1.0

1.1

1.25

1.4

1.6

1.8

2.0

2.2

2.5

2.8

3.2

3.6

4.0

4.5

5.0

5.6

CONTENTS:

SUMMER UNDERGRADUATE RESEARCH PROGRAM: ENVIRONMENTAL STUDIES

The purpose of the summer undergraduate internship program for research in environmental studies is to provide an opportunity for well-qualified students to undertake an original research project as an apprentice to an active research scientist in basic environmental research. The students are offered research topics in the areas of toxicology, risk assessment, environmental microbiology and marine sciences. During the ten week period that the students are on campus, several seminars are presented by participating faculty and invited lecturers highlighting aspects of research in the areas of environmental science. At the end of their ten week internship the students are required to give an oral presentation of their research and to turn in a written report on the project. By undertaking an independent research project the students will gain an understanding and appreciation of the process whereby scientific theory is developed, tested, and documented.

The program was very fortunate in this, its year of inception, to have attracted the interest of many qualified applicants. Ten well-qualified students from throughout the midwestern and eastern areas of the country were accepted into the program. These students selected projects in the areas of marine sciences, biostatistics and epidemiology, and toxicology. The research experience for all these students and their mentors was very positive. The seminars were well-attended and the students showed their interest in the presentations and environmental sciences as a whole by presenting the speakers with thoughtful and intuitive questions. The oral presentations and written reports by the students were well done and were approached with enthusiasm. Several students have stated that the introduction to and experience in research they gained from this program has provided them with the option of pursuing research in environmental sciences as a career goal. This summer program has shown itself to be a valuable educational tool.

SUMMER UNDERGRADUATE RESEARCH PROGRAM: ENVIRONMENTAL STUDIES

Summer 1993

Student List

Student: **SaraJane Carlson**
College/University: University of Michigan
                       Ann Arbor, MI
Major: Biology
Mentor: Karen Burnett, Ph.D.
         Marine Biomedical and Environmental Sciences
Project Title: *Assays of immune response in the red drum, Sciaenops ocellatus, as tools for monitoring "environmental health" in estuarine systems.*

Student: **Kimberly Frampton**
College/University: Presbyterian College
                       Clinton, SC
Major: Biology
Mentor: Susan Morrison, Ph.D.
         Biology Department, University of Charleston
Project Title: *Fit to eat? The effects of microwaving on the bacterial counts in oysters.*

Student: **Jill Huntington**
College/University: State University of New York - Buffalo
                       Buffalo, NY
Major: Undeclaired
Mentor: Peter Moeller, Ph.D.
         National Marine Fisheries Service and
         Marine Biomedical and Environmental Sciences
Project Title: *Isolation and purification of marine biotoxins produced by a Caribbean strain of Gambierdiscus toxicus.*

Student: **Lisa Johnson**
College/University: Long Island University - Southampton
                       Southampton, NY
Major: Marine Science/Biology
Mentor: Donald DiBona, Ph.D.
         Marine Biomedical and Environmental Sciences
Project Title: *Sister chromatid exchange as an indicator of environmental stress.*

Student: **Rachel Levine**
College/University: Wesleyan University
                        Middletown, CT
Major: Earth Science/Environmental Science
Mentor: Donald DiBona, Ph.D.
                Marine Biomedical and Environmental Sciences
Project Title: *Use of diatom distributions to monitor environmental health.*

Student: **Katherine Maze**
College/University: Georgetown College
                        Georgetown, KY
Major: Biology/Environmental Science
Mentor: Greg Doucette, Ph.D.
                Marine Biotoxin Program, National Marine Fisheries Service and
                Marine Biomedical and Environmental Sciences
Project Title: *Bacterial production of PSP toxins.*

Student: **R. Blaine McClesky**
College/University: College of Charleston
                        Charleston, SC
Major: Biochemistry
Mentor: Fran Van Dolah, Ph.D.
                Marine Biotoxin Program, National Marine Fisheries Service and
                Marine Biomedical and Environmental Sciences
Project Title: *Growth inhibition in GH4C1 cells: effects of MTX and TGFα.*

Student: **Matthew Mitchell**
College/University: Cedarville College
                        Cedarville, OH
Major: Biology
Mentor: Daniel Lackland, Ph.D.
                Department of Biostatistcs, Epidemiology and System Sciences
Project Title: *An epidemiological study of leukemia in South Carolina:*

Student: **B. Charlotte Smith**
College/University: College of Charleston
                        Charleston, SC
Major: Applied Mathematics
Mentor: Zhen Zhang, Ph.D.
                Department of Biostatistics, Epidemiology and System Sciences
Project Title: *Pharmacokinetics - physiological vs mathematical, a systems science approach.*

Student: **Amy Weaver**
College/University: College of Charleston
                        Charleston, SC
Major: Mathematics
Mentor: JoEllyn McMillan, Ph.D.
                Department of Cell and Molecular Pharmacology and Experimental Therapeutics
Project Title: *Toluene toxicity: metabolism by isolated rat hepatocytes.*

SUMMER UNDERGRADUATE RESEARCH PROGRAM: ENVIRONMENTAL STUDIES

Summer 1993

Seminar Speakers

Dr. Fred Holland
Director
South Carolina Wildlife and Marine Resources Research Institute
Title: *Overview of Coastal Environmental Issues*

Dr. Michael Schmidt
Assistant Professor
Department of Microbiology and Immunology
Medical University of South Carolina
Title: *Environmental Microbiology*

Dr. Daniel Lackland
Assistant Professor
Department of Biostatistics, Epidemiology and Systems Science
Medical University of South Carolina
Title: *Epidemiological Assessments of Environmental Hazards*

Assays of Immune Response in the Red Drum, *Sciaenops ocellatus*, as Tools for Monitoring "Environmental Health" in Estuarine Systems.

Researcher:

        SaraJane V. Carlson
        University of Michigan
        Summer Undergraduate Research Program, 1993
        Medical University of South Carolina

Mentor:

        Karen G. Burnett, Ph.D.
        Associate Professor
        Department of Marine Biomedical and Environmental Sciences
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Assays of Immune Response in the Red Drum, *Sciaenops ocellatus*, as Tools for Monitoring "Environmental Health" in Estuarine Systems.

INTRODUCTION

The quality of a habitat greatly impacts the health of the organisms which are found within it. Animal health itself can be measured in many ways, and the selection of an assay which might be diagnostic for habitat quality must be validated for this particular application. Teleost fish have a sophisticated immune system similar to that of other vertebrates which may be sufficiently sensitive to reflect environmental quality. In one early study, Goncharov and Mikryakov (1970) found a correlation between the concentrations of phenols and antibody production in carp and subsequently proposed that the immune systems of fish might be useful in monitoring the levels of toxins in their environment. Additional studies have shown immunosuppression of the humoral responses of fish due to other pollutants such as polycyclic aromatic hydrocarbons (Spitsbergen et al., 1986: Payne and Fancey, 1989.) In light of these data, it may be possible to determine the environmental health of an area by monitoring immune responses of the indigenous fish.

Fish normally carry and are exposed to an array of bacteria with which they live commensally (Bullock and Snieszko, 1969). Healthy, immunocompetent fish might be expected to produce humoral responses against their co-occurring bacteria. The present studies utilized *Sciaenops ocellatus*, commonly known as the red drum or spot-tail bass, which is a teleost fish native to the estuaries and near shore waters of the Southeastern United States. Two populations of red drum, one of apparently healthy and one of suspected unhealthy animals were sampled in an attempt to determine whether the health of individuals could actually be monitored by assays for humoral response against indigenous bacteria.

Materials and Methods:

Experimental Sites: Our study focused on two estuarine systems, the first in Charleston Harbor (Grice Cove), Charleston, South Carolina, and the second in Winyah Bay (Mother Norton Shoals), Georgetown, South Carolina. These fish populations were selected based on previous animal health observations by the staff of the South

Carolina Wildlife and Marine Resources Department during January and February, 1993. These workers noted generally healthy individuals in the Grice Cove area, while almost half of the individuals in Winyah Bay displayed open reddish sores on their flanks. For the present study, specimens were collected by stop net on May 10, 1993 and June 17, 1993 from Grice Cove, as well as by trammel net from Winyah Bay on May 19, 1993. In an additional netting attempt on July 14, 1993, no red drum were captured.

Specimen Collection: After capturing fish, we visually assessed the health of each individual and coded them according to the criteria in Table 1. About 4 ml of blood was obtained from all captured red drum of "2" or "3" health and from approximately ten fish of "1" health during each collection. The blood was placed in non-activated clot tubes and left on ice for at least 30 minutes before centrifugation at 1800 rpm for 10 minutes. The separated sera was aliquotted into microcentrifuge tubes, then stored at -20°C. We also swabbed the flanks of some red drum to collect mucosal slime with which we inoculated Trypticase Soy Agar (TSA) plates to promote growth of the harbored bacteria. The plates were incubated at 25°C for up to 72 hours.

Isolation and Identification of Bacteria: We picked isolated colonies of bacteria from the inoculated TSA plates to re-isolate them on TSA. Each originally picked colony was re-plated on TSA two to four times to ensure isolation. We distinguished isolates by morphological observations and labeled each with a type number. Backup vials of each isolate were stored in a freezing solution of 10% glycerol in phosphate buffered saline (PBS) by washing cultures from TSA slants after 48 hour growth and storing those suspensions at -70°C. We stored working slants of each isolate at 4°C. For use in identifying the bacteria, we gram stained all bacteria, then tested each isolate using O/F Dextrose tubed media, motility media, tubed, Triple Sugar Iron slants, and novobiocine disks which we obtained through BBL and using Pseudomonas F agar obtained from Difco Laboratories. Each isolate was also tested using selected biochemical tests in the Becton and Dickinson Minitech system for differentiation of bacteria in addition to cytochrome oxidase and indole spot test reagents. Some isolates (t1, 9, 12, 16, 17, 18, 19) were also tested on the api 20E System. Both systems were tested for equivalent results at temperatures of 25°C and 37°C.

Bacterial Antigen Preparation: To prepare antigens for use in ELISAs (enzyme-linked immunosorbent assays) we inoculated 5 ml of Trypticase Soy Broth (TSB) in a culture tube and incubated them at room temperature (RT) with agitation. After growth to maximal densities (between 8 to 36 hours depending on the growth rate of individual isolates) we inoculated 100 ml culture bottles containing 50 ml of TSB with 100ul or 250ul (volume was dependent on growth rate) of the 5 ml cultures. We incubated the 50 ml cultures at RT for 18 hours, or until they reached an optical density of 0.3 or greater at 660 nm. We then transferred the cultures and centrifuged them at 3500 rpm for 20 minutes. (Some variation in centrifugation occurred due to use of different centrifuges and because some species such as t7, were unable to be recovered from the supernatant after such high speeds of centrifugation.) We washed the bacteria twice by resuspending them in PBS and centrifuging at 3500 rpm for 20 minutes. After the second wash we resuspended the bacteria to an O.D. of between 0.25 and 0.34 to correspond to a density of about 5×10^8 cells/ml, as estimated using the McFarland Standard no. 0.5. We heated-killed the standardized bacterin preparations in a boiling water bath for 30 minutes, then froze 5 ml aliquots of these bacterin preparations at   -20°C.

ELISAs: ELISAs were run using the standard method with the following modification of volumes. We coated the wells by adding 50ul of the bacterins and letting the plates dry overnight in a 37°C incubator. We blocked the plates for 2 to 4 hours with Blotto Blocker in PBS obtained from Pierce which was also used for all subsequent antigen dilutions. Between coats, we washed the plate three times using PBS containing 0.1% Tween. For general screening we used the collected sera as the primary antigen at a 1:30 dilution (50 ul), the mouse-anti-red drum monoclonal antibody (previously developed in our laboratory) for the secondary antigen at a 1:5000 dilution (100ul), and a 1:500 dilution of goat-anti-mouse horseradish peroxidase conjugate (HRP) as the tertiary antigen (100 ul.) The color was developed using the Peroxidase Substrate Kit ABTS obtained from Bio-Rad, then the plates were read on a Molecular Devices spectrophotometer at 405 nm. We ran blanks for each antigen (1° coat Blotto Blocker) and subtracted these values from the appropriate wells. We ran positive controls to ensure working procedures by coating some wells with HSA/DNP and using serum from an HSA/DNP immunized fish which showed previous reactivity as the primary antigen. All other steps in the assay were conserved in the control wells.

<u>Results</u>:

Identification of Bacteria: All isolates were gram-negative short rods as observed through magnification of the gram-stained slides under oil-immersion . An identification scheme for bacterial fish pathogens and additional identification charts provided the basis for identifying the bacterial isolates (MacFaddin, 1980, Austin, B. and D. A. Austin, 1987). The Minitech and api 20E differentiation systems, both developed for use with human pathogens and designed for incubation at 37°C, showed varying results at the 25°C and 37°C incubations, therefore diminishing the reliability of the results. It was noted in the bacterial profiles of the estuaries, that Pseudomonas species were evident in Winyah Bay, but no Aeromonas species were found. In contrast, some Aeromonas species, but no Pseudomonas species were isolated from Grice Cove.

Serum Reactivities: The antibody reactivity profiles generated by the ELISA showed a striking difference between the immune responses of the two populations of fish. The optical densities of each serum reactivity against all isolates are listed in Table 3. In analyzing these data it is apparent that the "1" rated animals from Grice Cove show a wide range of reactivities against the isolates from Grice Cove (Figure 1) and Winyah Bay (Figure 2,) as well as against those from the recirculating tanks (not shown.) In the ELISAs, most sera from the Grice Cove red drum generated O.D.s greater than 0.500. In contrast, the sera from Winyah Bay red drum generated very low (< 0.500) O.D. readings.

It appears that the immune responses elicited by each bacterium varies greatly. Those species which mounted the greatest responses were the Aeromonads and Pseudomonads (excepting Pseudomonas fluorescens.) In general the sera all seem to show the lowest immune responses to the Enterobacter species.

A comparison of serum responses of the "1" and "2" rated fish from Grice Cove (Figure 3) shows no apparent differences in the magnitude of reactivities, or in the pattern of responses across the entire panel of bacteria. In making the same comparison on Winyah Bay animals (Figure 4), once again the pattern and magnitude of the ELISA responses between the "1," "2" and "3" health categories were not strikingly different. But, when comparing Figures 4 and 5, the difference between antibody responses in the two populations are visible.

Discussion:

The fish health assessment data does indicate that there may be a higher incidence of fish with external physical damage in the population at Winyah Bay, although, due to the small number of specimens captured in this area we are unable to make conclusions on the comparative visual health of the populations in these estuaries. From comparison of the immune responses measured in the different health categories within each population (Figures 3 and 4), we are able to infer that simple visual assessments are not sensitive indicators of the immunocompetence of a fish, and so it follows, would not be sensitive tools for the assessment of the environmental health of estuaries.

From these primary screenings of sera, it seems that the information from the ELISAs that could be most useful in indicating the health of individuals may be the magnitude of the optical densities when tested against a variety of bacterins. There is a wide range of individual responses even between two apparently healthy animals from the same area, as shown by the profiles of two animals from Grice Cove in Figure 5. The variable responses to a single bacteria across a population of healthy fish may be due to the degree of individual exposure to that bacteria. Even an immunocompetent fish will not show antibodies against bacteria that have not been introduced into their immune system, unless that bacteria has similar epitopes to another species which the fish has seen, causing cross-reactivity of an antibody. However, Figure 5 also suggests that it may be possible to select a panel of bacteria against which most immunocompetent animals show significant ELISA reactivity. It is interesting to note that Winyah Bay animals show a similarly high variance in response when ELISA readings are plotted on a log scale. This is evidence that the Winyah Bay animals may be eliciting very small responses to bacteria they are exposed to, but that because of their probable immunosuppression, their responses are very small. Alternatively, it is possible that this variation is due to reduced sensitivity of the ELISA at low levels of antibody.

For further use of the ELISA as a diagnostic tool in assessing animal and environmental health, it may be necessary to study animals with defined immunocompetence and immunosuppression. This data could be used to establish an optical density as a "cut-off" point between a true humoral response and background signals due

to the physical insensitivities of the assay. In previous studies involving purified protein antigens, 0.100 O.D. has been the "cut-off" point, above which animals were considered to show antibody response to the antigen. In the current study, though, it is not known how the bacterial antigens non-specifically react and affect the background signals.

Overall, the data show that populations of apparently healthy red drum are capable of mounting humoral immune responses against indigenous bacteria. The population of suspected unhealthy red drum studied failed to show humoral immune responses to these bacteria, leading to the conclusion that assays for the immune responses of the red drum may be useful indicators of environmental health. Additional work must be done to narrow the panel of bacteria for further screening, creating one to which most immunocompetent fish will show at least one antibody response and which will be useful in testing additional estuaries and microhabitats within the previously studied estuaries. It is also necessary to continue sampling at time points throughout the year in order to monitor possible seasonal fluctuations in antibody response due to temperature variations.

In the process of identification of the bacteria, the Minitech and api 20E systems showed variable responses at the 25°C and 37°C incubations. This may be due to the suppressed or inhibited growth of the fish bacteria at the higher temperature. Variable results in replicates run at 25°C in the Minitech system indicate that this system may be unreliable when used for bacteria with maximum growth rates at 25°C. It is possible, though, that this variance is due to differences in techniques or true variability in the biochemical reactions within the strain of bacteria. Duplicates were not tested in the api 20E system, so further tests need to be run to test this system for reliability and reproducibility at 25°C.

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Payne, J.F. and L. F. Fancey. 1989. Effect of polycyclic aromatic hydrocarbons on immune responses in fish: change in melanomacrophage centers in flounder (Pseudopleuronectes americanus) exposed to hydrocarbon-contaminated sediments. Mar. Environ. Res. 28: 431-35.
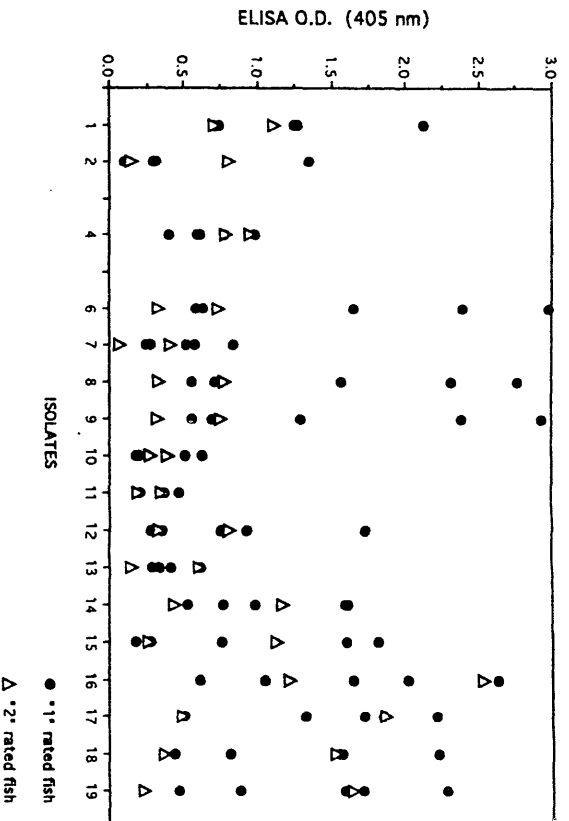
ELISA O.D. (405 nm)

GRICE COVE ISOLATES

Figure 1. The ELISA optical densities of "1" rated fish sera from Winyah Bay and Grice Cove (5/19/93 collection) against bacterial antigens from Grice Cove.

△ Winyah Bay
(5/10/93)

▲ Grice Cove
(5/19/93)

ELISA O.D. (405 nm)

WINYAH BAY ISOLATES

Figure 2. The ELISA optical densities of "1" rated fish sera from Winyah Bay and Grice Cove (5/19/93 collection) against bacterial antigens from Winyah Bay.

○ Winyah Bay
(5/10/93)

● Grice Cove
(5/19/93)

Figure 3. The ELISA optical densities of selected fish sera from Grice Cove (5/ 9/93 collection) against all bacterins. Data was plotted for specimen numbers 39964, 39965, 39967, 35144, 36199, 08645, and 08537.
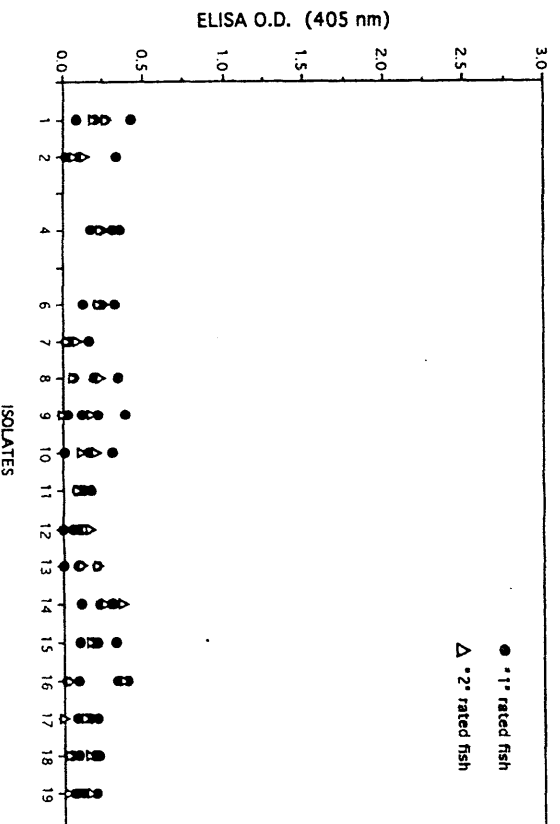
Figure 4. The ELISA optical densities of selected fish sera (5/10/93 collection) against all bacterins. Data was plotted for specimen numbers 36581, 39462, 41506, 41507, 41508 and 39962.
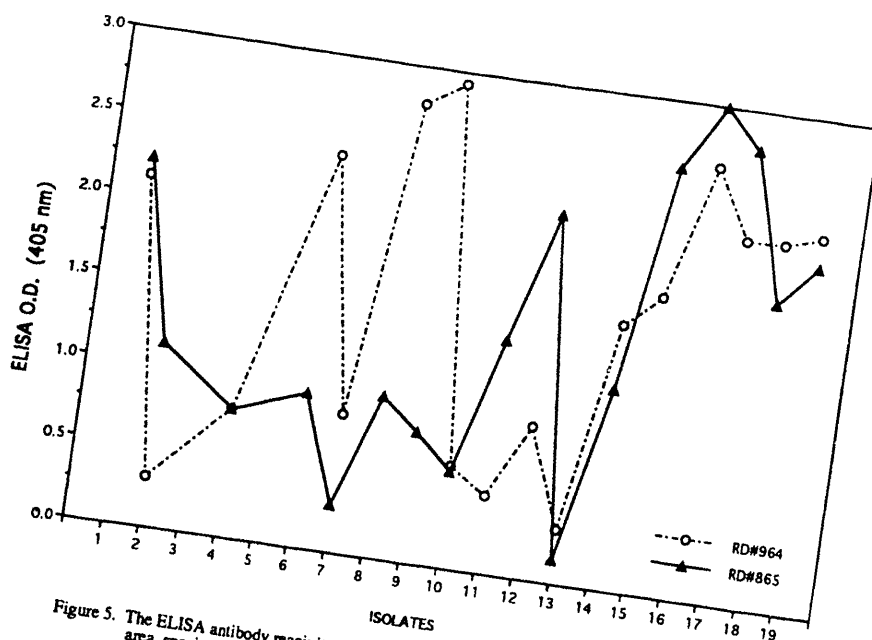
Figure 5. The ELISA antibody reacivity profiles of two apparently healthy red drum from the Grice Cove area, specimen numbers 39964 and 06865.

Table 1. Visual health assessment of feral red drum. Health categories were defined as follows: 1 = no external lesions or damage, 2 = some undefined external lesions or parasite, 3 = open reddish sore, 4 = hemorrhagic lesion (none found).

Health Code	Grice Cove (5/19/93)	Grice Cove (6/17/93)	Winyah Bay (5/10/93)
"1"	36/41	27/30	9/15
"2"	5/41	3/30	5/15
"3"	0/41	0/30	1/15

Table 2. Identifications of bacteria isolated and identified from both estuaries and from fish in mariculture tanks.

Bacterial Identification	Grice Cove	Winyah Bay	Mariculture
Acinetobacter calcoaceticus		t7	
Aeromonas hydrophila	t16		
Aeromonas salmonicida			t1
Aeromonas spp.	t17, t18, t19		
Edwardsiella spp.	t15		
Enterobacter spp.	t13	t11	
Flavobacterium spp.	t14		t4
Pasteurella piscidia	t12		
Pseudomonas fluorescens		t10	t2
Pseudomonas spp.		t6, t8	
unidentified		t9	

Table 3. Individual red drum specimens and their lengths, health code, and ELISA O.D. of sera tested against all bacterial isolates. *indicates that the ELISA 3° antibody used was 1:360 concentration instead of 1:500, so these data are not directly comparable to other O.D.s.

Collection	Specimen	Health	Length	Serum Reactivity against Isolates (ELISA O.D.)																
	(tag #)	Code	(mm)	t1	t2	t4	t6	t7	t8	t9	t10	t11	t12	t13	t14	t15	t16	t17	t18	t19
Winyah Bay	41501	1	405	0.45	0.2	0.21	0.34	0.05	0.07	0.08	0.29	0.12	0.08	0.17	0.24	0.12	0.14	0.06	0.06	0.04
5/10/93	39960	1	680	0.33	0.13	0.35	0.18	0.08	0.12	0.06	0.19	0.15	0.13	0.14	0.35	0.45	0.13	0.05	0.05	0.02
1st netting	36581	1	562	0.26	0.34	0.36	0.25	0.04	0.06	0.03	0.31	0.13	0.15	0.21	0.31	0.21	0.34	0.14	0.05	0.06
	39959	2	597	0.21	0.08	0.14	0.14	0.03	0.1	0.02	0.13	0.14	0.08	0.17	0.23	0.16	0.19	0.04	0.03	0.02
	41502	1	410	0.33	0.1	0.3	0.32	0.04	0.16	0.12	0.3	0.47	0.13	0.2	0.37	0.14	0.21	0.06	0.09	0.11
Winyah Bay	39462	1	688	0.2	0.1	0.31	0.33	0.03	0.19	0.22	0.17	0.08	0.1	0.1	0.3	0.33	0.09	0.16	0.22	0.08
5/10/93	39961	1	743																	
2nd netting	41503	2	325	0.23	0.1	0.27	0.18	0.06	0.1	0.08	0.17	0.3	0.08	0.14	0.3	0.3	0.13	0.09	0.12	0.18
	41504	2	371																	
	41505	1	396	0.18	0.08	0.11	0.2	0.12	0.41	0.31	0.1	0.04	0.04	0.05	0.15	0.05	0.1	0.09	0.14	0.05
	41506	1	447	0.43	0.11	0.23	0.22	0.16	0.21	0.12	0.16	0.11	0.06	0.09	0.23	0.18	0.4	0.21	0.19	0.2
	39962	2	549	0.27	0.14	0.26	0.26	0.09	0.24	0.18	0.21	0.11	0.15	0.22	0.26	0.21	0.38	0.14	0.16	0.17
	39963	3	591	0.36	0.17	0.25	0.28	0.02	0.09	0.03	0.28	0.14	0.09	0.14	0.22	0.32	0.13	0.11	0.08	0.05
	41507	2	382	0.19	0.07	0.24	0.23	0.03	0.06	0	0.12	0.09	0.17	0.12	0.37	0.17	0.04	0	0.04	0.03
	41508*	1	310	0.08	0.02	0.17	0.13	0.02	0.35	0.39	0	0.17	0	0	0.11	0.1	0.02	0.08	0.09	0.12
Grice Cove	39964*	1	582	2.12	0.31	0.78	2.39	0.84	2.77	2.93	0.62	0.47	0.93	0.33	1.61	1.81	2.63	2.21	2.22	2.28
5/19/93	6865*	1	688	2.22	1.12	0.78	0.94	0.29	0.98	0.8	0.59	1.42	2.22	0.15	1.22	2.6	3	2.76	1.86	2.11
	36203	1	564																	
	8537*	2	577	1.11	0.81	0.95	0.74	0.41	0.78	0.75	0.39	0.35	0.81	0.6	1.17	1.13	2.53	1.87	1.53	1.65
	39965	1	768	0.74	0.29	0.61	0.58	0.57	0.71	0.69	0.19	0.2	0.35	0.28	0.77	0.76	1.05	1.32	0.82	0.89
	35144	1	391	1.27	0.1	0.59	1.65	0.52	2.31	2.38	0.51	0.47	0.75	0.41	1.59	1.6	2.02	1.72	1.57	1.71
	33417	1	562	0.62	0.69	0.72	0.65	0.82	1.6	1.5	0.31	0.47	0.51	0.67	0.91	0.68	0.91	0.95	0.69	0.85
	34765	1	421	0.77	0.4	0.78	0.38	0.27	1.03	1.01	0.25	0.38	0.19	0.34	0.69	0.26	0.65	0.95	0.52	0.49
	36199	1	380	0.71	0.13	0.4	0.63	0.27	0.56	0.56	0.18	0.37	0.27	0.61	0.53	0.27	0.61	0.51	0.44	0.47
	8645	1	620	1.25	1.34	0.98	2.98	0.24	1.56	1.29	0.25	0.34	1.72	0.28	0.98	0.18	1.65	1.85	1.55	1.59
	39966*	1	610	1.34	0.36	0.63	1.86	0.6	0.42	0.48	0.19	0.33	0.25	0.47	0.72	0.5	1.77	2.65	0.55	0.98
	39967	2	696	0.71	0.15	0.79	0.33	0.07	0.33	0.32	0.27	0.19	0.33	0.15	0.44	0.26	1.22	0.5	0.37	0.23
Grice Cove	41618	1	430	1.12	0.07	0.53	1.16	0.34	1.35	1.31	0.3	0.58	0.74	0.32	0.91	0.63	0.96	1.9	1.11	0.97
6/17/93	41619	1	419	0.49	0.18	1.69	0.66	0.22	0.82	0.79	0.62	0.66	0.41	0.25	0.74	1.64	0.86	0.58	0.83	0.7
	41620	1	432	1.07	0.32	1.25	1.29	0.11	1.31	1.62	0.99	1.26	2.02	0.5	1.22	0.94	1.57	1.65	1.41	1.55
	41621	1	410	0.67	0.11	0.63	0.32	0.93	0.73	0.55	0.26	0.47	0.72	0.76	0.28	0.45	0.48	0.84	0.53	0.57
	8719	1	662	1.35	0.42	0.58	0.62	0.2	1.36	1.23	0.25	0.29	0.46	0.39	1.12	3.6	1.72	1.94	1.06	1.12
	39759	1	738	2.1	1.13	0.82	0.67	0.27	0.84	0.86	0.81	0.7	0.73	0.56	0.92	0.63	2.11	2	1.71	1.8
	32940	1	577	1.12	0.63	0.89	0.71	0.55	1.33	1.21	0.66	0.52	0.79	0.81	0.81	0.7	1.68	1.41	0.85	0.91
	41626	2	475	0.79	0.15	1.01	0.3	0.18	0.57	0.63	0.46	0.26	0.17	0.26	0.15	0.18	0.56	0.39	0.23	0.29
	33385	1	608	1.51	1	0.47	0.43	0.13	1.43	1.21	0.39	0.3	0.27	0.34	0.8	0.37	1.92	1.69	0.77	0.69
	39966*	2	611	1.49	1.26	0.84	2.98	0.63	1.09	1.12	0.21	0.55	0.42	0.42	0.85	0.64	3.83	3.85	1.48	2.33
	39970	2	747	1.26	0.32	1.04	1.38	0.37	1.97	2.08	0.71	0.5	0.76	0.49	0.8	0.5	1.97	2.14	1.12	1.25
	unknown	1		1.07	0.18	0.6	0.58	0.25	0.8	0.65	0.3	0.29	0.42	0.35	0.72	0.39	0.88	1.27	0.74	0.59

Fit To Eat?
The Effects of Microwaving on the
Bacterial Counts in Oysters

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Abstract

The effects of microwaving on the bacterial load in oysters were examined to determine the reliability of this cooking procedure as a means of rendering oysters safe for human consumption. Oysters were microwaved in the shell for 0,3,4,6,and 8 minutes, diluted, and blended before being plated in a dilution series from 10^0 to 10^{-6}. The general trend was a decrease in the number of viable bacteria, measured as colony forming units per gram of oyster tissue. However, consistent patterns did not occur due to a number of complicating factors.

Introduction

In the Lowcountry of South Carolina, a new culinary trend is appearing. People have begun microwaving oysters as a means of cooking them, or at least heating them and loosening the shells. This practice brings up the question of the reliability of this method in making the oysters safe to eat. The aim of this project is to prove or disprove that microwave cooking effectively reduces the bacterial load of the oyster to a level that is safe for human consumption while maintaining a desired level of edibility. If microwave cooking kills bacteria in oysters, then there will be fewer colony forming units per unit of oyster tissue following microwaving as determined by standard plate count procedures.

Studies on how microwaves heat foods have shown that the ability of the microwave to effectively cook the food depends on certain characteristics of the food itself. Moisture, salt content, and the physicochemical characteristics of the food affect the penetration depth of the microwave. Nonuniform heating rates can be caused by "spatial field intensity variations within the oven" (3). A study done on the survival of Salmonella species in various foods heated in the microwave found that sodium chloride was a significant factor in bacterial survival, leading to the conclusion that the presence of sodium chloride in food results in a lack of uniform heating during microwave cooking and an increased chance of survival by bacteria (3).

Through general conversation, it was discovered that many people microwave oysters as a means of cooking them. An interview with one person yielded more specific information. Loy Harn, a local businessman, has been microwaving oysters for many years. He piles nearly 2 dozen on a plate with a small amount of water under them to produce steam, then cooks them for 6 minutes. His aim is to crack open the shells just enough to make them easy to shuck and get the meat hot without drying it out. Harn commented that he has never gotten sick from this practice (2).

While some people eat oysters raw, it is not a safe practice. Oysters are filter feeders and easily accumulate microorganisms from their environment. These microorganisms can include pathogenic bacteria and viruses. It has been established that shellfish can carry bacteria such as <u>Vibrio cholerae</u>, <u>Vibrio vulnificus</u>, and <u>Salmonella typhi</u> as well as viruses such as hepatitis A, Norwalk virus, and a group of viruses referred to as "small round viruses" (1,4,7). Shellfish often collect these pathogens "through their ability to concentrate microorganisms from their environmental sea water" (4).

Previous studies done by Dr. Susan Morrison of the College of Charleston have shown that batches of oysters with artificially high bacterial loads are affected by microwave cooking. The starting load of between 10^7 and 10^9 showed a 2 log reduction after 5 minutes and a 4 log reduction after 10 minutes. The bacterial count was reduced, but the count overall still remained high. This may have been due to the artificially high

initial load. Therefore, it is hypothesized that microwaving oysters with naturally occurring bacterial loads would reduce the number of bacteria to negligible levels.

Materials and Methods

The procedures in this study were done using aseptic techniques. Handling and shucking of the oysters were done using techniques outlined in Laboratory Procedures For the Examination of Seawater and Shellfish, 5th ed (5). When the oysters were not being used or were between steps, they were kept in a refrigerator at 4°C. A flowchart of the procedure is depicted in Figure 1.

The oysters were bought from a reputable commercial seafood store. They were divided into batches of 8 to 11, depending on size and overall number available. The oysters were then scrubbed under running tap water until all loose dirt and debris was removed.

The batches were cooked for 0, 3, 4, 6, or 8 minutes at power level 6 in a General Electric Model JE1465G, 700 watt microwave on a mechanical turntable, immediately shucked, and placed in sterile jars. One at a time, the batches were weighed and diluted 1:1 with sterile phosphate buffer with magnesium chloride (5). The mixture was blended in a blender for 90 seconds, with 2 ten second pauses to aid in consistent mixing and to avoid further heating of the sample.

Immediately following blending, the oyster "soup" was measured out in a dilution series as diagrammed in Figure 2. Each sample was plated in duplicate or triplicate. The plates were inoculated with the appropriate dilution of the specimen. Melted standard plate count agar (Difco Laboratories, Detroit,

MI) was poured in and the plate gently swirled to attain even spreading of the inoculum. Once the agar solidified, an additional overlay of the same agar was added to the top to prevent overgrowth of colonies near the surface of the agar. Upon complete solidification, the plates were transferred to a walk-in incubator at 37°C and placed in an inverted position to prevent condensation from dropping on the surface of the agar.

After 48 hours, the plates were inspected for bacterial growth. A Quebec colony counter was used to count the number of colonies in each plate. The results from the series of plates with counts within the range of 30 to 300 colonies were then averaged and multiplied by the dilution power to attain the number of colony forming units per gram (cfu/g) of oyster meat.

Subcultures were done of some of the colonies present in the plates. Using standard procedures, various colonies were streaked onto solidified plate count agar (Difco Laboratories, Detroit, MI). From the subcultures, Gram stains were done. A few of the cultures were subsequently transferred to trypticase soy agar slants (BBL Microbiology Systems, Cockeysville, MD) and placed in a refrigerator at 4°C to keep for further study.

Results and Discussion

Observations of the oyster meat were noted during the
shucking procedure, based on personal judgement. The edibility
of the meat was judged by taking into account the temperature,
moistness, and texture of the meat after cooking. The results
are listed in Table 1.

The uncooked batches were all cold and very moist. The
three minute batches were slightly warm to the touch and moist,
with few of the shells popped open. The four minute batches were
generally hot to the touch, moist, and easy to open but not
popped. On average, the six minute batches were hot to the
touch, with some shells popped open and the meat rubbery. The
ones that remained closed were generally moist but not as soft as
the less cooked batches. The eight minute batches were very hot,
with most of the shells popped open and the meat very dry and
rubbery.

The data from the experiments are shown in Figures 3 - 10.
Log analysis of the data was also done, but did not significantly
change the pattern of the results and is therefore not presented.

Experiment A (Figure 3) showed a marked decrease in the
cfu/g in the batch cooked for 4 minutes, followed by a slight
increase at 6 minutes. The 8 minute batch increased to a level
nearly equal to the baseline count. The decrease at 4 minutes
was expected, but the results for the 6 and 8 minute batches were
not. Explanations for this are discussed later.

Experiment B (Figure 4) produced very different data from

Experiment A. The baseline count was much lower, and the cfu/g increased at 4 minutes instead of decreasing. The lower baseline count is most likely due to the variability in the bacterial load from oyster to oyster and batch to batch. This would also explain why the 4 minute batch had a higher number of cfu/g. Batches cooked for 6 and 8 minutes showed the expected decrease in the bacterial count.

The oysters used for experiment C (Figure 5) had a very low initial bacterial load, making any changes in that level difficult to see. There were more cfu/g of oyster tissue in the 8 minute batch. This could be due to the high variability among oysters or water weight loss, which are discussed later.

Experiment D (Figure 6) also involved oysters with low initial loads. There was little variability from batch to batch, but a small decrease is seen, with the exception of the 8 minute batch, as seen previously.

The final experiment was a double batch experiment. Two batches of oysters were processed for each time. The data produced curves that illustrate the gradual decrease in bacterial load in connection with longer cooking time (Figures 7 and 8). The decline correlates fairly well with the increasing length of microwaving time. This data supports the hypothesis that there will be fewer cfu/g following microwaving.

The results of this study were extremely variable. The baseline counts from the different experiments ranged from 2.6 x 10^3 cfu/g to 1.2 x 10^6 cfu/g, as seen in Figure 9, thus making

comparison of the results of the cooked batches difficult. A
comparison of all the data is presented in Figure 10. The line
represents the average trend for the experiments. While it does
show a slight decrease in the number of cfu/g of oyster meat in
conjunction with longer cooking time, the decrease is not as
sharp as expected. In more than one experiment, batches of
cooked oysters had higher counts than the uncooked batch. There
are several possible explanations for this phenomenon.

The most basic problem is the amount of variability in the
bacterial load of each individual oyster. In a study of the
bacterial accumulation by the Gulf Coast oyster, Kelly et al.
noted broad fluctuations in the coliform MPN [Most Probable
Number] of oysters that did not appear to be related to any
changes in the bacterial content of the surrounding water (6).
Sample sizes of at least eight oysters were used to diffuse this
effect somewhat. Another difficulty was judging how much the
shell affected heating. Often, the size of the shell belied its
actual thickness and the size of the oyster within. Batches with
more oysters or thicker shells tended to not get as hot as those
with less shell. Inconsistent heating by the microwave could
also have been caused by the presence of moisture and sodium
chloride in the oysters, thus increasing the variability of the
survival rates of the bacteria they contained (3).

In all but one of the experiments, the batch cooked for
eight minutes had higher counts than the batches cooked for less
time. One problem with the longer cooked oysters was their

rubbery consistency. The toughness of the oyster meat resulted in inconsistent mixing in the blender. The dry oyster meat also caused a discrepancy when comparing the grams of oyster meat to the number of bacteria. In the less cooked batches, water weight in the oyster was taken into account while in the eight minute oysters, the water evaporated, possibly leaving a higher concentration of bacteria per gram of oyster tissue. This resulted in a higher number of cfu/g of oyster tissue.

When Gram stains were done of some of the bacterial colonies subcultured from the original plates, many Gram positive and Gram negative rods were seen. There is a possibility that some of these rods are spore formers and are relatively unaffected by heat. This could help account for not seeing a total elimination of bacteria in the oysters.

Conclusion

While some of the data does show a decrease in the bacterial counts with cooking (Figures 6-8), other data does not (Figures 3-5). This discrepancy is most likely due to the high variability of bacterial counts among oysters, combined with the relatively low bacterial load, which may serve to exaggerate the actual difference between the batches. Water weight loss in the longer cooked batches is another complicating factor. When the overall trend of the experiments is graphed, an average decline in cfu/g can be seen (Figure 10), but bacteria remain present in all of the batches. The question that persists is: does microwave cooking kill the harmful bacteria found in raw oysters? If the bacteria that remain are not pathogens, the bacterial load that is left after microwaving may be safe for human consumption. Further study should be conducted in this area. Perhaps an experiment can be designed where the oysters are first treated with a pathogen, cooked, and then analyzed for survival of the target pathogen. Another approach would be to quantify the bacteria in another fashion. One strategy would be to analyze the bacterial load per gram of protein to reduce the effects of moisture on the results. While this will not completely eliminate the complicating factor of water weight loss in the longer cooked batches, it should decrease the impact. The results of this study do not decisively show that microwave cooking is a procedure that will reduce the bacterial loads of raw oysters to a level that is safe for human consumption.

Acknowledgements

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<u>Table 1</u>

Edibility Observations

Minutes Cooked	General Temperature	Meat Characteristics	Shell Condition
0	cold	very moist, soft	closed
3	warm	moist, soft	closed
4	hot	moist, soft	most closed
6	hot	moist, slightly tough	some closed, some open
8	hot	dry, rubbery	almost all open

<u>Figure 1</u>

Gulf Coast Oysters obtained
from local seafood store

$\downarrow$

Oysters scrubbed with sterile brush under
running tap water to remove dirt and debris

$\downarrow$

Oysters microwaved on clean paper plate
on mechanical turntable

$\downarrow$

Cooked and raw oysters aseptically shucked
Meat placed in sterile jars
Shell sizes measured
Edibility characteristics noted

$\downarrow$

Oyster meat weighed and diluted 1:1 with phosphate buffer
Mixture blended for 90 seconds

$\downarrow$

Serial dilution prepared
Samples plated out in duplicate or triplicate

$\downarrow$

Pour plates prepared with
melted standard plate count agar
Overlays poured with same type agar

$\downarrow$

Plates inverted and incubated at 37°C for 48 hours

$\downarrow$

Plates containing 30-300 colonies counted
Average of counts of duplicate or triplicate plates calculated
to give Colony Forming Units (CFU) per gram of oyster meat

Figure 2

Figure 3: Oyster Experiment A

CFU/G (Thousands) vs. Minutes Cooked
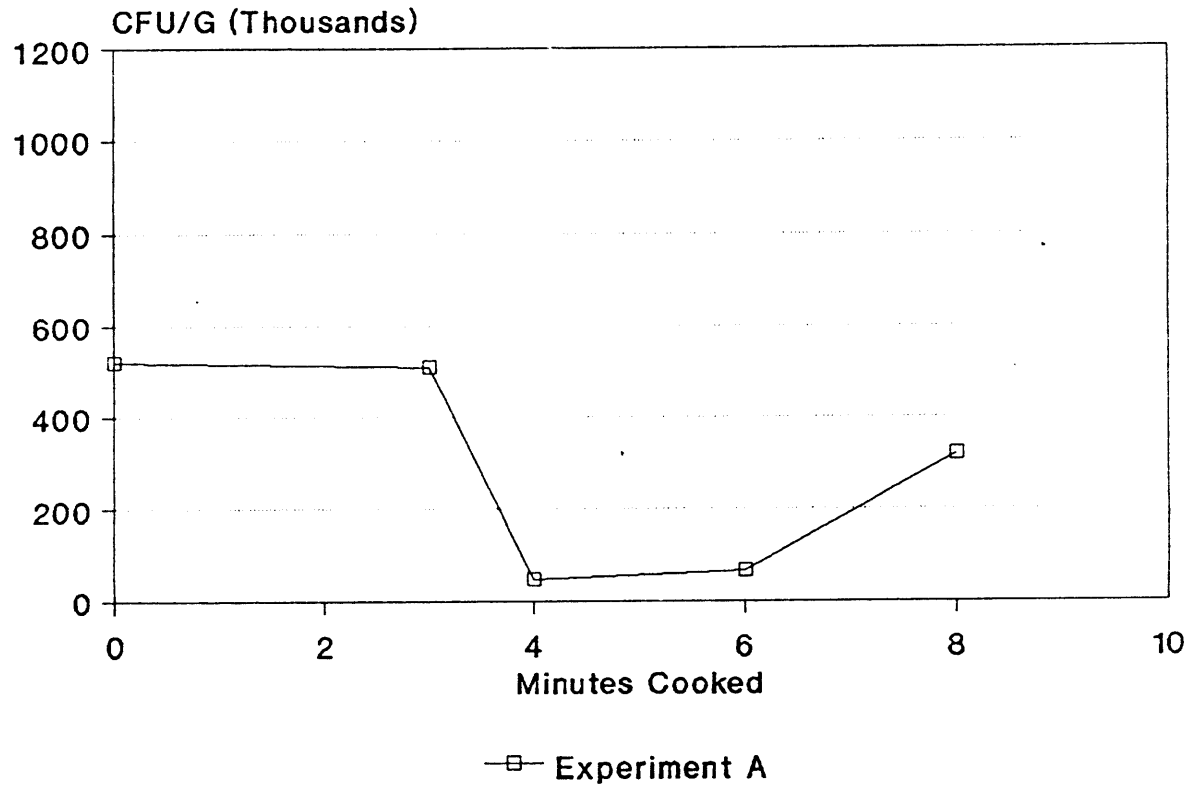
—□— Experiment A

Figure 4: Oyster Experiment B
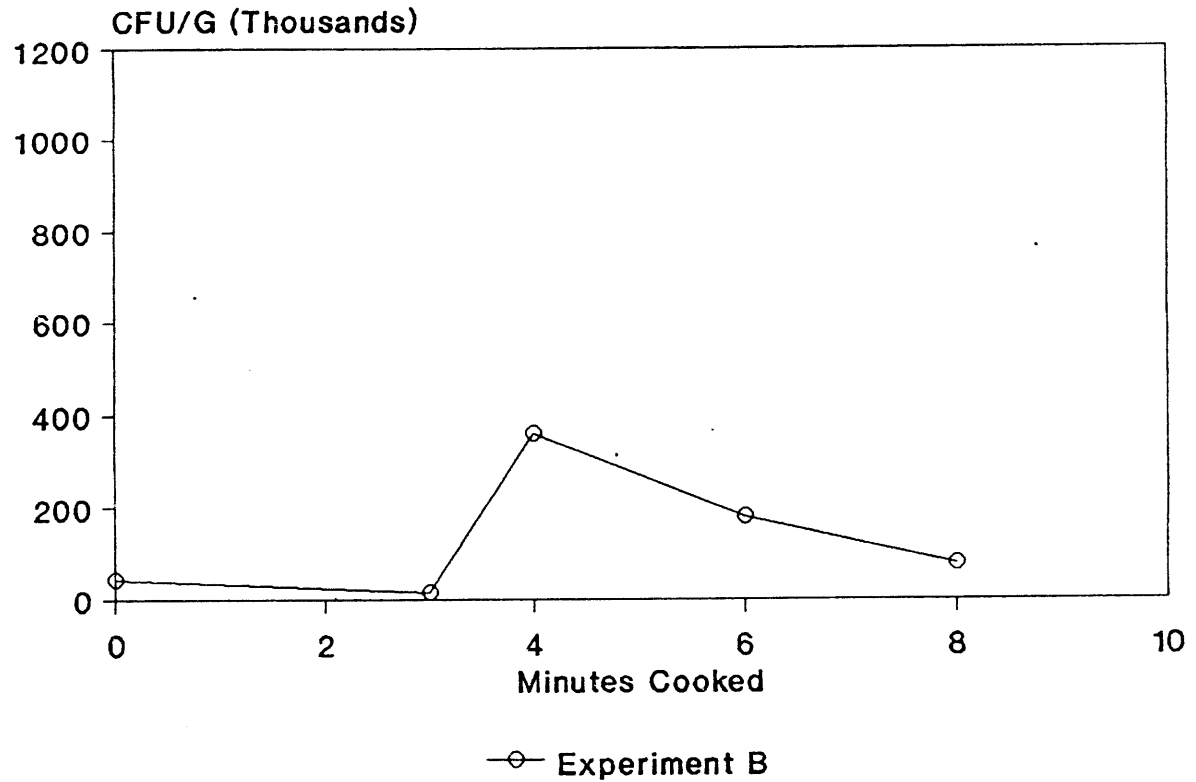
Figure 5: Oyster Experiment C

CFU/G (Thousands) vs Minutes Cooked
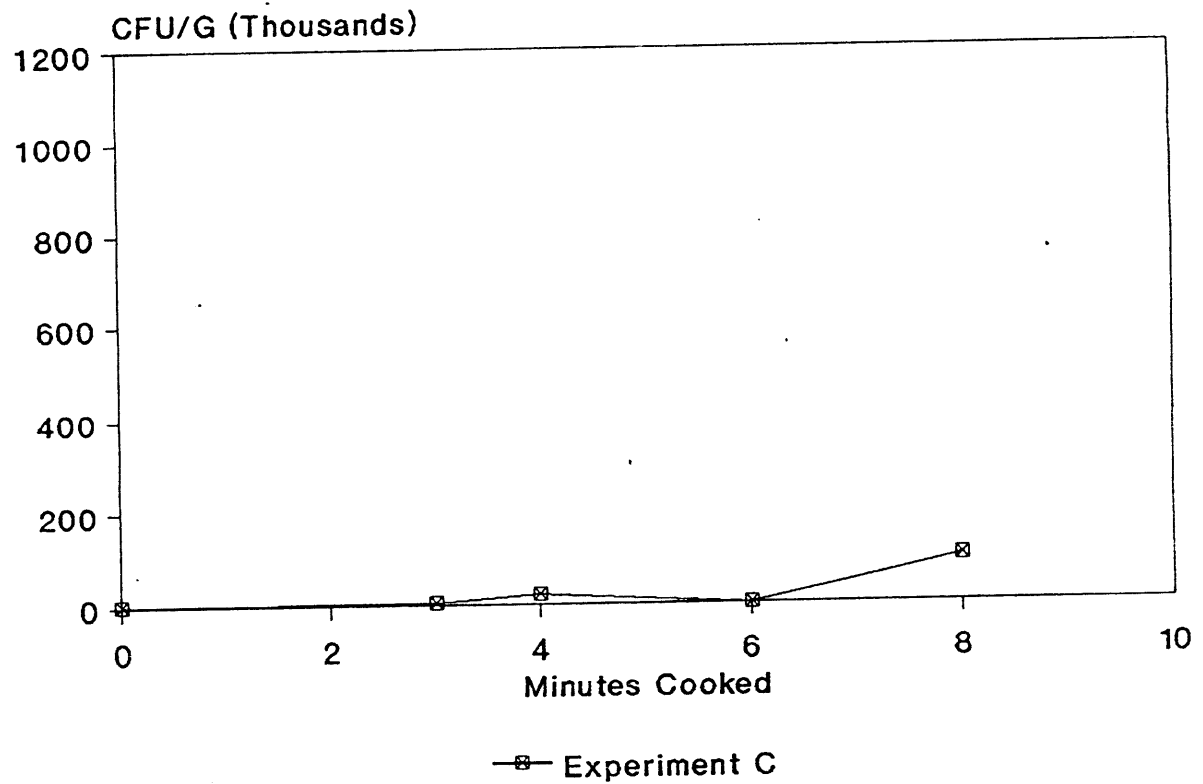
—⊠— Experiment C

Figure 6: Oyster Experiment D

CFU/G (Thousands) vs Minutes Cooked

Legend: ◇ Experiment D
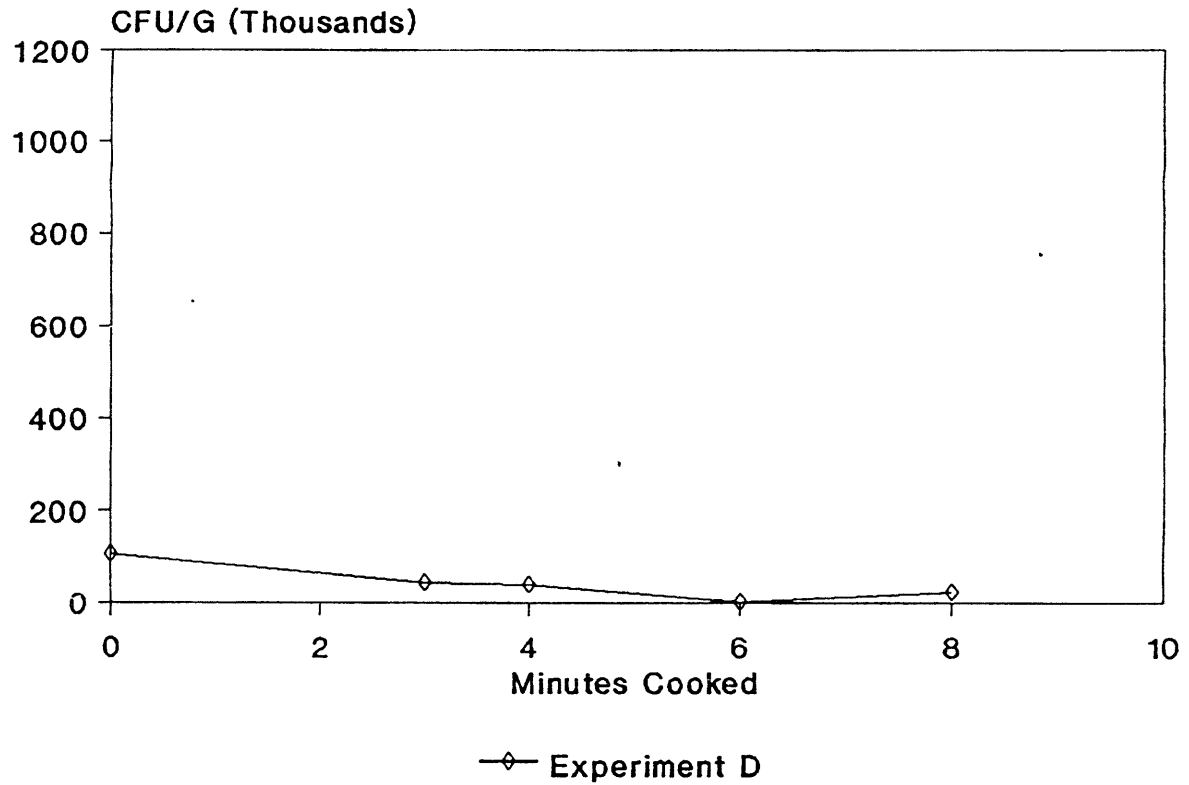
Figure 7: Oyster Experiment E-1

CFU/G (Thousands) vs Minutes Cooked
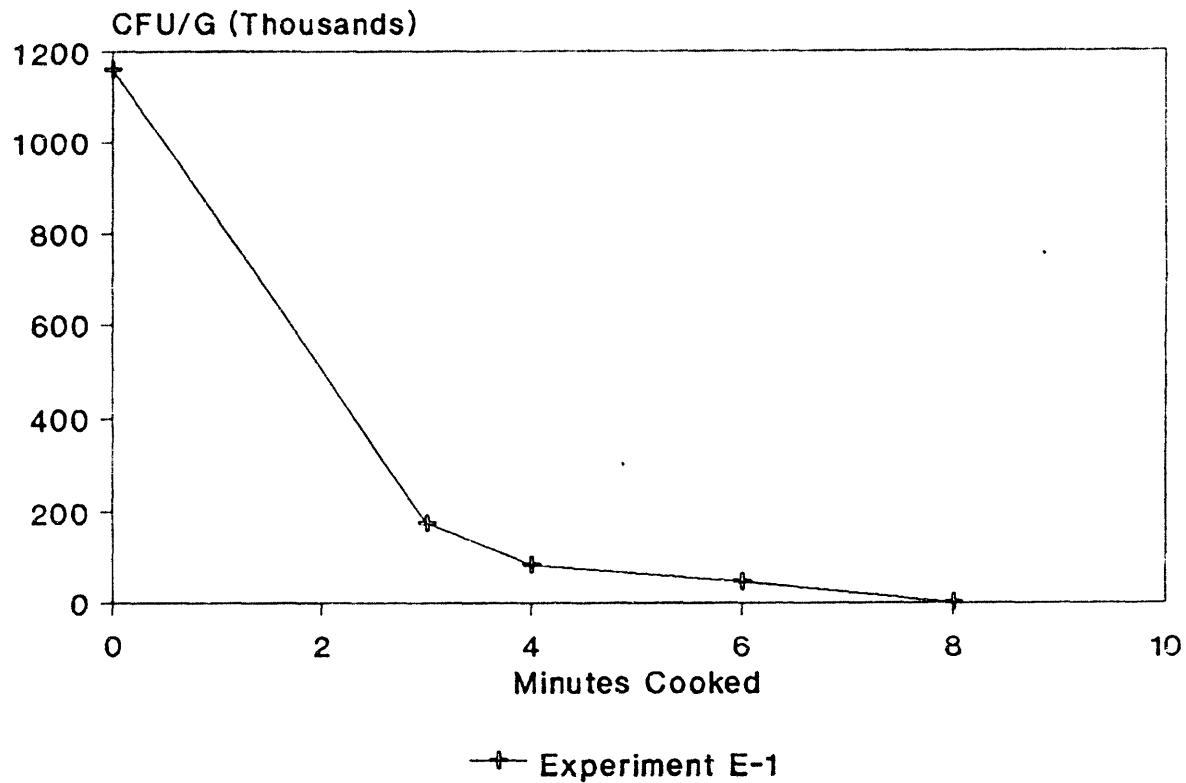
— Experiment E-1

Figure 8: Oyster Experiment E-2

CFU/G (Thousands)

Minutes Cooked
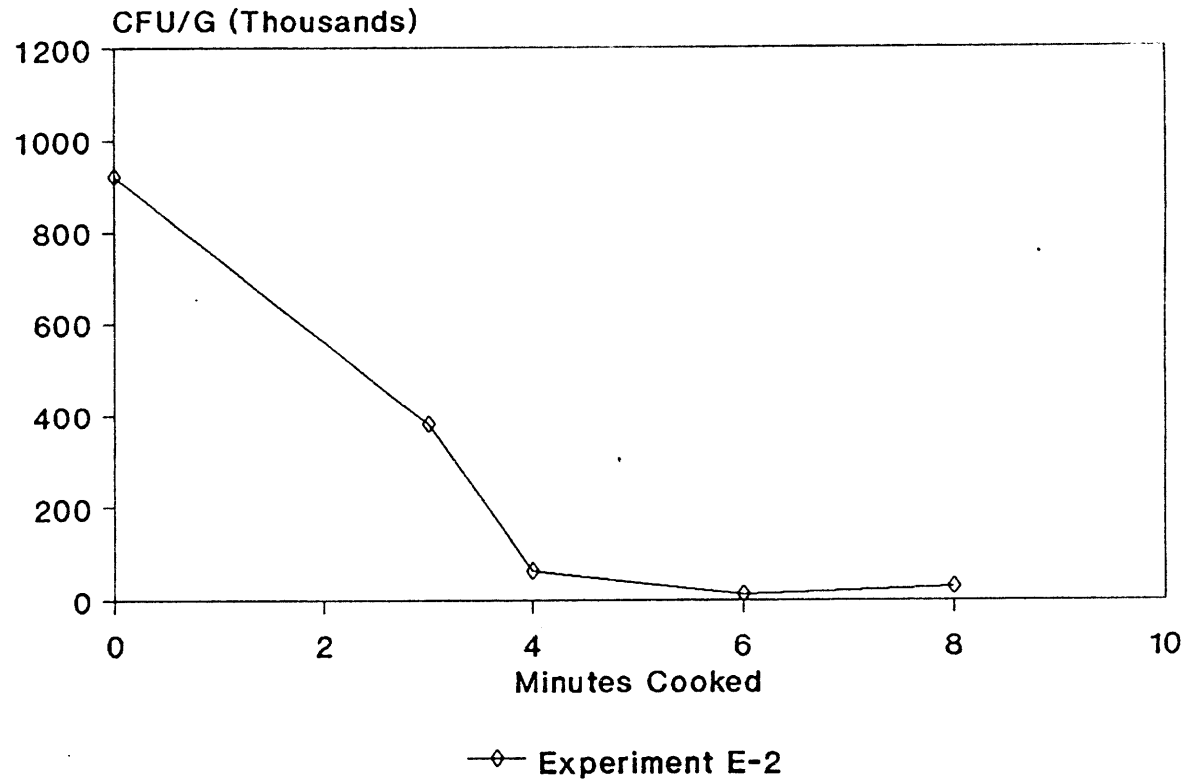
—◇— Experiment E-2

Figure 9: Baseline Counts

CFU/G (Thousands)

Bacterial Counts at 0 Minutes
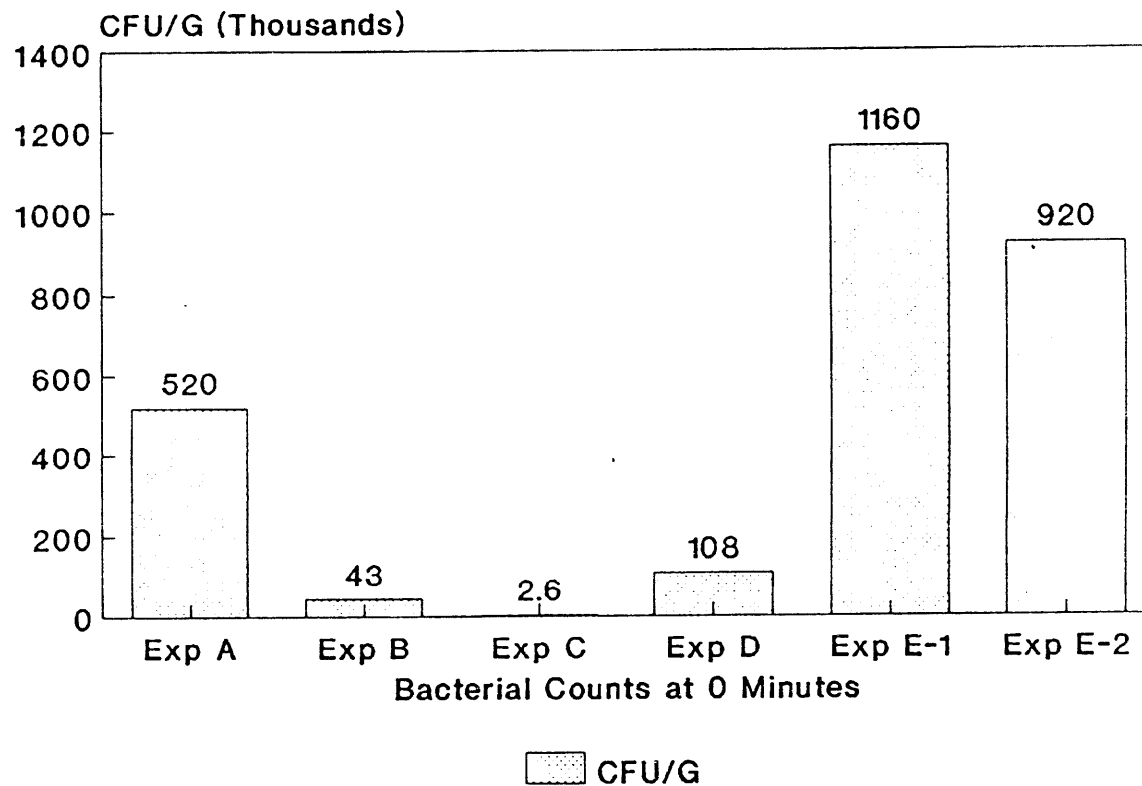
Figure 10: Average Bacterial Count
A Comparison of Selected Experiments
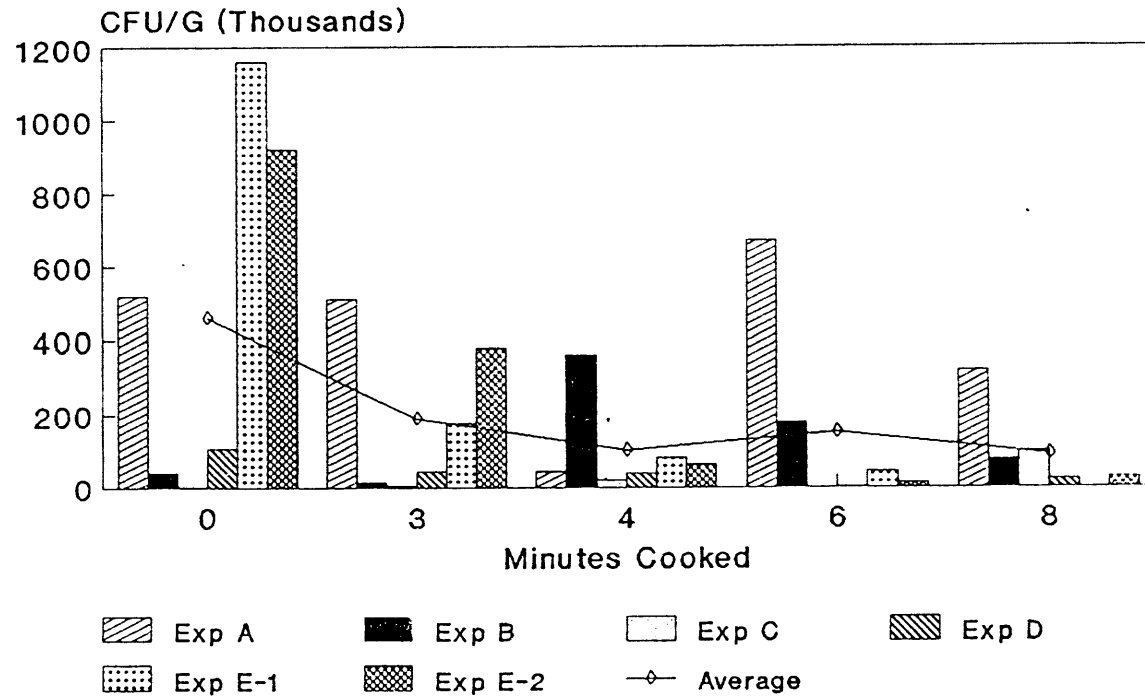
Kimberly A. Frampton
Fit To Eat?

Isolation and Purification of Marine Biotoxins Produced by a Caribbean Strain of Gambierdiscus toxicus.

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Abstract: A large scale, preparative isolation and purification protocol has been developed for maitotoxin (MTX) and ciguatoxic(CTX) congeners that were extracted from a Caribbean strain of _Gambierdiscus toxicus_. These two toxins have both been implicated in Ciguatera poisoning. The subsequent purification of a moderately polar family of maitotoxin, designated MTX-B, is described.

Introduction: Maitotoxin is a very potent marine biotoxin and is one toxin responsible for ciguatera seafood poisoning that results from eating contaminated reef fish. MTX was first isolated from the viscera of surgeon fishes, _Ctenochaetus striatus_ [1] and is believed to be produced, initially, by the marine dinoflagellate, _Gambierdiscus toxicus_ [2]. We have been working with a Caribbean strain of _G. toxicus_, isolated from the Martinique Islands, and propogated by mass culture. These cultures produce CTX-congeners, as well as highly polar and moderately polar families of MTX.

Maitotoxins, in general, are a valuable pharmacological tool because of their biological activity to activate voltage dependent calcium channels[3,4]. However, the important chemical features, such as molecular weight and chemical structure are only known for a highly polar MTX from a Pacific strain of _G. toxicus._ The molecular weight of this Pacific MTX is 3424 Da (as a disodium salt)[5], and its chemical structure (Fig.1) has only been recently elucidated, making it the largest known natural product that has been fully characterized[6]. This information if not yet available for the moderately polar MTX-B, or for any other Caribbean MTX.

Currently, there is a shortage of analytical samples of Caribbean strain MTX, thereby making further pharmacological evaluation and structural elucidation difficult. What we attempted to do was take MTX-B and develop a protocol to isolate the toxin and then take a simplified and

more direct approach to a preparative scale purification scheme that would circumvent the problems other research groups have encountered. Our purpose was to purify MTX-B to the point of obtaining an analytical sample, while in the process, to retain the maximum amount of toxic material free from degradation and/or oxidation.

Experimental Design: Before beginning the actual isolation procedure (Fig.2), we suspended a known, frozen wet-weight of cultured *G. toxicus* in 100% MeOH. The dinoflagellates were sonicated to ensure the maximum release of toxins with the use of a high intensity ultrasonic processor (GCA/Precision Scientific) for 10-12min per 5-7g.

The MTX-B sample that was used to determine the remainder of this protocol, was isolated using a C.!8 reverse phase chromotography column. The column was packed in 100% H_2O and the solvents began at 40% MeOH/H_2O and increased stepwise to 100% MeOH. At this point, the majority of the toxins had eluded off the column, and we were able to separate them into three families; CTX-congeners, MTX-B, and the highly polar MTX, based on their polarities and where they eluded off the column. The column was then washed with portions of ethyl acetate (EtAc) and chloroform (CCl₃) to remove any residual toxic material.

We have since begun using a more novel approach to toxin isolation with the Soxhlet extraction. This, however, is not the isolation method used for the MTX-B that was used in determining the purification protocol.

We began the Soxhlet extraction by applying the crude, sonicated sample onto a cellulose thimble and freeze-drying it, before using the standard Soxhlet apparatus and allowing 500mL of each solvent to reflux and condense over the thimble. The solvent scheme included nine solvents starting with a non-polar solvent, with each of the following solvents increasing in polarity. It began with hexane, followed by benzene, petroleum ether, ethyl acetate, acetone, acetonitrile, 2-propanol, methanol, and ending with water. One fraction per solvent was collected and submitted for cytotoxicity determination.

Toxicity of the collected fractions was determined by the CellTiter 96™ Non-Radioactive Cell Proliferation/Cytotoxicity Assay (Fig.3). Once cytotoxicity was determined, we were able to classify the toxins into families of CTX-congeners or MTX. This knowledge was obtained by using sodium channel activation and calcium influx[7,8] bioassays that were developed and used in-house. The maitotoxins were identified by a positive calcium influx assay, and CTX-congeners, known sodium channel activators, showed a positive result for that bioassay.

Our preparative purification protocol for MTX-B involved use of size-exclusion chromatography and HPLC (Fig.4). Purification began with size-exclusion chromatography using LH20 Sephadex packing (Pharmacia) and a column 200cm in length and 1cm in width. Run #1 used 45:45:10 MeOH:EtAc:H_2O. Toxic fractions were again identified by cytotoxicity bioassay. For run #2, we loaded toxic fractions from run #1 onto column using the same packing with 100% MeOH. Toxic fractions were again identified by cytotoxicity bioassay.

Our next step was HPLC, using ISCO ChemResearch, model 2350. We chose a semi-preparative (250mm x 10mm) silica column (Alltech, Econosil Silica 10u) with a linear gradient, dual pump, and single detector. Wavelength was set at 260nm, sensitivity at 1.0, and rise time at .80. Solvent B was 100% HPLC-grade ethyl acetate; solvent A was 100% HPLC-grade methanol. Injections were all 2.0mL.
Eluent scheme:

DUAL PUMP, LINEAR GRADIENT
NUMBER OF SEGMENTS: 4

SEGMENT #	TIME (MIN)	FLOW RATE (ML/MIN)	%SOLVENT [B]
0			100
1	15	5	100
2	52	5	10
3	75	5	10
4	85	5	100

We are currently in the process of developing the final puritive step to obtain an analytically pure toxin sample.

Results and Discussion: After the sonication of _G. toxicus_, approximately 85-90% of the cells were shattered and we felt a maximum amount of toxin had been released into solution. The results of the C.18 allowed us to form three toxic groups of CTX-congeners, MTX-B, and highly polar MTX. We found that the highly polar MTX would elude off the column first in 40% MeOH/H_2O, followed by MTX-B, in 70-90% MeOH/H_2O. CTX-congeners would elude off last in 100% MeOH. We were able to begin washing the column after this point, because we knew that the majority of the toxins had come off the column. During the wash, we did receive a few residual toxic fractions, but their quantities were small, and they were less potent.

The Soxhlet extraction procedure, however, has proven to be a more productive method of toxin isolation as was determined by the bioassay results that indicated very clear separations (Fig.5). In general, the toxin fractions off the Soxhlet extraction were purer than those received off the C.18, in that there was a more defined separation of toxins, and each fraction contained fewer pigments.

The Soxhlet extraction gave us two cytotoxic fractions with the non-polar solvents, hexane and benzene, that were also sodium channel active, indicating the presence of CTX-congeners. We also found two cytotoxic and calcium influx positive fractions with both the moderately polar solvents (ethyl acetate, acetone) and highly polar solvents (isopropyl alcohol, methanol, (water)). The fact that these fractions were calcium bioassay positive suggested the presence of maitotoxins. We focused the rest of our studies on the moderately polar toxic fractions.

Run #1 with the size-exclusion chromatography did little else but remove some pigments from the toxic fraction. Run #2, however, immediately separated into three distinct bands of color, and we collected one fraction that was both cytotoxic and calcium channel active.

We took the toxic fraction and applied it to the HPLC, using the eluent scheme described above. Fig.6 is a representative chromatogram for all HPLC injections with this particular sample of MTX-B. After again using the cytotoxicity bioassay, we determined that we had obtained two toxic fractions (highlighted areas along bottom axis of Fig.6), whose retention times remained consistent throughout all injections. We have arbitrarily labeled these toxic fractions B_1 and B_2, with their retention times 16-22 min and 36-75 min, respectively. We then took B_1 and B_2, and reinjected them, each individually, back onto the HPLC, following the same eluent scheme, with the intention of repurifying them, and again received two toxic fractions (Fig.7) with the same retention times of B_1 and B_2. To ensure that B_1 was not simply a carryover from a previous injection, we injected only solvent onto the column and ran a blank run, and after that, flushed the column with 300mL 100% MeOH before repurifying each B_1 and B_2. We verified these results by injecting the B_2 fraction obtained during the repurification of the original B_1. Again we obtained B_1 and B_2. What these results indicate is that an equilibrium process exists between B_1 and B_2 that is based on a conformational or configurational change of the MTX-B molecule. We are particularly interested in these results because such an equilibrium process has already been described for ciguatoxin[9].

The research into this matter is currently ongoing, but if we

continue to receive B_1 and B_2 during our verification process, then we will be able to eliminate any doubt that the MTX-B we are working with is anything other than a single entity, indicating that the analytical sample is only one or two steps away.

Summary: In the pursuit of obtaining analytically pure samples of Caribbean strain MTX and CTX-congeners we have developed preparative isolation and purification techniques that have brought us close to our goal.   Also, while in the process, we have indicated an apparent equilibrium process between isomers of MTX-B, that may be a clue to the relational biosynthetic origins of both CTX and MTX-B.

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Fragment C

Fragment A

Fragment B
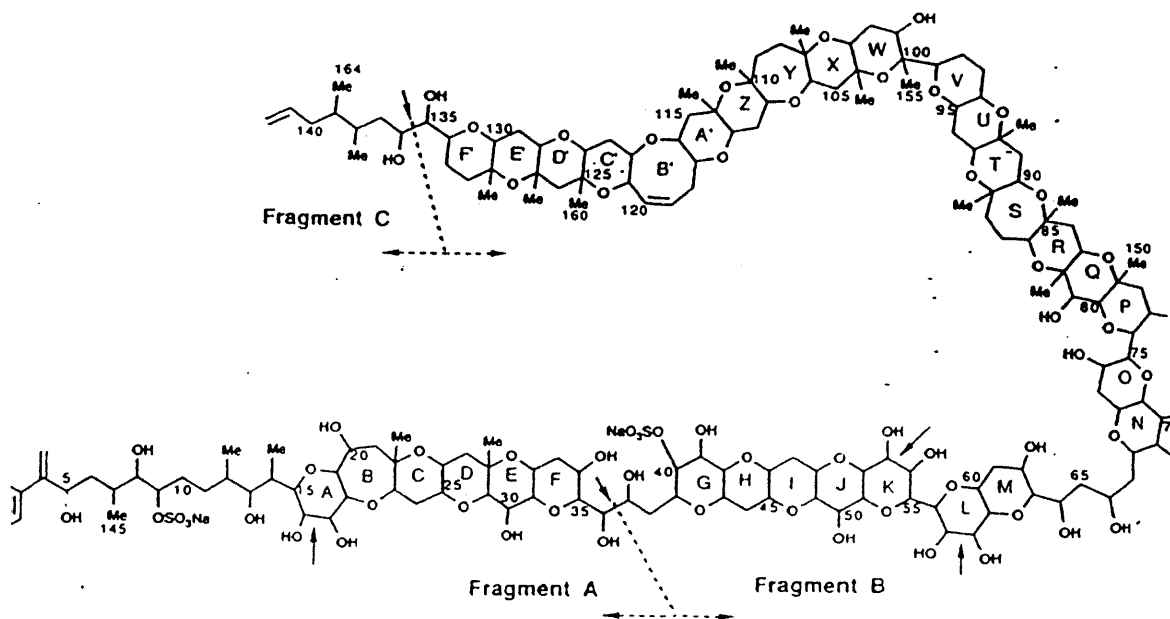
rows denote cleavage sites by periodate.

FIGURE.1 Structure of a Pacific strain of MTX.

ISOLATION

Gambierdiscus toxicus

↓

Sonication

```
          ┌──────────────┴──────────────┐
          │                             ↓
          │                      Freeze-dry
          │                             ↓
          ↓                          Soxhlet
        C.18                      
   ↙     ↓     ↘              ↙      ↓      ↘
CTX*  MTX-B  MTX-1⁺      CTX*   MTX-B   MTX-1⁺
```

C.18

CTX*   MTX-B   MTX-1$^+$

Soxhlet

CTX*   MTX-B   MTX-1$^+$

Bioassay

CTX*   MTX-B   MTX-1$^+$

MTX-B
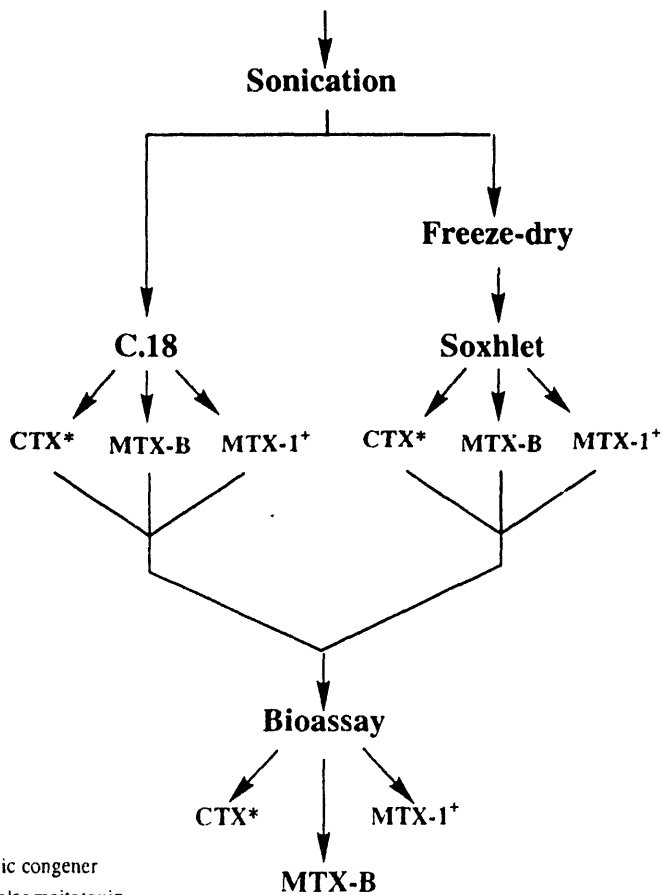
* Denotes a ciguatoxic congener
$^+$ Denotes a highly polar maitotoxin

FIGURE. 2. The isolation procedure including an alternative method.

CellTiter 96™ Non-Radioactive Cell
Proliferation/Cytotoxicity Assay
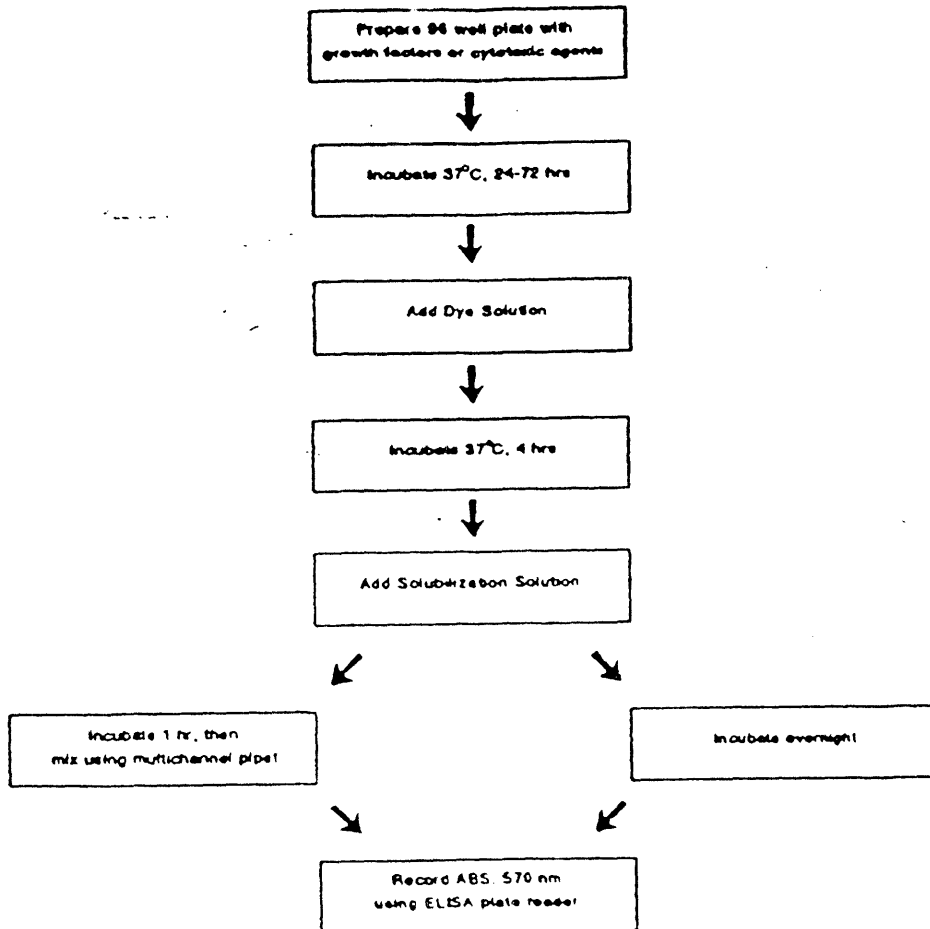
FIGURE.3 Standard protocol for cytotoxicity bioassay (Promega).

PURIFICATION

MTX-B

↓

Size-exclusion
LH20
(2X'S)

↓

Bioassay

↓

MTX-B

↓

HPLC
Silica

↓

Bioassay

↓

MTX-B

↓

In development

↓

Analytical sample
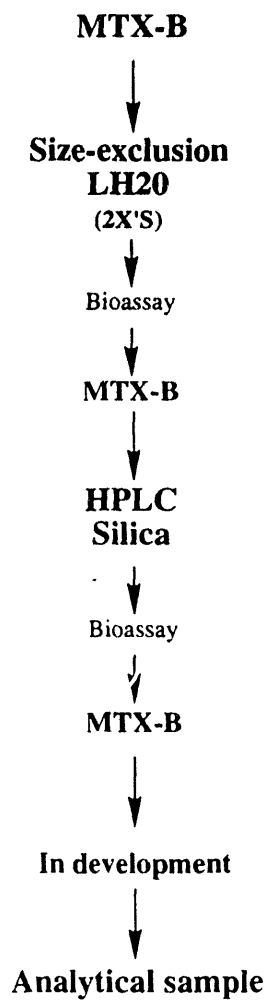
FIGURE.4 Protocol for purification of MTX-B.

SOXHLET EXTRACTION
BIOASSAY RESULTS

SOLVENT	CYTOTOX	Ca++	Na+
Hexane	+	-	+
Benzene	+	-	+
Pet. Ether	-	-	-
Ethyl Acetate	+	+	-
Acetone	+	+	-
Acetonitrile	-	-	-
Isopropyl Alcohol	+	+	-
Methanol	+	+	-
Water	(+)*	(+)*	-

*Indicates partial activity

FIGURE.5.   Results are for 10g frozen, wet-weight, of *G. toxicus*.
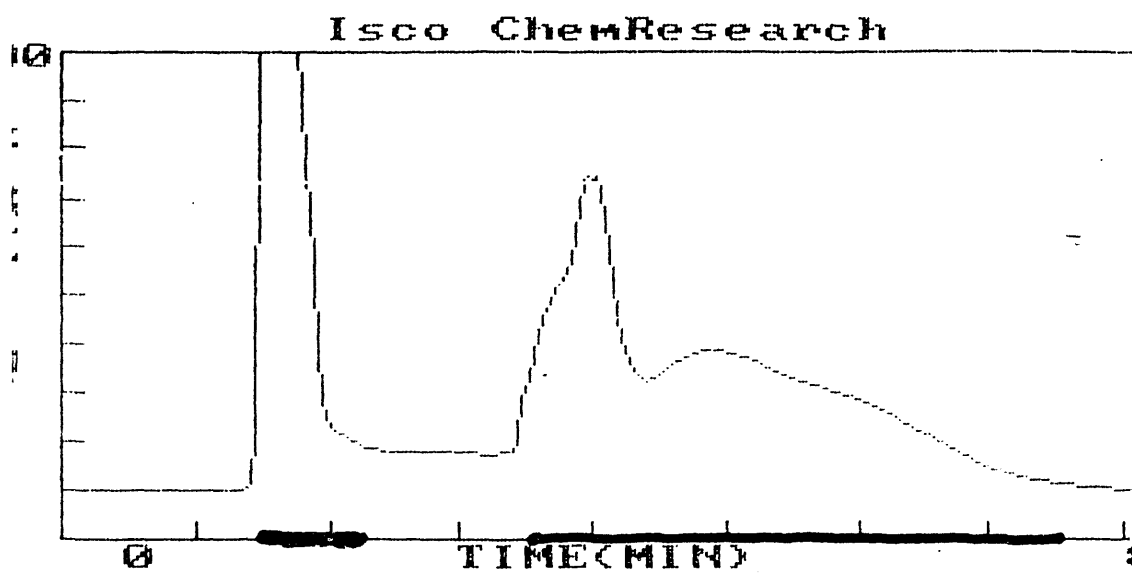
FIGURE.6 Chromatogram for HPLC. Darkened areas along bottom
axis signify times that toxic fractions were collected.

HPLC

B_1 B_2

B_1 B_2 B_1 B_2
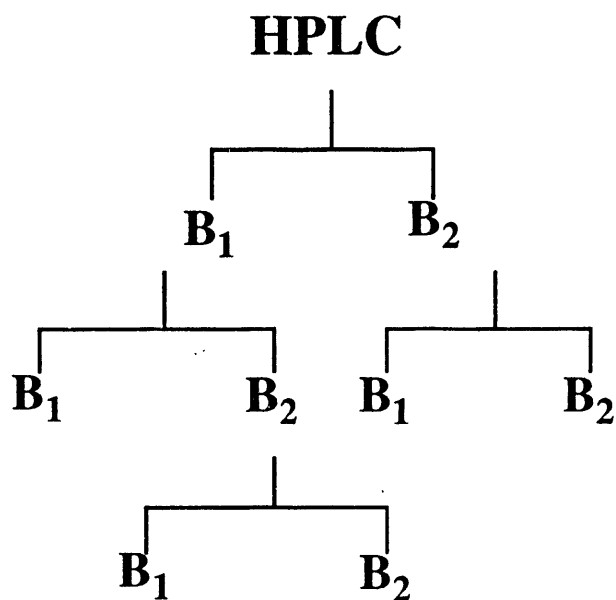
B_1 B_2

LEGEND

B_1 = MTX-B_1

B_2 = MTX-B_2

FIGURE.7 Repurification and verification scheme for two toxic
fractions that are believed to be in equilibrium with
each other.

Medical University of South Carolina
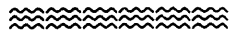
Marine Biomedical & Environmental Sciences

Sister Chromatid Exchange as an Indicator of Environmental Stress

by Lisa M. Johnson

Advisor: Dr. Donald R. DiBona

Summary

Environmental characterization requires the application of analytical procedures from a wide variety of disciplines. In the aquatic environment, resident fish reflect the surrounding stresses through a number of biological responses (biomarkers). Parallel investigations have been undertaken to: 1) examine the genetic basis of susceptibility to dioxin, and 2) to develop a laboratory assay of genotoxic stress. In each case experimental material has been collected, procedures developed, and assessment of results is underway. Consequently, this manuscript represents a progress report.

Introduction

Biomarkers are biochemical, physiological and histiological changes in organisms used to estimate exposure to chemicals or resultant effects. Here I explore the use of biomarkers from two perspectives: use of a sentinel species, *Rivulus marmoratus*, and testing a known assay, sister-chromatid exchange.

Rivulus marmoratus is the only internally self-fertilizing hermaphroditic vertebrate known. Clonal propagation of these fish eventually has resulted in homozygosity at every allele in their DNA. The benefit of this situation lies in the reliability of controls. *Rivulus* are also euryhaline, which makes them a good model because they can be used to test any environment from freshwater to two-three times marine water concentrations. Another advantage is that, with fish, we can completely control the environment of the experimental animal eliminating outside sources of variation between treatments. The only real disadvantage of using this organism is that propagation is slow, thus it takes a long time to get large enough numbers for experiments. Multiple strains of *Rivulus* were available at the marine biomedicine lab eliminating this problem. Spontaneous variation within the species accounts for the many different clonal strains of *Rivulus*. Different strains may vary with susceptibility to different stresses and offer a means to genetically characterize the basis of susceptibility.

In December 1992 the first experiment to begin and identify the genes that confer susceptibility to dioxin was made. Dioxin is a chemical produced in paper mills that is often a source of environmental contamination. The results of that preliminary experiment, in which several strains were screened, indicated that *Rivulus* is very sensitive to dioxin. The goal for the experiments that followed has been to verify suggested differences in susceptibility between strains and to use two strains that differ in susceptibility to try and identify the gene responsible for this difference. Gene sequencing of susceptible and nonsusceptible fish should locate the parts of the genome that are different between the two strains. Another goal of these experiments is to construct a dose-response curve for *Rivulus* exposed to different concentrations of dioxin.

In my experiment, I exposed three strains of *Rivulus marmoratus* to five different concentrations of dioxin. Through this experiment I hoped to detect some difference in susceptibility between the three strains and to begin construction of a dose-response curve for *Rivulus marmoratus*.

Sister-chromatid exchange can be used as an environmental biomarker because such exchanges are an indication of DNA damage (genotoxic stress). Using a special staining procedure it is possible to visualize recombination between sister chromatids and to calculate the frequency of such exchanges.

The Savannah River has been a site of public concern with respect to pollution generated by a defense plant (Savannah River Site) that is located on the river a few miles south of North Augusta. The production of nuclear materials has led to the release of both nuclear and other contaminants into the river. The extent of this contamination and its ultimate effect on man has been the concern of the studies done in this area. This concern was the reason for choosing the Savannah River as the location to be tested by SCE analysis.

Choosing an organism to use for my SCE experiment was a challenge. First of all, the organism had to be aquatic because of the environment I was testing and because of the ability to completely control an aquatic environment. However, not just any fish can be used for SCE analysis because most fish have many ($2n \geq 48$) very small ($<3\mu m$) chromosomes that make visualisation of SCE nearly impossible under light microscopy. The mudminnow, *Umbra* sp., has been- a common organism used for sister chromatid exchange tests[1-4] because of its low number ($2n=22$) of large ($3-8\mu m$) chromosomes. *Umbra* appeared to be ideal for my use because it is supposedly native to the Savannah River as well as to other local waters. Also, being a freshwater fish it is suitable to test areas from around the SRS. The availability of *Umbra* was not what I expected however, so I explored a couple of other options. One of these options was a small tropical fish, the chocolate gourami, *Sphaerichthys osphromenoides*. They can be conveniently purchased from pet stores, but the difficulty encountered with them is their extreme sensitivity to any changes in their environment. They were very difficult to keep alive in the laboratory even before any experimentation was tried with them. The only apparent alternative after that was the oyster toadfish, *Opsanus tau*. The oyster toadfish is found in estuarine to marine water and is locally available in Charleston harbor. They have been used for SCE tests[5], have a small number ($2n=$) of large chromosomes and are very hardy and easy to keep in a laboratory situation. All of these characteristics made the oyster toadfish my best choice. The only problem with use of this fish is its limited salinity range. It is not found in freshwater and therefore could not be a "resident" test for the whole area of interest.

Methods and Materials

Dioxin
Three strains of *Rivulus marmoratus* were exposed to different concentrations of dioxin in 50ml of 50% seawater. Three fish from each of the strains were exposed to each concentration (10ppt, 1ppt, .1ppt, .01ppt and controls with no dioxin). The fish were exposed over a 48 hour time period and water treatments were changed twice a day to ensure a semi-constant dioxin concentration. Fish were not fed during the exposure. After 48 hours the fish were rinsed three times with 50% seawater and one fish from each strain for each concentration was placed in a clean bowl, put back on a regular feeding schedule, and kept for observation. The other 2 fish per strain per concentration were rinsed three times with 50% seawater and immediately preserved in 10% formalin. Later the preserved fish were shipped to Dr. John Stegeman at Woods Hole Oceanographic Institution for analysis. In his laboratory, the generation of cytochrome P450 enzymes is directly assessed as an index of dioxin-derived tumorigenesis[6].

SCE
Collection of organisms
Much time and effort was spent in search of *Umbra pygmaea*, the eastern mudminnow, which seemed ideal for the type of experimentation I planned on doing. Unfortunately, a very substantial collecting effort only established that the fish is far from abundant. *Umbra pygmaea* are documented as being found in freshwater, slow moving, lowland creeks and streams along the Atlantic coast from Long Island, New York to Florida. Many techniques were used in my search for *Umbra*, including cast nets, minnow traps and dip nets. Although using a seine net appeared to be a popular technique used by others in the past[1], I was unable to seine in any of the areas I looked due to the geography of the creek bottoms and surrounding shore. I searched for *Umbra* in many sights along the Cooper and Savannah Rivers with no luck. A local expert in the field pointed me toward some sites throughout the state where *Umbra* were previously spotted. I tried a number of these sites plus anything along the way that looked promising. At two particular sites in Darlington county I was not only troubled with the absence of *Umbra*, but with the absence of water as well, as a local drought seemed to have dried up the small creek in which *Umbra* were previously found. I did finally locate *Umbra pygmaea* in a small pool of water isolated from the Mechaw creek in the Frances Marion National Forest. Dip netting was my successful procedure for catching the unsuspecting fish, however, only three fish were found at this site, two of which died before I got them back to the laboratory. With my options exhausted I decided to settle for using the oyster toadfish for my experiments. The oyster toadfish used for my experiment were collected from the Charleston harbor using a dredge. They were then brought back to the laboratory and kept in well aerated aquaria (salinity = ~23ppt) until use.

Injections and treatments

Two separate aquaria were prepared for Savannah River simulations. The two Savannah River simulations differed only in that one was prepared using sediment taken from a location above the SRS and the other sediment was taken from a location below the SRS. The Savannah River simulations consisted of sediment approximately one inch thick in a small aquarium with approximately 2.5L of 23ppt saltwater. The sediment and water were well mixed and allowed to settle for about three hours. Both aquaria were well aerated. Fish were first weighed and then injected with 500µg of 5-bromodeoxyuridine (BrdU) per gram body weight and placed into one of the aquaria prepared. Control fish were place back into the original aquarium after injection. Three fish were injected per treatment. After 5 days fish were injected with 10µg colchicine/g body weight to arrest dividing cells in metaphase. After 4-7 hours fish were sacrificed and various tissues were removed for possible analysis. Tissues were place in 5 ml .4% KCl solution for 30 minutes. They were then fixed in 3 changes of 3:1 methanol:acetic acid and refridgerated for later use.

Slide preparation

Tissues were prepared and stained using a modification of the procedure used by van der Gaag et. al. 1985[7]. The procedure involved the following:
1. Remove tissue from fixative and touch to filter paper to remove excess fixative.
2. Place in well of a depression slide and add 2-3 drops 50% acetic acid
3. Mince gently for one minute and return unsuspended tissue to fixative
4. With draw one capillary tube full of suspension and expel onto clean slide heated to 40-50° C and quickly withdraw suspension back into the capillary tube (repeat to produce 2-3 rings/slide)
5. Treat for 15 minutes with 33258 Hoechst (200µg/ml PBS solution)
6. Rinse with distilled water and wet mount in McIlvaine's buffer.
7. Expose to intense fluorescent light for ten minutes
8. Rinse with distilled water to remove coverslips
9. Treat with 5N HCl for 15 minutes
10. Rinse with distilled water
11. Stain slides in a 3% giemsa stain made up in 1/15M phosphate buffer for 7-8 minutes
12. Rinse in distilled water, air dry, and mount in permount
Slides were then viewed using light microscopy.

Results

Dioxin

Rivulus that were maintained and kept for observation were watched for survival rates. Only one fish died during the treatment. That fish was from the 1ppt treatment and from strain DS-352. The dates of fish mortality thus far can be seen in table 1.

Table 1. Mortality rate of three strains of *Rivulus marmoratus* after 48 hour exposure to various concentrations of dioxin.

Fish #	Strain	Treatment	Date Died
3	NA 318	10 PPT	June 25, 1993
6	024	10 PPT	July 12, 1993
9	DS-352	10 PPT	June 26, 1993
12	NA 318	1 PPT	
15	025	1 PPT	
17	DS-352	1 PPT	June 11, 1993
21	NA 318	.1 PPT	
24	021	.1 PPT	
27	DS-352	.1 PPT	
30	NA 318	.01 PPT	
33	024	.01 PPT	
36	DS-352	.01 PPT	
39	NA 318	CONTROL	
42	028	CONTROL	
45	DS-352	CONTROL	

Rivulus that were preserved and sent to Dr. Stegeman at WHOI have not yet been fully analyzed. Therefore no results from those fish can be included here, but should be received in the near future.

SCE

    Umbra pygmaea were looked for at many sights from the Cooper and Savannah Rivers as well as from small creeks around South Carolina (see maps 1-4 for location of sights explored). The only place where *Umbra pygmaea* were found can be seen on map 3. Under this bridge was a small isolated pool of water with a muddy bottom. Three *Umbra* were found living in the mud at the bottom of this pool. Two of these three *Umbra* died on the way back to the laboratory because of high water temperature in the bucket they were being kept in. The other *Umbra* was put into a freshwater aquarium, fed flaked food and appears to be healthy to date. No water was found at two of the sights on map 4. *Umbra* were previously found in good numbers at these two locations (Scott Lamprecht personal communications) however, a local drought seemed to have dried up these small creeks.

Discussion

Dioxin

    Results obtained by observation of *Rivulus* maintained after dioxin exposure indicate a possible difference in susceptibility between different strains. Both the strains DS-352 and NA 318 appear more susceptible than the strain 020-029. Not enough data was collected by this preliminary experiment to be conclusive. A second experiment is underway to further investigate this hypothesis. This experiment will test two of these strains in bulk. Five fish from the strains DS-352 and 020-029 will be exposed to dioxin at a

concentration of 10 ppt for a period of 48 hours. Fish will be maintained in the same manner as in this experiment and observed for rate of mortality. This should better enable us to determine any difference in susceptibility between these two strains.

SCE

No data were collected for the SCE test done using the oyster toadfish. I am presently having some difficulty with the slide preparation and viewing of the chromosomes. Plenty of tissue has been preserved for analysis and this work will be continued in the laboratory.

There are a couple of unanswered questions that need to be adressed regarding the SCE test. First, what is the proper method for testing for environmental contaminants? Is my method of putting contaminated sediments in uncontaminated water appropriate, or is their another method commonly used? I was unable to locate anything in the literature to help me answer this question. Eventual SCE results will address the issue of appropriate protocols for stress assessment.

Another question that has been asked with regard to the SCE test is whether or not fish will eventually become adapted to a contaminated environment and no longer show an increase in SCE frequency, or if fish can be sampled directly from the environment and analyzed for SCE frequency in a meaningful way. Adaptations within populations from various locations will exploit fingerprinting techniques.

Although there are a number of questions left unanswered, further data analysis of material collected during both the dioxin exposure and SCE experiment will continue. Through long distance communication I hope to be a part of this data analysis and continue to seek out the answers to these questions.

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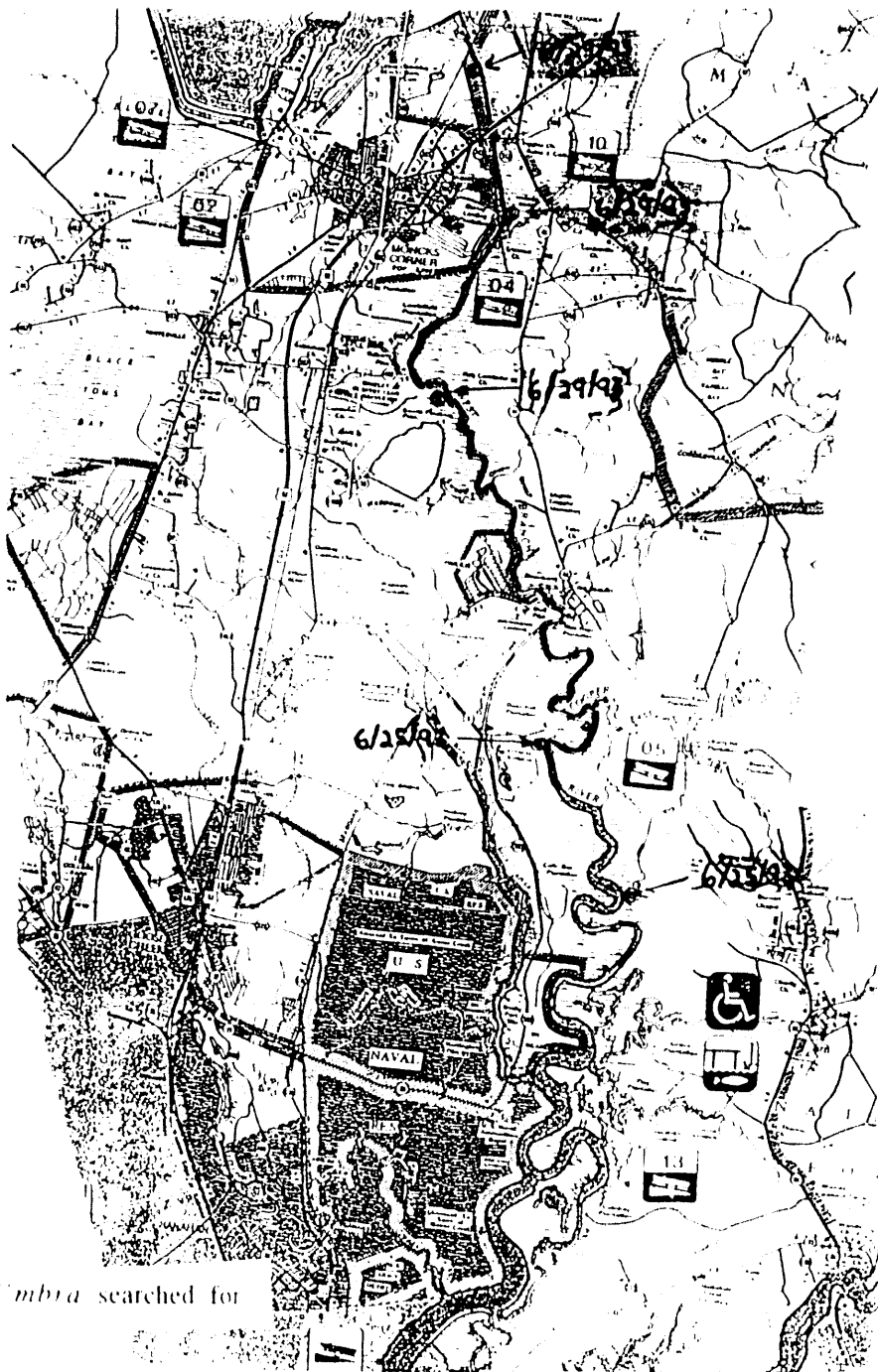
Sites *Umbra* searched for

Map 1 Sites explored for *Umbra pygmaea* along the Cooper River.

SRS

SITE 1

SITE 2

SITE 3
Pen Branch

Steel Creek

SITE 4

● Sites mud collected for Savann
River simulations

◐ Sites *Umbra* searched for

⊗ Allendale

HWY 301

South Carolina

Georgia

Savannah River

Miles

0   10   20   30

Beaufort-Jasper
Water Treatment
Plant

St. Helena
Island

Canal

Port Royal
Sound

Hilton Head
Island

N

Port Wentworth
Water Treatment
Plant

SITE 5

SITE 6

SITE 7

Savannah

Savannah
River Mouth

Map 2 Sites explored for *Umbra pygmaea* along the Savannah River.

Umbra found in past

Umbra searched for
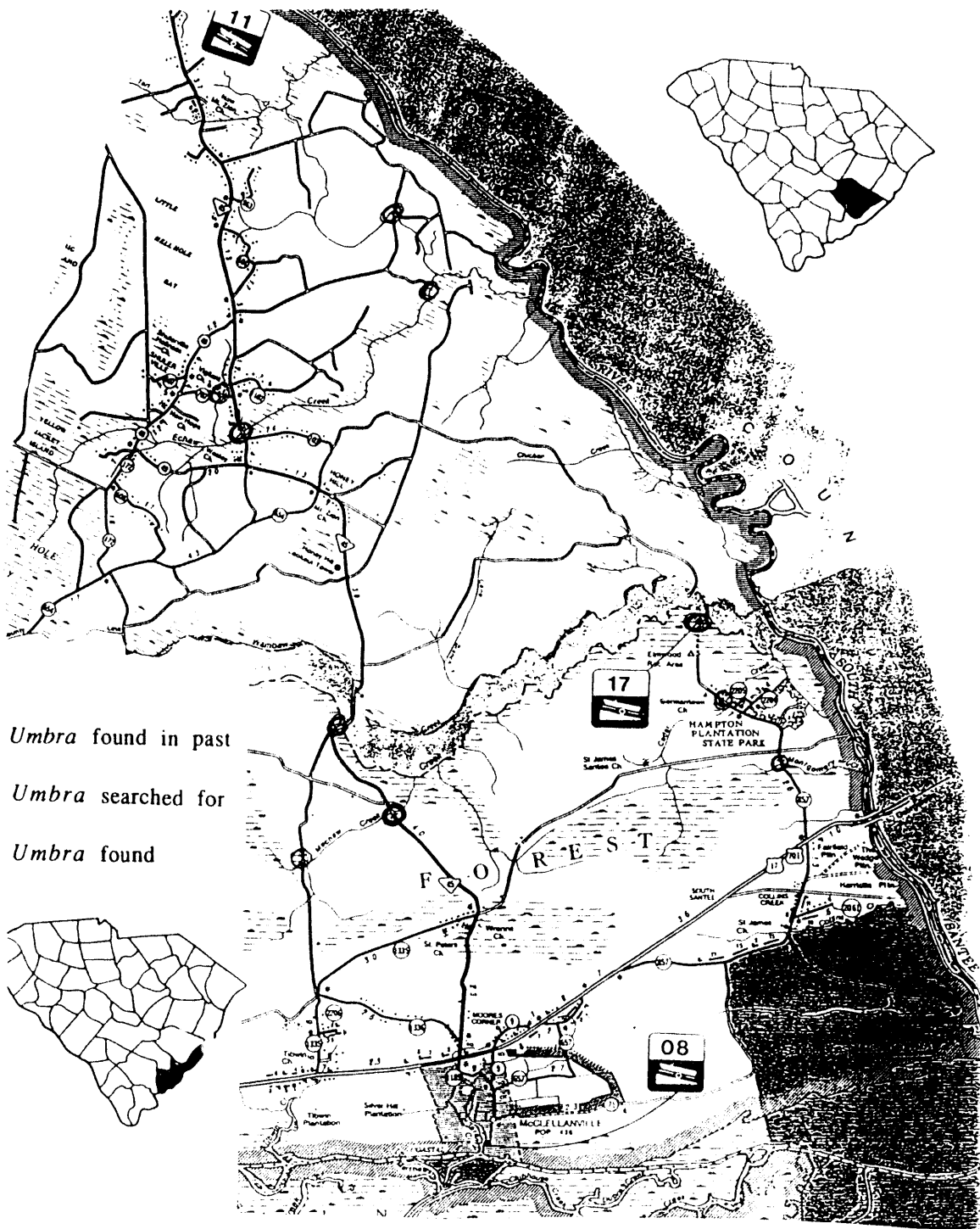
Umbra found

Map 3 Sites explored for *Umbra pygmaea* in Charleston and Berkley, counties.
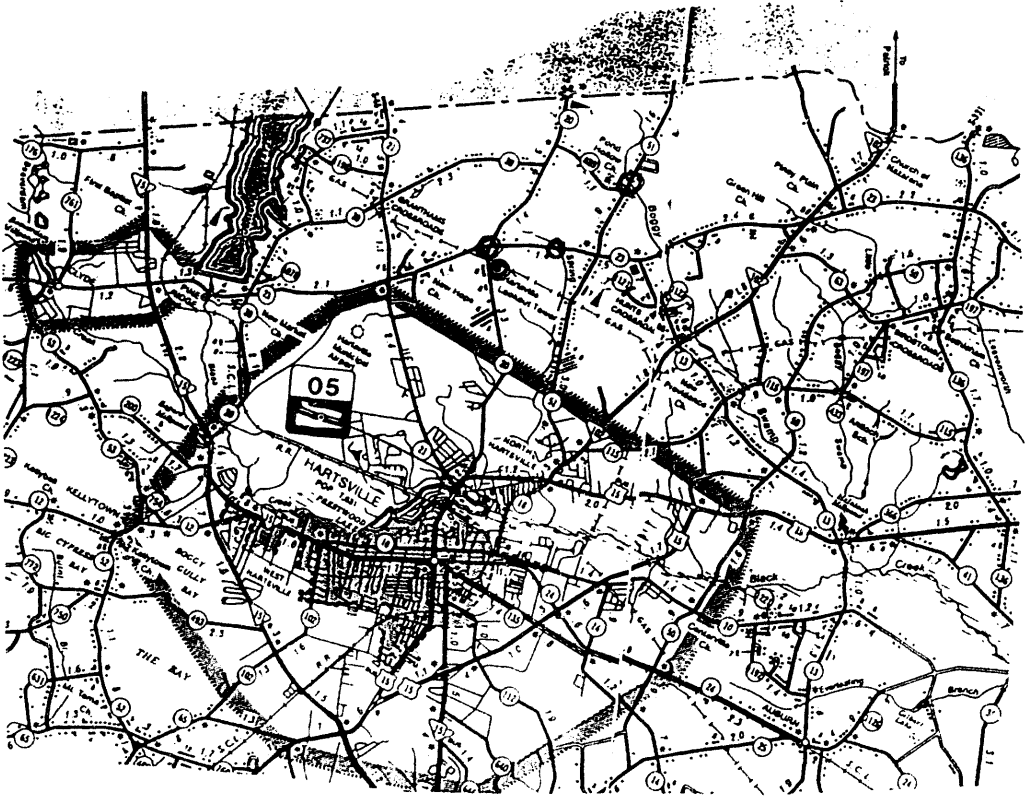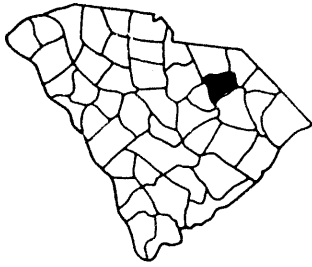
Map 4 Sites explored for *Umbra pygmaea* in Darlington county

Use of Diatom Distributions to Monitor Environmental Health

by Rachel H. Levine*
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Use of Diatom Distributions to Monitor Environmental Health

Summary: *A variety of approaches has been used in the past to assess the environmental impact of anthropogenic contaminants. One reliable index for aquatic environments is the analysis of diatom species distribution; the focus in this case being on the Savannah River. The completed objectives of this study were: A) the development and use of procedures for measuring diatom distribution in the water column and B) the development and evaluation of sediment sampling methods for retrospective analysis.*

Introduction

In the early 1950's, the Savannah River Site (SRS) was established on the bank of the Savannah River, near Aiken South Carolina, to begin production of nuclear materials. Over the years, various radiaoactive substances such as plutonium, tritium, cesium and strontium have been released into the ponds and creeks within the SRS plant (SRS 1991). Because of the continuous exchange of water between the Savannah River and its tributaries, much of the toxic waste has become dispersed throughout the approximately 160 miles of river between SRS and the river's mouth.

For the 40 years that SRS has been in operation, there exist only irregular records of both the amounts and types of waste materials released into the environment and their effects on the ecological systems of the Savannah River. In order to initiate a comprehensive history of the river's exposure to heavy metals and radionuclides, an analysis of diatoms was begun. It is possible that this approach will help in retrospective assessment of environmental contamination.

Diatoms are single-celled plants that are found both as phytoplankton and periphyton. Usually brown in color, and exhibiting a large range of shapes and sizes, diatoms exist in almost every aquatic habitat world-wide. Diatoms are unique in that they secrete silicon oxide, forming symmetrical shells, or frustules, which earn them the name "glass-houses." Along with the silicon

oxide, however, diatoms also incorporate into their frustules any elements accumulated from their aquatic surroundings. Materials enter the cell by diffusion (Rand and Petrocelli 1985), where they are packaged by various organelles and then secreted (Robinson and Sullivan 1987). The relative amounts of different elements in the frustule should therefore reflect the ratios of various materials in the water at the time of frustule formation.

When the diatom dies, the frustules sink and become part of the sediment. The cell decomposes, but there is little or no depuration from the frustule. All elements in the molecular structure of the shell remain intact and, by analyzing diatoms in the river sediment, it is possible to determine the ratio of toxic materials that were present in the water column over a long period of time. In this study, the initial aspects of that approach have been explored. Existing water column samples were analyzed, focusing on species composition to discover any present differences between the Cooper and the Savannah Rivers. Diatom distribution patterns in the water column will be used to initiate a database comparing past and present species composition as well as the amounts of different heavy metals and radionuclides that are suspected components of SRS effluent.

Materials and Methods
Field Procedures:

To collect field samples, both water column and sediment, from the Savannah River, two separate trips were made. The first, on July 1, 1993, began approximately at mile marker 10, north of the river's mouth, and continued upriver to mile 33, over which distance three sample sites were chosen (see figure 1). The second, on July 21, 1993, covered approximately 30 miles of river and 4 different sample sites, beginning 7 miles above SRS and ending close to the southern border of SRS territory (see figure 2). Three samples were also collected on each of two different days, June 25 and June 29, 1993, from the Cooper River as a control (see figures 3 and 4).

Sediment sample sites along both rivers were chosen where there was neither a strong current nor visible eddies and there appeared to be a lot of deposition, such as the inner curve of a bend in the river bed. A Wildco lexan hand corer (Wildlife Supply Co., Saginaw, MI), 2 inches in diameter, was used to take one core sample per site, core length ranging from 10 to 16

inches. Each core was then divided into three sections and placed into ziploc baggies.

Water column samples from both the Savannah and Cooper Rivers were taken in the same area as the sediment cores but in water at least 2 meters deep to avoid collecting bottom material. A submersible pump (Atwood 800 gpm, Attwood Corp.) attached to tygon tubing was lowered halfway into the water column and run for approximately 5 minutes per site (pump time was either increased or decreased depending on the amount of material collected). The water was run through a stack of 5 8-inch diameter sieves, with mesh sizes of 800, 270, 120, 70, and 37 microns (stacked downward from 800 to 37 microns) (Carolina Biological Supply Co.). The material from the 37, 70, and 120 micron sieves was then washed with river water from the mesh into separate collection bottles. Water column samples from the Cooper River, however, were combined by sieve size, i.e. all material collected in the 37 and 70 micron sieves from each site on the same day were washed into the same jar. A Hydrolab was used to measure the salinity/conductivity, pH, dissolved oxygen (DO), temperature, and depth of each water column site. Water column samples were maintained at 4 C during transportation to the lab.

Both water column and sediment samples were stored in a refrigerator when brought back to the lab without any preservation techniques, although 10% formalin was added to some of the water column samples a few days after collection in an attempt to preserve some of the organic material.

Laboratory Procedures: Water Column samples

In order to reduce the volume and concentrate the water column samples from both the Savannah River and the Cooper River, each sample was put into a 15 ml centrifuge tube and spun down at approximately 1000 rpm for 5 minutes. The supernatant was decanted, a few drops of distilled water was added, and the pellet was dispersed. Wet mounts were prepared from a few different samples and examined under a light microscope (Zeiss ICM-405 Inverted microscope equipped with video capabilities) both at 400x and at 1000x. Diatoms were visible at both magnifications and video thermal prints were made of some species to aid in classification (see figure 5).

Because of the ease with which diatoms were found under the scope, permanent slides were made. Three slides from each of the three sieve sizes collected (37, 70 and 120 microns) from all 7 sites sampled on the Savannah

River, and 4 slides from each sample size (37/70 and 120/270 microns) from each of the two Cooper River trips were prepared. A few drops of concentrated sample were placed on a glass slide and heated over an electric hot plate to dry. First Permount and then a coverslip were added to the dry samples before leaving them in a hood overnight for the Permount to harden. Completed slides were examined under the microscope at 400x, and the first twenty diatoms seen on each slide were classified by genus and tallied (see table 1A). Percentages were then calculated of each genus at each sample site (see table 2B).

Laboratory procedures: Sediment samples

Because of the density of the sediment cores and the large amount of material from each site, aliquots of each section of each core were filtered similar to the water column samples. Approximately 75ml of sediment from each sample was placed on the 800 micron sieve and washed through the 4 smaller sieves. Any material remaining on the 37, 70, and 120 micron sieves was then washed into separate sample bottles. After 3-5 spins at approximately 1000 rpm (beginning with 15 ml each time and then decanting the supernatant) for 5 minutes per spin, the samples were completely concentrated. A few drops of distilled water were added and the pellets were dispersed, then allowed to settle naturally.

The top layer of material from a few of the samples was pipetted onto glass slides and dried on a hot plate. Three slides were made from each of three samples (see table 2). When dry, the slides were examined under a dissecting scope at 50x. Diatoms appeared milky white among the other material on the slides. They were lifted off the slide with an eyebrow hair and a drop of distilled water and placed on a grid, each grid box representing a different shape of diatom.

Electron Microprobe Analysis

Special arrangements were made with Dr. Ann LeFurgey, Director of the Microscope Facility in the Department of Cell Biology at Duke University to use the lab's facilities and scanning electron microscope (SEM) to examine diatoms with electron probe analysis. That analysis would show the relative amounts of all the major elemental components in the diatom frustules.

To prepare the samples for examination in the SEM (JSM-6400), small aliquots of each sample were placed in petri dishes and left overnight under a hood to dry. Under a 10x dissecting scope, diatoms were identified, lifted by a fine brush with a drop of distilled water and placed on carbon stubs (made of spectroscopically pure carbon to avoid a false signal in the microscope) coated with colloidal graphite carbon paste. Approximately 4-10 diatoms were placed on a stub, then the stubs received a top layer of carbon. Completed carbon stubs were placed one by one in the SEM and examined. Three different types of images were used in the analyses: a Secondary Electron Image (SEI), a Backscattered Electron Image (BEI), and an Energy Dispersive X-ray (EDX). Black and white pictures were also taken of each diatom examined with Polaroid 4x5 Type 55 film (see figure 6).

Results

Diatom distributions from water column samples from the Savannah River as well as the Cooper River were calculated (see table 3). The most common diatom genus in the Savannah River is Melosira, comprising an average of 33% of the population. One interesting fact is that Melosira, while still the most abundant genus at each site, shows a definitive decrease in numbers, going from 50% of the diatom population at site 1 (farthest from the river's mouth) to only 22% at site 7 (at mile marker 10). Pleurosigma (also called Gyrosigma) (Heurck 1896) and Coscinodiscus are the next most common diatoms in the Savannah River, averaging 16% and 13% respectively. Except for Synedra (9%) and Navicula (8%), the majority of the other diatoms counted were classified in genera that each accounted for 4% or less of the total population.

The diatom distribution in the Cooper River was found to vary substantially from that of the Savannah. Although the most common diatom there is also Melosira at 32%, next on the list is Cyclotella, composing 21% of the diatom population, and Navicula at 15%. There is also a smaller variety of genera in the Cooper where only 13 genera were identified, compared to 19 in the Savannah River.

In the sediment samples taken from Savannah River sites 1 and 2, a large number of diatoms were found, including a high number of centric diatoms. This corresponds with the large percentage of centric diatoms (Coscinodiscus) found in the water column samples. In the slides from Savannah River site

4, however, insufficient numbers of diatoms were identified to analyze the population. Extracting diatoms from sediment is an exacting process, and considerable work will be required to develop reproducible procedures. Therefore, many more cores will have to be used for developing technical procedures before diatom populations of the past can be calculated on a routine basis.

SEI and BEI taken on the SEM were used for the photographs of the water column diatoms, clearly showing each frustule in detail (see figure 6). EDX data, printed in graph form, showed the relative amounts of each element present in a small section of each frustule. The most common elements were silicon, chlorine, potassium, calcium and barium (see figure 7). The chlorine peak is suspect, however, because there is a chlorine contamination factor from the SEM itself. An EDX was also done on the material inside one of the frustules, and much higher peaks of both aluminum and iron were recorded (see figure 8).

Discussion

The most common diatoms from mile markers 165-135 and 33-10 of the Savannah River are Melosira, Pleurosigma and Coscinodiscus (see table 3). When compared with previous calculations of diatom distributions, some interesting questions are raised.

For the most part, diatom species composition of the Savannah River has changed little over the past 40 years. In a study done by Louis G. Williams from 1960-1962 (Williams 1964), the most common diatoms at Port Wentworth, GA (near Savannah) were Melosira and Coscinodiscus. The Alvin W. Votgle Nuclear Power Plant's report on the algal populations at Port Wentworth from 1951-1968 also listed Melosira and Coscinodiscus as the most prevalent genera (Georgia Power Co. 1972). The results of this study agree with those results in that Melosira and Coscinodiscus comprise a large percentage of the total diatom population in the area; the presence of significant numbers of Pleurosigma is the striking difference to be accounted for.

Further upriver, near North Augusta, SC, Williams (1964) stated that Melosira was the most common diatom genus. The Votgle Nuclear Plant report (1972) concurrred, and also added Navicula to the list. This study has calculated that Melosira and Navicula are still a major portion of the area's

total diatom population. It deviates, however, in that Pleurosigma was also determined to be one of the most common genera, yet it is not prominent in the Savannah River in either of the 2 other studies mentioned above. Not enough information has been uncovered about diatoms as indicator species to be able to determine exactly what the increased appearance of Pleurosigma signifies, but future sediment analyses in areas where Pleurosigma exists in high percentages will hopefully be able to provide a history of the genus' population. That history may follow the rise and fall of the river's toxicity level, or it may show a general adaptation of different diatoms to changing environmental parameters.

On another level, the diatom populations of the Savannah River were compared to those of the Cooper River, and while there were some similarities, other variations were immediately visible (see table 3). For example, Melosira is the most common genus in each of the rivers but, where Pleurosigma and Coscinodiscus are also prominent in the Savannah River, they are not found in great numbers in the Cooper. On the other hand, Fragilaria and Cyclotella, two of the most common diatoms in the Cooper River, are very rare in the Savannah. While it is possible that these differences are due to the increased amount of toxic waste dumped into the Savannah (i.e from SRS) than the Cooper, there are numerous, non-anthropogenic differences between the two watersheds that must be considered.

More research must be completed before firm conclusions of environmental significance can be drawn from this data, although river quality assessment through diatom analysis appears to be a reliable procedure. Results agreed with assessments made previously, and sample sizes proved to be sufficient for statistical analysis. As more information continues to be collected, the database for Savannah River environmental health will be progressively enlarged.

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South Carolina
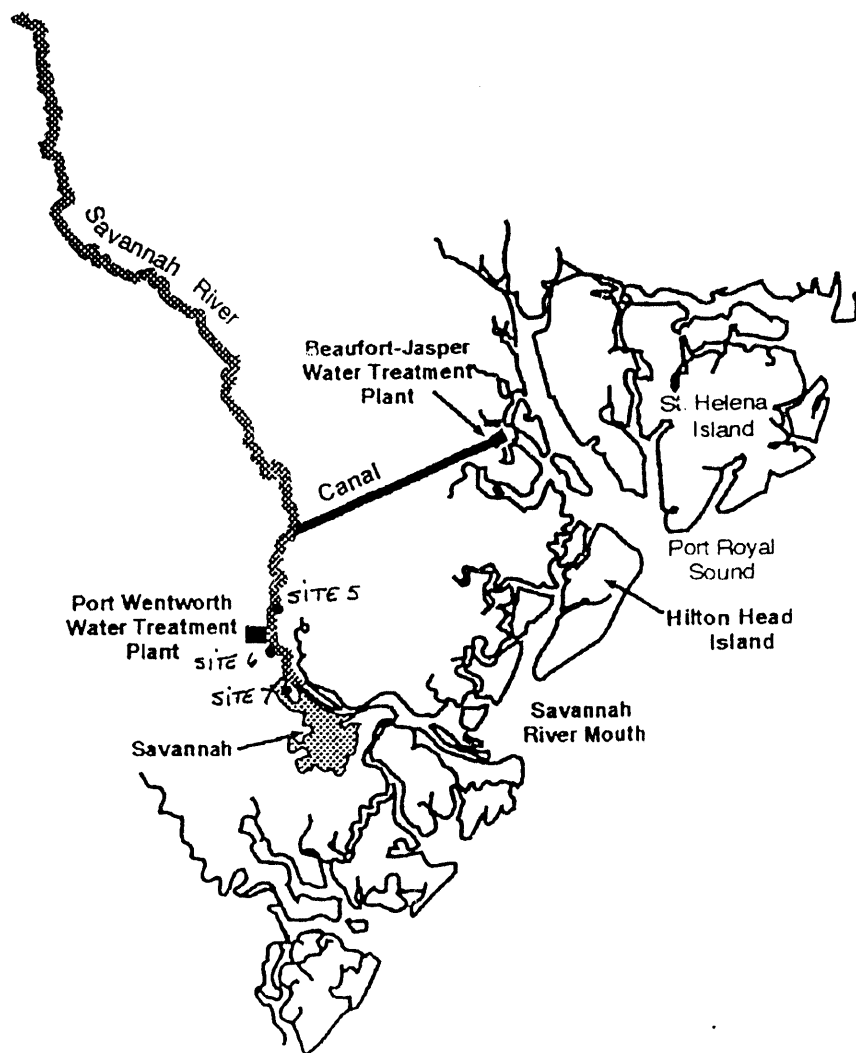
Figure 1. Sites 5-7 sampled on the Savannah River at mile markers 33, 20, and 10 on 7/1/93 are marked with a ●.

Figure 2. Sites 1-4 sampled on the Savannah River at mile markers 165, 155, 145, and 135 on 7/21/93 are marked with a ●.
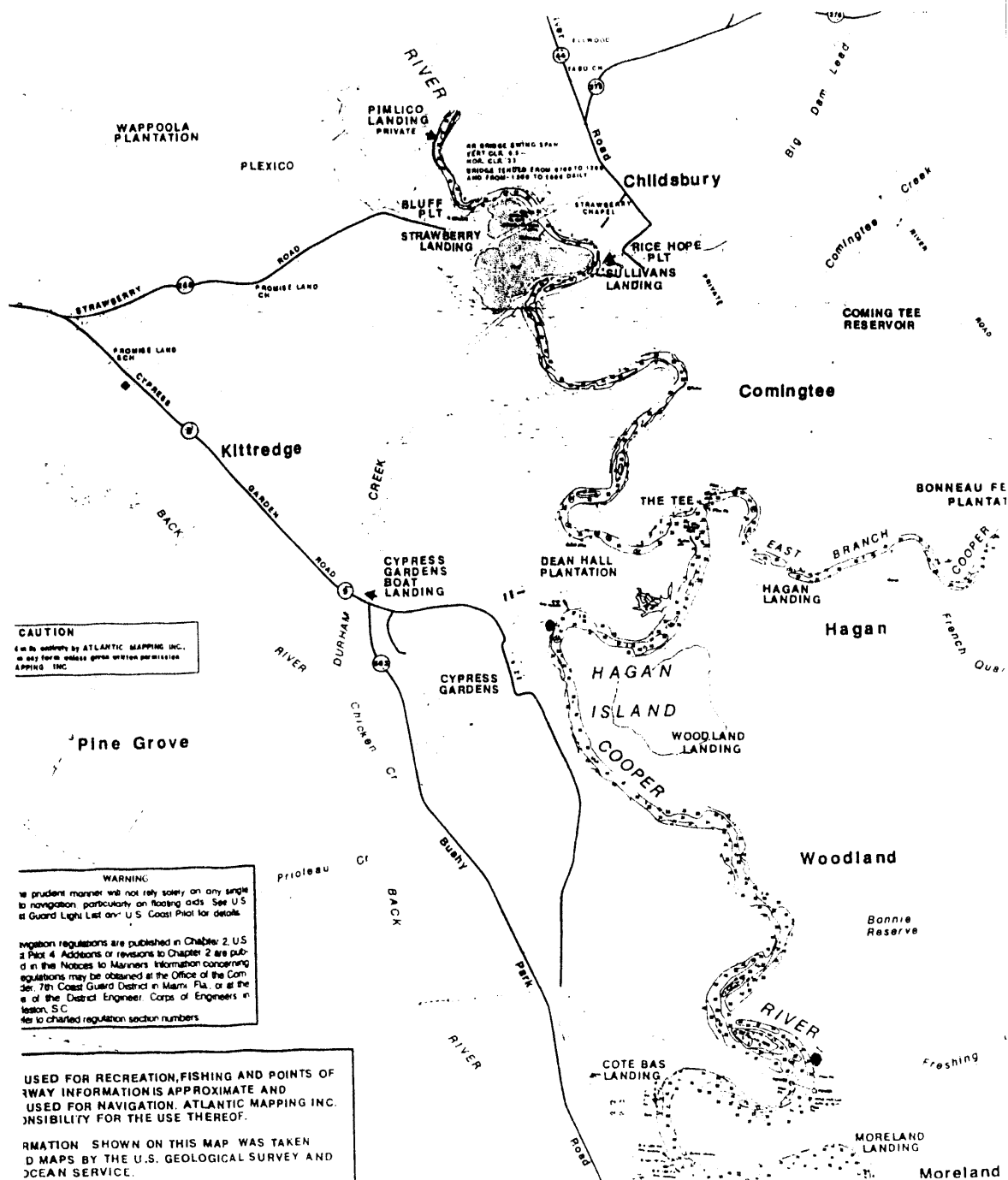
Figure 3. Sites sampled on the Cooper River on 6/25/93 are marked with a ●.
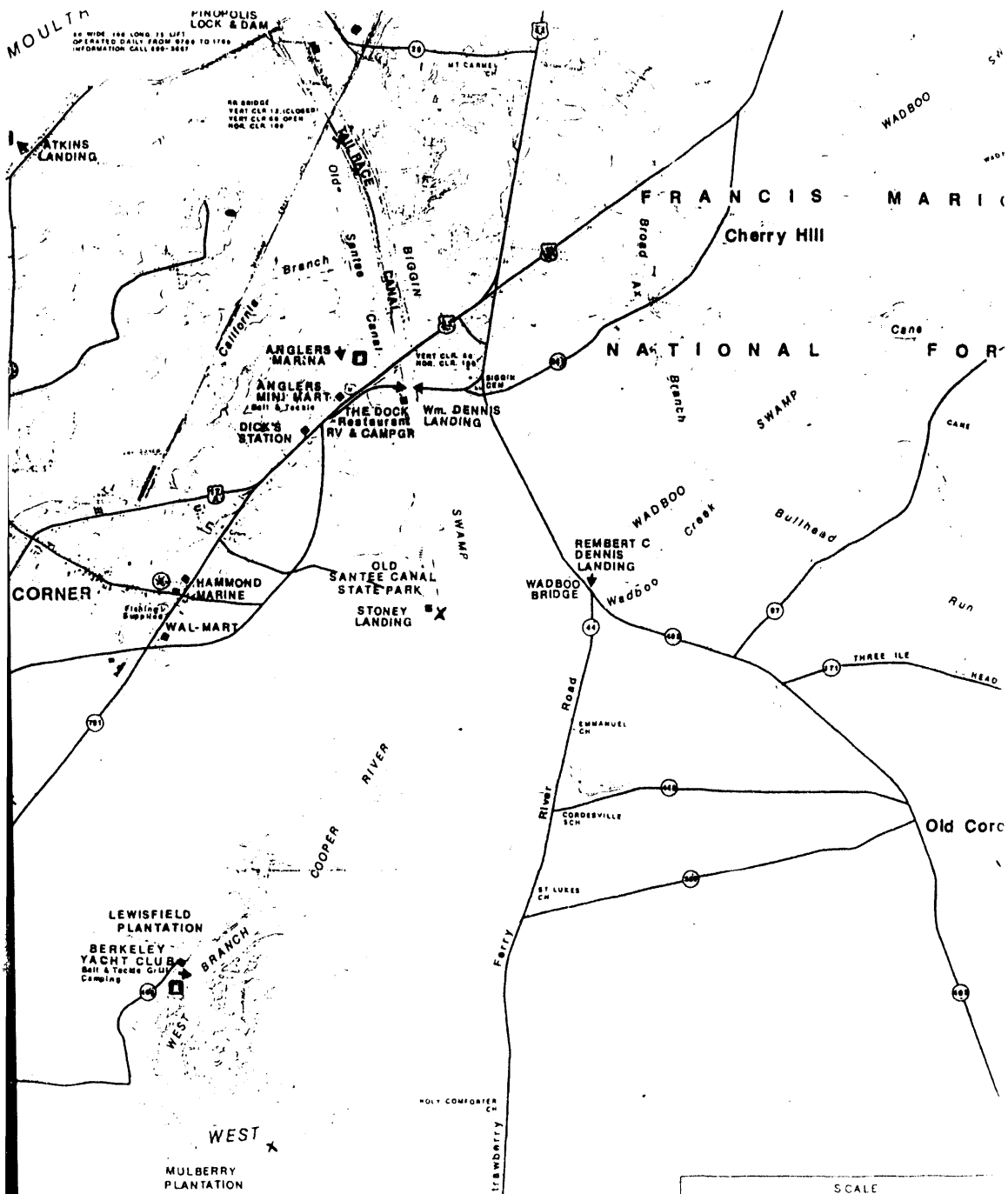
Figure 4.   Sites sampled on the Cooper River on 6/29/93 are marked with an X.
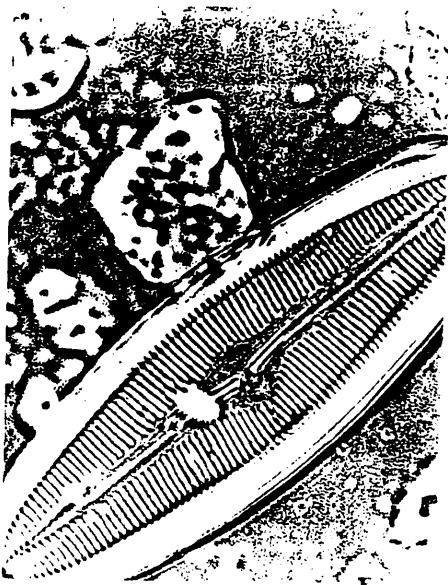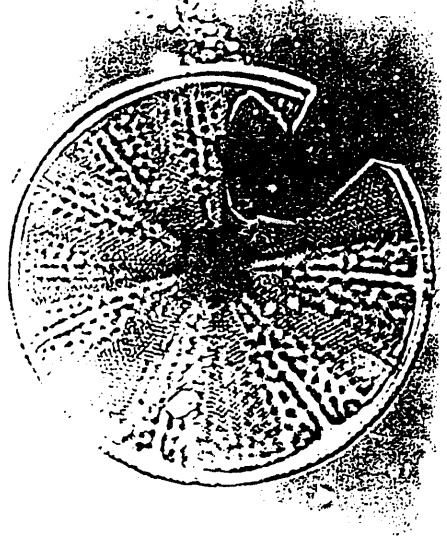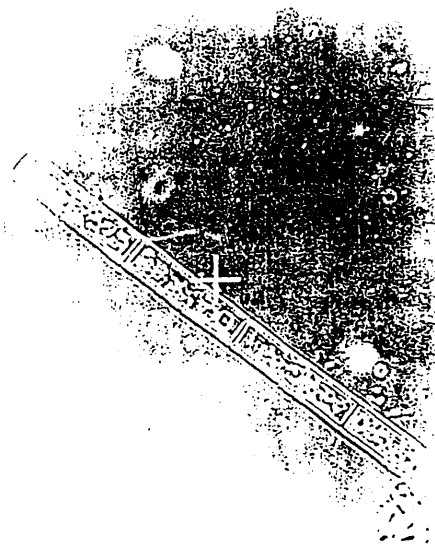
Figure 5 — Thermal points taken with video equipment hooked up to a light microscope.
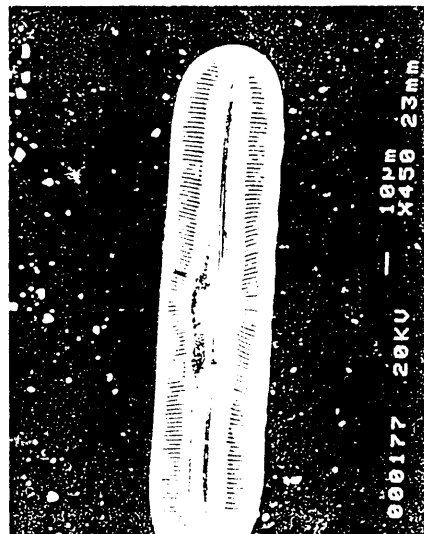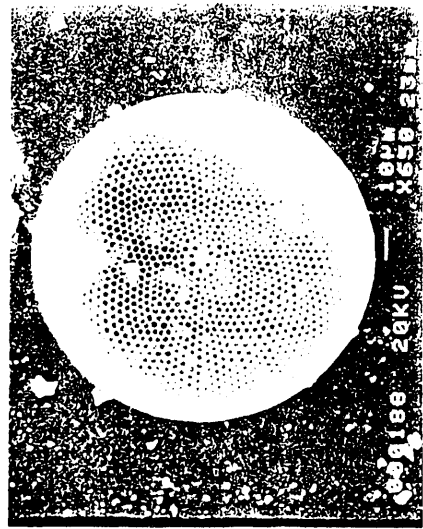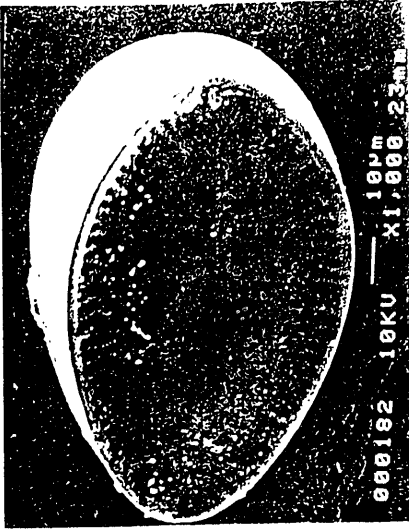
Figure 6. SEM SEI 45° photos
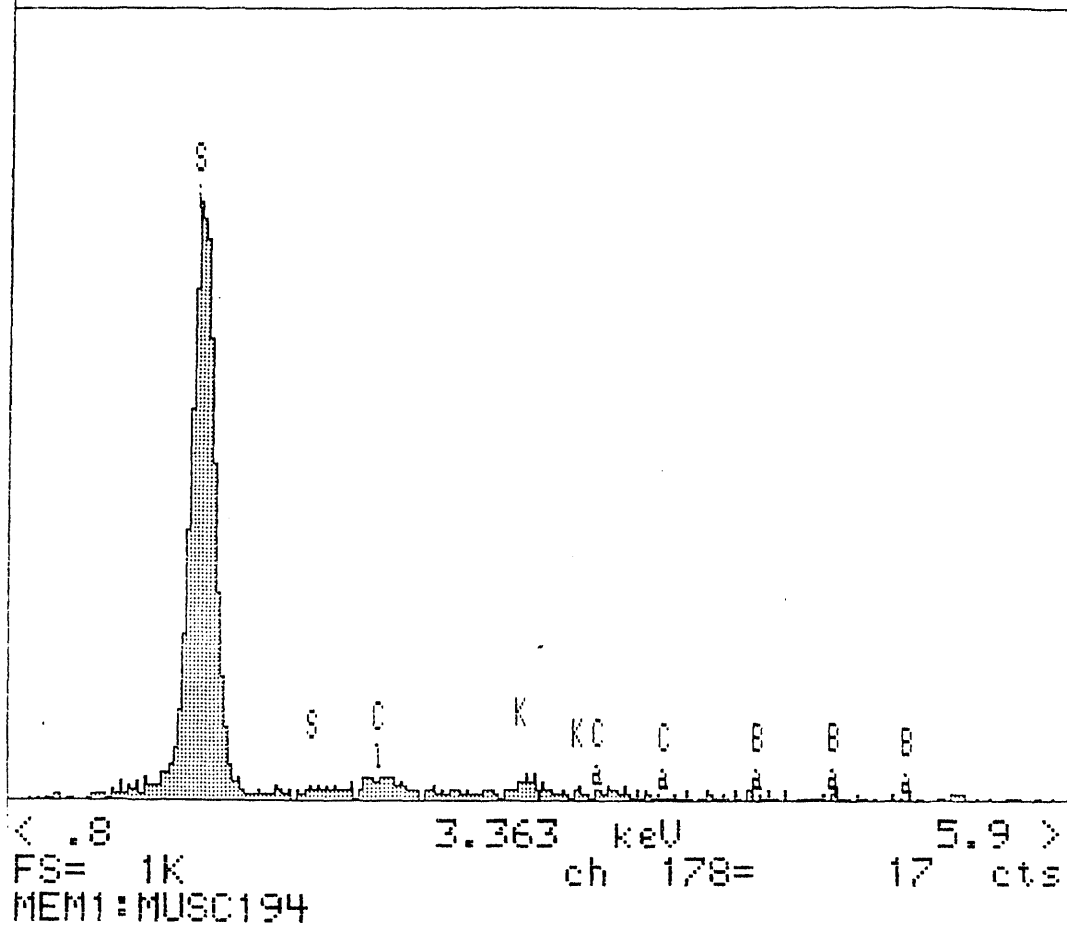
Figure 7. EDX data in graph form showing elemental composition of a diatom frustule. The most common elements are silicon, chlorine, potassium, calcium and barium.
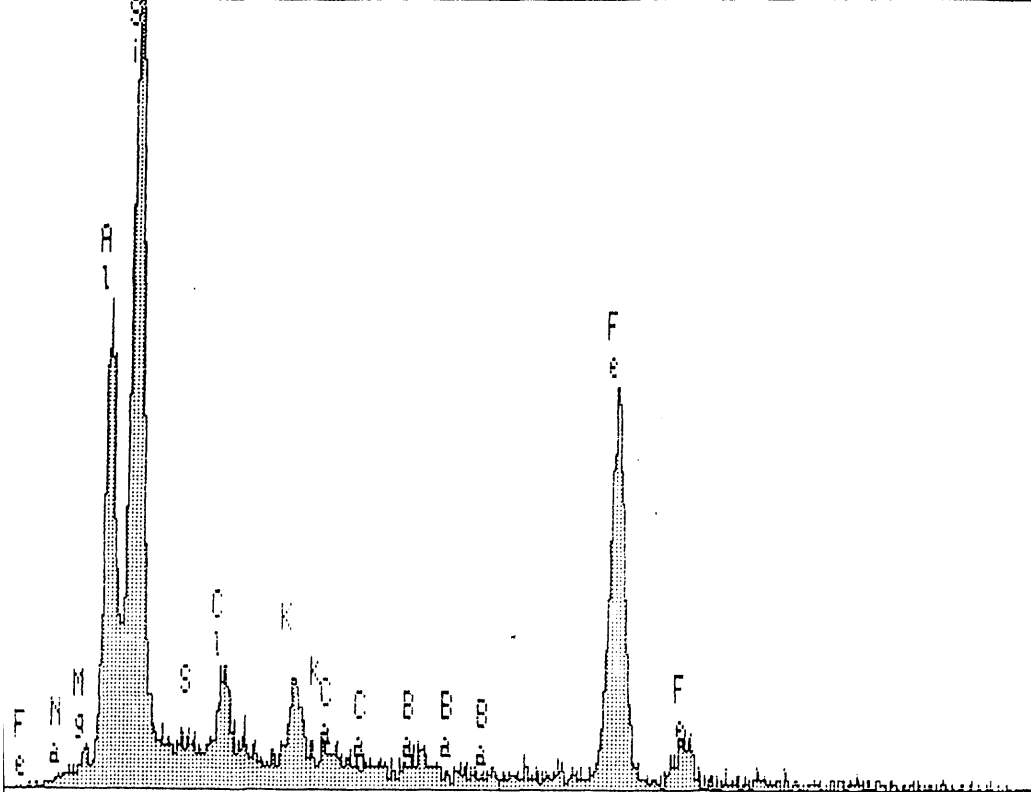
Figure 8. EDX data in graph form showing elemental composition of the material inside part of a diatom frustule. The most common elements are silicon, aluminum, and iron.

	7/21/93 SR1A 37	7/21/93 SR1B 37	7/21/93 SR1C 37	7/21/93 S1A 70	7/21/93 SR1B 70	7/21/93 SR1C 70	7/21/93 SR1A 120	7/21/93 SR1B 120	7/21/93 SR1C 120	7/21/93 SR1 TOT
MELOSIRA	14	9	10	3	9	10	14	10	11	90
PLEUROSIGMA	0	1	1	1	2	1	1	5	4	16
SURIRELLA	3	4	2	0	0	0	1	0	0	10
COSCINODISCUS	3	3	5	1	0	1	0	1	0	14
SYNEDRA	0	0	0	1	1	1	0	2	1	6
FRAGILARIA	0	2	0	4	0	4	0	1	0	11
CYCLOTELLA	0	1	0	0	1	0	0	0	1	3
TERPSINOE	0	0	2	1	1	0	4	1	0	9
NAVICULA	0	0	0	6	4	0	0	0	0	10
CLAVULARIA	0	0	0	2	1	2	0	0	0	5
NITZSCHIA	0	0	0	1	1	1	0	0	0	3
STARONEIS	0	0	0	0	0	0	0	0	1	1
ASTERIONELLA	0	0	0	0	0	0	0	0	2	2

A. Tallies of diatoms in water column samples from Savannah River sample site 1 were classified by genus.

	7/21/93 SR1 37	7/21/93 SR1 70	7/21/93 SR1 120	7/21/93 SR1 %
MELOSIRA	55%	37%	58%	50%
PLEUROSIGMA	3%	6%	16%	9%
SURIRELLA	15%	0%	2%	6%
COSCINODISCUS	18%	3%	2%	8%
SYNEDRA	0%	5%	5%	3%
FRAGILARIA	3%	13%	2%	6%
CYCLOTELLA	2%	2%	2%	2%
TERPSINOE	3%	3%	8%	5%
NAVICULA	0%	16%	0%	6%
CLAVULARIA	0%	8%	0%	3%
NITZSCHIA	0%	5%	0%	2%
STARONEIS	0%	0%	2%	1%
ASTERIONELLA	0%	0%	3%	1%

B. The percentage of each diatom genus in Savannah River sample site 1 was calculated from the data in table 1B.

Table 1

Savannah River Sediment Samples

SAMPLE #	RIVER	SITE	CORE LENGTH	SIEVE SIZE
1	Savannah	1	0-6"	120
2	Savannah	2	0-8"	120
3	Savannah	4	0-6"	120

Table 2

Savannah River and Cooper River Diatom % Comparison

	SR1 %	SR2 %	SR3 %	SR4 %	SR5 %	SR6 %	SR7 %	AVG.
MELOSIRA	50%	38%	42%	26%	28%	25%	22%	33%
PLEUROSIGMA	9%	18%	17%	16%	18%	24%	8%	16%
SURIRELLA	6%	3%	2%	6%	4%	3%	7%	4%
COSCINODISCUS	8%	15%	11%	12%	9%	9%	29%	13%
SYNEDRA	3%	9%	12%	9%	14%	11%	8%	9%
FRAGILARIA	6%	4%	1%	3%	0%	0%	1%	2%
CYCLOTELLA	2%	0%	2%	2%	3%	3%	4%	2%
TERPSINOE	5%	2%	1%	2%	1%	4%	0%	2%
NAVICULA	6%	8%	8%	11%	9%	8%	6%	8%
CLAVULARIA	3%	1%	4%	6%	7%	7%	3%	4%
NITZSCHIA	2%	1%	1%	0%	3%	2%	3%	2%
STARONEIS	1%	0%	1%	2%	1%	0%	3%	1%
ASTERIONELLA	1%	3%	0%	3%	1%	0%	1%	1%
CYMBELLA	0%	0%	1%	2%	0%	0%	0%	0%
EPITHEMIA	0%	0%	0%	1%	0%	0%	0%	0%
BIDDULPHIA	0%	0%	0%	0%	1%	2%	3%	1%
CHAETOCEROS	0%	0%	0%	0%	0%	1%	0%	0%
ACHNANTHES	0%	0%	0%	0%	0%	0%	1%	0%
RAPHONEIS	0%	0%	0%	0%	0%	0%	1%	0%
	CR 6/25%	CR 6/29 %						AVG.
MELOSIRA	28%	35%						32%
PLEUROSIGMA	2%	0%						1%
SURIRELLA	1%	0%						1%
COSCINODISCUS	3%	1%						2%
SYNEDRA	3%	3%						3%
FRAGILARIA	9%	18%						14%
CYCLOTELLA	22%	20%						21%
								0%
NAVICULA	15%	15%						15%
CLAVULARIA	2%	0%						1%
NITZSCHIA	10%	5%						8%
								0%
								0%
CYMBELLA	2%	0%						1%
								0%
								0%
CHAETOCEROS	0%	1%						1%
								0%
								0%
ODD	3%	0%						2%

Table 3

BACTERIAL PRODUCTION OF PSP TOXINS

by

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ABSTRACT

Paralytic Shellfish Poisoning (PSP) is a marine biotoxin syndrome caused by neurotoxins which are produced by unicellular algae known as dinoflagellates. These toxins act by blocking the voltage-sensitive sodium channels of the nervous system. This experiment tested whether the bacterium *Pseudomonas stutzeri*, isolated from the toxic dinoflagellate *Alexandrium lusitanicum*, was capable of autonomously producing PSP toxins. A defined minimal growth medium was developed for this bacterium using succinate as the carbon/energy source so that bacteria could be subjected to various types of nutritional stress. Several concentrations of growth limiting nutrient were tested in order to optimize the biomass yield of cultures grown under nitrogen or phosphorus stress. Using this information, large scale bacterial cultures were then grown under nutrient-replete, nitrogen-deplete, and phosphorus-deplete conditions. Finally, extracts from these cultures were tested for toxin using high performance liquid chromatography (HPLC) and a saxitoxin receptor binding assay. Extracts were analyzed for the presence of GTX1,2,3,4,and 5 using the HPLC. Samples tested positive for the presence of GTX1,2,4,and 5. The bacterium *Pseudomonas stutzeri* appears to be able to autonomously produce at least some of the PSP toxin derivatives, based on HPLC analysis. The GTX composition of *P. stutzeri* grown under different nutrient conditions with succinate as a carbon/energy source appears to be relatively constant. The experimental design used during this project proved to be a satisfactory approach to the study of PSP toxin production by bacteria associated with toxic dinoflagellates.

INTRODUCTION

The neurotoxins which cause Paralytic Shellfish Poisoning (PSP) are produced by a group of unicellular algae called dinoflagellates (for review, see Hall et al. 1990). When certain species of marine dinoflagellates bloom, filter-feeding bivalves become toxic by ingesting the algae as a source of food. Bivalves are not greatly harmed by the accumulation of PSP toxins. However, human poisoning results when bivalves containing sufficiently high levels of these toxins are consumed. The PSP toxins, comprised of saxitoxin and its derivatives, act by blocking voltage sensitive sodium channels of the nervous system.

One group of researchers has demonstrated that bacteria isolated from toxic strains of the dinoflagellate *Alexandrium tamarense*, can autonomously synthesize PSP toxins (Kodama 1990). However, their measurements of bacterial toxin production were at levels far below those documented for dinoflagellates. Other workers, unable to detect toxins in bacterial extracts, have suggested that the interference of intracellular bacteria in the metabolism of dinoflagellates mediates the algal toxicity (Silva 1990). Given this range of opinions, the question of bacterial involvement in PSP toxicity remains a very contentious issue.

The hypothesis tested was that the bacterium *Pseudomonas stutzeri*, isolated from the toxic dinoflagellate *Alexandrium lusitanicum*, can autonomously produce PSP toxins. The approach taken involved growing bacterial cultures in artificial medium under conditions of nutritional stress. Since it is known that PSP toxin production by dinoflagellates is enhanced

under such conditions (Anderson et al. 1990), we anticipated that the bacteria would exhibit a similar response. As a result, our ability to detect these toxins would be optimized. In addition, the growth rate of nutrient-limited bacteria would be comparable to that of the associated dinoflagellate, and would thus be more representative of natural conditions than growth rates in a rich nutrient broth.

Manipulation of bacterial nutrient physiology requires the use of a defined growth medium so that the concentrations of the growth-limiting nutrients can be precisely determined. Therefore, a defined minimal growth medium was first developed for the cultivation of *P. stutzeri*. This growth medium provided a means by which the bacteria could be subjected to various types of nutritional stress. Second, several concentrations of growth-limiting nutrients were tested in order to optimize the biomass yield of cultures grown under conditions of nitrogen or phosphorus stress. Third, large scale bacterial cultures were grown under nutrient-replete, nitrogen-deplete, and phosphorus-deplete conditions, providing large quantities of material for toxin extraction. Finally, extracts from the bacterial cultures were tested for the presence of toxin using the high performance liquid chromatography (HPLC) and a saxitoxin receptor-binding assay.

MATERIALS AND METHODS

Experimental organism: The bacterial strain used in this study, *Pseudomonas stutzeri* (strain SF2P) was isolated from the PSP-producing dinoflagellate *Alexandrium lusitanicum* (strain 18-1; culture collection of S. Franca, Inst. Nacional de Saude, Lisboa, Portugal), and was a gift of S. Franca. The bacterial isolation techniques used were designed to eliminate all bacteria external to the dinoflagellate cells. Therefore, this strain of *P. stutzeri* is presumed to have originated from within the dinoflagellate cells.

Experimental growth and maintenance: Bacterial cultures were maintained at 20°C on agar slants based on a complete seawater medium (SWC; Haygood and Nealson 1985). All experiments used an artificial seawater-based medium (ASW; Haygood and Nealson 1985). Both SWC and ASW media were modified from the original formulation and their composition is given in Tables 1 and 2, respectively.

In order to identify a suitable carbon/energy (C/E) substrate for the growth of *P. stutzeri*, 3mL cultures of ASW medium containing one of 17 substrates were inoculated with cells growing exponentially on SWC. Equimolar concentrations of carbon (20mM) were achieved by adjusting the amount of C/E source added. Figure 1 shows the substrates tested. Two controls, one receiving no C/E source addition and one of SWC were included. Maximum growth rates were calculated based on changes in optical density (OD) measured at 660nm over the exponential phase of growth, and using the following equation :

Growth Rate (doublings/day)= ((log (N1/N0)) x 3.322) / (T1 - T0),

where $N1 = OD_{660}$ at time 1 (i.e. T1)

$$N0 = OD_{660} \text{ at time } 0 \text{ (i.e. T0)}$$
$$T1 = \text{time 1 (in days)}$$
$$T0 = \text{time 0 (in days)}.$$

A Bausch & Lomb Spectronic 21 spectrophotometer was used to measure OD. Based on the results of this experiment and the desire to produce data comparable to those obtained previously for bacterial strains isolated from other toxic dinoflagellates, succinate was chosen as the C/E source for the remaining experiments.

  Bacterial cultures (3mL) used in nutrient depletion experiments were grown in 13x100 mm Fisherbrand Borosilicate Disposable Culture Tubes in triplicate under conditions of either nitrogen or phosphorus depletion. The following nitrogen concentrations were tested: 0, 200, 400, 600, 800, 1200, and 1600uM. The phosphorus concentrations tested were: 0, 1, 2, 4, 8, 16, and 32uM. ASW containing full nitrogen and phosphorus enrichments (=Replete) and SWC cultures were used as controls. Tests were done with and without 50mM Hepes buffer (pH 7.4) additions. Culture tubes were maintained in Dubnoff Metabolic Shaking Incubator at 20°C. Bacterial growth was measured at 12 hour intervals by taking OD readings as described above.

  Large scale bacterial cultures (300mL) were grown in 500mL Bellco flasks under nutrient-replete, nitrogen-deplete, and phosphorus-deplete conditions. Cultures were maintained in New Brunswick Scientific Innova 4330 Refrigerated Incubator Shaker at 20°C and 175 rpm. Growth was monitored daily as previously described. Nutrient-replete cultures were harvested during the log phase of growth. Phosphorus- and nitrogen-deplete cultures were harvested at or near the stationary phase of growth.

  At the time of harvesting, triplicate 0.5mL samples were collected for total cell protein analysis. Protein samples were spun in an IEC Centra MP4R centrifuge at 12,000 rpm for 12 min at 4°C. After centrifugation, protein samples were covered with parafilm, holes were punched for gas exchange, and samples were stored at -20°C until lyophilized. The remaining bacterial culture was harvested by centrifuging 250mL for 15 min. at 10°C at a force of 12,000 x g. Bacterial pellets were resuspended in 0.5mL of 0.05N glacial acetic acid. Samples were transferred to 2mL screw-capped tubes and stored at -20°C until extraction.

  Toxin extractions were done by splitting each sample into four tubes, each containing 600uL of 0.05N acetic acid. Zirconium beads (0.1mm diam.) were added to each tube. Cells were disrupted by shaking the tubes in a Biospec Products Mini-Bead Beater five times for 45 seconds each. Holes were punctured in the bottom of each tube with a hot needle, and tubes were inserted into 1.5mL microcentrifuge tubes. Samples were spun for 20 minutes at 2500 rpm in an IEC Centra-7 centrifuge in order to separate the cell homogenate from the beads. Bead-beater tubes were removed from the microcentrifuge tubes. The microcentrifuge tubes were then spun again for 15 min. at 8°C and 12,000 rpm in an IEC Centra MP4R centrifuge to remove cell debris. The supernatants were pooled and placed in a 0.5mL microcentrifuge tube and stored at 4 °C until analyzed. Extracts prepared in this way were first passed over a Sep-pak C_{18} cartridge (Millipore Corp.) to clean the sample, and then analyzed for PSP toxins according to the method of Oshima et al. (1988). Standards for the analysis and

quantification of these toxins were kindly provided by Y. Oshima (Tohoku University, Japan). Toxin extracts were also tested using a saxitoxin receptor binding assay being developed at the National Marine Fisheries Service laboratory by the Marine Biotoxins Group. Briefly, any toxin present in a sample competes against a known concentration of radiolabeled saxitoxin for toxin receptor binding sites present in a rat brain synaptosome preparation. Low radioactivity in the final synaptosome preparation (i.e. after incubation and washing) therefore reflects the presence of high (unlabeled) toxin concentrations in the sample, and vice versa.

RESULTS

Carbon/energy substrates. Growth of *Pseudomonas stutzeri* on various carbon/energy substrates is shown in Figure 1. The highest maximum growth rate was observed with the SWC medium. As a group, the organic acids succinate, malate, fumarate, α-ketoglutarate (αKG), and citrate represented the substrates most favorable for growth in ASW-based media. Other compounds tested supported far lower growth rates or no growth.

Nutrient depletion. The growth of *P. stutzeri* with nitrogen as the limiting nutrient, in the absence of buffer, is shown in Figure 2. As nitrogen concentration increased, a higher biomass yield was observed. The final yield for the 1600 µM N culture was over five times that of the 200 µM N culture. By comparison, the biomass yield increased only about 1.5 times as the phosphorus concentration increased from 1 to 16 µM (Figure 3). In both of these cultures, a precipitate developed that made it difficult to measure the OD. The pH of the cultures was measured at the end of the experiment and found to be approximately 9. Since the pH of seawater is closer to 8, it was decided that the medium should be buffered in order to minimize the increase in pH. A test was done to determine if buffer (50 mM Hepes, pH 7.4) could be added to the cultures without adversely affected growth (Figure 4). In SWC and in ASW without buffer, the growth rates were similar. However, the growth rate in ASW with buffer slowed somewhat compared to the other two media after the exponential phase of growth. Bacteria in all three media demonstrated the same yield.

Following the test of buffer effects on growth (Figure 4), curves for nitrogen- and phosphorus-deplete cultures with 50mM Hepes buffer (pH 7.4) additions were performed. As previously, the biomass yields increased directly with nitrogen and phosphorus concentration (Figures 5 and 6). More importantly, the yields of both the nitrogen- and phosphorus-deplete cultures with buffer were greater than those without the buffer addition, with this difference being most pronounced under phosphorus depletion. The P-deplete cultures with buffer also showed a far greater difference in biomass yield as a function of increasing phosphorus concentration when compared to those cultures without buffer.

Toxin production. From the data shown in Figures 5 and 6, nitrogen and phosphorus concentrations were chosen for growing large scale nitrogen- and phosphorus-deplete cultures. Figure 7 shows the growth of the large scale toxin production experiment. The final data points for each curve represent the time at which the respective cultures were harvested. The highest growth rate was observed in the nutrient-replete culture. The 16uM P culture produced a higher yield than either of the 4uM P cultures. The yield of the 4uM P culture

with the arginine addition was the same as that without arginine. The 400uM N culture produced a slightly lower yield than the phosphorus-deplete cultures. A representative chromatogram of the HPLC analysis of toxin extract from the nutrient-replete culture and a trace of the corresponding analytical standard are presented in Figure 8. Time permitted only the analysis of toxins GTX1, 2, 3, 4, and 5, and these data are summarized in Table 3. In all cultures, irrespective of nutritional status, GTX5 was the predominant toxin, accounting for approximately 95% of the GTX toxins present. The relative abundance of GTX4 varied between treatments, and was highest in the nitrogen-deplete culture (near 6%). Toxins GTX1 and GTX2, when present, represented less than 1% of the total GTX toxins. Results of the saxitoxin receptor binding assay (data not shown) were negative for all samples tested.

DISCUSSION

The most important observation to result from the present study is that *Pseudomonas stutzeri* appears to be capable of autonomous production of at least some of the PSP toxin derivatives, as determined by HPLC analysis. This work represents only the second HPLC-based confirmation of toxin synthesis by a bacterium associated with a PSP-producing dinoflagellate. Although the actual amounts of toxin produced by bacteria under different nutrient conditions was not determined, toxins in the extracts were easily detectable and frequently present in substantial quantities. Also, there did appear to be little variation in the GTX toxin composition between experimental treatments.

Since the actual production of PSP toxins by bacteria remains a contentious issue, the HPLC analysis of bacterial extracts warrants specific consideration. A set of chromatograms for the nutrient-replete culture is given in Figure 8. Also shown are traces of the toxin standards used to quantify the experimental run. The HPLC method employed requires three separate analyses to detect the twelve PSP toxin derivatives contained in the standard mixtures. While only one of these analyses was performed (that for GTX toxins), the cumulative results for all samples (Table 3) appear to show the presence of four of the five GTX toxin derivatives. The retention times of the sample peaks correspond either identically to, or are separated at most by 0.5 min from, the associated standard peak. However, it must be pointed out that these data do not represent unequivocal proof of PSP toxigenesis by this bacterium, and can only be considered as preliminary until other analytical methods, such as mass spectroscopy, can be used to confirm these findings.

The results obtained during this work demonstrate the usefulness of the experimental approach employed. The development of a defined minimal growth medium not only permitted growth of the bacteria under conditions more similar to those found in natural seawater, but also allowed the manipulation of bacterial nutritional status. In addition, the organic acids tested as potential carbon/energy sources represented the substrates most favorable for growth in ASW-based media. These organic acids are intermediates of the Krebs cycle, and from which arginine, a biosynthetic precursor of the PSP toxins, is derived. For this reason it is desirable for future work to examine the remaining organic acids as growth substrates for their effects on toxin production..

All samples tested negative for toxin by the saxitoxin receptor binding assay. This competitive binding assay relies on the toxins being active and able to bind to rat brain synaptosomes at the sodium channel. The negative results may be due to the toxin being present, but in an inactive form. Another possibility is matrix effects of the bacteria on the toxin or the receptor. The samples tested may not have been concentrated enough for the toxin to be detected. Further concentrating the samples may result in a positive test for toxin. The possibilities of matrix effects will be looked at further in future research.

Although the buffer addition may have caused a slight decline in growth rate following the exponential phase of growht. for the slight slowing after the exponential phase of growth of the ASW with buffer culture, all cultures demonstrated the same yield. Therefore, buffer was added to all cultures. The buffer alleviated the precipitate problem by preventing a rise in pH, allowing the cultures to grow for a longer time period and achieve a greater yield for use in toxin tests.

There are several areas that remain to be investigated by future research. The extracts need to be tested for the remaining saxitoxin g derivatives using the HPLC, and toxin content of the extracts needs to be normalized to total cellular protein of the culture samples in order to compare the toxin content of the different samples. Also, the entire experiment can be repeated using the remaining organic acids of the Krebs cycle as C/E sources to determine whether they have an effect on toxin production.

In summary, the bacterium *Pseudomonas stutzeri* appears to be capable of autonomous production of at least some of the PSP toxin derivatives, based on HPLC analysis. This is important because this work represents only the second HPLC-based confirmation of toxin synthesis by a bacterium associated with a toxic dinoflagellate. The GTX composition of *P. stutzeri* grown under different nutrient conditions with succinate as a C/E source appears to be relatively constant. Finally, the experimental design used during this project proved to be a satisfactory approach to the study of PSP toxin production by bacteria associated with toxic dinoflagellates.

This is a standard resolution test target (USAF/NBS style) with resolution bars and numerical markings: 1.0, 1.1, 1.25, 1.4, 1.6, 1.8, 2.0, 2.2, 2.5, 2.8, 3.2, 3.6, 4.0, 4.5, 5.0, 5.6, 6.3, and similar values.
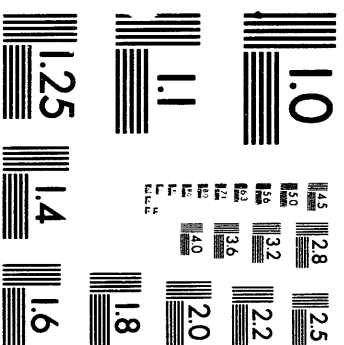
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Table 1. Composition of SWC medium.

Reagent	weight or volume/L
Tryptone	5 g
Yeast Extract	3 g
Glycerol (50%)	6 mL
Natural Seawater (30ppt--Vero Beach, FL)	1 L
Agar (1%); used only for slants	10 g

Table 2. Composition of ASW medium.

Reagent	Concentration
Sodium chloride	265 mM
Potassium chloride	10.06 mM
Magnesium sulfate heptahydrate	50.13 mM
Calcium chloride dihydrate	19.7 mM
Ammonium chloride	18.7 mM
Sodium phosphate dibasic	0.46 mM
Ferric sequestrene	54.58 uM
Sodium bicarbonate	2 mM

Table 3. Concentrations (μM) of PSP toxins GTX1, GTX2, GTX4, and GTX5 in extracts of *Pseudomonas stutzeri* (strain SF2P) cultures grown under different nutritional regimes. The relative abundance of each toxin (i.e. percent composition) is also given for purposes of comparing the response to different experimental treatments. See text for further explanation.

Sample	GTX1	GTX2	GTX4	GTX5
Replete	0.019 (0.2%)	0	0.311 (2.9%)	10.356 (96.9%)
Nitrogen Deplete	0	0	0.024 (6.1%)	0.369 (93.9%)
Phosphorus Deplete (4uM)	0	0	0.044 (4.2%)	1.003 (95.8%)
Phosphorus Deplete (4uM)+ Arginine	0	0.004 (0.2%)	0.043 (2.4%)	1.748 (97.4%)
Phosphorus Deplete (16uM)	0	0.003 (0.2%)	0.055 (2.9%)	1.800 (96.9%)

GROWTH OF *PSEUDOMONAS STUTZERI* ON CARBON/ENERGY SUBSTRATES
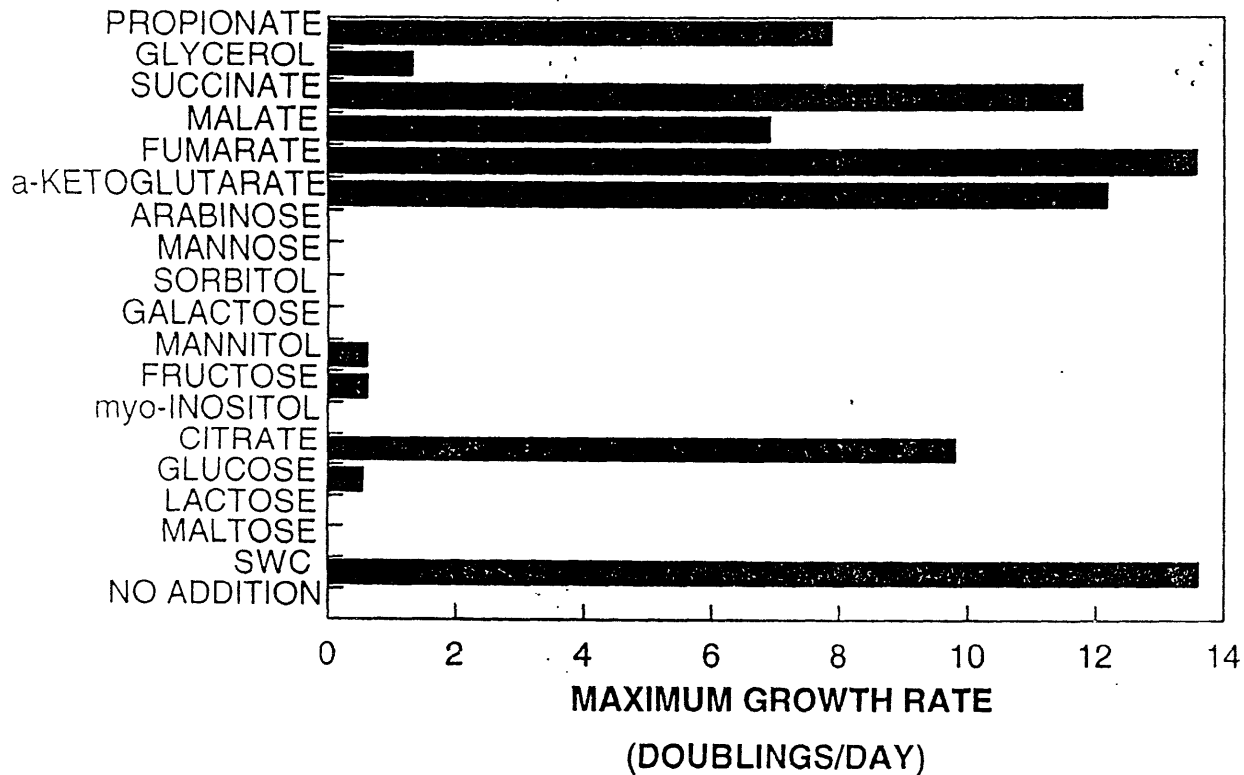
Figure 1. Maximum growth rates of the bacterium *P. stutzeri* in ASW-based media each containing one carbon/energy source. A culture with no **carbon/energy source** and **SWC medium were** included as controls.

NITROGEN DEPLETION / WITHOUT BUFFER
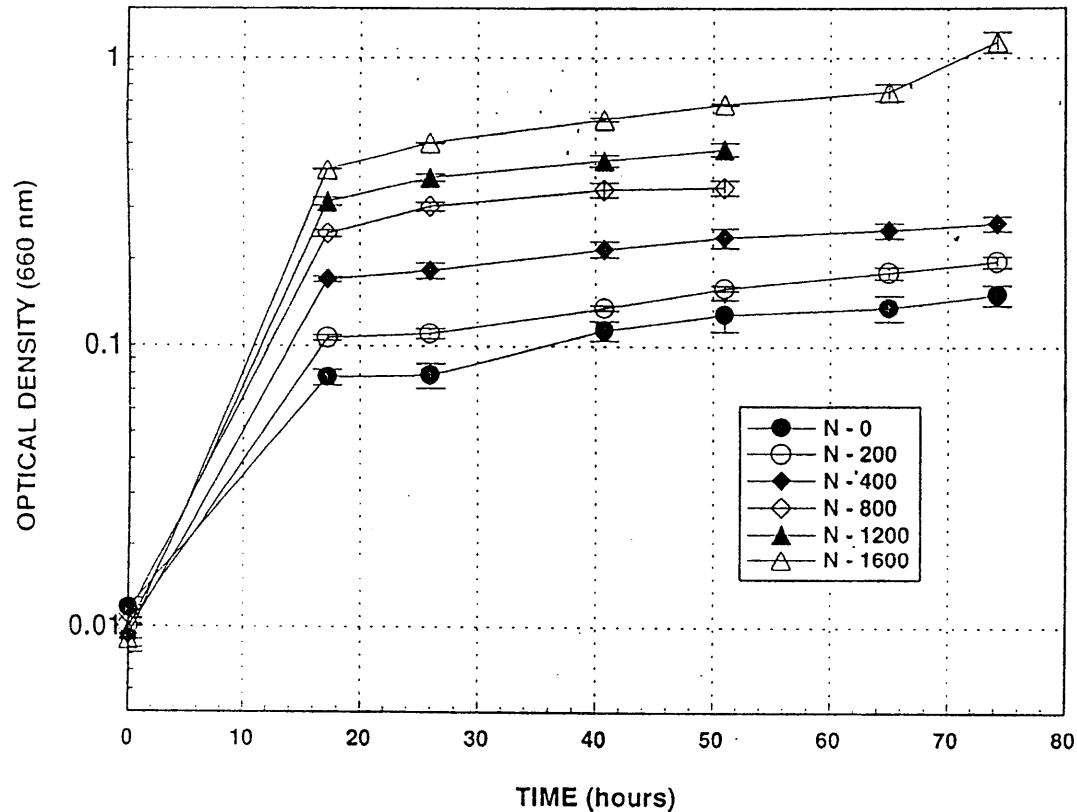
Figure 2. Growth curves for the bacterium *P. stutzeri*. Cultures were grown under nutrient replete conditions except for the level of nitrogen. Each curve represents a different nitrogen concentration: 0, 200, 400, 800, 1200, and 1600 uM. No buffer was added to the cultures.

PHOSPHORUS DEPLETION / WITHOUT BUFFER
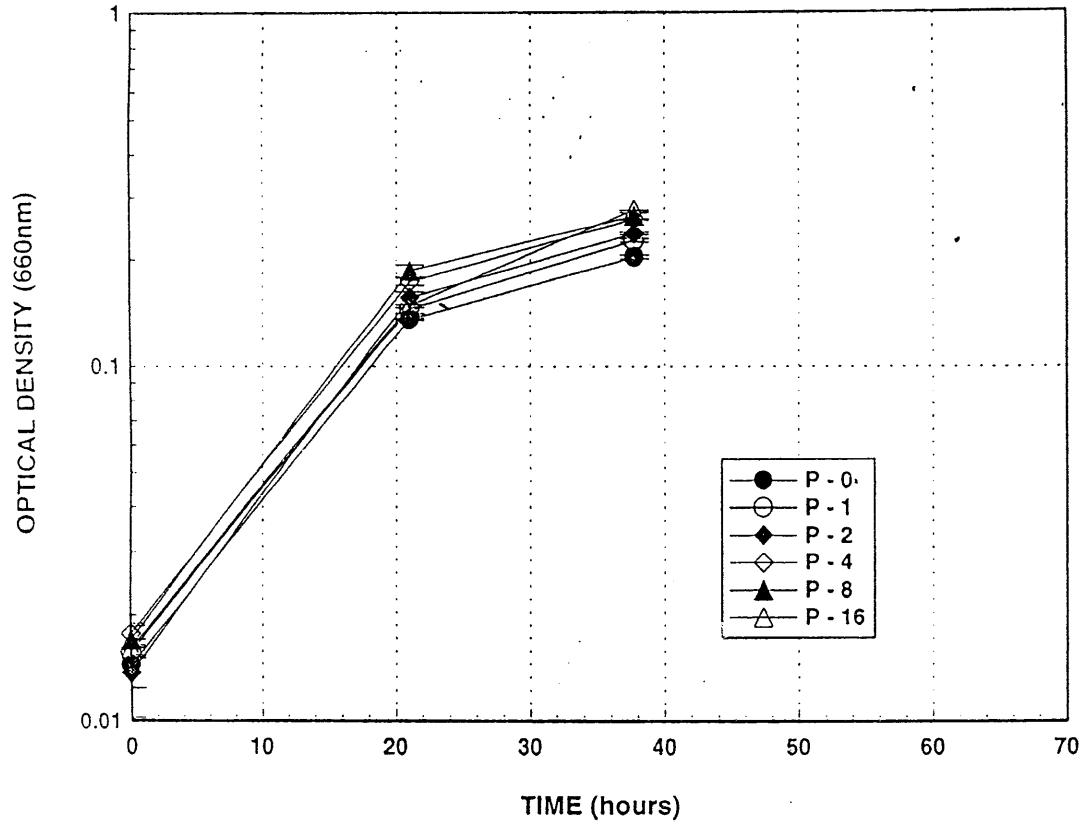
Figure 3. Growth curves for the bacterium *P.stutzeri*. Cultures were grown under nutrient replete conditions except for the level of phosphorus. Each curve represents a different phosphorus concentration: 0, 1, 2, 4, 8, and 16 uM. No buffer was added to the cultures.

EFFECT OF HEPES BUFFER ON BACTERIAL GROWTH
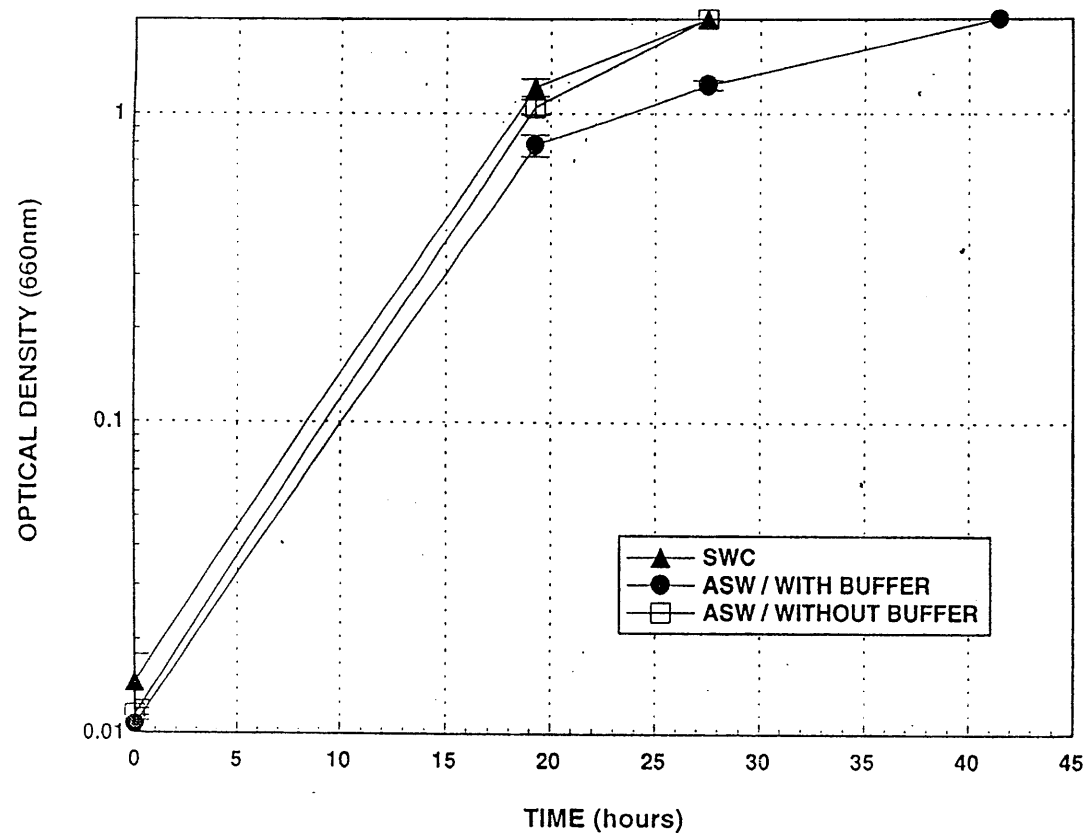
Figure 4. Growth curves for the bacterium *P. stutzeri*. This experiment compared growth in ASW with and without 50mM Hepes buffer(pH 7.4). An SWC culture was included as a control.

NITROGEN DEPLETION / WITH BUFFER
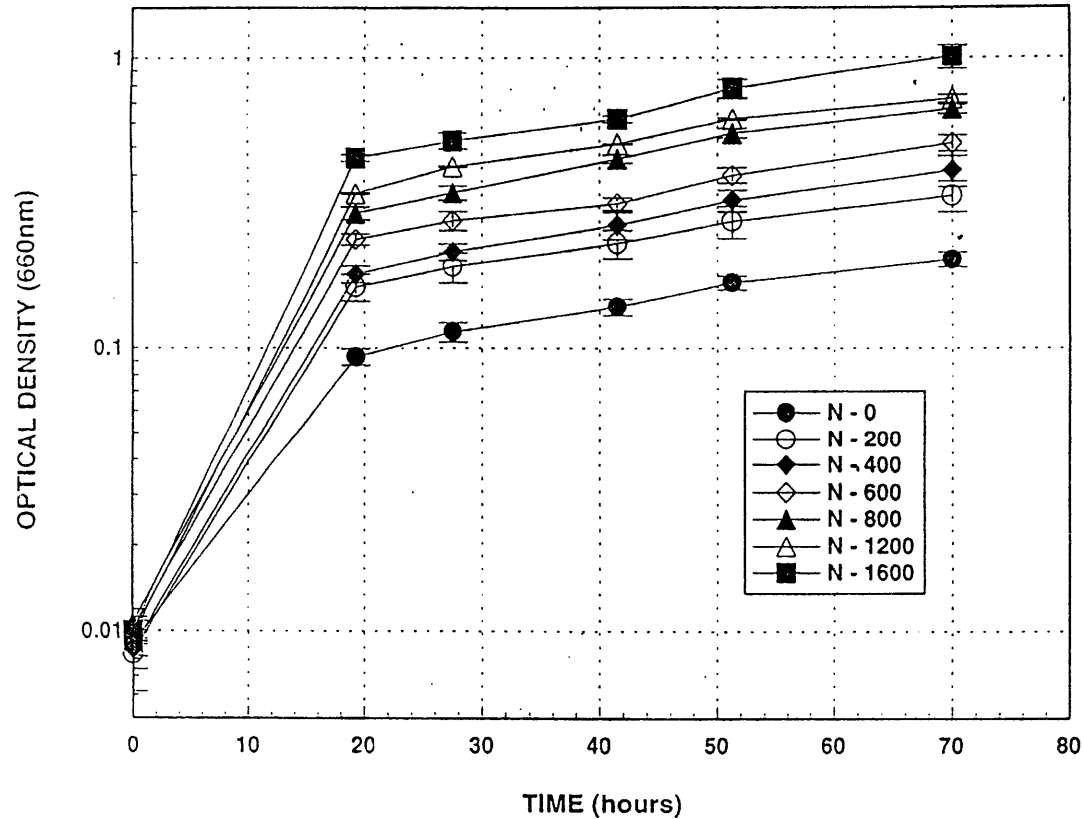
Figure 5. Growth curves for the bacterium *P. stutzeri*. Cultures
were grown under nutrient replete conditions except for the level
of nitrogen. Each curve represents a different nitrogen
concentration: 0, 200, 400, 600, 800, 1200, and 1600 uM. 50mM
Hepes buffer(pH 7.4) was added to all cultures.

PHOSPHORUS DEPLETION / WITH BUFFER
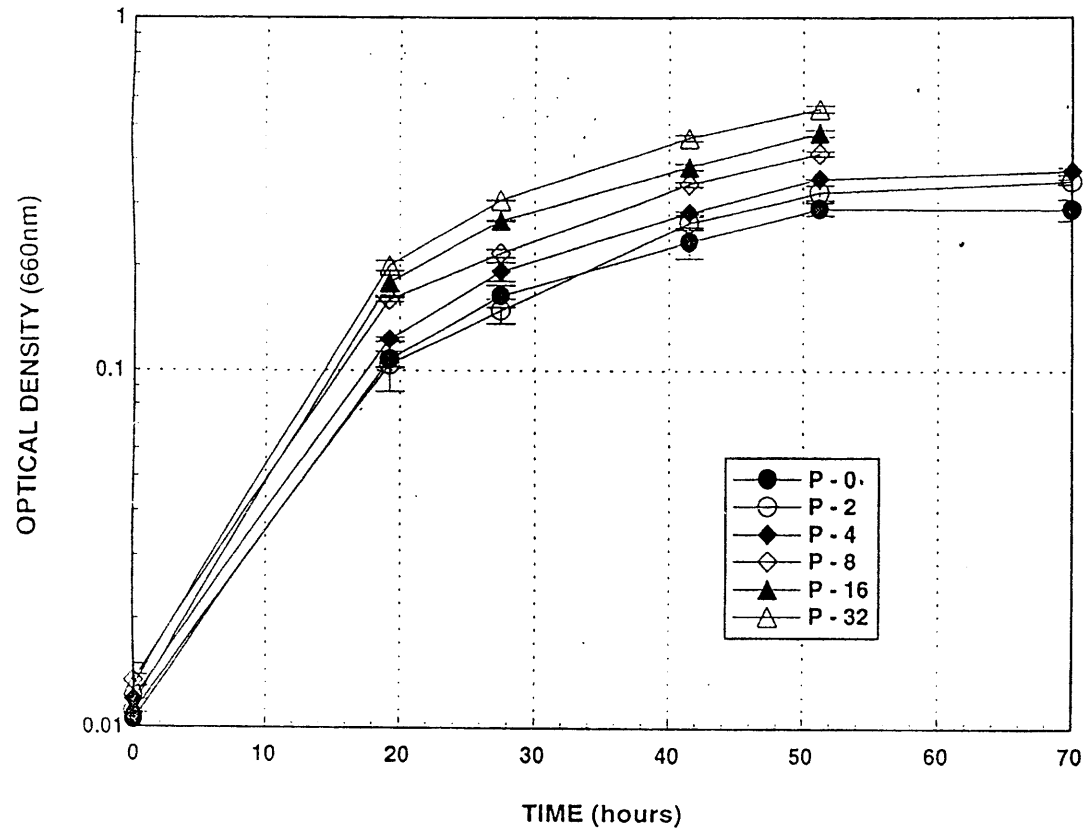
Figure 6. Growth curves for the bacterium *P. stutzeri*. Cultures were grown under nutrient replete conditions except for the level of phosphorus. Each curve represents a different phosphorus concentration: 0, 2, 4, 8, 16, and 32 uM. 50mM Hepes buffer (pH 7.4) was added to all cultures.

TOXIN PRODUCTION EXPERIMENT
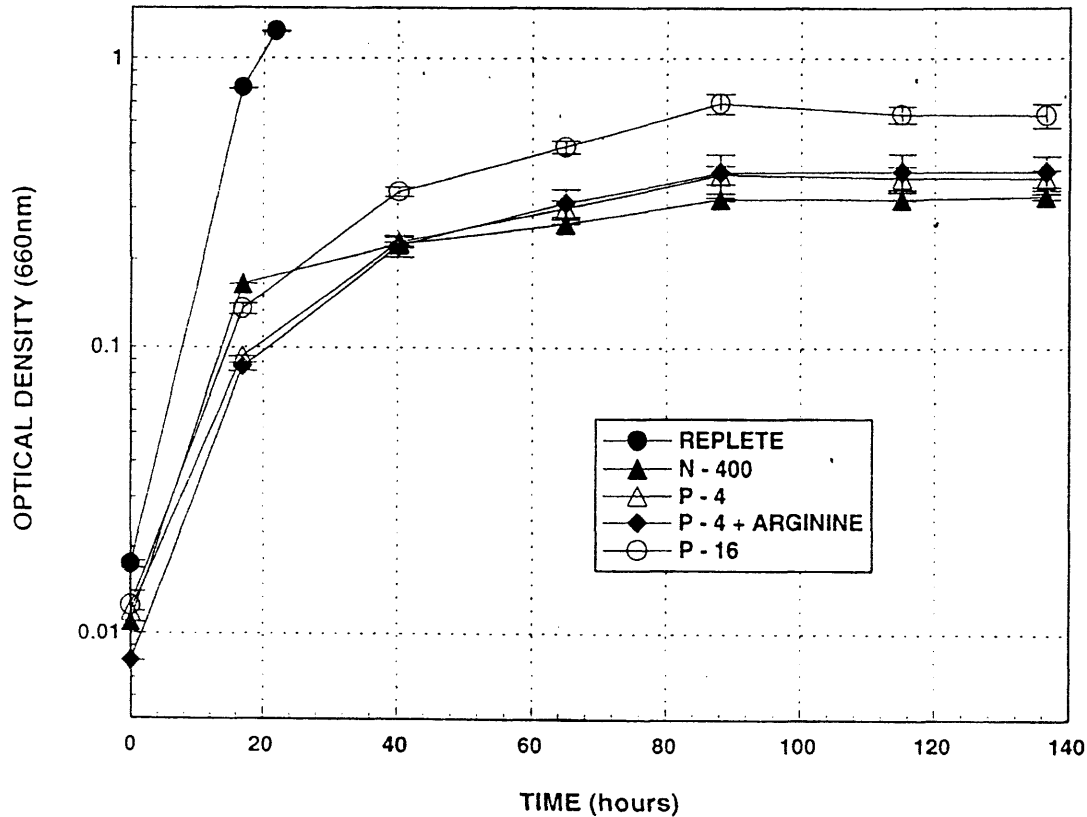
Figure 7. Growth curves for large scale cultures of the bacterium
P. stutzeri. One culture was grown under nutrient replete
conditions to serve as a control. The remaining four cultures
were grown into either nitrogen or phosphorus depletion. Each
curve represents a culture with a different concentration of a
growth-limiting nutrient. The concentrations of deplete nutrient
were as follows: 400uM N, 4uM P, 4uM P + 50uM Arginine, and 16uM
P.
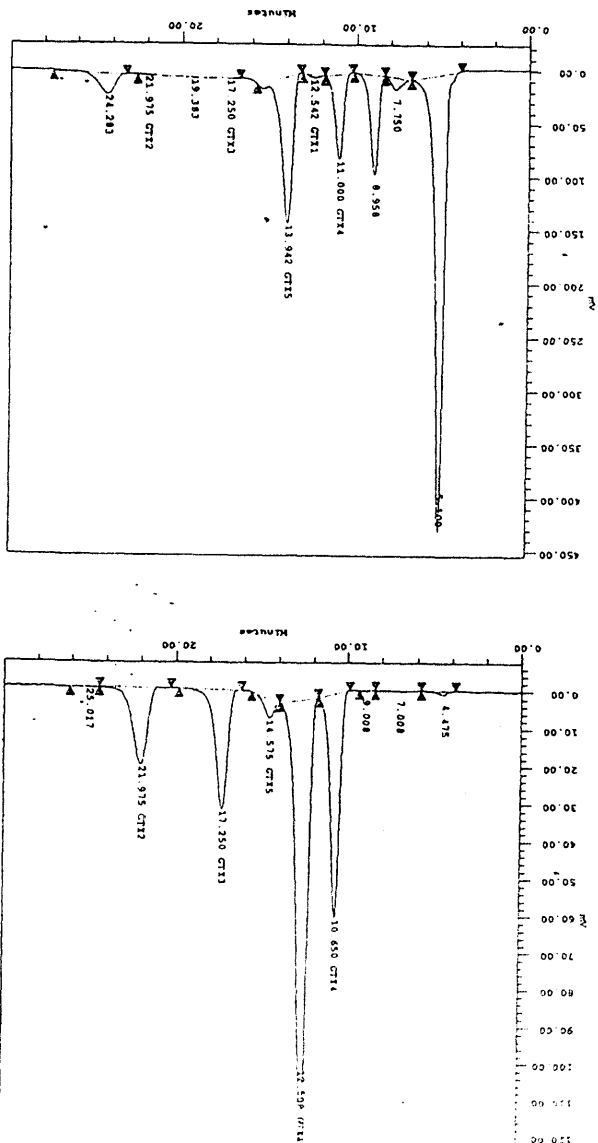
GROWTH INHIBITION IN GH4C1 CELLS:

EFFECTS OF MTX AND TGFα

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Cell proliferation is controlled by entry into and the rate of progression through the cell cycle. In somatic cells, the G1/S phase transition is one of the highly regulated transition points. It is currently believed that passage through the restriction point is dependent on the activation of the CDC2 protein kinase. MTX is a calcium channel activator, and in most eukaryotic cells an increase in intracellular calcium usually stimulates progression through the cell cycle at the G1 to S transition. However, in the GH4C1 cells it is a growth inhibitor. TGFα similarly inhibits cell proliferation at the G1 to S transition by means that are not fully understood. Therefore, investigations of the effects of these treatments in the G1 phase have been carried out.

INTRODUCTION

Cell proliferation is controlled by the entry into and rate of progression through the cell cycle. The cell cycle consists of four major phases: G1, S, G2, and M. The G1 phase is a time of intensive biochemical activity. The cell increases in size as its enzymes, ribosomes, mitochondria and other intercellular molecules and structures also increase in number. In early G1, the cell may opt to differentiate and enter G0 where the cell is in mitotic arrest. Entrance back into G1 represents reentry into the proliferative state. Just prior to the G1/S transition, the cell passes through the restriction point that commits it to replicate its DNA and allows it to continue on with the cell cycle. The key process of DNA replication occurs during the S phase of the cell cycle, a period in which many of the histones and other DNA proteins are also synthesized. During the G2 phase, the final preparations for cell division occur. The newly replicated chromosomes begin to condense which is necessary for the separation of the chromosomes that will occur in mitosis. In the M phase, the condensed chromosomes are arranged so that each new cell gets a full complement, one of each, forming what is known as the spindle. This process is divided into four phases: Prophase, metaphase, anaphase, and telophase. Finally, cytokinesis, the division of the cytoplasm forming two daughter cells, takes place.

In somatic cells, the G1/S phase transition is the most highly regulated transition point. It is currently believed that passage

through the restriction point is dependent on the activation of cell cycle regulatory protein kinases, CDC2 and/or CDK2. Treatments which stimulate or inhibit progression through the restriction point ultimately alter the activity of CDC2 and/or CDK2 prior to the G1/S transition; therefore, either activating or inhibiting the activity of cell cycle regulatory protein kinases. Calcium is an intracellular signal which has been demonstrated in most cell types to activate progression through the G1/S transition. In a few cell types calcium acts as an inhibitor at the G1/S transition; however, the signalling mechanism by which calcium modulates the G1/S transition is not well understood.

The GH4C1 cells utilized in this experiment are inhibited from progression through G1/S by agents which activate calcium signalling. In this study, we conducted some initial investigations to determine where in the G1 phase Maitotoxin, a calcium channel activator, inhibits cell cycle progression using flow cytometry and measurement of CDC2 and CDK2 activity.

EXPERIMENTAL PROCEDURES

Methods

Cell culture - GH4C1 cells were maintained at 37°C in F10+ medium (Hams F10 nutrient mixture supplemented with 2.5% fetal bovine serum and 15% horse serum in the absence of antibiotics. The cells were routinely passaged at weekly intervals to maintain their exponential growth, for a maximum of 10 passages. In order to

harvest cells, stock cultures were treated with 0.02% EDTA-PBS to detach cells from the dishes.

Cell synchronization - Cells were plated at a concentration of 0.5 x 106 cells per 100 mm dish with 8 ml of F10 medium (serum free) and then were incubated for at least 36 hours at 37°C (this caused the cells to be blocked in early G1). The cells were then released by removal of the serum free medium and subsequent addition of 8 ml of the F10+ medium. Synchronized cells were compared to asynchronized cell using flow cytometry.

Cell cycle analysis - Synchronized cells were released and were allowed to incubate for 22-24 hours with no treatments, 3 ng/ml MTX, or 10 ng/ml TGF-α which were added at various times. At the end of the incubation period, the cells were harvested and fixed with -20°C methanol. Cells were then pelleted by centrifugation at 1000 x g for 5 min, and resuspended in PBS containing 0.5% Tween 20, 1mg/ml heat treated RNase and 10 ug/ml propidium iodide (PI) for 15 min. Cell cycle distribution was analyzed on a Coulter Epics 753 flow cytometer using a 5W Argon laser at 488 nm excitation and 635 nm emission wavelengths. Cell aggregates were eliminated by gating all histograms within the linear range using a peak-area cytogram for PI fluorescence. Cell cycle distribution was analyzed using the "Multicycle" software program (Phoenix Flow Software, San Diego, CA).

Histone Kinase Assay - Histone kinase activity was measured by the

method of Van Dolah/Ramsdell. Synchronized cells were released in F10+ and allowed to incubate for 36 hours with no treatment, 3 ng/ml MTX, 10 ng/ml TGF-α, or 300 uM minosine. At the end of the incubation period, cells were harvested, pelleted by centrifuging at 1000 x g for 5 min, and then the supernatant was removed. Lysates were then prepared by addition of 100 ul lysis buffer [50 mM Hepes, pH 7.4, 0.1% Triton X100, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 50 mM NaF, 80 mM b-glycerophosphate, 0.1 mM Na^{3}VO^4, 1 ug/ml leupeptin, aprotinin 1:100 stock]. Lysates were then vortexed vigorously and incubated on ice for 15 minutes. Histone kinase activity specific to CDC2 or CDK2 kinase was immunoprecipitated from 150 ug protein from each lysate. For immunoprecipitation, 1 ug antibody was incubated with 30 ul IgG Sorb for 1 hour at 4°c in 30 ul lysis buffer. The capsules were pelleted, rinsed twice with 50 mM Hepes, pH 7.4, 150 mM NaCl, and 0.1% Triton X100, and then 150 ug lysate added and incubated for 3 hours on a rotator at 4°C. The capsules were again washed twice with 50 mM Hepes, pH 7.4, 1 mM dithiothreitol and then combined with a reaction mixture containing 40 ug Histone H1, 50 UM ATP, 10 mM uCi of gamma [^{32}P]-ATP. Samples were incubated 15 minutes at room temperature and the reaction stopped by addition of sample buffer for SDS/PAGE and heating for 5 minutes at 95 C. Lysates were then separated electrophoretically on 15% SDS polyacrylamide gels and relative histone kinase activity determined by autoradiography.

RESULTS

GH4C1 cells synchronization in G1

GH4C1 cells grown in F10 (serum free) medium for one length of the cell cycle (36 hours) arrested in early G1.

GH4C1 cells released from a F10 block

Adding serum (F10+) to the medium of serum starved cells that were synchronized in G1 allowed the cells to progress through the cell cycle in a synchronized fashion. It took 18-22 hours for cells release from a serum free block to reach S phase.

Both TGFα and MTX inhibit GH4C1 cell proliferation

The addition of 10 ng/ml of MTX and 10 nM TGFα at different times to synchronized cells which were released from a serum free (F10) block and then harvested at 22 hours determined the relative times MTX and TGFα block cell cycle progression. At 15 hours, TGFα began to lose its ability to block cells in G1 phase. MTX was somewhat later, and began to lose its ability to prevent S-phase entry at 18 hours.

MTX treated G1 cells block where CDC2 kinase is already activated

There was low CDC2 kinase activity in serum starved cells, cells in S phase had some what higher histone kinase activity. Cells treated with TGFα had approximately the same amount of histone kinase activity as the controls. MTX appeared to possess a much higher histone kinase activity.

DISCUSSION

GH4C1 cells grown in a serum free medium arrest in early G1. Addition of serum back to the cells allows the cells to proceed through the cell cycle in a synchronized fashion. Cells that have been released from a serum free block in early G1 take between 18 and 22 hours to reach S phase.

In a preliminary experiment, TGFα treated GH4C1 cells lose their ability to block S phase entry at about 15 hours after the serum addition. However, MTX lost its ability to block at about 18 hours. Also in a preliminary experiment, MTX had more CDC2 activity than TGFα. From previous experiments done at the lab, it has been determined that TGFα blocks at the restriction point. From these experiments, it appears that MTX blocks later than TGFα, and thus blocks after the restriction point.

It is important to point out that these are only preliminary results, and further experiments need to be performed before any definite conclusions can be made.

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Figure 1. a. Synchronized GH4C1 cells grown in serum free (F10) medium for 36 hours. Flow cytometry was then used for the cell cycle analysis. 90.8% of the cells are accumulated in G1.

Figure 1. b. Cell cycle analysis of unsynchronized cells. GH4C1 cells were grown in serum containing medium (F10+) and flow cytometry was used to determine cell cycle distribution. 50.7% of the cells were in G1, 38.2% were in S, and 11.1% were in G2 and M.

Figure 2. An experiment run previously which demonstrates the time it the cells to progress into S phase from a serum free block. At time 0 greater that 90% of the cells were in G1, and at 12 and 18 hours after addition of serum virtually none of the cells had entered S phase. However, between 18 and 24 hours after serum addition, nearly 50% of the cells had entered S phase.

Figure 3. Time for GH4C1 cells to reach S phase from a Serum free block. Until 18 hours, essentially no cells had entered S-phase, and between 18 and 22 hours after serum addition approximately 30% of the cells had entered S-phase.

Figure 4. Relative times that MTX and TGFα blocked cell cycle progression. Addition of serum to serum starved cells allowed the cell to progress through G1, then there was addition of 10ug/ml MTX or 10nM TGFα at different times after serum addition, and the cells were allowed to grow to 22 hours before being harvested. Cell cycle analysis was then used to determine when the cells entered S-

phase.

Figure 5. CDC2 kinase activity in control cells at 22 hours after serum addition, cells treated with MTX, TGFα, mimosine, and cells that remained in serum free medium. The serum free cells had no CDC2 activity where the control and the mimosine had about the same amount of activity. The TGFα had slight CDC2 activity, although there was a bit of degregation in this lane and the MTX had even more activity than the TGFα.

SYNCHRONIZED VS. UNSYNCHRONIZED CELLS

Figure 1
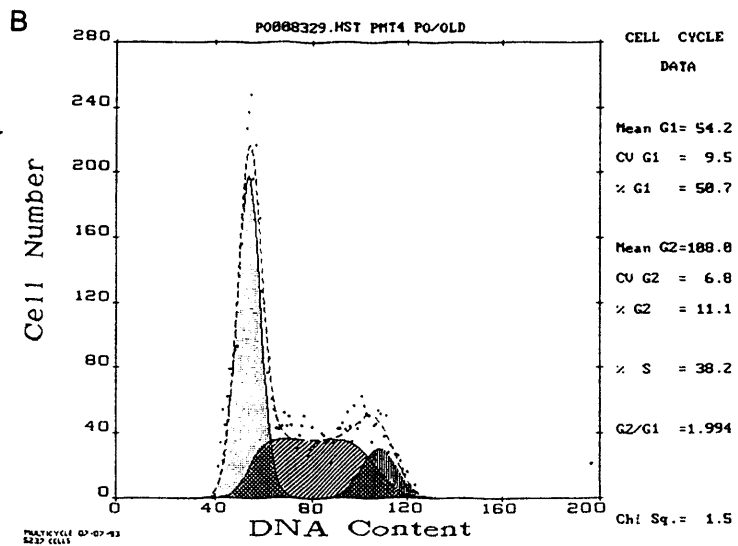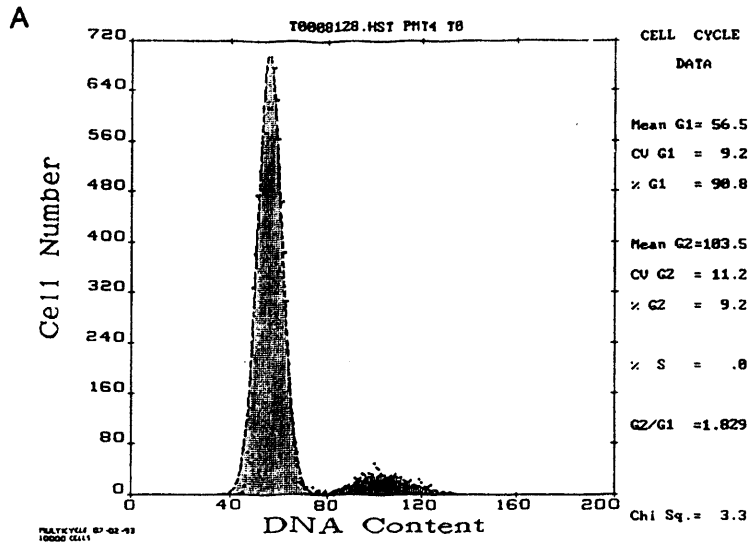
Figure 2
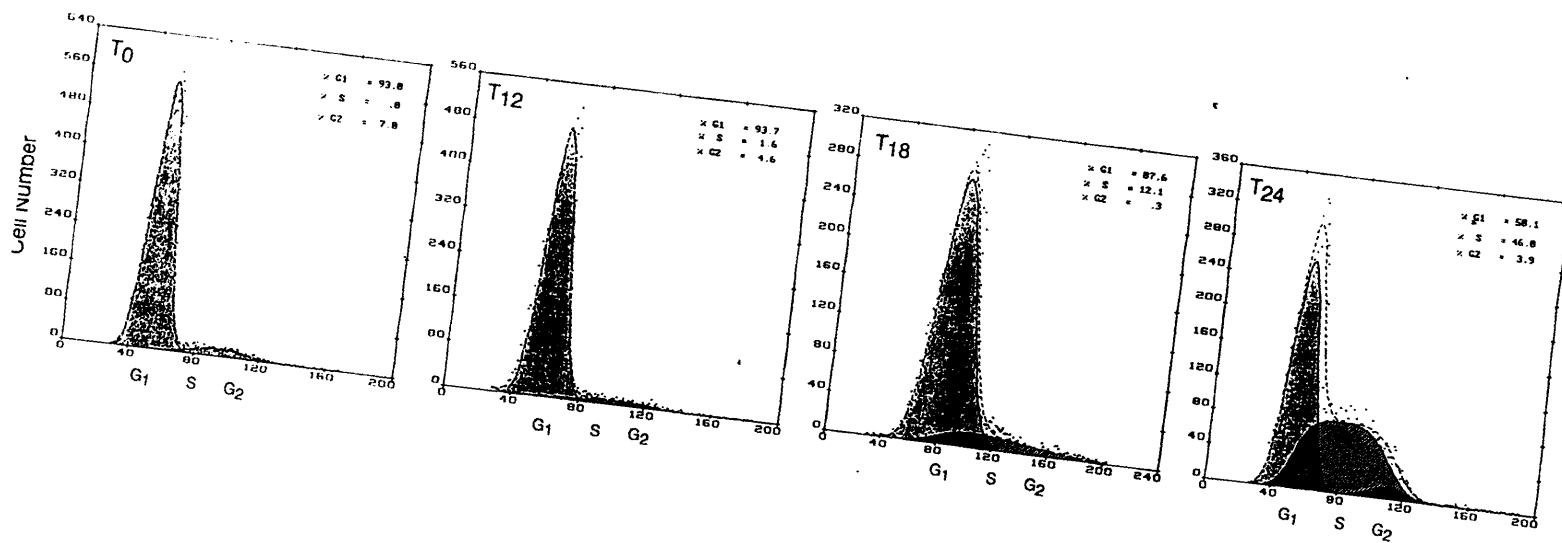
Figure 3

Time For GH4C1 Cells to Reach
S phase from a Serum Free Block

Figure 4

Time to MTX and TGF-α Block

Legend:
- TGF-α
- MTX
- Untreated Cells

Figure 5 CDC2 ACTIVITY

Histone H1 —

SF   MTX   TGF   MIM   CTL
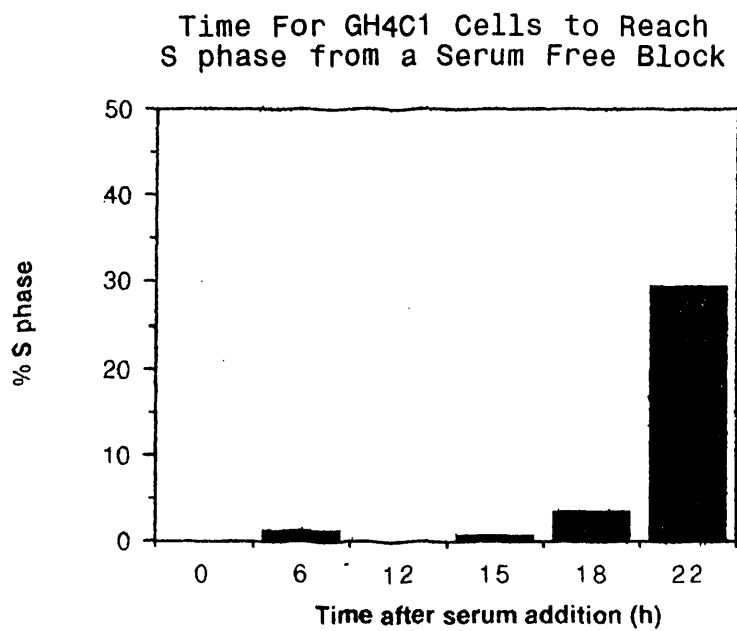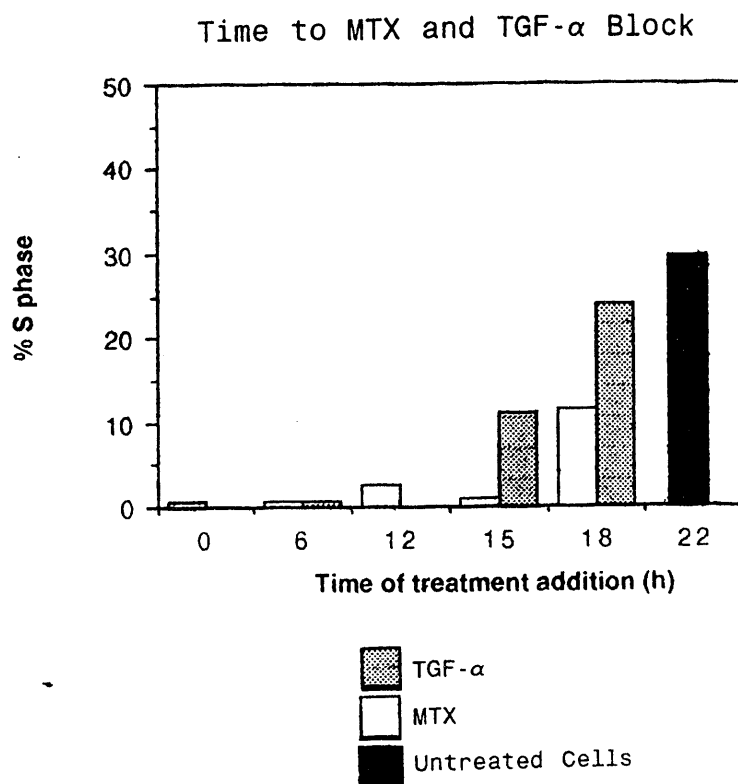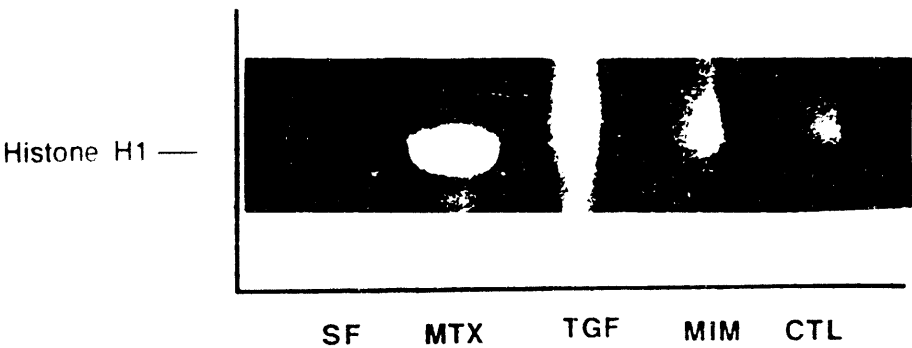
An Epidemiological Study of Leukemia
in South Carolina

Background

Over the past several years, numerous health studies have been carried out on populations living near nuclear facilities. The principle finding of many of these reports is an increase in mortality from leukemia between birth and age 24 (1-6). Although the reason for this apparent increase has been disputed, it is biologically plausible that proximity to radiation releasing facilities is a contributing factor, as it has been demonstrated that radiation produces differing responses in different age groups (7,8). Leukemia also lends itself well to an epidemiological study because it has a relatively short latency period between exposure and onset, allowing adverse conditions to be detected within a reasonable amount of time. My research project has been a study of leukemia in the 0-24 age group, focusing particularly on the counties on the southwestern side of the state that borders the Savannah River, where the Savannah River Plant is located. Built in Edgefield County by the Atomic Energy Commission in the early 1950's, the Savannah River Site occupies 312 square miles and has been the center of tritium production activity in the United States (9,10). Tritium is produced through the irradiation of lithium, and as an unwanted byproduct in the reactors at the plant (10). The main possible sources of environmental contamination are direct release of radionuclides into the river, and seepage from underground holding tanks that enters the river via streams. Slightly elevated levels of tritium have been detected downriver at treatment plants where the river is the main source of drinking water (11). It may be noted that the plant has maintained environmental monitoring and reported releases have been kept well below the recommendations of the International Commission on Radiological Protection (9,11,12).

Tritium is a radioactive isotope of hydrogen and is considered a health hazard (10,13-20). The main avenue of exposure to man is through ingestion, and possible routes include water vapor, drinking water, fish from polluted waters, contaminated milk, and others (11,21,22). Ingested tritium has a biological half life of 5-15 days in man (23,24).

Methods

The main focus of this report is on the thirteen counties covered by the Savannah River Region Health Information System (SPRHIS). This area embodies the South Carolina side of the Savannah River Region and includes the following counties (see map): Aiken, Allendale, Beaufort, Berkeley, Bamberg, Barnwell, Charleston, Colleton, Dorchester, Edgefield, Hampton, Jasper, and Orangeburg.

Mortality statistics from death certificates was the primary source of data used, supplemented with incidence rates (25). All rates are expressed as the number of people affected per 100,000 exposed. Where noted, rates are age adjusted to the 1970 U.S.

standard population to minimize skewing due to age variance
between populations. State and county population statistics for
the most recent years were available or projected from the 1990
census information, while population data for past years was
provided by the American Cancer Society. Due to the absence of
some very recent data in useable form, some rates have been
projected and are noted as such.

Limitations

The limitations inherent in reliance on mortality rates are
primarily caused by potential incorrect reporting of cause of
death, particularly in the earlier years when health care was not
as developed; therefore, the more recent data is generally
considered to be more reliable. Also, greater success in treat-
ment has increased the five year survival rate for leukemia to
over 50% in the younger age groups, making mortality a smaller
part of the overall picture. Incidence rates can be under-
reported where quality control is poor, or in areas without 100%
cooperation of hospitals. Since incidence information is pro-
cessed where the patient seeks treatment, geographical rates may
be slightly misleading for patients who travel out of the county
or state. (An effort has been made in this study to minimize
this problem by including cases from the Georgia cancer registry
with South Carolina addresses). Lastly, low numbers in some
categories, as recorded in the corresponding charts, in the age
specific rates present results within these categories with low
statistical power, requiring caution in interpretation.

Results

Mortality rates from the SRRHIS Region over the thirty-five
year period from 1953-1987 were compared to South Carolina and
U.S. rates (fig 1). Over that span, both the state and SRRHIS
rates were below that of the U.S., with no significant difference
between South Carolina and the SRRHIS region. The SRRHIS rate
was then recalculated subtracting the rates from the Trident
Health Region (Berkeley, Charleston and Dorchester counties), and
Colleton. These are the four furthest counties from the Savannah
River Site and are somewhat different from the other nine coun-
ties in the SRRHIS region in racial distribution and urbaniza-
tion. The resulting rate from the remaining counties was found
to be slightly lower than the others (fig 2).

The same comparisons for the years 1983-1991 (fig 3) indi-
cated that the SRRHIS region was noticeably lower than the state
and national rates, while subtracting the Trident area resulted
in a decrease to only slightly more than half of the national
figure. A breakdown into approximate ten year intervals demon-
strates that neither the SRRHIS region as a whole, nor the area
comprised of the non-Trident counties has ever been significantly
above the rest of South Carolina, and the non-Trident counties
have always followed the trend of being at or below the Trident
counties (fig 4).

To eliminate the possible skewing of statistics by racial
distribution, the data was divided into subcategories based on
race and separate rates were determined for whites and nonwhites

(figs 5-7). The pattern in both racial groups was quite similar, with high resemblance to the overall formation. The characteristic higher rate in whites was also present.

When incidence rates from 1991 (the only year that South Carolina data is currently available) were plotted against the U.S. rate (fig 8), the SRRHIS region was substantially lower, supporting mortality figures from the same year, which were more than 50% below the national mortality rate. The incidence rates were then broken down into five year age specific rates to determine if they were distributed normally (fig 9). All of the SRRHIS rates were uniformly below the U.S. rates for these categories with the exception of the 20-24 age group, which showed a slight elevation. SRRHIS region mortality rates for this age group matched with overall state rates over a 35 year interval showed some temporal variation, which is possibly due to random chance, while the totals for the length of the time period were basically identical (fig 10). Rates divided into racial categories were lower in the SRRHIS region for whites, but higher for nonwhites (fig 11). Also worthy of note is the fact that the normal trend for a substantially higher rate of leukemia in whites is not reflected here, but is slightly reversed. When the SRRHIS region was divided into those counties that actually border the river and those that don't (fig 12), the bordering counties consistently had higher rates than the non-bordering, with the relative difference increasing over time to a peak in 1983-1987 of a rate that was nearly three times higher in the bordering counties. Rates for whites and nonwhites demonstrate that the trend is exaggerated in whites, but reversed in non-whites (fig 13).

Conclusions

Neither overall rates nor race specific rates indicate any abnormal leukemia risks associated with the Savannah River. The rates are actually lower in the counties nearest the river, which may be due in part to lower overall urbanization. Parallel patterns in racial sub-groupings reinforce this, indicating a minimum of skewing from racial distribution. Incidence rates were also lower in the SRRHIS region than the U.S. for every age category except 20-24; however, mortality rates for this group were not elevated over state or national rates. Figures in counties bordering the river were shown to be somewhat higher than those in non-bordering counties, but the considerable difference in race specific rates, in the absence of a biologically plausible explanation, indicates that this may be due to chance distribution. Making inferences on this phenomenon will require the analysis of incidence data from future years, as well as demonstrable causation.
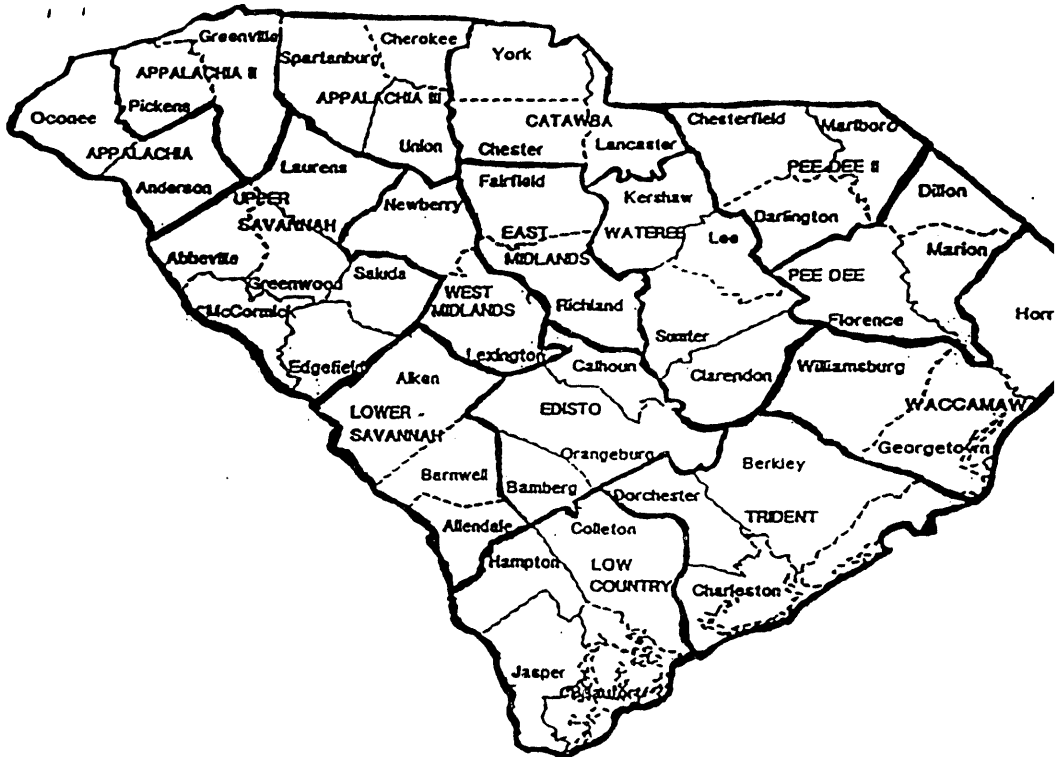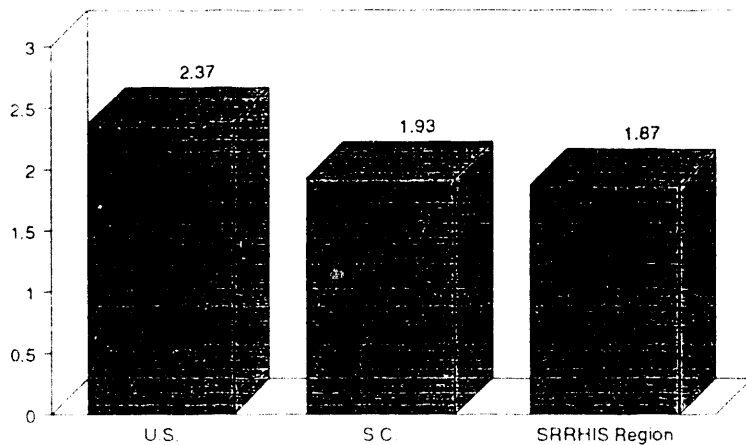
Health Districts: ▬▬▬
Counties: - - - -

Figure 1
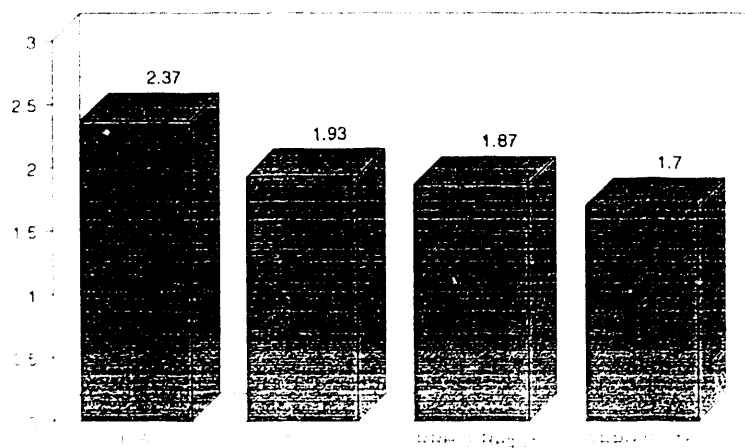
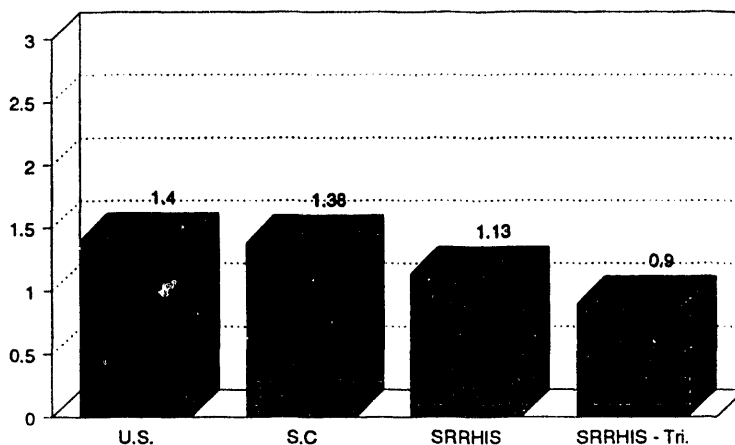# Mortality 1953-1987
Age: 0-24

Rates are age adjusted

Figure 2

Mortality 1953-1987
Age: 0-24

Figure 3

Mortality 1983-1991
Age: 0-24

Rates are age adjusted, U.S. rate is projected
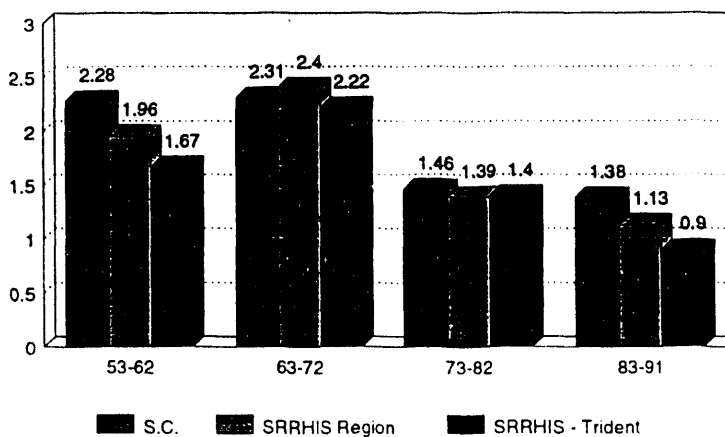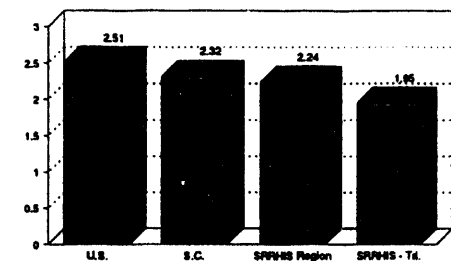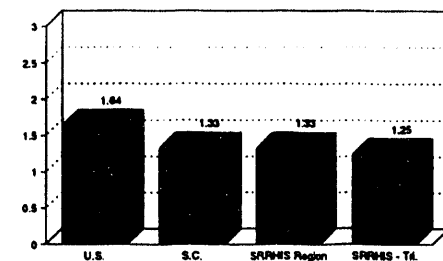
Figure 4

Trends in Mortality
Age: 0-24

Mortality By Race 1953-1987
Figure 7
Age: 0-24

2.51
2.32   2.24
1.95
2.37
1.93   1.87
1.64
1.7
1.33 1.33
1.25

White   Nonwhite   All

U.S.   S.C.   SRRHIS Region   SRRHIS - Tri.
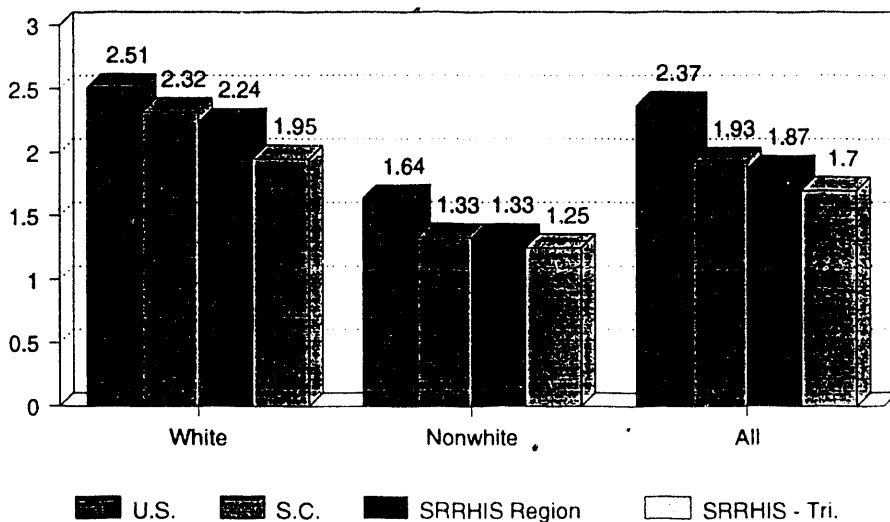
*Rates are age adjusted

1991 Incidence and Mortality
Figure 8   Age: 0-24
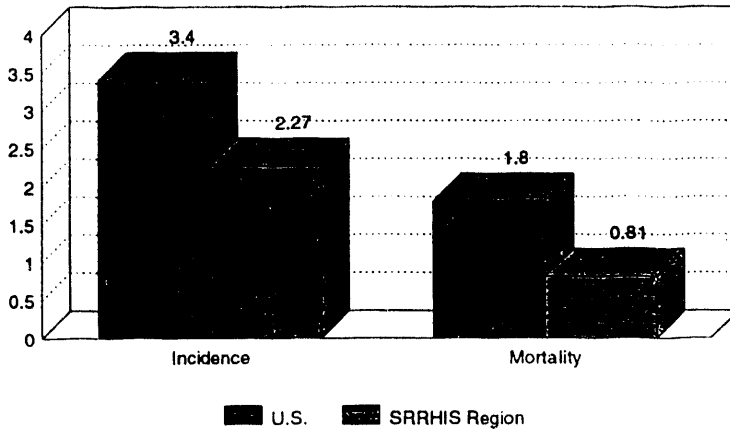
Rates are age adjusted, U.S. rates are projected

Incidence Rates By Age Group
Figure 9   1991 Cases: 12
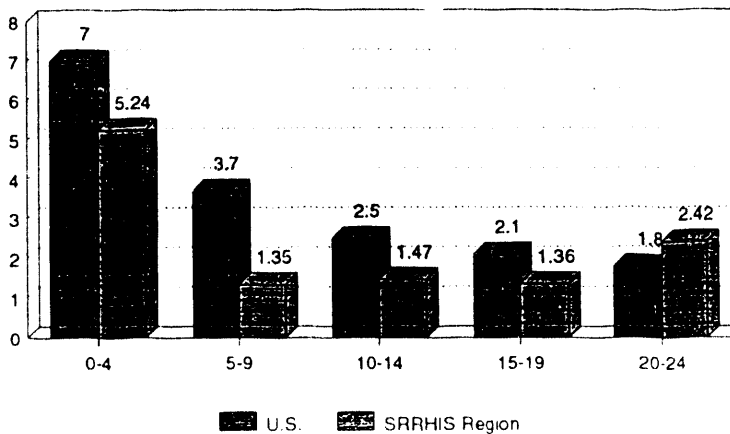
U S rates are projected

Figure 10

Age Specific Comparisons
Age: 20-24 Race: All
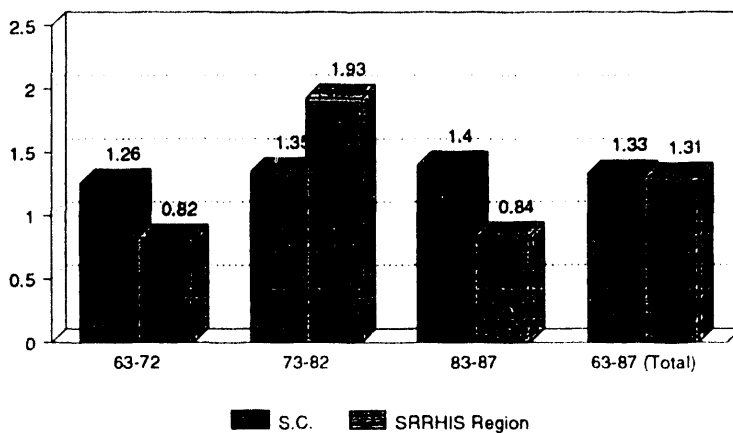
Total cases: 24

Figure 11

Age Specific Comparisons
Age: 20-24 Yrs: 1963-1987
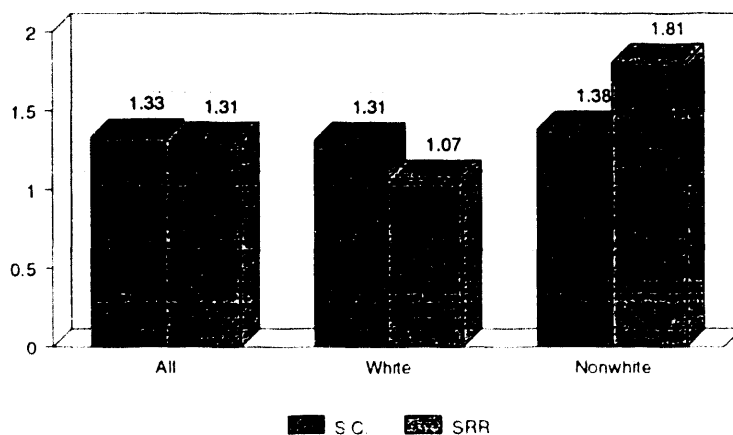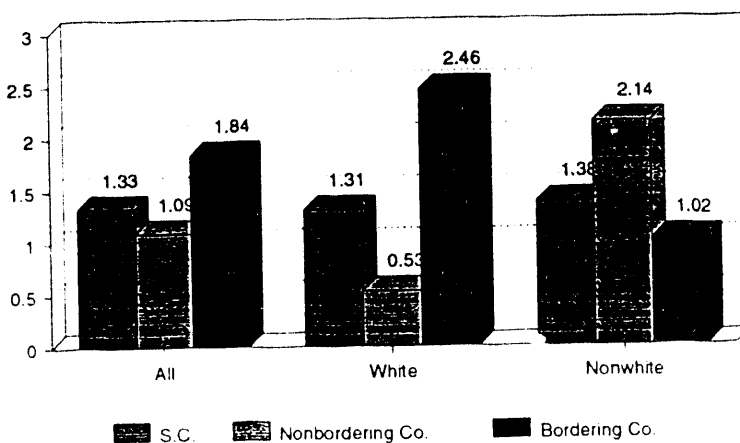

Total cases 24

Figure 12

Age Specific Comparisons
Age: 20-24
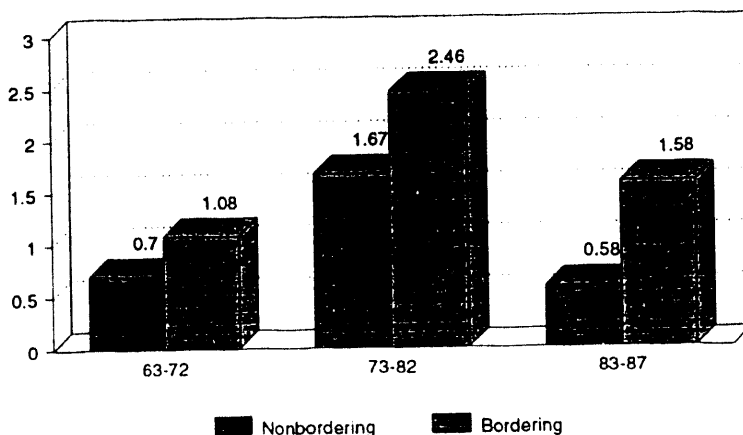
Number of cases: 24

Figure 13

Age Specific Comparisons
Age: 20-24

Total cases 24

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PHARMACOKINETICS—MATHEMATICAL VS. PHYSIOLOGICAL A SYSTEM SCIENCE APPROACH

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By: Beverly Charlotte Smith

Introduction

Physiologically-based pharmacokinetic (PB-PK) modeling and simulation have been an integral part of environmental exposure analysis and health risk assessment. PB-PK models rely heavily on known anatomical, physiological facts for the development of model structure and the determination of model parameters. Compared to the traditional mathematically-based multi-compartment pharmacokinetic models, the PB-PK models often have a higher order of complexity in terms of the number of variables and parameters, although they are used to model the same set of dose response data.

In the previous work by Drs. Z. Zhang, H: Zhang, and E. Voit, they derived a set of necessary conditions under which tissue regions in a PB-PK model can be "lumped" together without affecting the computation of the remaining part of the model. For a flow-limited PB-PK model if there is no more tissue region that can be further lumped together, the PB-PK model becomes a "minimum system." It was proved theoretically that such a minimum system and its mathematically-based counterpart model have the same level of complexity and are two possibly different "realizations" of the same system.

The goal for my summer research program is to use computer numerical simulation to experimentally verify the above two theoretical results, namely, for flow-limited PB-PK models, (1) the lumping of tissue regions using the derived necessary condition will not affect the computation of the remaining part of the model; and (2) for the same set of dose response data, a reduced minimal PB-PK model is equivalent to that of a mathematically based model.

Methods

1) Develop a PB-PK model with multiple tissue regions based on data from published literature. The model is so designed that parameters of some of the regions satisfy the derived necessary conditions for region lumping.

2) Conduct numeric simulation to verify that lumping tissue regions under the conditions does not affect the computation of the remaining tissues/organs in a reduced PB-PK model.

3) Use a known PB-PK model to generate a set of test data. A mathematically-based pharmacokinetic model is developed based on the test data and the graph of the data.

4) Derive the system transfer functions for both the mathematically-based model and a reduced model of the original model used to generate the test data. Show the similarity between the two system transfer functions.

5) Conduct numeric simulation to experimentally verify the results in 4).

DATA

MATHEMATICALLY BASED MODEL

PHYSIOLOGICALLY BASED MODEL

LAPLACE TRANSFORMATION

LAPLACE TRANSFORMATION

$=$
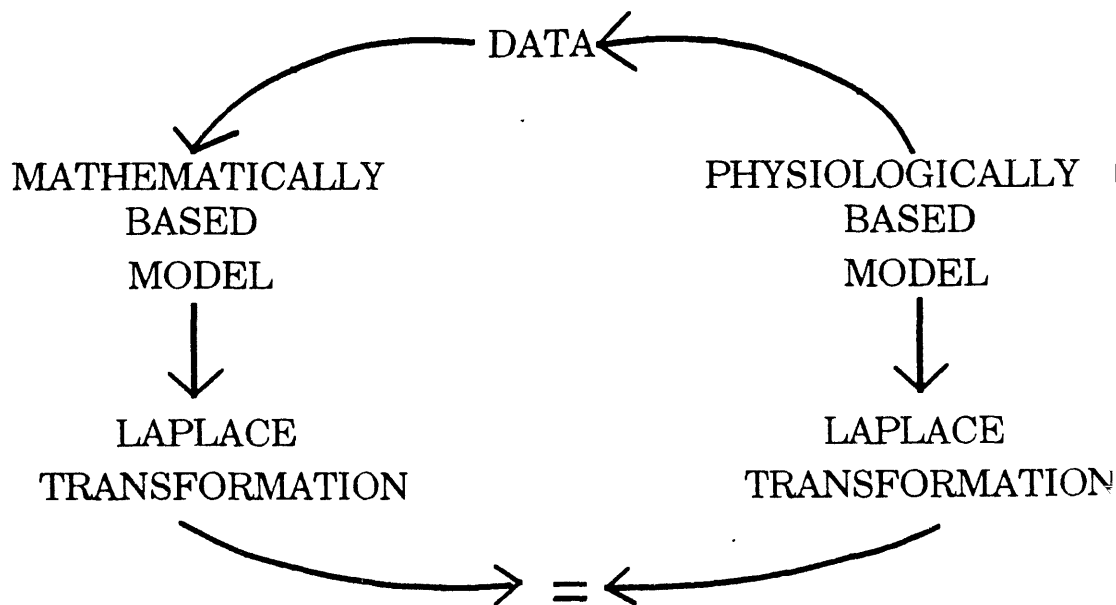
Results

The physiologically based model produced by my theoretical data set included the blood pool and three compartments: liver, kidney, and muscle. Each compartment has, associated with itself, several variables. The four most important variables in my study were R, the partition coefficient; Q, the flow rate of the metabolite within the compartment; V, the compartment volume; and C, the concentration of metabolite in the compartment. In this system two of the compartments satisfied the lumping condition

$$Q_k/(R_k{}^*V_k) = Q_m/(R_m{}^*V_m)$$

and could, therefore, be lumped or combined to form a minimal system, a system in which no compartments satisfy the lumping condition. The lumped compartment I called "tissue".

This compartment was given an R_t, Q_t, and V_t by combining those values of the unlumped compartments in the following manner

$$Q_t = \Sigma \ Q_i$$
$$V_t = \Sigma \ V_i$$
$$R_t = (\Sigma \ (V_i \ {}^* \ R_i))/(\Sigma \ V_i)$$

To obtain graphs of the concentrations in each compartment relative to time, differential equations are employed. The formula for any compartment i is

$$V_i \ {}^* \ (\delta C_i/dt) = Q_i \ {}^* \ (C_p - C_i/R_i)$$

where C_p is the blood pool concentration. The formula for the blood pool is

$$V_p \ {}^* \ (\delta C_p/dt) = (\Sigma \ (Q_i \ {}^* \ C_i/R_i)) - ((\Sigma \ Q_i) \ {}^* \ C_p)$$

where p denotes the blood pool, and i denotes a compartment.

In order to show that the two compartment system was equivalent to the three compartment system, I compared the graphs of the blood pool concentration. The two graphs overlay each other exactly.

The Laplace transformation is a formula used to demonstrate that two or more systems are equivalent regardless of how they appear. The final equations of each system involved should be equal even though they reached such a function by different means. The equation of the transpose is

$$b(sI-A)c$$

where b is the row matrix [1 0 0 ... 0]; s is the laplace operator; I, the identity matrix;

A, the coefficient matrix of the differential equations; and c, the transpose of b.

Depending on the size of your coefficient matrix, this equation can be very complicated. A simpler method is to simply recognize that

$$b(sI-A)c = det \text{ (minor of } (sI-A)) / det \text{ } (sI-A)$$

In order to create a mathematically based model, I graphed the natural log of the blood pool concentration from the minimal physiologically based system. The blood pool was chosen because that is the only data a study can accurately find. The chemical concentrations in organs and tissues is difficult, if not impossible, to measure. The laplace transformation is viewed slightly differently when working with mathematically based models because there are no differential equations from which to draw data. The disposition, d, is defined as

$$\Pi_i \text{ } (s + E_i) / \Pi_j \text{ } (s + \lambda_j)$$

$$j \in \{1..n\}; \text{ } i \in \{2..n\}$$

where n is the number of compartments in the minimal system (including blood pool); s is, again, the laplace operator; E is the exit rate constant; and λ is the disposition rate constant. The disposition rate constant is determined by the slopes of the lines from which the logarithmic plot is formed.

$$\lambda = -2.303 * m$$

where m is the slope. The exit rate constants are determined by the λ's and the y-intercepts (A_i).

$$A_i/A_{i+1} = (\lambda_i - E_{i+1}) / (E_{i+1} - \lambda_{i+1})$$

With the knowledge of these formulas and the appropriate λ's and E's from the physiologically based model, lines can be plotted on the plasma graph by computer aided linear regression. The formula for such a line is

$$y_i = a_i + b_i x_i$$

The y_i and x_i can be figured from the plot. The a_is, the y intercept, and the b_i, the slope, can be solved with a series of points or nodes.

Since it is critical that the mathematically based laplace transformation be as close to the physiologically based transform as possible, it is necessary to work backwards. From the physiologically based laplace transform it is known that

$$\lambda_1 = 9.098$$
$$\lambda_2 = 1.219 \qquad E_2 = 6$$
$$\lambda_3 = 0.000 \qquad E_3 = 1$$

The first line was to yield λ_1. By formula ###, the slope of such a line would have to be -####. By choosing appropriate starting and ending nodes on the plasma curve, a tangent with a slope of -3.23 (approximately accurate) was found. Thus $\lambda 1$ was ####. Each line was derived in this manner. The y-intercepts (A_1, A_2, A_3) could then be estimated and solutions E_2 and E_3 found. The mathematical laplace created was

$$[(s + 3.46) * (s + 0.118)] / [(s + 5.44) * (s + 0.209) * (s + 0.019)]$$

This transform is relatively close to the physiologically based one.

In order to get a better view of how close the mathematically based model was to the physiologically based model, the inverse laplace of each function was graphically compared.

The inverse laplace function is

$$f(t) = 0.372 * e^{-(t*5.44)} + 0.296 * e^{-(t*0.209)} + 0.333 * e^{-(t*0.019)}$$

for the mathematically based model and

$$f(t) = 0.35 * e^{-(t*9.098)} + 0.109 * e^{-(t*1.219)} + 0.541 * e^{-(t*0)}$$

for the physiologically based model.

Discussion

Overall, as I had originally asserted, the mathematically based pharmacokinetic model produces the same result as the physiologically based model. I was very surprised at how close to equivalent the models were. Although I expected such results, I also expected a large experimental error. I must assume the small error that I found was due to the method I used to choose the starting and ending nodes by which I derived the mathematical model. I simply eyeballed where I felt the lines should fall. In future tests of the equivalence between mathematically based models and physiologically based models, a better method of the node-choosing should be implored, and real data should be used rather than the experimental data I produced.

Although this experiment shows that the two modelling methods are mathematically equivalent, they are not equivalent in their usefulness. The Physiologically based model is used for extrapolation between species. It has also become a promising tool in environmental health risk assessment. The mathematically based model is only useful within a single species for interpolation and limited extrapolation. It is important that both methods be preserved for their different purposes.

Toluene Toxicity : Metabolism by Isolated Rat Hepatocytes

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Summer Undergraduate Research Program

Toluene is a popular organic solvent used worldwide in industrial settings. It is found extensively as a component in gasoline and refinery oils, as an ingredient in paint thinners and adhesives, and as an intermediate in the synthesis of many organic chemicals (Donald *et al.*, 1991). Because of its widespread use it has become the most abundant aromatic hydrocarbon contaminant at many waste sites. At DOE sites it is present in soil at levels up to 1,000 times and in water up to 10 times higher than that of the EPA's proposed maximum contaminant level in drinking water (Riley *et al.*, 1992).

Acute toluene toxicity is characterized by its central nervous system effects, which has led to the abuse of toluene for its intoxicating effects, *i.e.* "glue-sniffing" (Chapman *et al.*, 1990). Chronic effects of exposure to toluene have been observed in the central nervous system, liver, heart and kidneys of experimental animals and humans. Recent studies have implicated it as an embryotoxin and fetotoxin and a potential immunotoxin. In addition, toluene exposure has been shown to result in an increased number of micronuclei within polychromatic erythrocytes obtained from mouse femoral bone marrow, and this response could be increased by pre-treatment of the mice with hepatic cytochrome P-450 inducers (Chapman *et al.*, 1990).

Toluene is metabolized by the hepatic cytochrome P450 system to several oxidative products. Oxidation of the aromatic ring is thought to result in formation of a reactive intermediate(s) that binds covalently to biomolecules; covalent binding to a key protein(s) may lead to manifestation of toluene toxicity (Nakajima *et al.*, 1992).

In these studies, we wanted to examine the metabolism of toluene by rat hepatocytes and hypothesized that benzyl alcohol would be the major metabolite formed.

MATERIALS AND METHODS

Animals. Male Long Evans rats weighing 200 - 300 g. were used in Toluene treated rat hepatocyte experiments. All rats were maintained on pelleted feed and water.

Isolation of hepatocytes. Hepatocytes were isolated according to the Collagenase Perfusion Method (McMillan *et al.*, 1992).

Incubation and Assays The metabolism of Toluene was determined by measuring the rate of formation of benzaldehyde, benzoic acid, benzyl alcohol, hippuric acid, and o- and p- cresol. 1mM Toluene was added to the hepatocytes. Vials containing 1 ml of cells (8×10^6 cells / ml) were placed in a shaking water bath at $37.0°$ C for 2 hours. The hepatocytes were quick frozen to stop the reaction and then stored in the freezer. The scintillation vials were then taken out of the freezer, placed at room temperature, and allowed to thaw. The hepatocytes were centrifuged to separate out the proteins, and 20 µl of the supernatant was injected onto a high-performance liquid chromatography system (HPLC, Waters Associate Chromatography Pump) with UV detector (Waters 481). The conditions were as follows : a Resolve reverse phase C-18 radial compression module; mobile phase I, 6% methanol : 94% 0.01 M potassium phosphate at pH 3.5; mobile phase II, 50% methanol : 50 % 0.01 M potassium phosphate at pH 3.5 and a flow rate of 2.0 mL/ min; wavelength 254 nm.

β-glucuronidase To determine the extent of glucuronic acid formation, the major conjugate of benzyl alcohol in the hepatocytes, incubation with ß-glucuronidase solution was used. Incubation with ß-glucuronidase was carried out by centrifuging out the proteins, adding 4 volumes of ethanol for 1 volume of hepatocytes, and drying down the solution in an evaporator. The residue was then resuspended in 200µl of water. 200 µl of the ß-glucuronidase solution which was (a 1:10 dilution of ß-glucuronidase with .1 M Sodium Acetate at pH 5.2) was added to the resuspended solution. The solution was allowed to incubate in a water bath at $37.0°$ C for 16 hours. At the end of 16 hours, TCA was added to stop the reaction. The proteins were centrifuged out, and 20 µl of the supernatant was injected onto the HPLC.

RESULTS

Toluene metabolism in rat hepatocytes. Benzaldehyde, benzyl alcohol, hippuric acid, o- and p-cresol were not found in toluene treated hepatocytes. Benzoic acid was the main metabolite observed in the toluene treated hepatocytes; it had a retention time of 40 minutes (Fig. 1).
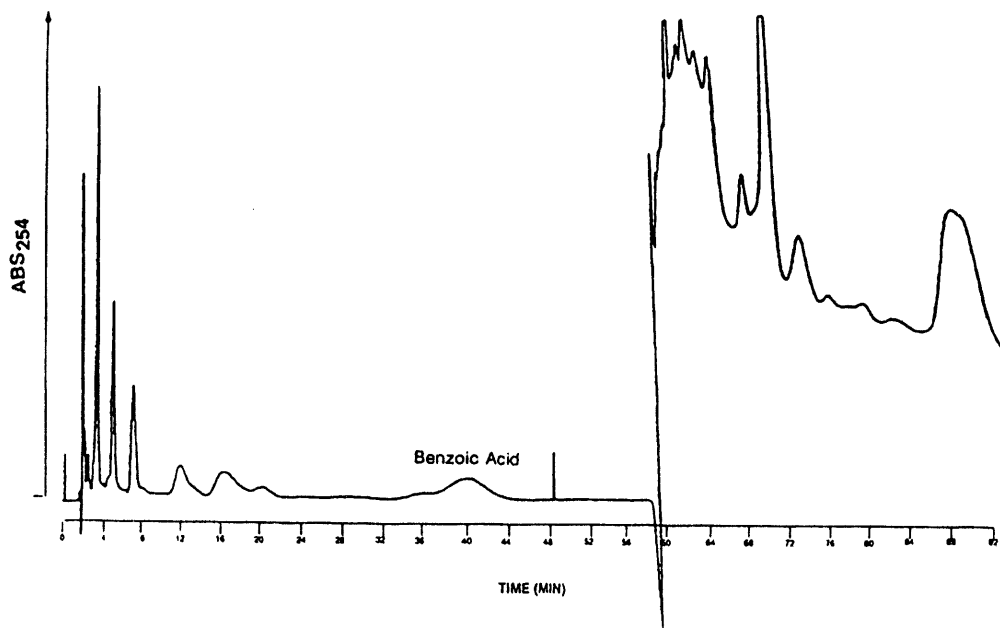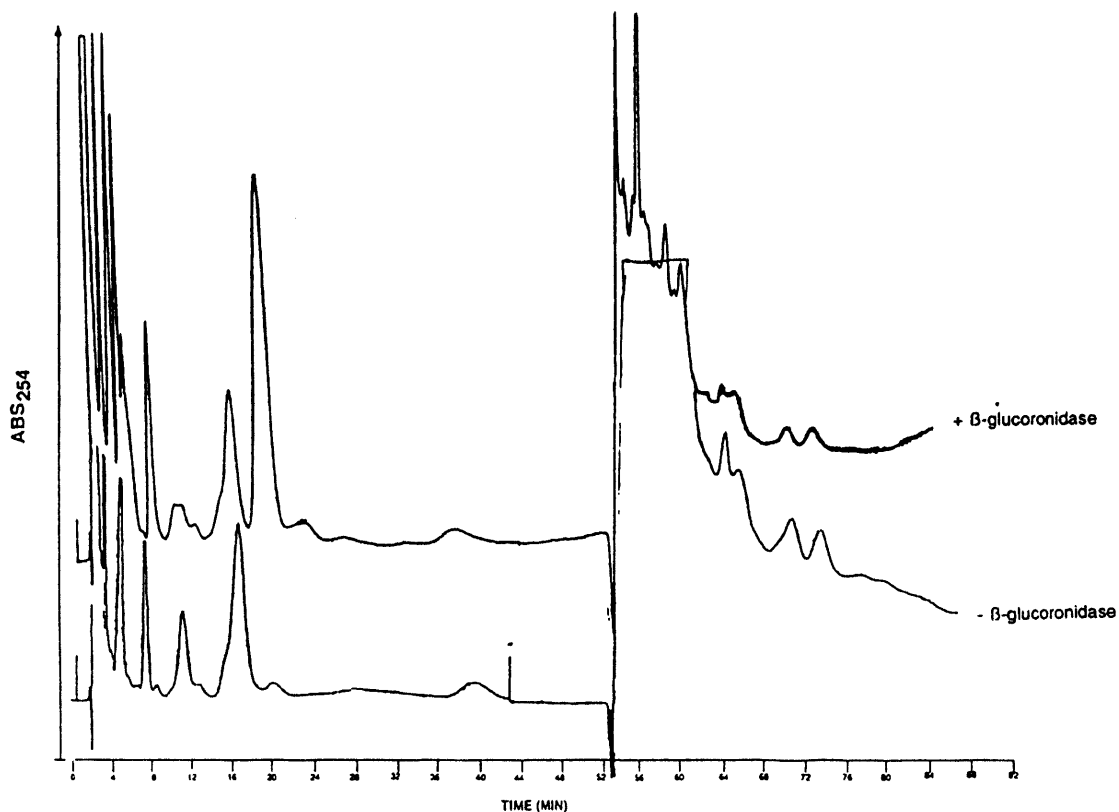
FIG.1. An HPLC Chromatogram of Toluene treated rat hepatocytes at the 2 hour time point. The only metabolite detected was benzoic acid with a retention time of 40 minutes. At 48 minutes, the mobile phase was switched from Mobile Phase I to mobile phase II. Un-metabolized toluene had a retention time of 60 minutes.

β-Glucuronidase digest of Toluene treated rat hepatocytes. To determine the presence of glucuronic acid conjugates, ß-glucuronidase digest was used. ß-Glucuronidase digest should cause a significant increase in the benzyl alcohol peak and a significant decrease in the glucuronide conjugate. A significant increase was observed at the retention time of benzyl alcohol, 20 minutes, but no significant decrease in any peak was observed when ß-glucuronidase was incubated alone; the increase at 20 minutes proved to be a contaminant in the ß-glucuronidase solution (Fig. 2).

FIG.2. An HPLC Chromatogram of Toluene treated rat hepatocytes with and without ß-glucuronidase digest at 16 hours. An increase is observed in the peak eluting at 20 minutes. A benzoic acid peak was observed again at 40 minutes. At 48 minutes, the mobile phase was switched from Mobile Phase I to Mobile Phase II. Un-metabolized toluene had a retention time of 60 minutes.

Metabolism of Benzyl Alcohol. Since there were no peaks corresponding to the retention time of benzyl alcohol at 20 minutes, which was hypothesized to be the major metabolite of toluene, an incubation with benzyl alcohol being added to the hepatocytes instead of toluene was carried. No peaks of benzyl alcohol or its glucuronic acid could be seen. Only a peak of benzoic acid could be seen at the corresponding retention time of 40 minutes (Fig. 3).
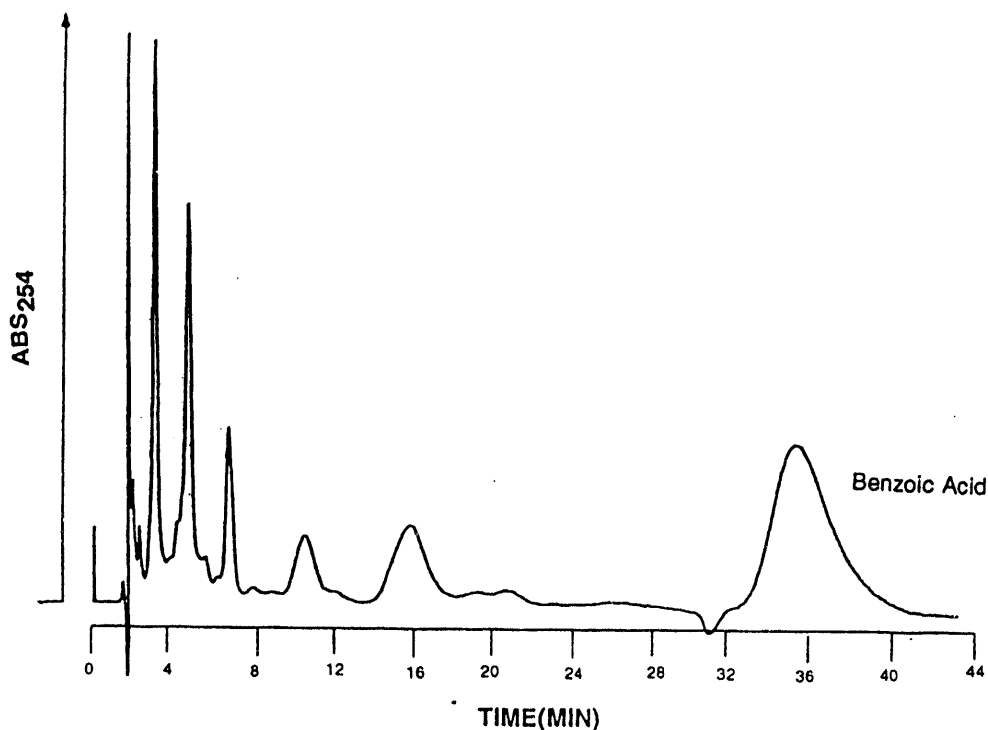
FIG. 3. An HPLC Chromatogram of Benzyl Alcohol treated hepatocytes at 2 hours. A peak corresponding to the 40 minute retention time of benzoic acid could be observed. A peak of benzyl alcohol could not be seen at its retention time of 20 minutes.

β-Glucuronidase digest with Benzyl Alcohol treated rat hepatocytes. ß-Glucuronidase of benzyl alcohol was done to see if the glucuronic acid conjugate could be seen. Once again digestion with ß-glucuronidase should show an increase in one peak height and a decrease in another. A significant increase was observed at the retention time of benzyl alcohol, 20 minutes, but no significant decrease could be seen. However, this significant increase in peak height proved to be a contaminant, as mentioned before, upon incubation of ß-glucuronidase alone (Fig. 4).
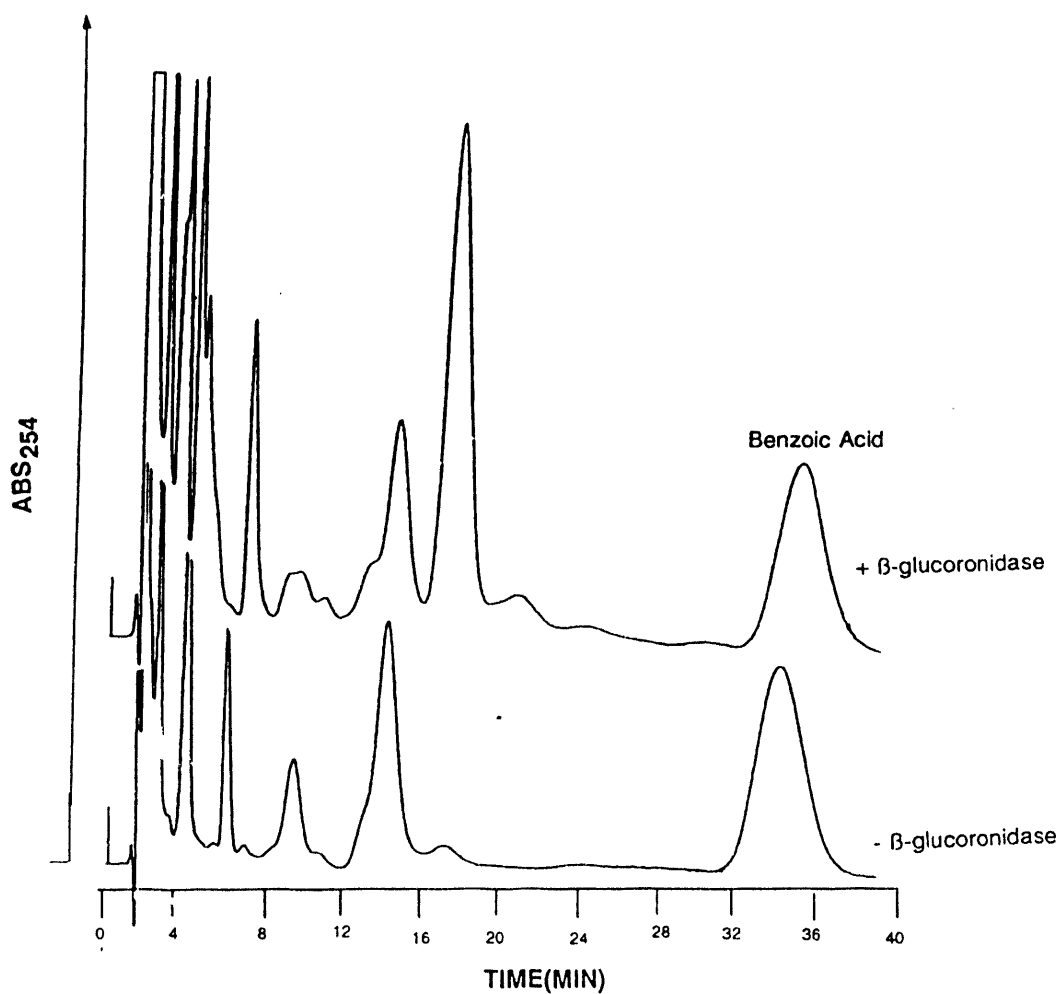
FIG. 4. An HPLC Chromatogram of Benzyl Alcohol treated rat hepatocytes with and without ß-glucuronidase digest at 16 hours. An increase in peak height is observed again at 20 minutes. Again, the benzoic acid peak is seen at 40 minutes.

Quantitative Study of Toluene Metabolism. Benzoic acid was injected onto the HPLC at various concentrations to determine quantitatively how much benzoic acid was being formed from toluene. The concentration of benzoic acid formed in the hepatocytes was 0.43 µM (Fig. 5).
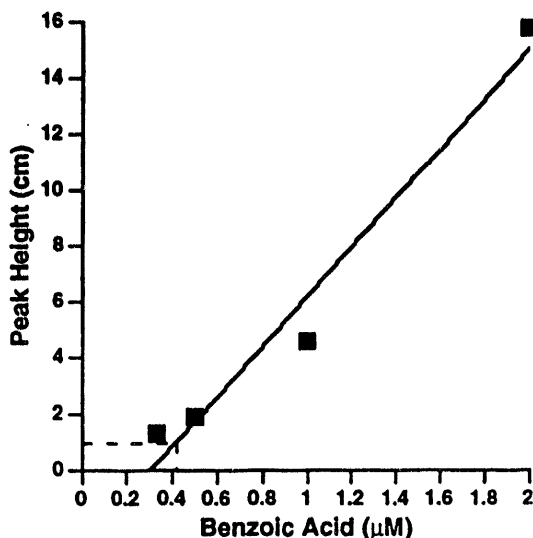
Fig. 5. Concentration vs. Peak Height for Benzoic Acid.
The dashed line represents the concentration of Benzoic
Acid in the rat hepatocytes after toluene treatment.

DISCUSSION

Results of HPLC analysis show that benzoic acid was the major metabolite formed from toluene in rat hepatocytes. The hypothesized major metabolite, benzyl alcohol, could not be seen. This could be due to one of two possibilities: either toluene was being metabolized to benzoic acid so quickly that benzyl alcohol could not be observed or toluene was being metabolized directly to benzyl alcohol glucuronic acid. The latter could be determined by ß-glucuronidase digest, but failed to show benzyl alcohol release. This failure was due to the contaminant found on incubation of ß-glucuronidase in a later experiment. However, the idea that benzoic acid was being formed

very quickly was further supported by a HPLC analysis on a sample treated with benzyl alcohol taken as a zero time point. For the time it took to quick freeze the sample, benzoic acid was already being formed.

Our results support the findings of Chapman *et al.* (1990). Chapman *et al.* found that benzyl alcohol was the major metabolite in microsomes while benzoic acid was the major metabolite in liver slices. Our results show that benzoic acid is the major metabolite in whole cell samples or liver hepatocytes. The enzymes alcohol and aldehyde dehydrogenase would carry out the conversion of benzyl alcohol to benzoic acid. These enzymes are soluble enzymes and would not be present in a microsomal suspension. In intact cells benzyl alcohol would be further metabolized to benzoic acid, whereas in microsomes only benzyl alcohol would be formed. Our findings indicate that rat hepatocytes can be used in studies if toluene toxicity.

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