blooded animals and ribotyped. An isolate of *E. coli* from a water source can then be ribotyped and compared to the host origin database to identify its host.

B. Objectives:

1. (Year 1) To construct a host origin database of *E. coli* ribotypes from a variety of warmblooded animals in Puerto Rico.
2. (Years 2 and 3) To identify the host origin of fecal contamination in the Yaguez and Grande de Añasco Rivers, both of which are impaired for fecal

contamination.

Methods, procedures, facilities, and timetable

A. Methods and procedures

Two Puerto Rican rivers will be selected, the Yaguez and Grande de Añasco. According to 1995–2000 USGS data (http://water.usgs.gov/pr/nwis/qwdata), the geometric means for the Grande de Añasco and Yaguez Rivers was 571 and 1,380 fecal coliforms per 100 mL, respectively. Therefore, both rivers are impaired with respect to fecal contamination. In the first year, *E. coli* isolates will be obtained only from host origin sources. In the second and third years, *E. coli* isolates will be obtained from both host origin and river sources.

A total of 400 isolates will be obtained each year. In the first year, the *E. coli* isolates will be obtained from (in order of priority, with the number of isolates in parentheses): humans (100), dogs (50), cattle (50), poultry (including roosters; 50), waterfowl (primarily ducks; 50), goats (50), and swine (50). Five isolates will be obtained from each animal, therefore, the number of animals tested will be (number in parentheses): humans (20), dogs (10), cattle (10), poultry (10), waterfowl (10), goats (10), and swine (10). Our experience suggests that this number of isolates is sufficient to be representative of resident *E. coli* subspecies. Because of strict regulations concerning human subjects, sampling of humans will be from septic tanks or sewer lines located as close as possible to homes. Therefore, for humans, this represents 25 septic tank or sewer sampling points. In the case of waterfowl, local wildlife specialists will help us with the sampling of these animals. In the case of river samples, there will be 10 sampling sites with 25 isolates obtained from each site. Ideally, each river will be sampled after a strong rainfall as this brings the maximum number of fecal sources into play. The remaining 150 isolates will be devoted to expanding the host origin database and will consist of 45 isolates from humans and 15 isolates from all the other categories of warm-blooded animals listed above. These isolates will be obtained from locations along the rivers that were not sampled in the first year.

In the case of host sources, fresh feces will be sampled with a culture swab containing Cary–Blair medium (Becton Dickinson, Sparks, Maryland). Swabs will be kept on ice for a maximum of 24 h before streaking the swab on 5-cm petri dishes containing mTEC medium (Difco Laboratories, Sparks, Maryland). In the case of river samples, duplicate 10- and 100-mL water samples will be filtered through separate sterile 0.45-µm membranes and the membranes will transferred aseptically to mTEC agar plates. All mTEC plates will be sealed inside triplicate

Ziploc bags and will be incubated submerged in a water bath

at 44.5±0.2 °C for 24 h according to standard methods (Clesceri et al., 1998). Yellow isolates will be randomly selected, streaked onto tryptic soy agar (Difco), and incubated at 35 °C for 24 h. The streaking will be repeated twice to ensure the purity of each isolate. Each isolate will be inoculated into a 24-multiwell tissue culture plate containing separate 1-mL slants of Simmons citrate and urea agar (both Difco). Three bacterial species from the American Type Culture Collection (ATCC; Manassas, Virginia) will be used as controls: *Escherichia coli* ATCC #11775 (citrate negative, urea hydrolysis negative), *Klebsiella pneumoniae* ATCC #13883 (citrate positive, urea hydrolysis positive), and *Enterobacter aerogenes* ATCC #13048 (citrate positive, urea hydrolysis negative). Isolates that are both citrate-negative on Simmons citrate agar and urea hydrolysis-negative on urea agar will be subjected to an oxidase test. Isolates that are oxidase negative will be considered *E. coli* and kept for long-term cryogenic storage at –70 °C.

For ribotyping, each *E. coli* isolate will be inoculated into Luria-Bertani broth contained in a test tube and incubated on a rotating shaker at 75 rpm at 35 °C. After 18 h, a 2-mL sample of the culture will be removed and the DNA will be extracted with a commercial kit (Qiagen DNeasy, Valencia, California). The DNA will be quantified with a fluorometer using standard DNA from *E. coli* strain B (Sigma Chemical Company, St. Louis, Missouri).

Two 1-µg samples of DNA from each isolate will each be separately digested overnight with the restriction enzymes *Eco*RI and *Pvu*II according to the manufacturer's directions (Roche Molecular Biochemicals, Indianapolis, Ind.). The digested DNA will be stained and electrophoresed in a 1.0% agarose gel at 58 volts for 3 h. Digoxigenin-labeled (DIG-labeled) Marker III (Roche) will be the molecular weight marker and will occupy every fifth lane of the gel. Additional lanes will contain no DNA (control) and DNA from *E. coli* ATCC #11775.

DNA will be transferred by Southern blotting to a nylon membrane with a vacuum blotting system (VacuGene, Pharmacia, Piscataway, New Jersey). The DNA on the membrane will be crosslinked with UV light. Following prehybridization at 42 °C for 2 h, the membrane will be hybridized at 42 °C overnight to DIG-labeled cDNA from *E. coli* total ribosomal RNA (Sigma).

Membranes will be prepared for chemiluminescence by a series of washing steps before a chemiluminescent substrate for alkaline phosphatase (Roche) will be added. Membranes will be placed in an imager (FluorChem 8000, Alpha Innotech, San Leandro, California) and images saved as a TIFF file. TIFF files will be imported into GelCompar II (Applied Maths, Kortrijk, Belgium) for analysis. Typically, gels showed 9 to 11 bands for *Eco*RI and 11 to 13 bands for *Pvu*II (Fig. 1; next page), and this will be considered sufficient for good discrimination among ribotypes. DNA fragments <1375 base pairs will be ignored because they are often indistinct.

Lanes will be normalized within the gel with the molecular weight marker and variations among the gels will be assessed with the *E. coli* ATCC #11775 strain. Optimization (shift between any two patterns) and tolerance (maximum distance between two band positions on different patterns) will each be set at 1.00%. Similarity indices were determined using Dice's coincidence index (Dice, 1945) and the distance among clusters calculated with the unweighted pair-group method using arithmetic averages (UPGMA). Based on variability of the inter-gel *E. coli* control, banding patterns will have to be ¡Ý90% similar to be considered the same ribotype.

B. Facilities

University of Puerto Rico

The Biotechnology and Nitrogen Fixation (BNF) Laboratory is located in the Finca Alzamora Research Building, and is the largest and best equipped laboratory of the Faculty of Agriculture. It is subdivided into a main laboratory, microbiology laboratory, sterilization and refrigeration area, storage room, and scientific and administrative offices. Equipment includes large- and medium-size autoclaves, plant growth chamber, shakers, balances, fermenter, microscopes, pH meters, centrifuges, gas chromatograph, microwave, and laminar flow hood.

Recently, equipment for molecular work has been acquired; this includes a digital imaging system, thermal cycler, spectrophotometer, and gel rigs for electrophoresis.

University of Georgia

The Soil Microbiology Laboratory consists of a 720 sq. ft. laboratory with all the necessary equipment to conduct ribotyping research. This includes gel rigs, microcentrifuges, UV crosslinker, blotting apparatus, hybridizing oven, and computer with GelCompar II software.

The FluorChem 8000 imager is kept in a separate room, but is considered part of the ribotyping laboratory. The laboratory also has a -70 °C freezer for maintaining the *E. coli* isolates. The laboratory also contains standard microbiology equipment including two clean benches and autoclave, and an attached light room for growing plants. The University provides standard support (instrument repair, computer repair, glassblowing shop, central research store) for all research laboratories in addition to an exceptional research library.

C. Timetable

Year 1 (1 Mar 02–28 Feb 03) (P = Puerto Rico ; G = Georgia; X = both)

Activity/Month

	M	A	M	J	J	A	S	O	N	D	J	F
Grant coordination (travel by)	G					P						
E. coli isolation from host animals		P	P	P	P	P						
DNA extraction and ribotyping			G	G	G	G	G	G				
Data analysis						G	G	G	G	G		
Manuscript/report preparation			P		P			P			X	X

Timetable explanation
In the first year, both Peter Hartel and Eduardo Schröder will travel. Peter Hartel will travel to Puerto Rico to coordinate the sampling with Eduardo Schröder. Later in the year, Eduardo Schröder will bring some of host origin isolates to the United States to learn ribotyping. Some of the host origin isolates will be sent to Georgia for ribotyping in the three months before Eduardo Schröder’s visit. In the second year, Peter Hartel will travel to Puerto Rico to coordinate the sampling of the Yaguez River. In this year, ribotyping and data analysis will also be conducted in Eduardo Schröder's laboratory. In the third and final year, Eduardo Schröder will travel to Georgia to coordinate the sampling of the Grande de Añasco River.

Related research
Introduction
In recent years, there has been considerable interest in developing phenotypic (characteristics expressed by the microorganism) and genotypic methods for determining the host origin of fecal bacteria in contaminated waters, a technique commonly referred to as microbial source tracking or bacterial source tracking. All of these methods are based on the assumption that specific markers or strains of bacteria are associated with specific animal species (e.g., Amor et al., 2000). While most of the research on phenotypic methods has concentrated on multiple antibiotic resistance (e.g., Wiggins et al., 1999), most of the research on genotypic methods has concentrated on ribotyping (e.g., Parveen et al., 1999) and various PCR methods (e.g., Dombek et al., 2000).
In this proposal, the method of microbial source tracking will be ribotyping. Ribotyping is based on an examination of the DNA that encodes for production of ribosomal RNA (rRNA). This portion of DNA is important because it is present in all bacteria and higher organisms and is highly conserved (does not mutate readily). For this reason, ribotyping is considered one of the most reproducible of the molecular typing methods (Farber, 1996). For ribotyping to work, a bacterial species must be selected. Among the best

indicators of fecal contamination are the fecal coliforms, a group of nonpathogenic bacteria commonly found in the feces of warm-blooded animals (Clesceri et al., 1998). Of the fecal coliforms, all microbial source tracking studies have been done with *E. coli*. Samadpour and Chechowitz (1995) matched 421 of 589 *E. coli* ribotype patterns (71%) from Little Soos Creek (in Washington State) to cows, deer, dogs, ducks, horses, humans, swine, and poultry. Subsequent studies in U. S. national parks and recreational areas also matched *E. coli* ribotypes to various animal hosts (Berghoff, 1998; Farag et al., 2001; Tippets, 1999). Ribotyping has identified the host origin of *E. coli* isolates in oyster beds (Simmons et al., 1995) and swimming areas (Simmons and Herbein, 1998) as well as differences between human and nonhuman sources of *E. coli* under conditions of a saltwater to freshwater gradient (Parveen et al., 1999).

We have done considerable microbial source tracking work with *E. coli* ourselves, but the manuscripts on this work are currently under review. In these manuscripts, we determined the effect of diet, flow rate, and biogeography on the diversity of *E. coli* ribotypes. In the case of diet, we determined its effect on diversity of *E. coli* ribotypes in penned and wild deer in a 13-ha forested watershed. A total of 298 *E. coli* isolates was obtained, 100 from penned deer, 100 from wild deer, and 98 from the stream in the watershed. The penned deer had 11 ribotypes and wild deer had 35 ribotypes, and this difference was significant ($p = 0.05$). No difference was observed between penned bucks and does. This suggests that diet, but not sex, affected ribotype diversity, and that a host origin database for microbial source tracking should contain isolates from wild rather than from captive animals. Also, 42 of 98 (42.9%) environmental isolates matched deer ribotypes. Microbial source tracking may have potential to delist watersheds that exceed total maximum daily loads for fecal coliforms where the sources of fecal contamination are predominantly from wildlife.

In the case of flow rate, we determined the diversity of *E. coli* ribotypes in a 77-km reach of the Chattahoochee River and its tributaries in metropolitan Atlanta (Georgia) under base flow and wet weather conditions. A total of 659 *E. coli* isolates was obtained from eight tributaries and four main stem sites during base flow and wet weather conditions, and 346 isolates were ribotyped and assessed for their similarity. During base flow conditions, 92 (123 isolates) of 162 ribotypes (239 isolates) were unique; during wet weather conditions, 57 (69 isolates) of 86 ribotypes (107 isolates) were unique. When the two conditions were combined, 72 of 92 (81.5%) unique ribotypes from base flow and 35 of 57 (61.4%) unique ribotypes from wet weather conditions remained unique. Therefore, most of the ribotypes were unique and were observed only once at one location during one flow condition. This large number of unique ribotypes suggests that considerable environmental

variability exists among ribotypes, and that a large number of *E. coli* isolates is needed from watersheds with complex land use patterns and varied flow conditions.

In the case of biogeographic variability, we determined the variability of *E. coli* ribotypes from one location in Idaho and three locations in Georgia for four animals (cattle, horse, swine, and poultry). This was done because it is not well understood to what degree host origin isolates are biogeographically variable (i.e., whether or not the *E. coli* isolates are cosmopolitan or endemic). Thus, a host origin database developed for Idaho may or may not be applicable to Georgia. A total of 568 *E. coli* isolates from Kimberly, Idaho (125 isolates), and Athens (210 isolates), Brunswick (102 isolates), and Tifton, Georgia (131 isolates) yielded 213 ribotypes.

The percentage of ribotype sharing within an animal species increased with decreased distance for cattle and horses, but not for swine and poultry. When the *E. coli* ribotypes among the hosts were compared at one location, the percent of unshared ribotypes was 86, 89, 81, and 79% for Kimberly, Athens, Brunswick, and Tifton, respectively. These data suggest that there is good ribotype separation among host animal species at one location, and the ability to match environmental isolates to a host origin database depends on a large number of isolates and a distance <175 km for certain host animal species. This research has direct applicability to the proposed research because, in addition to establishing an international host origin database of *E.coli* ribotypes, it also gives us an opportunity to confirm the biogeographic variability.

Summary

Microbial source tracking is still in its infancy. As the literature review suggests (e.g., Samadpour and Chechowitz, 1995), when a good host origin database exists, microbial source tracking works well. However, when only a limited host origin database exists (e.g., Berghoff,1998), microbial source tracking does not work well. The objective of our research is to begin 10 by building a strong host origin database of *E. coli* ribotypes in the first year, and then apply this database to the two rivers that are impaired for fecal contamination in the second and third years. We will continue to expand the host origin database in the second and third years as well.

Implicit in the development of strong host origin database is the idea that biogeographic separation of *E. coli* ribotypes occurs. This is likely to be even more so with an island like Puerto Rico. This means that Puerto Rico must develop its own host origin database for microbial source tracking and cannot rely on databases developed elsewhere.

References

Amor, K., D. E. Heinrichs, E. Frirdich, K. Ziebell, R. P. Johnson, and C. Whitfield. 2000.

Distribution of core oligosaccharide types in lipopolysaccharides from *Escherichia coli*.Infect. Immun. 68:1116-1124.

Berghoff, K. 1998. Beach sediment bacterial contamination and microbial source tracking study. 1997 Year End Summary Report, Glen Canyon National Recreational Area, National Park Service, UT.

Clesceri, L. S., A. E. Greenberg, and A. D. Eaton. 1998. Standard methods for the examination of water and wastewater, 20th ed. American Public Health Association, American Water Works Association, and Water Environment Federation Washington, D.C.

Dice, L.R. 1945. Measures of the amount of ecologic association between species. Ecology 26:297-302.

Dombek, P. E., L.-A. K. Johnson, S. T. Zimmerley, and M. J. Sadowsky. 2000. Use of repetitive DNA sequences and the PCR to differentiate *Escherichia coli* isolates from human and animal sources. Appl. Environ. Microbiol. 66:2572-2577.

Faith, N. G., J. A. Shere, R. Brosch, K. W. Arnold, S. E. Ansay, M. S. Lee, J. B. Luchansky, and C. W. Kasper. 1996. Prevalence and clonal nature of *Escherichia coli* O157:H7 on dairy farms in Wisconsin. Appl. Environ. Microbiol. 62: 1519-1525.

Farag, A., J. N. Goldstein, D. F. Woodward, and M. Samadpour. 2001. Water quality in three creeks in the backcountry of Grand Teton National Park, USA. J. Freshwater Ecol. 16(1):135-143.

Farber, J. M. 1996. An introduction to the hows and whys of molecular typing. J. Food Protect. 59:1091-1101.

Geldreich, E.E. 1970. Applying bacteriological parameters to recreational water quality. J. Am.Water Works Assoc. 62: 113-120.

Grimont, F., and P. A. D. Grimont. 1986. Ribosomal ribonucleic acid gene restriction patterns as potential taxonomic tools. Ann. Inst. Pasteur/Microbiol. (Paris) 137B: 165-175.

Parveen, S., K. M. Portier, K. Robinson, L. Edmiston, and M. L. Tamplin. 1999. Discriminant analysis of ribotype profiles of *Escherichia coli* for differentiating human and nonhuman sources of fecal pollution. Appl. Environ. Microbiol. 65:3142-3147.

Savageau, M. A. 1983. *Escherichia coli* habitats, cell types, and molecular mechanisms of gene control. Am. Naturalist 122

(6): 732-743.

Samadpour, M., and N. Chechowitz. 1995. Little Soos Creek microbial source tracking. Report to Surface Water Management Division, King County Department of Public Works, Seattle, Washington.

Simmons, G. M., Jr., and S. A. Herbein. 1998. Potential sources of *Escherichia coli* (*E. coli*) to Children's Pool in La Jolla, California. Final Report for the City of San Diego and the County of San Diego Department of Environmental Health.

Simmons, G. M., Jr., S. A. Herbein, and C. M. James. 1995. Managing nonpoint fecal coliform sources to tidal inlets. Universities Council on Water Resources. Water Resources Update, Issue 100:64-74.

Tippets, N. 1999. Backcountry water quality testing in Grand Teton National Park–1998 Summer Season. Report from the Environmental and Contaminants Research Center, U.S. Geological Survey.

Wiggins, B. A., R. W. Andrews, R. A. Conway, C. L. Corr, E. J. Dobratz, D. P. Dougherty, J. R.,Eppard, S. R. Knupp, M. C. Limjoco, J. M. Mettenburg, J. M.
Rinehardt, J. Sonsino, R. L., Torrijos, and M. E. Zimmerman. 1999. Use of antibiotic resistance analysis to identify nonpoint sources of fecal pollution. Appl. Environ. Microbiol. 65:3483-3486.

Training potential

Three undergraduate work/study students (one per year) will be trained in microbial source tracking over the proposed three-year lifespan of the grant. All the students will be at the University of Puerto Rico in Mayagüez. Two students will be from Agronomy and one from Biology.

Well and Interstitial Water Crop Protection Chemicals Study on the Salinas Fan

Basic Information

Title:	Well and Interstitial Water Crop Protection Chemicals Study on the Salinas Fan
Project Number:	2002PR5B
Start Date:	3/1/2002
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Descriptors:	None
Principal Investigators:	jose.dumas.1

Publication

Title: Well and Interstitial Water Crop Protection Chemicals Study on the Salina Fan Delta Aquifer

Statement of the critical water problems:
This project examines the groundwater and interstitial water quality, toxic substances and nonpoint contamination in the Jobos Basin Estuarine Reserve. The Jobos Bay is located between the Salinas and Guayama municipalities in the south coast of Puerto Rico and comprises more than 2,500 acres, including a forest and a mangrove. The Jobos Estuaries Ecosystem has been severely stressed since the late 80's by land and water channels alterations which have changed the water flow patters of the zone. One of the key issues that needs to be addressed in Puerto Rico and worldwide is the pesticide and phthalate esters movement in soil and groundwater and their effects over sensitive environmental zones, including flora, fish and wildlife.
Non-managed application of pesticides and other compounds that reach non target sites may result in leaving residues where crops will later be planted or where they may reach surface and ground water resources. No intensive research in Puerto Rico related to organic pollution in the Jobos mangrove zone has been carried out. The proposed field and laboratory research will be the first one that will help provide a more comprehensive view of water quality in the zone and will be complementary to another research project related to nitrate levels currently underway in the zone.

Statement of the results or benefits:
The expected benefits of the research proposed will be the development and advancement of new scientific information related to pesticide and organic toxic residues in the groundwater and interstitial water in the Salinas and Guayama municipalities. Agriculture is expected to continue in the zone, and water sampling for the presence of crop protection chemicals is necessary to ensure human health and the protection and conservation of the environment. The project will also provide data for risk/benefit decisions concerning chemical usage. Data for the year 2002-2003 will be analyzed, interpreted and results, findings and conclusions will be submitted for either technical papers on referred journals, oral presentations and posters during the following year. There will be one or two publications associated with the completion of the research.

Nature, scope and objectives of the research
Before the late 1970's, ground water contamination from field applied pesticides were virtually unexpected. It was assumed that pesticides in the natural environment would break down or be adsorbed into the soil, sand, gravel, and rock formations. Currently human activities such as organic compounds applied for crop protection can clearly lead to contamination of surface and groundwater and thus indirectly affecting estuaries and associated wetlands interstitial

water and soil quality (Panke, 2000; Snedaker, 1981). The water quality and humidity content of soil will affect first the microbial population of the zone, influencing nutritional chain, and many other biological and biochemical processes, including plant absorption, metabolism and microbial degradation of organic matter and other natural and anthropogenic chemicals. Therefore, pesticides and other toxic anthropogenic source compounds in water-soil environment are a public and environmental concern problems that deserve further investigation in this region.

The south coastal plains of Puerto Rico were used as agricultural lands for sugarcane cultivation until the mid 70's: After a diversification program established by the Puerto Rico Department of Agriculture, bananas and plantains, legumes, vegetables and tropical fruits production has intensified in the last twenty-five years. Groundwater in the Salinas municipality is used for potable water and agricultural purposes, extracting close to three million gallons per day (Molina, 1998) Some pesticides currently used in the zone are very soluble in water suggesting a high potential to move by runoff and leaching to reach surface and groundwater, hence affecting its quality and eventually reaching estuaries, wetlands and marine ecosystems. The Salinas Fan Delta is the major aquifer in the Guayama, Salinas and Santa Isabel zone and is recharging the water in the Jobos Estuarine Natural Reserve (Gomez, 1990; Gonzalez, 1999). Although intensive agricultural practices have been carried out in this zone, no comprehensive study has conducted to assess to the presence, runoff, leaching, degradation and fate of agrochemicals, and other anthropogenic source compounds.

The project’s objective is to determine the presence, levels and seasonal variability of agrochemicals in groundwater in the zone near the Jobos Basin. The magnitude of adsorption of the detected pesticides on the main agricultural soils and wetland soils found in the zone. This will serve as an index for selection of more adequate soils and agricultural management practices to avoid pesticide contamination and further disruption of the Jobos Estuaries Ecosystem. This research will help address pesticide groundwater contamination in Puerto Rico and pinpoint management practices, to save and protect Jobos Basin.

The specific objectives of the research are: 1) Survey water wells and interstitial water in the Jobos Basin zone to identify and quantify the major pesticides and phthalates esters, and the levels of nitrate-nitrite, phosphates, dissolved oxygen and other types of compounds from anthropogenic sources. 2) Determine the microbial mass present in the Jobos estuaries zone and its relationship to the mangrove health, organic and inorganic compounds present in interstitial
water and dissolved oxygen (DO).

Methods, procedures, and facilities

Objective 1

Sample collection, preservation and handling

All pollutants analysis will be conducted at the Agricultural Experimet Station Pesticide Laboratory. Water samples will be collected at a minimum of 15 wells from agricultural growing areas of the Jobos basin zone, and at a minimum of 15 piezometers and/or springs on the Jobos Reserve. Efforts will be made to have wells , piezometer and springs in the north, west and east zones of Jobos basin zones; near to the Rio Nigua, parallel to the abandoned stream channel, and in the Esmeralda fault, north to the bedrock hill at Aguirre Sugarmill. A preliminary sampling (screening) will be performed in the selected zone by using immunochemical methods for carbamates, s-triazines, organophosphorus and other pesticide residue analysis, and to delineate properly the water quality and variations within the affected area. A minimum of six wells and six piezometers or springs in the zone will be selected and used to achieve reliable results and findings. The selected sampling wells, piezometers and/or springs will be sited by GPS. Water samples will be taken monthly for one year. Triplicate samples will be collected in one-liter dark brown glass bottle with Teflon lined cap (pre-washed with detergent and hot tap water, rinsed with distilled and de-ionized water, and dried in an oven at 400 °C for 1 h). All water samples will be placed in an ice chest at around 4 °C and transferred to the Central Analytical and Pesticide Laboratories at Rio Piedras, within the same collecting day Sodium thiosulphate and copper metal (80 mg/L) will be added to remove a residual chorine and sulfur, respectively. The samples will stored at 0 to 4 °C in a refrigerator from the time of collection until extraction that will be within seven days after collection. All water samples will be filtered through a Whatman GB/F filter, followed by a Nylon membrane filter before chemical analysis.

Pesticides and other type of organic compounds analysis:

For carbamate analysis, samples will be filtered through a 0.22um polyvinylidene
difluoride filter, followed by direct injection into a High Performance Liquid Chromatograph (HPLC) with a C-18 column coupled to post column derivatization system and a fluorometric detector. The detection will be made by post-column derivatization and fluorescence detection (Foerst, 1987).

Other types of thermally stable compounds will be extracted following the US EPA method 8270 that include 259 semi-volatile organic compounds. A 1-L water sample, containing a surrogate, will be extracted in a continuous extractor, first under acid and then under basic conditions. Analysis will be performed by GC/MS using a DB-5 capillary column and an appropriate computer system.

Ammonium, nitrate, nitrite, pH, conductivity and phosphate
These chemical analysis will be conducted at the Central Analytical Laboratory .
Ammonium, nitrate and nitrite will be analyzed using the EPA method 353.2. Phosphate will be analyzed by the EPA method 365.1. The water sample will be preserved at 4 C with diluted sulfuric acid to pH <2 until the analysis.

Objective 2
Soil microbial biomass C and N
Soil core samples, in triplicate, from 0 to 10 and 10 to 20 cm will be collected from six plot areas selected by using the ground and surface water flow pattern map. These areas were selected due to the high hydraulic conductivity, and besides have the higher potential to be affected by dissolved solids that come from any human activities in the zone. The selected areas were in the north, west and east zones of Jobos basin zones; near to the Rio Nigua, parallel to the abandoned stream channel, and in the Esmeralda fault, north to the bedrock hill at Aguirre Sugar Mill. Soil samples will be placed in plastic bags, transferred to Rio Piedras, and then dried at 40 C. The 100 g of soil will be re-wetted to 15% moisture to stimulate microbial activity, and will be incubated at 25 C for 5 days. Soil microbial biomass C will be determined by fumigation incubation by exposing 40 g soil samples rewetted and incubated for 5 d to alcohol free $CHCl_3$ vapor for 24 h. The vapors will be evacuated and removed and the soil will be incubated on a 1-L gas tight glass container for 10 days at 25 C. Carbon dioxide evolved will be trapped in 1N KOH and determined by titration with 1N HCL. The quantity of evolved CO2-C will be divided by an efficiency factor of 0.41 to calculate microbial biomass C (Anderson, 1982, Haney, 2000).
Soil microbial biomass N will be determined by analyzing NH4-N concentration of fumigated samples following 10-d incubation period minus initial NH4-N prior fumigation, divided by an efficiency factor of 0.41. The NH4-N will be extracted from 7g soil sample using 28 ml of 2M KCl. Samples will be shaken for 30 min on a reciprocal shaker and filtered, and the extracts will be analyzed for NH4-N using an autoanalizer.

Bulk density, clay organic carbon content determination
Soil bulk density, clay, sand, silt and organic carbon content will be determined using standard method reported elsewhere (Blake, 1965). Data collected will be statistically analyzed by using variance method. The plot will be all area in which the soil samples for microbial analysis will be taken. The treatment will be wells and piezometers, and the factors will the soil type and their physical and chemical characteristics mentioned above.

Adsorption studies

Adsorption will be expressed as k-Langmuir values, and the more knowledge k-Freundlich values. A stock solution of 40 ppm in distilled and de-ionized water will be prepared for each selected compound by using each of the soil types collected in the selected plot area mentioned above. Concentrations of 0.5, 1,5,10,20,40 ppm will be prepared by dilution with distilled and de-ionized water. Batch equilibrium sorption isotherms will be obtained for each individual pesticide (Liu, 1997). The laboratory tests will be conducted treating 1g of air dried soil with 10 ml of the selected compound solution in a sealed test tube. The suspensions will be protected from light using aluminum paper, kept at 22 °C, and placed on a shaker for 24 h. The suspensions will be centrifuged for 10 min at 2500 rpm and a 5 ml aliquot of the clear supernatant solution will be extracted with three successive 15 ml portions of methylene chloride. The samples will be concentrated, with a smooth flow of high purity nitrogen gas, diluted with hexane to 5 ml, and analyzed using GC-FTD or GC-MS. The adsorption curves for pesticides and k- values will be determined using the Langmuir model.

Data generated will be compared with the bulk density, clay and organic carbon content of the surrounding soils to the sampled wells. The significance of the data will be statistically analyzed to find a relationship between these properties

Time Table (First year)

FY APROX DATE PROCEDURE

FY02/03

Obj. 1

March 2002 : Sampling piezometer adquisition or construction and placement in the watershed of Jobos

April 2002 : Wells and interstitial water sampling for screening organic toxic compounds by inmuno assay methods, nitrate-nitrite and DO levels

April 2002 : Selection of wells and piezometers to be sampled during one year period and beginning of monthly samplings, nitrate-nitrite-ammonia and DO levels determination, extraction and analysis of organic toxic compounds of water samples by GC-MS.

April 2003 : End of the first year water sampling and analysis by GC-MS.

Objective 2 April 2002 Soil microbial biomass C and N determination in selected soil in the water sampling zone.

April 2003: Soil microbial biomass C and N determination in selected soil in the water sampling zone.

Related Research

The presence of bacteria, organic solvents, nitrate and pesticides in spring and well water has been previously reported around the Island (Guzmán-Ríos and Quiñones-Marquez, 1985; Zack et al., 1986; Conde-Costas et al., 1997). Recently, Dumas et al., 1997a & b, conducted a one year study and found high levels of nitrate and traces of several pesticides in some wells in the Manatí quadrangle. Pesticides detected such as , dieldrin, ametryne, atrazine, ethoprop, diazinon and bromacil, have all been used in pineapple production in Puerto Rico (Dumas, 1996; Dumas et al., 1997a). In a one-year study, Montalvo and collaborators detected traces of atrazine, ametryne, diazinon, disulfoton, fenamiphos, ethoprop and bromacil in some wells in Salinas, Santa Isabel and Juana Diaz municipalities (Montalvo, 1998). In addition, levels about 10 ppm of N-Nitrate were recorded for at least two groundwater wells (Montalvo, 1999). Trace levels of atrazine and alachlor were found in Jobos basin zones in the south coast of Puerto Rico in 1999 (Artieri, 2001). In addition, close to two ppb of picloram were detected in one of four wells monitored during 1995 on the north east coast, suggesting the existence of picloram in the groundwater of Puerto Rico (Liu, 1997). The detection of pesticides at low levels suggests ongoing water pollution that deserves further studies related to detection and quantization, considering cumulative effect of several pesticides and also nitrate-nitrite levels in the water simultaneously.

The mangrove biota is the resident plant and animal assemblages in the water body (EPA, 2000). The condition of the biota definitely depends in part of the physical-chemical environment of the estuaries as showed some studies in the Puerto Rico red mangrove. These studies show that any activity or natural event affecting the groundwater quality and salinity of the mangroves may affect natural regeneration of the mangrove (Wier, 2000). Wier and collaborators found a high frequency of dieback and mortality of the red mangrove, caused by the fungus Cytospora rhizophorae (Wier, 2000). Other studied did in the coastal plain watersheds in Maryland showed the runoff and leaching movement of pesticide applied in corn, soybean and wheat farms such as atrazine, metribuzin, alachlor, nicosukfuron, cyanazine, metokachlor chlorimuron ethyl and thifensulfuron methyl vary greatly depending upon which herbicides are used. In addition, their movement is highly dependent in how they are applied. Although the risk associated with these herbicide concentrations was beyond the scope of the study the show a high herbicide movement through soil pore water in the watershed (Forney, 2001). Studies made to determine the effect of pesticides and metal pollutans in mangrove ecosystem showed that health mangroves are relatively insensitive to toxic materials in the parts per million range and lower (Snedaker, 1981). However, this toxic material may affect other animal assemblages and

coastal marine waters that may have eventually detrimental effect over all ecosystems. The estuary's ecosystems may be damaged by toxic organic compounds and physical stress (EPA, 2000). Toxic organic compounds at levels exceeded freshwater aquatic life guidelines were found in Chesapeake Bay,s Choptank River Estuary (Johnson, 2000). These toxic compounds including atrazine, chlorothalonil, chlorpyrifos, cyanazine, 2,4-D, diazinon, malathion, metolachlor simazine and trifuralin. Some of these pesticides are currently used in the south central coastal plain and their fate and movement deserve to be investigated.

Training Potential

One undergraduate and one graduate student from Chemistry will participate in this research. Also, one undergraduate and one graduate student from environmental engineering will benefit from the project.

References

Snedaker,SC; Brown,MS (1981): Water quality and mangrove ecosystem dynamics. (EPA-600/4-81-002) USEPA. Office of Pesticides and Toxic Substances, Gulf Breeze, Florida. 79 pages.

Conde-Costas, C., Rodriguez, G. 1997. Potentiometric surface and hydrologic conditions of the upper aquifer in the Manatí-Vega Baja area, north central Puerto Rico, March 1995. U.S. Geological Survey, Water-Resources Investigations Report 96-4184.

Dumas, J.A., Rosario, O. Montalvo-Zapata, R. and Casanova, P. 1997. Variación temporal de agroquímicos orgánicos en seis pozos localizados en siembras de piñas en el área de Manatí. Reunión Anual SOPCA, nov. 21, Lajas, P.R. (Poster).

Dumas, J.A., Rosario, O. Montalvo-Zapata, R. and Casanova, P. 1997. Extracción mediante fase sólida de plaguicidas y sus metabolitos en aguas. Reunion Anual SOPCA, Nov 21, Lajas, P.R. (Poster).

Guzmán-Ríos, S. and Quiñones-Márquez, F. 1985. Reconnaissance of trace organic compounds in ground water throughout Puerto Rico. October 1983. U.S. Geological Survey Open File Data Report 84-810, 1 sheet.

Zack, A., T. Rodríguez-Alonso, and A. Ramón-Mas. 1986. Puerto Rico groundwater quality. U.S. Geological Survey. Open File Report 87-0749. 8pp.

Liu, L. C., Dumas, J. A. and Cacho, C. 1997. Picloram groundwater contamination from pasture use. Journal of Agric., Univ. P. R. 81 (3&4), p. 211-218.

Montalvo, R. 1999. Solid phase extraction techniques in water samples. APR Project H-385 (S- 271).

Montalvo-Zapata, R., Dumas, J. A.. And Lugo, W. 1998. Screening of pesticides and determination of nitrate-nitrogen in six underground wells in Juana Dian, Santa Isabel and Salinas municipalities. Poster presented Annual

Meeting of SOPCA, San Juan, P.R.
Panke, M. and Quimby S. 2000. Pesticides. Caribbean Currents. Volume 8, Number 3, July 2000 EPA 220-N-00-008
Anderson, J.P.E. 1982. Soil respiration. Pages 837-871 in A.L. Page et. al., eds, Methods of Soil Analysis. Madison, WI: American Society of Agronomy and Soil Science Society of America.
Haney, R.L. Senseman, S.A., Hons, F.M. and Zuberer, D.A. 2000. Ffect of glyphosate on soil microbial activity and biomass. Weed Science 48:89-93.
Blake, G.R. Particle Density and Bulk density Pages 371-390 in C.A. Black et al, eds Methods of Soil Analysis. Madison, WI: American Society of Agronomy and Soil Science Society of America.
Foerst, D.L. 1987. Method 531.1, Measurement of N-Methylcarbamoyloximes and N-Methyl carbamates in GroundWater by Direct Aqueous Injections HPLC with Post Column Derivatization, National Pesticide Survey, EPA, 1987.
Gonazalez, C. 1998. Management Plan for the Jobos Bay National Estuarine Research Reserve, Guayama/Salinas, Puerto Rico.
Gomez-Gomez, F., Quiñones-Aponte, V. and Johnson, I.A.. 1990. Regional Aquifer Systems of the United States. Aquifers of the Caribbean Islands. American Water Resources Association. 5410 Grosvenor Lane, Suite 220. Bethesia, Maryland 20814-2192.
Johnson, W.E., Hall, L.W. Anderson, R.D. and Rice, C.P. 2000. A small agricultural Watershed Study on Maryland,s Outer Atlantic Coastal Plain. Pages 95-114 in T.R.
Steinheimer et al., eds, Agrochemical Fate and Movement: Perspective and Scale of Study.Washington, DC: American Chemical Society. ACS Symposium series 751.
Artieri, C. 2001. Northest Pesticide Education Meeting. Condado Plaza Hotel. San Juan, Puerto Rico. August 21-23, 2001.
Forney, D.R., Strahan, J., Rankin, C. Steffin, D., Peter, C.J., Spitter, T.D. and Baker, J.L. 2001.Monitoring pesticide runoff and leaching from four farming systems on field-scale coastal plain watersheds in Maryland. Pages20-43 in T.R. Steinheimer et al., eds, Agrochemical Fate and Movement: Perspective and Scale of Study.Washington, DC: American Chemical Society. ACS Symposium series 751.
EPA document number; Estuarine and Coastal Marine Waters: Bioassessment and Biocriteria Technical Guidance, (EPA 822-B- 00-024), dated December 2000.
Molina-Rivera, W.L., 1998, Estimated water use in Puerto Rico, 1995: U.S. Geological Survey Open-File Report 98-276, 28 p.
Wier, A.M., Tattar, T.A.,. Klekowski, E. J. 2000. Diseases of red mangrove (Rhizophora mangle) in southwest Puerto Rico caused by Cytospora rhizophorae Biotropica 32(2):299

FY 2003 State Water Resources Research Institute Program

Basic Information

Title:	FY 2003 State Water Resources Research Institute Program
Project Number:	2002PR16B
Start Date:	3/1/2002
End Date:	2/28/2003
Funding Source:	104B
Congressional District:	
Research Category:	Water Quality
Focus Category:	None, None, None
Descriptors:	
Principal Investigators:	Jorge Rivera-Santos, Walter F Silva

Publication

During this year the Institute has become more involved in attracting funds for research support. The director has developed and implemented an aggressive program to meet key personnel from different government agencies and create the necessary contacts to sell the Institute as an organization that may help finding the solution to many of the water related problems in Puerto Rico. This program has resulted in signed Memorandum of Understanding (MOU) between the Institute and other government or private organizations. As for example, the Institute signed an MOU with the Department of Natural Resources and the Environment of Puerto Rico (PRDNRE). This MOU, which covers a previous one signed with one of the divisions of the PRDNRE (Jobos Bay National Estuarine Research Reserve), allows the Institute to participate, jointly with the Department, in the solution of a gamma of water related problem. The designation of the Boquilla's Natural Reserve is one example. This is an inland estuary, which is being subjected to stress by the rapid urban development in its surroundings.

Other MOU was signed with the Environmental Quality Board (EPA state counterpart). Under this agreement the Institute has been contracted to develop TMDLs for the Mayagüez Bay Watershed. This watershed is comprised of three mayor rivers namely; Río Grande de Añasco, Río Guanajibo, and Río Yagüez. TMDLs are being developed for coliforms, and eventually, for lead, mercury, cooper, and arsenic.

This MOU has also been the vehicle to get funds for other research projects such as the establishment of water quality standards for nutrients in lakes of Puerto Rico.

MOUs have been signed with private and professional organizations too. Two of these are the professional organizations that joints almost all water professionals in Puerto Rico (PR Environmental Association and American Water Works Association - Puerto Rico Chapter. The MOU allows collaboration between both groups and the Institute and to share professional information such as the results of research projects. A one-week annual symposium is held in May in collaboration of these groups and other state and federal government agencies.

The institute has submitted research proposals to other federal government agencies, such as, the Corps of Engineers (CoE), FEMA, and EPA. At least one project each year has been contracted with the CoE resulting in funding of $160,000 annually. Other projects, including those mentioned above, are attracting funds in excess to $700,000 per year.

The Institute hired a full time assistant researcher. He is a GIS-specialized agronomer and is in charge of all GIS related task for all projects funded trough the Institute. A full time secretary has been hired too. With the increase in the number or projects, the paper work has increased proportionally.

The Institute has plans to expand his professional educational and training program to include new courses in areas of interest to the water professional in Puerto Rico and the Spanish spoken countries of the Caribbean, central, end South America.

Microbial Sources Tracking to Determinate the Host Origin of Fecal Contamination in two Puerto Rican Watershed

Basic Information

Title:	Microbial Sources Tracking to Determinate the Host Origin of Fecal Contamination in two Puerto Rican Watershed
Project Number:	2002PR17B
Start Date:	3/1/2002
End Date:	8/31/2003
Funding Source:	104B
Congressional District:	
Research Category:	Water Quality
Focus Category:	Non Point Pollution, Water Quality, Agriculture
Descriptors:	
Principal Investigators:	Eduardo Schroder

Publication

Coliform bacteria, “fecal coliforms” have been used traditionally as indicators of microbiological safety of water. Since they normally inhabit the intestinal track of warm-blooded animals, their presence in soil or water is a good indicator of contamination with pathogens. Therefore, assessing microbial contamination in the water constitutes a fundamental public health issue. Fecal coliform counts are typically used to monitor the microbiological quality of water in Puerto Rico.

One of the most vexing problems in isolating fecal coliforms from water samples is not knowing the host origin of these bacteria. In the past, the only way to identify the host origin of a bacterium was to observe the phenotypic markets (such as colony characteristics and antibiotic resistance). The main problems with using phenotypic markers are their lack of reproducibility and lack of discriminatory power (ability to distinguish two closely related strains). However, it is now possible to identify the host origin of a bacterium based on its DNA. This alternative method, called genotyping, not only has increased reproducibility, but also has increased discriminatory power. The most common of these genotypic methods include chromosomal DNA restriction analysis, plasmid typing, pulsed field gel electrophoresis, various polymerase chain reaction (PCR) method, and ribotyping. As a method for distinguishing a subspecies of a bacterium, ribotyping is considered to have excellent reproducibility, good discriminatory power, excellent ease of interpretation, and good ease of performance.

We will isolate and □ibotypes *Escherichia coli* for several reasons, but particularly because is a good indicator of pathogenic bacteria. However, further tests to consider alternative indicator bacteria will be performed. The specific objectives will be to isolate a large number of isolates to □ibotypes and track their origin. Initially methods will be tested and samples obtained from the Yaguez River, and in the future, Añasco River will be sampled.

Methodology:

The investigation is divided into three major parts:

A) Sampling of rivers in either base flow or storm flow
B) Using IDEXX Colilert for general survey and to obtain *E. coli* isolates
C) Conduct a targeted survey to determine if sources are persistent or transient, and if persistent, whether point or non point sources can be determined.

Principal Findings and Significance:

We developed a new targeted sampling protocol for microbial source tracking (Fig. 1). This new sampling protocol offers two advantages for microbial source tracking. First, all sampling is completed in one location (the Yaguez River) in one day. This reduces problems with geographic and temporal variability of *E. coli* isolates. Second, it identifies persistent sources of fecal contamination. Not surprisingly many nonpoint sources are subsequently identified as point sources. There is no need to do

microbial source tracking on point sources. Therefore, the amount of microbial source tracking needed is minimized (as are the associated costs). This changes the focus of the first year's work from developing an *E. coli* database for Puerto Rico to direct sampling of the Yaguez River, which was designated for the second year of the grant.

At this time, the Yaguez River has been sampled for the general survey during base flow conditions. Counts of *Escherichia coli* ranged from 1.4×10^2 to 1.2×10^5 CFU per 100 mL. Assuming that this is not a problem of *E. coli* regrowth under subtropical conditions, the data suggest that the Yaguez River is grossly contaminated with fecal material. Two hotspots were identified, one near the mouth of the river, and the other near a textile plant. (The textile plant is not suspected as a source and simply is a location point.) Counts of *E. coli* at both locations exceeded 10^5 CFU per 100 mL.

Because of these high counts, obtaining "local knowledge" about the sources is unnecessary, and, assuming these locations represent persistent sources, and they will be sampled in the next weeks. At least 50 *E. coli* isolates will be obtained from each of the two potential sources and the river itself. Ribotyping with the Qualicon RiboPrinter is expected to commence shortly thereafter.

First data are already analyzed and will be presented at the American Society for Microbiology meeting in May 2003. Additionally two research papers will be submitted for publication to peer review journals.

TRAINING ACCOMPLISMENTS

List all students participating in Section 104 projects.

Field of study	Academic Level				Total
	Undergraduate	MS	Ph.D.	Post Ph.D.	
Chemistry					
Engineering:					
Agricultural					
Civil					
Chemical					
Computer					
Electrical					
Industrial					
Mechanical					
Geology					
Hydrology					
Agronomy	4	1			5
Biology		4			4
Ecology					
Fisheries, Wildlife, and Forestry					
Computer Science					
Economics					
Geography					
Law					
Resources Planning					
Social Sciences					
Business Administration					
Other (specify)					
Totals		5			9

Well and Interstitial Water Crop Protection Chemicals in the Salinas Fan Delta Aquifer

Basic Information

Title:	Well and Interstitial Water Crop Protection Chemicals in the Salinas Fan Delta Aquifer
Project Number:	2002PR18B
Start Date:	4/1/2002
End Date:	7/1/2003
Funding Source:	104B
Congressional District:	
Research Category:	Water Quality
Focus Category:	Toxic Substances, Water Quality, Wetlands
Descriptors:	
Principal Investigators:	Jose Dumas

Publication

This project examines the groundwater and interstitial water quality, toxic substances and non point contamination in the Jobos Basin Estuarine Reserve. Jobos Bay is located between the Salinas and Guayama municipalities on the south coast of Puerto Rico and comprises more than 2,800 acres, including a forest and a mangrove. The Jobos Estuaries Ecosystem has been severely stressed since the late 80s by land and water channel alterations which have changed the water flow patterns of the zone. One key issue that needs to be addressed in Puerto Rico and worldwide is t he pesticide and phthalate ester movement in soil and groundwater, and their effects on sensitive environmental zones, including flora, fish and wildlife.

Non-managed application of pesticides and other compounds that reach non target sites may result in leaving residues where crops will later be planted or where these residues may reach surface and ground water resources. No intensive research in Puerto Rico related to organic pollution in the Jobos mangrove zone has been carried out. The proposed field and laboratory research will be the first to help provide a more comprehensive view of water quality in the zone. Besides will be complementary t o another research project currently underway related to nitrate levels in the zone.

Statements of the results or benefits:
The expected benefits of the proposed research will be the development and advancement of new scientific information related to pesticide and organic toxic residues in the groundwater and interstitial water in the Salinas and Guayama municipalities. Agriculture is expected to continue in the zone, and water sampling for the presence of crop protection chemicals is necessary to ensure human health and the protection and conservation of the environment. The project will also provide risk/benefit data for decisions concerning chemical usage. Data for the year 2002-2003 will be analyzed, interpreted, and results, findings and conclusions will be submitted for either technical papers on referred journals, oral presentations and posters during the following year. There will be one or two publications associated with the completion of the research.

Methodology:
Sample collection, preservation and handling
Pollutant analysis will be conducted at the Agricultural Experiment Station Pesticide Laboratory. Water samples have been collected from fifteen wells from agricultural growing areas of the Jobos basin zone, and from twenty-six piezometers on the Jobos Reserve (Table 1 and 2). Piezometers were at the north, west and east zones of Jobos Basin, parallel to the abandoned stream channel and in the Esmeralda fault, north of the bedrock hill at the Aguirre sugar mill. The selected sampling wells and piezometers were sited by GPS. Water samples were taken monthly for one year. Triplicate samples will be collected in one-liter dark-brown glass bottles with Teflon lined caps (pre-washed with detergent and hot tap water, rinsed with distilled and de-ionized water, and dried in an oven at 400° C for 1 h). All water samples were placed in an ice chest at around 4° C and transferred to the Central Analytical and Pesticide Laboratories at Rio Piedras on the same collecting day. Sodium thiosulphate and copper metal (80 mg/L) were added to remove residual chorine and sulfur, respectively. The samples were stored at 4° C in a refrigerator from the time of collection until extraction, which was done the next day

after collection. All water samples were filtered through a Whatman GB/F filter, followed by a Nylon membrane filter before chemical analysis.

Table 1. Wells identification, location and geographic position

			Geographic Position					
Well No	Well id	Sector	North			**West**		
W1	Aguirre 2	Aguirre	17	58'	049"	66	13'	524"
W2	Aguirre 3	Aguirre	17	57'	901"	66	15'	121"
W3	Esperanza	Esperanza	17	57'	632"	66	15'	271"
W4	Salich	Salich	17	57'	638"	66	16'	115"
W5	Vasquez	Las Mareas	ND					
W6	Soler1	Fortuna	17	58'	127"	66	15'	150"
W7	Jaguas 1	Jaguas	17	58'	696"	66	15'	690"
W8	Jaguas 2	Jaguas	17	58'	737"	66	15'	872"
W9	Vega	Fortuna	17	58'	264"	66	15'	437"
W10	Gonzalez	Fortuna	17	59'	363"	66	16'	275"
W11	Gonzalez Grua	Fortuna	17	58'	801"	66	15'	262"
W12	Gonzalez Antena	Fortuna	17	58'	683"	66	14'	961"
W13	Lanausse 1	Aguirre	17	58'	747"	66	14'	752"
W14	Lanausse 2	Aguirre	17	59'	160"	66	14'	757"
W15*	Gonzalez Antena 2	Fortuna	17	58'	801"	66	15'	262"

* well very close to W12
ND-No was determined

Analysis of pesticides and other types of organic compounds:
Organic compounds were extracted following the US EPA method 8270, which includes 259 semi-volatile organic compounds and/or SPE-disk method outlined by Muller. A 1-L water sample, was filtered through a glass fiber filter, and extracted. Analyses were performed by GC/MS using a DB-5 capillary column and an appropriate computer system.

Table 2. Piezometer identification, location and geographic position

			Geographic Position					
Piezometer id	Sector	Location	North			**West**		
P1S1	S1	Aguirre Forest	17	57'	442"	66	13'	141"
P2S1	S1	Aguirre Forest	17	57'	432"	66	13'	151"
P3S1	S1	Aguirre Forest	17	57'	421"	66	13'	149"
P4S1	S1	Aguirre Forest	17	57'	478"	66	13'	159"
P5S1	S1	Aguirre South	17	57	490	66	13	183"
P6S1	S1	Aguirre South	17	57	515	66	13	198"
P7S1	S1	Aguirre South	17	57	519	66	13	216"
P1S2	S2	Aguirre South	17	57'	110"	66	14'	853"
P2S2	S2	Aguirre South	17	57'	0"	66	14'	841"
P3S2	S2	Aguirre South	17	57'	19"	66	14'	870"
P4S2	S2	Aguirre South	17	57'	001"	66	14'	865"
P5S2	S2	Aguirre South	17	56'	984"	66	14'	846"
P1S3	S3	Las Mareas	17	57'	64"	66	15'	710"
P2S3	S3	Las Mareas	17	57'	29"	66	15'	672"
P3S3	S3	Las Mareas	17	57'	15"	66	15'	673"

P4S3	S3	Las Mareas	17	57'	068"	66	15'	735"
P5S3	S3	Las Mareas	17	57'	001"	66	15'	665"
P1S4	S4	Aguirre	17	57'	207"	66	14'	846"
P2S4	S4	Aguirre	17	57'	215"	66	14'	847"
P3S4	S4	Aguirre	17	57'	221"	66	14'	846"
P4S4	S4	Aguirre	17	57'	188"	66	14'	851"
P5S4	S4	Aguirre	17	57'	173"	66	14'	854"
P6S4	S4	Aguirre	17	57'	164"	66	14'	846"
P7S4	S4	Aguirre	17	57'	181"	66	14'	839"
P8S4	S4	Aguirre	17	57'	186"	66	14'	853"

Ammonium, nitrate, nitrite, pH, conductivity and phosphate

These chemical analyses were conducted at the Central Analytical Laboratory. Ammonium, nitrate and nitrite were analyzed by using the EPA method 353.2. Phosphate will be analyzed by the EPA method 365.1. The water samples were preserved at 4° C with diluted sulfuric acid to pH <2 until the analysis.

Soil microbial biomass C and N

Soil core samples were collected from the selected sampling sites for interstitial water monitoring (S1, S2, S3 and S4). These areas were selected because of the high hydraulic conductivity; besides they have the higher potential to be affected by dissolved solids that come from any human activities in the zone. The selected areas were in the north, west and east Jobos basin zones; near the Rio Nigua, parallel to the abandoned stream channel, and in the Esmeralda fault, north of the bedrock hill at Aguirre Sugar Mill. Soil samples will be placed in plastic bags, transferred to Rio Piedras, and then dried at 40° C. The 100 g of soil was re-wetted to 15% moisture to stimulate microbial activity, and then incubated at 25° C for 5 days. Soil microbial biomass C was determined by fumigation incubation by exposing 40 -g soil samples rewetted and incubated for 5 d to alcohol free $CHCl_3$ vapor for 24 h. The vapors were evacuated and removed. The soil was incubated in a 1-L gas tight glass container for 10 days at 25° C. Carbon dioxide evolved was trapped in 1N KOH and determined by titration with 1N HCL. The quantity of CO2-C was divided by an efficiency factor of 0.41 to calculate microbial biomass C (Anderson, 1982; Haney, 2000).

Soil microbial biomass N was determined by analyzing NH4-N concentration of fumigated samples

following 10-d incubation period minus initial NH4-N prior fumigation, divided by an efficiency factor of 0.41. The NH4-N was extracted from 7 -g soil sample using 28 ml of 2M KCl. Samples were shaken for 30 min on a reciprocal shaker and filtered, and the extracts were analyzed for NH4-N by using an autoanalyzer.

Determination of clay and organic carbon content:

Clay, sand, silt, plus inorganic and organic carbon contents were determined by using standard methods reported elsewhere (Blake, 1965). Sampling plots were all areas from which the soil samples for microbial analyses were collected.

Adsorption studies

Adsorption will be expressed as k-Langmuir values, and the more knowledge as k-Freundlich

values. A stock solution of 40 ppm in distilled and de-ionized water will be prepared for atrazine and ametryne by using each of the soil types collected in the selected plot area mentioned. Concentrations o f 0.5, 1,5,10,20,40 ppm will be prepared by dilution with distilled and
de-ionized water. Batch equilibrium sorption isotherms will be obtained for each individual
pesticide (Liu, 1997). The laboratory tests will be conducted by treating 1 -g of air dried soil with 10 -ml of the selected compound solution in a sealed test tube. The suspensions will be protected
from light with aluminum paper, kept at 22° C, and placed on a shaker for 24 h. Then, they will
be centrifuged for 10 min at 2500 rpm and a 5 -ml aliquot of t he clear supernatant solution will be extracted with three successive 15 -ml portions of methylene chloride. The samples will be
concentrated, with a smooth flow of high purity nitrogen gas, diluted with hexane t o 5 ml, and
analyzed by using GC-FTD or GC-MS. The adsorption curves for pesticides and k-values will be
determined using the Langmuir model. Data generated will be compared with the bulk density, clay and organic carbon content of the surrounding soils close to the sampled wells. The significance of t he data will be statistically analyzed to find a relationship between these properties.

Principal Findings and Significance:

Human activities in the municipalities of Salinas-Guayama adjacent to a threatened natural reserve, have increased concern regarding environment. Agriculture, dumping, Junker businesses, excessive ground water demand are polluting the Jobos Estuaries Reserve. A study was conducted in this zone to determine pollutant in ground and interstitial waters. Four sampling zones were selected for interstitial water monitoring. The central and west zones (S2, S3 and S4) are intensely cultivated with banana, plantain, papaya, sorghum, sunflower, soybean and other agricultural commodities upstream. The south central zone (S2) is severely affected by mangrove death, and the southern area is on a salt flat zone (S3) (Figure 1). The northern central selected area (S4) was intensely cultivated in sorghum.

Table 3. Summary of results of wells and piezometers sampled for recognized and suspected human hazard contaminants

Chemical	CASRN	use or type	frequency (%)	
			well[a]	piezometer[b]
1-Tetradecanol (ALFOL 14)	112-72-1	hair tonics	16	9
Acetophenone	98-86-2	fragrance	1	19
(Capric acid) Decanoic acid	334-48-5	pesticide	3	3
(Coraza, Shield) Benzeneamine, N-phenyl	122-39-4	pesticide	3	8
Caffeine	58-08-2	stimulant	0	11
(Muscalure) 9-tricosene, (Z)-	27519-02-4	pesticide	1	1

methanone. diphenyl	119-61-9	comestic	3	0
camphor	76-22-2	analgesic	1	0
(E)-tricos-9-ene	35857-62-6	pesticide	0	1
phenanthrene	85-01-8	PAH	0	1
Phosphoric acid, tributyl ester	126-73-8	herbicide	1	6
1,2-benzenwdicarboxylic acid, butyl phenol	85-68-7	plasticizer	5	7
1,2-Benzenedicarboxilic acid, diethyl ester	84-66-2	plasticizer	2	1
Bisphenol A	80-05-7	plastic and printing	0	5
nonylphenol	25154-52-3	detergent	6	8
phenol,2-(1,1-dimethylethyl)-4methyl)	2409-55-4	---	1	0
Phenol,4-chloro-3-methyl	59-50-7	pesticide	0	1
Di(2-ethylhexyl)adipate	103-23-1	plasticizer	0	1
(P-Cresol) Phenol,4-methyl	106-44-5	Paint	1	0
(-lindane) D-BHC	319-84-6	pesticide	0	2
Ametryne	834-12-8	herbicide	0	1
Benzaldehyde, 4-hydroxy-3-methoxy	121-33-5	---	1	1
1,2-Benzenediol, 4-(1,1-dimethylethyl)	98-29-3	printing	1	4
1,3-Isobenzofurandione	85-44-9	rubber and paint	0	1
O-Phenylphenol	90-43-7	Bactericides	0	2
Benzophenone	119-61-9	hair product, ink	0	1
Anthraquinone	84-65-1	---	0	1
Monuron	150-68-5	herbicide	0	1
Methanone (2-hydroxy-4-ethoxyphenyl)phenone	131-57-7	paint and plastic	0	1
Benzeneamine, N- nitroso-N-phenyl	86-30-6	rubber	2	0
formamide	123-39-7	---	3	4
octyl phenol isomer	1806-26-4	---	2	2
Atrazine	1912-24-9	herbicide	1	2
Amphetamine	300-62-9	stimulant	0	2
PCP	87-86-5	pesticide	0	2

[a]well samples No. - 77
[b]piezometer samples No. - 172

Nitrate was detected at levels between 0.11 and 5.00 µg/mL only in one piezometer in the Aguirre north zone (S4), which is close to the sorghum growing area. Phosphorus was detected only in the Aguirre Forest sampling zone (S1) at levels between 0.5 and 30 µg/ml, over the environmental background level of 0.03 µg/ml. These levels pinpoint hot point sources of nitrate and phosphate near these piezometers. Lead, cooper and zinc were detected in some interstitial waters. Lead levels were between 0.11 and 1.54, over environmental background of 0.015 µg/ml; copper levels were between 0.03 and 0.11 µg/ml; and zinc levels were between 0.05 and 0.37 µg/ml (Table 4).

Table 4. Summary of inorganic contaminants found in interstitial water

statistical	Pb (µg/ml)	NH4 (µg/ml)	NO3 (µg/ml)	Cu (µg/ml)	Zn (µg/ml)	Fe (µg/ml)	PO4 (µg/ml)
mean	0.78	1.43	0.72	0.15	0.07	0.58	3.85
std dev.	0.49	1.47	1.09	0.17	0.06	0.52	6.61
maximum	1.69	4.95	3.75	0.58	0.20	1.74	20.00

The water pH profile of the piezometer sampling areas showed higher values in interstitial water of the S4 area ranging between 8.0 and 8.4, whereas the remaining sampling zones had values ranging between 7.3 and 8.0. Strong differences in the amount of dissolved oxygen (DO) in the interstitial water were observed among the four

selected sampling zones. No detectable levels of DO were observed in sampling site S1. However, the sampling sites S2 and S3 had levels between 23 and 66, and 10 and 39 mg/L, respectively. The higher levels of DO was observed in the sampling site S4 with levels between 70 and 82 mg/L. The low levels of DO in the S1 sampling area are in accord with higher levels of dissolved organic matter expected in this zone (a mangrove forest), because the organic matter decreases levels of available oxygen in water. The other sampling areas look like salt flat zones (S2 and S3) and agricultural lands (S4).
Regarding groundwater analysis, the major contaminant found in the monitoring wells in agricultural land was nitrate, but levels varies throughout the months, probably because of the variability in rainfall and fertilizer application. Besides, phosphorus was not detected in the sampled wells in agricultural lands, thus suggesting that its presence in piezometers from Aguirre Forest is from others sources. In general lead, cooper and zinc were not detected in the sampled wells. Atrazine, a s-triazine herbicide was detected (below 0.1 μg/L) in some monitoring wells, and traces of pesticides such as diphenyl amine (Coraza) and 4-chloro-3methyl phenol (4-chlor-m-cresol) were detected (Table 3).

Soil was analyzed to determine nutrient levels and other types of inorganic pollutants at each of the selected interstitial water sampling places (Table 5 and 6). Higher lead concentration was detected on soil in S3 (Las Mareas) than in the other sampling zones. This is a potential threat to human health, because this is a highly populated area and must be carefully monitored. A very low organic matter content was found in S2 (mangrove threat area) compared to that of S1 (Aguirre Forest). This fact joined with lower values of potassium, calcium, magnesium and a higher pH value than in the S1 area is indirect data to confirm the shifting of microbial fauna and flora that is taking place in the S2 area.

Soil microbial biomass is an indicator of problems long before natural vegetation is lost or human health problems occur. Laboratory tests were done to measure C and N microbial biomass in the four sampling zones. Aguirre Forest, an area not affected by death of mangroves, had higher C microbial biomass than the other sampling areas in Jobos Bay. This finding is accord with healthy mangrove growing. However, N soil microbial biomass was lower in Aguirre Forest than in the S2 area, a mangrove death zone. This finding suggests a potential ecosystem health threat (Table 5). To confirm these findings, additional analyses were done. The second set of analyses showed higher levels of C microbial biomass, and had a N microbial biomass profile different fro, that of the first. The first sampling had C and N microbial biomass between 120 and 190 cg kg^{-1} and between non detectable and 25 mg kg^{-1}, respectively; whereas the second period had between 330 and 370 cg Kg-1 and between 5 and 24 mg kg^{-1}, respectively. High spatial variability and climatological condition during the second sample collection different from those of the first period may be responsible for the differences between the first preliminary sampling and the second (Table 7). The low range of N microbial biomass, especially in S2 and S3 zones, is not a characteristic sign of a healthy soil.

Table 5. Soil analysis in places where interstitial waters were monitored.

Sampling Place1/	P (ug kg^{-1})	Ca (%)2/	K (%)2/	Mg (ug/g)2/	Pb (ug/g)3/	Cd (ug/g)3/	Cu (ug/g)2/	NH4 (ug/g)4/	NO3 (ug/g)4/
S1	139 (±77)	0.63 (±0.11)	0.21 (±0.06)	0.57 (±0.20)	1.5	nd	0.61	14 (±4)	nd
S2	42 (±14)	0.53 (±0.05)	0.20 (±0.02)	0.53 (±0.07)	nd	nd	___	20 (±4)	nd
S3	44 (±16)	0.50 (±0.11)	0.13 (±0.03)	0.27 (±0.08)	160	nd	0.12	17 (±7)	12 (±17)
S4	24 (±4)	0.62 (±0.03)	0.04 (±0.01)	0.14 (±0.03)	0.87	nd	0.17	20 (±9)	12 (±6)

1/Samples collected from at least three areas for each sampling place. Each area was replicated six time; 2/exchangeable cation (NH4OAc pH 7); 3/total; 4/exchangeable KCl 2N.

Table 6. Particle size analysis of soils in places where interstitial waters were monitored .

Sampling Place1/	pH	M.O. (%)	N (%)2/	Cu (ug/g)2/	Zn (ug/g)2/	Fe (%)2/	Silt (%)	Lime (%)	Clay (%)
S1	7.7 (±0.3)	10.7 (±4.0)	0.25 (±0.11)	70 (±2)	102 (±51)	30 (±3)	30 (±19)	57 (±13)	13 (±6)
S2	8.00 (±0.2)	14.0(±8.50)	0.31 (±0.11)	44(±4)	53 (±9)	22 (±1)	22 (±9)	58 (±7)	20 (±6)
S3	8.3 (±0.1)	2.3 (±1.3)	0.13 (±0.07)	73 (±8)	121 (±38)	45 (±8)	24 (±14)	63 (±24)	13 (±10)
S4	8.4 (±0.5)	3.3 (±0.6)	0.18 (±0.03)	76(±3)	88 (±2)	44 (±2)	36 (±8)	23 (±11)	41 (±8)

1/amples collected from at least three areas for each sampling place. Each area was replicated six time; 2/total

Table 7. Microbial biomass-C and N in the four selected sampling sites

Place	Second samples set	
	Average (cgCO2/Kg)	Average (mgNH4/kg)
S1	349 (±8)	14 (±13)
S2	370 (±40)	5 (±5)
S3	360 (±32)	8 (±4)
S4	330 (±13)	24 (±21)

ND-non detectable; s- standard deviation

Information Transfer Program

USGS Summer Intern Program

Student Support

Student Support					
Category	**Section 104 Base Grant**	**Section 104 RCGP Award**	**NIWR-USGS Internship**	**Supplemental Awards**	**Total**
Undergraduate	5	0	0	0	5
Masters	5	0	0	0	5
Ph.D.	0	0	0	0	0
Post-Doc.	0	0	0	0	0
Total	10	0	0	0	10

Notable Awards and Achievements

Publications from Prior Projects

1. 2000PR10B ("Dynamic Simulation of Water Distribution System with Instantaneous Water Demands") - Conference Proceedings - Silva-Araya, WF; Artiles León, N, Romero Ramírez, M; 2002; "Dynamic Simulation of Water Distribution Systems with Instantaneous demands",Proceddings of the Ninth International Conference on Hydraulic Information Management, WIT Press, Southampton, Boston, pp 379-387.