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Final Report

on

ECLSS Medical Support Activities

by

The University of Alabama in Huntsville
Consortium for the Space Life Sciences
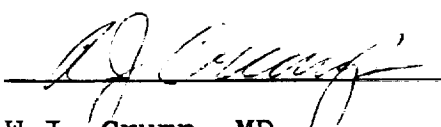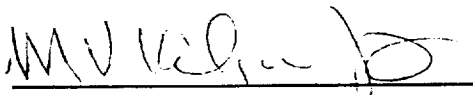**Environmental Laboratory Section**
Huntsville, AL   35899

for

The National Aeronautics and Space Administration
Marshall Space Flight Center
Marshall Space Flight Center, AL   35812
Contract # NAS8 - 36955 D.O. 76

Prepared by:

_____   _____
W.J. Crump, MD   and   M. V. Kilgore Jr. R.M.
Principal Investigator   Co-Principal Investigator

April 30, 1991

INTRODUCTION

During the period from April 10, 1990 to April 9, 1991, the Consortium for the Space Life Sciences provided technical assistance to the NASA/MSFC water recovery efforts. This assistance was in the form of literature reviews, technical recommendations, and presentations. This final report summarizes the activities completed during this period and identifies those areas requiring additional efforts. The tasks which the UAH water recovery team addressed were either identified by MSFC technical representatives or chosen from those outlined in the subject statement of work.

CONTRIBUTING PERSONNEL

During the period of this contract numerous personnel contributed toward the completion of the project tasks. These individuals are listed below:

William J. Crump, MD
Daniel S. Janik, MD
Alfred T. Mikell, PhD
Marian L. Lewis, PhD
David L. Elam, MS, CIH
Brian L. Benson, MS
Melvin V. Kilgore, Jr., RM
Tim L. Rhoads

SUMMARY OF ACCOMPLISHMENTS

During this contract period many separate efforts were initiated and completed. Some of the tasks completed were at the direction of NASA/MSFC technical monitor while others were from the typical tasks outlined in the statement of work. Individual monthly progress reports are included as Appendix A. Specific efforts completed are summarized in the following paragraphs.

1

During the first half of the contract period much effort was required to prepare and finalize procedures and requirements necessary to initiate the WRT activities. Through numerous meetings with NASA/MSFC personnel and NASA/MSFC contractor personnel several documents and subsequent revisions were completed and delivered under separate cover. The microbiological methods document and pertinent supplemental information requested by NASA/MSFC technical personnel is included as Appendix B. The Analytical Control Sample Procedures Document is included as Appendix C.

Participation and review of test protocols and concerns also required considerable effort. These tasks were supported by physical attendance and participation of UAH scientists at specific meetings to address pertinent issues related to IRB review, experimental design, test subject safeguards and data analysis. These efforts culminated in the issuance of Protocol B for final review. Specific written comments prepared are included as Appendix D.

Following completion of WRT 3A, UAH scientists participated in data review and analysis. Preliminary findings of the microbiological data collected during 1A, 2A and 3A were presented to NASA/MSFC personnel and contractors. Chemical data received by UAH consisted only of pH, TOC, Conductivity and Iodine determinations. These data were graphically illustrated but few conclusions could be drawn without additional supporting information. Each of these data review packages are included as Appendix E.

In addition to the above WRT related tasks UAH scientists also prepared additional research information related to the typical tasks outlined in the statement of work. These included recommendations regarding microbial contamination control measures, bioassay testing for non-specific toxicity, and characterization of chemically pretreated urine. These documents have been included as Appendices F, G and H, respectively. Finally, to help address the recent questions regarding the survival and detection of viruses in water a literature review was completed. Selected pertinent scientific articles have been included as Appendix I.

APPENDIX A

MONTHLY PROGRESS REPORTS

Account No: 5-32390

MONTHLY TECHNICAL PROGRESS

Report No. 1

FOR THE PERIOD OF: 4/10/90 - 5/9/90   PREPARED: 5/10/90

Contract No.: NAS8-36955	Delivery Order No.: 76

Delivery Order Title: "UAH/ECLSS Medical Activity"

Research Activities Performed:

During this reporting period, administrative functions associated with contract start-up were completed. A plan for the search for a Ph.D. Microbiologist was developed and initiated. Final revisions to the Microbial Methods document were made (Appendix A). Preparation of briefing material as requested by Dr. Humphries regarding microbial recommendations were completed.

Problems Encountered:

NONE

Research Activities Planned Next Month:

Begin recruitment for Ph.D. Microbiologist.

Develop Quality Control Plan for WRT. Check Sample Preparation.

_____
Principal Investigator   Date: 5/21/90

Prepared for: NASA/MSFC

ATTN: AP-29H/Marianne Campbell

Marshall Space Flight Center, AL 35812

(2)

cc:
CN-22D	(3)
AT-01	(1)
CN-01/Wofford	(1)
EM-13/L. Smith	(1)
ONRRR	(1)
ED-62/Mitchell	(2 + repro)
NASA/Sci & Tech Info. Fac.	(1 + repro)
Vaughan/UAH	(1)

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Report Documentation Page

1. Report No.	2. Government Accession No.	3. Recipient's Catalog No.
1	N/A	N/A

4. Title and Subtitle		5. Report Date
UAH ECLSS Medical Activity I First Monthly Progress Report		5/10/90
		6. Performing Organization Code
		N/A

7. Author(s)	8. Performing Organization Report No.
M.V. Kilgore, Jr., R.M. W.J. Crump, M.D.	1
	10. Work Unit No.
	N/A

9. Performing Organization Name and Address	11. Contract or Grant No.
University of Alabama in Huntsville Huntsville, AL 35899	NAS8-36955 D.O. 76
	13. Type of Report and Period Covered
12. Sponsoring Agency Name and Address	Monthly (4/10-5/9/90)
MSFC	14. Sponsoring Agency Code

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Appended documents sent under separate cover.

16. Abstract

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Account No: 5-32390

MONTHLY TECHNICAL PROGRESS

Report No. 2

FOR THE PERIOD OF: 5/10/90 - 6/10/90   PREPARED: 6/11/90

Contract No.: NAS8-36955	Delivery Order No.: 76

Delivery Order Title: "UAH/ECLSS Medical Activity"

Research Activities Performed: During this reporting period Mr. David L. Elam started full-time employment. Mr. Elam is a certified Industrial Hygienist with experience in air toxicology. Efforts have been initiated to bring Mr. Elam up to speed on toxicology issues. Likewise, a national search was conducted for a Ph.D. Microbiologist to support the MSFC efforts. Approximately 100 resumes were screened and resulted in four excellent candidates. Also the QC document for the preparation of WRT check samples was initiated and completed.(Appendix A). An outside review of current anaerobic procedures was completed by Dr. Tom Phelps at the University of Tennessee. Copies of the report will be sent when available.

Problems Encountered:

NONE

Research Activities Planned Next Month:

Interview Microbiology candidates.

Prioritize tasks and develop research plans.

Principal Investigator	Date:

Prepared for: NASA/MSFC

ATTN: AP-29H/Marianne Campbell

Marshall Space Flight Center, AL 35812

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cc:
CN-22D   (3)
AT-01   (1)
CN-01/Wofford   (1)
EM-13/L. Smith (1)
ONRRR   (1)
ED-62/Mitchell (2 + repro)
NASA/Sci & Tech
   Info. Fac.   (1 + repro)
Vaughan/UAH   (1)

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Report Documentation P

1. Report No. 2	2. Government Accession No. N/A	3. Recipient's Catalog No. N/A

4. Title and Subtitle	
UAH ECLSS Medical Activity II Second Monthly Progress Report	5. Report Date 6/11/90
	6. Performing Organization Code N/A

7. Author(s)	
M.V. Kilgore, Jr., R.M. W.J. Crump, M.D.	8. Performing Organization Report No. 2
	10. Work Unit No. N/A

9. Performing Organization Name and Address	
University of Alabama in Huntsville Huntsville, AL 35899	11. Contract or Grant No. NAS8-36955 D.O. 76
	13. Type of Report and Period Covered Monthly (5/10 - 6/10)

12. Sponsoring Agency Name and Address	
MSFC	14. Sponsoring Agency Code N/A

15. Supplementary Notes

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16. Abstract

17. Key Words (Suggested by Author(s))	18. Distribution Statement
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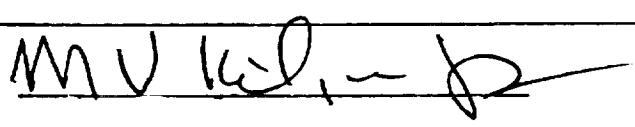| 19. Security Classif. (of this report) N/A	20. Security Classif. (of this page) N/A	21. No. of pages 2	22. Price N/A

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Account No: 5-32390

MONTHLY TECHNICAL PROGRESS

Report No. __3__

FOR THE PERIOD OF: __6/10/90 - 7/9/90__   PREPARED: __8/16/90__

Contract No.: NAS8-36955	Delivery Order No.: 76

Delivery Order Title: "Recycled Potable Water"

Research Activities Performed:

During this reporting period two Ph.D. Microbiology candidates were interviewed. Research plans and team leaders were identified in order to complete SOW research tasks. Upon completion of individual task items draft documents will be prepared and submitted for review by MSFC personnel. The review of anaerobic methodology was completed by Dr. Phelps and supplementary methods developed and submitted (Appendix A). Several meetings were attended regarding microbiological issues.

Problems Encountered:

None

Research Activities Planned Next Month:

Continued work toward completion of SOW tasks.

Date: 8/22/90

Prepared for: NASA/MSFC	cc:	
ATTN: AP-29E/Marianne Campbell	AT-01	(1)
Marshall Space Flight Center, AL 35812	EM-13/L. Smith	(1)
	ONRRR	(1)
	E.B.Rogers/EB	(2 + repro)
(2)	NASA/Sci & Tech	
	Info. Fac.	(1 + repro)
	Vaughan/UAH	(1)

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1. Report No.	2. Government Accession No.	3. Recipient's Catalog No.
3	N/A	N/A

4. Title and Subtitle	5. Report Date
UAH ECLSS Medical Activity II Third Monthly Report	8/16/90
	6. Performing Organization Code
	N/A

7. Author(s)	8. Performing Organization Report No.
M.V. Kilgore, Jr. W.J. Crump, M.D.	2
	10. Work Unit No.
	N/A

9. Performing Organization Name and Address	11. Contract or Grant No.
University of Alabama in Huntsville Huntsville, AL 35899	NAS8-36955 D.O. 76
	13. Type of Report and Period Covered
12. Sponsoring Agency Name and Address	Monthly (6/10 - 7/9)
MSFC	14. Sponsoring Agency Code

15. Supplementary Notes

Appended documents sent under separate cover.

16. Abstract

N/A

17. Key Words (Suggested by Author(s))	18. Distribution Statement
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19. Security Classif. (of this report)	20. Security Classif. (of this page)	21. No. of pages	22. Price
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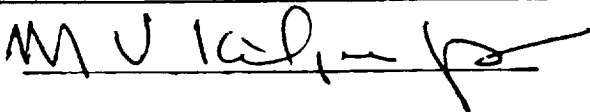
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MONTHLY TECHNICAL PROGRESS

Report No. 4

FOR THE PERIOD OF: 7/10/90 - 8/9/90   PREPARED: 8/16/90

Contract No.: NAS8-36955	Delivery Order No.: 76

Delivery Order Title: "Recycled Potable Water"

Research Activities Performed:

During this reporting period Protocol B was received for review. Medical and non-medical reviews were completed and submitted (Appendices A and B). Efforts continued toward completion of SOW tasks. Several meetings were attended regarding microbiological and Protocol B issues.

Problems Encountered:

None

Research Activities Planned Next Month:

Continuation of SOW task efforts and Review of WRT stage A data as requested.

Date: 8/22/90

Prepared for: NASA/MSFC	cc:	
ATTN: AP-29E/Marianne Campbell	AT-01	(1)
Marshall Space Flight Center, AL 35812	EM-13/L. Smith	(1)
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	Vaughan/UAH	(1)

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Report Documentation Page

1. Report No. 4	2. Government Accession No. N/A	3. Recipient's Catalog No. N/A
4. Title and Subtitle UAH ECLSS Medical Activity II Fourth Monthly Progress Report		**5. Report Date** 8/16/90
		6. Performing Organization Code N/A
7. Author(s) M.V. Kilgore, Jr. W.J. Crump, M.D.		**8. Performing Organization Report No.** 2
		10. Work Unit No. N/A
9. Performing Organization Name and Address University of Alabama in Huntsville Huntsville, AL 35899		**11. Contract or Grant No.** NAS8-36955 D.O. 76
		13. Type of Report and Period Covered Monthly (7/10 - 8/9)
12. Sponsoring Agency Name and Address MSFC		**14. Sponsoring Agency Code** N/A
15. Supplementary Notes Appended documents sent under separate cover.		
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17. Key Words (Suggested by Author(s)) N/A	**18. Distribution Statement**	
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Account No: 5-32390

MONTHLY TECHNICAL PROGRESS

Report No. 5

FOR THE PERIOD OF: <u>August 10 - September 9, 1990</u> PREPARED: <u>9/20/90</u>

Contract No.: NAS8-36955	Delivery Order No.: 76

Delivery Order Title: "FNAS UAH/ECLSS Medical Activities"

Research Activities Performed: During this reproting period WRT Microbiological and chemical data have been reviewed. A summary data package and presentation describing the preliminary findings and recommendations were completed regarding microbiological data (Attachment A). Chemical data package is still being reviewed. Work continued toward completion of other SOW tasks.

Problems Encountered: None

Research Activities Planned Next Month: Continuation of SOW tasks. A draft copy of completed items will be prepared and submitted as a six month progress report. Completicn of review of chemical data package is planned

M.V. Kilgore, Jr., Technical Studies Coordinator. Date: 9/21/90

Prepared for: NASA/MSFC	cc:	
ATTN: AP-29E/Marianne Campbell	AT-01	(1)
Marshall Space Flight Center, AL 35812	EM-13/L. Smith	(1)
	ONRRR	(1)
	E.B.Rogers/EH	(2 + repro)
(2)	NASA/Sci & Tech	
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	Vaughan/UAH	(1)

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Report Documentation Page

1. Report No.	2. Government Accession No.	3. Recipient's Catalog No.
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4. Title and Subtitle	5. Report Date
FNAS UAH/ECLSS Medical Activities Fifth Monthly Progress Report	9/20/90
	6. Performing Organization Code
	N/A

7. Author(s)	8. Performing Organization Report No.
M.V. Kilgore, Jr. W.J. Crump, M.D.	5
	10. Work Unit No.
	N/A

9. Performing Organization Name and Address	11. Contract or Grant No.
University of Alabama in Huntsville Huntsville, Alabama 35899	NAS8-36955 D.O. 76
	13. Type of Report and Period Covered
12. Sponsoring Agency Name and Address	Monthly (8/10 - 9/9/90)
MSFC	**14. Sponsoring Agency Code**
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15. Supplementary Notes

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4. Title and Subtitle	5. Report Date
FNAS UAH/ECLSS Medical Activities Sixth Monthly Progress Report	11/21/90
	6. Performing Organization Code
	N/A

7. Author(s)	8. Performing Organization Report No.
M.V. Kilgore, Jr.	6
W.J. Crump, M.D.	10. Work Unit No.
	N/A

9. Performing Organization Name and Address	11. Contract or Grant No.
University of Alabama in Huntsville	NAS8-36955 D.O. 76
Huntsville, AL 35899	13. Type of Report and Period Covered
	Monthly (9/10/90-10/9/90)

12. Sponsoring Agency Name and Address	14. Sponsoring Agency Code
MSFC	N/A

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16. Abstract

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ATTACHMENT A

MSFC Delivery Order 76
Statement of Work

Task Summary and Tenative Schedule for Completion

Presented below is a brief summary of task statements pro-
vided by the statement of work. A brief outline identifying the
team leader, a description of the progress made to date and
anticipated completion schedule is provided for each task state-
ment.

4.1.1 Delivery Order Activities

a. Review test protocols as necessary to support Institu-
tional Review Board.

As submitted

b. Recommend techniques to control microbial and/or chemi-
cal contamination of recycled air and water.

This task has been divided into two parts: microbiological
and chemical. Dr. Mikell has the lead on the microbiological
section and Mr. David Elam has the lead on the chemical section.
A draft report has been completed for the microbiological section
and is currently being reviewed. This report should be distrib-
uted in early December. A literature review has been completed
for the chemical section. This report should be distributed late
January or early February, 1991.

c. Suggest procedure for estimation of health risks of test
subjects participating in testing.

This task has been divided into two parts: exposure and
bioassay screening. Dr. Janik and Dr. Crump have the lead for
exposure and Dr. Lewis has the lead for bioassay screening. A
draft report recommending specific bioassay screening procedures
has been prepared and is current being reviewed. This report is
expected for distribution in late December, 1990 or early Janu-
ary, 1991. The exposure (including animal testing) part will be
completed toward the end of the performance period.

d. Identify and evaluate removal processes for various
drugs and/or pharmaceuticals by the urine reclamation systems.

Drs. Janik and Crump have the lead for this task. This task
will be completed toward the end of the performance period.

1

THE UNIVERSITY OF ALABAMA IN HUNTSVILLE

Account No: 5-32390

MONTHLY TECHNICAL PROGRESS

Report No. 6

FOR THE PERIOD OF: September 10 - October 9, 1990   PREPARED: 11/21/90

Contract No.: NAS8-36955	Delivery Order No.: 76

Delivery Order Title: FNAS UAH/ECLSS Medical Activities

Research Activities Performed:

Chemical data package (TOC, Cond. pH, turbidity) review was completed. Work continued toward completion of other SOW tasks. A progress report of SOW tasks is included as Attachment A.

Problems Encountered:

None, Request microbiological identification data required for completion of SOW tasks.

Research Activities Planned Next Month:

Continuation of SOW tasks (Refer schedule Attachment A).

_____

Principal Investigator

Date: 11/26/90

Prepared for: NASA/MSFC	cc:	
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Marshall Space Flight Center, AL 35812	EM-13/L. Smith	{1}
	ONRRR	{1}
	E.B.Rogers/EH	(2 + repro)
	NASA/Sci & Tech	
(2)	Info. Fac.	{1 + repro)
	Vaughan/UAH	{1}

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Attachments can be appended

4.1.2 System Design Activities

a. Preparation and review of test plans.

As submitted, to date two reviews have been provided.

b. Definition and interpretation of NASA/MSFC test design requirements related to medical issues and microbial and chemical contamination control.

As sumitted, to date no requests have been made.

c. Recommend development of new requirements for recycled air and water.

Mr. David Elam has the lead for this task. The current plan combines this task with 4.1.1 b above. This task report is expected to be delivered late January or early February, 1991.

d. Review documentation and make recommendations relative to design specifications.

As submitted, to date no request have been made.

4.1.3 Test Activities

a. Prepare test plans for future "man-in-the-loop" testing.

Drs. Crump and Janik have the lead for this task. This task will be completed toward the end of the contract perfomance period.

b. Recommend approach for developing a data base of all microorganisms recovered from past and future testing.

Mr. Tim Rhoads and Melvin Kilgore have the lead for this task. Initial thoughts and a review of information available for past recovered isolates has been completed. Following a review of data collected on current WRT isolates an plan can be devised and recommended.

c. Recommend plan to develop and characterize chemical constitutents of oxone/sulfuric acid treated urine including more complete organic carbon accountability.

Mr. Brian Benson has the lead for this task. A draft report has been prepared and is currently being reviewed. The task report should be distributed in early January, 1991.

d.   Assist in developing a plan to characterize the result of Iodine ingestion on microbial and chemical constituents of urine.

Drs. Scott Janik and Al Mikell have the lead for this task. This task will be completed toward the end of the contract period.

e.   Update and revise the Analytical Control program as necessary for upcoming ECLS test activities.

Mr. Kilgore has the lead for this task. To date, three revisions have been made to the Analytical Control Plan. Also, a disk copy of the lastest revision was provided in order for MSFC personnel to make changes quickly.

f.   Provide recommendations on methods development relative to chemical and microbiological analysis.

Mr. Kilgore has the lead for this task. To date, several recommendations have been provided for microbiological analysis. At this time no recommendations have been made for chemical analysis. In order to evaluate chemical analysis performance for specific parameters the data collected during WRT testing and associated control samples must be reviewed. In addition, the laboratory is currently preparing additional FAME library entries for water related bacteria not included in standard libraries available. These entries are being developed using ATCC reference cultures and include "type strains" only. This library will be provided to MSFC for distribution to contractors in late February or early March, 1991.

g.   Review medical significance of microorganisms recovered from ECLS process streams and hardware.

Mr. Tim Rhoads and Drs. Crump and Janik have responsibility for this task. A list of isolates has been prepared from WRT data received to date. Currently, medical information is being gathered for each isolate. Following completion of this process a medical review will be completed and medical significance assigned. We view this task as a on-going activity.
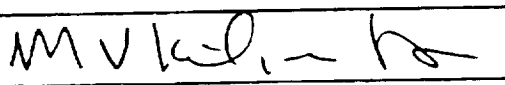
h.   Recommend appropriate investigations of biological enhancement of physical/chemical water recovery systems.

Drs. Lewis and Janik have the lead for this task. This task will be completed toward the end of the contract period.

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Account No: 5-32390

MONTHLY TECHNICAL PROGRESS

Report No. 7

FOR THE PERIOD OF: October 10 - November 9, 1990 PREPARED: 11/21/90

Contract No.: NAS8-36955	Delivery Order No.: 76

Delivery Order Title: FNAS UAH/ECLSS Medical Activities

Research Activities Performed:

A revised microbiological methods document (Rev. 3.0) was prepared to allow use of either spread plates or membrane filtration (Appendix A). In addition, new methods for staphylococci and fungi were included. A review of microbiological parameters for WRT stage 4 was also completed during this period (Appendix B). Work continued toward completion of SOW tasks.

Problems Encountered:

None

Research Activities Planned Next Month:

Continue work on SOW tasks.

_____
Principal Investigator

Date: 11 | 26 | 90

Prepared for: NASA/MSFC	cc:
ATTN: AP-29E/Marianne Campbell	AT-01 (1)
Marshall Space Flight Center, AL 35812	EM-13/L. Smith (1) ONRRR (1) E.B.Rogers/EH (2 + repro) NASA/Sci & Tech Info. Fac. (1 + repro)
(2)	Vaughan/UAH (1)

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Report Documentation Page

1. Report No.	2. Government Accession No.	3. Recipient's Catalog No.
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4. Title and Subtitle	5. Report Date
FNAS UAH/ECLSS Medical Activities Seventh Monthly Progress Report	11/21/90
	6. Performing Organization Code
	N/A

7. Author(s)	8. Performing Organization Report No.
M.V. Kilgore, Jr. W.J. Crump, M.D.	7
	10. Work Unit No.
	N/A

9. Performing Organization Name and Address	11. Contract or Grant No.
University of Alabama in Huntsville Huntsville, AL 35899	NAS8-36955 D.O. 76
	13. Type of Report and Period Covered
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N/A	N/A	2	N/A

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Acct. No: 5-32390

MONTHLY TECHNICAL PROGRESS REPORT
Report No.: 8

FOR THE PERIOD OF: November 10-December 9, 1990   Prepared: 1/15/91

Contract No.:	NASA-38958	Delivery Order No.:	73

Delivery Order Title: "ECLSS Medical Activities"

Research Activities Performed:

During this period work continued toward completion of sow tasks. In addition, efforts have recently been directed toward supplementing existing fatty acid libraries available for bacterial identification. The resulting supplemental library will be included as a deliverable when completed.

Problems Encountered:

None

Research Activities Planned Next Month:

Continue work on sow tasks.

Melvin V. Kilgore, Jr.

Date: 1/23/91

Prepared for:

NASA / MSFC
ATTN: Marlanne Campbell/AP 29E
Marshall Space Flight Center, AL 35812

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ONRRR	<1>
K. Mitchell/ED62	2 + repro>
NASA/Sci & Tech. Info. Facility	1 + repro>
Vaughan/ UAH	<1>

MONTHLY TECHNICAL PROGRESS REPORT
Report No.: 9

FOR THE PERIOD OF: December 10, 1990-January 9, 1991   **Prepared:** 1/15/91

Contract No:	NASB-36955	Delivery Order No.:	

Delivery Order Title: *"ECLSS Medical Activities"*

Research Activities Performed:

During this reporting period efforts continued toward completion of sow tasks and supplemental fatty acid library work was also initiated to review the medical signigicance of bacteria isolated from WRT activities.

Problems Encountered:

None

Research Activities Planned Next Month:

Continuation and completion of sow tasks and supplemental fatty acid library.

Melvin V. Kilgore, Jr.   **Date:** 1/23/91

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MONTHLY TECHNICAL PROGRESS REPORT
Report No.: 10

FOR THE PERIOD OF: January 10 - February 9, 1991   Prepared: 4/26/91

Contract No.:	NAS8-____	Delivery Order No.:	

Delivery Order Title: "ECLSS Medical Activities"

Research Activities Performed: Work continued on SOW tasks. Review of the Medical significance was suspended due to the lack of microbial information received to date. Work continued on the supplemental fatty acid library.

Problems Encountered: None

Research Activities Planned Next Month: Continuation and completion of tasks initiated to date.

M.V. Kilgore, Jr.   Date: 5/1/91

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MONTHLY TECHNICAL PROGRESS REPORT
Report No.: __11__

FOR THE PERIOD OF: __February 10 - March 9, 1991__   Prepared: __4/26/91__

Contract No.: NASB-36955	Delivery Order No.: 2

Delivery Order Title: *'ECLSS Medical Activities'*

Research Activities Performed: During this reporting period most tasks initiated were completed with the exception of the supplemental FAME Library.

Problems Encountered:
> None

Research Activities Planned Next Month: Completion of supplemental FAME library and preparation of final report.

M. V. Kilgore, Jr.   Date: 5/1/91

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APPENDIX B

MICROBIOLOGICAL METHODS

UAH POSITION CONCERNING DISCREPANCIES IN
MICROBIOLOGICAL METHODS
REVISIONS 1.4 AND 2.0

prepared by: Tim Rhoads

reviewed by: Melvin V. Kilgore, Jr., R. M.
Alfred T. Mikel, Jr., Ph. D.

approved by: W. J. Crump, M. D.

Introduction

This document outlines major differences between revisions 1.4 and 2.0 of the Microbiological Methods for the Water Recovery Systems Test. These issues are arranged and presented in order of priority, from most to least important. For each issue, the difference has been defined, the concerns and implications of the difference stated and possible remedies to resolve these differences have been suggested.

Discussion and revision are necessary whenever novel or unconventional methods are undertaken. The Water Recovery test is most certainly novel in several respects with regard to microbiological parameters and specifications. Some procedures are recognized as standard methods and must be adhered to whenever possible, as they represent the consenses view of noted experts in this field. Other procedures have had to be developed specifically to meet the requirements of the WRT. In these instances, although standard methods are not available, consenses views of leading experts must be followed. We would hope that this dialogue results in sound methods so that the highest quality data evolve from these efforts.

1) MAXIMUM SAMPLE HOLDING TIME

DISCREPANCY:

The procedure as presented in Revision 1.4, increases the maximum holding for all isolates to 24 hours.

CONCERNS:

The protocols in the Microbiological Methods for the Water Recovery Systems Test are designed to culture a variety of organisms, some of which are relatively sensitive to environmental conditions. Pathogenic and nonpathogenic organisms normally associated with the human body are especially sensitive due to the nutrient rich and thermally controlled environments from which they originate. These organisms are of the greatest concern due to potential health implications. Excessive sample holding times may damage or kill these organisms rendering them unculturable providing erroneous data.

Investigations by Mcdaniels et. al., revealed that numbers of the Enterobacteriaceae (includes several notable pathogens and indicators of fecal contamination) declined significantly after holding periods of 24 hours at 5 and 22 degrees Celsius. As part of these investigations, potable water samples analyzed upon collection contained bacterial coliform numbers in excess of limits defined in the Safe Drinking Water Act. These same samples after a 24 hour holding time were within acceptable quality limits (1).

Sample preservation and storage requirements for microbiological examination are clearly outlined in Section 9060 B in Standard Methods for the Examination of Water and Wastewater (2). Due to the relevance of these statements, the first two paragraphs from this document are quoted below in their entirety.

"Start microbiological examination of a water sample promptly after collection to avoid unpredictable changes. If samples cannot be processed within 1 hour after collection, use an iced cooler for storage during transport to the laboratory. If it is known that the results will be used in legal action, employ a special messenger to deliver samples to the laboratory within 6 hours and maintain chain of custody. Hold temperature of all stream pollution, drinking, and wastewater samples below 10° C during a maximum transport time of 6 hours. Refrigerate these samples upon receipt in the laboratory and process within 2 hours. When local conditions necessitate delays in delivery of samples longer than 6 hours, consider either making field examinations using field laboratory facilities located at the site of collection or using delayed-incubation procedures."

3

One exception was noted allowing 24 hour holding times in the case of, "individual potable water samples shipped directly to the laboratory by mail, bus, etc." This exception applies only to cases involving single family drinking water which require sample transport. The water recovery test has always been dependent on the capabilities and the close proximity of the Boeing laboratory, generating multiple daily samples and providing water to multiple persons. For these reasons, WRT samples are specifically excluded from this category.

Some clinically significant organisms included in the AEM's, are susceptible to environments and conditions found outside the human host. Species of Neisseria are among the most sensitive organisms to such conditions. The following are recommendations of clinical microbiology manuals concerning the handling of samples that may contain Neisseria.

The ASM Manual of Clinical Microbiology 4th ed. (3) states that in buffers such as Stuart and Amies, which are specifically designed for the maintenance of gonococci, Neisseria will survive well for 6-12 hours, if not exposed to temperature extremes. By 24 hours, their numbers may decrease to an extent that may prevent their recovery, especially if present in low numbers. It further states that such non-nutritive transport medias should not be used if the sample cannot be plated within 12 hours. The manual expresses a particular concern in the case of Meningococci which are very cold sensitive.

Bailey and Scott's Diagnostic Microbiology manual (4) states that a swab sample may be held for no longer than 3 hours at 4-6 degrees C without noticeable reduction in viable cell numbers of Neisseria. The conditions must be even more stringent in the case of Meningococci which are very cold sensitive.

Though species of Neisseria are sensitive to environmental conditions, most are not fastidious and can grow on common nutritive medias. This applies to some other clinically significant organisms as well. As these organisms comprise a portion of the total bacterial community represented in this test, the limited sample holding times which apply to heterotrophic bacteria is even more important to the recovery of AEM's. In addition to the aerobic human microflora (AEM's), many anaerobes are associated with the human body. Limited sample holding times are paramount for the recovery of those anaerobes having strict requirements for a reduced atmosphere. Environmental conditions detrimental to the AEM's as well as any oxygen residual, may damage strict anaerobes and make them uncultureable.

IMPLICATIONS:

A) Long holding times may result in erroneous data which underestimate actual microbial densities at the time of sampling. Resultant changes in populations may also be unpredictable.

B) If pathogens are involved, numbers may decrease below recovery limits. Furthermore, these organisms might not be identified and recognized as pathogens as these enumeration procedures are currently the only screening technique for the Water Recovery Test. Loss of detection might be further aggravated based on current confirmation procedures.

REMEDY:

Adopt shorter holding times. Empirical data to justify increasing holding times may be obtained in the laboratory by spiking sample waters with known numbers of stationary growth-phase bacteria and determining recovery at varying times. However, this will only provide information relative to test organisms and may not be applicable to other bacteria.

A joint study (UAH-Boeing) examining the potential value of a recently proposed medium for the preservation of bacteria in a water sample is being considered. This medium may make it possible to extend sample holding times when absolutely necessary.

2) HETEROTROPH AND AEM CONFIRMATION:

DISCREPANCY:

The procedures as presented in revision 1.4 requires that only 10% of the total morphological types be identified.

CONCERNS:

The criteria defined in revision 1.4 may result in the actual identification of very few colony types. It is certain that some samples will be very homogeneous presenting very few colony types. If less than 10 types were present in these samples, then according to revision 1.4, none would need to be identified. Conceivably, a plate presenting 30 colonies of identical morphologies may represent 30 taxonomically different bacteria.

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Many pathogens have unremarkable morphologies. This is particularly critical when applied to the AEM parameter. Many of the target organisms are fastidious and slow growing, producing small, colorless, translucent colonies. Many cannot even be distinguished from nonpathogens on the basis of their appearance (9, 10). Difco (manufacturers of the medium used in this procedure) states that various species of virulent pathogens including Neisseria, Brucella, and Hemophilus cannot be distinguished from various Pseudomonas and Flavobacterium spp. on the enriched chocolate agar chosen for this parameter (11).

The purpose of identifying isolates is to better understand the populations associated with these waste streams and to provide information to the medical officer as to the specific contaminants associated with water which is "out of specification." The current procedure accomplishes neither of these objectives.

The standard operating procedure at the UAH Medical Clinics which is accredited by the American Society of Clinical Pathologists (ASCP) is to identify a minimum of 3 of each colony type (12).

The standard operating procedure at the Humana Hospital bacteriology lab is: when dealing with a body site or sample that is expected to be relatively sterile, or when dealing with colonies or isolates suspected of being pathogenic all colonies are identified (13).

The conditions of the water recovery test in which the microbiological parameters will be utilized are unique. Existing and accepted clinical procedures are classically applied to the treatment of samples of human origin such as body surfaces or fluids. Pure water is not a common clinical sample. These water samples must therefore be treated as relatively pure clinical samples in order to conform to the accepted clinical procedures.

An additional concern is in the general method of identification referred to in the 3/19/90 Clean Water memo from MSFC. This memo stated that Gram stain and gross morphology would be used to identify isolated colonies. Morphology can be used in the characterization but not in the definitive identification of these isolates (14).

IMPLICATION:

Method may fail to identify many bacterial species present including important pathogenic microorganisms in test samples.

REMEDY:

Identification of all distinct colony types or a minimum of 10 colonies per plate (based on confirmation procedures recommended

by Standard Methods) will provide adequate information regarding the recoverable population from these samples. This does not have to be done for each and every sample. The Analytical Control Plan (ACP) states that all colony types (but not less than 10) from 10% of the plates for each parameter must be identified. This is adequate for most sample ports. All colonies from clean water ports (those which verify water quality for test subject use) should be identified.

This remedy will not provide information from all samples but it will provide adequate information to support current medical decisions and future design and control recommendations.

3) QUALITATIVE ANAEROBE PROCEDURE

DISCREPANCY:

This method has been included in revision 1.4 and not in 2.0.

CONCERNS:

This method was deleted in revision 2.0, as it has not been validated. Due to the limited experience with anaerobic procedures we could not recommend the inclusion of an anaerobe method unless it has been scientifically tested or designed by an experienced anaerobic microbiologist. We feel that the dangers involved in working with anaerobic bacteria and anaerobic methods warrant this caution.

Anaerobic microbiology methods traditionally employed often require contacting large numbers of pathogens with syringe needles and potentially high gas pressures in sealed glass containers (15). Many anaerobic bacteria produce copious amounts of gas. Broth cultures of anaerobes without sufficient head space or proper bottles could produce a "time bomb." Puncture wounds involving the introduction of anaerobes might result in serious consequences. Gangrene and tetanus are two examples of anaerobic wound infections (16). Issues concerning potential laboratory safety were not addressed in the anaerobe procedure. Due to the stringent and methodical procedures, as well as additional safety precautions which must be taken, a skilled microbiologist having direct experience using anaerobic techniques, is essential (17).

IMPLICATIONS:

A) Non-optimal laboratory safety

B) May miss important anaerobic indicator organisms and pathogens

A) Non-optimal laboratory safety

B) May miss important anaerobic indicator organisms and pathogens

REMEDY:

MSFC supplied us with a task statement to develop an applicable method which would satisfy all those concerned. We received such a request on 4/17/90. This has enabled us to contact an expert anaerobic microbiologist whose research specialty is the recovery of anaerobic bacteria from environmental sources.

The outcome of this communication will be a procedure which should minimize complexity and safety problems of the method presented in revision 1.4. This procedure will also replace 2.1.10 as it will provide quantitative results.

It has been the practice of UAH/CSLS from the outset of this task, that chemical methodologies which were not standard accepted procedures would be subject to validation before inclusion in the Water Recovery Test. We would suggest that the same criteria used to determine the need for validation of new chemistry methods be applied to new microbiological methods. This represents a sound scientific approach.

4) HETEROTROPH INCUBATION TIME

DISCREPANCY:

The procedure as presented in revision 1.4 specifies up to 7 days incubation time for Heterotrophs.

CONCERNS:

Heterotrophic bacteria represent a very comprehensive group. The variety of characteristics and requirements of the heterotrophs are broad. Among this group are some species requiring lengthy incubation times. The upper limit of 7 days for the incubation of the Heterotrophic bacteria may not be an adequate period of growth for the detection of some slow growing species. This is especially true for the R2A agar method which is specifically designed for the culture of oligotrophic bacteria characteristically requiring long incubation times.

Comments regarding the methods received from Johnson Space Center dated 12/11/89 recommended an incubation time of "up to 21 days" for heterotrophic bacteria. It was our understanding that this recommendation was made based upon past experience in similar testing.

8

For this reason, 24 and 48 hour heterotrophic plate counts on R2A agar has limited value.

IMPLICATIONS:

A) The limit of 7 days incubation for Heterotrophic bacteria may result in plate counts which underestimate the actual heterotrophic bacterial density of the sample.

B) Slow growing, heterotrophic species present may remain undetected and therefore unidentified.

REMEDY:

Each environment is unique. We can speculate as to the characteristics of the microbial population associated with the water recovery test but only after the test begins will we learn the facts. From the onset of the test, some of the heterotroph plates should be held for 21 days and counted at 14 and 21 days, in addition to the other specified periods. From these counts, we may learn if there are going to be bacteria in this population that require extended incubation times.

5) MEMBRANE FILTER POROSITY:

DISCREPANCY:

The procedures as presented in version 1.4 specify the use of membrane filters having a .45 um porosity.

CONCERNS:

From a microbiological standpoint, samples collected in the water recovery test will represent an oligotrophic, or low nutrient, environment. The presence of filterable bacteria (those that can pass through a .45 um filter) found in low nutrient environments has been documented in the articles referenced below. The use of .45 um porosity filters may allow some small diameter bacteria present in the water samples to pass through and be undetected.

M. T. MacDonell et. al. (5) collected samples of sea water around the Perdido Bay, Al. area. Viable filterable bacteria were found in each of the samples, some of which were filterable through a .2 um porosity filter. Electron micrographs supplied visual confirmation of various bacterial cocci and coccobacilli forms caught on the surface of a .2 um porosity filter, having a diameter approximately equal to that of the pores.

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P. S. Tabor et. al. (6) isolated species of filterable bacterial genera _Alcaligenes_, _Flavobacterium_, _Pseudomonas_, and _Vibrio_ from deep sea samples. Electron microscopy was performed to inspect the reduced size of these filterable bacteria in relation to the size of bacteria from more nutrient rich areas. Previously enriched isolates, exposed to a low nutrient, artificial sea water medium for 9 days, reduced in size to a point where 10 % became filterable through a .40 um porosity filter.

P. S. Amy et.al. (7) conducted a study in which a marine Vibrio sp. designated Ant-300 and _Escherichia coli_ were subjected to a low nutrient environment. Both isolates shrunk significantly within a period of 3 days. Starved Vibrio cells were subsequently inoculated into a nutrient rich medium and the effect was observed periodically via electron microscopy. It was determined that the cell size increased by 4 times in a period of 15 hours.

J. Shirey et al (8) used a filter tower with progressive .45 um and .22 um porosity filters to filter ground water samples. The investigators found that as total coliforms did not pass through to the .22 um filter, many other ground water bacteria did.

IMPLICATIONS:

The methods described in version 1.4 may result in plate counts which underestimate the actual bacterial density of the samples.

REMEDY:

The alteration of the procedure to conform with that specified in version 2.0 would alleviate the discrepancy. The use of the .2 um porosity filters is recommended for all methods in order to minimize confusion and difficulty.

6) PETRI DISH TYPE AND DIAMETER (MM)

DISCREPANCY:

The procedure as presented in revision 1.4 allows the use of 50 or 60 mm Petri dishes, and does not require tight fitting lids.

CONCERNS:

The submersion of the plates in the fecal coliform membrane filter procedure is an additional source of potential compromise of data validity.

The use of Petri dishes with loose fitting lids may allow moisture that gets into the sealed bag to alter the colony count from that plate. Excessive moisture may cause spreading of bacteria on the filter surface leading to a change in countable colonies or even confluency.

Fecal coliform bacteria are very susceptible to the effects of drying which are enhanced by the 44 degree C incubation temperature. Tight fitting Petri dish lids trap adequate moisture within the plate protecting the bacteria from this drying effect.

The 17th edition of standard methods for the examination of water and wastewater (2) specifies, in section 9-94, the use of 50 mm Petri dishes with tight fitting lids.

IMPLICATIONS:

The methods described in version 1.4 may result in inaccurate plate counts.

REMEDY:

The alteration of the procedure to conform with that specified in the 17th edition of Standard Methods would alleviate the discrepancy.

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M E M O R A N D U M

TO: Mr. Kenny Mitchell
           ED 62

FROM: Melvin V. Kilgore, Jr., R.M.

DATE: July 3, 1990

SUBJECT: Anaerobe Procedures

Due to our limited experience in Anaerobic Microbiology it was felt that the subject procedures should be reviewed by a Microbiologist having direct experience in this area. Microbiological Procedures Revision 2.0 and 1.4 were reviewed by Dr. Tom Phelps at the Institute of Applied Microbiology at the University of Tennessee. Dr. Phelp's comments are included as Attachment A.

Basically, his review indicates general areas within the Methods documents which are unclear or that require qualification and/or verification. Specifically, Dr. Phelps noted significant areas of concern with each of the anaerobic procedures. Based on these comments we have developed (in conjunction with Dr. Phelps) a new anaerobic procedure which will address these concerns and also provide a procedure more practical for NASA/MSFC's application (Attachment B). It is recommended that this procedure replace both the "qualitative" and "quantitative" procedures which currently exist.

In addition, the multiple tube method may be applicable to other WRT microbiological parameters and result in significant time savings to the laboratory. We would be interested to receive Dr. Wilson's comments regarding applicability to MSFC laboratory support as well as time and quality improvement over the current methods. If you have any questions concerning these comments feel free to contact Dr. Mikell or myself.

Tommy J. Phelps, Ph.D.
Inst. Appl. Microbiol.
10515 Research Dr. S. 300
Knoxville, TN 37932-2567

Tim Rhoads
SPMC Clinical Science Center
The Unversity of Alabama in Huntsville
201 Governors Dr.
Huntsville, Alabama 35801

Dear Tim and associates,

Enclosed please find my comments concerning the MB Manual 2.0 and 1.4 revision and my own ideas for sampling anaerobes from the water system. Please realize that there are many ways to accomplish a given task. I will try to communicate defieciencies, problems, limitations, and alternatives so that your organization may better accomplish goals. Please feel free to contact me for further explanations if so desired. Should you want further opinions from scientists familiar with anaerobic microorganisms I would suggest you contact Drs. Moore at VPI & SU. They worked with NASA in the 1970's and are experts in culturing and identifying anaerobes.

Background:
My review of the documents suggests that the intent of the specifications (Tables 2 and 7) was to show qualitative results suggesting that total anaerobes were less than 1 per 100 ml. (Also note that total aerobes are to be less than 1/100ml. As I stated previously, the proposed level for aerobes will not be attainable. For our purposes we will only consider the anaerobic specifications.) I sense the intent of the specifications was to insure that pathogenic anaerobes were absent from the water system. It is commonly accepted that most pathogenic anaerobes are norma flora of the intestinal tract, therefore it would be reasonable to assume that the intent of the specifications was to insure that intestinal flora and soil anaerobes would not be present in the water system. Bacteroides sp are the predominant genus in the colon and are opportunistic pathogens of infections and abdominal diseases. Other clinically important obligately anaerobic norma flora include Fusobacterium, Peptostreptococci, and Peptococci sp. which are common respiratory tract pathogens. Anaerobes from soil which are clinically important include Clostridium sp. observed in tetanus and gangrene infections. I feel it is important that any approved protocol be capable of effeciently culturing all of these important groups of anaerobes.

Effeciently culturing the clinically important anaerobes listed above requires strict anaerobic techniques and complex reduced media. Common procedures include the use of resazurin as a redox indicator, which turns pink at approximately -100 mV versus near 0 mv for methylene blue. Reductants such as cysteine-HCl or sulfide are advisable to thioglycollate because they have the ability to reduce media below -200 mV. Liquid culturing is preferred because of the lower attainable redox and lower solubility of oxygen. While colonies of anaerobes are often aerotolerant for

periods of time, individual vegatative cells are often killed after brief exposure to oxidized conditions. Defensible protocols should include these criteria.

Specific comments concerning Enumeration Procedures, revision 2.0.

1.p.2.   Sample preservation. Show the initial conc. of the thiosulfate solution as well as the final conc. of thiosulfate in the storage medium to avoid confusion.

2. p.2. Show the initial conc. of EDTA stock and final conc. of EDTA in the sample .

3.p.2 Sterilization: All ... are sterilized at 121... prior to each use.

4.p.2. Membrane filters: The 0.2 um filters must be checked with each of the targeted species on the proposed medium. Many microorganisms do not proliferate on filters and the low permeability 0.2 um filters are further inhibiting. In our experience less than 30% of aerobic cultures grew on 0.2 um filters vs >50% on 0.45 um filters.

5.p.2. Dilution and rinse water: The dilute peptone dilution rinse water is an excellent medium for growth. Many microorganisms would grow to dense turbidities on the peptone alone. Assumming 30% utilization of the peptone and 30% conversion of that to biomass could produce an optical density of >0.2 in a test tube examined at 660 nm (>75 mg biomass per liter medium). I would suggest your dilution media be a mineral salts soultion or a dilute gelatin solution as suggested in the VPI Anaerobe Manual. Furthermore I suggest you stipulate that no culture will be in contact with the dilution medium for greater than 1 hr.

6.p.3. Successive Filtrations: The UV sterilization needs to quantified in terms of uW cm2/sec delivered to the exact cm x cm areas requiring sterilization. While most vegatative cells are killed after 20,000 uW cm2/sec, spores will often survive. Doses of >200,000 may be required for 99% reduction of spores, which will necessitate eye protection.

7.p.4. Procedure step 2. Again I do not approve of peptone dilution solutions and particularly without time limitations.

8.p.4. step 3: 10 psi is not a partial vacuum.

9.p.4. Outline incubation conditions and times in procedure.

Section 2.1.10 Non-stringent heterotrophic anaerobic bacteria

1.p.25 Why not attempt to culture the clinically important anaerobes?

2.p.25. Many comments 1-9 above apply to this section as well.

3.p.25 Someone has drawn lines through the section on cysteine-HCL being added to the collection bottles, is it removed from the procedure? If it is removed then what is being done to remove the oxygen?

4.p.25. Transport: Again, how is oxygen being eliminated? A tremendous amount of oxygen dissolved in the water will be entering the collection bottle and some mechanisms need to remove the oxygen so the media will reduce to anaerobic conditions. A lot of cysteine HCl would be required and an alternative would be use of Oxyrase, a cell wall product which will enzymatically remove oxygen in the presence of lactate without destroying reductants. Such an oxygen scavenging system will reduce the oxygen tension of the initial sampling vessel so that when anaerobic media are inoculated the medium will remain reduced, as well as protecting the anaerobic microorganisms during processing and or transport.

5.p.25. Preparation: Autoclaving will remove oxygen from the medium. Reducing agents should be added immediately before autoclaving.

6.p.25. Any dilution solution should be prepared anaerobically as well.

7.p.26. Dispensing under a laminar flow hood will maximize oxidation of the media and minimize the ability to culture clinically important anaerobes.

8.p.26. Quality control: Are you using resazurin? If not you should be.

9.p.26. Why use Clostridium as a control? The spores will definitely survive the oxygenated treatments and grow later upon reducing the environment. Proper controls would include Bacteroides and clinically important nonsporing anaerobes.

10.p.26. Sample application: What reduced atmosphere are you using? Glove bags? Do you plan to test system with clinically important nonsporing anaerobes?

11.p.27. Dilution and rinse water: How will loosening caps result in outgasing of oxygen? Loosening of caps will equilibrate the oxygen between atmospheric, headspace and aqueous phase. I would recommend preparation of anaerobic dilution bottles.

Comments on Revision 1.4 for presence of non-stringent anaerobic bacteria. Pages 28-31.

1.p.28. I recommend focusing on clinically important anaerobes, and in so doing use reductants which will optimize results ie. cysteine-HCl and not thioglycollate.

2.p.28. Refer to previous comments and apply where appropriate.

3.p.28. Purging: can be done prior to autoclaving.

4.p.29. Silicone and teflon are very oxygen permeable. I much prefer butyl rubber. If butyl can not be used then make sure that all anaerobic solutions and incubations are stored inverted so that oxygen would have to go through the aqueous phase rather than gas phase exchange. This simple inversion will reduce oxygen diffusion into bottles 5 - 10 fold.

5.p.29. Enr. Quality Control: Resazurin should be reduced throughout the medium.

6.p.29. Again, use clinically significant nonsporing anaerobes as QC.

7.p.29. Caution: If the gas phase is greater than the liquid phase and thick walled glass used then media can be diluted so that gas production will not result in broken bottles and venting needle not required. For example if glucose and each nutrient are not more than 2 g / l growth will still be luxuriant but gas production will not explode the bottles. This will be easy to QC so that venting needles not required during growth.

8.p.30. Sample collection: Why are 18 g needles on the sample port? Can these be changed to 20-22g?

9.p.30. This vent is likely required, if butyl rubber bungs are used then use a 23 x 1 in needle.

10.p.30. I would consider 4x media so that sample plus media is less than 1/2 of bottle total volume.

11.p.30. This mess with plugging the hole would be unneccessary if butyl were used and a slightly smaller needle.

12.p.31. Incubation: I recommend holding for 10 days.

13.p.31. Diluents should be made and held anaerobically.

14.p.31. Isolation and identification: I recommend that isolates regardless of facultative or obligate anaerobes be examined by the MIS system, a library established and compared as a resource to known bugs and repeats of the bugs from your system. If the MIS system does not recognize a bug then it could be further identified and that id then cataloged in the MIC computer database.

I view the anaerobe testing as the only protocol examining contamination by anaerobic microorganisms form soil or human norma flora which can represent a pathological threat to water consuming inhabitants. Let us assume that the term "qualitative" in the specifications means that all anaerobes will not be examined. In fact, the suggested protocols will not culture sulfate reducing or methane producing anaerobes and many other groups which may be detrimental to the system. However, the protocols should at least examine those anaerobes which represent a health threat. The protocol 2.0 would successfully culture spores residing within the water system but would likely be unable to culture many vegatative cells of spore forming microorganisms as well as many nonsporing anaerobes of clinical significance. In the ensuing paragraphs I will attempt to describe alternatives which would prove successful at culturing the clinically significant anaerobes.

The rule of thumb in anaerobic microbiology is that the entire system should be anaerobic and below the redox of resazurin. Secondly, all media should be reduced with cysteine-HCl. If oxygen is a problem then one reduces its impact by performing initial growths in aquueous phase where oxygen solubility is lower. Since oxygen is a severe problem in the water system I recommend using MPN procedures rather than filtering. Furthermore, filtering results in considerable stress of cells and often death and reduced culturing effeciency. If you opt for filtering as proposed then you must demonstrate suitability of the techniques with nonsporing anaerobes of clinical importance. Sampling would be best by one of two methods; either adding aliquots of water directly to MPN media bottles or by taking one larger sample for subsampling into MPN tubes. Either way one must remove the oxygen soluble in the sampled water rapidly without creating toxic end products or utilizing all of the reducing potential of the reductants. This problem means that sulfide, a common reductant, is unsuitable because of the toxicity of elemental sulfur (oxygen plus sulfide yields sulfur). A lot of cysteine could be added but the best way to remove the large concentrations of oxygen from the sampled water is by use of Oxyrase (information enclosed). In the presence of 20 mM lactate, oxyrase (actually membrane components of E. coli) will remove nearly all of the oxygen so that trace additions of cysteine will reduce the media.

Another consideration of the MPN technique is the volume of sample to be incubated. Enclosed please find a copy of an MPN table from Vol 14 of Standard Methods (APHA) (this table is not in newest version) showing a three tube and five tube dilution series. At 10 ml sample per vial the three tube series could enumerate 4 bacteria /100ml. If one used 50 ml inoculum into three tubes followed by 5 and 0.5 ml inoculum into three tubes (with the 3 tube MPN series) then one of the 50 ml samples and another sample vial would have to exhibit growth before the bacterial concentration would be > 1 per 100 ml. (1-0-1 or 1-1-0 positive tubes for the MPN value to be 7 / 100 ml if 10 ml were used or 7/5 = 1.4 / 100 ml since 50 ml were used as inoculum volume.) Any combination of positive results not including one positive result from the 50 ml inoculum or just one positive result from the 50 ml inoculum and no other positive vials would result in MPN values less than 1 per 100 ml (3 or 4 per 100 ml if 10 ml used and 4/5 or 0.8 since 50 ml used. Consequently, the fewest number of tubes and smallest inoculum capable of measuring 1 anaerobe per 100ml (and still allowing one false positive due to contamination to not cause alarm) would be use of the three tube per

series MPN with 50, 5 and 0.5 ml inocula. Anything with more tubes or larger inocula would be added insurance.

Protocol recommended by T. J. Phelps.

1. Using 20 - 22 g needles inject sample from the water system into anaerobic containers. These containers could be media MPN bottles or a sample bottle. If a sample bottle is used it should be a 250 ml bottle containing Oxyrase plus lactate in 10 -20 ml anaerobic buffer containing a final concentration of 2 -5 mM phosphate and 2-10 mM bicarbonate at pH 7-7.3 (nitrogen gas phase). Volumes up to 175 ml could be injected into the sample bottle. The Oxyrase would then remove the oxygen dissolved in the water sample, decreasing the reducing capacity needed in the medium. After 30 min to 1 hr equilibration in the sample bottle aliquots would be withdrawn via syringe and inoculated into MPN media. Syringes would be flushed with the gas phase of the sample bottle to reduce oxygen additions into the MPN bottles.

2. I propose using a three tube MPN series at three dilutions. The first set of three bottles would use 50 ml inocula each. The bottles could be 125 ml serum vials with 25 ml of 5X concentrated medium pre-reduced with cysteine-HCl and nitrogen/CO2 gas phase. Upon addition of 50 ml of the anaerobic water the positive pressure would be relieved by withdrawing 50 ml of gas while removing the syringe. The 5ml and 0.5 ml bottles could be 25 ml serum vials or anaerobic tubes containing 10 ml medium.

3. All bottles would be inverted, and incubated for 10 days at 23-30C. Incubations would be better in an anaerobic incubator but insuring resazurin remains reduced is the important criteria, and incubations in anaerobic chambers should not be required if butyl rubber septa are used and all media incubated inverted.

4. Turbid bottles would be scored as positive, values calculated from the MPN table and recorded. Positive tubes would be streaked onto prereduced anaerobic agar (plates poured and stored in the glove bag) and incubated in the anaerobic incubator (also would help if they were in anaerobic pouches). All cultures would be screened on the MIS system and aerotolerance determined, and tentative ID stored on the MIS data library. Agar for streaking should be rather concentrated compared to the dilute MPN media. An excellent recipe medium would be BHIA supplemented with yeast extract and vit K-heme (recipe enclosed). Alternatively use the agar recommended for the MIS anaerobe system (contact Dr. Sasser or his MIS company, I have not used the anaerobe library and do not have a copy of his recipe).
   Each isolate should be streaked in the glove bag onto the rich agar plates, stored in anaerobic containers in an oxygen free environment with palladium catalysts plus hydrogen and or gas packs and examined after 3 but not discarded until 10 days.

Alternatives:

a. If water samples are to be added directly to the media I suggest that the media be prepared anaerobically without cycteine-HCl but with Oxyrase. 1/2 hr after sample added to media then cysteine-HCL added as a final reductant. This way the Oxyrase would remove most of the oxygen and the cysteine-HCl

would then reduce the media.

b.   Media could be prepared by you, you could modify these procedures and use media form Fisher page 1477, anaerobic blood culture bottles, or you could contact Oxyrase and have them get the media prepared to your specifications and quality controlled.

c. QC is essential. You need research to insure growth fo the following species; Bacteroides, Clostridium, Peptococcus, Peptostreptococcus, Fusobacterium and we may as well try desulfovibrio. Furthermore research ought to determine the effeciency of recovery of vegatative cells from oxygenated waters using the protocols.

d. Media; I recommend a complex medium containing a mineral salts solution, phosphate buffer (20 mM), and approx. 2 g / l each of glucose, trypticase, peptone, yeast extract, 25 mM lactate, 15 mM sulfate, and the final conc. of cysteine-HCl of 0.05% and resazurin at 0.5 ml /l of a 0.1% solution. The media should also contain vit K-heme as per instructions enclosed (p. 127 of VPI Anaerobe Laboratory Manual 2nd Ed.). I prefer media with 2-5 mM phosphate buffer and 2-10 mM bicarbonate buffer (or CO2 in the gas) at fianl pH of 7 - 7.3. NOTE THAT PH may change during autoclaving. QC will verify that the control bugs will grow but not explode the vials even when 50 ml sample plus 25 ml of 5X media are in the 125 ml vials (actually hold 158 ml).

e. Nitrogen is a cheap gas readily available and can be used in all vials. It is inert and does not alter pH. Unfortunately, many bacterai like a bit carbon dioxide. Therefore I suggest all anaerobic media contain 5% carbon dioxide in the nitrogen or 10 mM bicarbonate buffer. I use lot of gases so I keep 5% CO2/95% N2 in my lab as do most people doing lots of anaerobic studies but it can be alleviated by adding 10 mM bicarbonate buffer as long as all tubes are autoclaved as sealed conditions.

f. MPN dilutions could be done inthe anaerobic chamber but experience will likely lead to avoiding long working shifts in the chamber at all costs. syringe methods are still useful in the anaerobic chamber because bacterail aerosols are abundant in the bags. In the bags one sterilizes bungs and bottle caps by burning them (heating to excess) against a red hot heating element (Coy Man., Ann Arbor MI).

A section should also be included on safety:

Anaerobic microbiology can be dangerous, if explosive gases are used care must be taken, particulary if hydrogen gas is >7% in the glove bag and oxygen enters through a leak and the palladium catalysts glow; dive for the floor!!!!
Bottles will break and explode so I wear chain mail gloves (from meat packing industry) when making media and gassing tubes.
Needles will stab you so care must be used, particularly in transferring cultures.
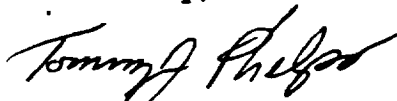Sulfide is highly toxic, and should only be used in hoods or via syringes.
Any toxic solutions should be maintained in 58 ml serum vials because these will often survive being knocked from a counter to the floor, larger vials will often break when dropped.

Prior to cleaning or autoclaving used media the overpressured gas must be releived by inserting a needle (23 x 1 in best) and venting in a hood away from your body. Aerosols from respiratory pathogens can be dangerous.

There are several items which should be adressed with some R & D:
a. QC with nonsporing anaerobes of clinical significance; and optimizing media for culturing anaerobes of importance.
b. effeciency of recovery of anaerobic vegatative cells
c. applicability of Oxyrase.
d. developing a turnkey operation.
e. library of isolates and characteristics.
f   examining other anaerobes such as sulfate reducers, methanogens, acetogens etc., which may be detrimental to the system integrity but not directly human pathogens, are these other bugs present, can the described protocols sereve as an indicator of when further detailed analyses are necessary (ie., when anaerobes > 100/ml should detailed examination for other bugs initiate?)
g. How well do these protocols accomplish tasks?
h. Are these or other useful as trouble shooting procedures?
i. correlation with water chemistry?

I hope these pages are of use. Please feel welcome to contact me if I can be of further assistance.

sincerely,

Tommy J. Phelps

DRAFT

2.4 Multiple Tube Fermentation (MTF) Method

The MTF method is based on the succesive dilution of a sample to the point of extinction of any organisms present in that sample.

Sample preservation and storage. Sodium thiosulfate (10% v/v) should be added to the samples (0.1 mL $Na_2S_2O_3$/100 mL sample) to give a final concentration of 0.01% where residual chlorine or other halogen is suspected.

Samples containing total metal (copper, zinc or heavy metals) at concentrations exceeding 10 ug/L should additionally be preserved using a chelating agent to reduce potential toxicity by these compounds. This may be acheived by the addition of 0.3 mL of a 15% solution of EDTA per 100 mL of sample giving a final concentration of 0.045%. The pH of the EDTA solution should be adjusted to 6.5.

Note: The sodium thiosulfate and EDTA should be added to the sample collection bottles prior to autoclaving if possible. These solutions may be combined and added as a single solution.

Samples not processed within one hour of collection should be stored between 4° and 10° C until analyzed. Samples should be analyzed within a maximum of eight (8) hours following collection.

Media preparation. Refer to individual procedure.

Dispensing. The appropriate medium for the test will be dispensed in the MTF tubes in two volumes. Three tubes containing 1.0 mL of the 10X stock media (except for the fecal coliform protocol which will require three tubes containing 10 mL of a 2X stock) and 21 tubes containing 9.0 mL of 1X or diluted stock media, will be needed per sample. Refer to the individual procedure for the proper tubes to be used.

Sterilization of apparatus and materials. All MTF tubes, glassware, pipette tips and utensils are presterilized at 121° C for 15 minutes.

Arrangement and inoculation. Three replicates of each sample volume (dilution) will be inoculated. The arrangement of the tubes is critical to determine the results of the procedure. The first series of replicate tubes will contain 1.0 mL of 10X stock media and will be inoculated with a sample volume of 10.0 mL. The next series of replicate tubes will contain 9.0 mL of 1X media and will be inoculated with a sample volume of 1.0 mL. The third series of replicate tubes will also contain 9.0 mL of 1X media but will be inoculated with a sample volume of only 0.1 mL. Each subsequent series of replicates will contain 9.0 mL of 1X media.

Starting with the third series of replicates, vortex the inoculated tube for a sufficient time and speed to facilitate adequate mixing and transfer 1.0 mL into the next replicate. Repeat this procedure changing to a sterile pipet tip at each dilution series.

The completed test will represent three replicate series of sample dilutions ranging from 10^{+1} to 10^{-7}. **Aseptic technique will be used for all above procedures.**

Quality control. Prepared MTF tubes should be incubated by conditions specified by the specific procedure and inspected for contamination. Before use, these tubes should be inspected again for turbidity or precipitaion that could alter the results of the test.

Incubation. Incubate all labeled replicates of MTF tubes as directed by the specific procedure.

Interpretation of results. Inspect the MTF tubes and count the number of turbid tubes in each series. The first tube without obvious turbidity should be examined closely for any trace of turbidity or precipitated growth. Note the most dilute set of sample tubes having all three tubes positive (turbid) for that dilution. Note also the number of positive tubes for the next two dilutions. Compare the three numbers for these three sets of dilutions to the 3 tube Most Probable Number (MPN) table found in the 14th edition of Standard Methods for the Examination of Water and Wastewater. The three numbers will correspond to a #CFU/100 mL sample, within the 95% confidence interval. The table will give a CFU value for initial sample dilutions of 10^1, 10^0 and 10^{-1} (the tubes with 10, 1 and .1 mL of sample added directly to the MTF tube). The actual value for the bacterial density must be calculated by considering the number of dilutions from the 10X tube to the most dilute set of tubes having all three tubes positive for that dilution. This number of dilutions will equal the log value that should be added to the value from the table. For example:

Volume Sample Added

● Turbid

○ Clear

Media Concentration

Final Sample Dilution

Serial Dilutions

| 10ml | 1ml | .1ml | | | | | | |

2X 1X

10^{+1} 10^{0} 10^{-1} 10^{-2} 10^{-3} 10^{-4} 10^{-5} 10^{-6} 10^{-7}

In the example above, the most dilute replicate series with all members turbid, or positive, (denoted by the diagonal lines) is followed by a replicate set with one tube positive and the next having all tubes clear (both replicate sets denoted by the grid lines). The three number code 3-1-0 corresponds to an MPN index value of 43 CFU/100 mL in the table. Since the most dilute replicate set with all members positive is 4 logs more dilute than the 10X tube, this amount must be added to the value from the table. Therefore, 43 CFU/100 mL becomes 4.3 X 10^8 CFU/100 mL.

THREE TUBE MPN TABLE

Sample (10 mL)	Sample (1.0 mL)	Sample (0.1 mL)	MPN INDEX CFU/100 mL
0	0	0	<3
0	0	1	3
0	1	0	3
1	0	0	4
1	0	1	7
1	1	0	7
1	1	1	11
1	2	0	11
2	0	0	9
2	0	1	14
2	1	0	15
2	1	1	20
2	2	0	21
2	2	1	28
3	0	0	23
3	0	1	39
3	0	2	64
3	1	0	43
3	1	1	75
3	1	2	120
3	2	0	93
3	2	1	150
3	2	2	210
3	3	0	240
3	3	1	460
3	3	2	1100
3	3	3	>2400

MTF-MPN SAMPLE DATA SHEET

SAMPLE #_____

SAMPLE LOCATION AND TYPE_____

DATE AND TIME COLLECTED_____

COLLECTED BY_____

DATE AND TIME ANALYZED_____

ANALYZED BY_____

10^1 10^0 10^{-1} 10^{-2} 10^{-3} 10^{-4} 10^{-5} 10^{-6}

THREE NUMBER CODE FROM FIGURE ABOVE_____

CORRESPONDING VALUE FROM MPN TABLE_____ CFU/100ml

LABORATORY DIRECTOR_____   DATE_____

2.4.1   Enumeration of Anaerobic Bacteria by the MTF Method

This procedure is designed for the cultivation and enumeration of stringent and nonstringent anaerobic bacteria from a water sample using the Multiple Tube Fermentation (MTF) method. The organisms will be cultured in a complex medium containing Cysteine-HCl as a reducing agent, and resazurin to indicate adequate reduction.

Procedure

Sample preservation and storage.   See section 2.4.

Sample collection.   Samples should be collected and incubated in a manner that will avoid any contact with oxygen. Contact with oxygen may significantly reduce the number of anaerobes culturable from the samples. Oxyrase (Oxyrase Inc., Ashland, OH) is added to the sample bottles to scavenge large amounts of oxygen that may be present in some samples upon collection.

Collection buffer.   Prepare 125 mL anaerobe sample bottles (Fisher cat. #06-406K) by adding 10 mL of anaerobic buffer containing the following ingredients per liter of distilled or deionized water:

Potassium phosphate monobasic	2.0 gm
Potassium phosphate dibasic	2.613 gm
Sodium lactate	1.681 gm
Resazurin (0.1% soln.)	0.5 mL
Sodium thiosulfate (10% soln.)	0.1 mL
*EDTA (15% soln.)	0.3 mL

*   Add only if concentrations of copper, zinc or heavy metals are greater than 10 ug/L.

The final pH of the buffer should be 7.2 $\pm$ 0.1 at 25° C. A Nitrogen-CO_2 (95:5, oxygen free) head space should be applied to the bottles just prior to sealing. Bottles should be fitted with a grey flanged, slotted plug, 13 X 20 mm stoppers (Fisher cat. #06-406-12) and 20 mm aluminum seals (Fisher cat. #06-406-14B). Sealed sample bottles should be autoclaved at 121° C for 15 minutes. Sterile sample bottles should be cooled to 50° C and 2 mL of presterilized Oxyrase (Oxyrase Inc., Ashland, OH) added via 20-22 gauge syringe aseptically. Allow sample bottles to incubate at room temperature, in an inverted position, for 24 hours and inspect for contamination prior to usage. Record the number of contaminated bottles in the Media Preparation Log Book. Discard all contaminated bottles. If more than 10% of the bottles are contaminated, discard the entire batch. The prepared sample bottles should be stored in the dark in an inverted position.

Samples (50 mL) should be collected in these sample bottles from sample ports fitted with hypodermic needles.

Ideally, samples should be processed on-site 30 minutes to one hour after collection. All samples must be processed **within four (4) hours following collection.**

<u>Media preparation.</u> Prepare the 10X MTF media by adding the following ingredients per liter of distilled or deionized water:

Potassium phosphate monobasic	13.61 gm
Potassium phosphate dibasic	17.42 gm
Sodium lactate	28.02 gm
Sodium sulfate	36.77 gm
Glucose	20 gm
Trypticase	20 gm
Peptone	20 gm
Yeast extract	20 gm
Magnesium Sulfate	5.0 gm
Ammonium Chloride	5.0 gm
Sodium Chloride	5.0 gm
Cysteine-HCl	0.5 gm
Resazurin (0.1% soln.)	5.0 mL
Vitamin K Heme soln.	100 mL

The Vitamin K Heme solution is prepared by adding 1 mL menadione stock solution to 100 mL hemin stock solution. Menadione and Hemin are supplied by the Sigma Chemical Company and are prepared as follows:

Menadione stock solution: Add 100 mg menadione to 20 mL 95% ethanol. Filter sterilize.

Hemin stock solution: Dissolve 50 mg hemin in 1 mL of 1 N NaOH, add 100 mL of distilled water. Autoclave at 121° C for 15 minutes.

Prepare 1X media by diluting 1 part of the 10X stock with 9 parts of distilled or deionized water.

Heat with continuous stirring allowing the medium to boil in order to completely dissolve the ingredients, then promptly remove the media from the heat. The final pH of the medium should be 7.2 $\pm$ 0.1 at 25° C.

Dispense the media into Bellco 18 X 150 mm aluminum seal type anaerobic culture tubes with caps and septa (cat. #2048). This tube size is necessary for a head space volume of no less than 50%. Prepare tubes containing 9 mL of the 1X media and 1 mL of the 10X media in a ratio of 7:1 respectively (refer to section 2.4). Before capping, apply a nitrogen-CO_2 (95:5, oxygen free) head to the tubes.

<u>Sterilization.</u> Autoclave tubes and bottles at 121 degrees Celsius for 15 minutes. Do not allow the medium to stand for more than 1 hour between preparation and autoclaving.

MTF tubes and sample bottles should be inverted when they are removed from the autoclave and remain in this position until use to minimize reoxidation.

Quality control. Allow MTF tubes and sample bottles to incubate at room temperature, inverted, in the dark for 24 hours and inspect for contamination prior to usage. Record the number of contaminated tubes or bottles in the Media Preparation Log Book. Discard all contaminated tubes or bottles. If more than 10% are contaminated, discard the entire batch.

Inoculate a randomly selected positive control MTF tube with Bacteroides fragilis (ATCC 23745) and incubate in an inverted position. Development of turbidity in this tube upon incubation will demonstrate the ability of this procedure to culture vegatative anaerobic cells. Inoculate a second randomly selected negative control MTF tube with Xanthomonas maltophilia (ATCC 13637) and incubate in an inverted position. No turbidity should develop in this tube. Record the results obtained for each batch of tubes in the media preparation log book.

Storage. The prepared tubes may be stored at 4° C, inverted, in the dark for up to 14 days. Tubes should be allowed to equilibrate to room temperature just prior to use. Any red color that may develop is due to reaction of oxygen with the resazurin. Discard any tubes or bottles that exhibit a red color.

Sample application. Once the sample has had 30 minutes to one hour to equilibrate in the sample collection bottle, aliquots can be withdrawn via 20-22 gauge hypodermic needle and transfered to the MTF tubes. The syringe should be flushed out with the N-CO$_2$ gas of the sample bottle to reduce addition of oxygen to the MTF tubes. Upon addition of the 10 ml aliquots into the 10X MTF tubes, the positive pressure should be relieved from the MTF tube by withdrawing 10 mL of gas before withdrawing the needle. Refer to section 2.4 for further instructions.

Note: An alternative method for this protocol would be to conduct all sample manipulations within an anaerobic glove bag. This would alleviate the need for syringes in sample transfer but the glove bag would add some degree of difficulty and inconvenience.

Incubation. Incubate the inverted culture tubes in an inverted position at 28 ± ° C for 10 days. Tubes do not have to be incubated in an anaerobic environment. This procedure insures the production and maintenance of a reduced atmosphere within the sample bottles and MTF tubes.

Interpretation of results. Refer to section 2.4.

Confirmation. Aliquots from turbid tubes should be applied to prereduced BHIBLA anaerobic agar plates (specified for use with the MID system) by the Spread Plate Method (refer to section 2.3).

Incubate the inoculated plates anaerobically at 28° C and examine after 3 but do not discard until after 10 days.

All cultures should be screened by the MID system, aerotollerance determined, and tentative ID stored on the MID data library. BHIBLA agar may be prepared in the lab as follows:

Brain Heart Infusion agar (Gibco #M06600B)	53 gm
Yeast Extract (Difco #0127-01)	5 gm
Hemin Chloride 0.1% solution (Sigma #H2375)	5 mL
Vitamin K 11.0% solution (Sigma #V3501)	0.1 mL
Distilled water	1 liter

Heat with continuous stirring allowing the medium to boil in order to completely dissolve the agar, then promptly remove the medium from the heat. The final pH of the medium should be 7.2 $\pm$ 0.2 at 25° C.

Sterilization. Autoclave at 121 degrees Celsius for 15 minutes. Do not allow the medium to stand for more than 1 hour between preparation and autoclaving.

Cool the sealed flask containing the media to 50° C in a water bath. In an anaerobic glove bag, add 50 mL of aseptic defibrinated sheep blood while continuously stirring flask. Dispense into 15 X 100 mM Petri plates and allow to solidify. Store plates in the anaerobic glove bag.

Note: BHIBLA agar may be obtained in prepoured form from Carr-Scarbourough (pdt. #01-1128-03)

2.4.2 Enumeration of Total Heterotrophs by the MTF Method

This procedure is designed for the cultivation and enumeration of heterotrophic bacteria from a water sample using the Multiple Tube Fermentation (MTF) method. The media used, R2A, is a low nutrient medium designed to culture heterotrophic bacteria. This includes oligotrophic bacteria, characteristically found in these aquatic environments.

Procedure

Sample preservation and storage. See section 2.4.

Preparation. Suspend the following ingredients in 1 liter of deionized or distilled water. This will result in a 10X stock solution of R2A broth. Preformulated R2A agar medium (Difco Laboratories, Detroit, MI) is available, but the broth form must be prepared in the laboratory.

Bacto Yeast Extract	5.0 gm
Proteose Peptone #3	5.0 gm
Bacto Casamino Acids	5.0 gm
Bacto Dextrose	5.0 gm
Soluble Starch	5.0 gm
Sodium Pyruvate	3.0 gm
Potassium Phosphate, Dibasic	3.0 gm
Magnesium Sulfate	0.5 gm

Heat with continuous stirring allowing the medium to boil in order to completely dissolve the ingredients, then promptly remove the medium from the heat. The final pH of the medium should be 7.2 ± 0.2 at 25° C.

Prepare 1X media by diluting 1 part of the 10X stock with 9 parts of deionized or distilled water.

Dispense the media into 16 X 150 mm slip cap tubes. Prepare tubes containing 9 mL of the 1X media and 1 mL of the 10X media in a ratio of 7:1 respectively (refer to section 2.4). Apply slip caps to all tubes before autoclaving.

Sterilization. Autoclave at 121 degrees Celsius for 15 minutes. Do not allow the medium to stand for more than 1 hour between preparation and autoclaving.

Quality control. The prepared 1X medium should be very light amber in color and is translucent. Darkened medium may indicate an increased time of temperature in sterilization and should not be used.

Allow MTF tubes and to incubate at room temperature, in the dark for 24 hours and inspect for contamination prior to usage. Record the number of contaminated tubes in the Media Preparation Log Book. Discard all contaminated tubes.

If more than 10% are contaminated, discard the entire batch.

Inoculate a randomly selected positive control MTF tube with _Staphylococcus epidermidis_ (ATCC 12228) and incubate under aerobic conditions. Development of turbidity in this tube upon incubation will demonstrate the ability of this procedure to culture this heterotrophic control organism. This is not a selective medium, therefore a negative control is not required. Record the results obtained for each batch of tubes in the media preparation log book.

Storage. The prepared tubes may be stored at 4 degrees Celsius in the dark for up to 14 days. Tubes should be allowed to equilibrate to room temperature just prior to use.

Sample application. Refer to section 2.4, MTF procedure for instructions.

Incubation. Incubate the culture tubes aerobically at 28 ± 0.2° C for up to 21 days. Examine the tubes for turbidity after 24 and 48 hours, after 7 days then weekly as some oligotrophic bacteria may require long incubation periods.

Interpretation of results. Determine the Most Probable Number (MPN) of Heterotrophic bacteria present as described in section 2.4.

Confirmation. Aliquots from turbid tubes should be applied to full strength R2A agar plates by the Spread Plate Method (refer to section 2.3) and incubated aerobically at 28 ± 0.2° C for up to 21 days. Pick all colonies representing different morphological types or a minimum of 10 colonies per plate. Isolates should be Gram stained and identified using standard biochemical tests and/or FAMES profiling.

2.4.3 Enumeration of Fecal Coliforms by the MTF Method

This procedure is designed for the cultivation and enumeration of fecal coliforms from a water sample using the Multiple Tube Fermentation (MTF) method. The A-1 media used is designed to differentiate between coliforms of fecal origin and coliforms from other sources.

Procedure

Sample preservation and storage. See section 2.4.

Preparation. Suspend the following ingredients in 1 liter of deionized or distilled water. This will result in a 2X stock solution of A-1 broth (17th edition of Standard Methods for the Exmination of Water and Wastewater).

Lactose	10 gm
Tryptose	40 gm
NaCl	10 gm
Salicin	1.0 gm

Heat with continuous stirring allowing the medium to boil in order to completely dissolve the ingredients, then promptly remove the medium from the heat. Cool the media to 50° C before the addition of 2.0 mL of Polyethylene glycol p-isooctylphenyl ether (Triton X 100, or equivalent). Adjust the final pH of the media to 6.9 $\pm$ 0.1 at 25° C.

Prepare 1X media by diluting 1 part of the 2X stock with 1 part of deionized or distilled water.

Dispense 10 mL of 2X media into 18 X 150 mm slip cap tubes and 9 mL of 1X media into 16 X 125 mm slip cap tubes. Prepare the tubes containing the 1X media and the 2X media in a ratio of 2:1 respectively (refer to section 2.4). Insert an inverted Durham tube and apply slip caps to all tubes before autoclaving. Durham tubes will be submersed and all air removed upon autoclaving.

Sterilization. Autoclave at 121 degrees Celsius for 10 minutes. Do not allow the medium to stand for more than 1 hour between preparation and autoclaving.

Quality control. Allow MTF tubes to incubate at room temperature, in the dark for 24 hours and inspect for contamination prior to usage. Record the number of contaminated tubes in the Media Preparation Log Book. Discard all contaminated tubes. If more than 10% are contaminated, discard the entire batch.

Inoculate a randomly selected positive control MTF tube with Escherichia coli (ATCC 25922) and incubate as directed below. Development of turbidity in the MTF tube and accumulation of gas in the Durham tube within 24 hours or less demonstrates the ability of this procedure to culture and detect coliforms of fecal origin.

Inoculate a second randomly selected negative control MTF tube with *Proteus vulgaris* (ATCC 13315). This MTF tube should become turbid but should not exhibit gas production. Record the results obtained for each batch of tubes in the media preparation log book.

Storage. The prepared tubes may be stored at 4 degrees Celsius in for up to 14 days. Tubes should be stored in the dark and allowed to equilibrate to room temperature just prior to use.

Sample application. Only three series of replicates will be prepared for the fecal coliform MPN tests (sample volumes of 10 mL, 1.0 mL and 0.1 mL added directly to MTF tubes). The necessary vortexing of sample in any further dilutions would cause air to accumulate in Durham tubes resulting in a false positive tube. Refer to section 2.4, MTF procedure for additional instructions. **Note: apply samples to MTF tubes in a manner that will avoid the collection of air bubbles in inverted Durham tubes.**

Incubation. Incubate the culture tubes aerobically at $35 \pm 0.5°$ C for 3 hours. Transfer the tubes to a water bath at $44 \pm 0.2°$ C and incubate for an additional 21 ± 2 hours.

Interpretation of results. Gas production in any of the tubes within 24 hours or less is a positive reaction indicating coliforms of fecal origin. Determine the Most Probable Number (MPN) of fecal coliforms present as described in section 2.4.

Confirmation. Aliquots from turbid tubes should be applied to full strength TSB agar plates by the Spread Plate Method (refer to section 2.3) and incubated aerobically by the instructions above. Pick all colonies representing different morphological types or a minimum of 10 colonies per plate. Isolates should be Gram stained and identified using standard biochemical tests and/or FAMES profiling.

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MEMORANDUM

TO: Mr. Kenny Mitchell
           ED/62

FROM: M. V. Kilgore, Jr.

DATE: November 2, 1990

SUBJECT: Microbiological Methods for the Water Recovery Systems
           Test, Revision 3.0

Enclosed is the latest draft of the subject document. This revision represents our most current recommendations based on the latest scientific information available to us. The procedures have been revised to be consistant with findings from recent WRT test data, noted problems and adherence to Standard Methods, 17 th edition.

The procedures previously outlined for Gram positives, Gram negatives, enterics, yeast and molds and anaerobes have been deleted. New procedures for yeast and molds and anaerobes have been included as appropriate substitutes. In addition, a membrane filtration procedure for staphylococci and a broth procedure for heterotrophs have been included based on previous recommendations following the preliminary review of the microbiological WRT data. A highlighted copy identifying the specific changes has been included for your convienence.

If you have any questions please do not hesitate to call.

cc: Ms. Mary Traweek

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MICROBIOLOGICAL METHODS

FOR THE

WATER RECOVERY SYSTEMS TEST

REVISION 3.0

MICROBIOLOGICAL METHODS FOR THE WATER RECOVERY SYSTEMS TEST

UAH Research Report Number 835

Revision 3.0

Prepared For:

National Aeronautics and Space Administration
Marshall Space Flight Center

Prepared By:

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October 24, 1990

MICROBIOLOGICAL METHODS FOR THE WATER RECOVERY SYSTEMS TEST

1.0 INTRODUCTION

Current microbiological parameters specified to verify microbiological quality of Space Station Freedom water quality include the enumeration of total bacteria, anaerobes, aerobes, yeasts and molds and fecal coliforms. In addition, other parameters have been identified as necessary to support the Water Recovery Test activities to be conducted at the NASA/MSFC later this year. These include: aerotolerant eutrophic mesophiles, legionellae and an additional method for heterotrophic bacteria.

If inter-laboratory data are to be compared to evaluate quality, analytical methods must be eliminated as a variable. Therefore, each participating laboratory must utilize the same analytical methods and procedures. Without this standardization, data can be neither compared or validated between laboratories.

Multiple laboratory participation represents a conservative approach to insure quality and completeness of data. Invariably, sample loss will occur in transport and analyses. Natural variance is a reality on any test of this magnitude and is further enhanced because biological entities, capable of growth and death, are specific parameters of interest. The large variation due to the participation of human test subjects has been noted with previous testing. The resultant data might be dismissed as "out of control" unless intra-laboratory control is included as part of the method or if participating laboratories are not available for verification.

The purpose of this document is to provide standardized laboratory procedures for the enumeration of certain microorganisms in water and wastewater specific to the water recovery systems test. At the present time, the document consists of ten separate cultural methods and one direct count procedure. It is not intended nor is it implied to be a complete microbiological methods manual.

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2.0 ENUMERATION PROCEDURES

2.1 Membrane Filtration Method

Sample preservation and storage. Sodium thiosulfate at a concentration of 10% should be added to samples (0.1 mL $Na_2S_2O_3$/100 mL sample) where residual chlorine or other halogen is suspected.

Samples suspected of containing copper, zinc or heavy metals should be additionally preserved using ethylenediaminetetraacetic acid (EDTA) as a chelating agent. 0.3 mL of a 15% solution of EDTA, adjusted to pH 6.5, is used per 100 mL of sample.

Note: The sodium thiosulfate and EDTA solutions should be added to the sample collection bottles prior to autoclaving. These solutions may be combined and added as a single solution.

Samples not processed within one hour of collection should be stored between 4° and 10° C until analyzed. Samples should be analyzed **within a maximum of eight (8) hours** following collection.

Media preparation. Media for this test is prepared by dispensing 5 mL of sterile molten media into 50 x 9 mm petri dishes with tight fitting lids. See the appropriate method in the following sections for specific media preparations.

Sterilization of apparatus and materials. All glassware, filter units, filter holders and utensils are presterilized at 121 degrees Celsius for 15 minutes.

Membrane filters. 47 mm diameter, 0.2 um porosity, white gridded (MSI, Irving, CA) membranes are used for this procedure except where indicated otherwise by a specific method. These filters should be purchased presterilized.

Volume requirements. When less than 20 mL of sample (including diluent) is to be filtered, add approximately 10 mL of sterile buffered dilution water to the funnel before filtration. Unequal distribution of bacteria may occur if less water is filtered. Plates which lack uniform distribution cannot be counted.

The membrane filtration test has a range of limits for colonies per plate. Depending on the specific parameters of interest, acceptable countable ranges are between 20 to 80 colonies per filter.

Dilution and Rinse Water. Sterile peptone dilution and rinse water will be used for all dilution and rinse procedures except where specifically noted. A 10% peptone water stock solution is prepared and autoclaved. The dilution/rinse water is prepared by diluting a measured volume of the sterile stock solution to a final concentration of 0.1%. The final pH is adjusted to 6.8 $\pm$ 0.2 at 25°C. The dilution water is dispensed to provide 99 $\pm$ 2

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mL after autoclaving at 121° C for 15 minutes. The rinse water is dispensed in quantities convenient for handling (approximately 1 L). The prepared rinse water is autoclaved at 121° C for 20 minutes prior to storage and/or use. All peptone dilution and rinse water must be checked for sterility prior to use. Any bottles demonstrating turbidity should be discarded. If more than 10% of the bottles are contaminated the entire batch must be discarded. Record these results in the media preparation log book.

Sample aliquots. Duplicate aliquots of 100 (or the maximum filterable volume), 10 and 1 mL are filtered for each sample. Duplicates for sample dilutions of 0.1, 0.01 and 0.001 mL are also prepared and filtered.

Note: Additional dilutions may be required for some samples to obtain countable plates. Dilutions prepared using peptone dilution water or phosphate buffered water must be used within thirty (30) minutes of preparation.

Stressed or damaged bacteria. Samples containing urine pretreatment and/or brine mixtures should be pretreated with buffer prior to membrane filtration. This will reduce the possible inhibition or damage of microorganisms on the filter surface due to the low pH and/or ionic concentration of these samples. Sample volumes of 10 and 1 mL are added directly to 99 mL of sterile phosphate buffer (Standard Methods, 9-xx), shaken as recommended and the entire contents filtered. Sample dilutions of 0.1, 0.01 and 0.001 mL are also prepared using sterile phosphate buffer and filtered as described above. Rinse funnels as described above substituting phosphate buffered water for peptone water. Sample volumes exceeding 10 mL cannot be analyzed using this procedure.

Quality control. Prepare at least two replicate plates for each sample volume or dilution used. Insert a sterile dilution water blank (99 mL) at the start of each sample analysis to check for contamination. Record the results on the appropriate worksheet.

Successive filtrations. Decontaminate the funnels between successive filtrations by using an ultraviolet sterilizer. Irradiate 2 minutes with the funnel upside down, then invert and irradiate 3 minutes. Filtration equipment should be autoclaved between filtration series. A filtration series ends when 30 minutes elapse between successive filtrations or every ten (10) samples.

Note: Do not expose the medium or membrane filter culture preparations to random UV radiation leaks that might emanate from the sterilization cabinet. Eye protection is recommended.

Incubation. Incubate the plates inverted as directed in the specific procedure used.

<u>Interpretation of results.</u> Count the plates as directed in the appropriate procedure using a Quebec type colony counter. **Do not use electronic counting probes to count colonies since many of the isolates must be subsequently identified.** The results are then recorded on the appropriate Worksheet.

If counts from all membrane filters are zero, calculate the number of colonies per 100 mL that would have been reported if there had been one colony on the filter representing the largest filtration volume for that sample. Report as less than ($<$) that number of colonies per 100 mL.

If counts are less than the acceptable countable range (<20) but greater than zero, count the actual number of colonies on the plates representing the lowest dilution. Calculate the number of colonies per 100 mL and report this number.

If colonies are too numerous to count, use the upper limit count from the smallest filtration volume (highest dilution) for that sample. Report as greater than ($>$) that number per 100 mL.

If there is no result because of confluency, lab accident, etc., report as "No Result" and specify reason.

Counts obtained from the sterile dilution blank must be less than or equal to 1 CFU per plate. If the number exceeds this limit the entire sample analysis is labeled as "suspect" and appropriate action is taken to identify and resolve the problem.

Procedure

1) Aseptically place a sterile membrane filter over porous plate of receptacle. Carefully place the funnel unit over receptacle and lock it in place.

2) Shake sample bottle vigorously (approximately 25 times in 7 seconds) to evenly distribute the bacteria. Take care to secure the screw-cap to prevent leakage during shaking.

3) Filter sample under partial vacuum ($\leq$ 13 kPa). Higher vacuum may result in cell damage/death and erroneous counts.

4) With filter still in place, rinse funnel by filtering three 20-30 mL volumes of sterile peptone rinse water.

5) Unlock and remove the funnel, immediately remove membrane filter with sterile forceps, and place it on the agar media with a rolling motion to avoid entrapment of air.

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2.1.1 Enumeration of Aerotolerant Heterotrophic Bacteria Using R2A Agar

This procedure is designed for the isolation and enumeration of heterotrophic bacteria from water samples containing low concentrations of organic carbon. The medium used, R2A, is a low nutrient medium designed to culture heterotrophic bacteria. This includes oligotrophic bacteria characteristically found in these aquatic environments.

Procedure

Sample preservation and storage. See Section 2.1.

Preparation. Suspend 18.2 grams of Bacto R2A medium (Difco Laboratories, Detroit, MI) in 1 liter of deionized or distilled water. The final pH of the medium should be 7.2 $\pm$ 0.2 at 25 degrees Celsius.

Heat with continuous stirring allowing the medium to boil in order to completely dissolve the agar, then promptly remove the medium from the heat.

Sterilization. Autoclave at 121 degrees Celsius for 15 minutes. Cool medium to 50 degrees Celsius in a preheated water bath. Do not allow the medium to stand for more than 1 hour between preparation and autoclaving.

Dispensing. Within a Laminar Flow hood, aseptically dispense 5 ml of the sterile medium into sterile 50 x 9 mm Petri dishes with tight fitting lids.

Allow the agar to solidify and cool in the laminar flow hood with the lids slightly ajar to prevent the excess accumulation of moisture in the plates.

Quality control. The prepared medium should be light amber in color and is translucent. Darkened medium may indicate an increased time or temperature in sterilization and should not be used.

Allow plates to incubate at room temperature, in the dark for 24 hours and inspect for contamination prior to use. Record the number of contaminated plates in the Media Preparation Log Book. Discard all contaminated plates. If more than 10% of the plates are contaminated, discard the entire batch.

Prepare a positive control using Staphylococcus epidermidis (ATCC 12228). The resultant colonies should appear small, round and smooth with a creamy white color. Record the results in the media preparation log book. This is not a selective medium, therefore a negative control is not required.

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<u>Storage.</u> The prepared plates may be stored at 4 degrees Celsius in sealed bags for up to 14 days. Plates should be stored in the dark and allowed to equilibrate to room temperature just prior to use.

<u>Membrane filters</u>. See Section 2.1.

<u>Sample application.</u> Follow the Membrane Filtration Method described in Section 2.1. Aseptically transfer the filters to plates.

Note: Sample and dilution aliquots of 0.1 mL may be plated using the Spread Plate technique described in Section 2.3.

<u>Incubation.</u> Incubate the cultures aerobically at 28 $\pm$ 0.5 degrees Celsius for up to 14 days. Examine the plates after 48 hours, 7 and 14 days as some oligotrophic bacteria may require long incubation periods. Count the colonies at the time of each observation.

<u>Interpretation of results.</u> There may be a variety of colony morphologies and pigmentation as this procedure will culture many bacterial types. Count all colonies present. The countable range of colonies is between 20-80 colonies per plate. Plates should be counted using the magnifying lens of a Quebec type colony counter.

Note: If the spread plate technique is used the countable range per plate is extended to 300 colonies per plate.

<u>Confirmation</u>. All colonies or a maximum of 10 representative colonies from each countable plate will be identified.

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2.1.2 Enumeration of Aerotolerant Heterotrophic Bacteria using Plate Count Agar

This procedure is designed for the isolation and enumeration of heterotrophic bacteria from water and wastewater samples by membrane filtration. The medium used, Plate Count Agar (PCA), is a non-selective nutrient medium designed to culture heterotrophic bacteria. This medium will 1) provide an excellent medium for the isolation of copiotrophic bacteria, 2) provide a continuum for comparison of CMIF test data with previous subsystem tests and 3) allow for corelation of the Gram positive, Gram negative and enteric procedures.

Procedure

Sample preservation and storage. See Section 2.1

Preparation. Suspend 8.5 grams of Bacto m-Plate Count Broth and 10 grams of Bacto Agar (Difco Laboratories, Detroit, MI) in 1 liter of deionized or distilled water.

Heat with continuous stirring allowing the medium to boil in order to completely dissolve the agar, then promptly remove the medium from the heat. The final pH of the medium should be 7.0 $\pm$ 0.2 at 25 degrees Celsius.

Sterilization. Autoclave at 121 degrees Celsius for 15 minutes. Cool medium to 50 degrees Celsius in a preheated water bath. Do not allow the medium to stand for more than 1 hour between preparation and autoclaving.

Dispensing. Within a Laminar Flow hood, aseptically dispense 5 mL of the sterile medium into sterile 50 x 9 mm Petri dishes with tight fitting lids.

Allow the agar to solidify and cool in the laminar flow hood with the lids slightly ajar to prevent the excess accumulation of moisture in the plates.

Quality control. The prepared medium should be light amber in color and slightly opalescent. Darkened medium may indicate an increased time or temperature in sterilization and must not be used.

Allow plates to incubate at room temperature, in the dark for 24 hours and inspect for contamination prior to use. Record the number of contaminated plates in the Media Preparation Log Book. Discard all contaminated plates. If more than 10% of the plates are contaminated, discard the entire batch.

Prepare a positive control using Staphylococcus epidermidis (ATCC 12228). The resultant colonies should appear small, round and smooth with a creamy white color. Record the results in the media preparation log book. This is not a selective medium, therefore a negative control is not required.

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Storage. The prepared plates may be stored at 4 degrees Celsius in sealed bags for up to 14 days. Plates should be stored in the dark and allowed to equilibrate to room temperature just prior to use.

Membrane filters. See Section 2.1.

Sample application. Follow the Membrane Filtration Method described in Section 2.1. Aseptically transfer the filters to plates.

Note: Sample and dilution aliquots of 0.1 mL may be plated using the Spread Plate technique described in Section 2.3.

Incubation. Incubate the cultures aerobically at 28 $\pm$ 0.5 degrees Celsius for up to 14 days. Examine the plates after 48 hours, 7 and 14 days as some heterotrophic bacteria may require long incubation periods. Count the colonies at the time of each observation.

Interpretation of results. There may be a variety of colony morphologies and pigmentation as this procedure will culture many bacterial types. Count all colonies present. The countable range of colonies is between 20-80 colonies per plate. Plates should be counted using the magnifying lens of a Quebec type colony counter.

Note: If the spread plate technique is used the countable range per plate is extended to 300 colonies per plate.

Confirmation. All colonies or a maximum of 10 representative colonies from each countable plate will be identified.

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2.1.3 Enumeration of Aerotolerant Eutrophic Mesophiles

This procedure is designed for the isolation and enumeration of bacteria associated with the human body. This includes normal flora, opportunistic and pathogenic bacteria. Fastidious organisms with a variety of growth requirements will grow on this medium. The hemoglobin component of the chocolate agar provides iron and the supplement provides preformed factors (X and V) which are essential for the culture of some fastidious organisms associated with the normal human flora.

Procedure

Sample preservation and storage. Sodium thiosulfate at a concentration of 10% should be added to samples (0.1 mL $Na_2S_2O_3$/100 mL sample) where residual chlorine or other halogen is suspected.

Samples suspected of containing copper, zinc or heavy metals should be additionally preserved using ethylenediaminetetraacetic acid (EDTA) as a chelating agent. 0.3 mL of a 15% solution of EDTA, adjusted to pH 6.5, is used per 100 mL of sample.

Note: The sodium thiosulfate and EDTA solutions should be added to the sample collection bottles prior to autoclaving. These solutions may be combined and added as a single solution.

Samples not processed within one hour of collection should be stored between 4° and 10° C until analyzed. Samples should be analyzed within a maximum of four (4) hours following collection.

Preparation. Suspend 71 grams of Bacto Chocolate Enriched Agar (Difco Laboratories, Detroit, MI) in 1 liter of deionized or distilled water.

Heat with continuous stirring allowing the medium to boil in order to completely dissolve the agar, then promptly remove the medium from the heat. Final pH of the medium should be 7.2 $\pm$ 0.2 at 25° C.

Sterilization. Autoclave at 121 degrees Celsius for 15 minutes. Cool medium to 50 degrees Celsius in a preheated water bath. Do not allow the medium to stand for more than 1 hour between preparation and autoclaving.

Aseptically add 10 mL of Bacto Supplement C per 1 L of precooled medium. Bacto supplement C is presterilized and heat-labile. Return the flask to the magnetic stirrer and mix so that the Supplement C is homogeneously dispersed throughout the medium. Bacto supplement C is rehydrated by aseptically transferring 5 mL of sterile deionized or distilled water per vial of supplement.

Note: Bacto Supplements B, C and VX are interchangable for this method.

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Dispensing. Within a Laminar Flow hood, aseptically dispense 5 ml of the sterile medium into sterile 50 x 9 mm Petri dishes with tight fitting lids.

Allow the agar to solidify and cool in the laminar flow hood with the lids slightly ajar to prevent the excess accumulation of moisture in the plates.

Quality control. The prepared medium should appear chocolate brown in color.

Allow plates to incubate at room temperature, in the dark for 24 hours and inspect for contamination prior to use. Record the number of contaminated plates in the Media Preparation Log Book. Discard all contaminated plates. If more than 10% of the plates are contaminated, discard the entire batch.

Prepare a positive control using Haemophilus hemolyticus (ATCC 33390). The resultant colonies should appear as small, semi-opaque and be gray-white in color. Record result in the media preparation log book. This is not a selective medium, therefore a negative control is not required.

Storage. The prepared plates may be stored at 4 degrees Celsius in sealed bags for up to 14 days. Plates should be stored in the dark and allowed to equilibrate to room temperature just prior to use.

Membrane filters. See Section 2.1.

Sample application. Follow the Membrane Filtration Method described in section 2.1. Aseptically transfer filters to plates.

Note: Sample and dilution aliquots of 0.1 mL may be plated using the Spread Plate technique described in Section 2.3.

Incubation. Incubate the cultures aerobically under an increased CO_2 tension of 5% and relative humidity of 90%, at 35 ± 0.5 degrees Celsius for 72 hours.

Interpretation of results. Examine plates and record results after 48 and 72 hours incubation. Magnification may be required to detect small opaque to transparent colonies possibly representing fastidious human pathogens. Count all colonies present on the plate. The countable range of colonies is between 20-80 colonies per plate. Plates should be counted using the magnifying lens of a Quebec type colony counter.

Note: If the spread plate technique is used the countable range per plate is extended to 300 colonies per plate.

Confirmation. All colonies or a maximum of 10 representative colonies from each countable plate will be identified.

2.1.4 Enumeration Of Staphylococci

This procedure is designed for the isolation and enumeration of <u>Staphylococcus</u> <u>spp</u>. from a water sample using the membrane filtration technique. The medium used is Mannitol salt agar which consists of beef extract for growth factors, 7.5% NaCl to select for staphylococci (which are halotolerant), D-mannitol and peptone as substrates and phenol red as a pH indicator.

Procedure

<u>Preparation.</u> Suspend 111 grams of Difco Mannitol salt agar (Difco Laboratories, Detroit, MI) in 1 liter of deionized or distilled water.

Heat with continuous stirring allowing the medium to boil in order to completely dissolve the agar, then promptly remove the medium from the heat. The final pH of the medium should be 7.4 ± 0.2 at 25 degrees Celsius.

<u>Sterilization.</u> Autoclave at 121 degrees Celsius for 15 minutes. Cool medium to 50 degrees Celsius in a preheated water bath. Do not allow the medium to stand for more than 1 hour between preparation and autoclaving.

<u>Dispensing.</u> Within a Laminar Flow hood, aseptically dispense the sterile medium into sterile 50x9 mm Petri dishes with tight fitting lids.

Allow the agar to solidify and cool in the laminar flow hood with the lids slightly ajar to prevent the excess accumulation of moisture in the plates.

<u>Quality</u> <u>control.</u> The Difco Mannitol salt agar should appear red in color and be very slightly opalescent.

Allow plates to incubate at room temperature, in the dark for 24 hours and inspect for contamination prior to usage. Record the number of contaminated plates in the Media Preparation Log Book. Discard all contaminated plates. If more than 10% of the plates are contaminated, discard the entire batch.

As a positive control, streak a randomly selected plate using <u>Staphylococcus</u> <u>aureus</u> (ATCC 25923). The resultant colonies should be circular, entire, convex, smooth, and be yellow in color or be surrounded by a yellow zone due to the fermentation of Mannitol. Also as a positive control, streak a second randomly selected plate using <u>Staphylococcus</u> <u>epidermidis</u> (ATCC 14990). The resultant colonies should have a similar morphology but be red in color. As a negative control, streak a third randomly selected plate with <u>Escherichia</u> <u>coli</u> (ATCC 25922). There should be little to no growth on this plate. Record the results in the media preparation log book.

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Storage. The prepared plates may be stored at 4 degrees Celsius in sealed bags for up to 14 days. Plates should be stored in the dark and allowed to equilibrate to room temperature just prior to use.

Membrane filters. See section 2.1

Sample application. Follow the Membrane Filtration Method described in Section 2.1. Aseptically transfer filters to plates.

Note: Sample and dilution aliquots of 0.1 mL may be plated using the Spread Plate technique described in Section 2.3.

Incubation. Incubate the cultures aerobically at 35 ± 0.5 degrees Celsius for 72 hours.

Interpretation of results. Examine the plates after 24 and 48 hours. Colonies should either appear yellow and/or be surrounded by a yellow zone, or they should be red in color with no yellow zone formation. Those colonies exhibiting yellow color formation should be considered pathogenic staphylococci whereas red colonies may be condidered non-pathogenic. Count all colonies present for a total staphylococci determination. Count only yellow colonies and/or colonies surrounded by a yellow zone for a determination of pathogenic staphylococci. The countable range of colonies with this medium is between 20-80 colonies per plate.

Note: If the spread plate technique is used the countable range per plate is extended to 300 colonies per plate.

Confirmation. Pick a minimum of 10 suspected colonies of staphylococci from the countable plates and perform Gram stains. Staphylococci are characteristically Gram positive and exist in grape-like clusters. Pathogenic staphylococci can be distinguished from non-pathogenic staphylococci by their ability to coagulate rabbit blood plasma.

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2.1.5 Enumeration of Fecal Coliform Bacteria

This procedure is designed for the isolation and enumeration of fecal coliforms from a water sample using the membrane filtration technique. The medium consists of a tryptose, lactose, peptone, yeast extract base enrichment with Bile Salts No. 3 to inhibit Gram positive bacteria. Rosolic acid is added for the color reaction of the fecal coliform bacteria. The elevated incubation temperature enhances selectivity and gives 93% accuracy in differentiating between coliforms from warm-blooded animals and those from other sources.

Procedure

Sample preservation and storage. See Section 2.1.

Preparation. Suspend 37 grams of dehydrated Bacto mFC Broth Base and 10 grams Bacto Agar (Difco Laboratories, Detroit, MI) in 1 liter of distilled or deionized water. Add 10 mLs of a 1% solution of Bacto Rosolic acid (Difco Laboratories, Detroit, MI) in 0.2N NaOH.

Heat with continuous stirring allowing the medium to boil in order to completely dissolve the agar, then promptly remove the medium from the heat. **Do not autoclave this medium.**

Dispensing. Within a laminar flow hood, aseptically dispense 5 ml of the medium into sterile 50 x 9 mm Petri dishes with tight fitting lids.

Allow the agar to solidify and cool in the laminar flow hood with the lids slightly ajar to prevent the excess accumulation of moisture in the plates.

Quality Control. The mFC agar should be cranberry red in color and be slightly opalescent. The medium should appear blue before the addition of the Rosolic acid.

Allow plates to incubate at room temperature, in the dark for 24 hours and inspect for contamination prior to usage. Record the number of contaminated plates in the Media Preparation Log Book. Discard all contaminated plates. If more than 10% of the plates are contaminated, discard the entire batch.

Streak a randomly selected plate with _Escherichia coli_ (ATCC 25922) to serve as the positive control. The resultant colonies should be umbonate in elevation, have a rough edge, and be blue in color. Streak a second randomly selected plate using _Xanthomonas maltophilia_ (ATCC 13637). This plate will serve as the negative control plate. The resultant colonies should appear round, smooth and be gray in color. Record the results obtained for each batch in the media preparation log book.

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<u>Storage</u>. The prepared plates may be stored at 4 degrees Celsius in sealed bags for up to 14 days. Plates should be stored in the dark and allowed to equilibrate to room temperature just prior to use.

<u>Membrane filters</u>. See Section 2.1.

<u>Sample application</u>. Follow the Membrane Filtration Method described in section 2.1. Aseptically transfer filters to plates containing mFC agar.

Note: Sample and dilution aliquots of 0.1 mL may be plated using the Spread Plate technique described in Section 2.3.

<u>Incubation</u>. Place the prepared cultures in watertight plastic bags. Submerge the bags containing plates in a water bath at 44.5 ± 0.2 degrees Celsius for 24 ± 2 hours. The bags should be anchored under the water to maintain the critical temperature requirements.

Note: Place all prepared plates containing filters in the water bath within 30 minutes after filtration.

<u>Interpretation of results</u>. Examine the plates after 24 hours. Fecal coliform colonies should appear blue in color and nonfecal colonies should appear gray. Few nonfecal colonies should be present on the plates due to the inhibitory effect of the medium and incubation temperature. The countable range of colonies with this medium is between 20-60 colonies per plate.

Note: If the spread plate technique is used the countable range per plate is extended to 300 colonies per plate.

<u>Confirmation</u>. Verify fecal coliforms by transferring a minimum of ten (10) isolates exhibiting a blue color and transfer to EC broth. Incubate at 44.5 ± 0.2 degrees Celsius for 24 hours. Confirmation of fecal coliforms is indicated by gas accumulation in the Durham tube.

Note: EC broth is prepared by dissolving 37 grams Bacto EC broth base (Difco Laboratories, Detroit, MI) in 1 L of distilled or deionized water. The medium is brought to a boil with continuous stirring, dispensed into 15 x 125 mm screw cap tubes in five mL quantities and autoclaved at 121 ° C for 15 minutes.

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2.1.6 ENUMERATION OF YEASTS AND MOLDS

This procedure is designed for the enumeration of yeasts and molds. The medium modified streptomycin-terramycin malt extract agar (MSTMEA) consists of a low nutrient base with anti-bacterial antibiotics. Since solid media do not permit the growth of all yeasts, a low estimation of yeast density may result using this procedure.

Procedure

Preparation. The medium formulation of modified streptomycin-terramycin-malt extract agar (MSTMEA) is as follows:

malt extract	30.0	g
peptone	5.0	g
streptomycin	200	mg
terramycin	200	mg
agar	15	g
Distilled water	1	liter

Prepare seperate solutions of each antibiotic in advance by dissolving 200 mg of the antibiotic in 100 ml of distilled water. Filter sterilize each solution.

Suspend the malt extract, peptone and Agar in 800 mL of distilled or deionized water. Heat with continuous stirring allowing the medium to boil in order to completely dissolve the agar, then promptly remove the medium from the heat.

Sterilization. Autoclave the agar base at 121 degrees Celsius for 15 minutes. Cool the medium to 50° C in a preheated water bath. Do not allow the medium to stand for more than 1 hour between preparation and autoclaving.

Antibiotic addition. Aseptically add each of the 100 mL portions of antibiotic solution to the precooled agar base. The final pH of the medium should be 5.4 $\pm$ 0.2.

Dispensing. Within a Laminar Flow hood, aseptically dispense 5 mL of the sterile medium into sterile 50x9 mm Petri dishes with tight fitting lids.

Allow the agar to solidify and cool in the laminar flow hood with the lids slightly ajar to prevent the excess accumulation of moisture in the plates.

Quality control. The Yeast and Mold medium should appear amber and clear in color. Record the result in the Media Preparation Log Book.

Allow plates to incubate at room temperature, in the dark for 24 hours and inspect for contamination prior to usage. Record the number of contaminated plates in the Media Preparation Log Book.

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Discard all contaminated plates. If more than 10% of the plates are contaminated, discard the entire batch.

Streak a randomly selected plate using Rhodotorulla rubra (ATCC 2510) as a positive control. The resultant colonies should appear round, smooth and be white with a yellowish tint. Prepare a negative control using Pseudomonas maltophilia (ATCC 13637). There should be little to no growth on this plate.

Storage. The prepared plates may be stored at 4 degrees Celsius in sealed bags for up to 14 days. Plates should be allowed to equilibrate to room temperature just prior to use.

Sample application. Follow the Membrane Filtration Method described in Section 2.1. Aseptically transfer the filters to plates.

Note: Sample and dilution aliquots of 0.1 mL may be plated using the Spread Plate technique described in Section 2.3.

Incubation. Incubate the plates aerobically at 20 $\pm$ 0.5 degrees Celsius for 5 days. Examine the plates and record the results after 48 hours and then daily.

Interpretation of results. Yeast colonies will have a smooth surface and color may vary. Mold colonies will have rough to downy appearance with a variety of colors. Colonies may discolor the medium slightly. The acceptable countable range of colonies is between 20-80 colonies per plate.

Note: If the spread plate technique is used the countable range per plate is extended to 300 colonies per plate.

Confirmation. Confirm molds by colonial morphology under binocular disecting scope (10X). Yeast may be confirmed based on cellular morphology.

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2.1.7 Enumeration of Legionellae

This procedure is designed for the isolation and enumeration of __Legionella__ spp. This method relies on membrane filtration for the initial concentration of legionellae from samples. The filters are subsequently acid treated and aliquots plated directly on a selective enriched agar using the spread plate technique. The BBL BCYE Agar base consists of a basal medium containing ACES buffer, charcoal, ferric pyrophosphate (0.25 g/L) and alpha-ketoglutarate. A special lyophilized enrichment containing L-cysteine (0.4 g/L) is added. Legionellae are fastidious bacteria which are easily overgrown by other bacteria in the environment. As a precaution against this an acid treatment step is used to reduce the numbers of competing bacteria. A mixture of antibiotics (BBL PAV supplement) which contains Polymyxin B (100,000 units), Vancomycin (5 mg) and Anisomycin (80 mg) is added to each liter of medium to further minimize overgrowth of unwanted bacteria.

Note: Special precaution should be taken when working with any sample suspected of containing Legionellae as all species are potential human pathogens. This includes spicific precautions to prevent aerosol formation.

Procedure

Sample preservation and storage. See Section 2.1.

Note: If the addition of EDTA is necessary use the potassium salt form since legionellae are sensitive to free sodium ions.

Preparation. Suspend 38.3 grams of BBL BCYE base (Baltimore Biological Laboratory, Cockeysville, MD) and 3 grams glycine in 900 mL of deionized or distilled water. The final pH of the medium should be 6.9 ± 0.2 at 25° C. If necessary adjust the pH using 1N KOH. **Do not use NaOH since legionellae are sensitive to free sodium ions.** Care should be taken to adjust the pH of the medium in order to obtain optimal recovery. After adjusting the pH, bring the volume of the medium to 1 L using distilled or deionized water.

Heat with continuous stirring allowing the medium to boil in order to completely dissolve the agar, then promptly remove the medium from the heat.

Sterilization. Autoclave at 121 degrees Celsius for 15 minutes. Cool medium to 50 degrees Celsius in a preheated water bath. Do not allow the medium to stand for more than 1 hour between preparation and autoclaving.

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Enrichment and antibiotic addition. Aseptically rehydrate and add a vial of the BBL cysteine (0.4 g/10 mL) using sterile distilled or deionized water per liter of prepared and cooled media. Also rehydrate and add a vial of BBL PAV antibiotic supplement using sterile distilled or deionized water per liter of prepared and cooled media.

Dispensing. Within a Laminar Flow hood, aseptically dispense the sterile medium into sterile 15 x 100 mm Petri dishes. Continually agitate the flask while pouring plates to keep charcoal from settling out of the media. Allow the agar to solidify and cool in the laminar flow hood with the lids slightly ajar to prevent the excess accumulation of moisture in the plates.

Quality control. The prepared BCYE Agar should appear gray-black in color.

Allow plates to incubate at room temperature, in the dark for 24 hours and inspect for contamination prior to usage. Record the number of contaminated plates in the Media Preparation Log Book. Discard all contaminated plates. If more than 10% of the plates are contaminated, discard the entire batch.

Prepare a positive control using Legionella gormanii (ATCC 33297). Colonies of Legionella spp. should be visible after 2 to 3 days incubation and appear light blue to blue-gray in color and should not discolor medium. Older colonies will become larger, smoother and gray-white in color. Typical cultural response of Legionella should be evident after 48-72 hours. Prepare a negative control using Escherichia coli (ATCC 25922). This control organism should grow but not produce a blue pigment. Record the results obtained from each batch in the media preparation log book.

Storage. The prepared plates may be stored at 4 degrees Celsius in sealed bags for up to 14 days. Plates should be stored in the dark and allowed to equilibrate to room temperature just prior to use.

Sample preparation. Concentrate maximum amount of water (up to 100 mL), in duplicate, through a 47 mm white, ungridded, Nuclepore membrane (Nuclepore Corporation, Pleasanton, CA) having a pore size of 0.2 um. Multiple membranes may be used, if necessary, and combined to obtain required detection limits. Following concentration place filter(s), soiled side down, in a sterile 50 mL centrifuge tube (or similar vessel) with a screw cap containing ten (10) mL of sterile water. Disperse bacteria from filter by vortexing (3 x 30 seconds) or place in a sonic bath for 10 minutes. Repeat the above procedure concentrating duplicate 10 mL aliquots.

Acid treatment. Place 1 mL of each suspension in a sterile 13 x 100 mm screw cap tube containing 1 mL acid treatment reagent (LATR) and vortex for 10 seconds. Let stand for 15 minutes at

room temperature and immediately neutralize by adding 1 mL of the alkaline neutralizing reagent (LANR) and vortex for 10 seconds.

Acid treatment reagent:

Solution A: 0.2M KCl (14.9 g/L in distilled or deionized water)
Solution B: 0.2M HCl (16.7 mL/L 10N HCl in distilled/deionized water)

Mix 18 parts solution A with 1 part solution B. pH of this solution should be 2.2 at 25° C. Check pH against a pH 2 standard buffer. Dispense in 1 mL volumes into 13 x 100 mm screw capped tubes and sterilize by autoclaving at 121° C for 15 minutes. Label tubes as Legionella Acid Treatment Reagent (LATR).

Alkaline neutralizer reagent:

Stock solution: 0.1N KOH (6.46 g/L in distilled/deionized water)

Dilute 10.7 mL of stock solution using distilled or deionized water to 100 mL. Dispense in 1 mL volumes into 13 x 100 mm scew capped tubes and sterilize by autoclaving at 121° C for 15 minutes. Label tubes as Legionella Alkaline Neutralizing Reagent (LANR)

Note: Equal volumes of LATR and LANR when mixed should result in a pH of 6.9 at 25° C.

Sample application. Inoculate 0.1 mL of the acid treated suspensions onto the BCYE agar and spread over the entire surface using a glass rod and turntable (See Section 2.3). Sterilize the rod between plating aliquots by soaking in alcohol and then flaming prior to each use. Also prepare serial dilutions, if necessary, from the 1 mL sample aliquots and plate as described above.

Incubation. Incubate the cultures aerobically in an atmosphere containing 2.5% CO_2 and a relative humidity of 90%, at 35 ± 0.5 degrees Celsius for at least 10 days. Examine daily for evidence of growth and record results.

Interpretation of results. Legionellae colonies will appear blue to grey-blue in color and should not discolor this medium. Any colonies fitting this description should be picked for confirmation. The countable range of colonies is between 30-300 colonies per plate. Determine the number of CFUs/100mL using the formula below:

$$CFUs/100mL = \frac{333 \times \text{number of colonies}}{\text{volume of sample filtered (mL)}} \times 100$$

Note: Although this medium is designed for the cultivation and isolation of Legionella spp., other organisms may grow and must be differentiated from the target organism. Also, due to the variety of nutritional requirements of the genus, some strains may be encountered that fail to grow or grow poorly on this medium.

Confirmation. Colonies suspected of being Legionella spp. should be Gram stained and subcultured to a fresh BCYE Agar plates with and without L-cysteine.

Gram negative organisms that grow on BBL-BCYE agar with cysteine but fail to grow on BBL-BCYE agar without cysteine may be presumptively identified as Legionellae.

Definitive identification is performed on all positive presumptive isolates on the basis of growth, morphology, fatty acid profiles and biochemical and immunological reactions.

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2.2 Direct Epifluorescent Filter Technique (DEFT)

The following procedure describes the direct microscopic count of bacteria using a fluorochrome and the membrane filtration technique. This technique is designed to determine the total number of bacteria in water samples.

Procedure

1) Select a slide with the appropriate fluorescent stain. Check to see that the proper filter pack is in place in the scope.

2) Low fluorescence immersion oil and the 100x objective are used for counting.

3) Randomly select a field and count all the bacteria seen in that field, starting at the top of the field, counting left to right. Be sure to count only those objects with "bacterial shapes". Do not count fluorescent debris. Record results.

4) Change randomly to another field and count again in the same manner.

5) Determine the average number of bacteria per field.

6) Determine the total number of fields to count using the following table:

Average number of bacteria per field	Minimum number of fields to count
≥ 15	10
10-14	25
6-9	50
3-5	75
≤ 2	100

7) Calculate the total number of bacteria in the water sample using the following equations:

Total Count = Mean bacteria count /field x Microscopic Factor (MF)

where,

$$MF = \frac{\text{Area of membrane through which sample is filtered (mm}^2\text{)}}{\text{Microscopic field [or grid] area (mm}^2\text{) x Sample volume (ml)}}$$

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2.2.1 Acridine Orange Staining For Epifluorescence Microscopy

This procedure uses the fluorochrome acridine orange to stain nucleic adids within microbial cells. When coupled with membrane filtration and epifluorescence microscopy, a rapid total direct count of microbial cells in aqueous solutions is feasible.

Sample fixation. Prepare the gluteraldehyde stock fixative solution fresh daily. The fixative stock solution is 5.0% (w/v) gluteraldehyde in phosphate buffer (see composition below). At the time of collection, add the glutaraldehyde fixative solution to the sample equivalent to 10% (v/v). The final concentration of glutaraldehyde is 0.5%.

Sample storage. Preserved (fixed) samples may be stored at 4° C for up to 21 days.

Procedure

1. Prepare phosphate buffer for use in this procedure by dissolving 13.6 g KH_2PO_4 in 500 mL distilled or deionized water. Adjust to pH 7.2. Bring volume to 1 L using distilled or deionized water. Autoclave and store prepared buffer at 4° C between uses. **Filter daily aliquots through a 0.2 um filter prior to use.**

2. Prepare fluorochrome as 0.1% (w/v) acridine orange in phosphate buffer. Filter this solution just prior to use through a 0.2 um disposable sterile syringe filter unit. Store in a light-proof container at 4 degrees Celsius.

3. Use a 25 mm cellulose backing-filter (Millipore Corporation, Bedford, MA) having a 0.45 um porosity. Dampen this filter first with filtered rinse water so that it adheres to the filtration base. Place a Nuclepore (Nuclepore Corporation, Pleasanton, CA) pre-stained black polycarbonate filter, 25 mm diameter having a 0.2 um porosity on top of the backing-filter. Assemble the filtration apparatus.

4. Determine the volume of sample to be filtered that is required for direct counting. Twenty milliliters of clean or potable water is usually sufficient. Volumes up to 25 ml may be added directly to the filter within the apparatus. For larger volume requirements 25 mL aliquots may be successively added and filtered through the same filter. If dilutions need to be made the phosphate buffer previously described should be used.

5. Add Acridine Orange stock solution directly to the sample (or dilution) to be analyzed at a ratio of 1:1 (v/v). Stain for 2 minutes.

6. Add phosphate buffer to the stain/sample mixture equivalent to a final ratio of 3:1:1 (3 parts buffer to 1 part stain to 1 part sample).

7. Filter with vacuum (approximately 13 kPa).

8. Rinse with a volume of phosphate buffer equivalent to one-half the total volume of the: stain solution + sample + buffer.

9. Filter with vacuum (approximately 13 kPa).

10. Remove the Nuclepore filter from base and backing-filter by its edge and air dry.

11. Place a small drop of low fluorescence immersion oil on a clean slide. Place the filter on the slide **so that the sample side faces the objective lens.** Add a small drop of oil to the filter and overlay with coverslip.

12. Examine filter surface with an epifluorescent microscope and oil immersion objective utilizing low fluorescence oil.

13. Determine average number of cells per field and calculate the number of cells/100 mL as specified in Section 2.2.

2.3 Direct Enumeration Using the Spread Plate Technique

Procedure

1. Label petri dishes with the sample ID, date, dilution, analyst's initials and any other pertinent information as specified in the laboratory's standard operating procedures (SOP) document. Media type and batch number should have previously been recorded on the plates.

Note: The plated media must be predried by incubating the plates with the lids on at 55° C for 12-18 hours. A 2-3 gram weight loss is not uncommon.

2. Vortex the sample to be plated for 10 seconds to evenly distribute the bacteria.

3. Pipette 0.1 mL of the sample directly onto the agar surface.

4. Using a bent sterile glass rod, distribute the inoculum over the surface of the medium by rotating the dish on a turntable. Allow the inoculum to be completely absorbed into the media before transferring the plates to the incubator. The spreading rods are sterilized between sample applications by immersing in 90% (v/v) ethanol and flaming prior to use.

5. Incubate at the specified temperature and time as dictated by the procedure.

6. Count colonies as prescribed by the specific procedure (see Section 2.1.7). The countable range using the spread plate technique is between 30-300 colonies per plate (15 x 100 mm).

7. Calculate the number of colony forming units (CFU) per 100 mL as described by the procedure and sample volume (or dilution) using the formula below:

$$CFU/100 \text{ mL} = \frac{\text{Number of Colonies}}{\text{Volume of Sample}} \times 100 \text{ mL}$$

Quality Control

Prepare at least two replicate plates for each sample aliquot and dilution being used.

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2.4 Multiple Tube Fermentation (MTF) Method

The MTF method is based on the succesive dilution of a sample to the point of extinction of any organisms present in that sample.

Sample preservation and storage. Sodium thiosulfate (10% v/v) should be added to the samples (0.1 mL $Na_2S_2O_3$/100 mL sample) to give a final concentration of 0.01% where residual chlorine or other halogen is suspected.

Samples containing total metal (copper, zinc or heavy metals) at concentrations exceeding 10 ug/L should additionally be preserved using a chelating agent to reduce potential toxicity by these compounds. This may be acheived by the addition of 0.3 mL of a 15% solution of EDTA per 100 mL of sample giving a final concentration of 0.045%. The pH of the EDTA solution should be adjusted to 6.5.

Note: **The sodium thiosulfate and EDTA should be added to the sample collection bottles prior to autoclaving if possible. These solutions may be combined and added as a single solution.**

Samples not processed within one hour of collection should be stored between 4° and 10° C until analyzed. Samples should be analyzed **within a maximum of eight (8) hours** following collection.

Media preparation. Refer to individual procedure.

Dispensing. The appropriate medium for the test will be dispensed in the MTF tubes in two volumes. Three tubes containing 1.0 mL of the 10X stock media (except for the fecal coliform protocol which will require three tubes containing 10 mL of a 2X stock) and 21 tubes containing 9.0 mL of 1X or diluted stock media, will be needed per sample. Refer to the individual procedure for the proper tubes to be used.

Sterilization of apparatus and materials. All MTF tubes, glassware, pipette tips and utensils are presterilized at 121° C for 15 minutes.

Arrangement and inoculation. Three replicates of each sample volume (dilution) will be inoculated. The arrangement of the tubes is critical to determine the results of the procedure. The first series of replicate tubes will contain 1.0 mL of 10X stock media and will be inoculated with a sample volume of 10.0 mL. The next series of replicate tubes will contain 9.0 mL of 1X media and will be inoculated with a sample volume of 1.0 mL. The third series of replicate tubes will also contain 9.0 mL of 1X media but will be inoculated with a sample volume of only 0.1 mL. Each subsequent series of replicates will contain 9.0 mL of 1X media.

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Starting with the third series of replicates, vortex the inoculated tube for a sufficient time and speed to facilitate adequate mixing and transfer 1.0 mL into the next replicate. Repeat this procedure changing to a sterile pipet tip at each dilution series.

The completed test will represent three replicate series of sample dilutions ranging from 10^{+1} to 10^{-7}. **Aseptic technique will be used for all above procedures.**

Quality control. Prepared MTF tubes should be incubated by conditions specified by the specific procedure and inspected for contamination. Before use, these tubes should be inspected again for turbidity or precipitaion that could alter the results of the test.

Incubation. Incubate all labeled replicates of MTF tubes as directed by the specific procedure.

Interpretation of results. Inspect the MTF tubes and count the number of turbid tubes in each series. The first tube without obvious turbidity should be examined closely for any trace of turbidity or precipitated growth. Note the most dilute set of sample tubes having all three tubes positive (turbid) for that dilution. Note also the number of positive tubes for the next two dilutions. Compare the three numbers for these three sets of dilutions to the 3 tube Most Probable Number (MPN) table found in the 14th edition of Standard Methods for the Examination of Water and Wastewater. The three numbers will correspond to a #CFU/100 mL sample, within the 95% confidence interval. The table will give a CFU value for initial sample dilutions of 10^1, 10^0 and 10^{-1} (the tubes with 10, 1 and .1 mL of sample added directly to the MTF tube). The actual value for the bacterial density must be calculated by considering the number of dilutions from the 10X tube to the most dilute set of tubes having all three tubes positive for that dilution. This number of dilutions will equal the log value that should be added to the value from the table. For example:

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In the example above, the most dilute replicate series with all members turbid, or positive, (denoted by the diagonal lines) is followed by a replicate set with one tube positive and the next having all tubes clear (both replicate sets denoted by the grid lines). The three number code 3-1-0 corresponds to an MPN index value of 43 CFU/100 mL in the table. Since the most dilute replicate set with all members positive is 4 logs more dilute than the 10X tube, this amount must be added to the value from the table. Therefore, 43 CFU/100 mL becomes 4.3×10^5 CFU/100 mL.

THREE TUBE MPN TABLE

Sample (10 mL)	Sample (1.0 mL)	Sample (0.1 mL)	MPN INDEX CFU/100 mL
0	0	0	<3
0	0	1	3
0	1	0	3
1	0	0	4
1	0	1	7
1	1	0	7
1	1	1	11
1	2	0	11
2	0	0	9
2	0	1	14
2	1	0	15
2	1	1	20
2	2	0	21
2	2	1	28
3	0	0	23
3	0	1	39
3	0	2	64
3	1	0	43
3	1	1	75
3	1	2	120
3	2	0	93
3	2	1	150
3	2	2	210
3	3	0	240
3	3	1	460
3	3	2	1100
3	3	3	>2400

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MTF-MPN SAMPLE DATA SHEET

SAMPLE #_____

SAMPLE LOCATION AND TYPE_____

DATE AND TIME COLLECTED_____

COLLECTED BY_____

DATE AND TIME ANALYZED_____

ANALYZED BY_____

10^1 10^0 10^{-1} 10^{-2} 10^{-3} 10^{-4} 10^{-5} 10^{-6}

THREE NUMBER CODE FROM FIGURE ABOVE_____

CORRESPONDING VALUE FROM MPN TABLE_____ CFU/100ml

LABORATORY DIRECTOR_____   DATE_____

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2.4.1 Enumeration of Anaerobic Bacteria by the MTF Method

This procedure is designed for the cultivation and enumeration of stringent and nonstringent anaerobic bacteria from a water sample using the Multiple Tube Fermentation (MTF) method. The organisms will be cultured in a complex medium containing Cysteine-HCl as a reducing agent, and resazurin to indicate adequate reduction.

Procedure

Sample preservation and storage. See section 2.4.

Sample collection. Samples should be collected and incubated in a manner that will avoid any contact with oxygen. Contact with oxygen may significantly reduce the number of anaerobes culturable from the samples. Oxyrase (Oxyrase Inc., Ashland, OH) is added to the sample bottles to scavenge large amounts of oxygen that may be present in some samples upon collection.

Collection buffer. Prepare 125 mL anaerobe sample bottles (Fisher cat. #06-406K) by adding 10 mL of anaerobic buffer containing the following ingredients per liter of distilled or deionized water:

Potassium phosphate monobasic	2.0	g
Potassium phosphate dibasic	2.613	g
Sodium lactate	1.681	g
Resazurin (0.1% soln.)	0.5	mL
Sodium thiosulfate (10% soln.)	0.1	mL
*EDTA (15% soln.)	0.3	mL

* Add only if concentrations of copper, zinc or heavy metals are greater than 10 ug/L.

The final pH of the buffer should be 7.2 $\pm$ 0.1 at 25° C. A Nitrogen-CO$_2$ (95:5, oxygen free) head space should be applied to the bottles just prior to sealing. Bottles should be fitted with a grey flanged, slotted plug, 13 X 20 mm stoppers (Fisher cat. #06-406-12) and 20 mm aluminum seals (Fisher cat. #06-406-14B). Sealed sample bottles should be autoclaved at 121° C for 15 minutes. Sterile sample bottles should be cooled to 50° C and 2 mL of presterilized Oxyrase (Oxyrase Inc., Ashland, OH) added via 20-22 gauge syringe aseptically. Allow sample bottles to incubate at room temperature, in an inverted position, for 24 hours and inspect for contamination prior to usage. Record the number of contaminated bottles in the Media Preparation Log Book. Discard all contaminated bottles. If more than 10% of the bottles are contaminated, discard the entire batch. The prepared sample bottles should be stored in the dark in an inverted position.

Samples (50 mL) should be collected in these sample bottles from sample ports fitted with hypodermic needles.

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Ideally, samples should be processed on-site 30 minutes to one hour after collection. All samples must be processed **within four (4) hours following collection.**

Media preparation. Prepare the **10X** MTF media by adding the following ingredients per liter of distilled or deionized water:

Potassium phosphate monobasic	13.61	g
Potassium phosphate dibasic	17.42	g
Sodium lactate	28.02	g
Sodium sulfate	36.77	g
Glucose	20	g
Trypticase	20	g
Peptone	20	g
Yeast extract	20	g
Magnesium Sulfate	5.0	g
Ammonium Chloride	5.0	g
Sodium Chloride	5.0	g
Cysteine-HCl	0.5	g
Resazurin (0.1% soln.)	5.0	mL
Vitamin K Heme soln.	100	mL

The Vitamin K Heme solution is prepared by adding 1 mL menadione stock solution to 100 mL hemin stock solution. Menadione and Hemin are supplied by the Sigma Chemical Company and are prepared as follows:

Menadione stock solution: Add 100 mg menadione to 20 mL 95% ethanol. Filter sterilize.

Hemin stock solution: Dissolve 50 mg hemin in 1 mL of 1 N NaOH, add 100 mL of distilled water. Autoclave at 121° C for 15 minutes.

Prepare 1X media by diluting 1 part of the 10X stock with 9 parts of distilled or deionized water.

Heat with continuous stirring allowing the medium to boil in order to completely dissolve the ingredients, then promptly remove the media from the heat. The final pH of the medium should be 7.2 $\pm$ 0.1 at 25° C.

Dispense the media into Bellco 18 X 150 mm aluminum seal type anaerobic culture tubes with caps and septa (cat. #2048). This tube size is necessary for a head space volume of no less than 50%. Prepare tubes containing 9 mL of the 1X media and 1 mL of the 10X media in a ratio of 7:1 respectively (refer to section 2.4). Before capping, apply a nitrogen-CO_2 (95:5, oxygen free) head to the tubes.

Sterilization. Autoclave tubes and bottles at 121 degrees Celsius for 15 minutes. Do not allow the medium to stand for more than 1 hour between preparation and autoclaving.

MTF tubes and sample bottles should be inverted when they are removed from the autoclave and remain in this position until use to minimize reoxidation.

Quality control. Allow MTF tubes and sample bottles to incubate at room temperature, inverted, in the dark for 24 hours and inspect for contamination prior to usage. Record the number of contaminated tubes or bottles in the Media Preparation Log Book. Discard all contaminated tubes or bottles. If more than 10% are contaminated, discard the entire batch.

Inoculate a randomly selected positive control MTF tube with Bacteroides fragilis (ATCC 23745) and incubate in an inverted position. Development of turbidity in this tube upon incubation will demonstrate the ability of this procedure to culture vegatative anaerobic cells. Inoculate a second randomly selected negative control MTF tube with Xanthomonas maltophilia (ATCC 13637) and incubate in an inverted position. No turbidity should develop in this tube. Record the results obtained for each batch of tubes in the media preparation log book.

Storage. The prepared tubes may be stored at 4° C, inverted, in the dark for up to 14 days. Tubes should be allowed to equilibrate to room temperature just prior to use. Any red color that may develop is due to reaction of oxygen with the resazurin. Discard any tubes or bottles that exhibit a red color.

Sample application. Once the sample has had 30 minutes to one hour to equilibrate in the sample collection bottle, aliquots can be withdrawn via 20-22 gauge hypodermic needle and transfered to the MTF tubes. The syringe should be flushed out with the N-CO$_2$ gas of the sample bottle to reduce addition of oxygen to the MTF tubes. Upon addition of the 10 ml aliquots into the 10X MTF tubes, the positive pressure should be relieved from the MTF tube by withdrawing 10 mL of gas before withdrawing the needle. Refer to section 2.4 for further instructions.

Note: An alternative method for this protocol would be to conduct all sample manipulations within an anaerobic glove bag. This would alleviate the need for syringes in sample transfer but the glove bag would add some degree of difficulty and inconvenience.

Incubation. Incubate the inverted culture tubes in an inverted position at 28 $\pm$ $^{\circ}$ C for 10 days. Tubes do not have to be incubated in an anaerobic environment. This procedure insures the production and maintenance of a reduced atmosphere within the sample bottles and MTF tubes.

Interpretation of results. Refer to section 2.4.

Confirmation. Aliquots from turbid tubes should be applied to prereduced BHIBLA anaerobic agar plates (specified for use with the MID system) by the Spread Plate Method (refer to section 2.3).

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Incubate the inoculated plates anaerobically at 28° C and examine after 3 but do not discard until after 10 days.

All cultures should be screened by the MID system, aerotollerance determined, and tentative ID stored on the MID data library. BHIBLA agar may be prepared in the lab as follows:

Brain Heart Infusion agar (Gibco #M06600B)	53	g
Yeast Extract (Difco #0127-01)	5	g
Hemin Chloride 0.1% solution (Sigma #H2375)	5	mL
Vitamin K 11.0% solution (Sigma #V3501)	0.1	mL
Distilled water	1	liter

Heat with continuous stirring allowing the medium to boil in order to completely dissolve the agar, then promptly remove the medium from the heat. The final pH of the medium should be 7.2 ± 0.2 at 25° C.

<u>Sterilization.</u> Autoclave at 121 degrees Celsius for 15 minutes. Do not allow the medium to stand for more than 1 hour between preparation and autoclaving.

Cool the sealed flask containing the media to 50° C in a water bath. In an anaerobic glove bag, add 50 mL of aseptic defibrinated sheep blood while continuously stirring flask. Dispense into 15 X 100 mM Petri plates and allow to solidify. Store plates in the anaerobic glove bag.

Note: BHIBLA agar may be obtained in prepoured form from Carr-Scarbourough (pdt. #01-1128-03)

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2.4.2 Enumeration of Total Heterotrophs by the MTF Method

This procedure is designed for the cultivation and enumeration of heterotrophic bacteria from a water sample using the Multiple Tube Fermentation (MTF) method. The media used, R2A, is a low nutrient medium designed to culture heterotrophic bacteria. This includes oligotrophic bacteria, characteristically found in these aquatic environments.

Procedure

Sample preservation and storage. See section 2.4.

Preparation. Suspend the following ingredients in 1 liter of deionized or distilled water. This will result in a 10X stock solution of R2A broth. Preformulated R2A agar medium (Difco Laboratories, Detroit, MI) is available, but the broth form must be prepared in the laboratory.

Bacto Yeast Extract	5.0 g
Proteose Peptone #3	5.0 g
Bacto Casamino Acids	5.0 g
Bacto Dextrose	5.0 g
Soluble Starch	5.0 g
Sodium Pyruvate	3.0 g
Potassium Phosphate, Dibasic	3.0 g
Magnesium Sulfate	0.5 g

Heat with continuous stirring allowing the medium to boil in order to completely dissolve the ingredients, then promptly remove the medium from the heat. The final pH of the medium should be 7.2 ± 0.2 at 25° C.

Prepare 1X media by diluting 1 part of the 10X stock with 9 parts of deionized or distilled water.

Dispense the media into 16 X 150 mm slip cap tubes. Prepare tubes containing 9 mL of the 1X media and 1 mL of the 10X media in a ratio of 7:1 respectively (refer to section 2.4). Apply slip caps to all tubes before autoclaving.

Sterilization. Autoclave at 121 degrees Celsius for 15 minutes. Do not allow the medium to stand for more than 1 hour between preparation and autoclaving.

Quality control. The prepared 1X medium should be very light amber in color and is translucent. Darkened medium may indicate an increased time of temperature in sterilization and should not be used.

Allow MTF tubes and to incubate at room temperature, in the dark for 24 hours and inspect for contamination prior to usage. Record the number of contaminated tubes in the Media Preparation Log Book. Discard all contaminated tubes. If more than 10% are contaminated, discard the entire batch.

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Inoculate a randomly selected positive control MTF tube with _Staphylococcus epidermidis_ (ATCC 12228) and incubate under aerobic conditions. Development of turbidity in this tube upon incubation will demonstrate the ability of this procedure to culture this heterotrophic control organism. This is not a selective medium, therefore a negative control is not required. Record the results obtained for each batch of tubes in the media preparation log book.

Storage. The prepared tubes may be stored at 4 degrees Celsius in the dark for up to 14 days. Tubes should be allowed to equilibrate to room temperature just prior to use.

Sample application. Refer to section 2.4, MTF procedure for instructions.

Incubation. Incubate the culture tubes aerobically at $28 \pm 0.2^\circ$ C for up to 21 days. Examine the tubes for turbidity after 24 and 48 hours, after 7 days then weekly as some oligotrophic bacteria may require long incubation periods.

Interpretation of results. Determine the Most Probable Number (MPN) of Heterotrophic bacteria present as described in section 2.4.

Confirmation. Aliquots from turbid tubes should be applied to full strength R2A agar plates by the Spread Plate Method (refer to section 2.3) and incubated aerobically at $28 \pm 0.2^\circ$ C for up to 21 days. Pick all colonies representing different morphological types or a minimum of 10 colonies per plate. Isolates should be Gram stained and identified using standard biochemical tests and/or FAMES profiling.

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2.4.3 Enumeration of Fecal Coliforms by the MTF Method

This procedure is designed for the cultivation and enumeration of fecal coliforms from a water sample using the Multiple Tube Fermentation (MTF) method. The A-1 media used is designed to differentiate between coliforms of fecal origin and coliforms from other sources.

Procedure

Sample preservation and storage. See section 2.4.

Preparation. Suspend the following ingredients in 1 liter of deionized or distilled water. This will result in a 2X stock solution of A-1 broth (17th edition of Standard Methods for the Exmination of Water and Wastewater).

Lactose	10 g
Tryptose	40 g
NaCl	10 g
Salicin	1 g

Heat with continuous stirring allowing the medium to boil in order to completely dissolve the ingredients, then promptly remove the medium from the heat. Cool the media to 50° C before the addition of 2.0 mL of Polyethylene glycol p-isooctylphenyl ether (Triton X 100, or equivalent). Adjust the final pH of the media to 6.9 $\pm$ 0.1 at 25° C.

Prepare 1X media by diluting 1 part of the 2X stock with 1 part of deionized or distilled water.

Dispense 10 mL of 2X media into 18 X 150 mm slip cap tubes and 9 mL of 1X media into 16 X 125 mm slip cap tubes. Prepare the tubes containing the 1X media and the 2X media in a ratio of 2:1 respectively (refer to section 2.4). Insert an inverted Durham tube and apply slip caps to all tubes before autoclaving. Durham tubes will be submersed and all air removed upon autoclaving.

Sterilization. Autoclave at 121 degrees Celsius for 10 minutes. Do not allow the medium to stand for more than 1 hour between preparation and autoclaving.

Quality control. Allow MTF tubes to incubate at room temperature, in the dark for 24 hours and inspect for contamination prior to usage. Record the number of contaminated tubes in the Media Preparation Log Book. Discard all contaminated tubes. If more than 10% are contaminated, discard the entire batch.

Inoculate a randomly selected positive control MTF tube with Escherichia coli (ATCC 25922) and incubate as directed below. Development of turbidity in the MTF tube and accumulation of gas in the Durham tube within 24 hours or less demonstrates the ability of this procedure to culture and detect coliforms of

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fecal origin. Inoculate a second randomly selected negative control MTF tube with _Proteus vulgaris_ (ATCC 13315). This MTF tube should become turbid but should not exhibit gas production. Record the results obtained for each batch of tubes in the media preparation log book.

Storage. The prepared tubes may be stored at 4 degrees Celsius in for up to 14 days. Tubes should be stored in the dark and allowed to equilibrate to room temperature just prior to use.

Sample application. Only three series of replicates will be prepared for the fecal coliform MPN tests (sample volumes of 10 mL, 1.0 mL and 0.1 mL added directly to MTF tubes). The necessary vortexing of sample in any further dilutions would cause air to accumulate in Durham tubes resulting in a false positive tube. Refer to section 2.4, MTF procedure for additional instructions. **Note: apply samples to MTF tubes in a manner that will avoid the collection of air bubbles in inverted Durham tubes.**

Incubation. Incubate the culture tubes aerobically at 35 ± 0.5^{o} C for 3 hours. Transfer the tubes to a water bath at 44 ± 0.2^{o} C and incubate for an additional 21 ± 2 hours.

Interpretation of results. Gas production in any of the tubes within 24 hours or less is a positive reaction indicating coliforms of fecal origin. Determine the Most Probable Number (MPN) of fecal coliforms present as described in section 2.4.

Confirmation. Aliquots from turbid tubes should be applied to full strength TSB agar plates by the Spread Plate Method (refer to section 2.3) and incubated aerobically by the instructions above. Pick all colonies representing different morphological types or a minimum of 10 colonies per plate. Isolates should be Gram stained and identified using standard biochemical tests and/or FAMES profiling.

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3.0 APPLICABLE DOCUMENTS

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APPENDIX C

PROCEDURES FOR PREPARATION OF ANALYTICAL CONTROL SAMPLES

SAMPLE PREPARATION PROCEDURE

NASA/MSFC WRT QUALITY CONTROL SAMPLES

Supplement A

to the

Analytical Control Test Plan

for the

Water Recovery Systems Test

UAH Research Report # 813

Prepared for:

National Aeronautics and Space Administration
George C. Marshall Space Flight Center
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June 6, 1990

SAMPLE PREPARATION PROCEDURE

NASA/MSFC WRT QUALITY CONTROL SAMPLES

1.0 GENERAL

The purpose of this procedural document is to define
specifications for quality control (QC) sample preparation for
water samples used in the analysis of certain chemical and
physical parameters. Stock certified reference materials have
been purchased from Environmental Resource Associates (ERA) in
Arvada, Colorado. Each standard has been assigned a lot number
by ERA. ERA has also provided certified values and advisory
ranges for each lot and instructions on how to prepare the
standards for analysis. The procedures for preparation used in
this document are taken directly from the ERA instructions. A
series of QC samples for chemical and physical parameters will be
prepared to correspond to each batch of water samples collected
in the NASA Water Recovery Testing (WRT) effort. When testing is
in progress a daily sample schedule will be developed by the
Analytical Control Coordinator (ACC). This schedule will specify
the number and types of QC samples required. The QC samples will
be prepared by a trained analyst capable of making dilutions and
volumetric measurements. Very strict laboratory technique must
be followed at all times.

2.0 GLASSWARE

All glassware used in QC sample preparation must be clean
and free of contamination to the maximum extent possible. All
pipets, graduated cylinders, and volumetric flasks must be rated
Class A. Volumetric flasks have been purchased specifically for
this program. These are to be dedicated for use on this program
only. Furthermore, volumetric flasks are to be segregated for
each standard category and be re-used only in that particular
category. No volumetric flasks are to be used other than those
dedicated specifically to the WRT program and the correct
standard category. Scratched or broken glassware must be
discarded.

2.1 Glassware Specifications

Borosilicate glassware will be used for all laboratory
operations, except where individual methods specifically indicate
use of alternate materials. These include Kimax and Pyrex brands

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or equivalent. It should be remembered that borosilicate glassware is not completely inert. Considerration must be given for storage of standard solutions of boron, silica, and the alkali metals in preferred polyethylene containers. Laboratory glassware will serve three basic functions: storage of reagents and samples, measurement of solution volumes, and confinement of reactions.

Dilute metal solutions are prone to plate out on container walls over time while being stored. Therefore, dilute standard metal solutions should be freshly prepared prior to analysis.

All volumetric glassware purchased by the laboratory will meet Federal specifications for designation as Class A glassware. Class A glassware does not require recalibration by laboratory personnel prior to use. Should it become necessary to recalibrate glassware, directions are included in the EPA Handbook for Analytical Quality Control in Water and Wastewater Laboratories (EPA-600/4-79-019), March, 1979.

Laboratory personnel must be thoroughly familiar with and consistently demonstrate approved techniques for usage of volumetric glassware. Proper techniques are illustrated in the EPA Handbook for Analytical Quality Control in Water and Wastewater Laboratories (EPA-600/4-79-019), March, 1979.

2.2 Glassware Cleaning

Methods for cleaning glassware should take into consideration both the substances which are to be removed and the laboratory analyses to be performed. Special cleaning requirements may exist for particular types of vessels and for glassware to be used for specific determinations. Special requirements for specialized glassware, fritted ware, and filters are included in the EPA Handbook for Analytical Quality Control in Water and Wastewater Laboratories, (EPA-600/4-79-019), March, 1979.

Removal of Water Soluble Substances

Glassware and porcelain vessels should be cleaned with a non-depositing soap, i.e. Alconox, and rinsed a minimum of three times in tap water, followed by two rinses in deionized water.

Removal of Water Insoluble Substances

Laboratory glassware may require the use of detergents, organic solvents, dichromate cleaning solution (11 ml concentrated H_2SO_4 added slowly with stirring to 35 ml of saturated sodium dichromate solution), nitic acid, or aqua regia (25% by volume concentrated HNO_3 in concentrated HCL). The use of NoChroMix is gaining wide acceptance as a substitute for dichromate cleaning solution due to the growing concerns of potentially carcinogenic residues which can be produced by the latter. Note: NoChroMix should not be stored in a sealed

container. Greasy spots may also be removed by using acetone, alcoholic potassium hydroxide, or a warm solution of NaOH (1g per 50 ml water, left to stand 10 - 15 minutes, followed by a water rinse, a dilute HCL rinse, and final deionized water rinses).

This laboratory will generally use the following method:

(1) Soak in dichromate cleaning solution or substitute.

(2) Wash with a non-depositing detergent.

(3) Rinse with tap water (3x).

(4) Rinse with deionized water (2x).

Cleaning for Trace Metals Determinations

For trace metal determinations one primary concern is contamination. Dust in the laboratory environment, impurities from laboratory equipment, and residues from primary cleaning procedures are sources of potential contamination. The sample bottle, whether borosilicate glass, polyethylene, polypropylene, or Teflon, should be thoroughly washed with detergent and tap water, rinsed with 1:1 Nitric Acid (trace), deionized water, 1:1 Hydrochloric Acid (tract), and finally three rinses with deionized distilled water, in that order. After washing, containers should be used immediately or for short term storage covered with lids or parafilm to prevent atmospheric contamination.

Cleaning of Glassware for Organic Determinations

Glassware used for trace organic constituents should be washed at least 15 minutes in chromic acid or nochromix to destroy organic residues. They should then be rinsed thoroughly in tap water and finally distilled deionized water. Glassware may be dried with redistilled nanograde acetone when needed for immediate use; otherwise it is oven or drip dried. This glassware should be stored inverted or covered with aluminum foil to prevent dust contamination. Sample bottles should also be rinsed several times with redistilled solvent (e.g., acetone, hexane, petroleum ether, chloroform).

Specialized Cleaning

Special cleaning requirements may exist for particular types of vessels and for glassware to be used for specific determinations. Special requirements for specialized glassware, fritted ware, and filters are included in the EPA Handbook for Analytical Quality Control in Water and Wastewater Laboratories, (EPA-600/4-79-019), March, 1979. Some of the most frequently occurring special cleaning requirements are listed in Table 5-1.

TABLE 5-1. SPECIAL CLEANING REQUIREMENTS

	Washing	Rinsing	Drying/ Storage	Precautions
Absorption cells	Detergents/ organic solvents. Do not use dichromate solution.	HNO rinse, alcohol, or acetone.	Remove any film. Store protected from dust.	Soaking may produce etching. Cells must be checked for equivalence after washing. Compare transmittance or OD values.
Trace metals/ Lead analysis		1:1 Nitric acid, followed by 10 -12 tap water rinses, followed by 4 deionized water rinses.		
Phosphate determination		Rinse thoroughly with distilled water.		Do not use phosphate-containing detergent.
Ammonia/Kjeldahl nitrogen analysis		Rinse with ammonia free water.		
Trace organic substituents/ chlorinated pesticides	15 minute wash with dichromate solution.	Rinse with acetone.	Dry at 375 C for 4 hours.	Store inverted and pro- tected from dust.
Collection bottles and liners for organic analysis	Wash with dichromate solution.	Rinse with distilled water, then several rinses with redistilled solvent such as acetone, hexane, ether, or chloroform.	Store inverted and pro- tected from dust. Store liners & caps in sealed containers.	
Caps for collec- tion of organics	Detergent wash.	Dist. HOH & redis. solv.	Sealed Containers.	

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2.3 SAMPLE BOTTLES

Special sample bottles have been purchased for the QC samples to be prepared for the WRT program. Bottle sizes of 40mL, 250mL, 500mL and 1000mL were obtained from I-Chem Research (city, state) and meet cleanliness standards published by the U.S. Environmental Protection Agency (EPA). Bottles are packaged in cases and are accompanied by chain-of-custody documentation originated by the vendor. Chain-of-custody is maintained in the laboratory by (i) storing opened cases in restricted access areas (ii) inventorying and documenting partial cases prior to storage and subsequent use and (iii) sealing opened cases with tamper-indicating seals. Bottles from cases showing evidence of unauthorized use are not used.

Chain-of-custody documentation is accomplished in the laboratory by assigning each case of glassware a unique number. This case number is recorded in the logbook for each QC sample along with the corresponding bottle volume (eg. 40-001) as used. Bottles are for one use only and are not washed and re-used. Open cases are re-sealed, dated and initialed with tape after removing the bottles required for daily use.

3.0 WATER PURITY

The quality of reagent grade water used for this program is assured by a rigorous maintenance schedule for distillation, deionization and reverse osmosis equipment. Confirmation of reagent grade water quality is accomplished by weekly analysis of the water for pH, conductivity and total organic carbon (TOC). Documentation regarding water system maintenance and analytical results are recorded in a log book.

4.0 SAMPLE DOCUMENTATION

Prepared samples are identified using numbers assigned by the data custodian, a representative of MSFC Test Laboratory (EL/64). The ACC is responsible for ensuring that the proper sample numbers are affixed to the proper sample bottle.

The ACC maintains sample number integrity by entering assigned sample numbers and other relevant data such as ERA lot number, I-Chem lot number and required dilution volume into a logbook. The ACC then relinquishes the logbook to the laboratory technician who then prepares the samples in accordance with logbook instructions.

Once samples have been prepared, the laboratory technician originates the chain-of-custody documentation which will accompany the samples in transit to the data custodian. Figure 1 illustrates the chain-of-custody form which is used for all samples prepared for this program.

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4.1 QC SAMPLE LOGBOOK

The ACC will maintain a logbook for all QC samples used in the WRT program. The information recorded in the logbook includes: sample number, ERA standard lot number bottle code, ERA standard category, sample volume, sample bottle volume and case code number, the initials of the analyst who prepares the sample, the date prepared, and the date delivered to NASA/MSFC.

4.2 CHAIN-OF-CUSTODY FORM

An internal chain-of-custody form (Figure 1) will be maintained for each daily allotment of QC samples. The preparer will initiate the form as samples are prepared. The ACC will deliver the samples to the Date Custodian at NASA, who will sign the form upon assuming custody. The ACC will return the form to the internal filing system. The information on the form includes: date, sample numbers, preparers signature, ACC signature, and Data Custodian signature.

4.3 QC SAMPLE LABELS

Sample labels will be provided to the ACC by the Data Custodian 24 hrs before test stage start up. The preparer will affix the appropriate label to each bottle prior to preparation. Labels will then be covered with clear plastic tape to prevent smearing. The labels will be pre-printed with the following information: sample number, date/time, preservative, remarks, and parameter type. The sample number and parameter type are filled in on the pre-printed label. The ACC or analyst will enter the following information on the sample labels prior to affixing them to the sample bottles: sampler's initials, sample date/time, field tracking number (to be provided by Data Custodian), and preservative (where required).

5.0 STORAGE OF CONCENTRATED AND PREPARED STANDARDS

All concentrated and prepared standards are organized by lot number and parameter and stored in a locked refrigerator at $4^{o}C$. The ACC maintains possession of refrigerator keys and is responsible for verifying that the refrigerators are locked when leaving the laboratory facility.

Prepared standards (samples) are returned to the refrigerator immediately following preparation. Once all necessary samples have been prepared, the samples are removed from the refrigerator and packed into plastic coolers for shipment to NASA/MSFC. Only "blue ice" is used in coolers to maintain shipping temperatures of $4^{o}C$. Because many samples have limited holding times, all samples are delivered to the NASA/MSFC data custodian within 12 hours of preparation.

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6.0 PREPARATION OF QC SAMPLES

Quality control samples are prepared by diluting ERA stock solutions with chilled, ultra pure water in accordance with ERA instructions. Sample aliquots are transferred to labelled sample bottles immediately following dilution and mixing. Chemical preservatives are added if samples collected during water recovery system testing receive a chemical preservative.

For selected parameters, dilution of ERA materials is not required. In these instances, measured volumes of ERA solutions are transferred directly to labeled sample bottles.

6.1 MINERALS AND HARDNESS

The minerals and hardness standards furnished by ERA do not require dilution prior to use. The laboratory technician prepares quality control samples for minerals and hardness by measuring 100 mLs of solution into a graduated cylinder and pouring the cylinder contents into a labelled sample bottle. The capped sample bottles are placed in the refrigerator at $4^{O}C$ pending shipment to the NASA/MSFC Data Custodian. Any unused solution is discarded.

6.2 DEMAND

Preparation of Demand Quality Control Samples is accomplished by volumetrically pipetting 5.0 mL of ERA Demand Concentrate into a 500 mL volumetric flask. Chilled ultra pure water is added to volume. The flask is inverted several times to facilitate mixing. Following thorough mixing, Demand samples are withdrawn from the volumetric flask and transferred to labelled sample bottles. Sample bottles are sealed and stored at $4^{O}C$ until transfer to the NASA/MSFC Data Custodian.

6.3 NUTRIENTS

Preparation of Nutrient Quality Control Samples is accomplished by volumetrically pipetting 5.0 mL of ERA Nutrient Concentrate into a 500 mL volumetric flask. Chilled ultra pure water is added to volume. The flask is inverted several times to facilitate mixing. Following thorough mixing, Nutrient samples are withdrawn from the volumetric flask and transferred to labelled sample bottles. Sample bottles are sealed and stored at $4^{O}C$ until transfer to the NASA/MSFC Data Custodian

6.4 CYANIDE

Preparation of Cyanide Quality Control Samples is accomplished by volumetrically pipetting 5.0 mL of ERA Cyanide Concentrate into a 500 mL volumetric flask. Chilled ultra pure water is added to volume. The flask is inverted several times to facilitate mixing. Following thorough mixing, Cyanide samples are withdrawn from the volumetric flask and transferred to labelled sample bottles. Sample bottles are sealed and stored at

4°C until transfer to the NASA/MSFC Data Custodian.

6.5 PHENOL

Preparation of Phenol Quality Control Samples is accomplished by volumetrically pipetting 5.0 mL of ERA Phenol Concentrate into a 500 mL volumetric flask. Chilled ultra pure water is added to volume. The flask is inverted several times to facilitate mixing. Following thorough mixing, Phenol samples are withdrawn from the volumetric flask and transferred to labelled sample bottles. Sample bottles are sealed and stored at 4°C until transfer to the NASA/MSFC Data Custodian.

6.6 TRACE METALS

Preparation of Trace Metals quality control samples is accomplished by volumetrically pipetting 5.0 mL of ERA Trace Metals concentrate into a 500 ml volumetric flask. Chilled ultra pure water is added to fill the flask to approximately 80 percent volume. Trace nitric acid is then added dropwise to yield a pH of less than 2. Once acidification is complete, the flask contents are mixed, additional ultra pure water is added to volume. The flask is inverted several times to facilitate mixing. Trace Metal samples are withdrawn from the flask and transferred to labelled sample bottles. Sample bottles are sealed and stored at 4°C until transfer to the NASA/MSFC Data Custodian.

6.7 RESIDUAL CHLORINE

Preparation of residual chlorine quality control samples is accomplished by volumetrically pipetting 1.0 mL of concentrate into a 1 liter volumetric flask. Chilled ultra pure water is added to volume. No preservative is required. The flask is inverted several times to facilitate mixing. Following through mixing samples are withdrawn from the flask and transferred to labeled sample bottles. Sample bottles are sealed and stored at 4°C until transfer to the NASA/MSFC Data Custodian.

6.8 TURBIDITY

Preparation of turbidity quality control samples is accomplished by volumetrically pipetting 5.0 mL of turbidity concentrate into a 500 mL volumetric flask. Shake the turbidity concentrate well before withdrawing an aliquot. Chilled ultra pure water is added to volume. No preservative is required. The flask is inverted several times to facilitate mixing. Following through mixing samples are withdrawn from the flask and transferred to labeled sample bottles. Sample bottles are sealed and stored at 4°C until transfer to the NASA/MSFC Data Custodian.

6.9 VOLATILES

Equilibrate the concentrate to ambient temperature. Using a calibrated 5 microliter fixed volume pipetter, transfer five microliters of the concentrate into a 100mL volumetric flask

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containing approximately 80 mL chilled ultra pure water. Mix by inverting the flask one time and add chilled ultra pure water to volume. Thoroughly mix the sample and place 40 mL into labeled sample bottles filling to zero headspace. Sample bottles are sealed and stored at 4°C until transfer to the NASA/MSFC Data Custodian.

6.10 BASE/NEUTRAL ACID EXTRACTABLE SEMI-VOLATILES

Base neutral/acid extractable quality control samples must be prepared as a single sample. Equilibrate each of the concentrate to ambient temperature. Volumetrically pipet 1.0 mL of each concentrate into a 1 liter volumetric flask containing approximately 800 mL of chilled ultra pure water. Add the concentrate with the pipet tip held 1 cm below the surface of the water. Bring to volume using chilled ultra pure water. Thoroughly mix the sample by inverting flask several times. Place entire 1 liter volume in a labeled 1 liter sample bottle. Sample bottles are sealed and stored at 4°C until transfer to the NASA/MSFC Data Custodian.

7.0 SAMPLE TRANSPORTATION

QC samples will be removed from the locked refrigerator and transfered to a locked cooler. The ACC, or his designee, will transport the QC samples to NASA/MSFC by automobile.

8.0 SAFETY

ERA samples are for laboratory use only. If any sample is ingested, notify the laboratory safety officer and contact a doctor immediately. Properly discard sample containers after use. For further information refer to individual Material Safety Data Sheets and/or the laboratory SOP document.

9.0 TRAINING

All technicians who prepare samples for this program will have at least 2 years of laboratory experience preparing reagent grade solutions. Technicians will work under the immediate supervision of the Analytical Control Coordinator until they demonstrate complete proficiency in the preparation of quality control samples.

APPENDIX D

REVIEW OF TEST PROTOCOL

August 6, 1990

Ms. Wendy Williams
NASA/MSFC
ED/62
George C. Marshall Space Flight Center
Marshall Space Flight Center, AL   35812

Dear Ms. Williams:

I have attached a copy of the Protocol "B" review comments generated by the Consortium for the Space Life Sciences technical staff. I hope you find our criticisms constructive.

We appreciate the opportunity to contribute to this important effort. Should you have questions or require additional information, please contact me.

Sincerely,

Melvin V. Kilgore, Jr.
Technical Studies Coordinator

PROTOCOL B REVIEW

I. BACKGROUND INFORMATION

A document package entitled "Protocol for the Participation of Human Research Subjects in the Environmental Control and Life Support System Phase III Test Program: Water Recovery Test Stage 4" dated May, 1990 was received for review on July 17, 1990. The document package consisted of the following: a cover memorandum dated July 6, 1990, Title page, signature page, table of contents, table of tables, table of figures, table of appendices, listing of acronyms, 28 text pages, 10 tables, 19 figures and Appendix E. In addition, the data package contained previous comments from UAH, Sverdrup Technology, NASA/JSC and specific NASA/MSFC responses to these comments. On July 24, 1990, UAH requested Appendices A-D which were received on July 27, 1990.

II. SPECIFIC COMMENTS

1. Page 3, Section 3.2: Include B. L. Benson as a technical advisor.

2. Page 4, Section 5.2: We suggest that the section title be changed to "Previous Tests" because this section actually identifies the objectives of Protocol A. With this change the reader will not expect actual results to be included here. After the results from Protocol A are analyzed an appended section might include a brief discussion of how each of the test objectives were attained.

3. Page 6, Section 7.0, Paragraph 1: The listed objectives imply that all data analyses will be conducted prior to initiation of Stage 4. We recommend that criteria to evaluate configuration performance and establish the Phase III configuration be identified.

4. Page 7, Section 7.3, First sentence: If there are any non-contractor volunteers "contractor" should be deleted from the sentence.

5. Page 8, Section 8.1.1, Paragraph 3: What temperature is the water cooled to? Is it cooled sufficiently to prevent damage of polyethersulfone membranes? Can any additional compounds be volatilized from system components upon contact with the heated water? Is the water temperature actually verified at 250°C or is this measured via external sensors?

6. Page 9, Paragraph 1: Paragraph does not clearly articulate the criteria for activating the alternative heating system nor does it identify the individual with responsibility for this task.

7. Page 9, Paragraph 5, Last two sentences: We suggest that

"doesn't" be changed to "does not" and that "created" be changed to "resulting". We acknowledge that there is no technical benefit associated with the proposed revisions.

8. Page 10, Paragraph 1: Text should address the instance where conductivity = 150 umho/cm.

9. Page 10, Paragraph 2, Line 7 & 8: Please note that some contaminants will volatilize at 1 psia.

10. Page 12, Paragraph 1: We have the same concerns here that we identified in comment number 5.

11. Page 13, Paragraph 1: If water in test tank fails the purity test, what is the criteria to determine how the tank will be cleaned prior to subsequent use?

12. Page 13 & 14, Section 8.1.4: Do all the monitors identified perform satisfactorily? If not, reference to them should not be made. Alternative configurations should be described. If the instruments are not performing consistently with standard off-line methodologies or if the results are not verifiable, then we suggest that such monitors not be emphasized. The frequency at which the instrumentation captures data should be identified.

13. Page 14, Section 8.1.6: The text should identify clean room classification.

14. Page 15, Section 8.2: We suggest that the text identify the procedures which will be used to ensure that the facility water treatment system produces water of the quality specified in Table 2. We also suggest that references to conductivity and/or resistivity be consistent throughout the document.

15. Page 15, Section 8.3.1: We suggest that the sentence be changed to read "The proper execution of all pretest activities cited in the following subsections is the responsibility of the Test Conductor."

16. Page 16, Section 8.3.1.1: Is a subject training curriculum submitted to the IRB for review and approval? If not, it is suggested that this material be included as supporting material.

17. Page 16, Section 8.3.1.2: Comment number 14 regarding verification of water quality is applicable here. Cleaning efforts focus on microbial contamination but do not address residual chemical contamination. Also, the concerns we identified previously (comment number 5) for verification of water temperature are applicable here.

18. Page 18, Section 8.3.1.4.1: Comment number 14 regarding verification of water quality is applicable here.

19. Page 19, Paragraph 5: Reference to NASA/BA Biomedical Personnel is inconsistent with similar paragraph on page 20

regarding recycled water.

20.   Page 21,   Section 8.3.2:   We suggest that the sentence be changed to read "The proper execution of all post-test activities cited in the following subsections is the responsibility of the Test Conductor."

21.   Page 22:   We recommend   that dishes be autoclaved prior to reuse.

22.   Page 24,   Section 8.4:   We could not find a schedule which illustrates that group Q (biofilms) samples are being collected and analyzed.   This sample group should be added to the sampling schedule or all references to biofilm should be deleted throughout the document.   If the only biofilm samples collected are hardware and expendibles described later in section 8.4.2 it is suggested that the reference to sample group Q be deleted.

If biofilms are present and shedding occurs, conventional plate count methods are inappropriate because of the potential for colony growth from a clump of organisms rather that a single cell.   Epifluorescence examination of water samples in conjunction with conventional plate count methodologies may alert analysts to the aforementioned problem.

23.   Page 24,   Section 8.4.1,   Second paragraph: All documentation regarding the use of non-standard procedures should be appended to the Analytical Control Document.

24.   Page 25,   Second paragraph: Holding times for microbiological samples might be reduced by collecting and transporting microbiological samples first.

25.   Page 26,   Section 8.4.2:   The reference to the biofilm procedure should be included with the microbiological methods and appended to the Analytical Control Document.

The text does not describe how "extent of biofilms" will be determined. The presence/absence of biofilms in certain sections or components seems more realistic.

26.   Page 26,   Section 9,   Item number 1: We suggest that ground fault interruption for electrical service to wet locations be specifically identified.

27.   Page 26-27,   Section 9:   There should be some discussion about how to contact medical or fire suppression assistance in the event of an emergency.   The text should also indicate the information the caller should provide emergency response personnel.   This information can be summarized on stickers that are adhered to phones in the test facility.

While there may be a concern about the burden on test conductors, it would still be appropriate for test conductors to receive

basic first aid and CPR training.

28.   Page 27,   Item Number 6, Line 17:   Delete period following Table 4.

29.   Page 28,   Section 11.1:   In addition to a first aid kit, it is suggested that a) a complete written procedures document and b) a written safety procedures and response document be available on-site.

30.   Page 28,   Section 12,   Item Number 2:   We   suggest the sentence be edited to read "The identity of no test subject will be released to..."

31.   Table 2.

A.   The maximum detectable concentration of total bacteria by epifluorescence using the current method is approximately 10^5-10^6 cells/100 mL.

B.   "Legionellae" should not be capitalized.

C.   The detection limit for legionellae is approximately 333 CFU/100 mL by the specified method.

D.   The plural of virus, viruses, should be used.

32.   Table 4.

A.   The method detection limits for total counts by epifluorescence and halogenated hydrocarbons will not allow determination of the maximum allowable concentrations specified.

33.   Table 7.

A.   Standard Methods referenced in Table 7 are from the 16th edition.   For instance leuco crystal violet method for iodine is now 4500-IA and TOC by persulfate/UV is 5310 C.   These more recent, updated, and in some cases, improved methods, should be considered. See Standard Methods for the Examination of Water and Wastewater, 17th edition, 1989.

The University
Of Alabama
In Huntsville

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MEMORANDUM

TO: Dr. Paul Hornyak

FROM: Dr. Bill Crump

DATE: August 2, 1990

RE: Protocol "B" Comments, May, 1990 Version

As suggested by MSFC, my comments on medical issues of Human Subjects Protocols are to be directed to you for your review. Those that you choose to include are then routed to MSFC, as was done on the enclosed fax dated 11/20/89. On this enclosed 11/20/89 document, the items with asterisks in the margin are my comments which I think are still pertinent to the current version of this protocol. Also enclosed are my comments on the current informed consent form, based on a checklist I abstracted from the current HHS IRB guidelines.

In addition to these more specific comments, I think the following are pertinent:

(1) The informed consent form says that the Medical Monitor will explain and sign the consent form and do the physical exam (E4). With Dr. Aten's new role, we may want to re-word this.

(2) All components of the medical exam (History, PE, Lab) for Protocol "A" were established with little knowledge of the real risks involved. Now that we have some data from "A", the entire medical protocol may be changed to focus on the risks identified.

(3) The IRB's work would be facilitated by a medical summary of Protocol "A". This could include the surveillance data from the subjects and a "problems encountered and solutions implemented" summary of what Dr. Walley has done so far when medical issues arose.

(4) As discussed in the 11/20/89 review, if the "Pre-Test Evaluation" is deleted, then all the exclusion criteria on attachment D, sheet 1 (2.14) Appendix C must be applied to the

initial evaluation or a later non-medical assessment, and their validity is lessened.

(5) Medical risk determination is based in part on water quality assessment. The validity of this assessment depends on a rigorous analytical control (AC) plan. For these reasons, the AC plan should be part of the human subjects protocol.

(6) As discussed in the 11/20/89 review, a standard procedure is needed for management of an ill or injured subject. Figure 12 was added in an attempt to handle the daily screening problem, but the question remains as to what the test conductor is to do with a subject who answers "yes" to question 6.

Enc. (2)
cc: Mr. Kenny Mitchell

INFORMED CONSENT CHECKLIST

                                                            Comments

A. 1. Purpose, Duration, Identify   X Yes __No   Duration of surveillance is a little unclear, But
       Experimental Procedures   careful explanation of Table E-1 should address
                                                   adequately.

    2. Risks or Discomforts   __Yes X No   First line of 9.6 on page 27 should be repeated in
                                                   informed consent form - ie. Gu, respiratory, GI
                                                   left off - too much emphasis on skin.

    3. Benefits   __Yes X No   Suggest add "Although no direct benefit is expected
                                                   for you from your involvement, your participation
                                                   could benefit all future space travellers".

    4. Alternative Treatments   N/A

    5. Confidentiality of Records   X Yes __No   But only in part 2 of form.

    6. Compensation for Participants X Yes __No   But only in part 2 of form.

       Compensation for Injury   __Yes X No   Unclear who will pay for any care needed - May be
                                                   clarified by reviewing "MSFC Guidelines".

       Whom to Contact for Injury   __Yes X No   Cannot evaluate this without seeing a copy of the
                                                   "MSFC Guidelines" for obtaining care.

    7. Whom to Contact for Questions __Yes X No   Not clear enough <u>how</u> subject contacts M.D.,
       (How to obtain acute care)   and who answers questions in "off" hours.

    8. Voluntary, No Loss of Benefits __Yes X No   Would suggest that add "No Loss of Benefits will
       Withdraw Anytime   Occur if you Withdraw".

B. (When Appropriate)
    1. Risks if Pregnant   __Yes X No   Suggest you add "If You Think You May Be Pregnant,
                                                   You Should Not Participate in the Test   - Notify
                                                   the test conductor, but you do not need to tell him/
                                                   her why you are withdrawing".

    2. Circumstances whereby PI can   X Yes __No
       Withdraw Subject

    3. Costs to Subject   __Yes X No   Not clear who pays travel costs to M.D. office, or
       (Travel, Time)   to 4755; it should be clear that subject must be
                                                   available outside of regular working hours to be in
                                                   4755.   Table E-1 must be part of informed consent
                                                   form.

4

PRECEDING PAGE BLANK NOT FILMED

4. Method and Consequences for   XYes __No
    Subject's Withdrawal

5. New Findings will be told to   __Yes XNo   Add "If at any time unsuspected toxicity of your
    Subject (How?)   previous exposure is discovered you will be
                                                  notified by the P.I.".

6. Number of Subjects Involved   XYes__No
    (at each site)

As a general comment: Consent forms are usually not two part - I think this could be confusing. A
single form, including Table E-1, should be signed by P.I. and Subject, and Subject should be given
a copy.

ADDED 8/2/90

Bill Crump

JSC comments

COMMENTS ON PROTOCOL B: WATER RECOVERY TEST STAGE 4
November 9, 1989

The current protocol is much clearer than the previous one especially regarding definition of risk(s) to participants. The specific risks to human research subjects, however remain largely unidentified. In order to comment on the appropriateness of the medical, historical, clinical, laboratory and procedural methodologies incorporated by reference in Appendices, these risk(s) will have to be identified and clearly stated.

JSC

JSC/WJH

Title Page: Add "Protocol B" under Water Recovery Test Stage 4

3.2 Technical Advisors,

Add:
J. Boycel   JSC/SD2   (713) 483-7890
L. Cioletti   KRUG/SD4   (713) 483-7298
C. Doarn   KRUG/SD4   (713) 483-7182

Delete:
J. Scarlett
S. Beck

7.0, page 4, para 3

Reword first sentence to read "The WRT Stage 4 test will investigate the performance of a closed-loop preprototype hygiene water reclamation system with man-in-the-loop and an open-loop preprototype potable water reclamation system" for clarification.

7.3, page 5

How many individual test subjects are required as a minimum? This page says 18, pg. 17 says 17 people, and Fig. 14 says 20 people. Please make these pages consistent.

* What daily medical screening will be provided? These need to be defined.   JSC/WJH

8.1.2, page 9, para 3   The use of heat to control microbial load
of the potable water prior to multifiltration processing is of concern. If the water is not cooled to ambient temperature prior to entering

the unibeds, excess iodine will be released from the MCV iodinated resin.

8.1.5 page 12 How will the stair stepper be secured to surroundings? From the experience of one of the reviewers these machines need to be restrained.

8.3.1.2, page 13, para 3 MCV's are only temperature sensitive in an actively flowing system.

What type of filter will be used? Why does it need to be disinfected? (Is it not purchased pre-sterilized?) How will hydrogen peroxide affect the porosity of the filter? Can the filter be autoclaved?

8.3.1.2 page 13 para 4 Non-saprophytic plate count should be replaced with aerotolerant eutrophic mesophilic (AEM) plate count.

JSC 8.3.1.3 page 13/14 The process and criteria for excluding an ill JSC/GAH subject needs to be specified, e.g. if a subject has had gastroenteritis since the last physical exam, how will that subject be excluded? (see attachment QQ, subject controls and comment to para 7.3 page 5)

JSC (e) Why not use prototype SSF cosmetics? List from Phyllis

(a) Add "and Medical Monitor**" following the Principal Investigator to ensure that he is informed and his approval is obtained for any such decisions

**We feel Medical Officer would be a better choice than Medical Monitor, but do not feel it is critical to change the terminology.

Sentence #2 Add: "...every 24 hours *if experience from Protocol A testing indicates stable operation and concurrence to reduce screening is received from the PI and the Medical Monitor*"

Sentence #3 Add: "The Principal Investigator *and the Medical Monitor...*"

Sentence #5 Add: "...without the approval of the Principal Investigator *and the Medical Monitor.*"

8.3.1.4 page 14

Paragraph 1 Sentence #2   Add: "...every 24 hours *if experience from Protocol A testing indicates stable operation and concurrence to reduce screening is received from the PI and the Medical Monitor*"

Paragraph 2 Add "and Medical Monitor" to last sentence.

✳ **8.3.2 page 16** 1. Add: "..will be contacted immediately and appropriate medical care be provided as designated by the Medical Monitor"

JSC What are the PI and Medical Monitor to do with an ill subject? (see JSC/UAH later comments to p. 22)

8.3.3 page 16/17/18   Determination of specific risk(s) to human participants is still the requisite step in preparing human participant proposal for IRB review and approval. The baseline and tentative test configurations as described in this section are insufficient to allow analysis and determination of the specific risk(s) to human participants which must be listed in a subsequent medical section, and will subsequently determine necessary medical preparations (e.g. specific medical history questions or laboratory tests) and the content of the necessary informed consent form(s).

Page 17,   Paragraph 1   See comment to Section 7.3, page 5 - please make numbers of subjects consistent in all sections.

JSC **Paragraph 2** See comment to page concerning need for daily medical screening.

JSC **Figure 12** does not address recent illness.

✳ **Para 3**   By what mechanism/device do female participants collect urine? Describe device and cleaning procedure. The word "samples" should be replaced with "void".

Para 4   It is probable that few subjects will spend more than 30 minutes of the hour actually exercising but they will continue to perspire during rest periods. Will there be a place to sit? Will subjects be required to stay in the area to permit sweat to evaporate?

8.4 page 18-20   See above comments regarding determination of risk(s) to human participants. For example, using these sampling and analysis methods, less than 50% of the specific contaminants comprising reclaimed water TOC have in the past, been identifiable. The specific risk(s) have not been adequately identified to avoid a detailed discussion of existing methods, validations and their limits when applied to this situation.

8.4.1 page 19   The micro sampling procedure and equipment needs to be defined and must be a closed sampling system similar in concept to that used to sample STS (See LS 10048 previously provided).

In order to assure that the micro sample is representative of the system rather than the sample port, either the port needs to be flushed prior to collection of the micro sample, or the chemical sample needs to be obtained prior to the micro sample. This last procedure is what is used on STS. On cleaning the port with alcohol, there should not be a problem if only the outside of the luer lock is cleaned and a period of air drying is allowed before the interfacing luer with hose is attached.

Hydrogen peroxide as the disinfectant should be considered as an alternative.

Page 20 Para 3 Please provide us with a copy of the analytical control plan.

✳ Page 21 By what mechanism will test subjects be informed of out-of-spec water? (A written informed consent is suggested).

✳ It is suggested that some data be collected on "twice washed" dishes before they are used by subjects (e.g. swab cultures of a small random set early in the test) or that proper operations of the autoclave be verified e.g. spore strips.

✳ Is any exclusion of drug ingestion by subjects planned? (see attachment QQ subject controls).

JSC 10.0   Infection is regarded by most authorities as a clearly risky situation rather than an inconvenience or discomfort and should be treated as such. Change 10.0 to Possible Inconveniences, Discomforts or Exposure Risks to Test Subjects.

JSC **11.0** **page 22** Section 11 and Appendix C are internally inconsistent. The introduction to Appendix C states there will be a pre-test examination one week before involvement. This allows for disqualification for recent illness, menstrual history, or abnormal lab (2.14 Attachment to Protocol D sheet 1/). Clarification is needed as to whether this is required before each involvement, if subjects participate repetitively. JSC/UAH

※ JSC What is entailed with the daily health screen? JSC/UAH

JSC Paragraph 12.1 states that a standing procedure will exist for illness/ injury care. It is suggested that this is critical to IRB consideration, and should be specified as a written Appendix (see Attachment SS, Potential Functions of Physicians...) JSC/UAH

14.4 page 23 The MSFC facility safety letter of approval must be available for IRB review before IRB approval can occur.

Table 2. (continued) 1, 4-dichlorobenzene should be 1,2-dichlorobenzene

Table 4 Use of the term "specific toxic organics" is inappropriate. Change to "specific organics"

Table 7 The method for potassium should be EPA 300.7, not EPA 300.0. The MDL for sodium should be 0.03 mg/L not 0.002 mg/L.

Table 8 Sample Volumes See attached sheet for sample volumes required for JSC analyses.

Table 8 **Microbiologicals** Include heterotrophic plate count (R2A) in Sample Group A. Recommend fecal group be deleted from Sample Group A.

Non-saprophytic plate count should be referred to as Aerotolerant Eutrophic Mesophilic (AEM) plate count.

Figure 5 The input locations for the facility water need to be shown. It is not clear how the system is to be initially filled with facility water.

Appendix C **Attachment A**

JC The Cornell Medical Index Health Questionnaire incorporated by
reference is a good initial attempt to define a screening tool to
correctly select "nominal" test subjects. However, it is not specifically
designed for this test and may be inferior to a questionnaire which
addresses the problem of specific risk and risk co-factor
identification.

Appendix C Attachment B

JC It should also be recognized that a "screening" health examination of
otherwise expected normal test subjects requires much more
experience and skill than diagnosing and evaluating even the most
exotic or severe diseases in an ill subject. It would therefore seem
prudent that review of the history (including symptoms and signs)
and physical examination be performed by the most experienced
medical person available to this test. After medical certification, most
of the subsequent problems can be delegated at least initially to a
health care extender if necessary.

JSC In the introduction to Appendix C, it is specified that a nurse obtain
informed consent, with implied access to PI to answer questions. This
is strongly recommended. The symptom diary (Attachment B to
Appendix C of protocol) should be deleted.

JC Will the Environmental Health Physician be a civil servant or a
contractor?

JC If subjects are repetitively involved, 10 or more follow-up visits may
be required (see attachment RR, Questions likely to be asked...).

Appendix E

see
Attachment
TT
Previously an informed consent was not available. Since these NASA
tests are subject to HHS Regulations (see Attachment TT, Approved
by Fletcher p62 11/88), there are major concerns with the current
consent form. This would also apply to Protocol A. Specifically :

Page E2   C does not include a statement that subjects will be
showering in reclaimed or recycled water. See previous comment to
Section 7.0 page 4.

C does mention reuse of clothing. Is anything known of this? The reference to the dishes (being reused) is not clear.

Page E3 The definition of clothing to be worn, what is to be washed, spare clothing by test subjects needs to be clearly described.

Will test subjects be allowed to shower before they participate? How long before they participate?

Page E5 Generally, the test subject and the PI sign the same document, on the same page.

D. para 2 The clothing exchange (both street and exercise) is not clear - please elaborate.

JSC **F. 3.** Man Systems provided cosmetics should be used if available.

para 6 If the water has not passed necessary checks then it will not be used -- Change to " You will be informed *that* the water has passed ..."

When is the last time participants are permitted to shower prior to the test?

✳ **para 7** It is unclear whether the test subject will be informed of the water quality prior to use. There should be a clear statement THIS WATER IS NOT MEANT FOR NOR SHOULD YOU INGEST (DRINK) IT, included here. What if this should occur?

JSC Risk(s) should be listed separately and clearly delineated. Some assessment of the risk(s) should be included. Physical conditions and situations which would increase the risk(s) should be specifically identified so that a test subject can intelligently refrain or remove themself from participating and/or seek help. Instructions on what to do should an increased risk situation occur need to be delineated or referenced.

✳ JSC There is no information on release of medical information either from the test subject's physician. It would be almost impossible for most private physicians to assess whether subsequent problems the patient might be seen for are in any way related to this test. Should the individual's private physician be advised that his/her patient

JSC 3. On site physicians coverage and medical liability for tests is largely ~~JSC/UAH~~
unaddressed, and could result in major risks and costs to govt and
BAC/test personnel if not adequately covered.

4. IRB approval should not proceed until rationale behind Option A/B
is clear.

JSC 5. Informed consent form needs major revision, with inputs of Bill
Crump MD of UAH incorporated, along with description of exams, ~~JSC/UAH~~
(Genitalia included? should be). Definition of acceptable medication
ingestion (if any) should be clear. Pregnancy should be clearly noted
as DQ.

6. A statement comparing water test's water standards to those of
the MSFC/ Huntsville community would allay many subject fears.

7. Protocol should clearly state that oral ingestion of water is not in
test plan and subjects will be instructed to avoid - why is "taste" a
question on post shower questionnaire?

8. Who adds the urine reagents to the urine? IF subject or test
monitor, so state.

9. Risks for other than subjects in tests, if any, is not clearly stated.
Are there any risks to test monitors or engineers?

10. Sample testing of hypodermic needle stubs and flaming needs
better definition and addressing of contamination avoidance
procedures. (Page 11)

JSC 11. Exercising of subjects can result in major liability if adequate
screening is not done beforehand. Suggest pre test medical exam
include exercise history, testing (possibly including treadmill), and
inclusion of specific guidelines for exercise in test - suggest sub-
maximal heart rate limits at most for subjects, with better definition
of medical capabilities at site and plans for contingencies.

12. What micro testing of MCVs will be done? How often will filters
in various areas be changed? What criteria or in line tests can be
done on filter beds?

13. Is pumping gas a DQ? Page 13. Why?

14. Is it a test objective to study and use equipment (dishwasher,etc?) when will these be tested if no? Will shower walls and other contact points be micro tested?

15. A description and standardization of shower (one minute rinse, one minute lather, etc) should be done to standardize results. Same for handwashing and exercise.

JSC 16. Pre test screening of test subjects for infectious disease needs to be better defined. Any during test screens should also be defined, if any. *JSC/UH*

17. What water is being used for handwashing by Type II participants? (Page 17). Why split the showers for Type I participants other than subjective reasons? If subjective tests are being used, standardizing criteria for tests should be clearly given to subjects.

18. How will you determine adequate sweat volume from subjects? Are subjects to bring own shoes for exercise?

19. Nit-picking - description of dinners, and preparation, etc should be better. I assume meals won't be frozen as protocol states when subject eats.

JSC 20. Standby physician plan (Kelsey or UAH, etc) must be better defined. Need for on site medical monitor from JSC for test medical rep should be defined. MSFC standard coverage is not defined nor understood at JSC. *JSC/UH*

JSC 21. Cornell health questionnaire is ponderous, might consider using JSC astronaut exam physical questionnaire. Some areas need better coverage - family hx, drug history/use, smoking history, and allergy history aren't covered adequately on present history or consent forms, for instance. Statement from subject on general health and recent health status over past year should be incorporated. Specific question on iodine should be included.

JSC 22. Daily health record could be simplified and still get more useful information. This should be done by JSC, along with # 21 and 20.

3C 23. Medical evaluation forms for test subjects is good, but should include allergies (attach. C).

24. References were very complete and thorough. Enormous amount of work that has gone on in the past (and will be done in the future) is obvious.

25. Subject "bill of rights" type and better explanation of exact time and performance requirements should be placed in consent form.

26. Legal disclaimer in consent form should be reviewed by NASA and BAC legal for better resolution prior to any testing. Subject testing (i.e. AIDS and drug testing) is a hot bed of legal problems.

Resolution of these and others comments should help clarify protocol and minimize risks to subjects and NASA/BAC. Continued consistent inputs from medical personnel is essential for success, we stand

JSC ready to assist.

1655 Arlington Blvd
El Cerrito CA 94530-2004

W. Crump MD
Consortium for Space Life Sciences
Univ of Alabama Huntsville
201 Governers Drive
Huntsville AL 35801   1 Sept 90

Dear Dr. Crump,

This report is in response to your request of 18 July 90 for critical review of the NASA-MSFC draft entitled **Protocol for Participation by Human Research Subjects in the Environmental Control and Life Support System Phase III Test Program: Water Recovery Test Stage 4** dated May 1990.

General Comments:
The draft received included approximately 36 pages of text and 118 pages of miscellaneous figures, tables, appendices (consecutively marked E, A, B, C and D) and additions. The additions include 6 pages of "Specific Responses to Comments for Protocol 'B', 7 pages of "UAH Comments: Review of the Draft Protocol...", 16 pages of "Sverdrup Comments: Comments to..." and 11 pages of "JSC Comments: Comments on..."
The draft text, figures, tables and appendices are not serially paginated. For purposes of this report, I have retained pagination as begun in the "List of Appendices" (page 0). The attached, referenced figures, tables, appendices and other information are not paginated except for Appendices E and D. I have arbitrarily assigned page numbers to all other items as they were received beginning with text page 30 to coincide with text pagination. All comments in this report will refer to page numbers as I have assigned them above. As stated in past reports, **to aid review, future drafts including appendices, tables and figures should be paginated in a single consistent manner prior to dissemination.**
Virtually all of my comments on CMIF protocols 1-3/6 and 6 apply to this protocol and are incorporated by reference.

Specific Comments:

Page 5, paragraph following item #11
See previous comments my review of CMIF protocols 1-3/6 and 6
Page 6, 6.0 Justification

That reclaimed potable water will not be recycled (i.e. consumed, metabolic wastes collected and metabolic wastes reintroduced into the loop) does not mean that test subjects will not consume it. It would be clearer to say that test subjects will not consume it. This clearly also precludes recycling.

Page 6, Test Plan...

First paragraph: If the purpose of the first 7 stages was indeed "to demonstrate the readiness to proceed to WRT Stage 4 described in this document" then the data, results and conclusions should be reported or appended in order for the IRB to make a correct determination of the human risks involved in this stage. If not, delete or reword the purpose(s) of previous stages.

Second paragraph: If the "system configuration to be used will be determined from results obtained from the first seven stages" the above comment applies here also. If not, delete or reword the sentence.

Last paragraph: After "hygiene water reclamation" insert "and recycle" as this better characterizes the nature of the system to be tested in this stage.

Last paragraph, last sentence: While dishes that have been washed in reclaimed hygiene water is technically true, it is deceptive because on page 27 it is stated that "indirect exposure through the reuse of dishes washed in recycled hygiene water will be minimized by rewashing all dishes using a commercial dishwasher...before reuse by any individual...Dishes will be autoclaved after rewashing." I take this to mean that after dishes are washed in reclaimed hygiene water, they will be rewashed and autoclaved before test subjects use them to eat off of, however, this requires clarification.

Page 8, First Paragraph, Underlined Sentence: See my comment regarding Page 5, paragraph following item #11.

Pages 8-11: As stressed in my comments on CMIF protocols 1-3/6 and 6, the primary purpose of a IRBWG review is to determine whether the proposed research activity warrants the risk to the test subjects. Determination of such depends almost entirely on assessing the relative risk of exposure to hazardous, dangerous and toxic substances. **Wherever and to the best extent possible detailed quantitative, and where impossible qualitative, descriptions of specific risk factors**

should be enumerated, referenced and discussed. This is the single most important part of the IRBWG and is entirely missing from this particular document. For instance, it is clear from Page 8, second paragraph, that the following exposure characteristics are likely: **Showering** involves direct skin, mucous membrane (e.g. nasopharyneal), and pulmonary exposure to contaminants in water and offgassed into the shower stall in normally low but potentially high concentrations (e.g. if failures such as primary treatment system membrane or mechanical breakage, or "channelling " of contaminants through a failed adsorptive device should occur) for moderate durations. This statement reflects consideration given to both anticipated "normal" and failure modes, and allows discussion of failure mode protection of test subjects. Similarly **washing** involves direct skin and possible mucous membrane exposure to contaminants in water for minimal durations. **Clotheswashing** involves direct skin and possible mucous membrane contact with dried, potentially concentrated contaminants for long durations. **Dishwashing** and **urinal flushing** should be similarly characterized. Furthermore specific types of contaminants can be identified, e.g. bacteria, bacterial spores and fungal spores in showering. In many cases specific agents can be identified such as specific bacterial species identified in previous NASA shower water reclamation experiments. All such data should be clearly identified, likely exposures calculated, and probably and possible medical effects of each discussed. **References are absolutely essential where poorly defined but potentially significant human exposures are concerned.** Similar discussion should center around the medical effects of exposure to iodine disinfection products and iodinated organic contaminants. On page 10, it is stated that "Distillates from the TIMES and VCD typically contain levels of trace contaminants. **It is absolutely imperative that the contaminants be identified at the most specific level possible, that their medical effects be identified, that exposure levels causing various levels of medical effects be determined or calculated, and that likely "normal" and failure mode exposures to test subjects be determined and discussed.** For instance, there is no discussion at all regarding either dissolved or volatile organic contaminants, including whether or not the contaminant sampling, screening and/or identification methods proposed in subsequent sections are even relevent! Standard organic contaminant identification methods used for nonrecycled, terrestrial, surface water specifically ignore dissolved organic

will be a test subject? Would this physician have something to say to his patient regarding this patient's specific risks?

It would be timely to involve the Medical Officer in the above decisions before they are fixed by IRB certification.

More Generally:

A checklist Dr. Crump developed for the UAH IRB based on the HHS Guidelines is enclosed as Attachment UU. Generated from the checklist is Attachment RR. Some questions likely... Most, if not all of these questions should be answered in the informed consent document. You might want to obtain a legal opinion with IRB experience as this is critical to the success of the project, and all comments apply to Protocol A as well.

see Attachment RR and UU

The following general comments were provided by J. Boyce, MD :

Overall, a good thorough presentation and obvious volume of work has been done. Protocol and explanations were generally well written, schematics were adequate, and engineering details were adequate for IRB. Schedule and overall timeline appear tight but achievable, assuming no major problems at any stage. Scientific results of study will be most interesting and should result in much better definition of issues and areas for future work. Protocol doesn't fully define overall "game plan" for ECLSS, which would help somewhat in understanding how tests fit in the big picture. Several areas need further clarification for IRB review, however, and some safety factors need better solutions. Comments will be on entire document, with focus on medical aspects. <u>Lack of involvement to date from JSC medical personnel is notable problem that should be resolved ASAP. The resulting minor "disconnects" are obvious in several areas.</u>

JSC

1. Huge number of lab tests and study criteria will be very difficult to do, expensive, and labor intensive. Ability to perform such tests should be checked before tests begin with BAC labs.

JSC 2. Micro. standards for water may not be reasonable, necessary, or achievable with present technology. Microbiology meetings at JSC of Nov 6-8 recommendations to raise CFU limit to 10/ml should be entertained at some point, possibly after initial tests.

contaminants other than chlorinated priority pollutant hydrocarbons.

Page 11, Third Paragraph: Delete "Through the use of" in items #1 and #2, as the devices and their function are specifically being evaluated.

Page 13, Last Paragraph and Page 14, First Two Paragraphs: While it is clear that three experimental types of organic monitors would be employed, it is not clear exactly how they as individually or as a group will be evaluated with any standard reference method and how without this, the proposed comparison will have any relevance to medical toxicology.

Page 14
    8.1.5 Water Sample Ports, Second Paragraph: Is there any way to document whether contaminants isolated from the ports were introduced upstream or in the process of sampling either immediately or in the past?
    8.1.6 End-Use...: What is a "clean room environment" and what does it mean in regard to possible contaminant exposures to test subjects?

Page 15, First Paragraph: "Urine contributions from female test subjects will be collected privately" does not sufficiently document the procedure. Does this mean that individuals will collect the urine any way they choose? For microbial purposes will it be by "clean catch?" What about fecally contaminated urine? What about wipes?

Page 17, Item #2: No mention is made of smoking or alcohol, vitamin, birth control pill, illicit drug or other medication use. Since astronauts are not expected to smoke, be exposed to smoke, consume alcohol, or use illicit drugs these at least should be absolute contraindications to inclusion of exposed test subjects, unless they will be used as special test substances. In either case identifying their use and where appropriate excluding users is of great importance. Will these items be discussed with test subjects each day? Will oral probing or objective tests (e.g. serum test for smoking byproducts or urine test for illicit chemicals) be done?

Page 19
    Assessment..., Third Paragraph: See my comments regarding Pages 8-11.

8.3.1.4.2 Reclaimed: The parameters listed in Table 4 are appropriate for nonreclaimed, nonrecycled, terrestrial, surface water, and as such represent only "best guesses" based on virtually no data. This is not clear in this section and deserves discussion at least with regard to risk(s) to test subjects using water meeting these requirements and careful referencing. This section also lacks discussion and risk assessment due to the limitations of the specific sampling method(s) which may be employed.

Page 20, Assessment, Third Paragraph: See my comments regarding Pages 8-11.

Page 22, 8.3.3.1 Test..., Second Paragraph: Some drugs may require more than 24 hours to clear and would therefore have to be specifically excluded.

Page 23, 8.3.3.2: Replace "Test" by "Sub", "System Configuration and" by "Systems Integration" as individual subsystems are described in 8.0. Much of this section is redundant and can be eliminated.

Page 24, Second Paragraph: Why are chemical analyses done after microbial analyses instead of reverse. Isn't microbial contamination more difficult to control following multiple stabs verses chemical contamination?

Page 26
8.4.2 Biofilm: Sampling procedure deserves much more detailed discussion as any sampling during the test will "break" the closure and directly introduce contaminants, while sampling only after the test is over will not provide information on whether the system reached equilibrium with regard to biofilm formation. What is ED62 73-90?
8.4.3 Air: Replace "available by "specified in". What are ED 62 (59-90) and (60-90)?
9.0 Hazards: The first sentence is superfluous and should be deleted. Replace "Particular attention" by "Specific safety precautions" and "be directed to" by "include".

Page 27, Item #7: See my comments regarding Page 6, Test Plan..., Last paragraph, last sentence.

Page 28, 11.1 Physician: Reference the specific MSFC Guidelines mentioned.

Appendix E, Informed Consent: Form(s) and discussion regarding them were not included. Specific comments can therefore not be provided on this portion of the document.

"Specific Responses to Comments for Protocol B": It is very difficult to comment on this section without a corresponding copy of the original comments. Specific comments are therefore not provided on this portion of the document.

Appendix C, Medical Protocol: See my comments on pages 8-11 and Page 17 Item #2 above. I have commented before that the Cornell Medical Index is a good starting point for designing a health questionaire that addresses health and safety risks to test subjects. However, without significant modification is both inadequate and inappropriate for use for this test. **Again I strongly recommend that a meeting of experts be held to specifically address this issue, as it is perhaps the single most important part of this document and the very reason why tests like this must be reviewed and approved by an IRBWG!**

Appendix D, References: Add the following references:
1-Crump WJ, Thomas LD and Janik DS: Human Subjects Concerns in Ground-based ECLESS Testing: Managing Uncertainty in Closely Recycled Systems SAE Tech Paper #901251 (Society of Automotive Engineers, Warrendale PA, 1990).
2-Janik DS et al: Problems in Water Recycling for Space Station Freedom and Long Duration Life Support SAE Tech Paper #891539 (Society of Automotive Engineers, Warrendale PA, 1989).
3-Janik DS, Sauer RL and Thorstenson YR: Medical Effects of Iodine Disinfection Products in Spacecraft Water SAE Tech Paper #871490 (Society of Automotive Engineers, Warrendale PA, 1987).
4-Janik et al: Quality Requirements for Reclaimed/Recycled Water NASA Technical Memorandum #58279 (NASA-Johnson Space Center, Houston TX, 1987).

I hope the above comments and suggestions are helpful. Some discussion may be required on some of the above. If so, please do not

hesitate to contact me. Thank you for the opportunity of reviewing this protocol.

Sincerely,

Daniel S. Janik

APPENDIX E

WRT DATA REVIEW AND ANALYSIS

PRELIMINARY OVERVIEW OF WRT 3A

DATABASE

o Difficult to work with

o Size

    o Time requirements
    o Hardware requirements
    o Software requirements/compatibility
    o Number and description of parameters reduced

o Holding times should be included as data parameters

o Internal process for data verification is needed

o Data acquisition

o Consistant format for entering ID info into dbase

PRELIMINARY OVERVIEW OF WRT 3A

MICROBIOLOGICAL DATA

o Preliminary Nature

    o Stage 3A

    o Limited Data Points

    o No operational parameters/anomolies available

    o No physical/chemical data reviewed

    o Incomplete data reporting

    o Limited Quality Control

    o Critical Data not Collected

PRELIMINARY OVERVIEW OF WRT 3A

MICROBIOLOGICAL DATA

o Observations

- o Facility HOH indicates a trend of 6-7 days following sterilization

- o Potable and Hygiene tanks demonstrate similar trends regarding microbial density

- o Both subsystems demonstrated spikes at days 2 and 15

- o Potable loop is slightly higher than hygiene loop and both demonstrate decrease in microbial quality over time

- o Both subsystems demonstate excellent reduction of viable organisms at storage tanks

- o Cultural data and AODC data is often inconsistant & represents an extremely low recovery

- o Some data suggests that microbial numbers may be increasing within the system

- o Incubation times appear to be significant and may be a source for substantial false negatives or low estimation of numbers

- o All anaerobic samples collected were negative

- o Gram positive bacteria demonstrate greater survivabilty within both potable and hygiene subsystems

- o Good indication that bacteria recovered from the hygiene loop were derived from a human source and did survive the treatment

- o Transportation and/or holding times may have significant effect on recovery

- o No comparative data available to evaluate recovery with regards to pore size

- o Emmon's media consistantly reduced bacterial numbers as compared to non-selective media

- o Significant number of data not reported to date

- o Critical cultural data was not collected

FACILITY WATER SUPPLY
Port 38

DAYS FOLLOWING STERILIZATION
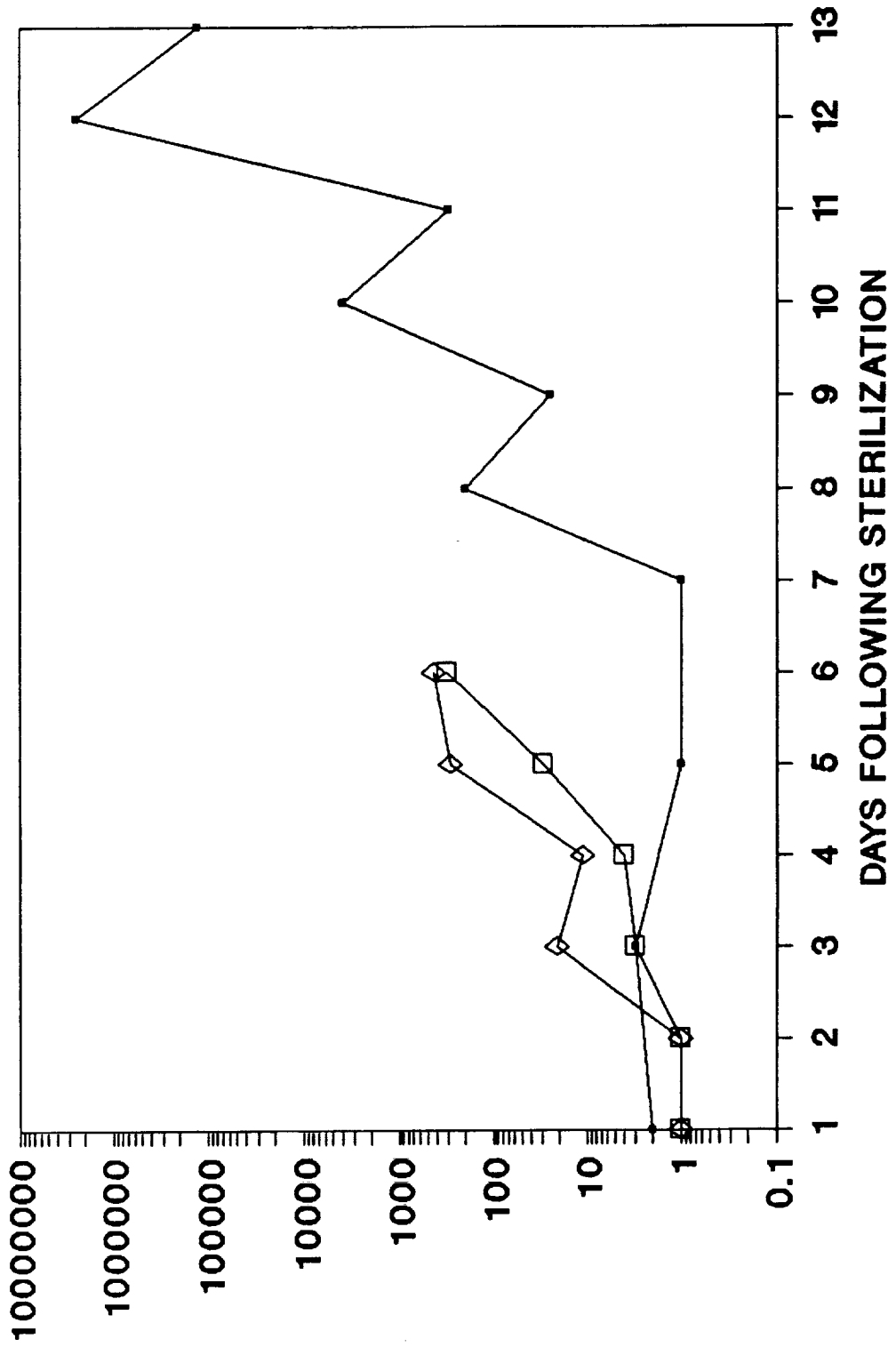
CFUs/100 mL

FACILITY WATER SUPPLY
Port 38

DAYS FOLLOWING STERILIZATION
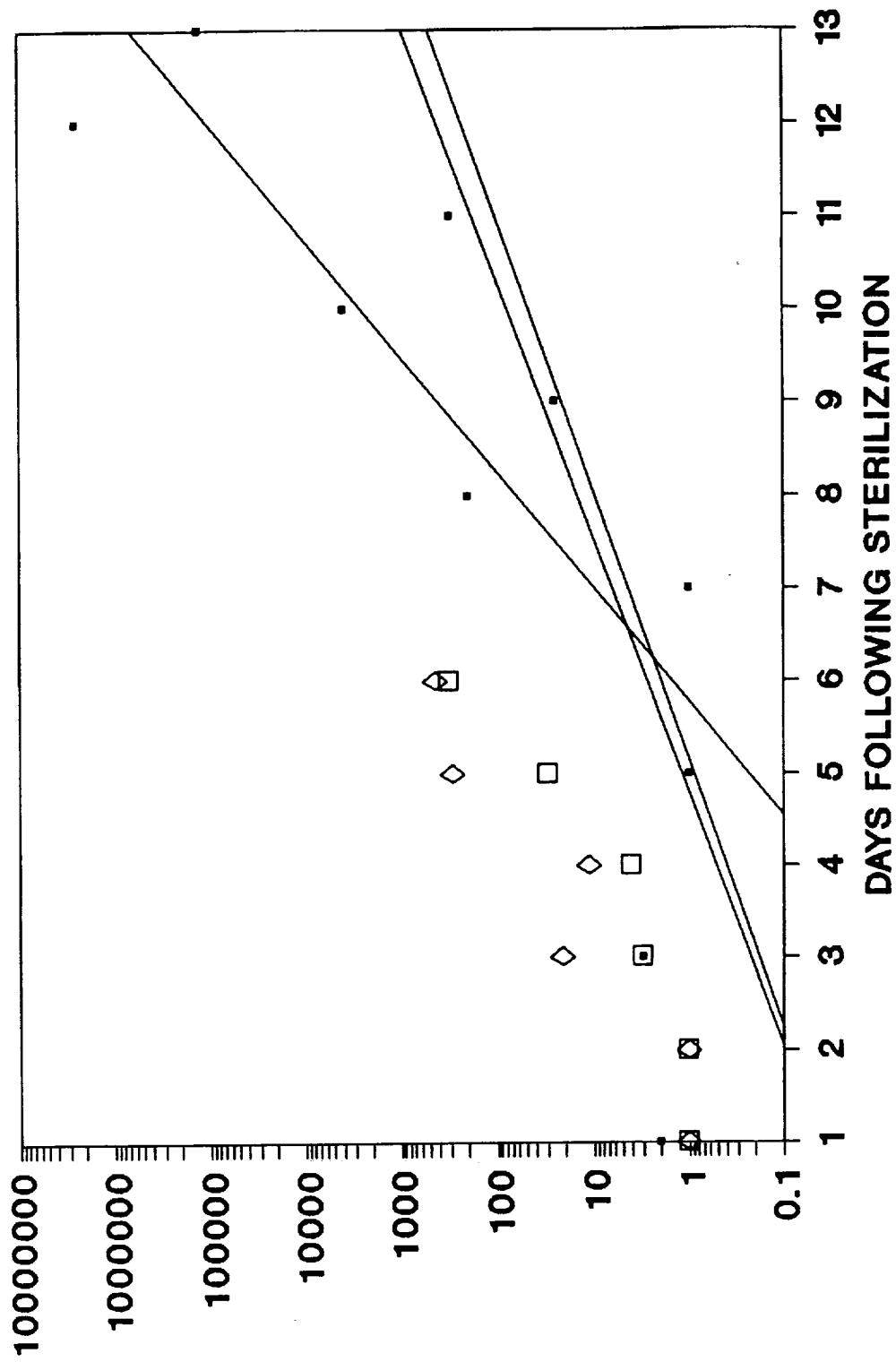
CFUs/100 mL

WRT 3A Microbiology Results
Hygiene Tanks

Total Count (Millions)

Test Day

HT-1 — HT-2 — HT-3 — HT-4

WRT Microbiology Results
Potable Tanks

Total Count (Millions)

Test Day

— PT-1   —△— PT-2   —◇— PT-3   —□— PT-4
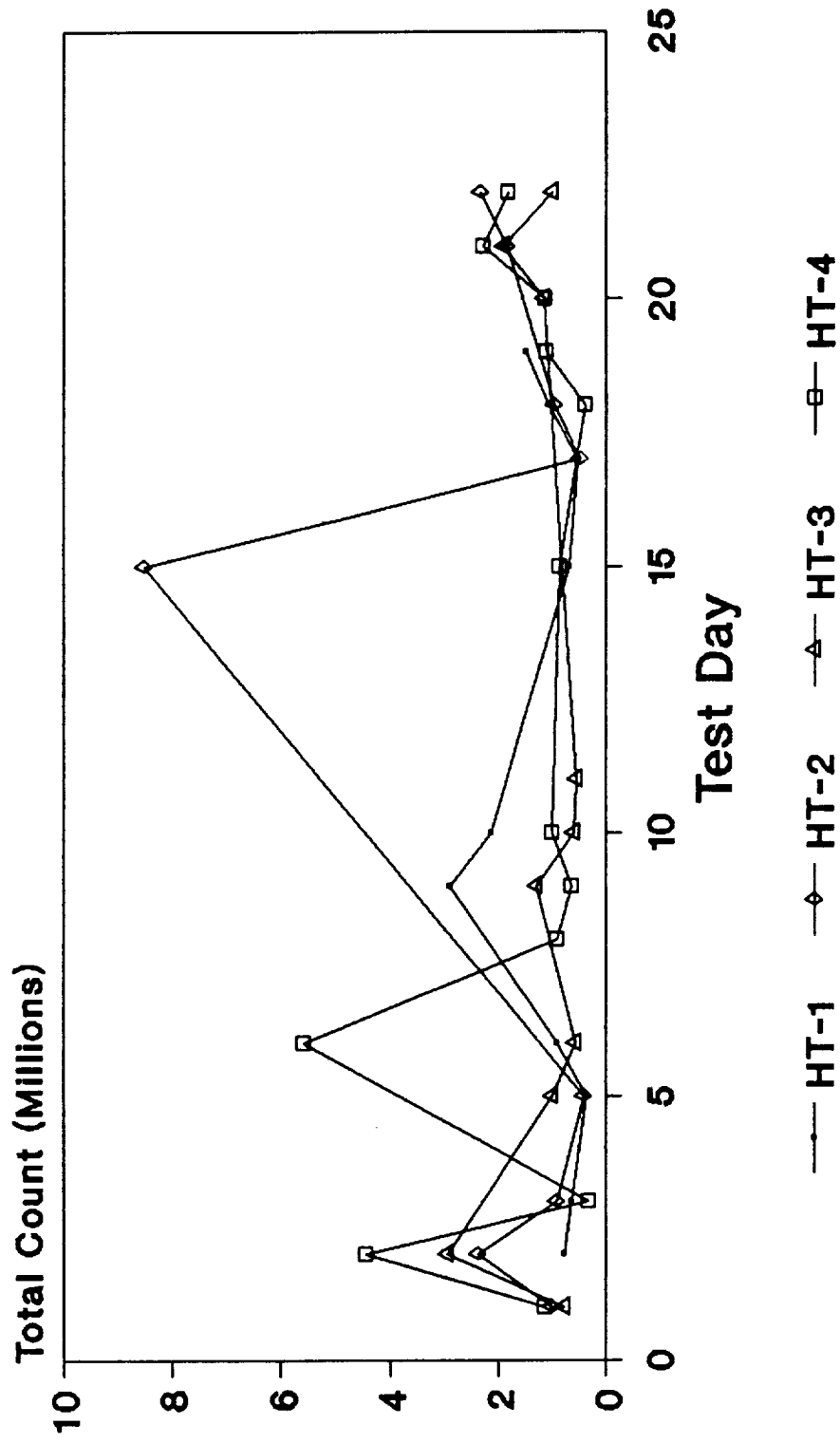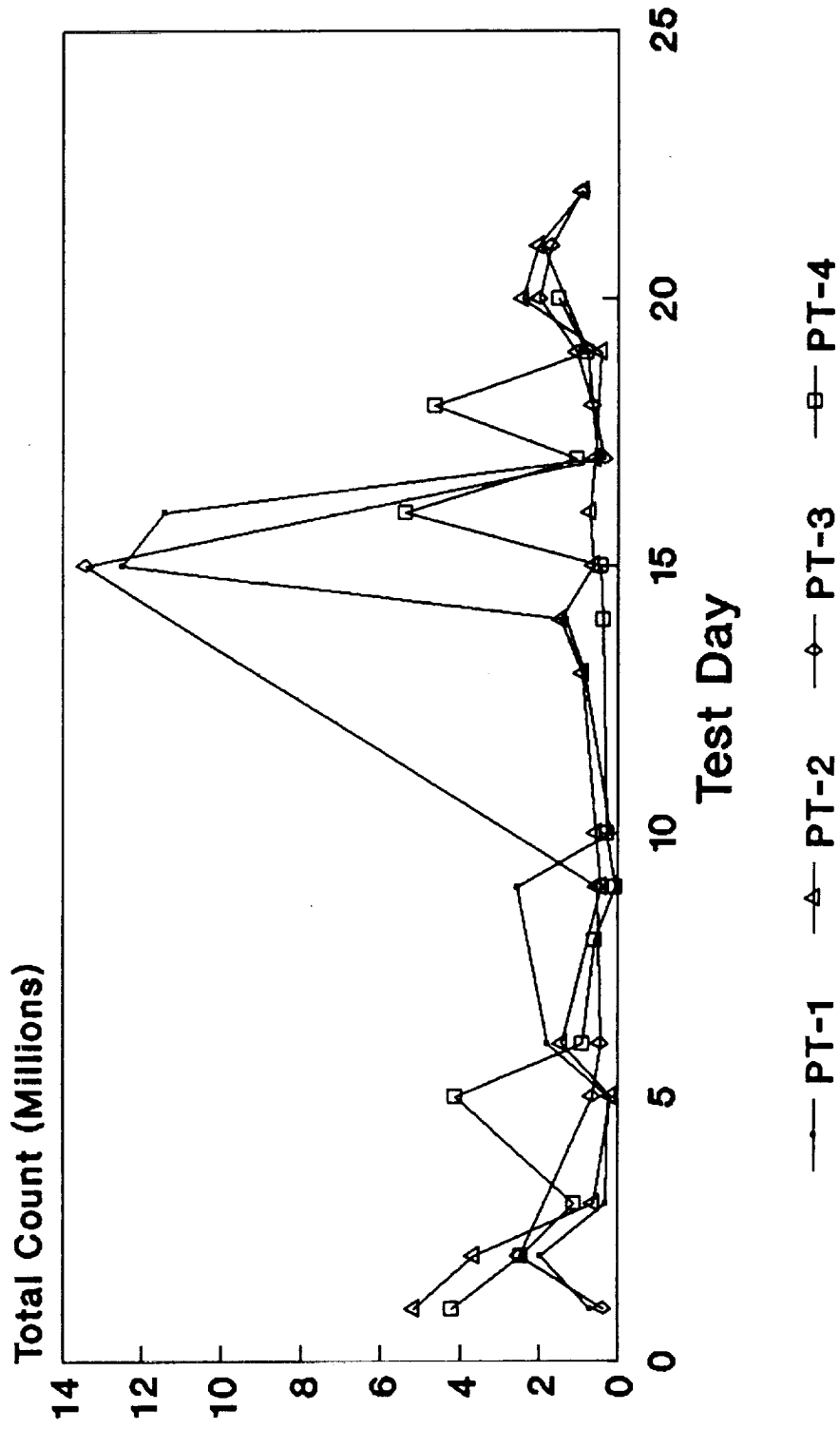
Cells/100 mL

WRT Microbiology Results
Potable and Hygiene Tanks

Total Count (Millions)

Test Day

— PT-1   — PT-3 —   — HT-2
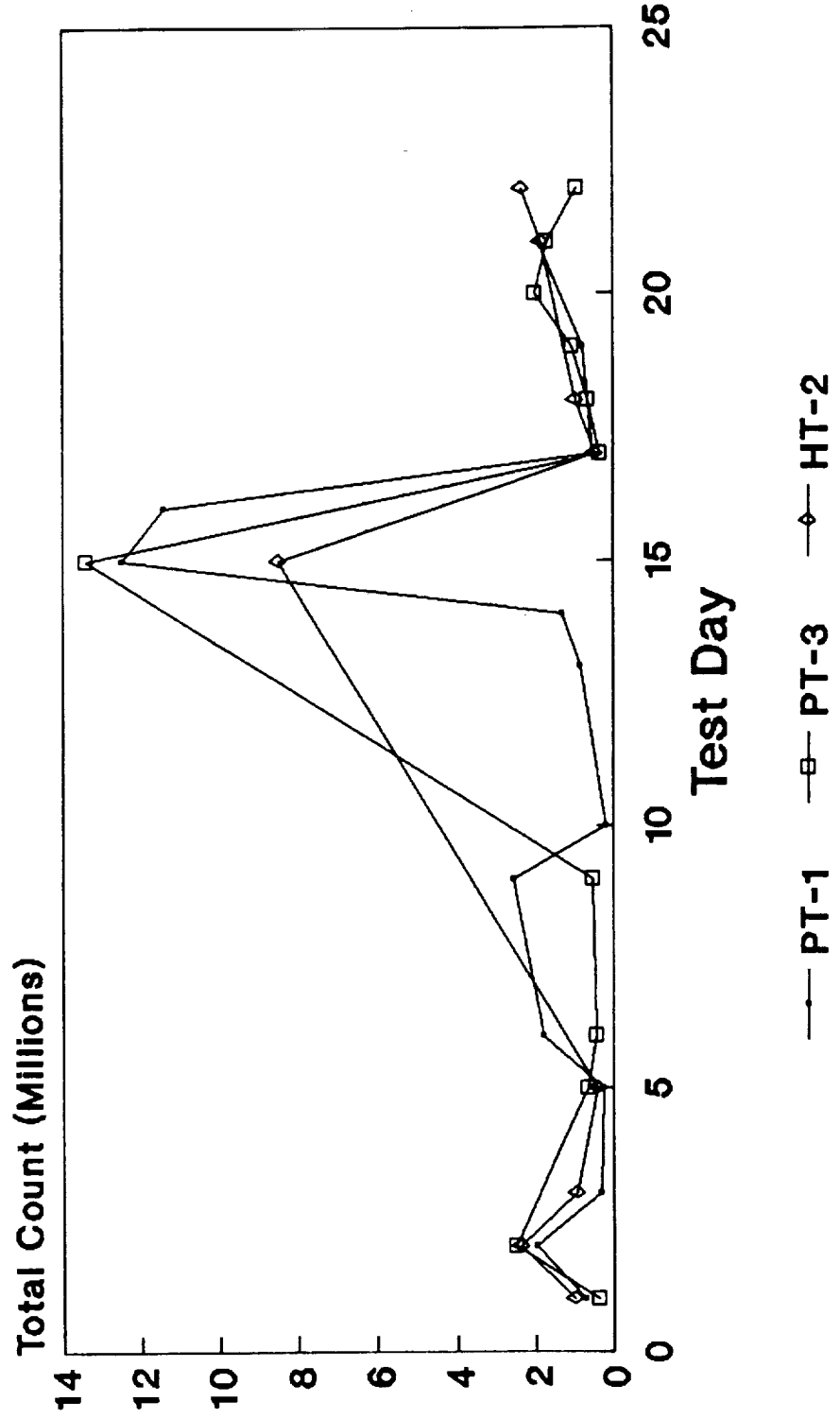
Cells/100 mL

WRT Microbiology Results
Cummulative Results (1A, 2A & 3A)

Total Count (Millions)

Days

■ PORT 9   ▨ PORT 10
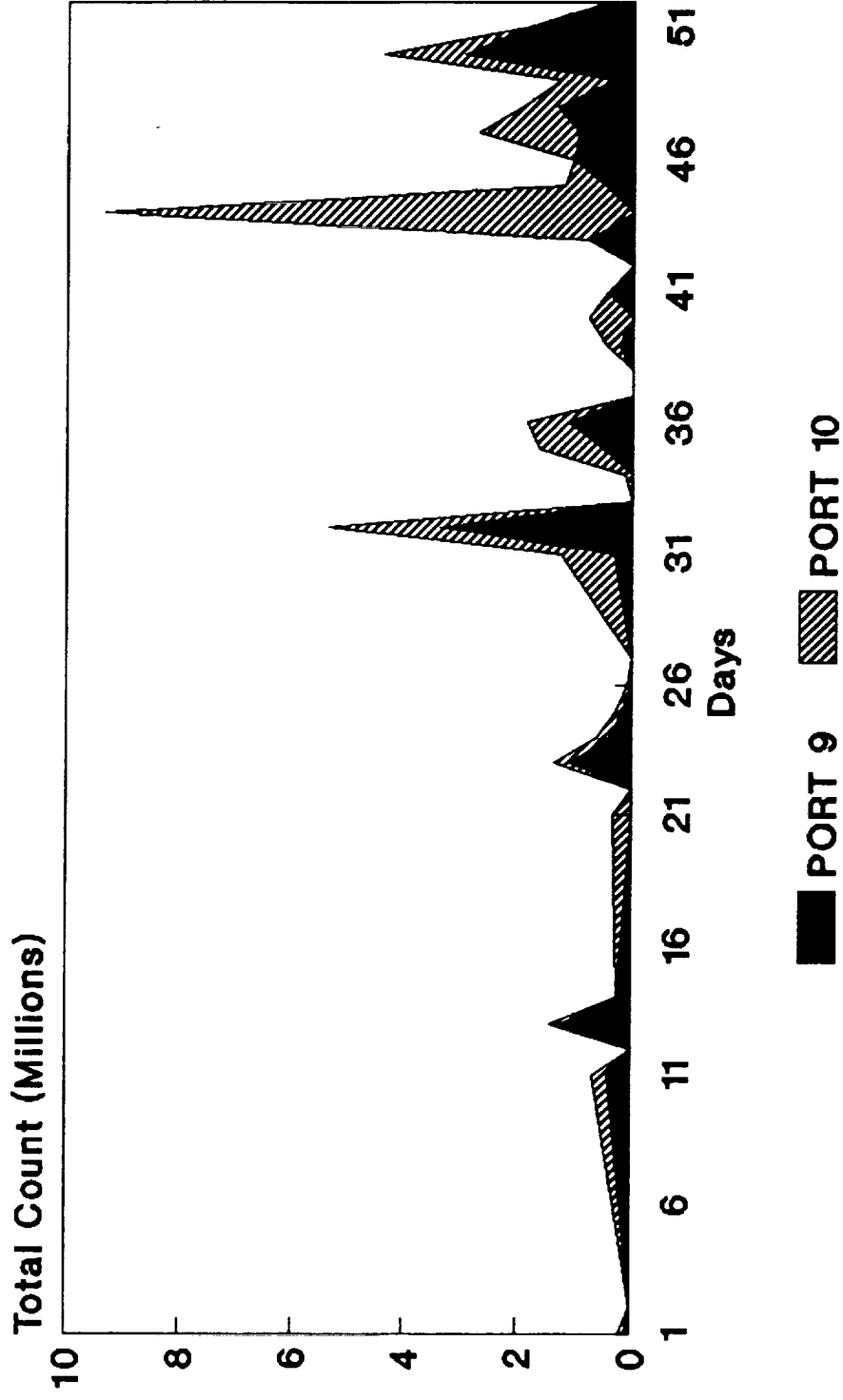
POTABLE AND HYGIENE MANIFOLDS

Hygiene Loop
Total Counts versus HPC
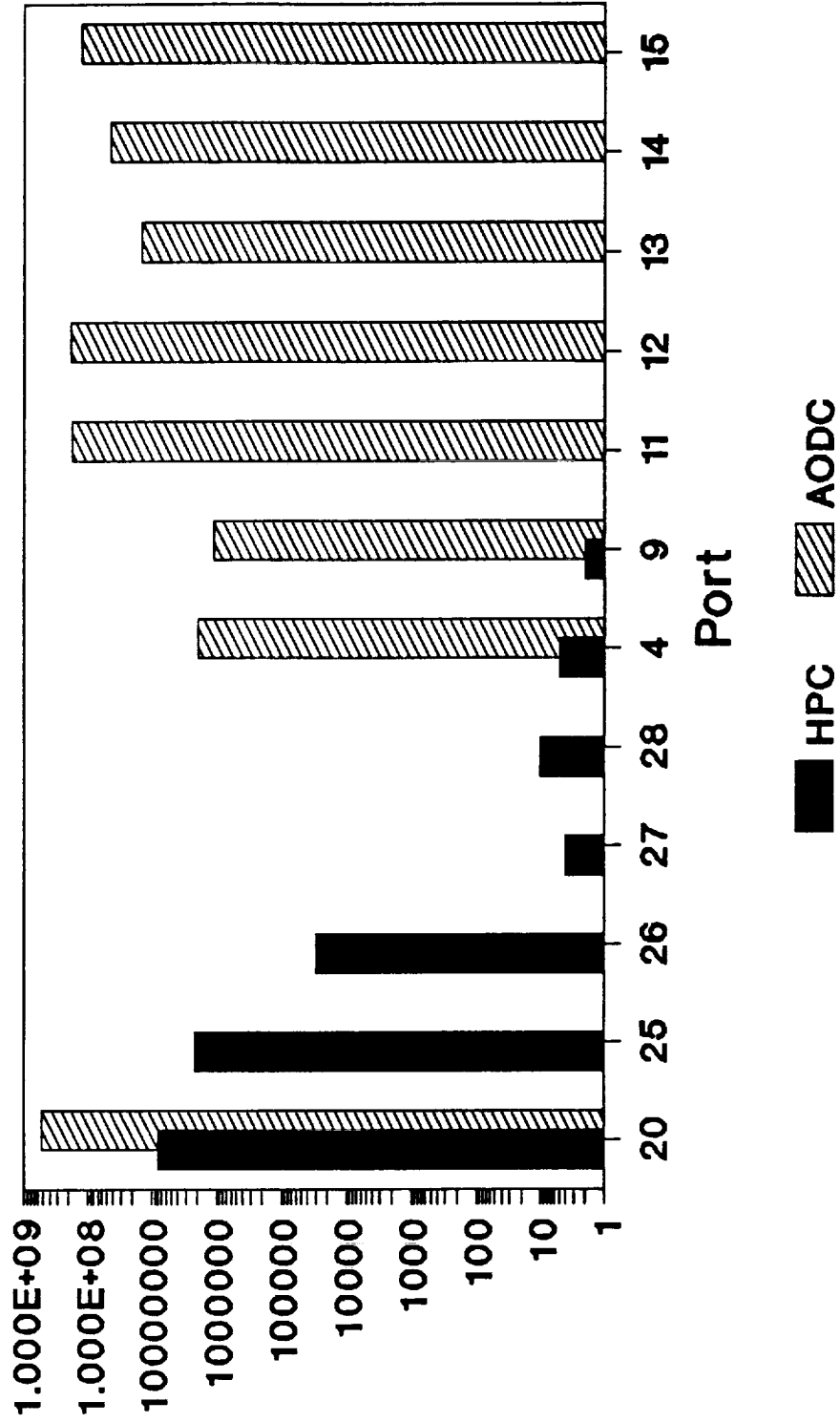
Cells/100mL

Potable Loop
Total Counts versus HPC
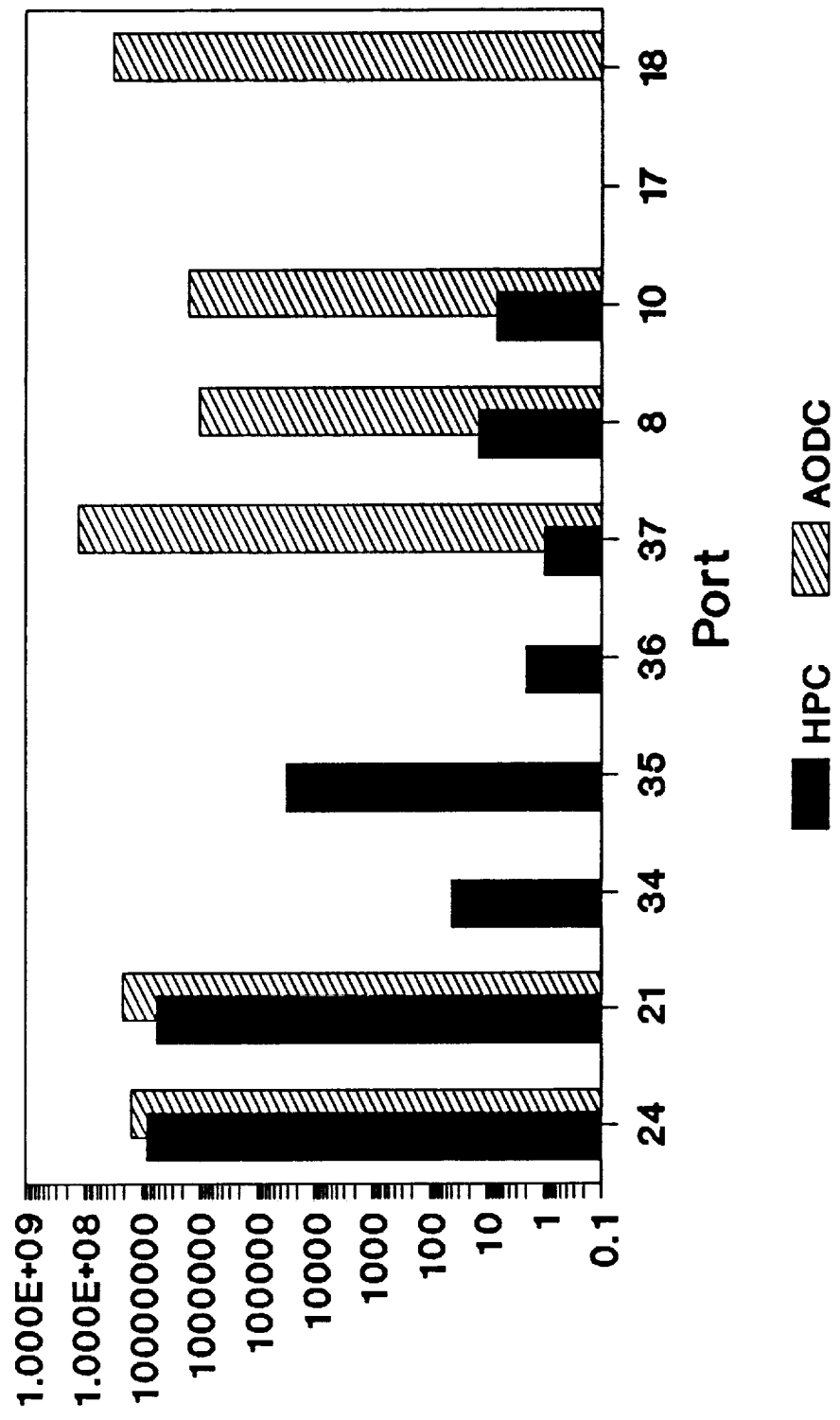
Cells/100mL

SUMMARY OF ANAEROBE RECOVERY

PORT	NUMBER OF ATTEMPTS
2	4
6	3
9	6
20	10
29	6
38	2
total attempts	31

HYGIENE WASTEWATER TANK
Yeast and Mold versus AEMs

CFU/100 mL

DAY

■ Y&M   ▨ AEMs
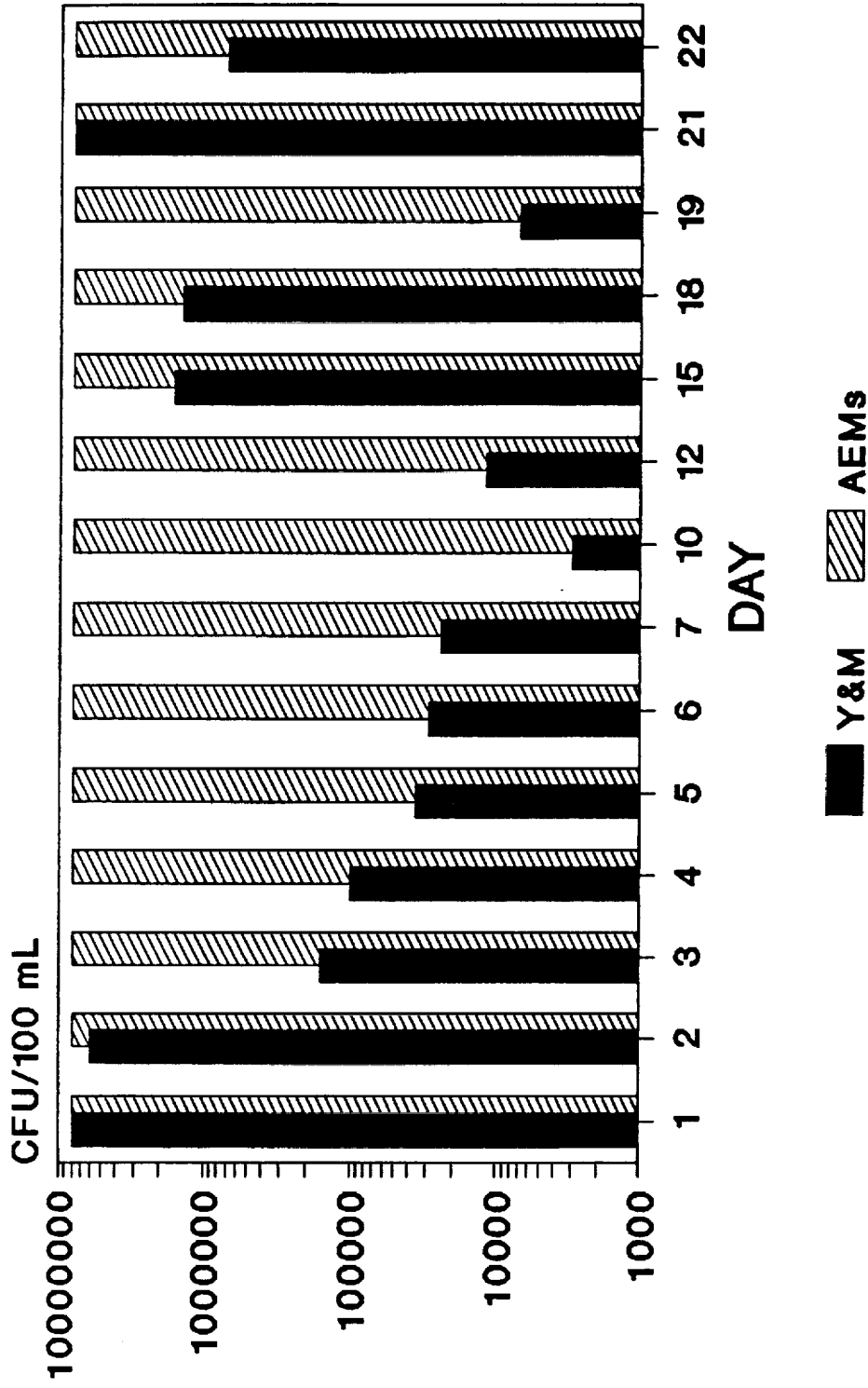
Counts are based on maximum incubation times.

SUMMARY OF MICROBIOLOGICAL DATA REPORTED
Ports 1-38

Parameter	(reported/expected)	%Reported
AODC	332/462	72
AEMs	243/471	52
HPC	174/326	53
Y&M	66/131	50
FC	76/118	64
ANA	34/113	30
LEG	1/6	17
TOTAL	926/1627	57

PORT	EPI	AEH	HPC	Y&M	FC	ANA	LEG
	332/462(72)	243/471(52)	174/326(53)	66/131(50)	76/118(64)	34/113(30)	1/6(17)
1	21/15(100)	17/23(74)	0/3(0)	0/3(0)	0/3(0)	0/3(0)	-
2	22/19(100)	22/24(92)	7/1(100)	7/3(100)	9/5(100)	7/3(100)	-
3	6/31(19)	4/24(17)	0/3(0)	0/3(0)	0/3(0)	0/3(0)	-
4	23/30(77)	18/24(75)	0/9(0)	0/9(0)	0/3(0)	0/9(0)	-
5	18/25(72)	14/24(58)	0/6(0)	0/6(0)	0/3(0)	0/6(0)	-
6	22/34(65)	20/27(74)	4/3(100)	4/3(100)	4/9(44)	4/3(100)	-
7	21/28(75)	17/24(71)	0/6(0)	1/6(17)	0/3(0)	0/6(0)	-
8	23/36(64)	18/24(86)	0/9(0)	0/6(0)	0/3(0)	0/6(0)	0/3
9	22/24(92)	19/21(90)	11/15(73)	11/15(73)	12/12(100)	10/15(67)	-
10	21/20(100)	17/21(81)	-	-	-	-	-
11	16/15(100)	-	-	-	-	-	-
12	16/15(100)	-	-	-	-	-	-
13	16/15(100)	-	-	-	-	-	-
14	16/15(100)	-	-	-	-	-	-
15	7/6(100)	-	-	-	-	-	-
18	16/15(100)	-	-	-	-	-	-
19	6/6(100)	0/9(0)	-	-	1/0	-	-
20	13/26(50)	18/35(51)	13/35(37)	14/35(40)	19/35(54)	15/35(43)	-
21	6/6(100)	14/15(93)	14/15(93)	13/15(87)	14/15(93)	0/15(0)	-
22	2/2(100)	0/13(0)	-	-	-	-	-
23	2/2(100)	0/13(0)	-	-	-	-	-
24	2/2(100)	13/15(87)	13/15(87)	13/15(87)	13/12(100)	0/3(0)	-
25	0/2(0)	1/13(8)	10/15(67)	-	-	-	-
26	0/6(0)	0/9(0)	11/15(73)	-	-	-	-
27	1/9(11)	0/9(0)	13/18(72)	-	-	-	-
28	2/12(17)	0/9(0)	14/21(67)	-	-	-	-
29	-	5/6(83)	4/6(67)	5/6(83)	4/6(67)	5/0	-
30	1/6(17)	0/9(0)	13/15(87)	1/0	-	-	-
34	0/4(0)	1/14(7)	9/18(50)	-	-	-	-
35	0/1(0)	1/14(7)	13/15(87)	-	-	-	-
36	1/4(25)	0/14(0)	15/18(83)	-	-	-	-
37	2/7(29)	0/14(0)	12/21(57)	-	-	-	-
38	27/24(100)	29/24(100)	4/3(100)	4/3(100)	7/6(100)	4/3(100)	-
45	-	-	0/6(0)	0/3(0)	-	0/3(0)	1/3(33)

RECOMMENDATIONS

o Incorporate routine heat treatment every 4-5 days when using facility water

o Eliminate Gm+/Gm- requirement

o Eliminate PCA or R2A counts

o Replace filtration with Spread plates for all dirty ports

o Add staphylococci parameter

o Increase incubation times

    AEMs -   48 & 72 hours
    FC   -   24 & 48 hours
    R2A   -   7 & 14 days

o Replace anaerobe procedure

o Minimize holding times

o Initiate studies to determine poor plating efficiency noted

    MTF/MPN
    Anaerobes
    Chemolithotrophs
    Direct viable counts
    Alternative media/conditions

o Reduce sample number and parameters to be analyzed

o Re-evaluate sample groups so that they are consistant for tracking purposes and they supply necessary information

o Consider splitting work load based on sample preservation and holding requirements

    AODC   - UAB
    LEG   - UAB
    LAL   - UAB
    Cultural - BAC

UAH PROPOSED SAMPLE SCHEDULE - WRT STAGE 3A/FIRST WEEK

PORT #	LOCATION	1	2	3	4	5	6	7
1	hygiene tank (fill)	-	-	-	-	-	-	-
2	hygiene tank (test)	A	-	A	-	A	-	A
3	hygiene tank (backup)	A N	B	A N	B	A N	B	A N
4	hygiene tank (use)	B	A N	B	A N	B	A N	B
5	potable tank (fill)	-	-	-	-	-	-	-
6	potable tank (test)	-	A	-	A	-	A	-
7	potable tank (backup)	B	A N	B	A N	B	A N	B
8	potable tank (use)	A N	B	A N	B	A N	B	A N
9	hygiene dist'n manifold	B	B	B	B	B	B	B
10	potable dist'n manifold	B	B	B	B	B	B	B
11	shower inlet	B	A	B	A	B	A	B
12	handwash inlet	B	B	B	B	B	B	B
13	clotheswash inlet	-	B	-	B	-	B	-
14	dishwash inlet	-	B	-	B	-	B	-
15	UCPU flush inlet	-	B	-	B	-	B	-
16	SFE inlet (hygiene)	---------	---------	---------	--not in use---------	---------	---------	---------
17	SFE inlet (potable)	---------	---------	---------	--not in use---------	---------	---------	---------
18	potable dispenser	A	B	A	B	A	B	A
19	pretreated urine tank outlet	-	D	-	D	-	D	-
20	waste hygiene tank outlet	-	L	-	E	-	E	-
21	condensate tank outlet	L	-	F	-	F	-	F
22	RO brine tank outlet	-	D	-	D	-	D	-
23	EEF waste hygiene accum.	-	E	-	L	-	E	-
24	EEF condenser accum.	F	-	L	-	F	-	F
25	RO permeate outlet	-	L	-	E	-	E	-
26	RPTA outlet	-	E	-	L	-	E	-
27	RPSA outlet	-	M	-	M	-	M	-
28	RMCV outlet	-	M	-	M	-	M	-
29	RO brine outlet	-	H	-	H	-	H	-
30	TIMES/VCD distillate outlet	-	E	-	E	-	E	-
31	TPTA outlet	---------	---------	---------	--not in use---------	---------	---------	---------
32	deleted							
33	deleted							
34	MF outlet	F	-	F	-	L	-	F
35	PTOC outlet	H	-	H	-	H	-	H
36	MPSA outlet	M	-	M	-	M	-	M
37	MMCV outlet	M	-	M	-	M	-	M
38	FACILITY WATER SUPPLY	C	B	A	B	A	B	C
39	Special Analysis							
40-60	Surface/Air Samples*							
45	Shower #1 nozzle	L	M	M	M	M	M	M

UAH   Proposed   Sample   Schedule   -   Individual   Analyses

PARAMETER

SampleGroup/Number	AODC	HPC	AEM	Y&M	FC	ANA	LEG	STA
A - 90	90	90	90	90	90	--	--	90
B - 144	144	--	144	--	--	--	--	--
C - 4	4	4	4	4	4	4	4	4
D - 6	6	6	6	--	--	--	--	--
E - 45	40	40	40	--	--	40	--	--
F - 24	21	21	21	--	--	21	--	--
H - 21	21	21	21	--	--	21	--	--
M - 60	60	60	60	--	--	--	--	--
L - 11	11	11	11	11	11	11	11	11
TOTAL	397	253	397	105	105	76	15	94
% Reduction[1]	-14%	-22%	-16%	-20%	-11%	-33%	0	0

[1] 15%   Overall   Reduction   in   Analyses

Hygiene Tank 1 Iodine Trends

Iodine (ppm)

Test Day

□ Column 2

Hygiene Tank 1 TOC Trends

Figure: Scatter/line plot titled "Hygiene Tank 1 TOC Trends" with x-axis labeled "test day" (0 to 40) and y-axis labeled "Total Organic Carbon (ppm)" (0 to 140). Legend: Column 2.
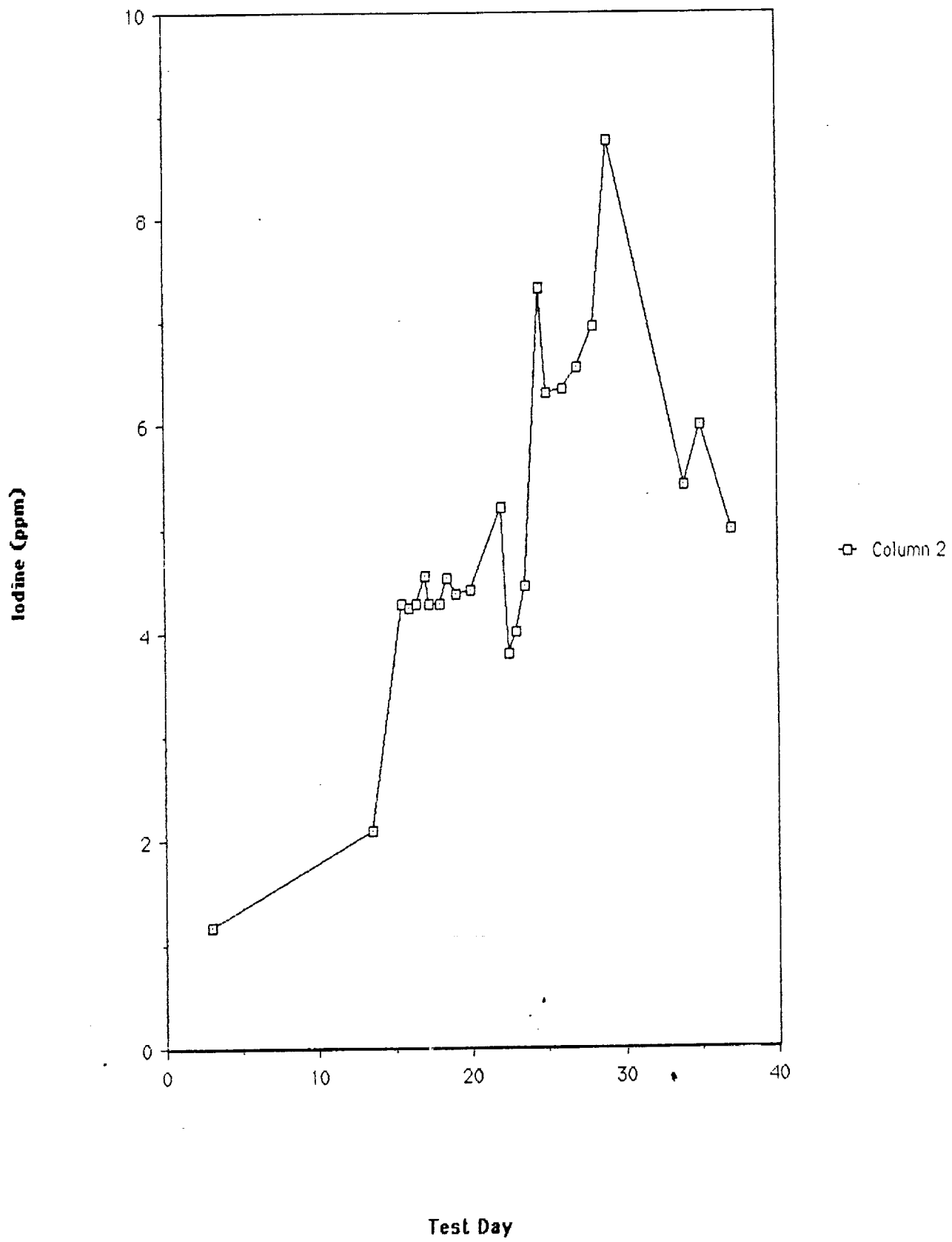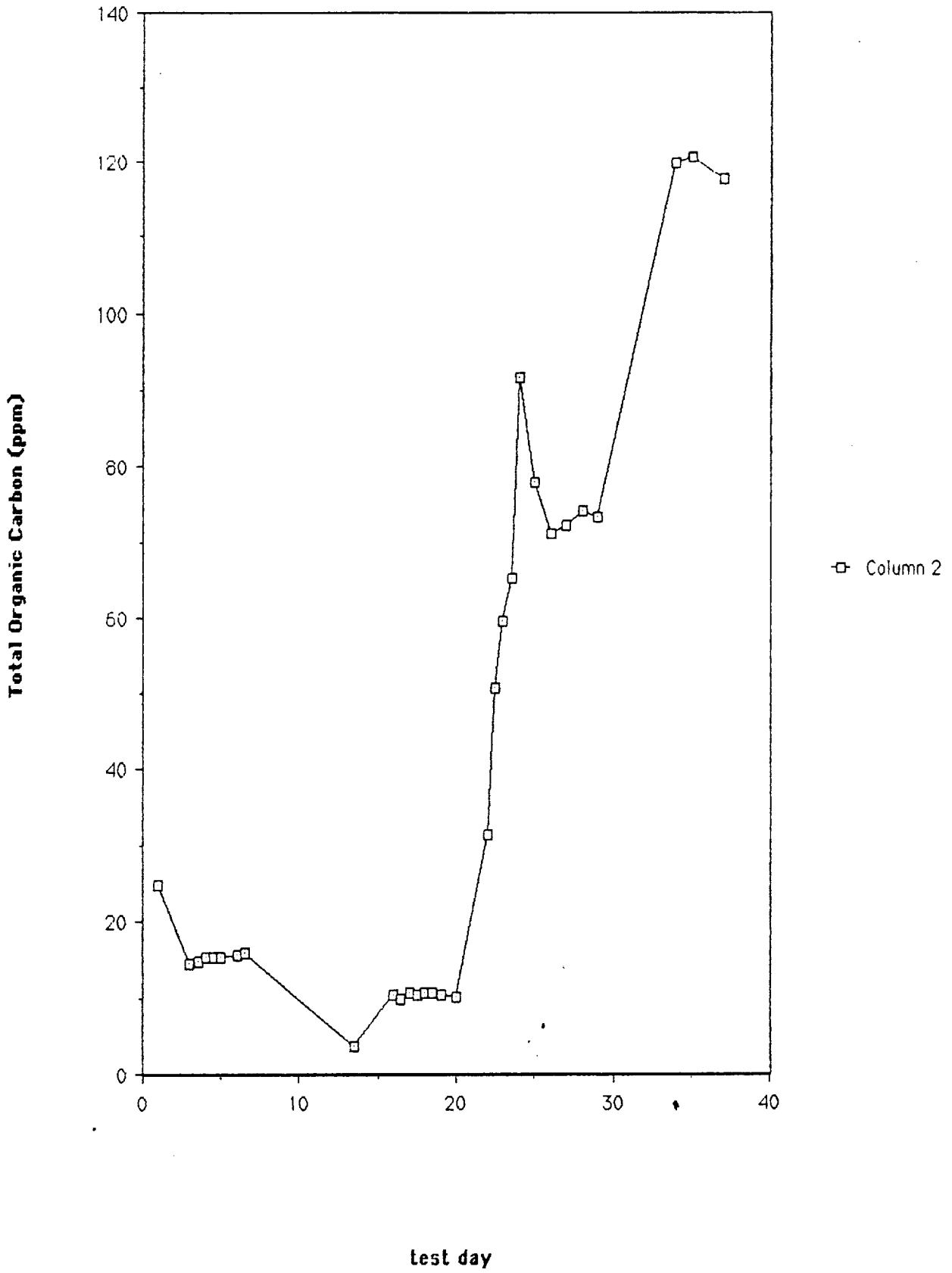
Hygiene Tank 1 pH Trends

pH

Test Day

HT1PH
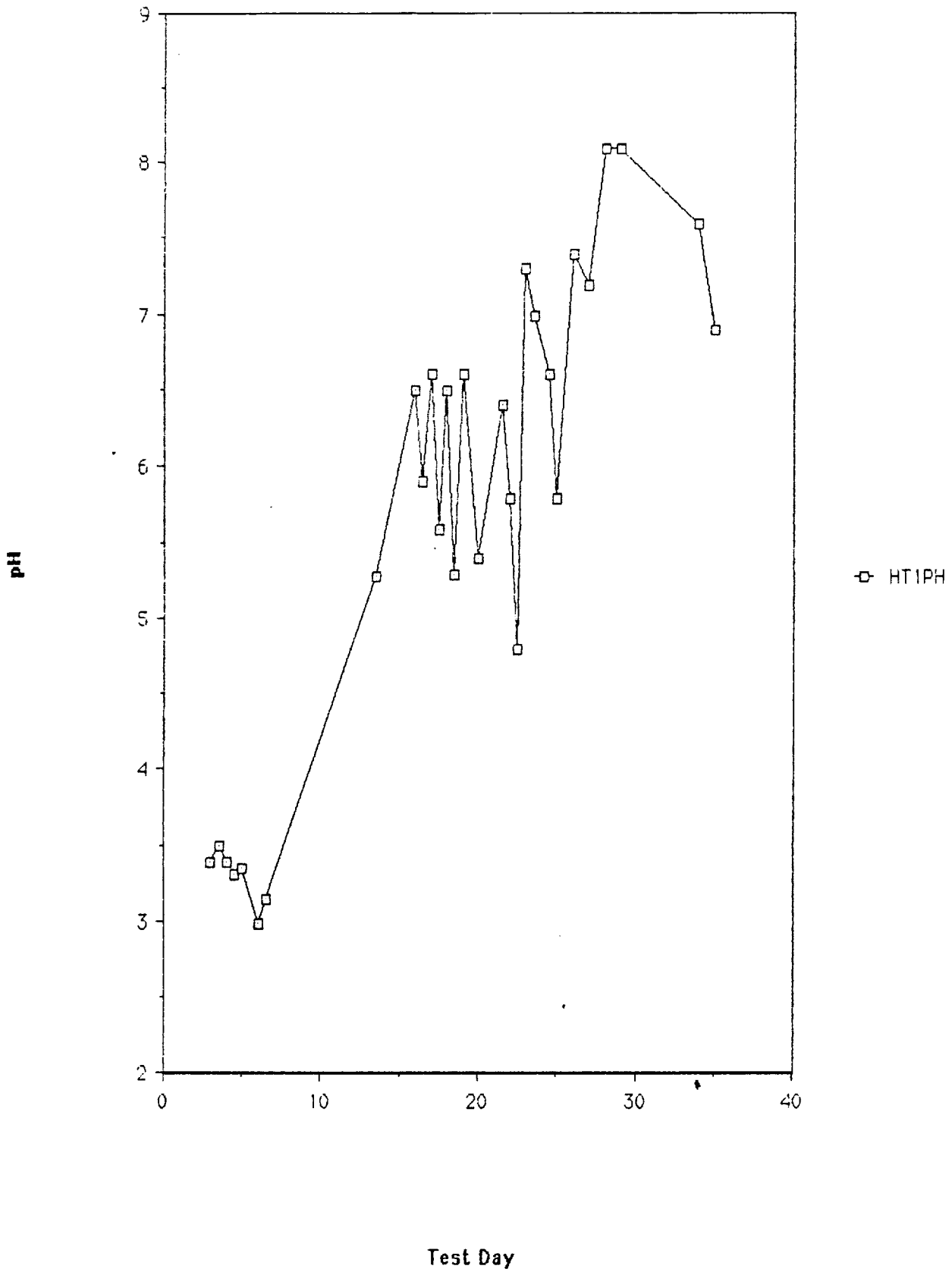
Hygiene Tank 2 Iodine Trends

Iodine (ppm) vs Test Day

Legend: Column 2
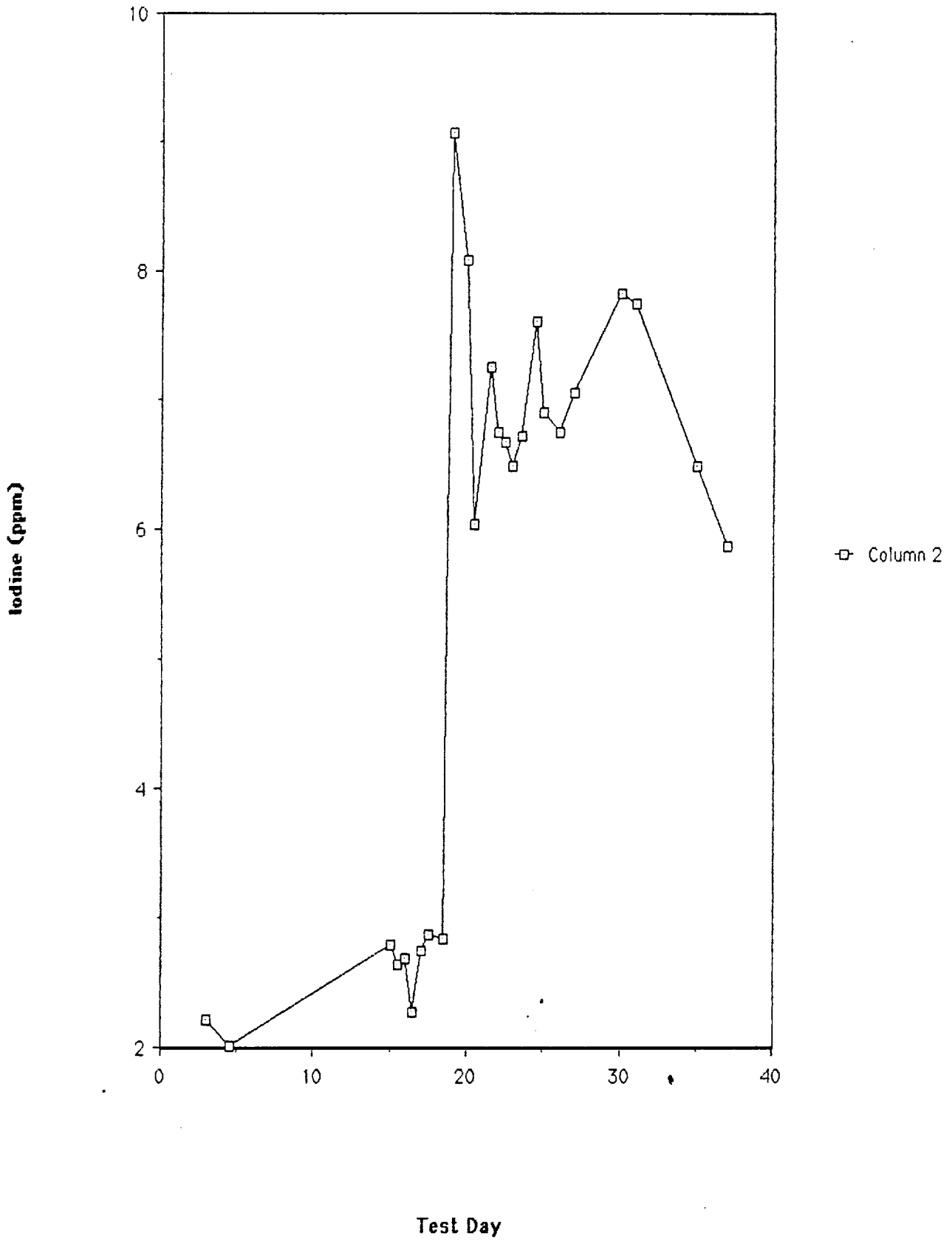
Hygiene Tank 2 TOC Trends

Plot with Y-axis labeled "Total Organic Carbon (ppm)" ranging 0 to 120, X-axis labeled "Test Day" ranging 0 to 40. Legend: Column 2.
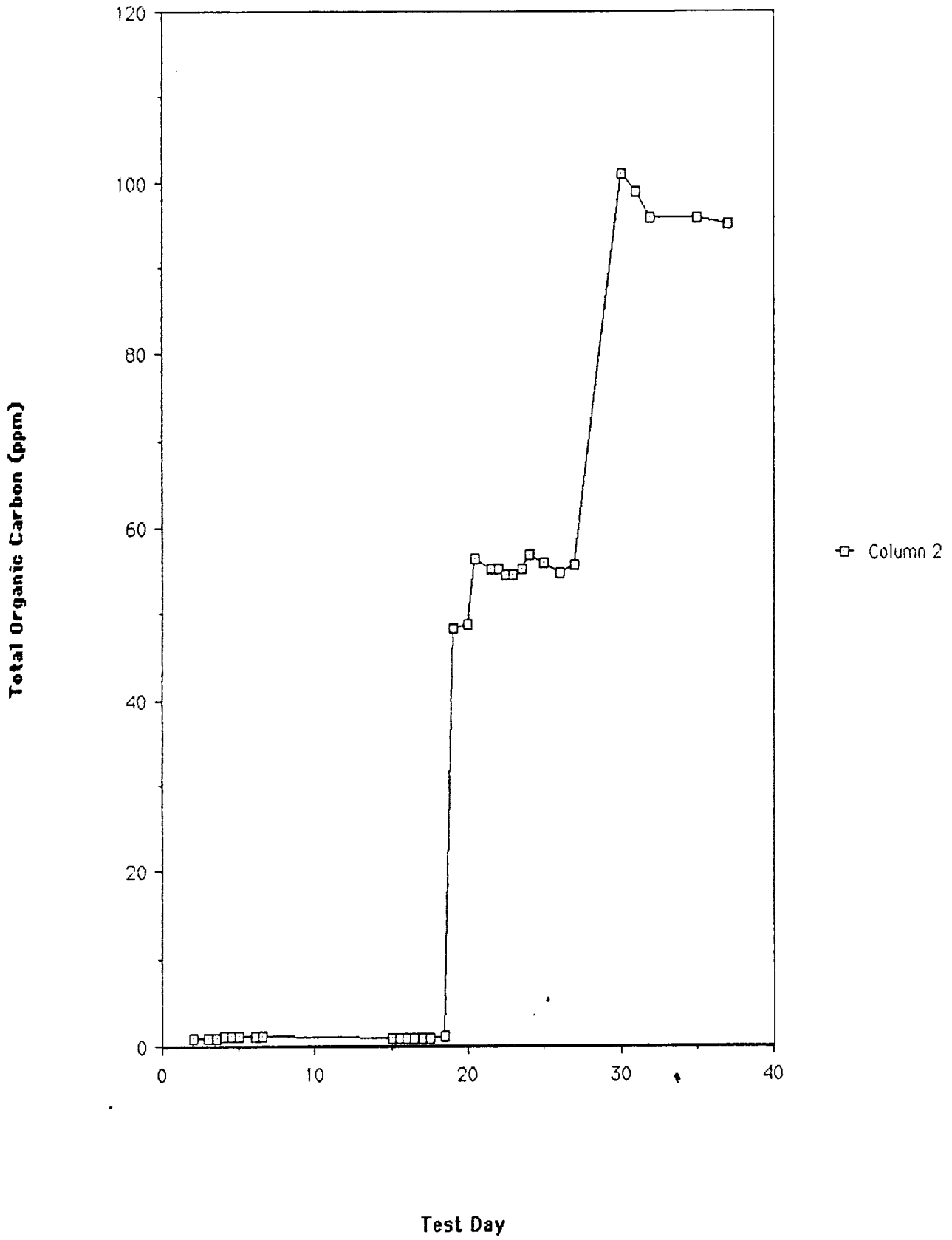
Hygiene Tank 2 pH Trends

Test Day
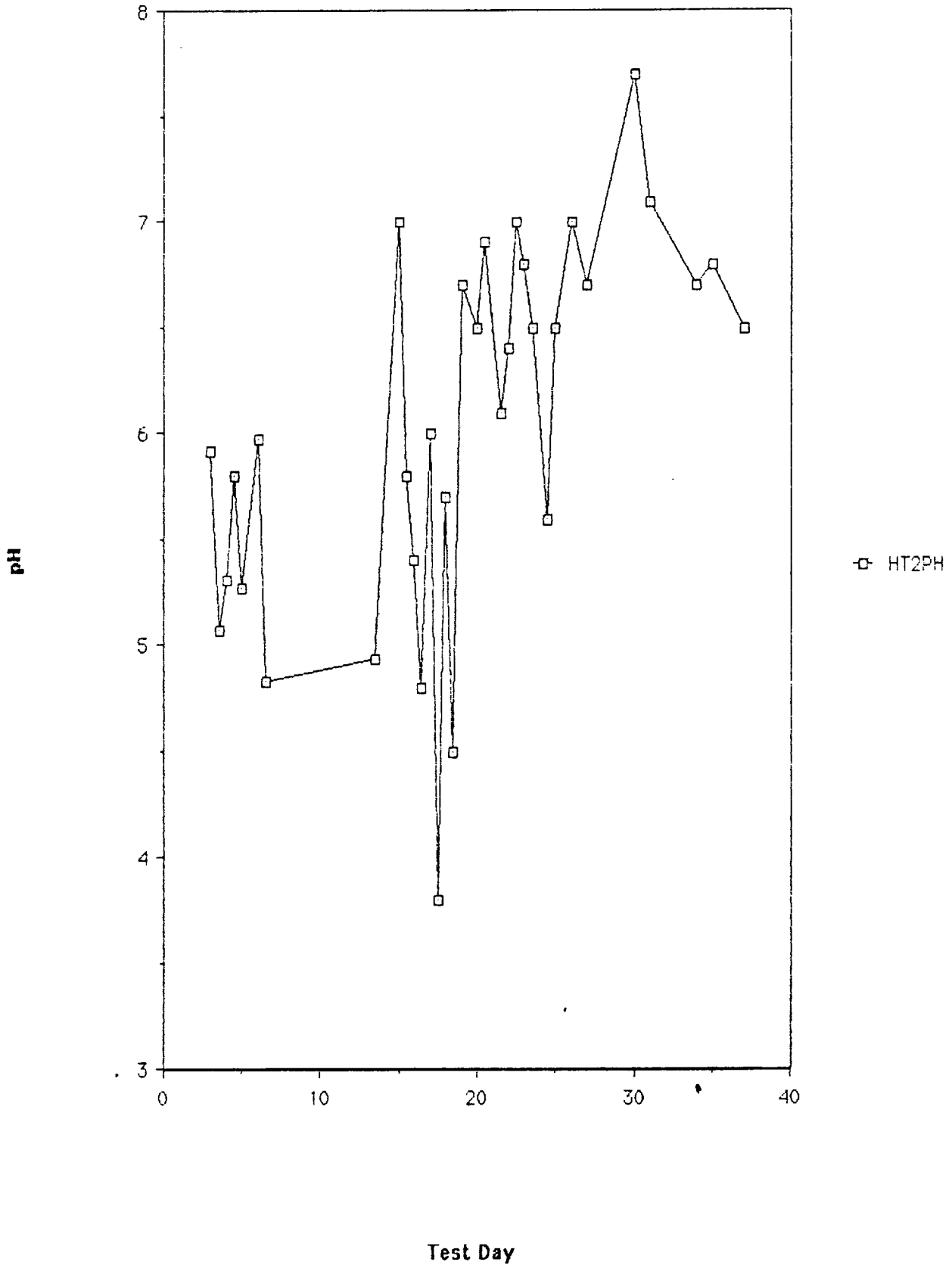
Hygiene Tank 3 Iodine Trends

Iodine (ppm)

Test Day

-□- Column 2
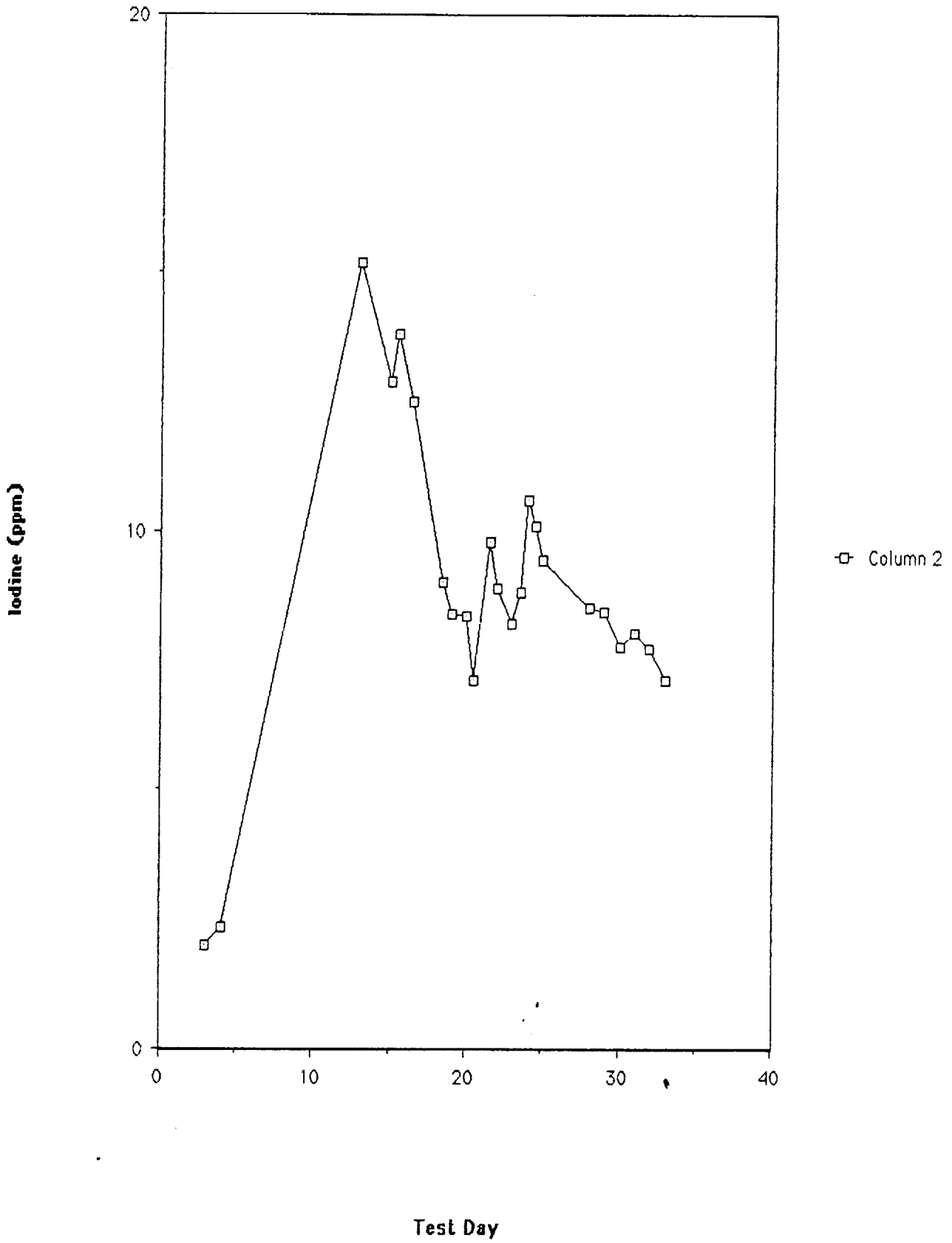
Hygiene Tank 3 TOC Trends

Total Organic Carbon (ppm) vs. Test Day

-□- Column 2

Hygiene Tank 3 pH Trends

pH (y-axis), Test Day (x-axis), legend: HT3PH
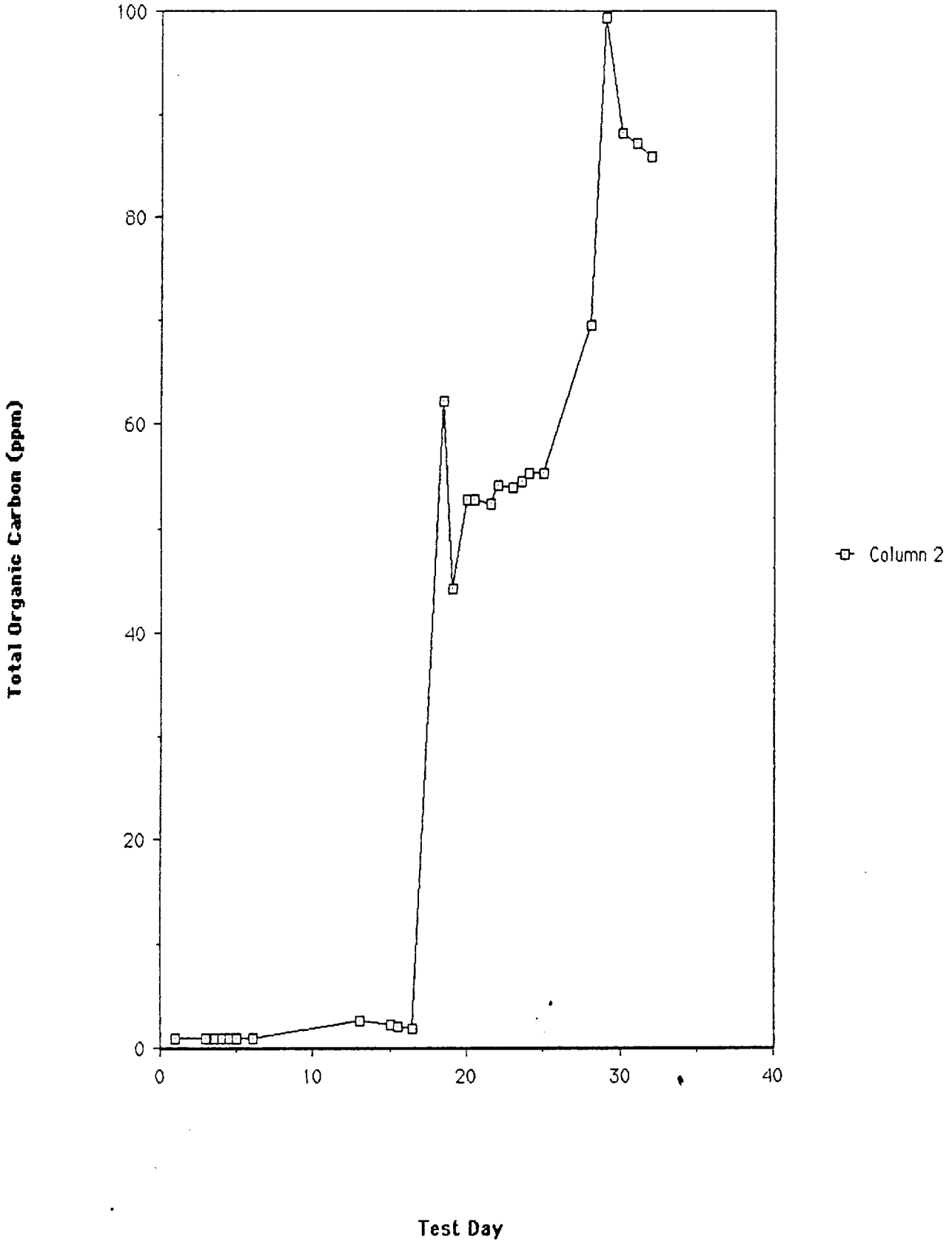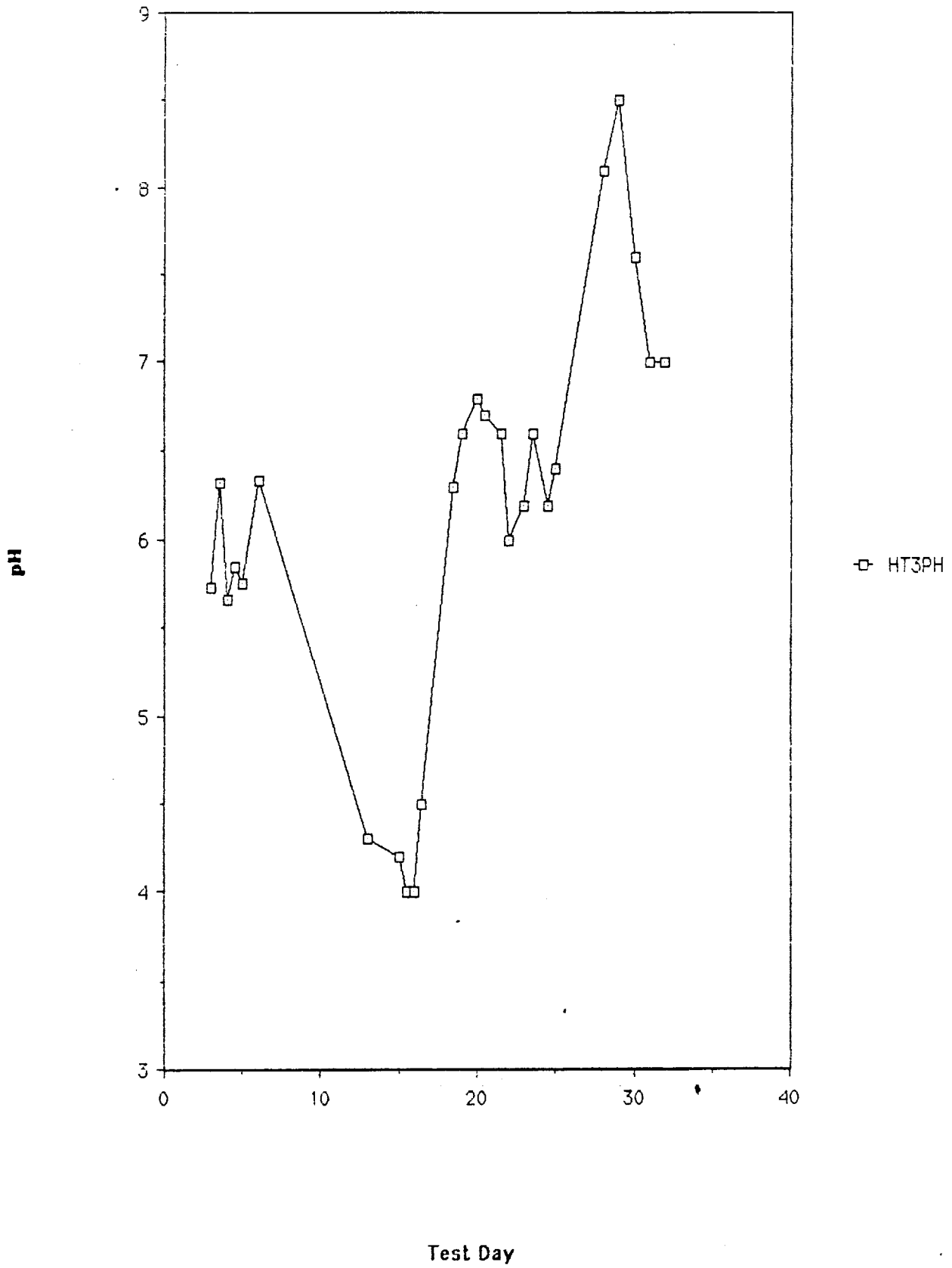
Hygiene Tank 4 Iodine Trends

Iodine (ppm) vs Test Day
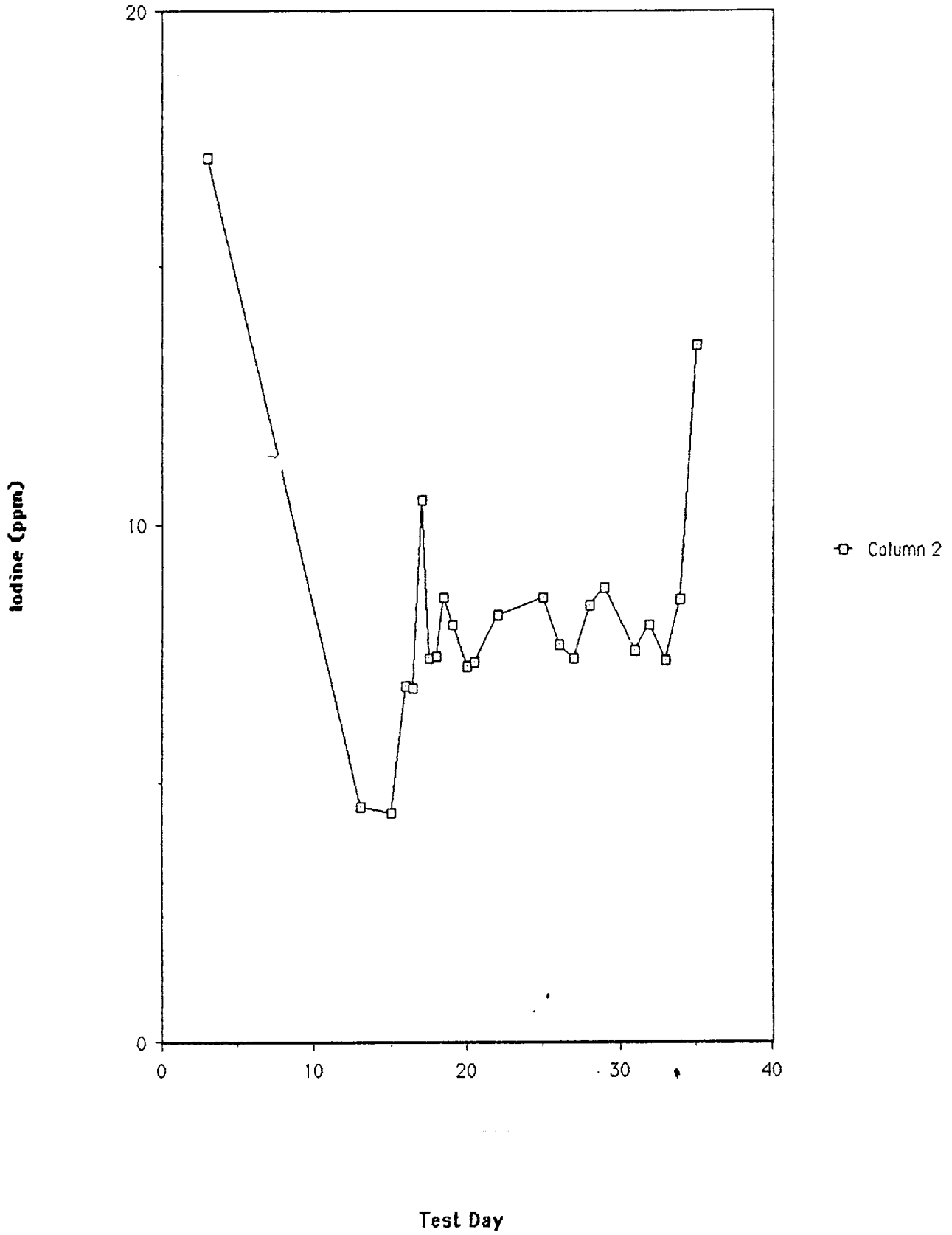
Hygiene Tank 4 TOC Trends

Total Organic Carbon (ppm)

Test Day

-□- Total Organic C
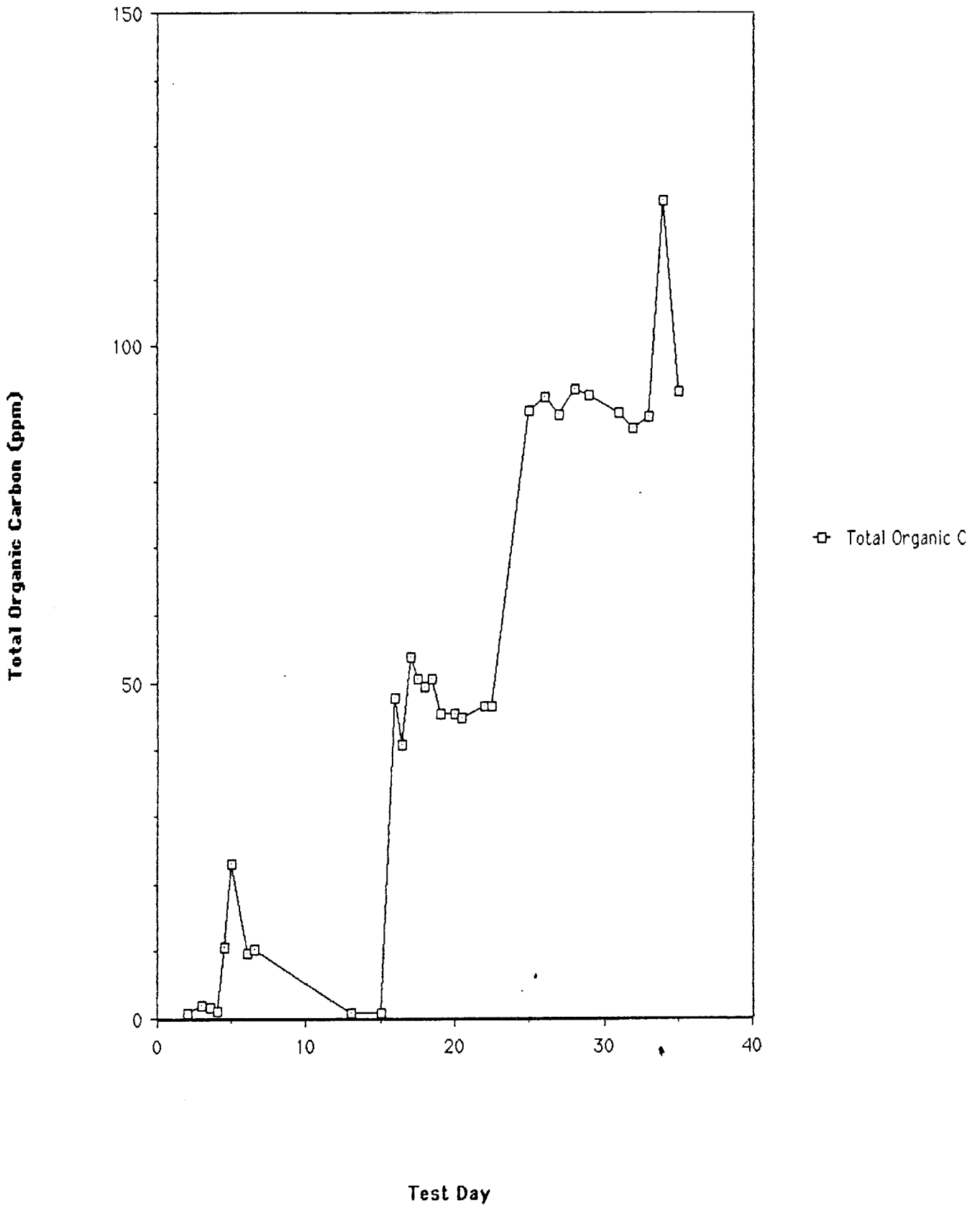
Hygiene Tank 4 pH Trends

Test Day
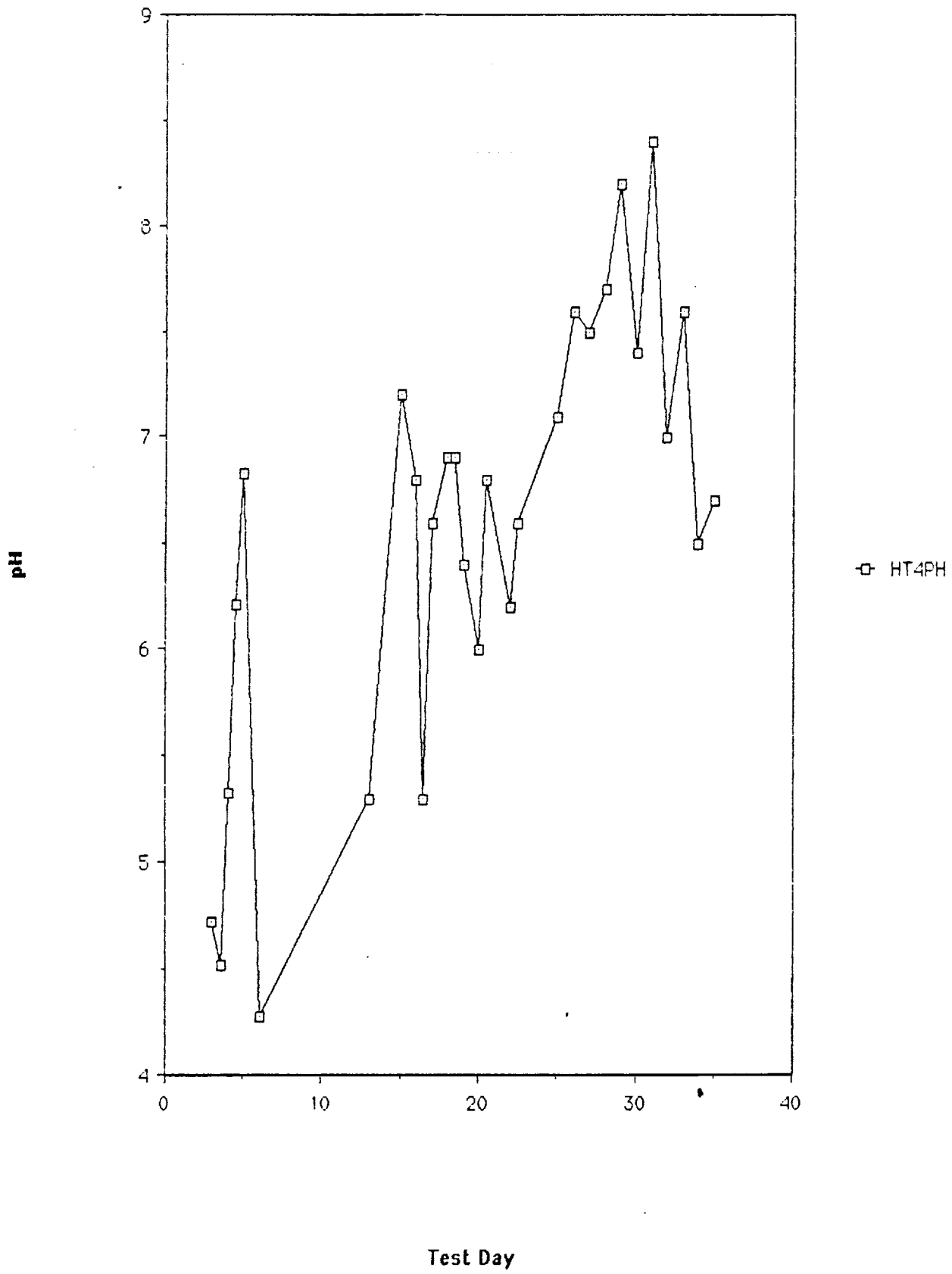
Potable Tank 1 Iodine Trends

Iodine (ppm) (y-axis)

Test Day (x-axis)

-□- Column 2
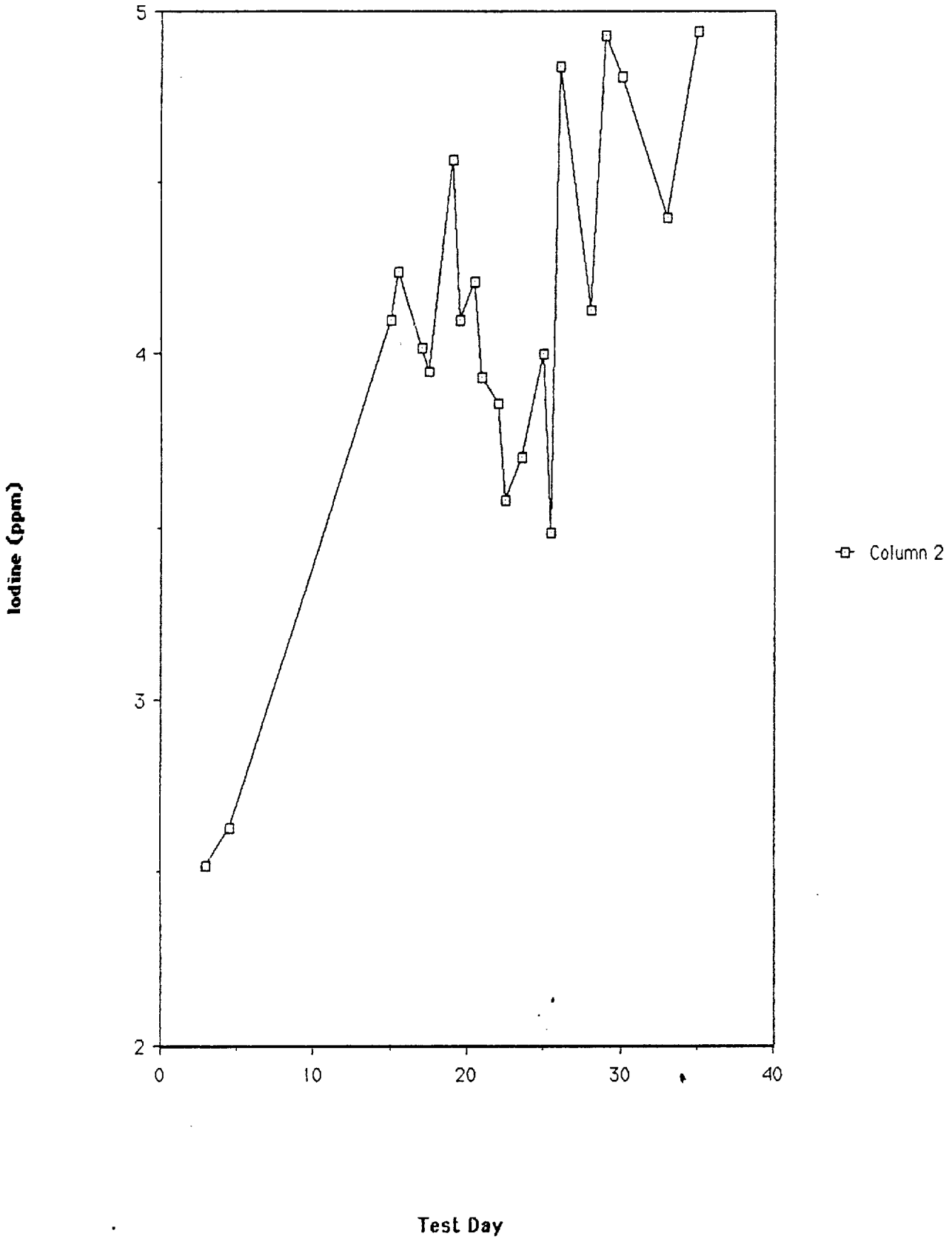
Potable Tank 1 TOC Trends

Total Organic Carbon (ppm)

Test Day

-□- Column 2
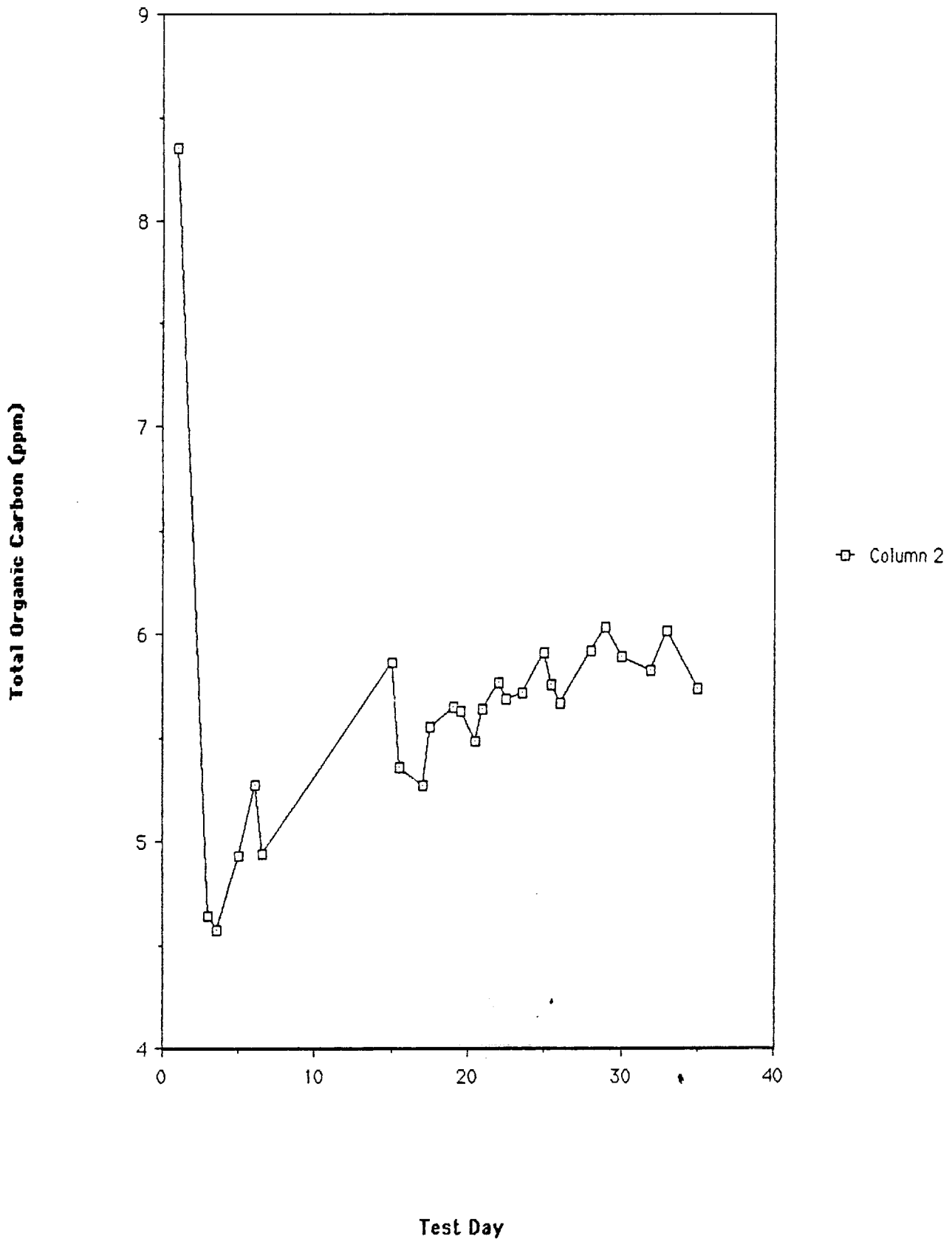
Potable Tank 1 pH Trends

Test Day
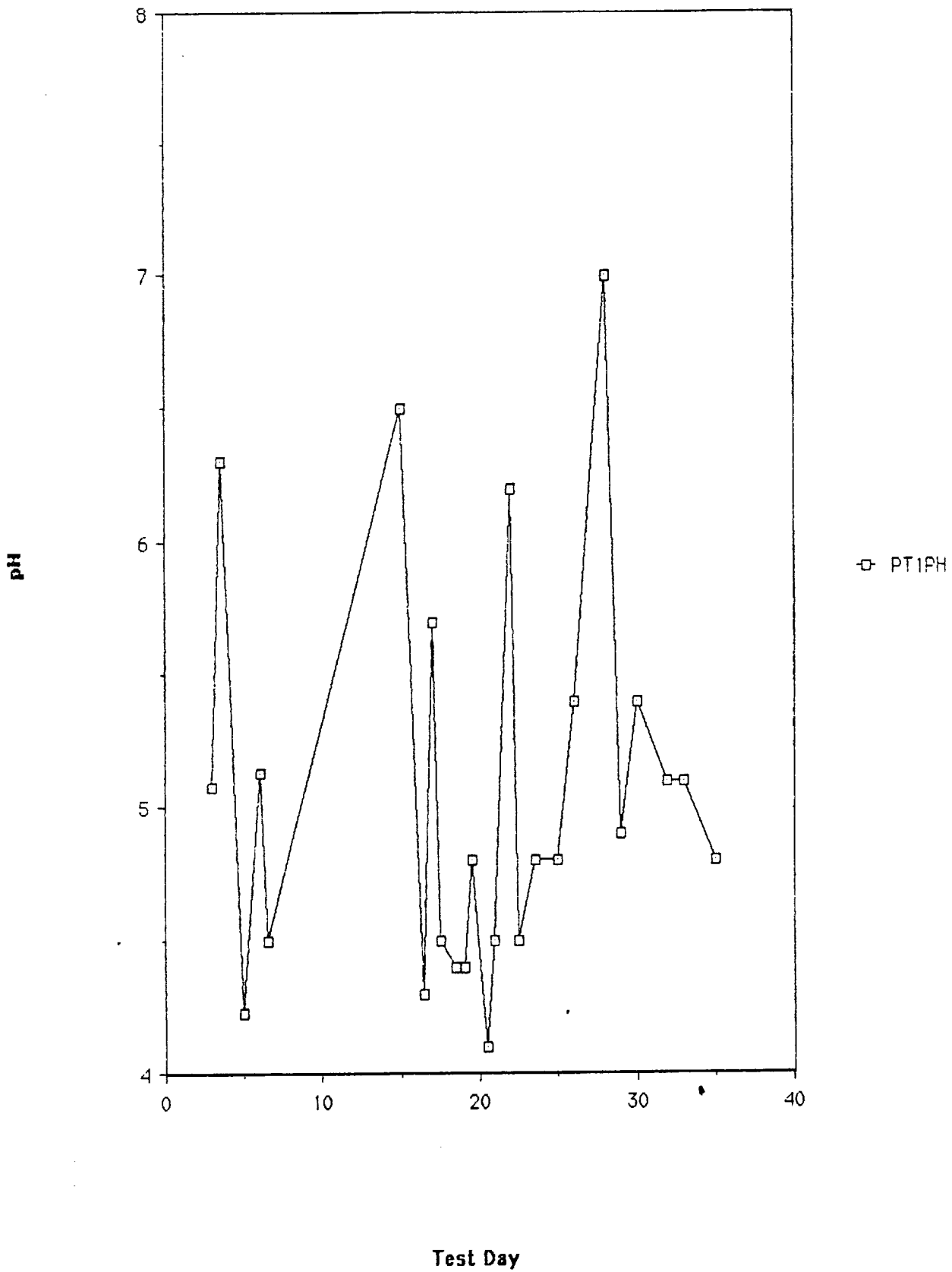
Potable Tank 2 Iodine Trends

Iodine (ppm) vs. Test Day

-□- Column 2

Test Day
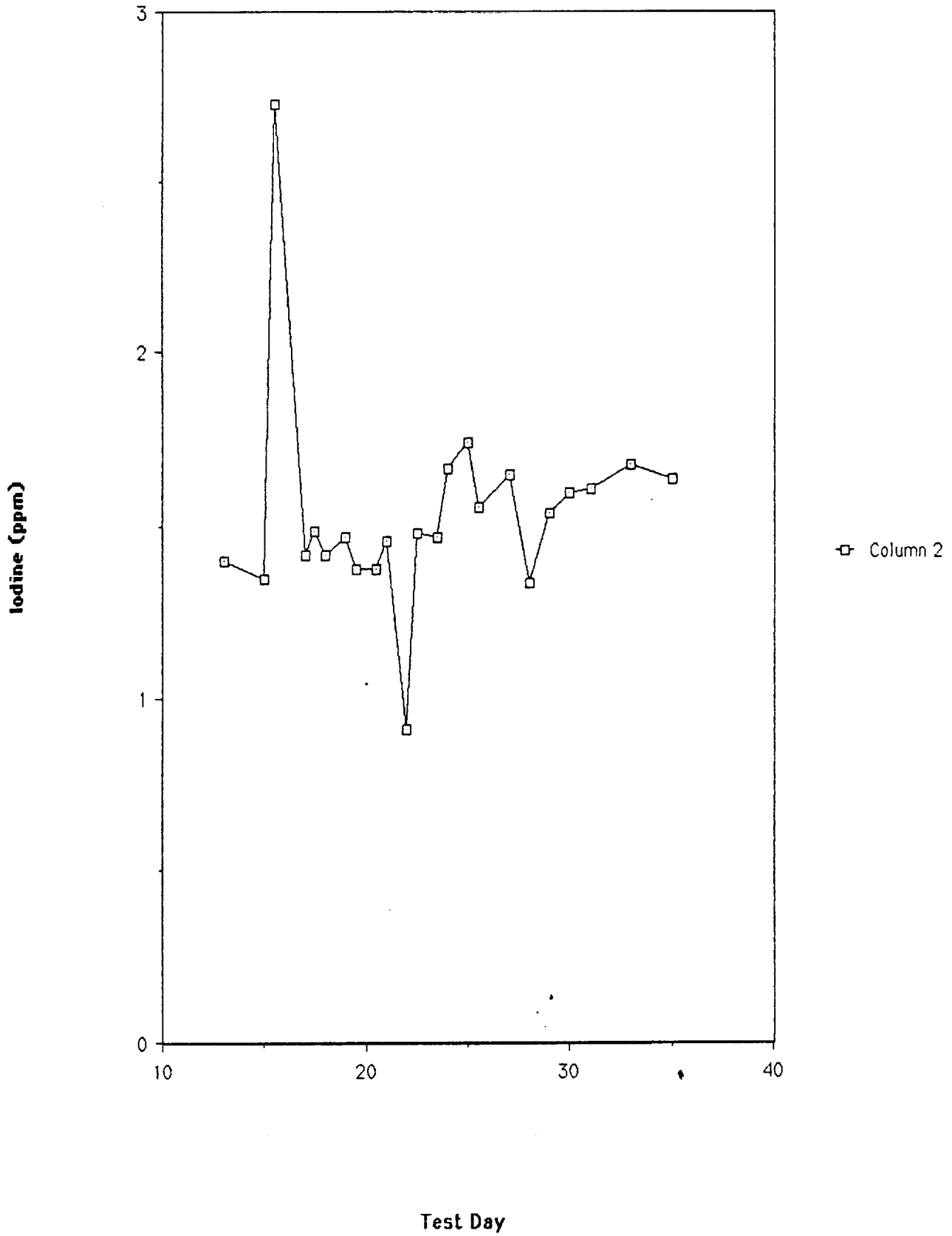
Potable Tank 2 TOC Trends

Total Organic Carbon (ppm)

Test Day

-□- Column 2
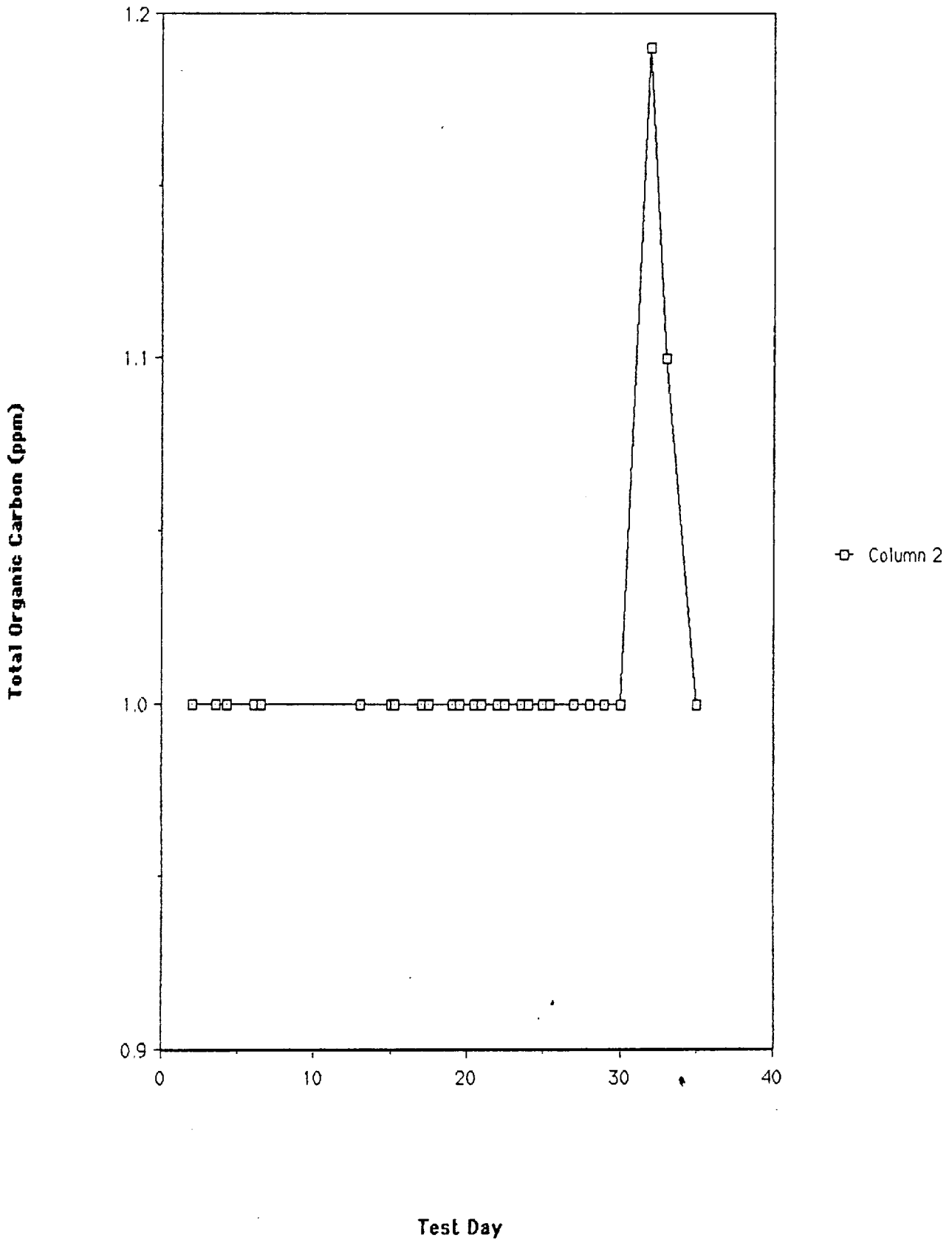
Potable Tank 2 pH Trends

pH (y-axis)

Test Day (x-axis)

Legend: -□- PT2PH
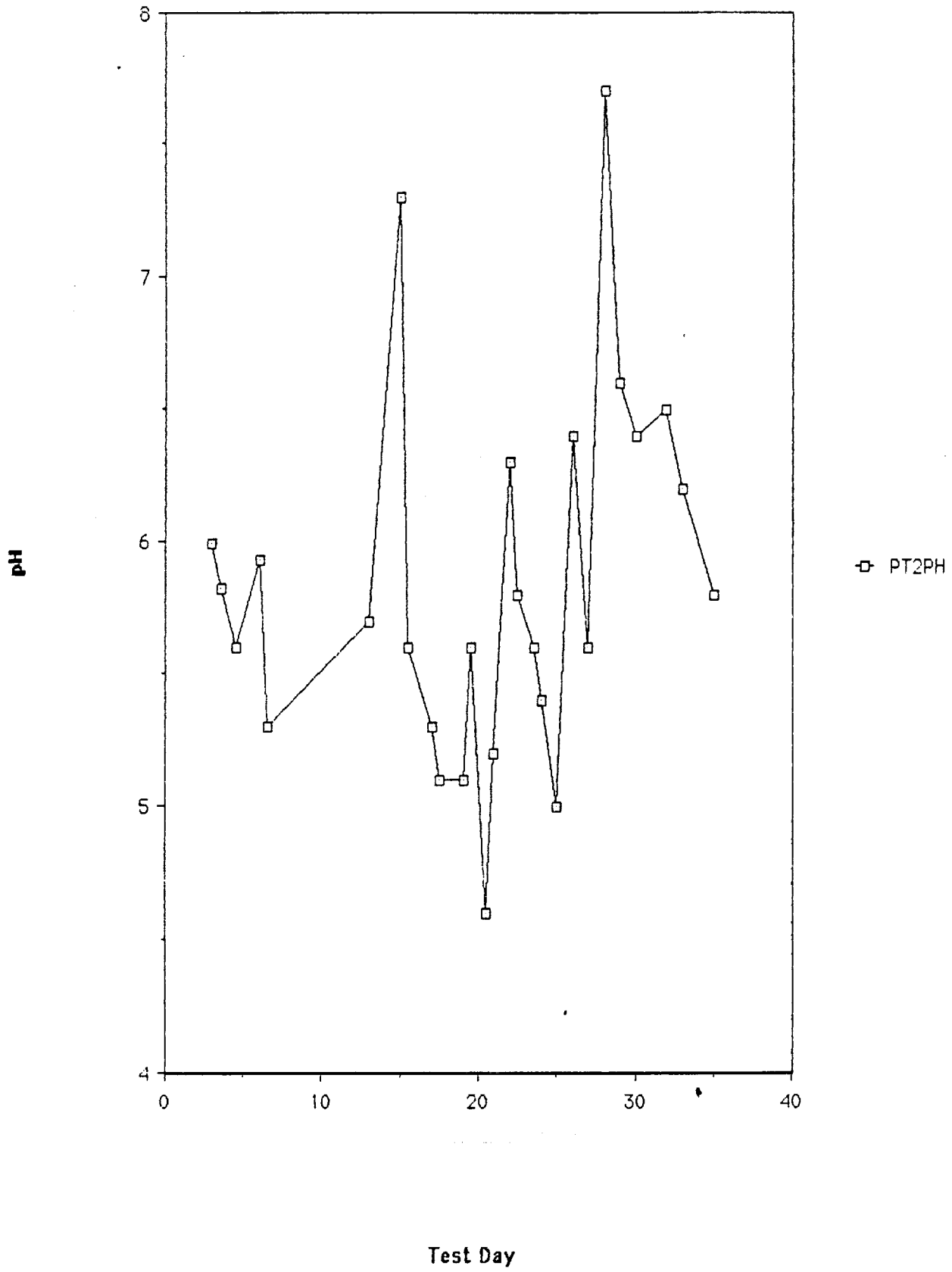
Potable Tank 3 Iodine Trends

Iodine (ppm) vs Test Day

Legend: -□- Column 2
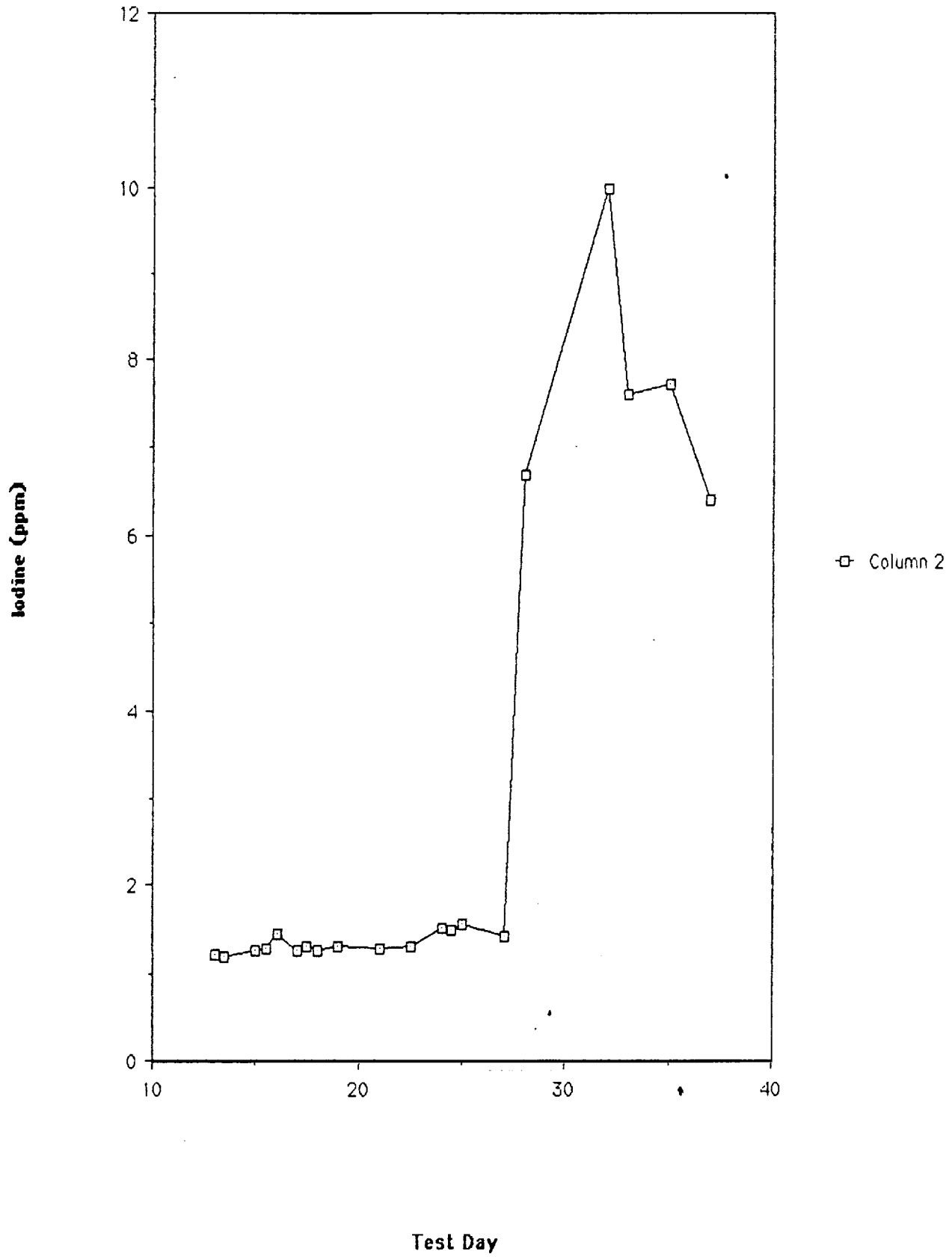
Potable Tank 3 TOC Trends

Total Organic Carbon (ppm)

Test Day

-□- Column 2
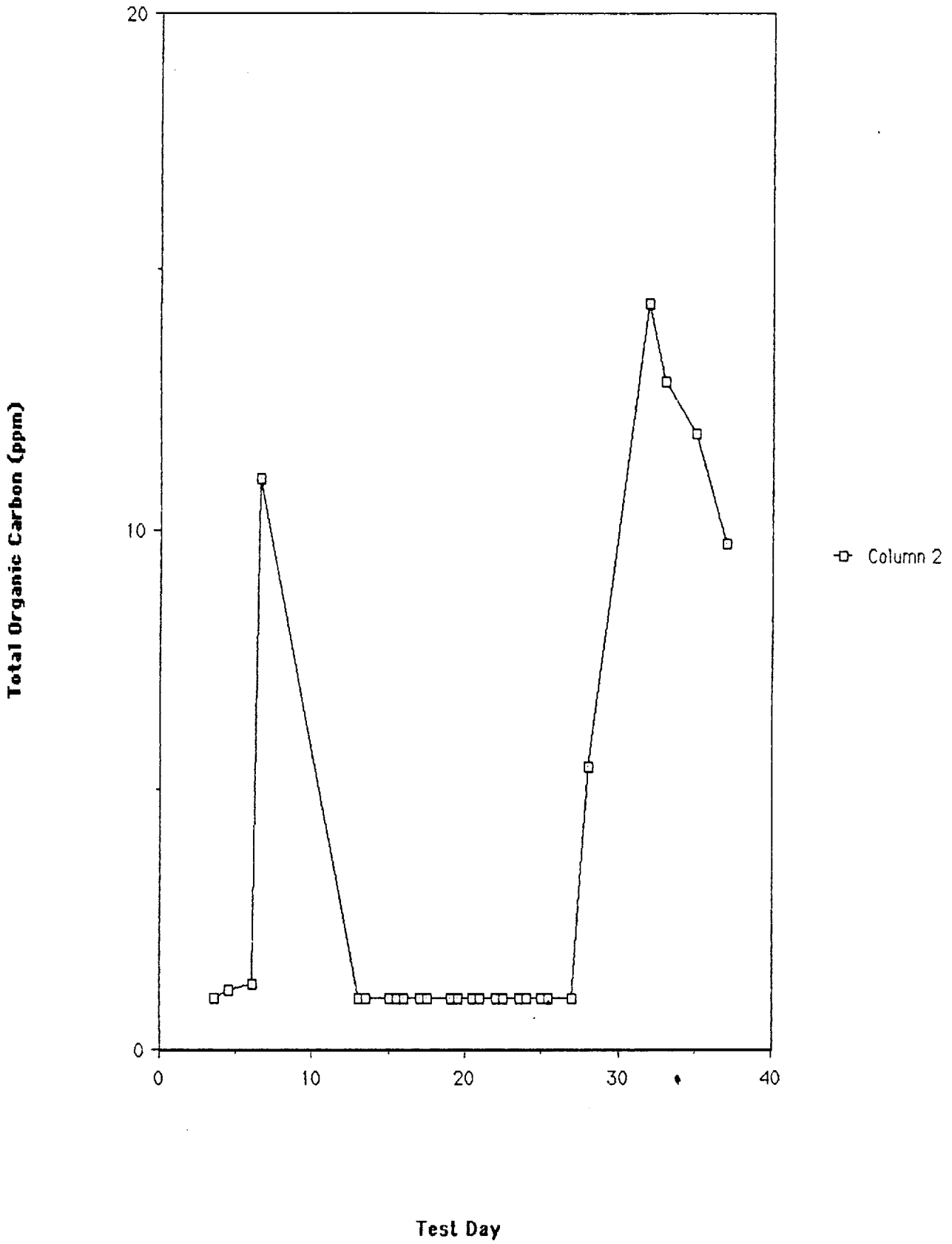
Potable Tank 3 pH Trends

pH vs Test Day

PT3PH
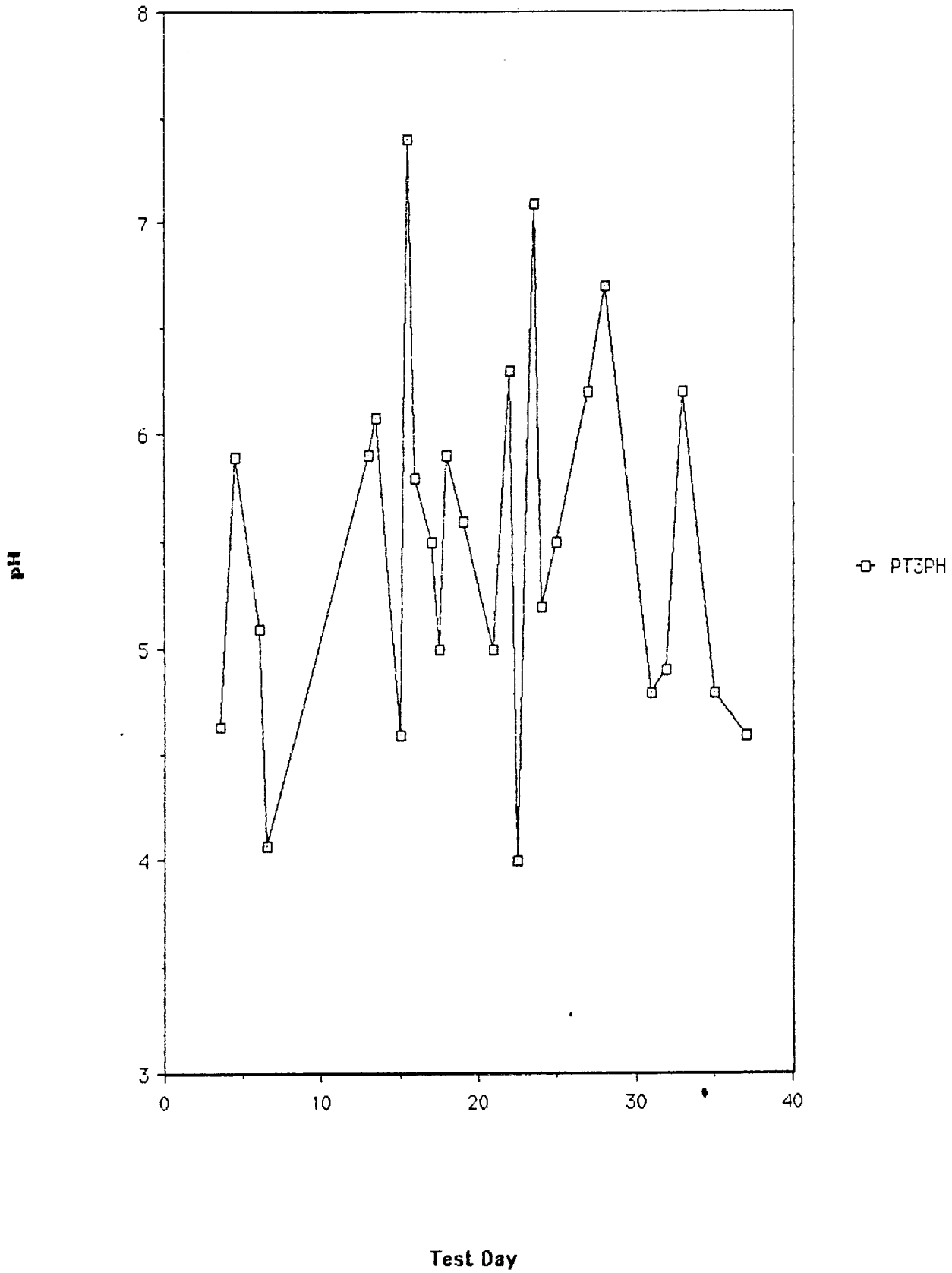
Potable Tank 4 Iodine Trends

Legend: □ Column 2

Y-axis: Iodine (ppm)

X-axis: Test Day
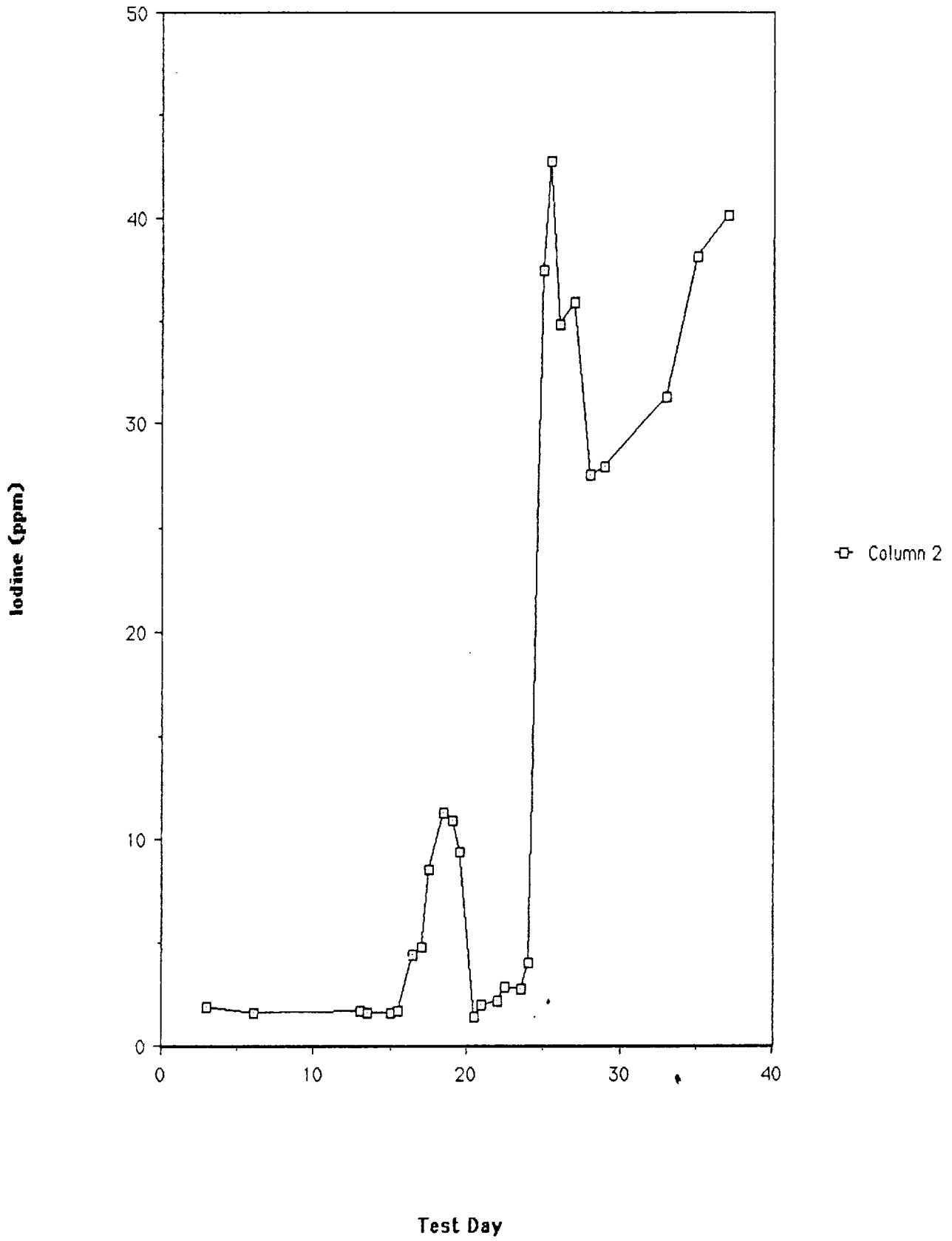
Potable Tank 4 TOC Trends

Total Organic Carbon

Test Day

-□- Column 2
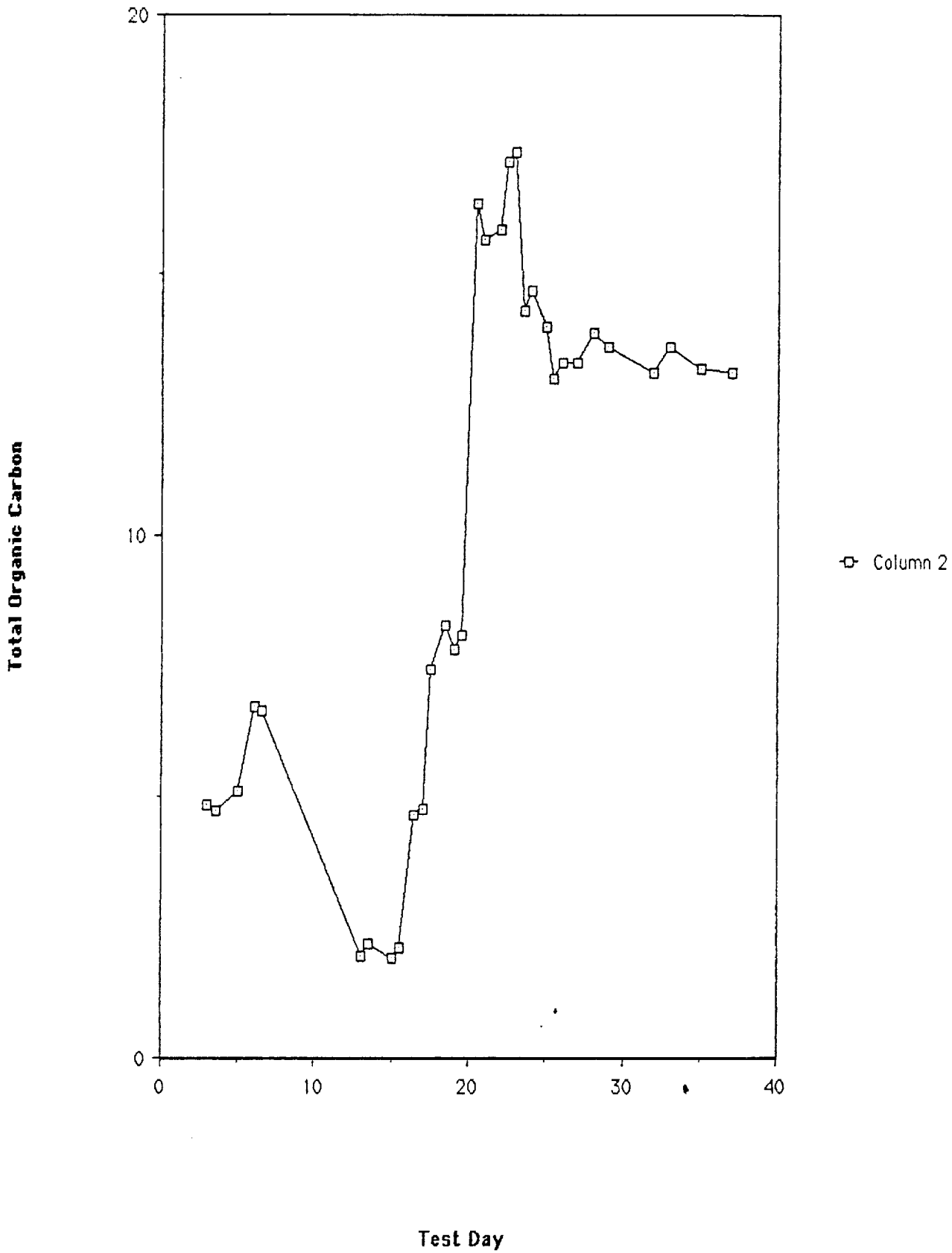
Humidity Condensate TOC Trends

pH

Test Day

-□- TNK24TOC
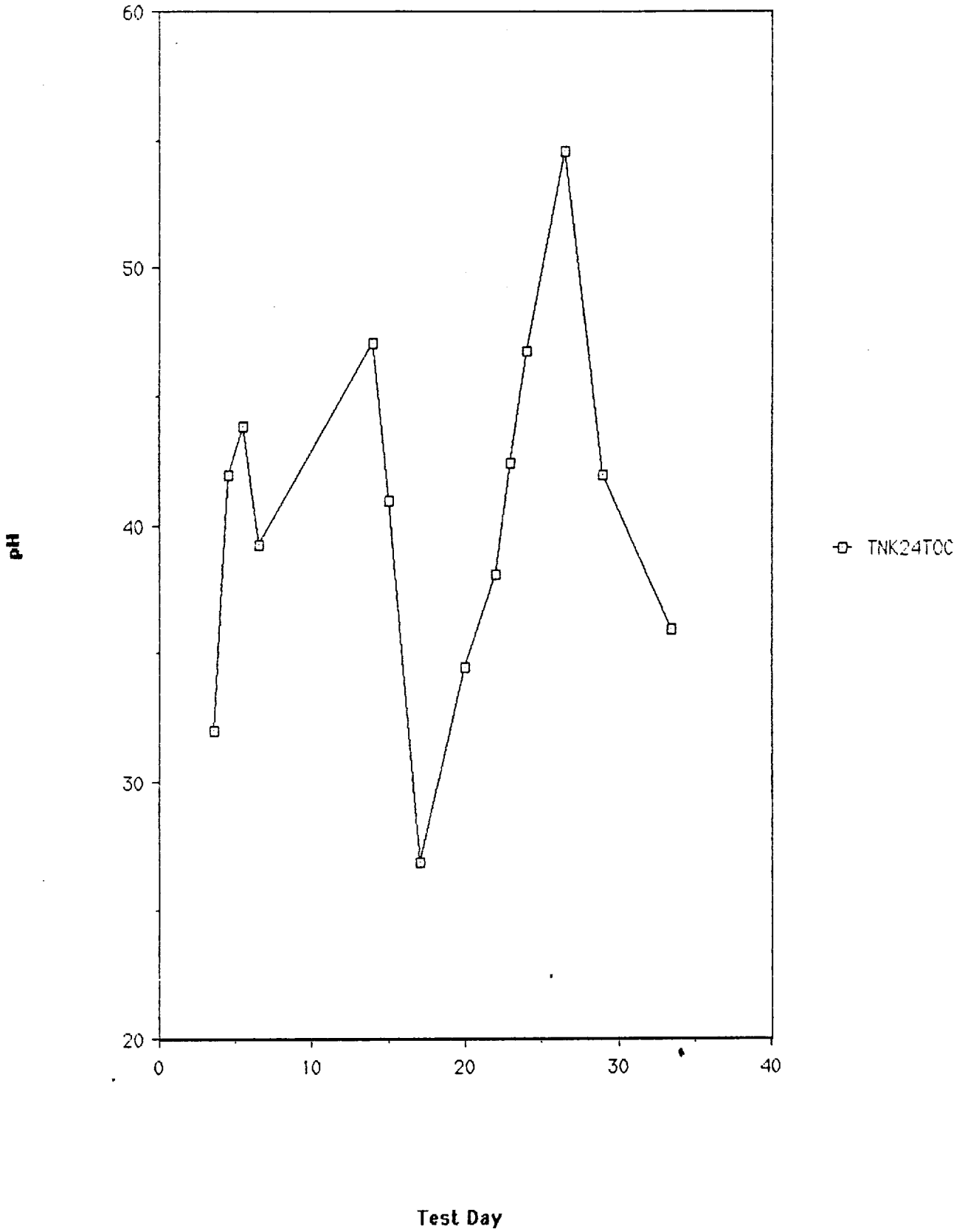
Potable Tank 4 pH Trends

Test Day
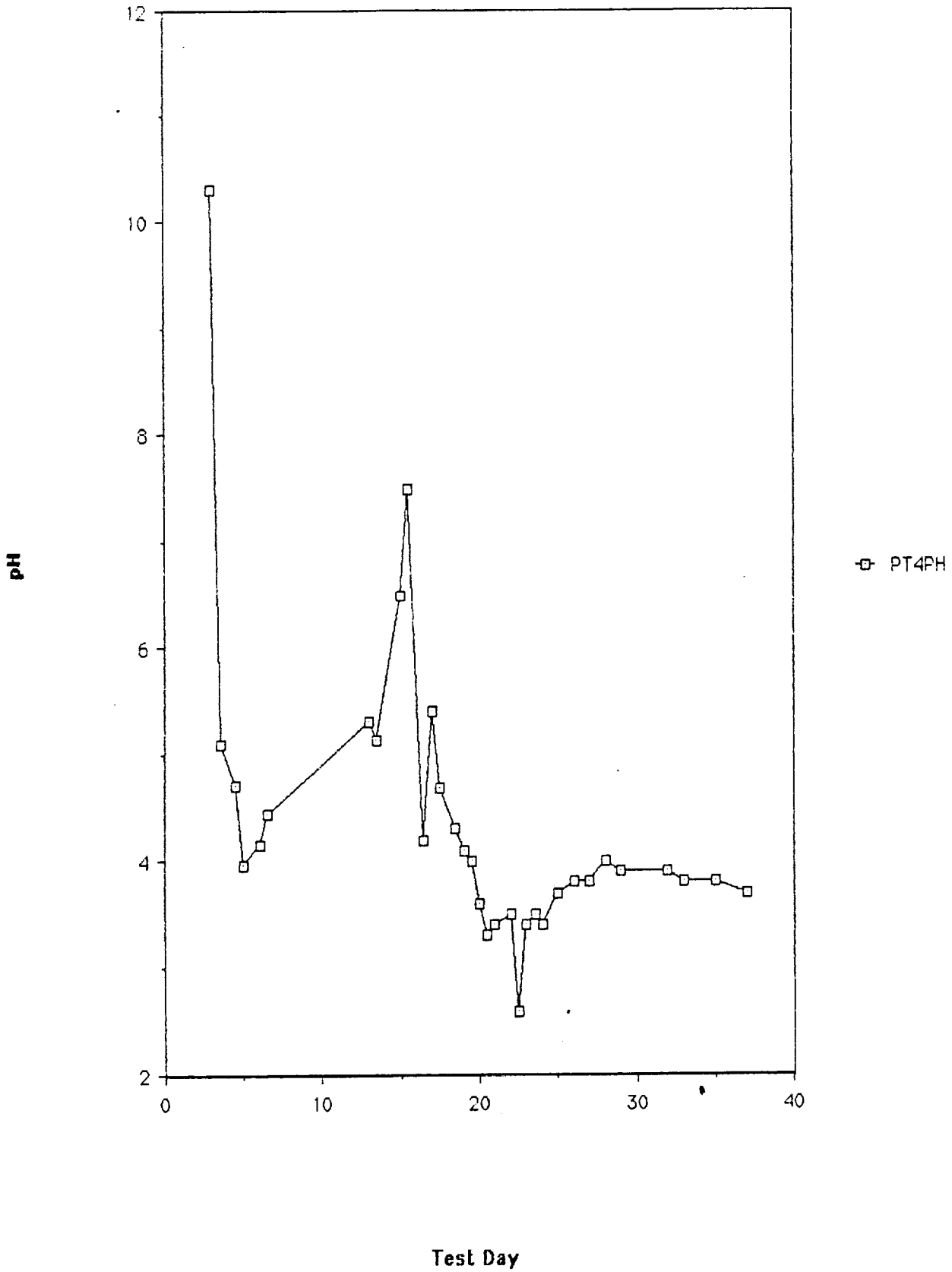
Humidity Condensate pH Trends

pH (y-axis) vs Test Day (x-axis). Legend: □ TNK24PH
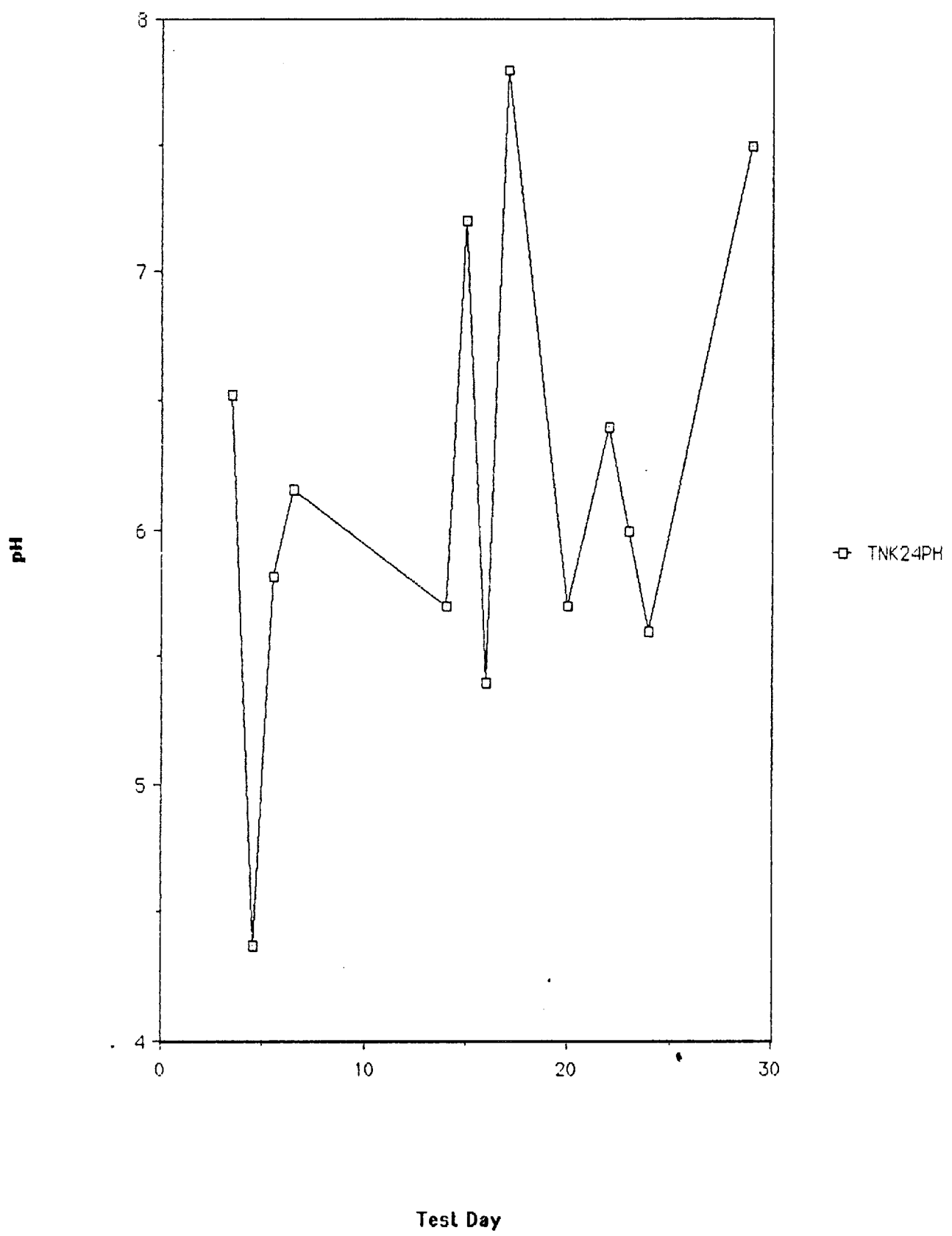
APPENDIX F

RECOMMENDATIONS REGARDING MICROBIOLOGICAL CONTAMINATION CONTROL

INTRODUCTION

There is little doubt on space missions of long duration, microorganisms will play a vital positive role as a component of the life support system, recycling minerals, gases; and in the manufacture of drugs, foods and materials. There are, however, two basic negative aspects of microorganisms which necessitate their control or management: crew health maintenance and damage to hardware. The first concern is of course immediate, threatening the safety and health of the crew. The second aspect threatens the crew indirectly through deprecation of system performance as hardware components are affected (e.g. corrosion, fouling). In actuality, hardware and health are not neatly compartmentalized as certain organisms may assume either role, or may change roles.

Types, locations and numbers of microorganisms will require control, or more broadly, management on-board Space Station Freedom. This document will review current technologies for microbial control as well as a general background of principles related to bacterial growth and death. For the most part this document will concentrate on control of microorganisms in water systems. Treatments effective for surface and atmosphere applications will also be discussed as these environments are often difficult to isolate from the water system.

Microbiological control or management does not necessarily imply total elimination, or sterilization. The term "sterilized" should be used with great care, as it means the process leading to total absence of life. This indeed is difficult to achieve

and very hard to evaluate statistically. It is much more realistic to establish a specification and the probability associated with obtaining that number.

The final section of this document addresses factors to prevent microbial growth. A knowledge of how and where microorganisms may grow, should enable engineers to select materials and designs which do not promote growth. This may eliminate (or minimize) energy, health and safety requirements of treatment alternatives.

Several questions follow which should be addressed before instituting a microbial control plan.

1.Is it necessary that all organisms be eliminated (sterilization)? If so, what is the desired probability?

2.If reduction is a sufficient objective, what is the resident population in the raw water and what is the degree of reduction required?

3.Do all types of organisms need to be controlled? Or is the specification of some group sufficient (e.g. pathogens, anaerobes, fungi, Gram negative bacteria)?

4.Is death necessary, or will suppression of growth suffice?

5.What material is to be treated and will the agent selected in any way harm the material or create unwanted byproducts?

6.What is the toxicity to humans of the agent chosen at the concentration and time for treatment?

DEFINITIONS

Chemical or physical agents which kill bacteria are referred to as bactericides, or biocides. Bacteriostatic agents are compounds which suppress the growth of bacteria. Bacteriostasis is not equivalent to death. Table 1 provides definitions of common-

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ly used terms regarding microbial control which will be helpful
as a precursor to this review.

TREATMENT EFFICACY STUDIES

After microbial requirements/specifications are determined
for surfaces, water and atmosphere; agents and stratagems to
achieve those goals may be selected. This involves designing and
conducting experiments to establish the effectiveness of various
agents, or evaluation of data and publications. Limitations of
testing control must be understood to select the optimum treat-
ment regime.

The effectiveness of agents on the survival of bacteria is
determined by carefully controlled efficacy studies. These
studies involve challenging an agent with bacteria to optimize
treatment for the application desired. Efficacy studies may be
conducted on pure cultures in the laboratory, or empirically
within the system. Factors directly related to control include:
agent concentration or intensity; time of exposure; presence of
exogenous material; type and initial number of organisms. Unless
there is a strong reason to adhere to standard test procedures,
in vitro studies should mimic the environment (i.e. temperature
and pH) of the application. For any study, both the initial and
final number of organisms should be statistically defined.
Hence, effective experimental treatment should not result in the
total elimination of microorganisms.

Both laboratory (in vitro) and empirical (in situ) studies
have advantages and disadvantages. Efficacy studies using pure
cultures may not be extrapolated to another type of microorganism

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TABLE 1 Definitions of Commonly Used Terms Regarding Microbial Control

Sterilization	-Any process that completely destroys all forms of microbial life.
Disinfection	-Any process designed to remove or destroy pathogenic micro-organisms. Disinfection reduces the number of microorganisms in the material but does not eliminate all.
Pasteurization	-A process designed to eliminate unwanted microorganisms in a material by heating the material for a brief period of time. Pasteurization does not sterilize.
Decontamination	-A process in which both living pathogens and their toxic products are removed from a material.
Sanitizing	-The systematic cleansing of inanimate objects to reduce the microbial population to a safe level.
Antiseptic	-Term commonly used for topical agents, may be either bacteriocidal or bacteriostatic.
-cidal	-A suffix referring to a treatment that causes irreversible cellular changes resulting in cell death.
-static	-A suffix referring to treatments that halt microbial growth as long as the inhibitory substance or state is present.

or another species. Factors such as the age of the culture and physiological condition of the test organism may significantly impact the results. Evaluations of biocide efficacy in situ would entail determining the resident viable (live) microbial count in the "raw" water. It is often very difficult to ascertain the number of living cells.

If killing microorganisms is a control objective, it is also critical to use techniques which encourage the recovery of damaged/stressed organisms, as death is defined as irreversible. Failure to do so will result in false positive values, as many chemicals or processes which appear to kill microorganisms only prevent microbial growth. These compounds are bacteriostatic, not bacteriocidal. Once these chemicals or processes are eliminated, or removed, microorganisms may resume growth and multiplication.   Biofilms. Where water systems are involved, biofilms may develop. These biofilms may induce corrosion of metals or degradation of other system materials. Biofilms may harbor bacteria capable of producing disease which can contaminate the system water as they have a tendency to slough cells. If their mass becomes extensive, they may reduce flow. This is termed biofouling.

Biofilms have many implications in treatment regimes. Organisms may shed from these films contaminating the system water. Any microbicide, or microbiostatic agent, will be much less effective to organisms within the biofilm as this community offers protection to its members (Costerton, et. al., 1981).

As previously discussed, sterilization is defined as the

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destruction or removal of **ALL** microorganisms present. In many instances, it is impossible to verify that a procedure has resulted in _bona_ _fide_ sterilization. In those instances where sterilization is warranted, one can rely on certain characteristics of microbial death and apply methods which will result in a very low probability of any microorganism surviving the treatment. In order to understand this concept, a brief discussion of the kinetics of microbial death is required.

Kinetics _of_ _death._ Microorganisms die as they grow, exponentially. If a given treatment results in a one log (90%) decrease in viable cell number in ten minutes, doubling the treatment time will result in a decrease in viable cells in the population by 99%. This is referred to as the decimal reduction, or, D-value. Microbiologists have defined the D-value as the time required for a treatment to kill 90% of the organisms in the population (i.e., a one log decrease in viable numbers). D-values can be determined empirically for any treatment in question and for any organism. Once the D-value is known, the time required to lower the microbial population to acceptable levels can be calculated. It should be emphasized that a D-value determined for one species of bacteria can not necessarily be applied to a different species. Figure 1 illustrates a typical death curve and calculation of the D-value.

An excellent analogy of exponential death has been developed (Pelczar, et. al., 1986). In this analogy, each cell represents a target, and a large number of bullets (i.e., units of a physical or chemical agent) are being sprayed randomly with a machine gun. The probability of hitting a target is proportional to the

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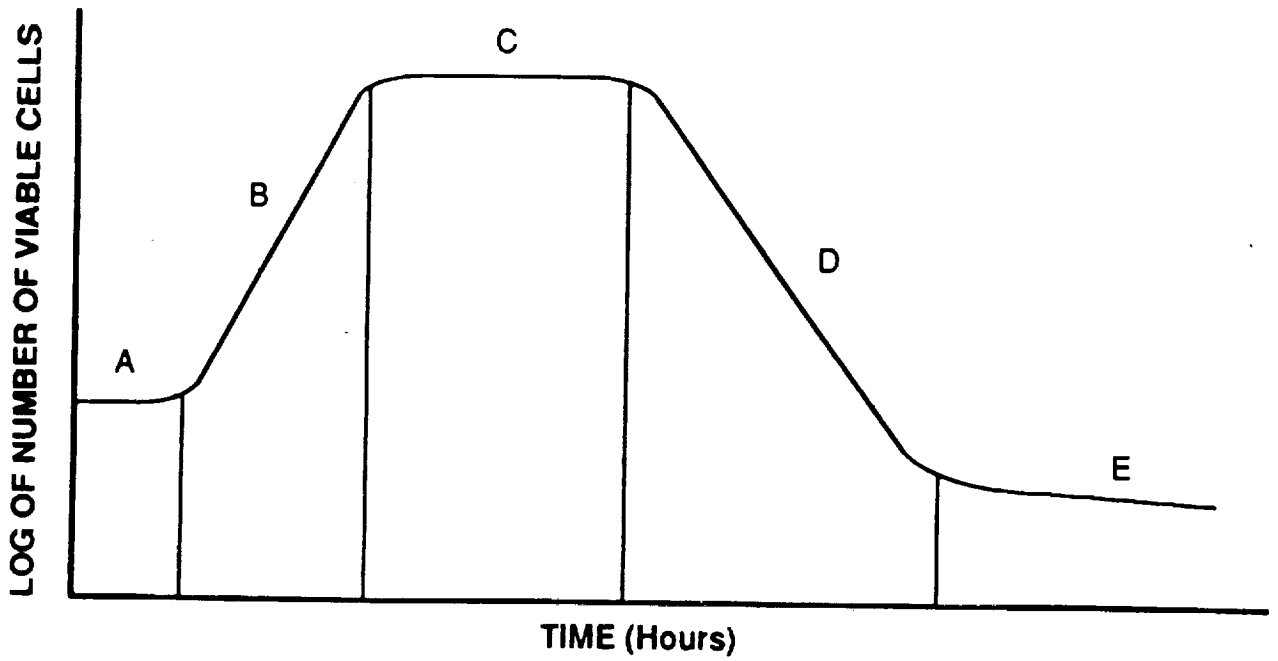
Figure 1. Typical Growth Curve which illustrates the various phases of microbial growth and death: (A) Lag Phase; (B) Exponential Growth Phase; (C) Stationary Phase; (D) Death Phase; and (E) Survival Phase
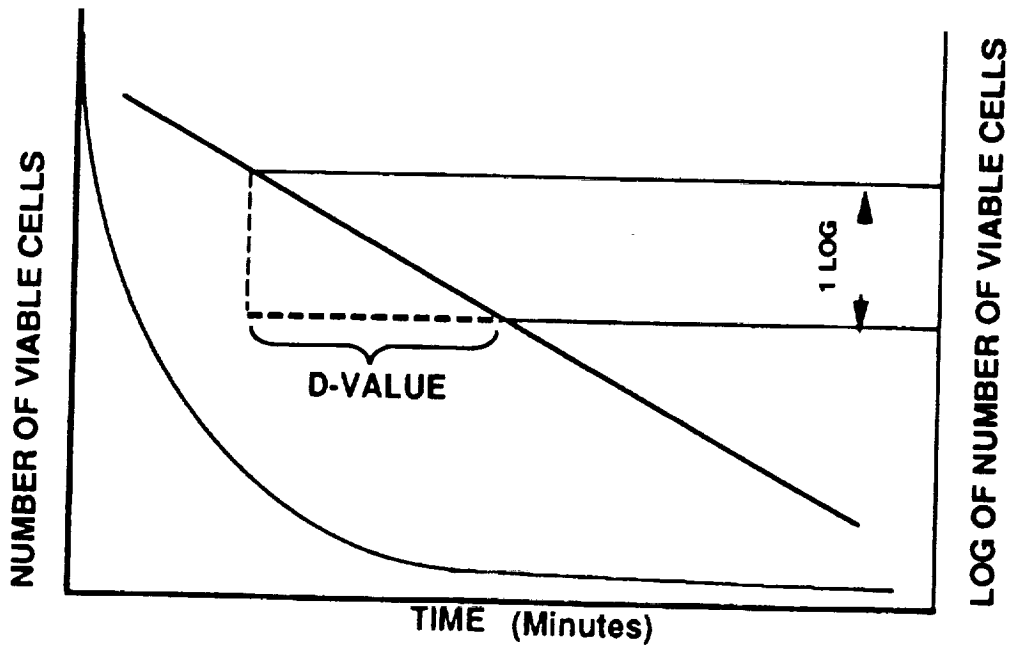
Figure 2. Typical Death Curve which illustrates microbial death. The D - value is the time required to decrease cell number by 90% (a 1log decrease in viable cells).

number of targets, or bacteria, present. Hitting a target a second time does not count, as bacteria only die once. As time goes on, the number of targets decreases so that it becomes harder and harder to hit the remaining ones. To illustrate, assume that there is an initial population of 1 million targets. If they are showered with bullets for 1 minute and 90% are hit, there are now 100,000 survivors left. They are then showered with bullets 1 minute more, but since there are only one-tenth as many targets as in the first round, only one-tenth as many are hit. In other words, this time 90,000 of the targets are hit and 10,000 survive. This pattern repeats itself until there are no targets left. However, there is always some degree of uncertainty that the last bacterium has been killed. The common practice is to give the targets enough overkill for there to be a high probability that the last target has been hit.

Phenol coefficient. Whereas D-values express treatment time (kill rate), effective concentrations are usually based on relative performance. One such standard for comparing the potency of chemical disinfectants is determined by comparing their effectiveness relative to phenol. These comparative evaluations allow for the assignment of what is referred to as the phenol coefficient. The procedure requires the use of standard conditions and known reference strains of bacteria. If a disinfectant has a phenol coefficient of 10, then that disinfectant is 10 times more effective than phenol. Likewise, if a disinfectant has a phenol coefficient of 0.5, then that disinfectant is one-half as effective as phenol. The higher the phenol coefficient, the less time and concentration is required to kill microorganisms.

Two other relevant control terms will be briefly discussed. The first is LD_{50} which is commonly used in toxicological testing. This represents the concentration, or intensity, of a physical or chemical factor resulting in the death (LD=lethal dose) of 50% of the test population. A second term is the minimum inhibitory concentration (MIC). This is the lowest concentration of an agent which will suppress the growth of a test population of microorganisms. MIC's are routinely applied to antibiotic sensitivity testing.

Treatment used to lower viable numbers of microorganisms on or in a material may be either physical, chemical or biological in nature. Each specific method has its unique properties and applicability.

PHYSICAL CONTROL METHODS

As previously discussed, microorganisms have a wide range of tolerance to extreme physical conditions. Generally, pathogenic microorganisms have a higher sensitivity to extremes than do environmental organisms. Physical methods which are successfully used to control microorganisms include heat, radiation, and filtration. Ultrasonic treatments may also have applicability to the space environment. Table 2 summarizes commonly used physical methods for sterilization and disinfection.

HEAT

Pasteurization. Pasteurization, as classically defined, involves the exposure of material to moderately high temperatures (60-70° C) for short periods of time. This method has been em-

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ployed to prevent the transmission of human pathogens via dairy products. Pasteurization results in the elimination of defined human pathogens from the material but only reduces the total numbers of microorganisms present. Pasteurization does not sterilize. Power requirements to provide the heat necessary may be prohibitive in employing this method within space habitats. Applicability only to liquids, restricts its general use. Pasteurization is not a broad spectrum method since it takes advantage of the greater sensitivity of specific pathogenic bacteria.

Tyndallization. Intermittent heating to 100°C for 3 successive days can provide an effective means to lower the population of viable microorganisms in liquids. This process is known as tyndallization, or fractional sterilization. Power requirements to provide the necessary heat and subsequent cooling may be prohibitive on the Freedom Station. However, requirements may be lower than subsequent heating methods discussed. Restricted applicability to liquids limits applications, as does the time required for effective processing.

Boiling. Liquid water heated to boiling point at zero elevation (100° C) kills most vegetative microbial cells. However, this temperature must be maintained for hours to be effective against bacterial spores and some viruses such as the hepatitis virus. This method is probably impractical for general application on Freedom due to power restrictions, water availability, and hardware and systems incompatibility.

Steam. The use of pressurized steam within a sealed chamber (autoclaving) is the most effective use of heat. Treatment using pressurized steam allows temperatures in excess of the boiling

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TABLE 2   PHYSICAL METHODS FOR STERILIZATION AND DISINFECTION

Method	Description	Advantages	Disadvantages
Heat			
Unpressurized Moist Heat	Using heat to increase the temperature of water to any point including boiling.	Kills most vegetative cells	Ineffective against bacterial spores and some viruses such as hepatitis. Power restrictions, water availability, and hardware and systems incompatibility may be prohibitive for space applications
Pressurized Steam	The use of pressurized steam within a sealed chamber (autoclaving)	Most effective use of heat. Allows temperatures above boiling point of water. Applicable in sterilization of steam penetrable heat stable materials.	Reduced pressure will lower boiling point and maximum temperature. Power requirements to produce steam, weight of hardware, and water availability may be prohibitive in space applications.
Pasteurization	Exposure of material to moderately high temperatures (60-70°C) for short periods of time.	Eliminates defined human pathogens.	Does not sterilize, only reduces numbers of microbes present. Power requirements may be prohibitive in space applications. Limited to use on liquids. Not a broad spectrum method.
Tyndallization	Intermittent heating and cooling to lower the population of organisms in liquids.	Effective means to lower the population of viable microbes in liquids. Power requirements lower than with pressurized steam or pasteurization methods.	Power requirements for heating and cooling and may be prohibitive in space application. Limited applicability to liquids. Time required for effective processing.
Dry Heat	Dry material is sterilized by heating at 160°C for one to three hours.	Lower temperatures may be used to reduce viable numbers of organisms.	More time must be used with the lower temperature.

TABLE 2 (Continued)

Method	Description	Advantages	Disadvantages
Incineration	Complete combustion of biologically contaminated materials results in sterilization.	All microorganisms in material will be destroyed.	Applied only to non-reusable materials. May have limited application for the processing of bio-hazardous material generated.
Ultra-rapid Heating	Sterilization of fluids by heating the material very rapidly.	Very fast sterilization times. Viruses are also killed.	May be impractical in space applications due to power requirements.
Radiation			
Ionizing Radiation	Electromagnetic radiation of wavelengths shorter than UV light form highly reactive chemical species that alter resulting in cell death.	Quick kills possible. Good penetration of most materials.	Applicability limited due to safety concerns.
Ultraviolet Radiation	Damage to DNA from alteration of nucleic acid molecular structure by UV light, causing cell death.	Highly effective. Can be used with surface and air disinfection.	UV light is harmful to humans. The mercury vapor lamps which produce the UV light are a potential source of toxic substances. UV bulbs must be made of quartz which is costly. Ozone, an unwanted chemical by-product, is produced. The UV source diminishes over time. Power requirements may be restrictive.
Ultrasound	High frequency sound waves rupture and kill microbial cells	Useful in lowering microbial populations in liquids or on surfaces submerged in liquids.	Limitations poorly understood.
Filtration	Filters physically entrap, adsorb, or exclude microbial cells in or on the filter.	Does not alter the chemistry of the solution filtered. Energy efficient.	Filtration efficiency. Microbial grow-through. Filter capacity. Filter failure.

point of water to be achieved. This method has wide applicability in the sterilization of heat stable materials. This method would have no application to the disinfection of exposed surfaces or heat labile materials, however. The use of pressurized steam may have specific application for sterilizing biological and/or clinical wastes produced in the health maintenance laboratory and other specific areas within space habitats. Power requirements to provide the steam, weight considerations of the necessary hardware to withstand the pressure, and water availability may be prohibitive in employing this method for space-related applications.

Dry heat. Dry material may be sterilized effectively by heating at 180° C for one to three hours. Applications may require alterations in both the time and temperature. A decrease in one factor must be compensated by an increase in the other. Power requirements to provide the necessary heat may be prohibitive in employing this method in space environments. Applicability only to dry, heat stable materials restricts generalized use.

Incineration. Complete combustion of biologically contaminated materials results in sterilization. All microorganisms in incinerated material will be destroyed. However, this method obviously can be applied only to non-reusable materials. Incineration may have limited application for the processing of biohazardous material provided that power requirements and system configurations are acceptable.

Microwave radiation. Radiant energy of radio frequency excites water molecules rapidly to produce heat in the process.

If the magnetron (source) is properly shielded so as not to interfere with electronics, this may represent a much more efficient means of imparting heat to water when compared with thermal resistance. All the above limitations (time and temperature requirements) to heat apply as kill is dependant on heat not radiation.

SONICATION

High frequency sound waves (ultrasound) can be used to rupture or damage microbial cells so they are unable to recover, resulting in death. Ultrasound may be useful in lowering microbial populations in liquids or on surfaces submersed in liquids. Many previous studies evaluating the use of ultrasound to kill bacteria have not considered or utilized heat controls properly. In one study, the effects of ultrasound using bacteria of different structural cell types was evaluated independent of heat (Rhoads, et al., 1990). These results indicate that significant reductions in viable cell numbers may be achieved using ultrasonic treatment. In addition, Gram negative bacteria were found to be more susceptible to ultrasonic treatment when compared to Gram positive bacteria.

RADIATION

Ionizing radiation. Electromagnetic radiation of wavelengths shorter than ultraviolet light, results in the formation of highly reactive ions. These ions non specifically alter proteins and nucleic acids which results in cell death. The applicability

of this method at this point is limited. This is primarily due problems due to health/containment and the necessary weight requirements of proper shielding. Nevertheless, its consideration cannot be ignored as it is extremely effective on surfaces and liquids, requiring no additional power inputs. As ionizing radiation produces very little heat, it is applicable to heat labile materials.

Ultraviolet radiation. Certain chemical bonds in living cells specifically absorb ultraviolet radiation including nucleic acids and proteins. The absorption of the energy associated with the UV photons results in chemical reactions which alter the nascent molecular structure. Extensive damage to DNA will result in death of the cell due to numerous irreparable mutations. Ultraviolet irradiation is used on Earth in many applications where the sterilization of surfaces or compartments is required and may have limited but important application for microbial control in space habitats. The major disadvantages regarding UV light are: 1) UV light exposure is harmful to humans, being responsible for some skin cancers and eye damage; 2) the source of this radiation is traditionally mercury vapor lamps which would provide a potential source of a toxic substance within the closed environment; 3) UV radiation does not readily penetrate glass or plastic and thus UV bulbs must be manufactured using expensive quartz as the transparent encasement; 4) ozone is produced at some wavelengths providing another source of chemical by-products; and 5) the UV source continually diminishes over time. The advantages of this method include its effectiveness and its applicability to surface and air disinfection. UV irra-

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diation also is used in water treatment to lower the number of bacteria and eliminate organic compounds responsible for unpleasant tastes and odors. Power requirements may be restrictive for widespread use of this method.

FILTRATION

Filtration results in the removal of microorganisms from liquids or gasses. Two general types of filters have applicability to disinfection or sterilization of certain media. Depth filters result in the removal of microorganisms by physical entrapment in the matrix of the filter or by adsorption of microbial cells to the porous filter material as the medium passes through the filter. Sieve filters are composed of very thin membranes containing numerous holes or pores of a defined size through which the medium can pass. Microorganisms, being too large to pass through the pores, are retained on the filter surface. There are four major concerns regarding the use of filters for sterilization or disinfection. These are 1) filtration efficiency, 2) filter capacity, 3) microbial grow-through and 4) filter failure.

Filtration efficiency. When filters are applied for the purpose of sterilization or disinfection, filtration efficiency is directly related to microbial retention. With sieve filters, this parameter is directly related to the pore size of the filter. Generally, the smaller the pore size, the more efficient the filter in microbial retention. The efficiency of a filter can be empirically determined and vendors of such products gener-

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ally include microbial retention tests in their quality control evaluations. Some of the currently available membrane filters can reliably achieve a seven log reduction in bacterial numbers when filters of the appropriate pore size are used. In choosing the appropriate filter for microbial removal, the size of the microorganisms which must be removed is a major consideration. The pore size of filters applied for microbial removal must be chosen with care. Even using filters of very small pore size (0.2 um), some bacteria of the mycoplasma group cannot be reliably removed with sieve filters. Many bacteria shrink in size under low nutrient conditions.

Filter capacity. Filter capacity relates to the maximum amount of material that can be retained by a filter without unacceptable decrease in the flow of liquid or gas through the filter. In general, sieve filters have a lower capacity than do depth filters but high capacity sieve filters are available. A filter with a high capacity is desirable as this decreases maintenance and the risk of system contamination.

Grow-through. The potential for some microorganisms to grow through microbial filters is an obvious concern, particularly if it is desirable to have filters remain in place in a system for long periods of time. Microbial grow-through is more likely to occur with depth filters. Thus, depth filters are not the filter of choice where this parameter is a concern. Both pore size and filter composition affect microbial grow-through. Microbial grow-through was delayed with filters composed of cellulose, polypropylene, or polysulfone when compared to nylon or PVDF

(Simonetti and Schroeder, 1984). In this study, no grow-through was detected when polypropylene filters of 0.2 um pore size were tested over a 300 hour time period with 6×10^9 total cells of Pseudomonas diminuta as the challenge organism. The hazards associated with microbial grow-through due to prolonged use of filters is documented (USP, 1979).

Filter failure. Any decrease in the integrity of a filter may result in filter failure. With membrane filters failure may occur after a filter has clogged. The resultant increase in pressure on the filter may cause the filter to break. Thus, as discussed above, information regarding the filter capacity and the microbial/particulate load encountered must be determined in order that filter change-out schedules can be safely predicted. With an adequate change-out schedule and the incorporation of pre-filters, the potential for filter failure can be reduced drastically. However, the use of dual filters in tandem may be incorporated to reduce the potential for contamination should a filter fail.   Filtration is relatively inexpensive and is an efficient, predictable process, when the correct filter or filter combination is employed.

CHEMICAL ANTIMICROBIAL AGENTS

The chemical antimicrobial agents which have various application for microbial disinfection and sterilization can be placed into several groups. The mechanisms of action of these agents are diverse. Chemical agents may affect cell membranes, cell walls, proteins, interfere with cellular functions or damage

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nucleic acids. In addition to the factors affecting physical treatments, pH and temperature will also affect the action of chemical disinfectants. Table 3 summarizes commonly used chemical antimicrobial agents.

PHENOLICS

The precise mechanism of the mode of action by which the phenolics kill microbes is not known. However, it appears that the these compounds damage the cell membrane, which is followed by further cellular deterioration. These compounds are generally stable and retain effectiveness against susceptible microorganisms for long periods of time. Depending on the concentration used, phenolics can be bacteriocidal or bacteriostatic. As in the case of other agents used for microbial control, bacterial spores and some viruses are less susceptible than are vegetative microbial cells and enveloped viruses. Due to the general stability of phenolics, the cleanup required after disinfection with these compounds may restrict their use in closed environments. In addition, the potential human toxicity of phenolics and current Freedom water quality requirements for phenols (<1 ppb) make these compounds undesirable for application.

HALOGENS

The halogens most frequently used for disinfection are iodine and chlorine. Halogens catalyze the oxidation of organic compounds. The oxidation of essential molecules within microbial cells will result in cellular damage and ultimately cell death.

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TABLE 3 CHEMICAL ANTIMICROBIAL METHODS FOR STERILIZATION AND DISINFECTION

Method	Description	Advantages	Disadvantages
Phenolic Compounds	Mechanism not well known.	Generally stable and maintain effectiveness for long periods of time. Can be bacteriocidal or bacteriostatic.	Bacterial spores and some viruses are less susceptible than vegetative cells or enveloped viruses. Cleanup of phenolics after use may restrict use in closed system. Potential human toxicant.
Halogens	Catalyze the oxidation of essential molecules within microbial cells, killing them. Iodine can also inactivate enzymes, resulting in cell death.	Some compounds so effective, can be used in cold sterilization. Some decompose to non-toxic products.	Some compounds have a level of toxicity that may be higher than tolerable in space environments. Iodophores may not be suitable as surface disinfectants due to intense cleanup procedures. Potential of selection for iodine resistant microorganisms. Molecular chlorine and iodine irritate tissues. Long term physiological effects of iodine use not known.
Alcohols	Microbiocidal for some organisms by coagulating cellular proteins thereby disrupting cell membranes.	The volatility of ethanol and isopropanol leaves no residual on surfaces. Limited toxicity and biodegradable potential.	Bacterial spores and some viruses not effectively killed. Limited effectiveness because time of treatment short due to volatility. Increased load to air from volatilization. Limited spectrum of alcohol sensitive microorganisms.
Metal Ions	Ions of heavy metals inhibit certain enzymes containing sulfhydryl groups.	Broad range of effectiveness as a biocide.	Toxicity to mammals as well as microorganisms. Heavy metals bioconcentrate and are extremely stable and difficult to remove. Potential to evoke hypersensitive responses in sensitive individuals even at non-toxic concentrations.

TABLE 3 (Continued)

Method	Description	Advantages	Disadvantages
Gases	Ethylene oxide and formaldehyde chemically react with cellular proteins and inactivate them. Ozone is bactericidal.	Ozone processes are controllable and by-products are less toxic than halogen treatments.	Gases are difficult to contain, require specialized equipment, and are toxic for humans.
Peroxides	Hydrogen peroxide produces reactive oxygen species which oxidize cellular proteins and inactivate enzymes.	3% aqueous hydrogen peroxide is non-toxic, and requires only brief time applications. Broad spectrum of effectiveness. Decomposition produces harmless oxygen and hydrogen.	30% hydrogen peroxide solution is caustic and irritating to the skin and eyes.

Chlorine. Chlorine has traditionally been used to reduce the number of microorganisms in drinking water. The introduction of chlorination was a key aspect of public health programs greatly reducing the incidence of cholera and typhoid fever. Chlorine, hypochlorites, chloroamines, and some other chlorine-based compounds, when in aqueous solution, give rise to hypochlorous acid. Hypochlorous acid is a strong oxidizing agent. The recently developed chloramine compounds may also have potential application for use in closed environments (Williams, et al., 1988). Some of these compounds are so effective that they can be used for cold sterilization. In addition they decompose yielding apparently non-toxic products, thus clean-up would be minimal.

Iodine. Iodine is one of the oldest and most effective germicidal agents. Iodine, in addition to its oxidizing properties, can result in the iodination of tyrosine residues of proteins. This iodination can lead to the inactivation of the enzymatic capabilities of some enzymes and ultimately to cell death. Tincture of iodine (an alcohol solution of iodine and either sodium or potassium iodide) is a traditional antiseptic. Since iodine solutions stain and have irritant properties, compounds containing iodine bound to organic compounds, known as iodophores, have been developed. These compounds have a greater applicability as disinfectants due to their non-staining and low irritability properties. Iodine at low concentrations in aqueous solution have been employed in previous U.S. space applications for water disinfection with success (Willis and Schultz, 1987). Iodine seems a probable candidate for water disinfection in

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closed environments. The use of iodophores as surface disinfect-
ants may not be suitable in these environments due to the poten-
tially intense cleanup procedures which may be required. Since
all currently proposed space habitats are projected to be func-
tional for twenty years or more, the potential for selection of
iodine resistant microorganisms is a serious concern (Favero, et
al., 1976 and McFeters and Pyle, 1987).

Both molecular chlorine and iodine can irritate tissues.
Halogens will also react with organic to produce halogenated by-
products that may be carcinogens. The physiological effects on
plants, animals and humans of long term iodine use is not known
(Janik, et al., 1989).

ALCOHOLS

Alcohols are microbicidal for some microorganisms due the
fact that cellular proteins are coagulated and cell membranes are
disrupted. Bacterial spores and some viruses are not effectively
killed by alcohol disinfection. The most commonly used alcohols
for disinfection on Earth are ethanol and isopropanol in aqueous
solution at concentrations of 70% to 90%. Since ethanol and
isopropanol are volatile, little residue is left on surfaces
disinfected with these compounds. This provides both an advan-
tage and a disadvantage in the application of alcohols as disin-
fecting agents. The advantage is that these compounds can be
used when it is desirable to have the disinfected item free of
biocide in a short period of time after treatment. The disadvan-
tage is that the time period of treatment of surfaces is gener-
ally limited and thus, the effectiveness limited. Also, since

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these compounds are volatile, they will enter the air and will increase the load on the both the air and water purification system. Although alcohols are limited with respect to the spectrum of sensitive microorganisms, the limited toxicity of dilute ethanol and high potential for biodegradation may warrant consideration of ethanol as a disinfectant for closed environments.

HYDROGEN   **PEROXIDE**

Hydrogen peroxide at a concentration of 3% (v/v) is an effective antimicrobial agent due to its oxidizing properties. Hydrogen peroxide has a mechanism similar to ozone. Since 3% aqueous hydrogen peroxide is non-caustic to human skin, it has broad application as an antiseptic. This compound is used to clean wounds, surgical implants and soft contact lenses because it requires only brief periods of time of exposure when proper concentrations are employed.

Decomposition can be retarded by keeping 3% hydrogen peroxide solutions in a dark and cool environment. Stored in this fashion, hydrogen peroxide solutions will retain their antimicrobial effectiveness for at least one month. Hydrogen peroxide solutions of 30% are caustic to the skin and eyes. These concentrated solutions are not recommended for direct application but may be a desirable concentration to store the disinfectant until it is diluted for use.

Hydrogen peroxide has been shown to be an extremely effective oxidant in the removal of taste and odor compounds. Studies have been conducted demonstrating the efficacy of combining

hydrogen peroxide and ultraviolet radiation to convert organic contaminants to carbon dioxide (Baozhen and Jun, 1988, Wallace, et. al., 1988). Hydrogen peroxide is one of several reactive oxygen species generated upon ozonation. Recently, ozone and peroxide have been combined for municipal water treatment as a process termed PEROXONE (Wolfe, et. al., 1989).

METALS

Ions of the heavy metals such as mercury, silver, arsenic and copper are toxic generally by inhibiting certain enzymes containing sulfhydryl groups. These compounds have a broad range of effectiveness as biocides but currently have limited use due to their toxicity to mammals as well as microorganisms. Recent generation Soviet water reclamation systems have used silver as a residual biocide. The use of heavy metals for microbiological control within closed system environments has several disadvantages. First, heavy metals may be concentrated within living organisms (bioconcentration). The further use of this material (plant or animal) by humans results in a much higher level of exposure than the initial treatment concentration. Second, heavy metals are extremely stable and are difficult to remove from the environment, especially a closed environment with little dilution and limited treatment options. Third, their general toxicity and their potential to evoke hypersensitive responses in sensitive individuals even at non-toxic concentrations must be considered.

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Gases such as ethylene oxide and formaldehyde are microbicidal because they chemically react with cellular proteins and inactivate them. Both of these gases are highly toxic and as such this may preclude their use for Space Station Freedom.

Ozone has long been known to be one of the most potent drinking water disinfectants available. Ozone is unstable, and during its degradation to ground-state oxygen, highly reactive oxygen species such as the hydroxyl radical and the superoxide anion are generated. These reactive oxygen species in turn oxidize cellular proteins and inactivate enzymes. Ozonation has represented a viable alternative to halogenation (chlorination) in many municipalities (Mignot, 1982, Lesbros, 1982). Ozonation has even been employed to recycle swimming pool water (Legeron, 1982). This is the result, in part, of the increased awareness of efficacy limitations (Anderson, et. al., 1990) and health concerns regarding by-products of chlorination (Miller, 1986).

BIOLOGICAL METHODS

With the exception of antibiotics, biological methods for control of microorganisms have not had widespread application on earth. These methods may be useful in the space environment where physical treatments are limited by power requirements and chemical methods are limited by environmental constraints. Table 4 summarizes common biological treatment methods.

TABLE 4 BIOLOGICAL METHODS FOR STERILIZATION AND DISINFECTION

Method	Description	Advantages	Disadvantages
Antibiotics	Antibiotics are natural chemical substances produced by certain microorganisms which are active against other microorganisms. They vary in their mode of action.	Bactericidal and bacteriostatic. Used for in-vitro and topical treatment of bacterial and fungal infections.	Not effective against viruses.
Bacteriocins	Bacteriocins are proteins, produced by most bacteria, which inhibit or kill closely related species of bacteria.	Bactericidal selectivity to particular groups may be beneficial. No potential for chemical contamination. Non-toxic to humans and animals.	Not broad spectrum. Not effective against algae, protozoa and fungi.
Bacteriophage	Bacteriophages are viruses that infect bacterial cells. One type is lytic and causes bacterial cell lysis. Temperate types cause the viral nucleic acid to recombine with that of the host and replicate.	Bactericidal selectivity to particular species may be beneficial. No potential for chemical contamination. Non-toxic to humans and animals.	Type specific. Not effective against algae, protozoa and fungi. May transfer genetic material and increase mutation. Bacteriophage may enter temporal stage.
Predation	Ingestion of bacteria by protozoa.	Self maintenance.	Potential adverse effects to humans and/or animals. Difficult to control.

ANTIBIOTICS

Antibiotics are natural chemical substances produced by certain microorganisms which are active against other microorganisms. A large number of antibiotics have been discovered, of which approximately only 1% have significant medical value in their natural form. Soil microorganisms, including bacteria such as _Streptomyces_ and _Bacillus_ and fungi such as _Penicillium_ and _Cephalosporium_ are the principal producers of antibiotics. Antibiotics vary in their mode of action, affecting essential cellular activities such as cell wall synthesis, membrane function, protein synthesis, and nucleic acid synthesis. Antibiotics affect a range of microbial species referred to as their activity spectrum. For example, cephalosporin (the product of the fungus _Cephalosporium_) is effective in the control of both Gram positive and Gram negative bacteria and is therefore known as a broad spectrum antibiotic. This is contrary to a narrow spectrum antibiotic such as bacitracin, affecting only Gram positive bacteria. Other antibiotic agents are effective against mycobacteria and still others are antifungal. Tetracycline is commonly used to treat mycoplasmal, chlamydial and rickettsial infections. An antibiotic agent may be either bactericidal or bacteriostatic. Generally, antibiotics are used for systemic and topical treatment of bacterial and fungal infections. Antibiotics are not effective against viruses. In nature, antibiotics probably function to inhibit competing organisms within specific niches.

BACTERIOCINS

Bacteriocins are proteins produced by most bacteria which inhibit or kill closely related species of bacteria. The bacte-

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riocins of <u>Escherichia</u> <u>coli</u> are called colicins, and those of <u>Pseudomonas</u> <u>aeruginosa</u> are known as pyocins. The ability to produce these agents is inherited via plasmids. The plasmids of <u>E</u>. <u>coli</u> responsible for colicin production are called col factors. Some col factors are readily transferred between organisms via conjugation so that only a few organisms would need to be introduced into the system.

BACTERIOPHAGE

Bacteriophages are viruses that infect bacterial cells. There are two main types of bacterial viruses: lytic (virulent) and temperate (avirulent). The effect of a lytic infection is cell lysis due to the production of large numbers of progeny phage within the cell. In a temperate infection, the viral nucleic acid is recombined with that of the host and replicated within the host cells from generation to generation, with no lysis of the cells. The temperate life cycle can, however, spontaneously revert to the virulent form and lyse the host cells. This can often be induced by conditions which stress the host bacteria. There are also some filamentous phages which "leak" out of the host cells without killing them.

PREDATION

Most microorganisms are saprozoic within the environment defined. Saprobes depend on the uptake of dissolved nutrients. Certain algae (i.e. euglenoids) and many protozoa are holozoic. Holozoic organisms ingest solid or particulate nutrients as food. Microinvertebrates and even some bacteria (ie.g., <u>Bedelivibrio</u> and <u>Vampirovibrio</u>) may play a role in bacterial control.

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Predation (ingestion) of bacteria by protozoa has increased the catabolic activity of the bacteria in the environment (Stout, 1980). Predation also influences the community structure of bacteria in solution as well as on adjacent surfaces, or biofilms. This selectivity may be due to specific feeding behavior. Some protozoa, most notably the paramecia, feed on free-living bacteria. Other protozoa may be classified as grazers as they are adapted to feed on detritus and bacteria from a surface interface (Curds and Cockburn, 1970). Further selectivity may be based on actual prey recognition. This has been documented in bactivorous protozoa, as well as protozoa: protozoa predation (Berger, 1980). One additional example of selective feeding would be amoeba which prefer algae. Technically, this amoeba could be classified as a herbivore (Sleigh, 1973). The physiological basis for this feeding selectivity is unknown, but may entail the secretion of repellents by the prey as a protective mechanism (Berger, 1980). Protozoa may be present in the water treatment systems in space habitats. Many types produce resting stages or cyst, which are resistant to desiccation or starvation. These cysts may result in contamination of the atmosphere as well as the water in space habitats (Neff and Neff, 1969). Any type of biological waste treatment would most probably be contaminated with protozoa. It has been documented that their presence in waste treatment may actually improve the efficiency of the process (Curds and Cockburn, 1970) and therefore would likely be encouraged if pathogenic protozoa can be controlled or eliminated.

SYNERGISTIC APPLICATIONS

Combinations of various control methods may increase overall effect. In many cases, combinations may result in a synergistic effect in which the combined action is greater than the sum of each single agent. Other potential benefits of combining treatments may result in: 1) use of lower concentrations of chemical agents, which is important in closed habitats where removal from or concentration in the environment is a primary concern; or 2) reduced intensity of a physical treatment where a reduced power requirement is a significant advantage. Combining treatments will also significantly reduce the probability of selecting organisms resistant to a particular agent or treatment.

PREVENTION OF MICROBIAL GROWTH

As evident in the previous sections, all known methods to kill or inhibit microorganisms are limited. It is much more reasonable to control microorganisms by prevention. All known microorganisms conduct chemical reactions which are referred to as metabolism. These reactions follow all laws of thermodynamics. So it is known that microorganisms do not appear from nowhere (spontaneous generation) but there must be building blocks, or nutrients, to construct cells and energy to organize and maintain their structure. There of course must be viable cells present, however, due to microbial "omnipresence", contamination of any system is almost inevitable.

It can also be stated that a finite amount of energy and materials will produce, given a favorable environment, a finite

amount of cells or biomass. Both factors, energy and materials, must be present and are both subject to control.

Prevention ideally starts with design. Materials should be selected so as to contribute minimal energy and nutrients. A partial list of known energy resources for bacteria includes: organic carbon, light, ammonium, cyanide, hydrogen, sulfur and iron. Where possible, environments should be inhospitable. Given the range of extreme environments these organisms inhabit on earth this is doubtless a difficult task. Nevertheless, stressful environments will restrict potential colonizing species.

PRESENT RECOMMENDATIONS

As obvious in the previous sections, there is no ideal control method. To increase efficiency, the ideal system will no doubt be configured from several methods or subsystems. This will permit the flexibility needed for long-term effective treatments.

Now that there is a considerable volume of data available from the Water Recovery Test, certain modifications or appraisals may be conducted relative to the present generation life support system. A summary of these items are listed below.

1) With regards to iodine, continue to compile data to evaluate the development of resistant bacterial strains. These isolates could be obtained from the ECLSS test waters.

2) Examine data from the water recovery test to determine if the production of toxic iodinated hydrocarbons occurs during treatment.

3) Filters and microbial check valves may now be evaluated relative to log reduction data.

4) Present control strategies could be altered to include fractional sterilization based on periodic heating which may be very effective and require less energy than present pressurized high temperature schemes.

Additionally, developmental programs should be instituted to evaluate the efficacy of alternative strategies (ozonation, UV, hydrogen peroxide, sonication) and synergistic strategies.

SUMMARY   STATEMENTS

The following points represent logical considerations of any microbial   control   plan.

-State reasonable objectives for each system or subsystem with regards to microbial requirements.

-Where possible, consider design options to minimize bacterial contamination and control.

-Select agents, treatments, or combinations to effectively meet specifications.

-Implement a microbial monitoring program to evaluate control performance.

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APPENDIX G

RECOMMENDATIONS REGARDING NON-SPECIFIC TOXICITY TESTING

RECOMMENDATION OF BIOASSAY TYPES FOR NON-SPECIFIC TOXICITY TESTING OF SPACE STATION TESTBED ENVIRONMENT WATER

The assays described in this report are recommended for testing water from the Space Station testbed for the presence of potentially toxic substances, both organic and inorganic, which may pose a health risk to humans. The tests described will utilize cultured cells. Cells in culture provide an excellent model system for bioassay of non-specific toxicity because variations due to hormonal, circulatory and other factors characteristic of whole animals are avoided, low levels of toxicity can be detected, results are obtained rapidly (two to three days), cells are much less expensive than whole animal assays, adequate replicates can be easily set up and in many cases, human cells may be used.

While the choice of cultured cells must be carefully considered, animal cell models have been shown to be acceptable for certain compounds. For instance, rat hepatocytes are a valid model for predicting genotoxic effects of some substances in humans (1). Neoplastic transformation is another aspect of toxicity. Initiation and promotion of neoplastic growth can be monitored by use of primary epidermal keratinocytes. When animal cells in vitro are used for screening substances potentially toxic to humans, it is important to use cells from tissues of common target organs and more than one cell type should be used (Tyson and Stacey 1989). For example, hepatocytes in suspension or in monolayer culture were found to be more sensitive to 14 hepatotoxic substances than either of the established cell lines, HeLa and Chang Liver (Elkwall and Acosta 1982). These researchers suggest that the chemicals which produced low responses in the cell lines probably require metabolic activation for manifestation of toxicity in the intact animal. Primary hepatocytes apparently possess sufficient metabolic activation potential to allow sensitivity to toxicity due to these substances.

Several toxicity assay types using cells in vitro have been proposed (See UAH Research Proposal No. 90-368, "Development of an In Vitro Mammalian Cell Assay to Monitor Toxic Substances in the Space Station Environment", M.L. Lewis, Ph.D.). A summary of candidate methods is listed below.

1. Leaky cells - measurement of lactic dehydrogrnase (LDH), glycogen and potassium from liver cells and glucose and LDH loss from kidney cells.

2. Metabolic activation - production of active metabolites by

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cytochrome P-450-dependent microsomal mixed function oxidase system in hepatocytes.

3. Cytotoxicity - inhibition of spreading of HeLa cells, leakage of LDH in Chang liver cells and rat hepatocytes.

4. Ability to release glutamic oxaloacetic transaminase (GOT) from hepatocytes.

5. Albumin secretion from a human hepatoma cell line, Hep G2, co-cultured with adult rat hepatocytes.

6. Cellular glutathione content - rat hepatocytes.

7. DNA repair - by autoradiography in hepatocytes.

8. Cell growth, glucose and oxygen use - any proliferative cell type.

9. DNA synthesis/repair - tritiated thymidine pulse labelling and scintillation counting. This can be used with any proliferative cell type.

The recommendations described for selection of cell types and methods for toxicity testing of the water from the MSFC Space Station testbed facility are made from the list above based on the following criteria. 1) Expected sensitivity to a wide range of potentially toxic substances 2) Expected target organ cell type and 3) Availability of equipment and expertise to conduct the assays.

Recommendation of Cell and Assay Type

The following bioassays are recommended for detection of non-specific toxicity due to inorganic metals and organics which may be present in recirculated water from the space station testbed facility.

A) Cell growth, viability and metabolic evaluations (oxygen and glucose use, carbon dioxide production and pH of the culture medium).

B) DNA synthesis/repair using tritiated thymidine pulse labelling and scintillation counting.

C) Cytotoxicity - leakage of lactic dehydrogenase (LDH) and other enzymes from cells and cell death.

A) The method of choice of these three is the first, cell growth and metabolic evaluations. The advantages of this method are that many different cell types (from several target organs) can be used and the results are quickly and easily obtainable. Most cell types, except primary hepatocytes which do not actually grow in culture, can be monitored by simple growth curve and viability evaluations by counting cells daily and testing for viability by dye exclusion. There are a number of other

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advantages inherent in this method. For instance, screening tests can be adapted to microcarrier wells and thus utilize few cells while still providing a number of replicate evaluations. Coupled with these evaluations, changes in glucose use rate can be used to show direct effect on cell metabolism for a battery of potentially toxic substances. For selected samples, metabolic evaluations can be determined for cells in closed chambers by monitoring pH and oxygen and carbon dioxide levels daily. Non-specific toxicity may first affect the metabolic competence of the cells before cell death is obvious. Measuring glucose and oxygen levels over several days can give an early indication of a toxic substance effect which may not be detected by early cell count and viability evaluations alone. Both attachment dependent and suspension cell types and primary cultures and cell lines can be used in this method, thus several host systems can be tested simultaneously with the same water samples.

The main disadvantage of this method is that some of the toxic substances may require metabolic activation in order to show toxicity. Freshly prepared hepatocytes are usuallly the cell of choice for metabolic activation assays and since hepatocytes do not grow in culture, they cannot be used for growth curve studies. A screening assay to detect cytotoxicity in hepatocytes should be used in addition to growth curves if metabolically inactive toxins are suspected in the samples.

B.   The second method of choice is evaluation of DNA synthesis/repair using tritiated thymidine incorporation as a means to detect cell growth. The advantages of this method are the same as for growth curve assays in that a number of different cell types can be utilized for toxicity screening. The DNA labeling method is more sensitive that cell counts since it is a quantitative way to detect cell cycle progression. The main disadvantage of this method is that it requires the use of radioactive precursors and disposal of radioactive material.

C)   While the methods to measure release of LDH, glycogen and potassium, and other enzymes from hepatocytes, and albumin secretion and glutathione content are sensitive methods used to detect cellular toxicity, they are not straightforward either in assay technique or interpretation of results.

One of the best documented methods is evaluation of the P-450 mixed function oxidase system. The main disadvantage of the metabolic activation method (activation of metabolites by cytochrome P-450 dependent microsomal mized function oxidase system in hepatocytes) is that the hepatocytes remain viable for only a short time after removal from the animal and the enzymes of the cytochrome P-450 system are short lived outside the body.

Therefore, the most direct, measurable and easily interpreted screening procedure to simply detect the presence or absence of toxic substances in test bed water is growth curve and viability assays, morphology changes, and metabolic assay for glucose use. Three cell types are recommended including a hematopoietic suspension cell line and attachment dependent human

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kidney and liver cell lines. If more sensitive tests are indicated after these primary screening assays are conducted, primary rats hepatocytes obtained by perfusion of adult rat liver can be used to assess membrane damage detected by leakage of enzymes from the cells.

The choice of cell types for growth curve studies should include those from expected target organs such as liver, kidney and hematopoietic cells. It is recommended tht the cell types be acquired from the American Type Culture Collection (ATCC) as established cell lines because of economic feasibility of using cell lines as opposed to processing primary cultures from animals for each test. Cell lines of liver, human kidney, and suspension cultures of several types of hematopoietic are available throught ATCC.

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APPENDIX H

RECOMMENDATIONS REGARDING THE CHARACTERIZATION OF CHEMICALLY

PRE-TREATED URINE

Characterization of the Chemical Constituents of Oxone/Sulfuric Acid Pretreated Urine, Including more Complete Organic Carbon Accountability

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Prepared for

The National Aeronautics and Space Administration
George C. Marshall Space Flight Center
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2.0 TECHNICAL APPROACH AND RESEARCH PLAN

3.0 DETAILED TEST PLAN

1.0 Introduction

Acidic oxidative pretreatment is an excellent method of urea destruction and when oxidant is used in sufficient concentration is an acceptable biocidal treatment as well, but there is another issue which requires further investigation. The principle organic chemical species present in oxone^R pretreated urine have not been identified. The oxidation products of the major portion of organic carbon containing compounds in raw urine are unknown. Validated government environmental standard methods necessary to study these issues do not exist.

The sole purpose of a life support system is to insure the health and safely of the spacecraft crew. In keeping with this purpose, current Space Station Freedom Water Quality requirements dictate that the components in hygiene water be sufficiently characterized to assure that the total concentration of toxics is less than 1000 ppb when reported as Total Organic Carbon (TOC).

A relatively large number of low molecular organics are produced by oxone pretreatment of urine and free radical halogenations are known to occur. Cyanogen chloride has been isolated from pretreated urine distillate as well as chloroform, methylene chloride, and several other halogenated organics. Many halogenated organics are EPA monitored industrial pollutants as they are used in industrial processes or as pesticides. The EPA regulates levels of trihalomethanes in municipal drinking water supplies due to their high chronic toxicity. The toxicity of many halogenated organic compounds in humans is considerable and well documented[4].

Quite a few EPA regulated compounds have been detected in urine (Table 1) or hygiene process water (Table 2) from spacecraft or related tests . This list appears extensive but TOC accountabilities have never been sufficient to assure that water quality requirements are met.

Urine Chemistry

Historically the use of analytical chemistry techniques and equipment to answer questions of biological and clinical importance has closely paralleled the development of these techniques and equipment, and has often served as the driving force for such development. The clinical importance of understanding the biochemistry of health and disease is obvious, and health care professionals have exploited chemical analytical techniques to better understand the chemical basis and effects of many health threatening conditions as soon as these techniques became available[3].

The chemical constituents of a person's urine offer a great deal if information about that persons health. Diabetes, liver disease, drug abuse and pregnancy are but a few of the conditions which cause changes in the urine chemical profile. Thus the chemistry of urine has been the subject of considerable study. In 1964 Webb and Associates, under NASA contract, compiled a list of over 150 chemical constituents of urine and their concentration ranges from five referenced sources[13]. The referenced work took place before modern trace analytical techniques such as capillary gas chromatography, high pressure liquid chromatography, or mass spectrometry were commonly

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TABLE 1. SPECIFIC TOXICANTS IN URINE

PRIMARY REQUIREMENTS

Compound	Source	Amt detected (ug/L)	Reference
VOLATILES			
Alkylbenzene	4	50	3
Benzene	8	4	3
Bromoform	8	4	3
Chloroform	4	10	
methylene chloride	8	16	3
propa-di-ene chloride	4	30	
1,1,1-trichloroethane	8	8	3
ACID COMPOUNDS			
O-cresol	8	160	
phenol	4	460	
	8	150	
dichlorophenol	4	55	
2,4,6-trichlorophenol	4	25	
chloromethylphenol	4	30	
2-nitrophenol	4	265	
methylphenol	4	1000	
* phenol, 2,6-di -t-butyl-4-methyl-	8	150	
* phenol cmpds (9)	4	2200	3
* phenol cmpds (8)	6	600	3
* phenol,2,6-di -t-butyl-4-methyl-	8	150	3
phenol, chloro- adduct	4	100	
phenol, dichloromethyl	4	100	
phenol, t-butyl	4	150	
BASE/NEUTRALS			
* phthalate esters (2)	6	350	3
* phthalate, bis(2-ethyl hexyl)-	8	103	3

TABLE 1.   SPECIFIC TOXICANTS IN URINE (Cont.)

Compound	Source	Amt. (ug/L)	Toxicity	Reference
Acetaldehyde			orl rat LD_{50} 1950mg	
acetaldehyde, trichloro-	4	20		
acetic acid	4	300-31200	orl hmn TDLo 1470 ug GIT	
acetic acid, phenyl	4	400		3
Acetone			orl rbt LD_{50} 5300mg	
hydroxyacetone	4	150		
acetone cmpds (2)	4	250		3
Butyric Acid	8	30	orl rat LD_{50} 2940mg	
benzoic acid	4	2000	orl rat LD_{50} 3040mg	
benzoic acid, 2-hydroxy	4	1000		
benzoic acid cmpds (2)	4	3000		3
	6	150		3
benzaldehyde	4	20	orl rat LD_{50} 1300mg	
	6	100		3
benzaldehyde, 4-methyl	4	100		
benzaldehyde cmpds (3)	4	150		3
butyldihydroxybenzene	4	10		
carboxylic acids (5)	4	40800		3
carboxylic acids cmpds (5)	6	52000		3
Decanal			orl rat LD_{50} 3730mg	
n-decane	4	150		
decan cmpds (2)	4	280		3
ether, p-chlorophenyl methoxyisobutyl	4	400		3
ethyl alcohol	4	7000	orl man LDLo 50mg	3
	6	1500	(est amt)	3
furfuryl alcohol	6	200	orl rat LD_{50} 275mg	3
	4	10		
furan, 2,5-di-methyltetra-hydro	4	10		
formic acid	4	2678	orl rat LD_{50} 1210mg	
heptanoic acid			orl mus LD_{50} 160mg	
4-heptanone	6	100		3
	4	25		
heptanone cmpds (2)	4	60		3
heptyl alcohol	4	1		
Hexanol			orl rat LD_{50} 720mg	
hexanol, 2-ethyl-1-	6	100		3
hydrocarbon, unsaturated	4	30		
methyl alcohol	4	7000	orl hmn LDLo 340mg	
	6	1500	(est amt)	3

TABLE 1. SPECIFIC TOXICANTS IN URINE (Cont.)

SECONDARY REQUIREMENTS (CONT.)

Compound	Source	Amt. (ug/L)	Toxicity	Reference
morpholine	4	5		
	6	100		3
myristic acid	8	10	ivn mus LD_{50} 43mg	
nitrile, methyl benzo	4	400		3
octanol	4	1		
palmitic acid	8	10	ivn mus LD_{50} 57mg	
propionic acid	8	1500	orl rat LD_{50} 1510mg	
salicylic acid	6	300	orl rat LD_{50} 891mg	3
sulfone, methyl-	4	20		
toluonitrile	4	400		
n-undecane	4	200		3
vanillan	6	200	orl rat LD_{50} 1580mg	3
	4	30		
Xylenol			orl mus LD_{50} 1070mg	
xylene	4	8		

TABLE 2. SPECIFIC TOXICANTS IN HYGIENE WATER

PRIMARY REQUIREMENTS

Compound	Source	Amt detected (ug/L)	Reference
VOLATILES			
* Acetyl benzene	3	30	
Alkylbenzene	4	50	3
Benzene	8	4	3
Bromoform	8	4	3
dibromomethane	3	10	
Chlorobenzene	3	5	
Chloroform	4	10	
	3	4	
Ethane, tetrachloro	7	4	3
Methane, dichlorobromo	7	4	3
methylene chloride	7	200	3
	8	16	3
propa-di-ene chloride	4	30	
1,1,1-trichloroethane	8	8	3
ACID COMPOUNDS			
0-cresol	8	160	
phenol	3	10	3
	4	460	
	8	150	
dichlorophenol	4	55	
2,4,6-trichlorophenol	4	25	
chloromethylphenol	4	30	
2-nitrophenol	4	265	
methylphenol	4	1000	
* phenol, 2,6-di -t-butyl-4-methyl-	8	150	
phenol cmpds	7	350	3
* phenol cmpds (9)	4	2200	3
* phenol cmpds (8)	6	600	3
* phenol,2,6-di -t-butyl-4-methyl-	8	150	3
phenol, chloro- adduct	4	100	
phenol, dichloromethyl	4	100	
phenol, t-butyl	4	150	
BASE/NEUTRALS			
* phthalate esters (2)	6	350	3
phthalate ester cmpds (4)	3	75	
phthalate ester cmpds (3)	7	20	3
* phthalate, bis(2-ethyl hexyl)-	8	103	3

TABLE 2.   SPECIFIC TOXICANTS IN HYGIENE WATER (Cont.)

SECONDARY REQUIREMENTS

Compound	Source	Amt. (ug/L)	Toxicity	Reference
Acetaldehyde			orl rat LD_{50} 1950mg	
acetaldehyde, trichloro-	4	20		
acetic acid	4	300-31200	orl hmn TDLo 1470 ug GIT	
	3	35800		2
	7	940		
	7	500		2
acetic acid, phenyl	4	400		3
acetone	3	1000	orl rbt LD_{50} 5300mg	3
hydroxyacetone	4	150		
acetone cmpds (2)	4	250		3
alcohol cmpds (2)	3	5000		
alkene ($C_{29}H_{58}$)	7	10		3
Butyric Acid	8	30	orl rat LD_{50} 2940mg	
benzoic acid	4	2000	orl rat LD_{50} 3040mg	
	3	848		3
benzoic acid, 2-hydroxy	4	1000		
benzoic acid cmpds (2)	4	3000		3
	6	150		3
benzaldehyde	3	1000	orl rat LD_{50} 1300mg	3
	4	20		
	6	100		3
benzaldehyde, 4-methyl	4	100		
benzaldehyde cmpds (3)	4	150		3
butyldihydroxybenzene	4	10		
carboxylic acids (5)	4	40800		3
carboxylic acids (11)	3	81500		3
carboxylic acids (5)	7	3000		3
carboxylic acid cmpds	3	500		
carboxylic acids cmpds (5)	6	52000		3
Decanal			orl rat LD_{50} 3730mg	
n-decane	4	150		
decan cmpds (2)	4	280		3
decanoic acid	3	5000		
Dodecyl alcohol	3	100	ipr rat LD_{50} 800mg	
dodecanol	3	100		3
dodecanoic acid	3	5000		1
decanol, ethoxy-do-1	3	50		
decanol, hexa-	3	20		
dioxalane cmpds	3	600		3
dioxalane, 2,4-dimethyl-1,3-	3	100		
dioxalane, 2-ethyl-4-methyl-1,3-	3	500		

TABLE 2.   SPECIFIC TOXICANTS IN HYGIENE WATER (Cont.)

SECONDARY REQUIREMENTS (CONT.)

Compound	Source	Amt. (ug/L)	Toxicity	Reference
ether, ethylene glycol monobutyl-	3	200		
ether, p-chlorophenyl methoxyisobutyl	4	400		3
ethers, glycol (2)	3	900		3
ethyl alcohol	4	7000	orl man LDLo 50mg	3
	3	2500	(est amt)	3
	7	1000	(est amt)	3
	6	1500	(est amt)	3
furfuryl alcohol	6	200	orl rat LD_{50} 275mg	3
	4	10		
furan, 2,5-di-methyltetra-hydro	4	10		
formic acid	4	2678	orl rat LD_{50} 1210mg	
	3	48100		2
heptanoic acid	3	200	orl mus LD_{50} 160mg	
4-heptanone	6	100		3
	4	25		
heptanone cmpds (2)	4	60		3
heptyl alcohol	4	1		
Hexanol			orl rat LD_{50} 720mg	
hexanol, 2-ethyl-1-	6	100		3
Hexanal			orl rat LD_{50} 4890mg	
hexanoic acid	3	2000		
hydrocarbon, unsaturated	4	30		
2,5-hexanedione	3	50		
3-hexane-2,5-dione	3	100		
lactic acid	3	1000		
lactone cmpds	3	130		3
lactone, delta	3	10		
lactone, gamma	3	60		
lactone, gammahepta	3	30		
levulinate, methyl	3	30		
methyl alcohol	4	7000	orl hmn LDLo 340mg	
	7	1000	(est amt)	3
	3	2500	(est amt)	3
	6	1500	(est amt)	3
morpholine	4	5		
myristic acid	8	10	ivn mus LD_{50} 43mg	
nitrile, methyl benzo	4	400		3
	6	100		3

TABLE 2.   SPECIFIC TOXICANTS IN HYGIENE WATER (Cont.)

SECONDARY REQUIREMENTS (CONT.)

Compound	Source	Amt. (ug/L)	Toxicity	Reference
nonanolic acid	3	2000	orl rat LD_{50} 3200mg	
octanol	4	1		
octanoate, methyl-	3	200		3
oxalic acid	3	370	orl hmn LDLo 700mg	
octanoic acid	3	20000		
octatetraene, formylcyclo	3	30		
palmitic acid	7	10	ivn mus LD_{50} 57mg	3
	3	30		
	8	10		
propionic acid	3	7000	orl rat LD_{50} 1510mg	2
	8	1500		
	7	100		2
salicylic acid	6	300	orl rat LD_{50} 891mg	3
sulfone, methyl-	4	20		
toluonitrile	4	400		
undecanoic acid	3	30	ivn mus LD_{50} 140mg	
n-undecane	4	200		3
vanillan	6	200	orl rat LD_{50} 1580mg	3
	4	30		
Xylenol			orl mus LD_{50} 1070mg	
xylene	4	8		

Number key for the sources:

HUMIDITY CONDENSATE	1
HUMIDITY CONDENSATE (BENDS PERMEATE)	2
TIMES SHR	3
TIMES URINE RAW DISTILLATE	4
VCD URINE RAW DISTILLATE	6
POST TREATED SHR RAW DISTILLATE	7
POST TREATED VCD/TIMES RAW DISTILLATE	8

Key to abbreviations:

Orl = oral
Ivn = intravenous
Ipr = intraperitoneal
Hmn = human
Mus = mouse

Key to toxicity information:

LDLo = Lowest published lethal dose (mg/Kg)
TCLo = Lowest published toxic concentration (mg/Kg)
TDLo = Lowest published toxic dose (mg/Kg)

Papers

1. VCDS Component Enhancement, Testing and Expert Fault Diagnosis
   Development Final Report, Volume II, E.S. Mallinak, December, 1987.

2. Chemical and Microbial Impurity Analysis Results on Recycled Shower
   Water.

3. Recovered Wastewater Impurity Characterization, C.E. Verostko, May, 1986.

4. Chemical Analysis of One Sample Project TDDAE4540F (Spacelab 3)

5. Analysis of Spacelab Humidity Condensate (Spacelab 3) by UMPQUA RESEARCH CO.

6. Chemical Impurity Analyses Results on Spacelab 3 ARS Humidity Condensate

7. Spacelab Condensate Analysis

available. Additionally, as Paul Webb points out, "... in many instances the data in standard handbooks and textbooks have been repeated over and over from one rather skimpy original source."

With the development of modern chromatographic and detection equipment, the list of chemical components in urine has grown considerably. In 1971, Mrochek reported 150 chromatographic peaks from urine samples using a form of liquid chromatography with multiple detectors[9]. By 1982, Tanaka reported 163 organic acids alone were detected in human urine[12]. These two accounts are merely illustrate the vast amount of information being generated regarding physiology in general and urine chemistry in particular using modern analytical, clinical, and physiological techniques[8]. An intensive review of the literature since 1970 would almost certainly reveal many hundreds if not thousands of chemical compounds which are known to occur in urine.

Although hundreds (or more) of compounds have been isolated from urine, Putnam pointed out that a relatively small number (45) make up 98% of solids on a per weight basis[10]. Modern techniques may continue to identify trace components for many years to come, but it is unlikely that the major components were unknown even as early as 1964.

Oxone[R] Chemistry

Oxone[R] is a commercial dry oxidizing agent commonly used in bleaches, cleaning compounds, and the manufacture of certain organic chemicals. Oxone is composed of potassium monopersulfate, potassium hydrogen sulfate, and potassium sulfate in 2:1:1 proportion. Potassium monopersulfate is the active oxidizing

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agent, which decomposes in aqueous solutions to yield mainly O_2 and SO_4^{2-} with small amounts of H_2O_2 and $S_2O_8^{2-}$. The peroxydisulfate ion $S_2O_8^{2-}$ is one of the most powerful and useful of oxidizing agents.

$$S_2O_8^{2-} + 2e^- <=> 2SO_4^{2-} \quad E^O = 2.01 \text{ V}$$
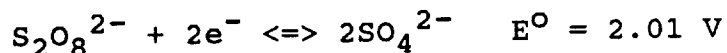
Compare the reduction potential value for the peroxydisulfate ion above with the value for hydrogen peroxide, widely recognized as a very strong oxidant.

$$H_2O_2 + 2H^+ + 2e^- <=> 2H_2O \quad E^O = 1.77 \text{ V}$$
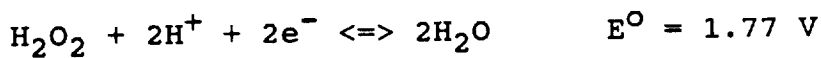
Oxone is known to oxidize phenols to quinones, cyclic ketones to lactones, toluene to benzoic acid, diphenylmethane to benzophenone, olefins to glycols or glycol esters, primary aryl amines to nitroso compounds, and toluene to benzyl halides. Oxone almost certainly oxidizes Chloride ion (Cl^-) to chlorine radicals ($Cl\cdot$) in solution. Given sufficiently strenuous conditions (time, temp. and oxone conc.) oxone is capable of oxidizing almost any organic compound to CO_2 and water. However, the conditions used for oxone pretreatment of urine are obviously not this severe.

The actual oxidation products of the organic components of human urine have not been adequately characterized. Compounds in the urine pretreatment mixture will be oxidized according to their relative concentration and oxidation potential. As a general rule abundant easily oxidized compounds will react (oxidize) to a greater extent than a difficult to oxidize species present in trace amounts. Free radical oxidations are powerful and non-specific, but are generally not the preferred

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tool for organic synthesis as many secondary, undesired products are often produced. As late as 1987 R.J. Bull stated "virtually nothing is known about the by-products formed from the use of ... the oxone process ..." Furthermore, "reaction products arising in water as it is being treated ... cannot be predicted solely on the basis of the nature of the treatment chemical (or its formulation)"[4].

The "detailed analytical study of urine distillate" reported by Jolley in 1990 [7] identified a little more than one fourth (12 of 44 and 20 of 77) of the gas chromatographic peaks produced using a Hall detector selective for halogenated organics in oxone pretreated distillate. A "strongly absorbing chromaphore" detected in HPLC analysis was not identified even though it could represent a significant portion of the 293 ppm TOC measured . TOC accountabilities for this study ranged from 29% to 292%. This may be in part due to sample variability but the lack of detail regarding the methods and analytical control provisions employed makes this difficult to evaluate. There is no mention of how calculated TOC values were produced for compounds reported as <2 or <50 ppm. It appears that the maximum value was used to give the illusion of better TOC accountability. For example, it seems unlikely that 75% of TOC can be accurately accounted for in the 1st distillate sample of Oxone II pretreatment (table 3-17) when only 8 of 37 gas chromatographic peaks were accounted for. Chromatograms with large numbers of unidentified compounds must be interpreted carefully regarding relative concentrations of unknowns. A small amount of a strong chromaphore may produce a larger UV Absorbance peak than a large amount of a very weak

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absorber. There are analogous effects for many other kinds of detectors. A small amount of an unknown with a high refractive index may produce a larger refractive index peak than a large amount of another unknown component with a refractive index almost identical to that of the mobile phase[11].

In a Center for Life Support methods development feasibility study[2], over 30 organic acids and related compounds were detected in a relatively simple HPLC analysis of urine (Figure 1). Oxone pretreatment removed some compounds (peaks) but produced a few new compounds (peaks) not originally present (Figure 2). It appears that the total concentration of urinary acids decreases after pretreatment, but a significant number are still present in detectable amounts. It is important to note that relative peak size is not necessarily an indication of the relative concentrations of unknown compounds.

2.0 Technical Approach and Research Plan

Recent development work by the Center for Life Support suggests the use of broad methods development techniques designed to detect, identify, and quantify entire chemical classes is problematic. Complete characterization of samples of unknown composition is a notoriously difficult problem in analytical chemistry. The problem is compounded in this case since the sample is a complex mixture with many components at very low levels and a few components at high levels. A more specific methodical approach is called for.

A more thorough review of clinical literature is essential

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Figure 1
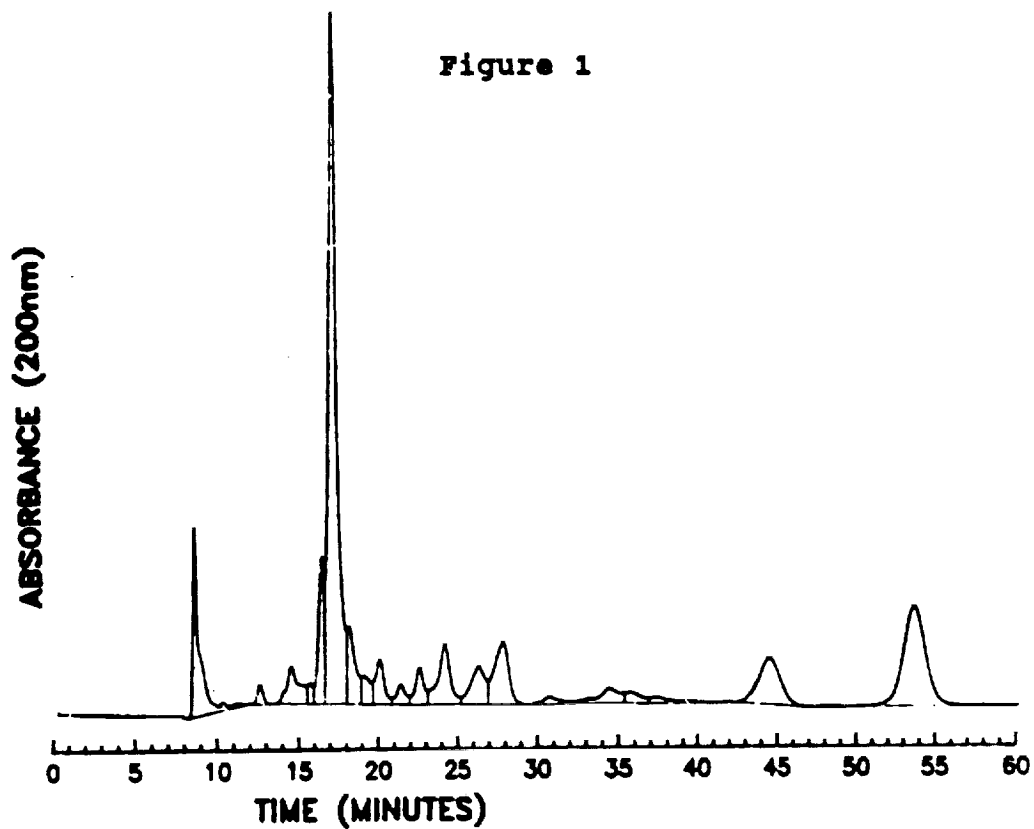
Urinary organic acid profile of a urine sample prior to oxone/sulfuric acid pretreatment

Figure 2
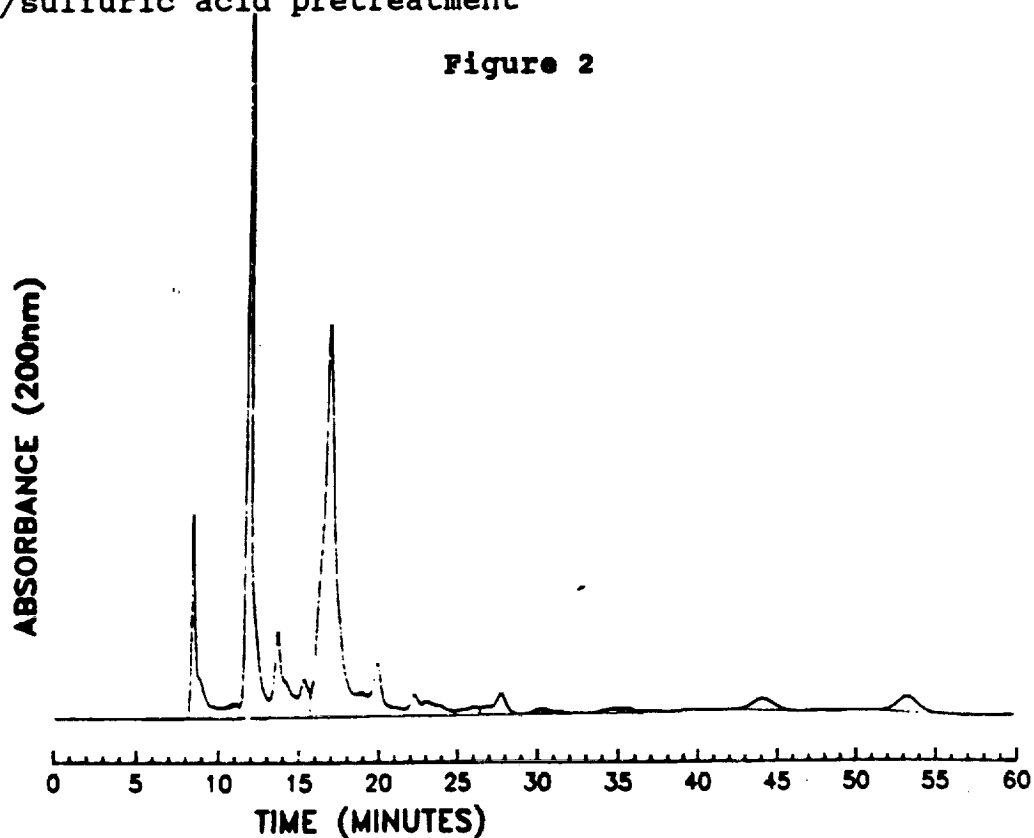
Urinary organic acid profile of a urine sample after oxone/sulfuric acid pretreatment

to this type of analytical effort. A good deal is known about the chemistry of human urine, but this knowledge has not been sufficiently exploited in addressing the problem of organics characterization in oxone pretreatment.

As Putnam pointed out[10], the majority of the TOC content in urine may be due to relatively few compounds with many more present in trace quantities. The major constituents should be targeted in order of occurrence for detailed chemical study.

3.0 DETAILED TEST PLAN

Objective 1 - Target, analyze, and quantify the major organic components in urine before and after pretreatment

(1) Urea - The most abundant chemical species, urea constitutes approximately 50% of the total dissolved solids in human urine. The concentration of urea is approximately 14 g/l. Urea hydrolyzes readily in acid or base or thermally in neutral solution, and is probably converted quantitatively to CO_2 by oxone pretreatment.

(2) Creatinine - The second most abundant organic compound in urine, excreted at a rate of approximately 1.5 g/l, polar very water soluble, cannot be analyzed be GC without derivatization but standard clinical methods exist. Creatinine has not been isolated in raw distillate and the amount oxidized in pretreatment is not known. Predict what compounds may result when creatinine is partially oxidized based on literature review. Determine creatinine concentration before and after oxone pretreatment. Review the chemical and analytical literature and target creatinine oxidation products.

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(3) Hippuric acid - The glycine conjugate of benzoic acid, concentration is approximately 1.25 g/l in urine, most likely converted to benzoic acid during oxidative pretreatment. Hippuric acid is not amenable to simple GC analysis, but LC methods exist.

(4) Citric acid - A six carbon tricarboxylic acid important to the Krebs cycle for carbohydrate metabolism in resting muscle tissue. The concentration in urine is about 0.75 g/l. A review of the organic chemistry literature may reveal likely oxidation products.

(5) Glucuronic acid- This sugar acid is present in urine in the free state but primarily as the hydrolysis product of various glucuronide conjugates produced by the body to make toxic elimination products water soluble. Its concentration in urine is about 0.6 g/l.

(6) Uric acid - The chief product of purine metabolism, uric acid is present at about 0.5 g/l in urine.

(7) Uropepsin - A group of proteolytic enzymes for the hydrolysis of proteins. Present in urine at approximately 0.4 g/l. Uropepsin and other proteins would likely be hydrolyzed to short peptides and free amino acids with acidic pretreatment.

(8) Creatine - The precursor of urinary creatinine, creatine, largely in the form of phosphocreatine, plays a role in muscle contraction and carbohydrate metabolism. Creatine is converted to creatinine on treatment with acid. The concentration in raw urine averages 0.4 g/l.

(9) Glycine - The simplest amino acid, glycine conjugates are common elimination products. The concentration is

approximately 0.3 g/l in urine.

(10) Phenols - Hydroxylated benzenes, these compounds are about the only abundant chemical class in urine amenable to GC analysis without derivatization. Total phenols are present at about 0.3 g/l.

(11) Lactic acid - Major by-product of carbohydrate metabolism in muscle tissue during exertion. Lactic acid is responsible for causing muscle soreness after exercise. The average concentration in urine is about 0.3 g/l depending on activity level.

(12) Histidine - Essential amino acid, eliminated at a concentration of approximately 0.2 g/l in urine depending on protein intake.

These 12 compounds contribute about 90% of the organic carbon containing compounds in "average" human urine. Water quality requirements seem to require that at most 90% of TOC be accounted for to unsure total toxic organics less than 1000 ppb.

Although the types and especially the amounts of compounds present in urine, pretreated urine, raw distillate, and hygiene product water may be different it is reasonable to propose that compounds found in pretreated urine (the oxidation products of the raw urine constituents) will be the major organic constituents of hygiene product water. Distillation, filtration, disinfection and deionization are not expected to produce as many new compounds as oxidative pretreatment. Certainly distillation and post-treatment will essentially remove some compounds present in pretreated urine, and change the relative concentrations of others. Disinfection involving iodine and high temperatures most

likely produce iodine derivatives of some compounds. However for the purposes of methods development planning and emphasis it may be helpful to assume that compounds not present in pretreated urine will not be found in raw distillate or hygiene product water in significant quantities. The most effective means of assessing the chemical consequences of oxone pretreatment may be to analyze for these major constituents and their most likely oxidation products based on a thorough literature review. Oxidation/Reduction potential data for organics in the literature may provide a basis for postulating likely oxidation products formed during pretreatment.

Objective 2 - Research and Develop Analytical Methods

Reliable standard wet chemical methods are available for many major urine constituents[5]. These clinical methods, with established detection limits and interferences and may serve in a first approximation assessment of the fate of these compounds during oxidative pretreatment. Sample preparation and concentration techniques in these methods may be adapted for use in more general chromatographic methods.

Gas Chromatography/Mass Spectrometry (GC/MS) methods for volatile and semi-volatile organics are well developed and excellent standard methods are available from EPA[1], APHA[6] and others. Using EPA Method 624 or possibly the new 524 quality assurance, tuning, and sample preparation procedures with a modified temperature program a wide variety of unknown volatile purgables could be identified.

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HPLC is rapidly becoming an important and well developed tool for the analysis of non volatile organics especially those of biological origin. Standard methods promulgated by regulatory agencies are not as common for HPLC analysis but many are featured in the analytical and clinical chemistry literature[8]. Organic acids and proteins are the most common chemical classes eliminated in urine, and the prospects for eventually developing broad LC methods for these two groups is good.

Objective 3- Identification of Unknowns

The identification of chromatographic peaks in unknown samples is never simple and becomes quite difficult in complex mixtures with many analytes present in near trace amounts. In simple samples where the compounds to be identified are known, identifications are often made on the basis of retention time comparison with reference compounds. This is most effective for ruling out a particular compound, since if there is no peak at the retention time of the reference compound (in the sample matrix by "spiking", not in reagent solvent) that compound is not present in the sample above the detection limit. However, retention time is not certain proof that a peak which is present was produced by the reference compound. Coelution is rather common for closely related compounds, especially when chromatographic conditions were selected for a much broader range of compounds and in liquid chromatography in general where peak shape and absolute retention time are more variable that in capillary GC. Use of a "confirmation" analysis with different chromatographic conditions can remove most of the ambiguity in

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retention time based identifications, since different compounds are unlikely to coelute by both methods. This approach is very laborious, however, with many separate analytical runs of samples spiked with each reference compound in addition to blanks and quality control samples by each of two different methods.

Spectrophotometric (UV/Vis, fluorescence, diode array) and electrochemical detectors (potentiometric) measure physical/chemical properties of the analyte rather than the difference in bulk properties of the mobile phase and analyte (e.g. Refractive index, conductivity). This can be exploited to provide identification information. It is unlikely that two coeluting compounds will have the same ratio of uv absorbance at two wavelengths, and they could not possibly have the same uv/vis spectrum as measured with a diode array detector. In fact, the diode array spectrum of a analyte can be can be used to identify an unknown for which there is no reference compound, by comparison with spectral data in the literature and software libraries. UV/Vis and fluorescence spectra are dependent on solvent (mobile phase) composition and other factors, like pH, so identification by matching with reference spectra is not as straight forward as in mass spectrometry.

Collecting fractions and performing off line derivatization and other wet chemical methods of identification, or performing IR spectroscopy on each compound as it elutes is still the most powerful, versatile and certain of the identification methods. Unfortunately it talks a tremendous amount of very skilled manpower to perform this kind of characterization on a completely

unknown complex mixture. It also requires large amounts of sample when analytes are present at low levels to obtain enough purified analyte for subsequent wet tests or spectroscopy.

Liquid Chromatography/Mass Spectrometry (LC/MS) has perhaps the best identification potential of any on-line LC detector[2]. While the mass spectra produced by LC/MS are somewhat more complicated (especially thermospray spectra) than those produced by GC separation or Direct Insertion Probe (DIP) sample introduction, identification by spectral matching is simpler than with UV/Vis or fluorescence spectra. Unknowns may be identified by interpretation of their mass spectra by a skilled mass spectroscopist using established rules and techniques involving isotopic ratios and fragmentation. This identification from known principles is generally not possible in UV/Vis or fluorescence spectroscopy.

Objective 4 - Perform and report method validation studies and analytical quality control

The use of reference compounds, standards, ersatz or other knowns is essential to the successful efficient development and qualification of more elegant chromatographic methods suitable for whole classes of compounds. In addition to their role in establishing recoveries, detection limits and otherwise verifying method reliability, known reference compounds and mixtures of them are very helpful in developing and optimizing chromatographic conditions, extractions, and other phases of sample preparation and analysis. Blindly analyzing

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uncharacterized samples without a documented method qualification process involving known mixtures is not productive , and is not considered good practice. Without this information and proper analytical control data, it is impossible to determine the validity of data reported for actual samples.

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APPENDIX I

PERTINENT LITERATURE ARTICLES REGARDING VIRUS SURVIVAL

AND DETECTION IN WATER

CHAPTER 21

Application of Gene Probes to the Detection of Enteroviruses in Groundwater

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Nutrition and Food Sciences, University of Arizona, Tucson, Arizona

INTRODUCTION

Rapid and low-cost methods for the detection of enteric viruses in water have been sought by water virologists for many years. Virus detection in water requires passage of 400–1000 liters of water being sampled through a filter to which the viruses adsorb. Viruses eluted from these filters are then assayed in animal cell culture.[1] Although cell culture techniques are now relatively simple to perform, this assay system does have several drawbacks. One drawback is that incubation periods of three days to six weeks may be required before cytopathogenic effects (CPE) are observed. A second problem with this assay system is the lack of one cell line that will permit the replication of all enteric viruses.[1] In addition, some enteric viruses, such as hepatitis A virus, do not exhibit cytopathogenic effects in cell culture.[2] Other viruses, such as Norwalk virus, have not yet been grown in cell culture. In addition, cell culture is very expensive and can cost between $300 to $750 per sample.

More rapid techniques that are commonly used in the human clinical laboratory, such as fluorescent antibody (FA) or radioimmunoassay (RIA), lack the needed sensitivity necessary to detect the low numbers of viruses found in contaminated water.[3-5] Since low numbers of viruses in water are believed significant in the spread of waterborne disease, methods must be capable of detecting as few as 1–10 infectious viruses in 100–1000 liters of drinking water.[6]

Current advances in DNA technology using gene probes now provide a method for identifying the genes of any organism. Gene probes are small

pieces of complementary DNA or RNA that have been labeled with either an isotope or a nonradioactive compound such as biotin.[7] The development of gene probes against enteric viruses now makes possible the rapid detection of enteric viruses in water. Up to 96 concentrated water samples can be probed at one time providing results within 72 hours.

MATERIALS AND METHODS

Cells and Viruses

Poliovirus type 1 (LSc) was assayed and grown in the Buffalo Green Monkey (BGM) continuous cell line. All assays were by the plaque-forming unit (PFU) methods.[2] Hepatitis A virus (enterovirus type 72) strains HAS 15, CR326, and HM 175 were grown and assayed in the FRhK6 continuous cell line. The hepatitis A virus (HAV) was quantitated by radioimmunofocus assay.[8]

Probes and Hybridizations

Two different cDNA probes were used for enterovirus detection. A poliovirus type 1 (Mahoney) cDNA probe (base pairs 115–7440) inserted into the Pst 1 site of the plasmid pBR322 was provided by Drs. Rancaniello and Baltimore. The second probe contained the first 1380 base pairs from the 3' end of the hepatitis A virus. The poliovirus and hepatitis A virus cDNA probes were grown in transformed Escherichia coli HB-101. The cDNA probes were extracted from the E. coli and isolated on a cesium chloride/ethidium bromide gradient as previously described in Maniatas et al.[7]

The probes were labeled with ^{32}P dCTP and ^{32}P dATP (specific activity 3000 Ci/mmole) using nick translation to a specific activity of 2.0×10^9 cpm/μg of DNA or greater. The entire pBR322 plasmid along with the viral cDNA insert was used as the probe rather than the insert alone.

Prehybridizations and hybridizations were done at 44°C in sealable plastic bags according to the procedures of Thomas.[9] Approximately 1.0×10^7 counts were added to each hybridization bag and hybridizations were carried out for 24–36 hours in a water bath with constant agitation. Hybridization membranes were washed in a $2 \times$ SSC (0.3 M sodium chloride, 0.03 M sodium citrate) solution at room temperature for 10 minutes. This was then followed by a second wash using $2 \times$ SSC, 1% SDS (sodium dodecyl sulfate) at 52°C for 30 minutes. A final wash of $0.2 \times$ SSC was done at room temperature for 15–30 minutes. Results were visualized by autoradiography for 24–36 hours at −70°C.

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h of 0.2 × SSC was done at room
e visualized by autoradiography

Sample Analysis

Samples were first centrifuged to clarify and remove any large particles. To liberate the viral genomes, samples were originally phenol/chloroform extracted according to the methods of Maniatas et al.[7] This was followed by two chloroform extractions and then by a water-saturated ether extraction. Dissolved ether was removed by bubbling air through the mixture until traces of ether were gone.

Further research done in our laboratory demonstrated that the genome of poliovirus could either be liberated or exposed from the viral protein coat by heating the sample to 65°C for 30 minutes. RNasin was added prior to heating the sample to inhibit RNase activity and prevent RNA degradation from occurring once the genome had been liberated and/or exposed. Water samples (where indicated) were processed in this manner to liberate the viral genome rather than using a phenol/chloroform extraction.

RESULTS

Table 1 demonstrates that poliovirus and hepatitis A virus were detected in seeded tap water with sensitivities equal to the PFU and RIFA assays. To determine if beef extract used in viral elution would interfere with hybridization, 3% beef extract seeded with poliovirus and beef extract without poliovirus was concentrated by organic flocculation and then assayed by the PFU method and the gene probe assay for the presence of virus. Table 2 indicates that beef extract does not interfere with the sensitivity of the gene probe assay nor does it produce false positive results. Poliovirus was detected with equal sensitivities in both assay systems. Table 3 indicates that the sensitivity of poliovirus detection in seeded tap water was equal for the PFU method and the phenol/chloroform extractions, but there was an increased sensitivity in virus detection for the heat-treated sample. Table 4 shows the results of the different groundwater samples that were assayed for virus. Phenol/chloroform extraction of samples or heat treatment of samples are indicated in the table.

DISCUSSION

The results of this research describe the development of a cDNA probe capable of detecting as few as 1 PFU of poliovirus or 1 RIFA unit of hepatitis A virus within 72 hours. Beef extract, which was used to elute viruses from filters did not seem to interfere with the sensitivity of the assay nor did it create false positive results. Phenol/chloroform extractions liberated viral genomes and permitted the detection of viral RNA with sensitivities approxi-

Table 1. Sensitivity of the Dot Blot Assay for Hepatitis A Virus and Poliovirus Detection

Virus	Titer	Dot Formation (Virus Dilutions)					
		10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
Hepatitis A							
HAS 15	9.5×10^5 RIFA/mL	+	+	+	+	−	−
CR 326	2.0×10^6 RIFA/mL	+	+	+	+	−	−
HM 175	1.3×10^6 RIFA/mL	+	+	+	+	−	−
Polio 1							
LSc	1.2×10^4 RIFA/mL	+	+	−	−	ND[a]	ND

[a]ND = not done.

Table 2. Effects of Beef Extract on the Dot Blot Assay for Poliovirus Detection

Sample	Poliovirus PFU/mL	Dot Formation (dilutions)			
		10^{-2}	10^{-3}	10^{-4}	10^{-5}
Beef Extract Concentrate	4.2×10^4	+	+	+	−
Beef Extract	0	−	−	−	−

Table 3. Comparison of Phenol/Chloroform Extraction and Heat Treatment for the Detection of Poliovirus

Method	Dilution Series of Poliovirus						
	10^0	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
PFU	+	+	+	+	+	−	−
Phenol/chloroform extraction	+	+	+	+	±	−	−
Heat treatment	+	+	+	+	+	±	−

mately equal to tissue culture. Heat treatment of the samples seemed to increase the sensitivity of the assay, making it more sensitive than tissue culture.

The gene probe assay does not first require virus growth in cell culture. This allows samples to be probed directly and decreases the assay time. It also allows for the detection of viruses that may not be infectious, but still retains their genome. However, for untreated groundwater used for drinking water, this test is ideal, because it can screen many samples in a short time to determine if virus is present. Untreated groundwater should not contain any viruses, and hence the gene probe assay can be used as a rapid assay to determine if a sample is contaminated with viruses. Samples which were positive by gene probe assay but negative by tissue culture may have contained viruses which were below the detection limit of tissue culture but were

Hepatitis A Virus and Poliovirus

	Dot Formation (Virus Dilutions)				
	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
+	+	+	-	-
+	+	+	-	-
.	+	+	-	-
+	-	-	ND[a]	ND

t Assay for Poliovirus Detection

	Dot Formation (dilutions)		
10^{-2}	10^{-3}	10^{-4}	10^{-5}
	+	+	-
	-	-	-

Extraction and Heat Treatment for the

...les of Poliovirus

10^{-3}	10^{-4}	10^{-5}	10^{-6}
+	+	-	-
+	±	-	-
+	+	±	-

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...re virus growth in cell culture. ...nd decreases the assay time. It ...may not be infectious, but still ...ted groundwater used for drinking ...n many samples in a short time ...groundwater should not contain ...ay can be used as a rapid assay to ...viruses. Samples which were ...y tissue culture may have con- ...tion limit of tissue culture but were

Table 4. Detection of Naturally Occurring Enteric Viruses in Water Using Gene Probes vs Tissue Culture

Sample	Tissue Culture (CPE)	Gene Probe	
		Phenol/Chloroform	Heat Treatment
1	+	+	+
2	+	+	+
3	+	+	+
4	-	+	+
5	-	+	+
6	-	+	+
7	-	-	-
8	-	-	-
9	-	ND[a]	+
10	+	ND	+
11	+	ND	+
12	+	ND	+
13	+	ND	-

[a]ND = not done.

detected by the gene probe assay. Also, the samples may have contained inactive viruses which could not be replicated in cell culture but were detected by the gene probe assay. Since the samples in Table 4 are undisinfected drinking water samples, these samples should not contain any viruses. Sample 13 (Table 4) indicates tissue culture assay was positive, while gene probe assay was negative. Research in our laboratory has shown that the poliovirus cDNA probe will cross-hybridize with other enteric viruses such as coxsackie B and echoviruses but does so with almost a two-log reduction in sensitivity when compared to tissue culture. Such viruses may be detected by tissue culture but go undetected by the gene probes used in this study.

Upon comparison with tissue culture, the gene probe assay is more rapid and sensitive than cell culture. Phenol/chloroform extractions were one original drawback of the gene probe assay. These extractions required the user to be exposed to potentially harmful organic solvents and were often long and tedious when very proteinatious samples were encountered. Heat treatment of the sample increases the simplicity of the gene probe assay. Samples are treated by adding protease K and then incubating at 65°C for 30 minutes. Prior to this, RNasin is added to help prevent RNA degradation.

Current costs of water analysis for virus contamination can exceed $500 per sample and can take as long as 3–6 weeks for results. The gene probe assay was reliable and sensitive and provided results within 72 hours. Also, the gene probe assay will reduce the cost of testing water to under $150. This type of low-cost sensitive assay will permit water utility companies to monitor for the presence of viruses.

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Elimination of Viruses and Bacteria During Drinking Water Treatment: Review of 10 Years of Data from the Montreal Metropolitan Area

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INTRODUCTION

Pathogenic microorganisms, found in water to be utilized for the preparation of drinking water, should ideally be completely removed or inactivated by the treatment processes applied at the water filtration plant. However, many bacteria, viruses, and parasites have been found to be resistant to one or more of these treatments. The detection of viruses in drinking water meeting current bacteriological standards of quality is a rare occurrence, but since the advent of more reliable methods for their detection the number of reports describing their presence has been increasing. Our own interest in the dissemination and survival of human and animal enteric viruses in water has led us to study not only their presence in surface water, but also their survival during drinking water treatment as well as the health risk they may constitute. The present paper is a review of the results and experience accumulated in our laboratory since 1975.

METHODS

The methods used in our laboratory for the concentration of viruses in water have not been modified much since 1976. The water to be tested is conditioned to pH 3.5 and 0.0015 M aluminum chloride to enhance virus adsorption to the electronegative cartridge filters. After filtration of at least

1000 liters of water, the filters are eluted using an alkaline beef extract solution (1.5%, pH 9.75) that is then flocculated at pH 3.5 to obtain a final volume of concentrate of less than 50 ml, easily assayable in cell culture. The major differences over the years in the assay of these samples has been the use of more sensitive methods, increasing the number of virus types that can be detected. More susceptible cell lines as well as new assay methods have greatly enhanced the overall sensitivity. Our current preferred assay is the use of an immunoperoxidase method with Buffalo Green Monkey kidney cells (BGM) or MA-104 Rhesus kidney cells, which is up to 50 times more sensitive than the previously used cytopathogenic effect method on the same cell lines.

PRESENTATION OF RESULTS

In 1981, we published data showing that viruses were present in all raw and treated drinking water samples tested over a one-year period at a local drinking water treatment plant (plant PV). The plant was using a complete conventional treatment, including prechlorination, flocculation with alum, dynamic sedimentation, slow sand filtration, ozonation, and a final chlorination. At the time, such reports were rare and critiques were rapidly aimed at such causes as laboratory contamination of our samples. This plant was treating water abstracted from a river heavily polluted by untreated sewage discharges and, as discovered over a period of years, was not always properly operated. These poor operation procedures were compounded by an aging plant: flocculation was not always optimal, chlorination levels were not carefully monitored, the ozone generators were not functioning properly, and treatment basins had dead ends, resulting in under treatment. All these reasons were probably sufficient to explain the presence of viruses in the drinking water prepared from river water containing up to several thousand viruses per liter. However, because some treatments such as prechlorination and flocculation reduce dramatically the number of bacteria in water, this plant was still able to produce water meeting current bacteriological standards.

To demonstrate if similar observations could be made at other plants, a Canadian collaborative study among three laboratories was initiated with the financial support of Health and Welfare Canada. Nine drinking water treatment plants, located in the cities of Ottawa, Toronto, and Montreal, were sampled for two years: raw (100 liters) and finished water (1000 liters) were sampled monthly. No viruses were found, but the sensitivity of the cell line used for virus isolation was later found to be very low.

During the same period, we initiated a collaborative study with the Ministry of Environment of Quebec, and in 1982–83 we sampled seven drinking water treatment plants twice monthly for a year. At each plant, 100 to 1000

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liters of water were sampled after each treatment: raw water, prechlorination
(if possible), sedimentation, filtration, ozonation, and after the final chlori-
nation. Numerous bacteriological and physicochemical analyses were per-
formed. Essentially, all bacteria present in the abstracted water were elimi-
nated by the treatments and, more specifically, by prechlorination and floc-
culation, which will reduce to barely detectable levels most indicator micro-
organisms. Bacteria from the total coliform group, as well as some *Pseudo-
monas aeruginosa*, are occasionally detected in finished water, but they are
mostly below the limit of detection of the standard 100 ml test. These low
levels of bacterial contaminants are not likely to be of public health signifi-
cance because the minimal infective dose (the number of bacteria required
to initiate the infection) is usually high.

When these waters were tested for the presence of human enteric viruses,
the results were quite different (Tables 1 and 2). While the number of infec-
tious viral particles was rarely above 100 viral particles per liter in the river
water used, we were still able to detect viruses in finished water at levels of
0.003 to 0.020/liter (3 to 20 viruses/1000 liters).

Table 1. Virus Elimination During Drinking Water Treatment

Sample	Positive/Total	Virus Density[a]	Residual Virus (%)
Raw	120/152	3.36	100
Chlorinated	11/17	0.072	2.1
Sedimented	23/119	0.016	0.47
Filtered	17/119	0.001	0.03
Ozonated	4/45	0.0003	0.009
Finished	12/138	0.0006	0.018

[a]Virus density expressed as the most probable number of cytopathogenic units per liter
(mpncu/L) and as the average of all samples.

Table 2. Viruses Isolated During Drinking Water Treatments at Water Filtration Plants

Sample	Polio 2	Polio 3	Coxsackie B4	Coxsackie B5
Chlorinated	0	0	0	1
Sedimented	2	6	2	1
Filtered	1	7	3	2
Ozonated	2	2	0	0
Finished	0	9	0	2

The presence of these viruses in drinking water or at any step of treatment
did not correlate with the presence of any other bacterial parameter or any
physicochemical parameter: the only positive correlation was that if viruses
were present at detectable levels in the raw water, they could be detected in
the finished water (Table 3).

Thus, our conclusion at the time was: in order to obtain virus-free drinking
water, raw water as clean as possible should be used. More recent virological
analysis of drinking water at two plants has not revealed the presence of any

Table 3.   Correlation Analysis[a] of Virus Density with Bacteriological Data

Water	Plate Count	Total Coliforms	Fecal Coliforms	P. aeruginosa
Raw	0.56	0.68	0.59	0.41
Chlorinated	0.34	0.07	0.04	0.05
Sedimented	0.19	0.03	0.001	0.06
Filtered	0.09	0.09	0.12	0.20
Ozonated	0.11	−0.06	NA[b]	−0.05
Finished	0.06	−0.04	NA[b]	−0.02

[a]Correlation analysis of parameters by Pearson test.
[b]NA = not applicable.

virus; however, these two plants, which had been shown to produce virus-positive drinking water earlier, have been physically modified (new filters, better ozone generators, new settling tanks, etc.) or the operating procedures have been optimized (better control of chlorination and flocculation).

The presence of viruses in the finished water, even after complete treatment, could be explained by two hypotheses: they were resistant to disinfection by chlorine or they were protected by particulate matter. We tested all virus isolates that were detected in treated waters for their resistance to free chlorine. Their resistance to 0.5 mg/L free residual chlorine was highly different (Figure 1). Coxsackievirus B-5 isolates were the most resistant: even after two hours in the presence of residual chlorine more than 20% of the original virus was still infectious. For a similar period of treatment, all poliovirus isolates were reduced to barely detectable levels.

Their presence in drinking water had to be explained by another hypothesis. To determine the physical state of viruses in contaminated river water, we have used a filtration method that has shown that most viruses are free or harbored by particulates less than 0.2 microns in diameter (Figure 2).

VIRUS SURVIVAL WITH 0.5mg/l FREE CHLORINE

- Coxs. B-5
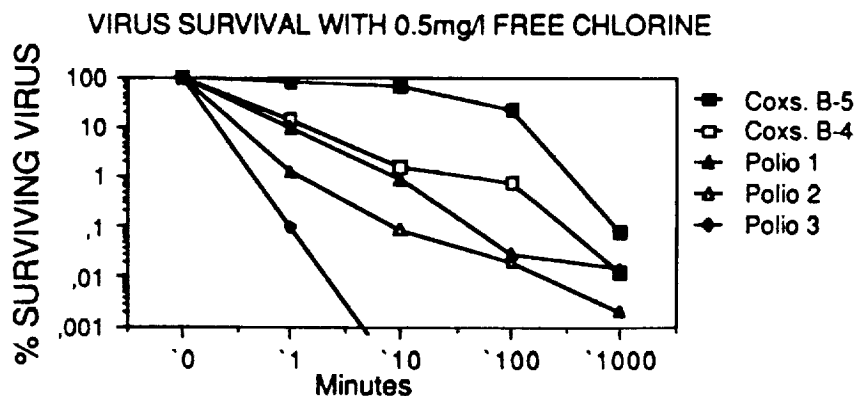- Coxs. B-4
- Polio 1
- Polio 2
- Polio 3

Figure 1.   Inactivation by free residual chlorine (0.4 mg/L) of viruses isolated from treated drinking waters. While poliovirus strains are reduced by more than 99.9% in less than two hours, coxsackievirus strains are much more resistant.
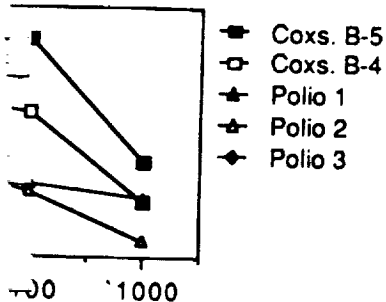
... with Bacteriological Data

...	Fecal Coliforms	P. aeruginosa
	0.59	0.41
	0.04	0.05
	0.001	0.06
	0.12	0.20
	NA[b]	−0.05
	NA[b]	−0.02

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...microns in diameter (Figure 2).

REE CHLORINE

- Coxs. B-5
- Coxs. B-4
- Polio 1
- Polio 2
- Polio 3

...00   '1000

...g/L) of viruses isolated from treated
...J reduced by more than 99.9% in less
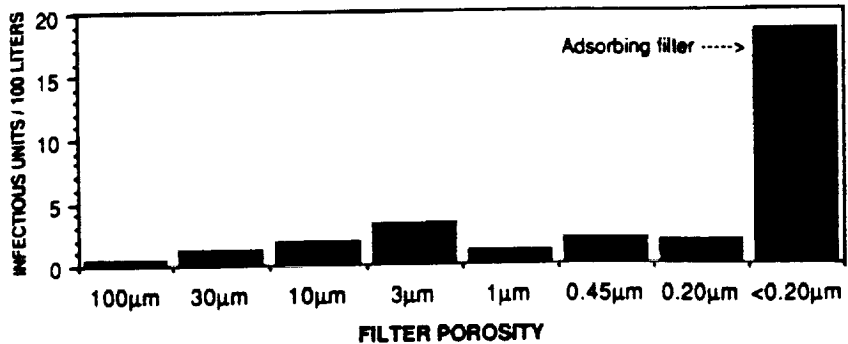... m... ...ore resistant.

ENTERIC VIRUSES

Figure 2. Distribution of human enteric viruses on non-virus-adsorbing filters of selected porosities and on a virus-adsorbent filter. The presence of a large number of viruses on the virus-adsorbing filter indicates that these viruses are embedded or adsorbed to particles that are less than 0.2 μm in diameter.

Because most viruses have diameters that are less than 0.1 micron, these viruses are probably free in the water. The presence of viruses in treated water thus remains to be explained.

As the minimal infective dose of viruses is near 1 infectious particle, the risk of human infection is probably more elevated than for bacteria, for which the number of cells required for inducing infection is more elevated. Dr. C. P. Gerba (University of Arizona), has prepared for the EPA/AAAS a report on the possible health effects of these low doses of viruses present in water.[1] Table 4 summarizes the possible risks estimated by Dr. Gerba according to the viral concentration in water and the infection rate of the virus.

From this table, it is evident that even very low levels of viruses could be the cause of an increased incidence of enteric viral illnesses. The average incidence of gastrointestinal illnesses in the North American population is about 50 episodes/week/1000 individuals, with 1.5 episodes/person/year. For a population of 190,000 individuals in the above example, this is equivalent to about 500,000 episodes per year. These values indicate that the fraction of illnesses due to viral contamination of water at a level of 1 virus/100 liters is low for rate of infection of 1% but can be high for high attack rates. Further analysis of the effects of these low virus levels remains to be evaluated; it is in this direction that our laboratory is now heading.

With the support of the U.S. EPA and of Health and Welfare Canada, we are hoping to initiate an epidemiological surveillance of several hundred families to determine if any health effect is attributable to the presence of these viruses. Preliminary data were obtained during a pilot project to evaluate the feasibility of such a study. Some of the results are presented in Tables

Table 4. Expected Infection Incidence In a Population with 190,000 individuals at Two Attack Rates

Virus Density	Infections/Year	
	Attack Rate 1%	Attack Rate 30%
1/10 liter	140,000	4,000,000
1/100 liter	14,000	400,000
1/1000 liter	1,400	40,000
1/2000 liter	730	20,000

5 and 6. Data obtained by telephone interviews include water perception in two areas during spring and autumn 1986 as well as the incidence of gastrointestinal symptoms in the spring period of 1986 for bottled water and tap water. From these results, it would appear that the effect of drinking water on the incidence of gastrointestinal illnesses in our area is small: bottled water drinkers have only a slightly reduced incidence of illness. This is, however, in agreement with the hypothesis that only about 5% of these illnesses would be attributed to water.

CONCLUSION

After ten years of experience in the field of environmental virology and particularly in water treatment virology, we have gained some knowledge of the behavior of human enteric viruses, not under laboratory conditions but directly at the water treatment plant. As expected, the theories elaborated from laboratory scale experiments do not always correlate with the experimental data. Viruses were detected after treatments that should theoretically have eliminated more than 12 log of viruses. The data obtained at several water treatment plants have convinced us that, except under optimal conditions, most filtration plants will not remove all human enteric viruses. The low-level viral contamination observed may not be a health problem, but

Table 5. Water Quality Perception in Three Different Cities In Spring and Autumn 1986

	Spring			Autumn		
	Rep.[a] (%)	T-M[b] (%)	Laval (%)	Rep. (%)	T-M (%)	Laval (%)
Excellent	2	4	5	5	8	8
Very good	4	15	19	14	24	23
Good	16	47	48	38	46	47
Poor	23	20	19	31	17	17
Very poor	54	13	8	12	5	5

[a]Rep. = City of Repentigny.
[b]T-M = Terrebonne-Mascouche.

TREATMENT

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Infections/Year	
%	Attack Rate 30%
	4,000,000
	400,000
	40,000
	20,000

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	Autumn		
	Rep. (%)	T-M (%)	Laval (%)
	5	8	8
	14	24	23
	38	46	47
	31	17	17
	12	5	5

(

Table 6. Two-week Incidence of Gastrointestinal Symptoms (Spring 1986)

Symptom	Repentigny		Terrebonne	Laval
	Tap Water (%) (N = 626)	Bottled Water (%) (N = 402)	Tap Water (%) (N = 863)	Tap Water (%) (N = 813)
Diarrhea	6.9	6.8	5.6	6.0
Nausea and vomiting	3.6[a]	2.5[a]	1.6	1.2
Nausea and cramps	7.0[a]	5.7[a]	4.3	3.4
Vomiting and cramps	3.1[a]	2.0[a]	1.3	1.2
All symptoms	11.4[a]	9.3[a]	7.5	8.1

[a]Significantly different.

only further studies will establish if such problems exist. Preliminary epide-
miological data have enabled us to show that the increased incidence due to
drinking water is small and will require the surveillance of large populations
to demonstrate any effect. Until then, because these viruses are so resistant
to actual water treatment, they can be used as indicators of appropriate water
treatment to detect deficiencies.

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Enteric Viruses and Coliphages in Latin America

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Abstract

Drinking and other types of waters in three Latin American countries were sampled for the presence of enteroviruses, rotaviruses, and coliphages. Large volumes of water and sewage were concentrated using a positively charged filter for the detection of enteric viruses. Statistical analyses indicated no correlation between the presence or absence of fecal coliforms, total coliforms, fecal streptococci, and viruses. Total coliforms and fecal streptococci were isolated in large numbers from pristine tropical rain forest streams, but no enteric viruses were detected in any of the same samples. All streams contaminated with sewage contained enteric viruses and high levels of indicator bacteria. These results indicate that at the present time there is no reliable indicator of the presence of viruses in waters. The presence of coliphages in waters seemed associated with fecal contamination. The large numbers of fecal streptococci and coliforms (both fecal and total) present in the waters sampled may not necessarily indicate that these waters are contaminated with fecal waste.

INTRODUCTION

Studies on the occurrence of viruses in waters in Latin America are scarce. This is possibly due to the high cost of sampling and assaying for their presence. Many (if not most) of the gastroenteritis outbreaks in these areas are probably of viral etiology. Studies on the incidence of rotaviruses in Colombian hospitals with children afflicted with gastroenteritis have indicated that rotavirus may be one of the most important agents of gastroenteritis (Colombian National Institute of Health, personnal communication). Water has been implicated in some outbreaks (Sutmoller *et al.*, 1982; Tulshinski *et al.*, 1982; Zamotin *et al.*, 1981). Water was also suspect in at least two outbreaks of gastroenteritis in 1983 and 1984 in Colombia (Toranzos, unpublished data). Most outbreaks seem to be associated with the consumption of contaminated

waters, yet there are very few studies on the incidence of enteric pathogens in these waters (Deetz et al., 1984; Herrero and Fuentes, 1977; Keswick et al., 1984; Toranzos et al., 1986a,b).

Numerous outbreaks of gastroenteritis linking water as a possible vector of Norwalk viruses and rotaviruses have been described, but yet little is known about their incidence in developing countries. The presence of even low numbers of infectious particles in the water is of great concern as a result of the low infectious dose (ID) of viral agents. It has been indicated that the ingestion of even one particle may result in overt symptoms of gastroenteritis (Akin, 1981). It has been estimated that up to five million people die of gastroenteritis each year worldwide (Evans, 1986). Most of these deaths are possibly a result of the consumption of waters contaminated with biological waste. Previous studies in Latin American countries have demonstrated the presence of enteric viruses in treated drinking waters (Deetz et al., 1984; Toranzos et al., 1986a,b; Gerba et al., 1984). These studies have also demonstrated the presence of viruses in the absence of indicator bacteria in waters meeting recommended standards for turbidity.

In the present study we examined several types of water for the presence of enteric viruses, coliphages, and indicator bacteria in three Latin American countries.

MATERIALS AND METHODS

Sampling Sites

One water treatment plant in the city of Cochabamba as well as several plants in various Colombian cities were sampled. Samples were obtained after different stages of treatment. The distribution network was monitored by taking samples from private houses. In Puerto Rico, sites in a tropical forest as well as source waters were sampled. Raw sewage, and sewage-contaminated rivers and streams, were also sampled in all three countries.

Bacterial Analyses

Grab samples were obtained at the same time as the viral samples. All analyses were performed as outlined in *Standard Methods* [American Public Health Association (APHA), 1985]. The media used and the makers were as follows: m-Endo, M-FC, and KF (Difco, Detroit MI). Any chlorine was immediately deactivated by the addition of crystalline sodium thiosulfate. Typical colonies were randomly picked and confirmed as follows: total coliforms were inoculated into lauryl sulfate

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broth followed by inoculation into brilliant green lactose broth. All colonies growing with the production of gas were considered positive. Fecal coliforms were confirmed in EC broth. Presumptive fecal strepto-cocci colonies were inoculated into azide-dextrose (AD) broth and subsequently reinoculated into KF agar. Several selected colonies from several plates were further identified using API-20E strips (Analytab Products, Plainview, NY).

Coliphage Analyses

One-liter samples of water were concentrated through two 1MDS filters and coliphages were eluted from the filters with 20 mL of 3% beef extract (pH 8.0). Eluate was neutralized and assayed. _Escherichia coli_ C3000 (ATCC 15597) was used as the host bacterium. All assays were done as outlined by Farrah and Preston (1985). Briefly, 0.1 mL of a 4-h-old culture of _E. coli_ (grown in trypticase soy broth) was mixed with a 0.5 mL of sample. This mixture was added to 4.0 mL of soft nutrient agar (0.7% agar w/v), mixed with 4.0 mL of nutrient agar. After an 18–24 h incubation period at 35°C, the plaques were counted. Only samples taken in Puerto Rico were assayed for the presence of coliphages.

Enteric Virus Detection

Different types of water such as treated and untreated potable water, untreated sewage, and sewage-contaminated streams and rivers were concentrated by passing the sample (1.0–100 L) through two layers of 1MDS Virozorb filters (AMF-Cuno, Meriden, CT) or one layer of a 50S Zeta-Plus depth filters (AMF-Cuno). Collection of samples was accomplished by the method described by Toranzos _et al._ (1984). Any chlorine present was neutralized by adding crystalline sodium thiosulfate directly to the collection vessel. Viruses adsorbed to the filters were eluted using a combination of 3% beef extract and 10% tryptose phosphate broth, pH 10.0. The eluent was immediately neutralized to avoid any virus inactivation and frozen until assay (whenever a freezer was not available, eluates were kept on ice).

Eluates were transported to the laboratory, where they were further concentrated by ultracentrifugation (27,000 rpm, 1.5 h). Eluates showing high turbidity were centrifuged at 10,000 rpm prior to ultra-centrifugation. By following this procedure we were able to largely reduce bacterial contamination, which in turn eliminated the need for using high concentrations of antibiotics in the cell culture assays. The pellets were resuspended in 2.0 mL of Na_2HPO_4 (0.15 M, pH 7.3). One milliliter was inoculated onto buffalo green monkey (BGM) cells in

order to detect any enteroviruses present. The inoculum was allowed to be in contact with the monolayer for 2 h with rotational agitation every 15 min. At the end of this period the monolayer was washed with minimum essential media (MEM) supplemented with 2% fetal bovine serum (FBS), 100 U/mL penicillin, 100 μg/mL streptomycin, and 100 μg/mL mycostatin. This medium was decanted and a fresh aliquot of the same medium was added to the bottles. These cells were observed for the appearance of cytopathic effect (CPE) for a period of one week. The medium was changed as needed or every third day. Any bottle showing CPE (90% of cells infected) was immediately freeze-thawed three times, the supernatant filtered (through a sterile 0.2-μm pore size filter), and the filtrate inoculated onto a fresh monolayer. The formation of CPE in these cells confirmed the presence of enteric viruses.

Enteric viruses were enumerated by following the same procedure as above, but the liquid MEM was supplemented with 3.0 μg/mL neutral red (Difco, Detroit, MI) and 1.5% Bacto-agar (Difco). The monolayers were observed daily for the presence of plaques, which were enumerated. Randomly chosen plaques were "plucked" with the help of a sterile Pasteur pipette. These plaques were then confirmed by passage onto a fresh monolayer of cells as described above.

The second milliliter of sample was inoculated onto an 18-h-old monolayer of MA104 cells grown in eight-chamber tissue culture slides (Lab-Tek Prod., Miles Labs. Inc., Naperville, IL), and indirect immunofluorescence (IIF) used for the detection of rotaviruses. All procedures used for rotavirus detection were as described by Smith and Gerba (1981).

RESULTS

Table I shows the results obtained from samples taken from a treatment plant in Bolivia at different stages of treatment. Total coliforms and fecal streptococci were isolated from the finished water. In this sample the turbidity was less than one nephelometric turbidity units (NTU), but the chlorine was extremely low (0.06 mg/L). The latter sample was the only sample that had any measurable chlorine. Total coliforms and fecal streptococci were isolated from all stages of treatment except one sample of raw well water. Raw well water ranges of coliforms and fecal streptococci were 0–103 and 0–18, respectively, depending on the day that samples were taken. The only sample that contained rotaviruses was a raw well water sample. None of the others assayed were positive for the presence of either rota- or enteroviruses.

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TABLE 1

Microbiological analyses of raw and treated water samples in Bolivia[a]

Type of water	Total coliforms (/100 mL)	Fecal streptococci (/100 mL)	Chlorine (mg/L) Total	Free	pH	Rotaviruses (fluorescent foci/L)	Enteroviruses CPE[b]	Turbidity NTU[c]
Finished	4.0	3.0	0.06	0.06	6.8	ND[d]	–	<1.0
Postfiltration	5.0	1.0	0.0	0.0	7.2	ND	–	<1.0
Prefiltration	28.0	5.0	0.0	0.0	7.0	ND	–	>1.0
Raw well water	0.0	0.0	0.0	0.0	7.3	0.0	–	>1.0
	5.0	1.0	0.0	0.0	7.4	0.0	–	>1.0
	9.0	10.0	0.0	0.0	7.1	4.0	–	<1.0
	2.0	0.0	0.0	0.0	7.4	0.0	–	<1.0
	1.0	1.0	0.0	0.0	7.0	0.0	–	<1.0
Raw surface water	103.0	18.0	0.0	0.0	7.4	0/0	–	<1.0
	13.0	3.0	0.0	0.0	7.4	ND	–	<1.0
	7.0	11.0	0.0	0.0	7.2	ND	–	>1.0

[a] All virus samples were processed through two 1MDS, 90-mm diameter filters.
[b] NTU: nephelometric turbidity units.
[c] CPE: cytopathic effect (–) indicates absence of CPE.
[d] ND: not determined.

TABLE II
Virus isolation from untreated sewage and sewage-contaminated waters in Bolivia[a]

Coliforms (/100 mL)	Fecal streptococci (/100 mL)	Enteroviruses CPE[b] (PFU/L)	pH of sewage	Rotaviruses (fluorescent foci/L)
4.2×10^6	$>1 \times 10^6$	+	6.5	250
7.0×10^5	$>1 \times 10^6$	+	7.8	ND[c]
9.0×10^5	$>1 \times 10^4$	+	7.7	139
$>1 \times 10^6$	$>1 \times 10^6$	+(2)	7.8	100
$>1 \times 10^{6d}$	$>1 \times 10^6$	+	7.9	0.0

[a] All samples were processed through two 1MDS filters and eluted as indicated in the text.
[b] CPE: cytopathic effect. (+) indicates presence of CPE.
[c] ND: not determined.
[d] Sample was obtained from a sewage-contaminated stream.

Table II shows the results of samples taken from sewage and a sewage-contaminated stream in Bolivia. All contained greater than 1×10^5 total coliforms/100 mL and greater than 1×10^6 enterococci/100 mL. A range of 100–250 rotaviruses/L were enumerated as immunofluorescent foci (IFF) in raw sewage samples. No rotaviruses were detected in the sewage-contaminated stream. All samples were positive for enteric viruses. One sample that was enumerated contained 2 plaque forming units (PFU)/L of enteric viruses.

Six water treatment plants were sampled in Colombia. The results are shown in Table III. Total coliforms were isolated from only one finished water sample from Plant F. Fecal streptococci were isolated from the same sample. Plant E also contained fecal streptococci, but no total coliforms. Enteric viruses were detected in Plant B at the postflocculation stage. Rotaviruses were isolated from finished waters in Plants E and F.

Thirty-three samples of drinking water taps were taken from different areas in Colombia (Table IV). Only six of the samples tested were free of total coliforms and all except one contained fecal streptococci. Levels ranged from 1 to >1000 total coliforms/100 mL and from 1 to >1000 fecal streptococci/100 mL. None of the samples contained enteroviruses. All of the samples assayed for rotaviruses were negative (Table IV).

All sewage and sewage-contaminated streams contained high levels of total coliforms and fecal streptococci (ranging from 8×10^5 to 1.1×10^7 per 100 mL and from 6×10^7 to 9×10^7, respectively) (Table V). Out of 21 samples assayed, 18 were positive for enteroviruses. The

...d sewage-contaminated waters
...a[a]

...uses ...(/L)	pH of sewage	Rotaviruses (fluorescent foci/L)
...	6.5	250
	7.8	ND[c]
—	7.7	139
)	7.8	100
	7.9	0.0

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TABLE III

Microbiological analyses at different stages at Colombian water treatment plants

Stage of treatment	Total coliforms (/100 mL)	Fecal streptococci (/100 mL)	Chlorine (mg/L) Total	Chlorine (mg/L) Free	pH	Rotaviruses (fluorescent foci/L)	Enteroviruses CPE[a]	Turbidity NTU[b]
Plant A								
Finished	0.0	ND[c]	2.2	1.5	6.9	0.0	–	<1.0
Postfiltration	2.0	ND	0.0	0.0	7.1	ND	–	1.0
Postflocculation	14	ND	0.0	0.0	6.9	ND	–	1.5
Raw intake	300	ND	0.0	0.0	7.7	ND	–	2.0
Plant B								
Finished	0.0	ND	0.7	0.6	6.3	0.0	–	1.0
Postfiltration	12.0	ND	0.0	0.0	6.1	ND	–	1.5
Postflocculation	>300	ND	0.0	0.0	6.4	ND	+	4.5
Raw intake	600	ND	0.0	0.0	6.6	ND	–	3.0
Plant C								
Finished	0.0	ND	0.8	0.7	7.0	0.0	–	3.0
Postfiltration	460	ND	0.0	0.0	7.0	ND	–	2.5
Postflocculation	690	ND	0.0	0.0	7.1	ND	–	6.0
Raw intake	1200	ND	0.0	0.0	8.0	ND	–	3.0
Plant D								
Finished	0.0	ND	0.5	0.3	6.2	0.0	–	<1.0
Postfiltration	10.0	ND	0.0	0.0	6.0	ND	–	ND
Raw intake	100.0	ND	0.0	0.0	ND	ND	–	ND
Plant E								
Finished	0.0	50.0	ND	1.2	8.3	0.46	–	7.5
Raw intake	460.0	320.0	0.0	0.0	8.7	ND	–	10.0
Plant F								
Finished	130.0	21.0	2.5	1.8	7.3	0.14	–	7.0
Postflocculation	3200.0	4000.0	0.0	0.0	7.6	ND	–	6.5
Raw intake	250.0	1200.0	0.0	0.0	7.5	ND	–	8.0

[a] CPE: cytopathic effect. (–) indicates no CPE.
[b] NTU: nephelometric turbidity units.
[c] ND: not determined.

TABLE IV
Microbiological analyses of drinking water samples from Colombia

Sample number	Total coliforms (/100 mL)	Fecal streptococci (/100 mL)	Chlorine (mg/L) Total	Free	pH	Rotaviruses (fluorescent foci/L)	Enteroviruses CPE[a]	Turbidity NTU[b]
1	0.0	ND[c]	0.0	0.0	5.8	ND	—	0.4
2	0.0	ND	0.0	0.0	5.8	ND	—	0.4
3	0.0	ND	ND	ND	7.1	ND	—	2.0
4	0.0	ND	0.0	0.0	7.2	ND	—	4.0
5	11.0	ND	0.0	0.0	ND	ND	—	ND
6	50.0	ND	0.0	0.0	7.0	0.0	—	4.0
7	ND	ND	0.0	0.0	ND	ND	—	ND
8	150.0	460	ND	ND	7.2	ND	—	2.0
9	ND	ND	0.0	0.0	4.2	ND	—	ND
10	ND	ND	0.3	0.2	8.5	ND	—	4.0
11	100.0	37	0.4	0.08	7.0	ND	—	2.5
12	ND	ND	0.25	0.15	7.2	0.0	—	3.5
13	ND	ND	0.15	0.1	6.9	0.0	—	ND
14	>300	>300	0.0	0.0	8.1	ND	—	ND
15	2040	>300	0.0	0.0	7.3	ND	—	ND

No.								
5	11.0	ND	0.0	0.0	ND	ND	—	4.0
6	50.0	ND	0.0	0.0	ND	ND	—	ND
7	ND	ND	0.0	0.0	ND	0.0	—	4.0
8	150.0	460	ND	ND	7.2	ND	—	ND
9	ND	ND	0.0	0.0	4.2	ND	—	2.0
10	ND	ND	0.3	0.2	8.5	ND	—	ND
11	100.0	37	0.4	0.08	7.0	ND	—	4.0
12	ND	ND	0.25	0.15	7.2	ND	—	2.5
13	ND	ND	0.15	0.1	6.9	0.0	—	3.5
14	>300	>300	0.0	0.0	8.1	0.0	—	ND
15	2040	>300	0.0	0.0	7.3	ND	—	ND

No.								
16	1.0	2.0	0.5	0.4	6.2	ND	—	>1.0
17	0.0	0.0	0.25	0.2	6.8	ND	—	>1.0
18	10.0	4.0	0.5	0.4	6.0	0.0	—	>1.0
19	0.0	1.0	0.08	0.03	6.2	ND	—	>1.0
20	15.0	>300	0.01	0.006	7.1	ND	—	>1.0
21	>300	3.0	0.7	0.4	5.5	ND	—	<1.0
22	45.0	1.0	0.9	0.75	5.9	ND	—	<1.0
23	16.0	2.0	0.0	0.0	6.2	ND	—	>1.0
24	9.0	1.0	0.4	0.3	5.8	ND	—	>1.0
25	>300	21.0	0.0	0.0	5.0	ND	—	<1.0
26	35.0	14.0	0.0	0.0	5.5	ND	—	<1.0
27	>300	40.0	0.35	0.25	7.9	ND	—	>1.0
28	162.0	5.0	0.9	0.8	5.5	ND	—	>1.0
29	ND	1.0	0.0	0.0	6.0	ND	—	>1.0
30	100.0	134.0	0.0	0.0	6.0	ND	—	<1.0
31	202.0	11.0	0.0	0.0	6.0	ND	—	<1.0
32	ND	ND	0.5	0.45	5.0	ND	—	<1.0
33	ND	ND	0.2	0.0	5.0	ND	—	<1.0

a CPE: cytopathic effect.
b NTU: nephelometric turbidity units.
c ND: not determined.

TABLE V

Microbiological analyses of sewage and sewage-contaminated waters in Colombia

Type of sample	Total coliforms (/100 mL)	Fecal streptococci (/100 mL)	pH	Rotaviruses (fluorescent foci/L)	Enteroviruses CPE[a]	PFU[b]/L (enteroviruses)
Sewage	8.0×10^6	ND[c]	8.1	ND	+	187
	1.2×10^6	ND	8.1	ND	+	287
	3.9×10^6	ND	ND	ND	+	ND
	1.0×10^6	ND	ND	ND	+	55
	1.0×10^6	1.0×10^6	7.2	0.0	+	2,214
	2.5×10^6	$>1 \times 10^6$	7.8	0.0	+	ND
	7.0×10^7	$>1 \times 10^6$	7.6	ND	+	125
	ND	ND	5.0	ND	+	ND
	ND	ND	7.8	ND	+	ND
	ND	ND	8.0	ND	+	ND
	ND	ND	7.8	ND	+	438
	2.2×10^7	3.8×10^4	ND	ND	+	ND
	3.8×10^6	2.7×10^4	ND	ND	+	39
	1.3×10^6	2.7×10^4	ND	ND	+	10
	4.0×10^7	4.8×10^4	ND	ND	+	144
Sewage-contaminated streams	ND	ND	7.8	ND	−	ND
	ND	ND	7.9	ND	−	ND
	1.1×10^7	9.0×10^7	8.3	ND	−	ND
	1.0×10^4	5.0×10^4	7.3	0.0	+	ND
	5.0×10^6	3.5×10^6	ND	ND	+	ND
	1.4×10^6	1.1×10^6	6.4	0.0	+	ND

[a] CPE: cytopathic effect.
[b] PFU: plaque-forming units.
[c] ND: not determined.

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Sewage-contaminated streams						
4.0 × 10⁷	4.8 × 10¹	ND	ND			39
ND	ND	ND	ND		+	10
ND	ND	ND	ND		+	144
1.1 × 10⁷	9.0 × 10⁷	7.8	ND		—	ND
1.0 × 10⁵	5.0 × 10⁵	7.9	ND		—	ND
5.0 × 10⁵	3.5 × 10⁵	8.3	ND		+	ND
		7.3	0.0		+	ND
1.4 × 10⁵	1.1 × 10⁵	ND	ND		+	ND
		6.4	0.0			ND

a CPE: cytopathic effect.
b PFU: plaque-forming units.
c ND: not determined.

TABLE VI

Microbiological analyses of water samples from rural Colombia

Sampling site	Total coliforms (/100 mL)	Fecal streptococci (/100 mL)	Chlorine (mg/L)		pH[a]	Rotaviruses (fluorescent foci/L)	Enteroviruses CPE[b]	Turbidity NTU[c]
			Total	Free				
Fusagasuga	71	100	0.0	0.0	5.0	ND[d]	—	>1.0
	34	1000	0.0	0.0	6.0	ND	—	>1.0
El Placer	>300	450	1.1	1.0	5.0	ND	—	<1.0
Lazaro Fonte	ND	ND	0.0	0.0	5.0	ND	—	>1.0
Utica (River)	ND	ND	0.0	0.0	6.0	4.0	—	>1.0
(Tap)	>300	>300	0.0	0.0	6.0	ND	—	>1.0
Quebrada-Grande (River)	1150	1170	0.0	0.0	6.0	ND	—	>1.0
(Tap)	1000	950	0.0	0.0	6.0	ND	—	>1.0
El Bagazal	>1000	98	0.0	0.0	6.0	0.0	—	>1.0
La Salada treatment plant:								
(Finished)	>1000	>1000	0.0	0.0	6.0	0.0	+	<1.0
Postsediment	>1000	>1000	0.0	0.0	6.0	1.2	+	>1.0
Raw water	>1000	>1000	0.0	0.0	6.0	6.0	+	>100

a pH shown is only an approximation since it was measured with pH paper.
b CPE: cytopathic effect.
c NTU: nephelometric turbidity units.
d ND: not determined.

TABLE VII

Microbiological analyses of recreational waters in Colombia

Type of sample	Total coliforms (/100 mL)	Fecal streptococci (/100 mL)	Chlorine (mg/L)		pH	Rotaviruses (fluorescent foci/L)	Enteroviruses CPE[a]	Turbidity NTU[b]
			Total	Free				
Swimming pool	670.0	300	<1.0	<1.0	8.3	0.0	—	2.5
	ND[c]	ND	1.7	1.2	ND	0.0	—	ND
	ND	ND	1.0	0.7	8.0	0.0	—	<1.0
	350.0	250	0.0	0.0	8.0	ND	—	<1.0
Marine	>300	>300	0.0	0.0	8.0	0.1	—	10.0
	33.0	1810	0.0	0.0	8.7	0.0	—	15.0

[a] CPE: cytopathic effect.
[b] NTU: nephelometric turbidity units.
[c] ND: not determined.

TABLE VIII

Bacteriological and coliphage analyses of pristine and sewage-contaminated waters in Puerto Rico

Type of sample	Total coliforms (/100 mL)	Fecal coliforms (/100 mL)	Fecal streptococci (/100 mL)	Coliphages[a] (/L)
Pristine waters	930	4.0	720	0.0
	1240	110	107	0.0
	890	75	183	0.0
Sewage-contaminated waters	840	15	268.0	60.0
	>300	>300	960.0	2.0
	1122	177	62.0	160.0
	289,000	2300	201.0	2856.0

[a] One-liter samples were concentrated as indicated in text.

concentrations ranged from 8 to 2214 PFU/L (Table IV). Only eight rotavirus assays were possible, as the inocula were toxic to the cell monolayer. In the latter assays, the number of fluorescent foci ranged from 0 to 287 IFF/liter.

Table VI shows the results of samples obtained from eight rural towns in the Departamento de Cundinamarca in Colombia (including three water treatment plants). All of the samples contained high levels of total coliforms and fecal streptococci, ranging from 34 to 4500 CFU/100 mL and from 98 to 1170/100 mL (several contained greater than 1,000 CFU/100 mL), respectively. Enteroviruses were isolated from all stages of treatment at one of the plants. Rotaviruses were isolated from the postsedimentation stage (1.2 IFF/L), and from the raw water (6.0/L) and from the raw water source (0.57 IFF/L) at the Utica plant. None of the other samples assayed were positive for enteroviruses.

Results from recreational water samples (namely hotel swimming pools and marine bathing beaches) are shown on Table VII. No enteroviruses were isolated from any of the samples. Rotaviruses (0.1 IFF/L) were isolated from a bathing beach, but all other samples assayed were negative.

Table VIII shows the results from samples taken at different sites in Puerto Rico. In spite of large numbers of total coliforms, fecal coliforms, and fecal streptococci, only those samples that were known to be contaminated with sewage were positive for coliphages. No enterovirus isolations were attempted in this country.

DISCUSSION

The treatment plant sampled in Bolivia (city of Cochabamba) is a conventional treatment plant. Alum is added to incoming water and

[a] CPE: cytopathic effect.
[b] NTU: nephelometric turbidity units.
[c] ND: not determined.

sedimentation, followed by sand filtration and chlorination done prior to distribution to the population. Most of the raw water is obtained from mountain streams [for a complete description of the plant, see Toranzos et al. (1986b)]. Only low levels of coliforms and fecal strepto-cocci are expected in the raw water since there is little chance of con-tamination. The treatment plant also uses well water as a raw source. These wells are located in an area near several septic tanks. Rota-viruses were detected in one of the latter samples. None of the other raw water samples were positive for either entero- or rotaviruses. These findings indicate that the source water is of very good quality. In a previous study, we isolated rotaviruses from the finished water at the same plant (Toranzos et al., 1986b). Thus the water quality seems to vary. This varying water quality may be the result of rains, which may facilitate the transport of viruses and bacteria from the point source to streams or wells. The treatment eliminates all bacterial indicators present in the raw water intake. In most cases, flocculation followed by filtration seems to get rid of all indicator bacteria. Chlorination was not done continuously as a result of the inconsistent availability of chlorine. Most of the time during the sampling periods, no chlorine was detected in the finished water. Nonetheless, as a result of the high-quality raw intake water, inconsistent chlorination seemed to be of little significance. Such is the case in many cities in the world that use well water with no treatment whatsoever for distribution to the popu-lation. Thus the quality of the raw water determines the type of treat-ment necessary. However, a thorough monitoring schedule should be implemented in plants (such as the one in Cochabamba) that do not go through all stages of treatment or do so depending on the availability of chlorine.

All raw sewage samples tested positive for the presence of en-teroviruses. The concentration of enteric viruses (2 PFU/L) was ex-tremely low when compared to other parts of the world (Rao and Melnick, 1986). The inocula were found to be extremely toxic to the cell monolayer, and thus it was possible to enumerate enteroviruses in only one of the samples. In all other cases, the monolayer was de-stroyed within 48 h, making enumeration impossible. We are cur-rently using complementary DNA (cDNA) probes on these samples. The latter technique will allow us to approximate the number of PFU without the worry of toxicity (Margolin et al., 1985). The presence of rotaviruses in the sewage was not surprising, and the concentrations at which they were present was comparable to those found in Houston, Texas (Rao et al., 1985), and those found in Bombay, India (Rao et al., 1978). The studies cited were done with treated sewage samples; the concentrations in untreated sewage (as in our study) are expected to be

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higher, and thus this would explain the $1-2$ $\log_{10}$ difference in concentration of rotaviruses.

Raw sewage is often used for irrigation in many countries, and Bolivia is no exception. One of the samples taken was from a stream used for such purposes. Enteroviruses were detected, but the sample was negative for rotaviruses. Although the raw sewage had been diluted in the stream, enumeration of enteroviruses was not possible due to cytotoxicity. The levels of total coliforms and fecal streptococci were expectedly high. Thus the danger of using untreated sewage to irrigate crops extends not only to the presence or absence of viruses, but to the presence of bacterial and parasitic pathogens as well. Epidemiological studies in Kibbutzim (collective communities) in Israel have shown no relation between enteric disease and treated wastewater irrigation (Shuval et al., 1986). Nonetheless, no studies have been conducted on the incidence of enteric disease as a result of the consumption of fresh vegetables irrigated with untreated sewage.

All drinking water treatment plants sampled in Colombia were successful in eliminating indicator bacteria present in the source waters. Only one plant was found to contain total coliforms and fecal streptococci. The latter plant did not practice sand filtration, but rather, alum was added to intake waters, which were then chlorinated and distributed to the population. Although the finished waters contained 1.2 mg/L free chlorine, the high levels of indicator bacteria were expected due to the high turbidity and the lack of contact time. The finished water tested positive for rotaviruses but negative for enteroviruses. The presence of only rotaviruses may be indicative of the seasonality observed in rotaviral gastroenteritis.

Most of the samples obtained from the distribution network (household taps) in Colombia were found to contain high levels of total coliforms and/or fecal streptococci. No enteric viruses were isolated from any of these sources. We previously reported isolating both entero- and rotaviruses from some of these areas, although the concentrations detected were low (Toranzos et al., 1986a). Some of the samples reported in the present article were taken from the same areas and yet were negative for the presence of enteric viruses. The pattern of virus isolation does not seem to correlate with water quality leaving the treatment plants.

The high concentration of total coliforms and fecal streptococci present in sewage is not unexpected. The differences in concentrations from sample to sample may be explained by the degree of dilution of sewage. Many major cities in Colombia dispose of their sewage by directly dumping it into canals, which eventually carry this sewage to rivers. Thus the concentrations of biological contaminants detected in

this sewage depends on several factors, such as flow rate of the waters, number of houses disposing of the sewage, and many others. The concentration of enteroviruses detected were similar to those in many other parts of the world. The maximum concentration was 2214 PFV/L. Previous studies have reported much higher numbers in Israel and similar concentrations in the United States (Rao and Melnick, 1986). Once again, the concentrations found in sewage from different parts of the world may vary because of several factors, including different filters and concentration methods used, which do not necessarily have the same efficiency of reconcentration. For most of the previous studies, negatively charged filters were used. In our study we use positively charged filters and a combination of eluents not used before. Thus the concentrations reported may not necessarily indicate more or less biological contamination.

All samples taken in Colombian rural areas contained high levels of total coliforms and fecal streptococci. Enteric viruses were isolated from the raw influent water at one of the plants, but none were detected in the finished water at this same plant. Enteroviruses were isolated from all stages of treatment at another plant assayed. Rotaviruses were also present in these waters. This plant obtains its raw water source from the River Bogota. This river goes through several cities and towns before arriving at the town where the treatment plant is located. Raw industrial and domestic sewage is dumped into this river upstream. Thus it is not surprising to find viruses in these waters. In fact, the concentration of detergents was so high in the finished waters that simply pouring the water created an enormous amount of foam. The intake water had a blackish color, which was completely removed by treatment. The plant was not practicing chlorination at the time of sampling. Rural areas obtain their water from forest streams, which have little chance of getting contaminated by human waste. All of the sites where the source waters were sampled were far from any houses and relatively isolated from roads and towns. Nevertheless, all of them were high in levels of coliforms and fecal streptococci. This is to be expected, since the tropical rain forest vegetation is expected to have total coliforms associated with them. The apparent presence of fecal streptococci may be an artifact, since KF media was used and several bacteria can give a positive reaction (pink to red colonies). Any nonfecal streptococci present in the waters sampled may also grow in this medium, giving a typical reaction.

All recreational water samples were negative for the presence of enteric viruses, except for a marine bathing beach where rotaviruses were detected. The absence of enteroviruses in these samples was unexpected, since at the time of sampling there was a nationwide polio

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vaccination campaign taking place in Colombia. All beaches sampled
had a large number of people swimming at the time of sampling (in-
cluding a large number of children). The concentrations of total coli-
forms and fecal streptococci were not unexpectedly high, considering
the large number of bathers.

Five sites in a rain forest were sampled in Puerto Rico. All con-
tained high levels of indicator bacteria. However, coliphages were de-
tected in only four of the samples and these four samples were obtained
from areas where there is fecal contamination. An urban area dumps
secondarily treated sewage into the stream from where the samples
were obtained. Thus the presence of coliphages seems to be linked to
fecal contamination. In samples taken upstream of the effluent site, no
coliphages were isolated. Several bacterial isolates identified as *E. coli*
by the API 20E techniques were used as hosts when trying to isolate
indigenous strains of coliphages. In the latter set of experiments no
phages were detected at all, which may indicate that coliphages only
affect *E. coli* strains originating from fecal sources. We are currently
trying to determine if the latter hypothesis holds true by using varying
temperatures and concentrating large volumes of water. Coliphages
may in fact be better indicators of fecal contamination in the tropics
than currently used bacterial groups.

All of the samples taken in all three countries seem to have one
thing in common, i.e., high concentrations of indicator bacteria. The
presence of indicator bacteria in potable waters is a cause for concern,
since treated waters leaving the plants are free of such bacteria. Thus
there has to be contamination of the waters after they leave the treat-
ment plants. This contamination may be due to cross-connections in
the distribution systems. A second possibility may be the existence of
indigenous coliforms and fecal streptococci in the water distribution
pipes. The latter hypothesis is corroborated by the presence of high
concentrations of total coliforms even when the waters contained high
levels of total and free chlorine. If this is the case, then biological
pollution in these countries is overestimated. The absence of enteric
viruses in the majority of samples taken also supports the hypothesis
that there may be coliforms (even fecal coliforms) that may be able to
survive and grow in extraenteral environments.

Studies conducted in Puerto Rico (Hazen *et al.*, 1987; López-Torres
et al., 1987; Rivera *et al.*, 1988) have indicated the presence of indige-
nous *E. coli*. It is possible that bacteria currently used to indicate fecal
contamination may not be the most appropriate in tropical areas of the
world. The present study demonstrates that the presence of total coli-
forms and fecal streptococci do not in fact correlate with the presence
or absence of enteric viruses in waters. Most of the samples were posi-

tive for indicator bacteria but negative for enteric viruses. Of all the samples assayed for the presence of coliphage, only those receiving treated sewage were positive for these viruses. The pattern of isolation of coliphages seems directly related to sewage contamination. If this is so, then coliphages may be better indicators of fecal pollution in the tropics. The presence of enteric viruses in waters can only be a result of fecal contamination, since these viruses can only originate from fecal sources. Viruses get inactivated over a period of time as a result of environmental stress. That no enteric viruses were isolated from most of the drinking waters further indicates that these waters are very possibly not contaminated with fecal waste.

The most important observation in this article is that in all cases the water quality leaving the treatment plant did not correlate with the water quality of the water in the distribution network. Thus, if any efforts are to be made to improve the water quality, it is not going to be enough to increase the efficiency of treatment. Rather, more efforts should be made to upgrade and upkeep already existing distribution lines. In the countries where the sampling took place, the distribution networks are very old. Also, the possible presence of naturally occurring coliforms in treated and untreated waters contradicts the use of these bacteria as indicators of water quality.

Thorough and long-term monitoring studies are needed in tropical and subtropical areas of the world. This will allow the determination of whether techniques developed elsewhere apply realistically to these areas.

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C-4

Detection of Enteric Viruses in Treated Drinking Water

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The occurrence of viruses in conventionally treated drinking water derived from a heavily polluted source was evaluated by collecting and analyzing 38 large-volume (65- to 756-liter) samples of water from a 9 m³/s (205 × 10⁶ gallons [776 × 10⁶ liters] per day) water treatment plant. Samples of raw, clarified, filtered, and chlorinated finished water were concentrated by using the filter adsorption-elution technique. Of 23 samples of finished water, 19 (83%) contained viruses. None of the nine finished water samples collected during the dry season contained detectable total coliform bacteria. Seven of nine finished water samples collected during the dry season met turbidity, total coliform bacteria, and total residual chlorine standards. Of these, four contained virus. During the dry season the percent removals were 25 to 93% for enteric viruses, 89 to 100% for bacteria, and 81% for turbidity. During the rainy season the percent removals were 0 to 43% for enteric viruses, 80 to 96% for bacteria, and 63% for turbidity. None of the 14 finished water samples collected during the rainy season met turbidity standards, and all contained rotaviruses or enteroviruses.

The isolation of viruses from treated drinking water (2, 3, 10, 12) has raised concerns that water treatment methods may not always adequately remove viruses from water designated for human consumption. In fact, viruses have been isolated from drinking water which met acceptable coliform, chlorine, and turbidity limits (T. R. Deetz, E. M. Smith, S. M. Goyal, C. P. Gerba, J. J. Vollet, L. Tsain, H. L. DuPont, and B. H. Keswick, Water Res., in press). The question is raised as to whether current drinking water standards ensure safe drinking water. In a previous report (Deetz et al., in press), we described the isolation of enteroviruses and rotaviruses from treated drinking water in a distribution system and at a water treatment plant. This paper reports the results of a more extensive investigation of the removal of viruses by water treatment processes, including clarification, filtration, and chlorination, at a full-scale water treatment plant.

MATERIALS AND METHODS

Water treatment plant. A 9 m³/s (205 × 10⁶ gallons [776 × 10⁶ liters] per day) water treatment plant was selected for study based on our previous results indicating that the raw water source contained high amounts of viruses. Raw water is withdrawn from a river and conveyed ca. 17 km by canal via two pumping stations. The river is fed by a shallow lake located in a watershed with a human population of 4 × 10⁶ to 8 × 10⁶, and untreated water and wastewater are discharged into the watershed. The treatment facility consists of five separate plants constructed over a 20-year period from 1955 to 1975. Each plant has a treatment sequence consisting of chemical addition (liquid alum and Catfloc or Superfloc [Calgon Corp., Pittsburgh, Pa.]), followed by hydraulic mixing, flocculation, clarification with pre- and postchlorination, filtration either through rapid sand filters or automatic valveless sand filters, and final chlorination. All samples were collected from three of the plants.

Water sample collection. Samples of 9.8 to 756 liters were collected by using 1MDS Virosorb filters (AMF CUNO, Meriden, Conn.), which eliminate the necessity to precondition the water. An electric 1-horsepower centrifugal pump was used to collect samples which could not be collected

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directly from taps. Where necessary, in-line injectors (DEMA, St. Louis, Mo.) were used to inject sodium thiosulfate into water to neutralize the chlorine before passage through the virus-adsorbing filters (11). Samples were collected at the canal intake delivering water to the plant, after clarification, after filtration, and after final chlorination. On each day that samples were collected, finished water samples were always collected first and intake water samples were always collected last. Separate filter housings and hoses were used for each sampling location. The filter housings were disinfected by soaking for 30 min in buckets filled with water containing more than 5 mg of free chlorine per liter. Hoses and pumps were disinfected by pumping water containing more than 5 mg of free chlorine per liter through them. Concurrent 1-liter grab samples were collected for bacterial, bacteriophage, and turbidity analyses.

Elution and reconcentration procedures. Adsorbed viruses were eluted by passage of 1 liter of 3% beef extract (Scott Laboratories, Fiskeville, R.I.), (pH 9.5) into the filter housing. The pH of the eluent solution was adjusted just before use by the addition of 1 N NaOH. Once the housing was filled, the beef extract solution was allowed to remain in the housing for 2 to 5 min and then was forced out of the housing with air. The pH of the eluate was adjusted to 7.0 to 7.5 by the addition of 1 N HCl. Samples were frozen for shipment to the laboratory at the University of Arizona. Thawed samples were reconcentrated to an average volume of 36 ml by the organic flocculation method (7), and the final concentrate was divided into three equal volumes, one for enterovirus analysis, one for rotavirus analysis, and one to be stored for future use.

Virus detection. All samples were processed under code. Samples for enterovirus analysis (2 ml) were inoculated onto monolayers of BGM cells in 75-cm² plastic flasks, overlaid with maintenance medium, and observed for cytopathic effect (CPE) for a period of 21 days. Samples which did not produce CPE were passed an additional two times on monolayers of BGM cells and observed for CPE to ensure that no positive samples were missed. Samples positive for CPE were confirmed by passage to a second BGM monolayer, and after development of CPE, they were plaqued by using an agar overlay method (9).

TABLE 1. Water quality in samples collected after successive drinking water treatment processes (dry season)

Treatment process	Sample no.	Date (1982)	Vol (liters)	pH	Total chlorine (mg/liter)	Free chlorine (mg/liter)	Turbidity (NTU)
None (raw)	5	3/2	76	7.6	0	0	ND[a]
	6	3/2	67	7.6	0	0	ND
	11	3/3	65	8.0	0	0	6.1
	12	3/3	111	8.0	0	0	6.1
	19	3/4	77	ND	0	0	7.1
Clarified	3	3/2	128	7.8	1.4	0.3	5.5
	4	3/2	111	7.8	1.5	0.4	6.1
	9	3/3	90	7.9	1.7	1.4	6.7
	10	3/3	116	7.9	1.7	1.5	6.7
	18	3/4	87	7.9	1.9	1.1	3.3
Finished[b]	1	3/2	525	6.8	7.5	1.0	0.73
	2	3/2	756	6.8	6.5	1.0	0.84
	7	3/3	416	7.8	ND	0.75	0.64
	8	3/3	416	7.8	ND	0.75	0.64
	15	3/4	756	7.7	1.9	0.5	0.95
	13	3/3	513	8.1	2.7	2.2	2.9
	14	3/3	549	8.1	2.7	2.0	2.9
	16	3/4	626	7.9	2.6	2.5	0.65
	17	3/4	691	7.9	2.5	2.1	0.65

[a] ND, Not done.
[b] Finished water was treated by clarification, sand filtration, and chlorination

Rotavirus was detected by inoculating the samples onto cultures of MA-104 cells (13) in Lab-Tek glass chamber slides (Miles Scientific, Div. of Miles Laboratories, Inc., Naperville, Ill.) in the presence of medium containing trypsin (0.5 µg/ml, Sigma type IX; Sigma Chemical Co., St. Louis, Mo.). Samples which showed toxicity (1) were filtered through 0.45-µm cellulose nitrate filters (Millipore Corp., Bedford, Mass.) or diluted 1:5 or 1:10. After 24 h of incubation at 38°C, the medium was aspirated off, the chamber was removed, and the monolayers were fixed in −20°C methanol for 5 min. The slides were then stained for the fluorescence assay described by Smith and Gerba (13). Primary guinea pig or rabbit antisera directed against human rotavirus was obtained from the National Institute of Allergy and Infectious Diseases reference reagents and from DAKO (Subsidiary of Accurate Chemical & Scientific Corp., Westbury, N.Y.). Secondary antisera conjugated with fluorescein isothiocyanate was obtained from Miles Laboratories (Elkhart, Ind.).

Bacteriophages in both grab samples and beef extract concentrates were analyzed by plaque assay on *Escherichia coli* Hfr host bacteria (4).

Bacterial measurements. Grab samples were tested by the membrane filter method (1) with Nalge Nutrient Pad kits (Rochester, N.Y.) for standard plate count, total coliforms, fecal coliforms, and fecal streptococci.

Water quality measurements. The pH of the water collected was measured on site with a portable pH meter. Samples for turbidity testing were frozen and returned to the laboratory where they were read on a Hach turbidity meter (Hach Chemical, Loveland, Colo.). Residual chlorine was measured with a DPD colorimetric kit (Hach Chemical).

Quality assurance. Stool specimens or rectal swabs were collected from each of the laboratory personnel coming in contact with the samples. All personnel wore gloves when handling filters or samples throughout the course of analy. The specimens were processed and inoculated onto cell cultures in designated areas physically separated from areas where laboratory strains of viruses are in use. Heavily chlorinated tap water (378 liters) collected in Tucson, Ariz., was also examined to control for false-positive specimens.

RESULTS

The first sampling trip was made during the dry season (March 1982); 19 samples were collected. The sample volumes ranged from 65 to 756 liters (Table 1). Five samples were collected from raw water, five after the clarification process, and nine after final chlorination (finished water). Overall, 12 of the samples were positive for enterovirus and 10 were positive for rotavirus. Significantly, five finished water samples contained enterovirus and two contained rotavirus. None of the finished water samples contained any total coliform bacteria, but fecal streptococci were detected in six of six samples examined. The total plate count bacteria ranged from 1 to 110 CFU/100 ml, with a mean of 28. The mean pH value of the finished water samples was 7.6, and the mean total chlorine and free chlorine contents were 3.7 and 1.42 mg/liter, respectively (Tables 1 and 2).

The five samples of raw water had a mean total plate count of 255 CFU/ml and a mean total coliform count of 870 CFU/ 100 ml (Table 3). The mean turbidity was 6.4 nephelometric turbidity units (NTU), and the mean pH was 7.6. Four of the five samples were positive for enterovirus and five of five were positive for rotavirus.

Samples collected after the clarification process contained a mean total plate count bacteria of 194 CFU/ml, a mean pH of 7.8, and a mean turbidity of 5.6 NTU. Three of five samples were positive for enterovirus and three of five were positive for rotavirus.

The percent reduction effected by each step in the water treatment process was calculated from the mean measurement of turbidity, total plate count bacteria, total coliform bacteria, fecal streptococcal bacteria, enteroviruses, coliphages, and rotaviruses (Table 3). It should be noted that the mean recovery was higher and the percent reduction was lower for finished water than for filtered water. Since these

TABLE 2. Isolation of bacteria and viruses after successive drinking water treatment processes (dry season)

Treatment process	Sample no.	Bacteria				Viruses			
		Total plate count (CFU/ml)	Total coliforms (CFU/100 ml)	Fecal coliforms (CFU/100 ml)	Fecal streptococci (CFU/100 ml)	No. of coliphages/10 ml (direct)[a]	No. of coliphages/1,000 ml (concentrate)	Enterovirus cultures[b]	No. of rotaviruses/100 liters
None (raw)	5	340	240	140	3,400	14	12,700	1/3	268
	6	340	240	140	3,400	ND[c]	ND	3/5	1,202
	11	170	1,500	140	1,820	0	21,000	3/3	1,163
	12	170	1,500	140	1,820	ND	17,300	0/4	324
	19	ND	ND	910	ND	21	292,700	3/3	94
Clarified	3	530	0	0	120	2	25,200	4/4	148
	4	120	0	1	27	3	0	1/5	36
	9	63	0	0	0	17	0	3/3	0
	10	63	0	0	0	0	300	0/4	586
	18	ND	0	ND	ND	0	0	0/7	0
Finished[d]	1	21	0	0	1	0	0	5/5	233
	2	110	0	0	27	0	100	0/5	0
	7	16	0	0	16	0	0	3/3	0
	8	16	0	0	16	0	0	1/5	126
	15	ND	0	ND	ND	0	0	0/5	0
	13	1	0	0	3	0	0	3/3	0
	14	1	0	0	3	ND	0	0/4	0
	16	ND	0	ND	ND	0	0	4/4	0
	17	ND	0	ND	ND	ND	0	0/5	0

[a] Water was plated directly for coliphages on *E. coli* Hfr host bacteria.
[b] Enteroviruses were detected by the production of CPE in monolayer cultures of BGM cells contained in 75-cm² flasks. Number positive/number tested.
[c] ND, Not done.
[d] Finished water was treated by clarification, sand filtration, and chlorination.

samples were not collected temporally, water quality fluctuations may have been responsible for these results.

Several factors may affect water quality and so water treatment (5). In the study area, raw water quality decreases during the rainy season, as turbidity increases due to storm runoff. To determine the effects of decreased water quality on virus removal by water treatment, the second sampling trip was conducted during the rainy season (July 1982). As indicated by the turbidity and bacterial measurements, a decrease in water quality was reflected by an increased frequency of virus isolation in samples collected during July 1982 (Tables 4 and 5). The mean counts of coliphages and rotaviruses were greatly increased over those obtained from dry season samples. In nine finished water samples, the

mean turbidity was 9.6 NTU, the mean pH was 6.6, the mean total residual chlorine was 8.8 mg/liter, and the mean free chlorine was 0.6 mg/liter. Means for total plate count bacteria (716), total coliforms (5,140), fecal coliforms (2), and fecal streptococci (3,726) were also higher than in the dry season. Data for raw water and clarified water are also presented. The percent removals were calculated as above (Table 3).

DISCUSSION

The analysis of samples collected during the dry season (Tables 1 and 2) confirms our earlier findings (Deetz et al., in press) that viruses may be present in water which meets acceptable limits of turbidity (< 1.0 NTU), residual chlorine

TABLE 3. Efficiency of virus removal by water treatment processes

Season and treatment process	Turbidity (NTU)	Bacteria[a]			Viruses		
		Total plate count (CFU/ml)	Total coliforms (CFU/100 ml)	Fecal streptococci (CFU/100 ml)	Rotavirus[b]	Enterovirus[c]	Phage[d]
Dry							
None (raw)	6.4	255	870	2,610	610	55	11
Clarified	5.6 (12.5)	194 (23.9)	0 (100)	36 (98.6)	154 (74.3)	35 (36.8)	4 (63.6)
Finished[e]	1.2 (81.3)	28 (89.0)	0 (100)	11 (99.6)	40 (93.5)	41 (25.0)	0 (100)
Rainy							
None (raw)	26	3,610	46,500	1.1×10^5	1,745	7	47
Clarified	10 (61.5)	76 (97.8)	740 (98.4)	510 (99.5)	3,417 (0)	29 (0)	16 (65.7)
Filtered	6.8 (73.8)	195 (94.5)	748 (99.6)	1,000 (99.0)	342 (80.4)	15 (0)	3 (93.6)
Finished[e]	9.6 (63.1)	716 (80.2)	5,140 (88.9)	3,726 (96.6)	990 (43.3)	7 (0)	4 (91.4)

[a] Mean recovery and (in parentheses) percent reduction from that in raw water.
[b] Rotavirus infectious foci per 100 liters.
[c] Percent cultures positive for enterovirus CPE.
[d] Coliphage PFU by direct assay of sample.
[e] Finished water was treated by clarification, sand filtration, and chlorination.

TABLE 4. Water quality in samples collected after successive drinking water treatment processes (rainy season)

Treatment process	Sample no.	Date (1982)	Vol (liters)	pH	Total chlorine (mg/liter)	Free chlorine (mg/liter)	Turbidity (NTU)
(raw)	26	7/19	15	7.6	0	0	23
	31	7/20	9.8	7.2	0	8	39
Clarified	25	7/19	65	6.6	5.0	0.1	17
	30	7/20	37.4	6.4	0	0.1	3.4
Filtered	29	7/20	378	6.8	8.5	0.8	8.6
	36	7/21	340	6.6	>10	1	6.5
	37	7/21	340	6.5	>10	0.6	7.8
	34	7/21	378	6.4	>10	>0.1	1.5
	35	7/21	359	6.9	>10	0.1	10
Finished[*]	24	7/19	412	6.9	>10	0.5	6
	38	7/22	378	6.8	6.5	0	13
	39	7/22	376	6.7	>10	0.4	8.9
	22	7/19	375	6.0	>10	1.1	8.6
	23	7/19	246	6.2	>10	1.2	15
	27	7/20	247	6.7	>10	0.8	15
	28	7/20	268	6.8	>10	0.6	8.5
	32	7/21	378	6.7	>10	1.4	7.2
	33	7/21	467	6.7	8.0	0.7	4.5

Finished water was treated by clarification, sand filtration, and chlorination

(2 mg/liter), and total coliform bacteria (<1 CFU/100 ml). ... of nine finished water samples collected met these criteria; four of these contained either enteroviruses or ...oviruses. Importantly, none of the samples containing ...ses had detectable total coliform bacteria. In contrast, ... of four samples containing viruses also contained fecal ...streptococci, and three of these met the criteria for turbidity, ... chlorine, and total coliforms ... were also interested in the ability of coliphages to ...ve as indicators of the animal virus content of water. The ...ults indicate that direct plating of water for coliphages did ... reflect the animal virus content of finished water. Furthermore, in several samples phages were detected in the concentrates but not by direct plating of the sample. A ...centration procedure may be necessary for coliphages to ...ve a useful function as indicators.

Since rotavirus is a major cause of gastroenteritis and may be waterborne (13), the ability of the water treatment process to remove rotavirus was of major interest. In this study, ... 94% of the rotaviruses present in raw water were removed by the treatment process during the dry season. As expected, during the rainy season, when the quality of the water declined, so did the removal of rotavirus. It is ...ly that adsorption to particulates which were not removed during the clarification and filtration steps protected the rotaviruses from final chlorination (6). Further studies on ... susceptibility of human and animal types of rotavirus to ...[_] action of disinfectants and the manipulation of water treatment processes for their enhanced removal are indicated.

... five of nine (56%) of the finished water samples collected ... ing the dry season contained enteroviruses. In only one ...These samples did the turbidity exceed 1 NTU; thus, high turbidity alone may not account for the failure to remove ...ses during water treatment. Furthermore, it should be ...ed that the lower mean turbidity and bacterial removal detected in finished water during the rainy season (Table 3) ... li_   ... ue to the fact that the samples were not collected ...y, and so short-term fluctuations in water quality ... accounted for. Statistical analysis of the relation-

ship of water quality measurement to the numbers of enteroviruses and rotaviruses recovered awaits the results of analyses on all 120 samples which have been collected in this study. Enteroviruses recovered from these samples have not yet been identified due to the recent inavailability of the Lim-Benyesh-Melnick antisera pools. Further efforts are being made to quantify the numbers of enteroviruses recovered and to identify the rotavirus isolates as either human or animal strains.

A further difficulty encountered has been the coconcentration of material which interferes with the assay of viruses in cell culture. This may have reduced the rate of recovery of enteroviruses in the raw water during the rainy season because of poor water quality. Since the assays for rotaviruses and enteroviruses are conducted under different conditions, this also may explain the differences in recovery of enteroviruses and rotaviruses during the rainy season. Alternatively, there may have been more rotaviruses in the water, as the rainy season appears to coincide with the peak period of rotavirus activity in the area (8; Deetz et al., in press). Preliminary testing indicated that the filtration process used to remove toxicity did not greatly affect the ability to detect viruses in these samples. However, this could not be determined for each sample since many were too toxic to be assayed unfiltered.

Drinking water treatment should and usually does produce microbiologically safe drinking water. As evidenced by the isolation of viruses from treated drinking water (3, 10), this is not always the case. Upon completion of this project we hope to be able to provide a prediction of the conditions, as measured by water quality parameters, under which viruses may survive water treatment. This study confirms that acceptable water quality measurements of turbidity, total residual chlorine, and total coliform bacteria do not necessarily reflect the virus content of treated drinking water. However, since not all finished water samples met each of these criteria, it is likely that operational difficulties were sometimes encountered as water quality fluctuated, indicating that the water treatment process in general is sound, but that under certain conditions viruses may survive treatment.

TABLE 5. Isolation of bacteria and viruses after successive drinking water treatment processes (rainy season)

Treatment process	Sample no.	Bacteria				Viruses			
		Total plate count (CFU/ml)	Total coliforms (CFU/100 ml)	Fecal coliforms (CFU/100 ml)	Fecal streptococci (CFU/100 ml)	No. of coliphages/10 ml (direct)[a]	No. of coliphages/1,000 ml (concentrate)	Enterovirus cultures[b]	No. of rotaviruses/100 liters
None (raw)	26	6,200	73,000	9,000	132,000	76	173,000	1/9	3,491
	31	1,020	20,000	0	89,000	18	110,000	0/5	0
Clarified	25	69	200	0	300	1	0	0/4	930
	30	82	1,280	0	720	31	0	2/3	5,904
Filtered	29	170	250	640	2,800	8	100	0/5	171
	36	620	120	0	160	1	0	2/3	463
	37	43	42	0	30	3	2,000	0/4	8x
	34	100	30	0	2,000	1	0	0/3	585
	35	43	300	0	10	6	800	1/5	40x
Finished[c]	24	53	70	10	540	9	13,000	0/4	10,44x
	38	ND[d]	ND	ND	ND	4	49,000	0/5	69
	39	ND	ND	ND	ND	3	50,000	0/5	10x
	22	>200	>2,000	0	640	4	0	1/4	24?
	23	>200	>2,000	0	1,152	4	300	0/4	11?
	27	>200	>2,000	ND	>200	6	0	0/5	5?
	28	240	>2,000	5	>2,000	4	100	0/3	2x?
	32	1,230	15,300	0	16,100	10	0	0/4	15?
	33	1,340	50	0	200	2	0	0/3	4?

[a] Water was plated directly for coliphages on E. coli Hfr host bacteria.
[b] Enteroviruses were detected by the production of CPE in monolayer cultures of BGM cells contained in 75-cm² flasks. Number positive/number tested.
[c] Finished water was treated by clarification, sand filtration, and chlorination.
[d] ND, Not done.

Common operational factors which may have contributed to these results include inadequate floc formation, floc breakdown, and filter overloading. These conditions can lead to increased amounts of particulates in finished water which can render terminal disinfection ineffective (6). The prediction of conditions which favor the survival of viruses in treated drinking water should enable improvements to be made in design and operation of water treatment facilities which will increase the likelihood that finished water is virus safe.

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CHAPTER 12

Water as a Reservoir of Virus in Nature and Means for Control

JOSEPH L. MELNICK

I. INTRODUCTION

Over 100 different viruses are known to be excreted in human feces. Many of them, particularly Picornaviridae, Adenoviridae, and Reoviridae, are sufficiently thermostable that they remain viable in water for long periods of time. A number also are resistant to conventional procedures, including chlorination, used in wastewater treatment plants and may be found far from the original source of contamination. With the recent recognition of hepatitis A virus and rotaviruses (the causative agents of type B viral gastroenteritis), methods for their detection in water are being developed so that they may be monitored together with the

TABLE 1
Human Enteric Viruses That May Be Present in Water[a]

Virus group	Number of types	Disease caused
Enteroviruses		
Poliovirus	3	Paralysis, meningitis, fever
Echovirus	34	Meningitis, respiratory disease, rash, diarrhea, fever
Coxsackievirus A	24	Herpangina, respiratory disease, meningitis, fever
Coxsackievirus B	6	Myocarditis, congenital heart anomalies, rash, fever, meningitis, respiratory disease, pleurodynia
New enterovirus types (68-71)	4	Meningitis, encephalitis, respiratory disease, acute hemorrhagic conjunctivitis, fever
Hepatitis type A (probably an enterovirus)	1	Infectious hepatitis
Gastroenteritis type A (probably an enterovirus)	2	Epidemic vomiting and diarrhea, fever
Reovirus (Reovirus family)	?	Epidemic vomiting and diarrhea, chiefly of children
Gastroenteritis type B		
Rotavirus	3	Not clearly established
Adenovirus	>30	Respiratory disease, eye infections

[a] Other stable viruses that might contaminate water are SV40-like papovaviruses, which appear in the urine. The JC subtype is associated with progressive multifocal leukoencephalopathy. Creutzfeld-Jakob disease virus. Like scrapie virus, the CJ virus resists heat and formaldehyde. The virus causes a spongiform encephalopathy, characterized by severe progressive dementia and ataxia. Parvoviruses. The adeno-associated satellite viruses that are excreted in stools of children survive heating for one hour at 60°C.

...onventional enteroviruses in sources of drinking water. Other human viruses that occur in fecally contaminated water, but for which suitable laboratory methods for detection in water have yet to be worked out, include the parvoviruses and the agent of type A viral gastroenteritis.

Removal of viruses from contaminated water includes processes of physical removal and those causing inactivation or destruction of the virus particle. Processes that bring about virus inactivation are preferable to those of simple removal since the latter presents problems in the disposal of infectious virus. The inability of chlorine to act as an effective viral disinfectant in wastewater and the concomitant production of toxic and carcinogenic chlorinated compounds in waters containing organic compounds are factors that are leading to the development of alternate disinfection procedures that can achieve large reductions in pathogenic microorganisms, particularly if wastewater is to be reclaimed and recycled as drinking water. One such method, photodynamic inactivation, in which solar energy can be used as a practical and inexpensive alternative to artificial light for the necessary energy, will be described.

II. OCCURRENCE OF VIRUSES IN WATER

Water contamination by human and animal excreta is the equivalent of varying contamination by bacteria and viruses found in feces and urine. Although viruses of lower animals may be contributing to this pollution, most of our knowledge is based on studies of viruses of humans (see Table 1). Since little can be added to John Fox's (1976) admirable review, I would like to quote relevant parts of it, with some editorial changes for which I take responsibility.

A. Picornaviruses[1]

These relatively hardy agents are capable of surviving for significant periods in the free state in water, such capability varying a bit with particular viruses and/or subgroups (e.g., the vulnerability of rhinoviruses to low pH). These latter, although by far the largest subgroup (over 100 serotypes so far recognized), primarily infect the nasal mucosa and are not commonly shed in feces, so that any possible waterborne spread would be virtually restricted to bathing situations. The remaining groups, polioviruses, group A and B coxsackieviruses, and echoviruses are known collectively as enteroviruses which multiply primarily in the alimentary tract and are excreted in substantial amounts in the feces for

varying periods of time, the longest being polioviruses, with a mean duration of 50 days and the shortest being echoviruses. The best studied of these are the polioviruses and the least studied are the many serotypes of group A coxsackieviruses that can only be isolated in suckling mice.

Numerous studies in developed countries have readily demonstrated the presence of enteroviruses in contaminated streams, in sewage, and in effluents from sewage treatment plants, and enterovirus surveillance of sewage has been used to monitor the impact of live poliovirus vaccination in the community. Although shellfish have not been implicated to date in the transmission of the conventional enteroviruses, clams and oysters

[1] Adapted from Fox (1976).

disease caused by the other enteroviruses. Meanwhile, the populations most vulnerable to such disease are those of developed countries. Data on the prevalence of immunity to the nonpolio enteroviruses is fragmentary but, in Fox's 1960–1965 study in New York, prevalence of neutralizing antibody among adults to selected viruses ranged from 14% for coxsackievirus B1 to 59% for coxsackievirus A9. In Seattle in 1968 only 10% of adults had antibody to type 30 echovirus. The occurrence in 1968 of a community-wide epidemic of aseptic meningitis due to this virus in Seattle with 44 cases recognized, chiefly in older children and adults, suggests the vulnerability of urban U.S. populations to these agents. Had extensive waterborne spread occurred, the epidemic probably would have been far more dramatic. The moral clearly is that, unless constant vigilance in protection of water supplies is maintained, nonpolio enteroviruses could cause major outbreaks of serious disease.

B. Adenoviruses

Although commonly thought of as respiratory viruses, adenoviruses almost invariably infect the alimentary tract and are abundantly shed in feces. While infections restricted to the alimentary tract cause little or no disease, disease commonly results when other sites, respiratory and conjunctival, are also infected. Fecal shedding of adenoviruses is extremely common among young children and may exceed that of wild enteroviruses in U.S. cities, although enteroviruses almost certainly predominate in developing countries.

Although efforts to detect viruses in sewage-polluted waters have commonly employed methods selected for detection of enteroviruses rather than of adenoviruses, the latter have been detected in a number of such studies. However, the only well-documented waterborne spread of adenoviruses has been in the epidemics of pharyngoconjunctival fever associated with swimming pools. In summary, waterborne spread of adenoviruses is theoretically possible but, for reasons cited, it has not resulted in recognized disease except in relation to swimming pools.

C. Parvoviruses

Most known members of this group of very small DNA viruses chiefly infect lower animals, e.g., rats, mice, cats (panleucopenia of cats), dogs, swine, and birds. The limited available evidence suggests that as a group these are very stable agents. The first recognized parvoviruses of human origin were the adeno-associated satellite viruses (AAV), which can replicate only with the help of adeno- or (less readily) herpesviruses. Seroepidemiologic studies indicated that antibodies to AAV, especially types 2 and 3, are widely prevalent in young children and that infection is

grown in contaminated water do acquire and harbor enteroviruses and viruses can persist in processed and even partially cooked oysters. Few similar studies of viral contamination of water have been published from developing areas but one can assume that, where sanitation is more primitive, gross fecal contamination of water is common and the viruses must be abundantly present.

A few words are now in order concerning the disease potential of enteroviruses. Wild polioviruses are significant entirely because they can cause serious central nervous system (CNS) disease (actually rather infrequently, from 1 in 100 to more than 1 in 1000 infections, depending chiefly on the virulence of the virus and the age of the host). Presently in the U.S. and other developed countries the frequently excreted polioviruses (the group encountered with the greatest frequency in Fox's virus watch studies) are almost entirely vaccine-derived and their pathogenicity is very low, although reversion with respect to neurovirulence during human passage is known to occur. The other enteroviruses are also often causes of CNS disease of usually benign nature (aseptic meningitis), but occasionally resulting in clinically typical paralytic poliomyelitis. While Fox's experience suggests that mild respiratory disease is by far the more typical result of these enterovirus infections and that serious syndromes including CNS involvement must be very infrequent, group B coxsackieviruses in particular do have the potential for causing other significant types of diseases. These include the very uncomfortable epidemic pleurodynia (Bornholm's disease), pericarditis (chiefly in older persons), serious and often fatal myocarditis in infants, and congenital defects (chiefly cardiac) in infants born of mothers experiencing infection during pregnancy. In summary, enteroviruses can cause very significant disease but, fortunately, do so in only a very small proportion of infections under most circumstances.

This infrequent association with serious disease may well help explain why documentations of waterborne spread of enteroviruses have been so few. A very important principle, best exemplified by the polioviruses, is that the severity of the outcome of infection in a nonimmune host is directly related to host age. In developing countries, where wild enteroviruses are highly prevalent, infections typically are acquired early in life when the risk of serious disease is minimal and most older children and adults are immune. In these areas waterborne spread undoubtedly occurs and may indeed be an important part of the process of natural immunization. As sanitation in some of these areas has improved, reflecting a slow increase in living standards, paralytic poliomyelitis has increased, presumably due to relative interference with virus spread sufficient to delay infections to a somewhat older age but not to prevent them. A similar trend should evolve (perhaps is already evolving) with respect to

cause disease. A recently discovered member of the Reoviridae, rotavirus, has been established as the major pathogen of nonbacterial infantile diarrhea throughout the world. The virus has been detected in approximately 40% of infants with diarrhea. However, during the winter months, when infection appears to be most common, the frequency of detection has averaged 70%. During the peak of the winter outbreak, the virus has been observed in specimens from 80–90% of infants and young children hospitalized with diarrhea. Large numbers of particles (10^9/g of feces) may be excreted by infected individuals, and so it would not be surprising if contamination of water supplies occurred.

The disease can be induced by the virus in experimental animals; newborn piglets, calves, and rhesus monkeys develop diarrheal illness when given a human stool filtrate containing the virus. The viral shedding patterns in feces of infected gnotobiotic piglets were consistent with those observed for infants with diarrhea.

The human virus has been shown to be related antigenically to Nebraska calf diarrhea virus, the virus of epizootic diarrhea of infant mice, the SA11 virus of monkeys and the O virus (isolated from water flowing from troughs in which the intestines, hence "offal," of both sheep and bovines were washed at the slaughterhouse). Malherbe isolated two other strains of similar agents: one from an algal furrow at the end of a conventional sewage purification plant in Pretoria, South Africa; and the other from a stream receiving effluent from another sewage plant, but at a point in the stream where agricultural land run-off occurred, so that the virus may have come from the land itself and not from the sewage effluent. Three of the viruses of this closely related group cause gastroenteritis in the natural host and some of these agents may find their way into surface water.

F. Papovaviruses[1]

These include the human wart virus plus one or more SV40-like agents associated with progressive multifocal leukoencephalopathy (PML). While this serious disease is extremely rare, seroepidemiological studies indicate that antibody to one of these agents, the JC virus, is widely prevalent (69% of Wisconsin adults) and, hence, infection must occur with some frequency albeit by unknown means and with equally unknown effects other than PML. Still another SV40-like agent, the BK virus, has been recovered from the urine of several renal allograft patients, which means contamination of wastewater. While its relation to human disease remains to be established, serologic studies indicate that primary infection usually occurs in early childhood and that adults commonly possess

associated with childhood respiratory disease. AAV, together with adenoviruses, have been recovered from feces and, hence, are almost certainly present in fecally contaminated water. However, data on frequency of excretion are lacking, as is an adequate evaluation of AAV impact on human health.

D. Hepatitis A Virus and Gastroenteritis Type A Virus[1]

The agents of type A viral gastroenteritis and type A viral hepatitis have recently been identified. Work is too limited to classify them with any certainty; their size and morphology indicate that they are either picornaviruses or parvoviruses. The basic fecal–oral transmission of such agents suggests that waterborne spread may be very important wherever sanitation is deficient (or a break occurs in the protection of a water supply).

Hepatitis A virus is excreted in the feces over a relatively extended period and, on the basis of many well-studied epidemics, this agent is often spread via water. Further, the tremendous 1955–1956 epidemic in Delhi, India, caused by gross sewage contamination of the water supply, provided inadvertent evidence for a highly relevant characteristic of the responsible agent, namely, its ability to withstand levels of residual chlorine (greatly raised to combat the emergency), which apparently were adequate to counteract all other enteric pathogens which must also have been present.

As regards gastroenteritis type A, viral particles have been demonstrated by use of immune electron microscopy (IEM) in stool specimens from patients with gastroenteritis. In one outbreak in Norwalk, Ohio, a virus was detected in stool specimens obtained during acute illness. The virus had a diameter of about 27 nm and a buoyant density of 1.38–1.41 g/ml, similar to that of other "pico" viruses.

Particles resembling the Norwalk agent in size and buoyant density were also visualized by IEM in stool filtrates derived from two of six other outbreaks of transmissible nonbacterial gastroenteritis. These two viruses were present in a lower concentration in stool than the Norwalk agent and were detectable by IEM only after stool filtrates were concentrated. The two viruses were distinct antigenically. However, one of the viruses shared antigenic determinants with the Norwalk agent. Thus, there appear to be at least two serotypes among the gastroenteritis type A viruses.

E. Reo- and Rotaviruses

Reoviruses have often been recovered from contaminated surface waters. Although highly infectious, little is known about their ability to

antibody. Papovaviruses as a group are unusually stable agents and, in theory, could remain viable in water over long periods.

G. Slow Viruses

The demonstrated existence in lower animals of several so-called slow viruses, especially visna and scrapie (both in sheep), suggests that analogous agents may affect man, a suggestion already supported by the demonstration that Creutzfeldt–Jakob disease and kuru can be experimentally reproduced in lower primates via cell-free filtrates from affected human brains. The kuru agent is highly stable and is thought to infect by ingestion. If these hardy agents should ever appear in water, they would be difficult to remove by any of our current practices.

III. THE PROBLEM

Water problems may vary in different countries, but all will agree that water problems exist now even in the most advanced countries and it takes little foresight to realize that the problems will become even more critical in the not too distant future. Harris and Brecher (1974) aptly point out:

Almost everyone supposes that community water systems are under continuous surveillance by competent state and local health officials, that water samples are scrupulously tested at frequent intervals, that any flaws in a water system will be soon discovered and corrected, and that the water we drink therefore must be safe. Unfortunately, almost everyone supposes wrong.

True, cities in the U.S. no longer suffer from large-scale recurring epidemics of typhoid, cholera, dysentery, and other waterborne bacterial infections. It was those epidemics, with their ghastly death tolls traceable to drinking water, that forced the establishment of community water supplies during the past century. The then-new water systems worked quite well against the hazards they were meant to alleviate. But today, many of the same water systems are overage, dilapidated, substandard in serious respects and barely able to meet peak demands.

Their design is primitive, and they are typically staffed by people trained in an outmoded tradition or not trained at all. As the level of pollution has risen in our sources of raw water, the techniques employed to make that polluted water safe for human consumption have become less and less adequate.

Experience has shown that community water supplies must be tested at frequent intervals, if intermittent bacterial contamination is to be spotted. Bacteriological tests are primarily designed to identify coliform bacteria, the types of bacteria found in feces and soil. The standards do not require a total absence of coliforms; a moderate count is permitted. A well-operated water treatment plant can do considerably better than the standards require.

Furthermore, we now know that coliform counts do not correlate with

virus counts. We have had many samples in our laboratory negative for coliforms that were positive for human pathogenic viruses.

The sanitary engineers who built the early community sewage and water systems did not know about viruses, which is understandable, but many modern sanitary engineers still do not know about viruses, which is neither understandable nor excusable. No accepted standards have been set for viruses in recreational or drinking waters, although standards have been proposed, based on present monitoring technology for virus detection. As our detection and monitoring methods improve, we have the obligation to assure members of society that the water they drink is free not only from pathogenic bacteria but from viruses as well.

Viruses occur in water, and waterborne outbreaks of a viral disease such as hepatitis have been well documented. If a virus infection has a short and uniform incubation period, and produces a characteristic, easily recognizable disease, carriage of the virus by water routes can be traced with a fair degree of accuracy.

In contrast to diseases whose spread can be traced in this fashion, the characteristics of many viral diseases are such that their transmission by water is very difficult to recognize. Viruses whose spread is difficult to trace include those viruses that produce clinically observable illness in only a small fraction of the persons who become infected, those that produce diseases with widely variable incubation periods, and those that are easily spread by direct human contact.

These reasons may account for the fact that almost 60% of all documented cases of disease attributable to drinking water in the U.S. were caused by agents of unknown etiology. In addition, at present no field-proven method exists for the detection of the agent of infectious hepatitis in water. These difficulties have led to an emphasis on the detection of enteric viruses in water as an indication of the presence of human pathogenic viruses and the possibility of contracting disease from such water.

Attention is being increasingly paid to problems of viruses in water. In the U.S. the number of waterborne cases of hepatitis type A virus, which behaves as an enterovirus, has been increasing in recent years and is now the most prevalent waterborne disease attributable to a specific etiologic agent. During this same recent period in the U.S. the annual number of waterborne cases of typhoid, a bacterial disease, has decreased at least fivefold.

In many parts of the world increased demands on available water resources due to the concurrent expansion of the world's population and industrial demand make recycling of domestic wastewater inevitable in the future. One of the major problems to be overcome is the development

of adequate methods to ensure the elimination of human pathogenic viruses from reclaimed water. This problem is also compounded by the concern that present water treatment procedures may not regularly be sufficient in preventing viruses from reaching community water supplies.

It has been 30 yr since the first studies on the presence of human enteric viruses in water began in earnest, but their public health significance in water has yet to be ascertained. This has been due in part to the inapparent nature of the infections caused by these viruses and the widespread lack of methodology for detection. Studies have shown that enteric viruses easily survive present sewage treatment methods and many can persist for several months in natural waters.

More than 100 different enteric viruses are known to be excreted in human feces. Table 1 lists the major groups of enteric viruses that have been found in raw sewage or are known to be present in the feces of infected persons, including healthy carriers. Enteric viruses are excreted in concentrations as high as one million viruses per gram of feces, and can persist for long periods of time in the environment. Reported survival times range up to 168 days in tapwater, seawater, or soil.

The expected average enteric virus density in domestic sewage has been estimated to be about 7000 viruses/liter, but as many as 500,000 viruses/liter have been detected in some parts of the world. The amount of virus present in raw sewage is highly variable depending on such factors as the hygienic level of the population, the incidence of disease in the community, socioeconomic level, and the time of year. In the U.S. peak levels occur in the late summer and early fall. Enteric viruses survive the customary secondary sewage treatment and chlorination as commonly practiced in sufficient numbers to be isolated easily by modern concentration procedures at all times of the year. Thus, it is not surprising that these viruses have been detected in several of the major rivers of the U.S. Since enteric viruses are considerably more resistant to various sewage and water treatment methods than either coliform or enteropathogenic bacteria, the absence of the latter organisms may not guarantee the absence of a viral disease hazard.

Little is known about the occurrence of viruses in drinking water, because until recently methods for the concentration of viruses from large volumes of water have been lacking. A few reports do exist in the literature. In a study during the 1960s in Paris, enteric viruses were detected in 18% of 200 samples, and the average virus concentration was estimated at one infectious unit per 300 liters. More recently, Russian investigators have reported the isolation of enteric viruses on several occasions from drinking water. The water treatment plant from which the water came was found to be functioning normally (the process included

chlorination) during the periods when viruses were recovered. Viruses also have been isolated from drinking water in South Africa. Virological surveys of drinking water supplies are sorely needed to determine if currently practiced water treatment methods are adequate, but unfortunately few laboratories at the present time are equipped or staffed to undertake such studies.

Figure 1 illustrates some of the routes by which viruses from human and animal wastes may find their way to susceptible human hosts (Gerba et al., 1975).

IV. THE SOLUTION

Processes available for virus removal from wastewater include those involving physical removal and those causing inactivation or destruction of the particle. Processes that involve actual removal include sedimentation, adsorption, coagulation and precipitation, and filtration. Conditions that cause inactivation are high pH, chemical oxidation by disinfectants such as halogens, and the recently described process of photooxidation by dyes and light. Processes that bring about virus inactivation are preferable to those of simple removal since the latter present a problem of the disposal of potentially infectious material.

Primary treatment of wastes, involving only settling and retention before discharge, removes little or no viruses. Any virus removal that occurs during this treatment probably results from the sedimentation of viruses associated with sewage solids. Virus removal of up to 90% has been observed after activated sludge treatment, but large variations in removal have been reported. The physical-chemical treatment of sewage can result in large reductions of virus. Alum (aluminum sulfate), lime (calcium hydroxide), and salts of iron compounds as well as polyelectrolytes, are capable of removing as much as 99.9% of virus suspended in water. It has been postulated that coagulation results in the formation of a coagulant-cation-virus complex that settles from solution. The virus is not inactivated by this process, and in fact such coagulation has been used as a method to concentrate viruses from water. The high pH that can be attained during lime treatment can also result in very large reductions of virus. If pH levels above 11 are maintained for sufficiently long periods, 99.9% inactivation of the viruses present can result. Large variations in the reported times required for inactivation exist in the literature, and there is a need for a much closer look at such factors as concentration of organics, time, and temperature.
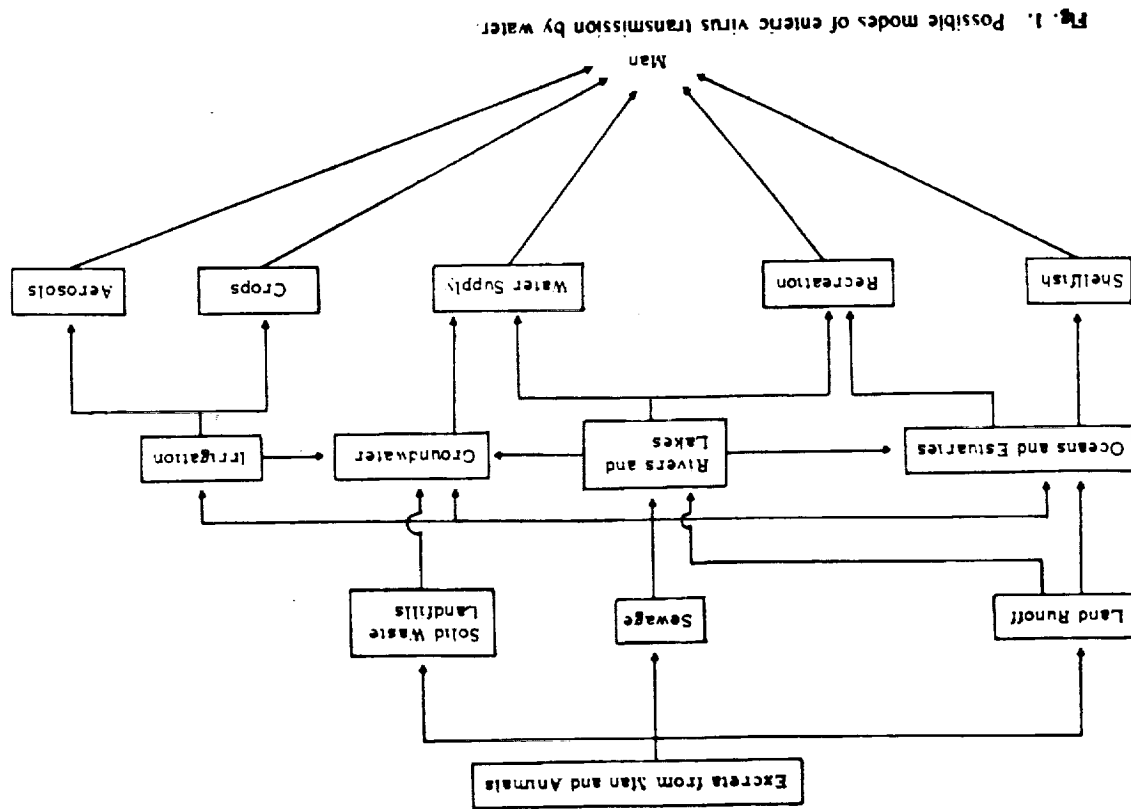
Under appropriate conditions, viruses are readily adsorbed to a wide

variety of surfaces, including activated carbon, diatomaceous earth, glass, membrane filters, colloidal organic matter, clays, and soil. Adsorption is reversible by alteration of ionic levels or pH or by the addition of competing organic matter. Activated carbon removes virus, but its capacity is soon reached and virus desorption often occurs as organic substances replace the adsorbed virus. Sand filters can remove virus by adsorption onto substances trapped by the sand, but little adsorption onto the sand itself occurs.

Although chlorine treatment has been the mainstay of water disinfection for over 50 yr in the U.S., too little is known about the mechanisms by which it renders the virus nonviable. Chlorine's effectiveness as a viral disinfectant is highly dependent on a number of factors including temperature, pH, the presence of organic matter, and the physical state of the virus (that is, whether it is adsorbed or aggregated). Because of the presence of large amounts of organic matter in the effluents from activated sludge plants, large reductions of virus are not possible because of the combination of the chlorine with ammonia and organics. Application of 8 mg of chlorine per liter of sewage effluent may not decrease the virus concentration. With very high doses (40 mg/liter for 10 min), 99.97% destruction of virus in sewage has been achieved, but then the problems of expense, chlorine toxicity to higher forms of life of such effluents are discharged, and the production of carcinogenic chlorinated hydrocarbons remain. Further complicating the problem is the wide variability in resistance of different enteric viruses to inactivation by chlorine. In a study of the resistance of 25 human enteric viruses, the time required for 99.99% inactivation of the viruses, under the same conditions, varied from 2 min to 2 hr.

Ozone has been widely used in many parts of the world as a disinfectant for water and wastewater, but little information is available to indicate its virucidal efficiency, especially under field conditions, where organic loads are present and act to protect virus from inactivation. One of the main arguments against the widespread use of ozone is that it lacks a residual effect since it has a half-life of only 25 min.

The inability of chlorine to act as an effective disinfectant against viruses in sewage and the possible production of toxic and carcinogenic chlorinated compounds has led to a call for the development of alternate disinfectants (Pietuch, 1975). In addition, disinfection methodology must be developed that is capable of achieving very large reductions of pathogenic microorganisms if reclamation of sewage is to become a reality. In previous work, the inactivation of bacteria, fungi, and viruses by sensitizing these agents with photoreactive dyes and exposing them to white light or monochromatic light to bring about photodynamic oxidation

Fig. 1. Possible modes of enteric virus transmission by water.

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been described (Wallis and Melnick, 1965; Wallis *et al.*, 1965). Recently, we have been exploring this method as a means of disinfecting sewage.

Photodynamic Inactivation Theory

The photodynamic oxidation reaction has been known since about 1900; however, detailed studies on the inactivation of proteins, nucleic acids, bacteria, and viruses were not performed until the late 1920s and early 1930s (Spikes and Livingston, 1967). During the late 1950s and early 1960s mechanistic studies were made on the inactivation of free nucleic acids and on intact viruses. The results of these latter studies indicated that the site of the attack was the nucleic acid and involved the guanine base, which is a heterodicyclic carbon–nitrogen compound. Also, the studies showed that the rates of inactivation of the guanine moiety of the nucleic acid was much faster in the presence of oxygen and that one mole of oxygen was consumed per mole of guanine photooxidized when sensitized with the dye methylene blue.

Laboratory Studies

The photoreactive heterocyclic dye, methylene blue, was chosen for these studies because of its low cost and because the wavelength at which it has maximum absorption (670 nm) was found to penetrate clarified sewage with 15 Jackson turbidity units (JTU) to a depth of 2 in. (5 cm) with a transmission of greater than 80%. The absorption spectrum of methylene blue is shown in Fig. 2. The dye also has a very low toxicity and, in fact, is administered orally or intravenously in treatment of certain diseases of humans and as an antidote (Arena, 1975).

The effectiveness of methylene blue as a photosensitizing dye using white light has been shown to increase with increasing pH. The rate of photooxidation of the nucleotide deoxyguanosine increased 30 times with a pH changes from 7 to 10.5.

In our study, the photoirradiation lamps were 4-ft long, 40-W monochromatic lamps (Westinghouse F 44T12/RB/SHO, 1500 M, lamps), and each lamp emitted 2000 μW/cm² of 670-nm light at 1 in. (2.5 cm) from the surface of the lamp. The emission spectrum of the lamp along with the emission spectrum for white light is also shown in Fig. 2. The emission spectrum of the RR lamp, as illustrated, is in the same range in which methylene blue absorbs most strongly.

A number of parameters of the system have been studied (Gerba *et al.*, 1977), using a simple flowthrough irradiation cell shown in Fig. 3. Preincubation of dye–bacteria mixtures resulted in large numbers of coliforms
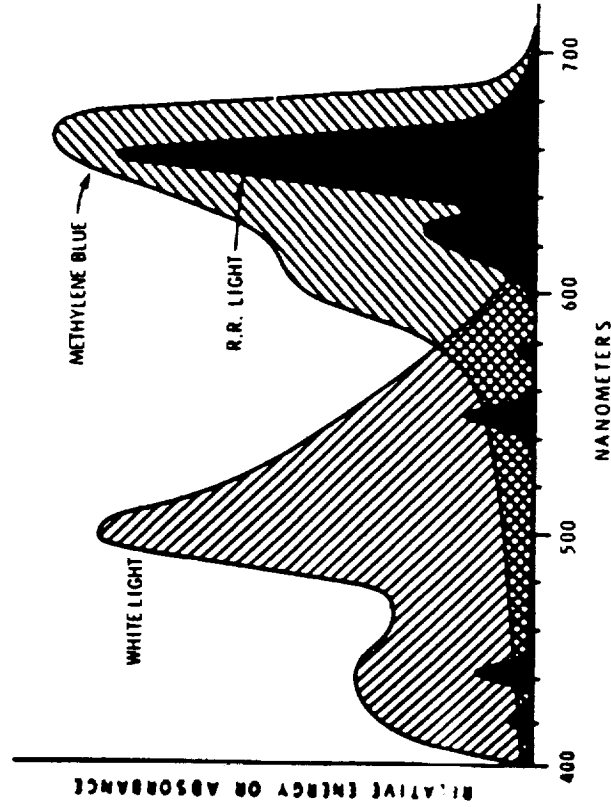

Fig. 2. Nomograph of the absorbance spectrum of methylene blue and the emission spectra of Westinghouse 40-W RR Special SHO tubes and to incandescent and fluorescent white lights.

that could be inactivated. Over 100 million bacteria could be inactivated with only a 60-sec exposure to 2000 μW/cm² of 670-nm light containing 1 mg liter of the dye after sensitization for 4 hr in the dark, while after 8 hr sensitization less than 10 sec was required to inactivate the same number of bacteria.

Poliovirus was found to be readily inactivated. The effect of various concentrations of the dye on the amount of poliovirus that could be inactivated after a 5-min exposure to the light source is shown in Table II. The amount of virus inactivated continued to increase until a concentration of 10 mg/liter was reached. Higher concentrations of dye resulted in a gradual reduction in the amount of virus that could be inactivated. This was attributed to the absorption of the light by the dye in the upper layers of the fluid.

pH and incubation temperature can dramatically affect the photoinactivation of enteroviruses by heterotricyclic dyes. The effect of pH on the photoinactivation of poliovirus using a 5-min exposure to light is shown in Table III. A marked increase in the amount of virus inactivated occurred between pH 9.5 and 10.0. The effect of incubation temperature of the dye–virus mixture on the amount of virus inactivated after exposure to the light source is shown in Table IV.
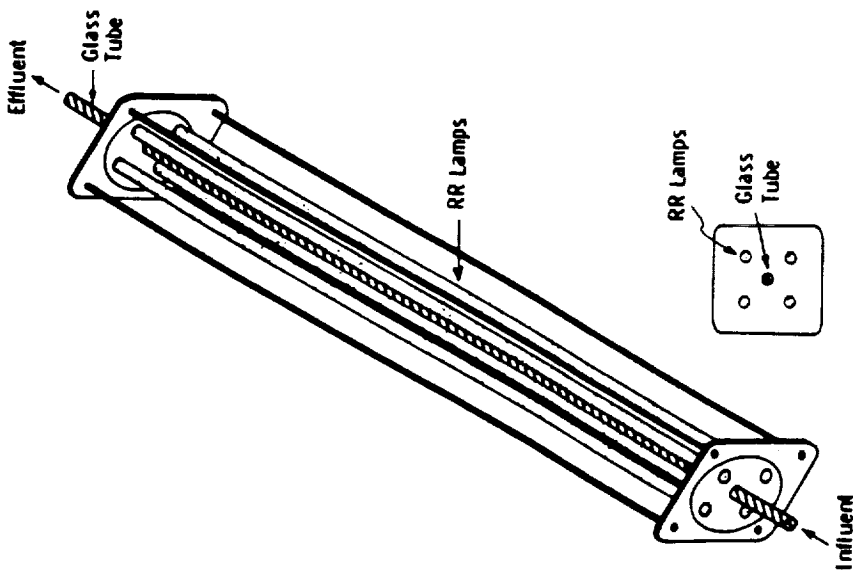
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TABLE II
Effect of Dye Concentration on the Photoinactivation of Poliovirus[a]

Dye concentration (mg/liter)	$-\mathrm{Log}_{10} N/N_0$[b] of virus inactivated after 5-min exposure to 2000 μw/cm² 670-nm light
0.1	0.04
1	0.65
5	1.83
8	1.89
10	2.28
15	2.12
20	1.72
50	0.17

[a] Samples were sensitized for 24 hr.
[b] $-\mathrm{Log}_{10} N/N_0 = \log$ of the number of organisms inactivated.

determine the effect of the photodynamic inactivation process scheme on poliovirus. Dye removal was accomplished when desired by adding the sterilized sodium form of Amberlite IR120 to the dye–virus solution and vigorously agitating the mixture while adjusting the pH to 8.5. Virus concentration was performed using 4 liters of the decolorized supernatant sample by the procedures already described (Wallis et al., 1976), which readily allows for concentration of the sample to 20–40 ml.

Secondary effluent was obtained from a package sewage treatment plant that processes municipal sewage. The effluent was pumped into the

TABLE III
Effect of pH on Photodynamic Inactivation of Poliovirus[a]

pH	$-\mathrm{Log}_{10} N/N_0$ of virus inactivated after a 5-min exposure to 2000 μw/cm² 670-nm light
10.0	2.07
9.5	1.11
9.0	0.98
8.0	0.49
7.0	0.20
5.5	0.00
4.5	0.00

[a] Virus was sensitized for 24 hr at pH 10.0 with 5 mg/liter dye in glycine buffer.

Fig. 3. Flowthrough irradiation cell.

C. Photoinactivation Using Solar Energy (Melnick et al., 1976)

A large part of the energy near the 670-nm wavelength contained in light from the sun passes through the atmosphere and reaches the surface of the earth (Gates, 1962). Photometer readings indicated that during midday the 670-nm energy reaching the Houston area exceeds 10,000 μW/cm². A range of 1000–2000 μW/cm² has been observed. Exposure of sensitized virus to sunlight indicated that this source of energy was very effective in the photoinactivation of virus in both sewage and tapwater (Table V).

D. Pilot Scale Operations (Hobbs et al., 1977)

The experimental procedures were designed to provide high quality water from either tapwater or from sewage secondary effluent and to

TABLE IV

Effect of Incubation Temperature on Photoinactivation of Poliovirus[a]

Temperature (°C)	$-\text{Log}_{10}\ N/N_0$ of virus inactivated	
	Before[b]	After[c]
4	0	1.2
24	0	1.74
37	0.3	2.55

[a] Virus was sensitized in the dark at pH 10.0 with 5 mg/liter dye at the indicated temperature for 24 hr before exposure to 2000 μw/cm² 670-nm light for 3 min.
[b] Before exposure to lighting system.
[c] After exposure to lighting system.

1000-gal storage–flocculation tank and was adjusted to pH 10 and flocculated with ferric chloride.

Photodynamic inactivation of poliovirus was performed in irradiation cells containing 12 and 23 monochromatic lamps. The period of irradiation was controlled by the flowrate. In one cell the 12 monochromatic lamps were arranged horizontally in three stacks so that six equal-size chambers surrounded by four lamps were obtained. In the center of each irradiation chamber, a 1-in. (2.5 cm) ID clear plastic tube was placed, and the tubes were interconnected in a series pattern to give a plug-flow reactor. Each lamp was 1 in. from any adjacent irradiation tube.

TABLE V

Use of Solar Energy for the Photoinactivation of Poliovirus[a]

Time (min)	670-nm light[b] (μw/cm²)	Temperature (°C)	$-\text{Log}_{10}\ N/N_0$ virus inactivated	
			Sewage (20 JTU)	Tap-water (< 1.0 JTU)
0	2700	25	0	0
3	2700	25	0.2	0.9
6	2700	25	0.3	2.4
10	2700	26	0.5	>3.1
30	1800	28	2.1	—

[a] Ten liters of sewage or tapwater were placed in a large metal pan with a depth of approximately 10 cm. The pH was then adjusted to 10.0 and stock poliovirus was added to a final concentration of 9.6 × 10² PFU/ml. Dye to a final concentration of 5 mg/liter was then added as the solutions were continuously mixed by a magnetic stirring bar. Amount of virus inactivated is the loss of titer difference between controls held in the dark.
[b] As measured horizontal to the surface of the liquid. The experiment was performed on a cloudy, overcast day in Houston, Texas.

The coliform results in the large unit corroborated the earlier data that the photoinactivation of the organisms at pH 10 with methylene blue at 5 mg/liter was very effective.

Continuous flow enterovirus inactivation studies were performed in the prototype flowthrough cell to determine the processing parameters for the photoinactivation process on a pilot plant scale basis. Poliovirus was sensitized at 23°C and pH 10.0 to methylene blue at concentrations of 1, 2, and 5 mg/liter for 4–24 hr. The sensitized virus was then photoinactivated in the large cell for various periods of time, which were accomplished by varying the flowrate to the cell. The results of the tests are shown graphically in Fig. 4 and indicate that the photoinactivation of poliovirus on a large-scale continuous flow basis is feasible and corroborate the earlier bench-scale study results.

The effect of methylene blue dye concentration as determined by this limited number of runs suggested that 1–2 mg/liter of dye was as effective as 5 mg/liter of dye for the photoinactivation of poliovirus. The lower
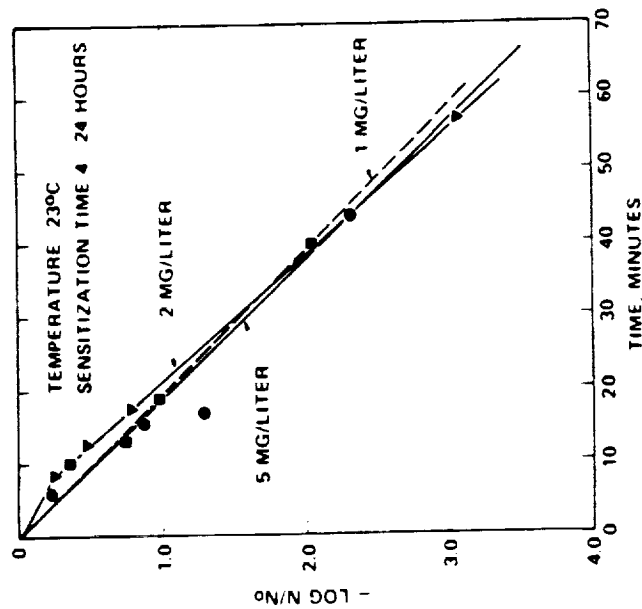

Fig. 4. Photoinactivation of poliovirus in a pilot-scale flowthrough cell using methylene blue at pH 10.0.

usage of dye would have a cost benefit not only from less reagent usage but also for a cost reduction on the process requirements for dye removal, if this is deemed necessary.

E. Process and Economic Evaluation

The proposed photodynamic inactivation process for the disinfection of sewage secondary effluent is shown schematically in Fig. 5 and consists of the following steps: (1) adjustment of secondary effluent to pH 10 using caustic soda or lime and ferric chloride addition to aid in flocculation of solids and removal of phosphorus; (2) clarifier for settling of solids; (3) additional step for nitrogen removal if desirable by air stripping; (4) sand or dual media filtration; (5) dye addition to tank for sensitization of the bacteria and viruses with the dye; (6) photodynamic irradiation cell; (7) dye removal by activated carbon, which will also result in effluent beneficiation due to the removal of refractory organic materials; and (8) pH adjustment to pH 9.0–9.5 prior to discharge.

The sequence of process steps proposed for the photodynamic inactivation process, except for the photocell, are the very unit operations for tertiary treatment of secondary effluents for phosphorus and ammonia removal. Also, they are the same sequence of steps used for both the low and high lime processes for raw sewage. Consequently, where physical-chemical operation or tertiary treatment of secondary effluent is being considered, the photodynamic inactivation technique can readily be incorporated into the process without addition of major capital equipment, other than a metering system for the dye, a holding tank for sensitization to occur, and the irradiation cell.

The photodynamic operation has been evaluated on the basis of an enterovirus content in sewage of 1000 plaque-forming units (PFU)/gal, which is to be reduced to 1 PFU/10 gal, or a reduction of 10,000× in virus
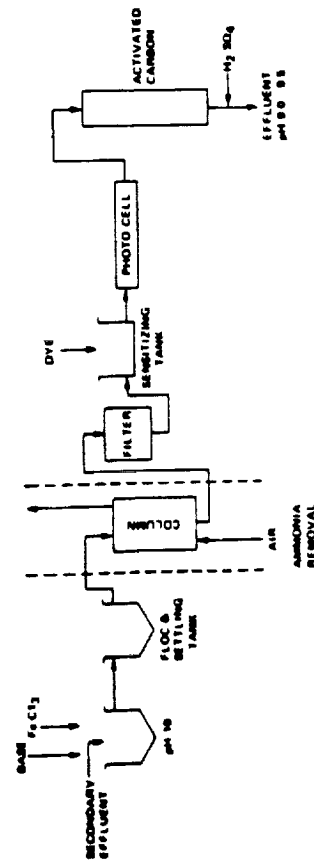
content. This level of enterovirus in secondary sewage effluents is now required by ordinance in Montgomery County, Maryland, and has been discussed at the International Conference on Viruses in Water at Mexico City (Berg et al., 1976) and at earlier meetings (Melnick, 1971).

The power requirement was determined for operating such a cell with a volume of 32.6 gal (0.123 m³). The estimated irradiation times at various temperatures, to achieve a 4 log reduction in virus for the flowthrough cell with the lamps in contact with the water were calculated by the FMC Corporation and are recorded in Table VI along with the costs for operating the cell in large and small plants. The chemical costs for the photodynamic oxidation process using 2 mg/liter of methylene blue were also calculated by the FMC Corporation and are given in Table VII.

The effects of photodynamic oxidation on organic molecules have been intensively studied for over a decade and much information is available on how it acts to destroy the integrity of these molecules. Such information is generally lacking for most disinfectants that are commonly used today. It has generally been established that photodynamic oxidation acts directly on the nucleic acid of the virus, resulting in its inactivation. All groups of animal viruses tested have been shown to be sensitive to photodynamic inactivation. Bacteria commonly found in sewage, such as coliforms, are easily photoinactivated as are pathogenic fungi such as *Candida albicans*.

Organic matter has been shown to greatly reduce the effectiveness of many disinfectants, especially chlorine and ozone. High concentrations of chlorine must be added to obtain small residuals in wastewater. It was shown in this study that sewage organics did not interfere with the action of the dye on microorganisms. Systems utilizing ultraviolet light would be

TABLE VI

Projected Power Costs for Photodynamic Inactivation of Virus[a]

Temperature (°C)	Rate				Cost ($/1000 gal)	
	t_1 (hr) (10⁴ Red)	t_2 (min.) (10⁴ Red)	gallon/min	gallon/day	Package plant (at 2.4¢/kWh)	Large plant (at 1.5¢/kWh)
22	5	45	0.72	1040	1.35	.85
28	5	36	0.90	1304	1.07	.67
38	5	27	1.21	1740	0.80	.50
22	24	22	1.48	2130	0.65	.41
28	24	18	1.80	2590	0.53	.34
35	24	13	2.51	3610	0.39	.24

[a] As of 1975, using 2 mg/liter methylene blue.

Fig. 5. Proposed process for photodynamic inactivation.

described in this paper. The major cost factors in photodynamic inactivation are the chemical cost for adjusting sewage secondary effluent to pH 10 and the electrical power cost. In physical–chemical and tertiary treatment systems, the chemical cost for pH adjustment is charged against nutrient removal; consequently, the processing costs will be reduced, as well as the reduction in capital costs to only the cost of the dye metering system and the photoinactivation cell. However, the major cost factor is the electrical power for operating the cell, and according to various lamp manufacturers, there does not appear to be any new lighting systems that will increase the efficiency of conversion of the electrical energy to light energy in the 600–700 nm range. The electrical power cost can be reduced by heat treatment during sensitization and by longer sensitization time. Heating the wastewater from 23 to 35°C reduces the irradiation time by half and would materially reduce the electrical power costs as indicated in Table VI. Moreover, solar energy has been shown to be a possible alternative to the use of the high intensity monochromatic lamps for photooxidation using methylene blue. The use of solar energy would eliminate the power costs for the photoinactivation cell, which would reduce the processing costs so that the photodynamic inactivation process would be competitive to the cost for chlorination and ozonation (Melnick et al. 1976).

The amount of 670-nm light in Houston, Texas, was found to be many times that available from the artificial light source that we used in the laboratory. Large reductions of poliovirus in both turbid wastewater and tapwater were easily accomplished, even under heavily overcast skies (Table V). Use of solar energy would greatly reduce operational costs of a system employing photoinactivation. Such a system should be feasible anywhere in the world where some daylight is available year round. It also should be pointed out that sensitization in the dark is not a necessity, since microorganisms can be inactivated by light in the process of becoming sensitized.

The solution to the problem of viruses in water is to prevent their contamination at the source. If sewage is properly treated, our surface waters can be made essentially free of viruses, which in turn reduces the potential of viruses entering our drinking water supplies. To prevent virus contamination of our surface waters, this report describes the feasibility of photodynamic oxidation for disinfection of sewage. Photoinactivation should be of greatest use in systems where large reductions in pathogenic bacteria and viruses are required, such as wastewater recycling systems. Optimal parameters for the photodynamic inactivation of coliforms and poliovirus in sewage by methylene blue have been determined. Temperature, pH, dye concentration, time of sensitization, and time of light

ineffective in such effluents because of their absorption by the organic matter at the surface of the fluid. Wavelengths in the 670-nm range, though, are not absorbed by the organics and can penetrate to much greater depths. Turbidity can also affect the penetration of visible light into fluids, but under the conditions of this study turbidities of up to 10 JTU did not appear to affect the amount of coliforms or poliovirus that could be inactivated.

Photoinactivation could be most advantageously used in advanced wastewater treatment facilities where a high quality effluent is required. Photoinactivation could easily be incorporated into advanced wastewater treatment plants that use lime flocculation, since alkaline pH levels optimal for sensitizing microorganisms are produced during this process. Facilities that use elevated pH levels for ammonia stripping could also readily incorporate photoinactivation. Such treatment plants also greatly reduce turbidity, allowing for further penetration of light into the effluent. Passage of final effluent from such facilities through granulated activated carbon to remove residual organics is also practiced. This could be taken advantage of for the removal of the dye, since it readily adsorbs to activated carbon. We also conducted studies at a land disposal site in which dye was added to wastewater, and we found that methylene blue quickly becomes undetectable and presumably degraded in the environment. Nevertheless, if desirable, it can be removed to ensure the absence of any potentially, although presently unknown, adverse environmental effects.

The photodynamic inactivation process is more costly as compared to chlorination, breakpoint chlorination, and ozonation using the system

TABLE VII

Chemical Costs for Photodynamic Treatment at pH 10

Reagent	(lb/1000 gal)	(¢/lb)[a]	(¢/1000 gal)
Caustic (50%)	3.4	3.5	11.9
Ferric chloride (anhydrous)	0.02	12.5	0.2
Sulfuric acid (93%)	0.5	2.3	1.2
Methylene blue (at 2 mg/liter)	0.017	202.00[b]	3.4
Activated carbon	0.04	32.0	1.3
Total chemical			18.0

[a] Chemical Market Reporter, June 20, 1975.
[b] FMC Corporation projected cost for large-scale production.

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exposure influence the inactivation of coliforms and virus. In contrast to other methods of disinfection, organics in sewage failed to interfere with the photodynamic inactivation of the microorganisms. At first glance, the process and capital costs are greater than for chlorination because of the high cost of electrical power for the lamps and because of the necessity to raise the pH. However, in water treatment plants where the lime-softening process is used for water purification, the added costs of the photodynamic process would be greatly reduced. Furthermore, solar energy was found to be a practical and inexpensive alternative to use of an artificial light source for the photodynamic process. Photodynamic inacti-vation should be of greatest use where large reductions in pathogenic bacteria and viruses are required, such as water recycling systems. This is particularly true where one wishes to avoid the use of large amounts of chlorine with the subsequent formation of chlorinated carcinogenic com-pounds, many of which are now appearing in our water supplies.

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CHAPTER 13

Sexually Transmitted Viruses

LAURE AURELIAN AND BRUCE C. STRNAD

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TX 1 OF 13.
       The following reports describe two outbreaks of viral
   gastroenteritis associated with contaminated *water.*
   2 OF 13.
       South Dakota. An outbreak of diarrhea occurred among the 331
   participants in an outing held at a South Dakota campground on August
   30 and 31, 1986. During the event, in which participants hiked 10 or
   20 km, *water* and a reconstituted soft drink were available at rest
   stands. The State Department of Health conducted a survey of 181
   participants: 135 (75%) of these persons reported a gastrointestinal
   illness. Symptoms most frequently reported were diarrhea (69%),
   explosive vomiting (55%), nausea (49%), headache (47%), abdominal
   cramping (46%), and fever (36%). None of the participants required
   hospitalization. Attack rates by sex and age of patients were
   virtually equal. Onset of illness occurred 35 hours (mean) after
   arrival at the campground, and duration of illness was about 33
   hours.
   3 OF 13.
       A biotin-avidin immunoassay performed at CDC yielded a fourfold
   rise in antibody titer to Norwalk *virus* in seven of 11 paired human
   serum specimens. No pathogenic bacterial or parasitic agents were
   identified from stool samples. Illness was strongly associated with
   the consumption of *water* or the reconstituted powdered soft drink
   made with *water.* No other foodstuffs were implicated. The
   implicated *water* came from a well at the campground. A yard hydrant
   was located next to a septic dump station, where sewage from
   self-contained septic tanks and portable toilets in the park was
   collected. *Water* from this hydrant had been used to fill *water*
   coolers and to prepare the powdered soft drink. Laboratory analyses
   of remaining *water* and reconstituted soft drink samples showed
   bacterial contamination (fecal coliforms greater than 1,600 cfu/100
   mL). Chlorine was stored in a tank and then drawn directly into the
   water system by a pump without a monitoring system. *Water* samples
   obtained from various locations in the campground had excess

Giardia was isolated. Convalescent-phase sera were submitted to CDC
for 13 cases and 26 controls (2 per case), matched for age within 5
years, gender, and city of residence. Controls were selected from
health department personnel who had not visited the lodge. No
difference in Norwalk titers was found between five cases and five
controls.

     Under the supervision of state environmentalists, the *water*
system was renovated before the lodge reopened, with particular
emphasis on filters, the chlorinator, and the storage tank.

     Reported by: PA Bonrud, MS, AL Volmer, TL Dosch, W Chalcraft, D
Johnson, B Hoon, M Baker, KA Senger, State Epidemiologist, South
Dakota State Dept of Health. CF Martinez, TO Madrid, MPA, RM
Gallegos, MS, SP Castle, MPH, CM Powers, JA Knott, RM Gurule, MS
Blanch, LJ Nims, MS, PW Gray, PA Gutierrez, MS, M Eidson, DVM, MV
Tanuz, HF Hull, MD, State Epidemiologist, New Mexico Health and
Environment Dept. Respiratory and Enteroviral Br, Div of Viral
Diseases, Center for Infectious Diseases, CDC.

     Editorial Note: The two outbreaks of *gastroenteritis* described
above are representative of those frequently reported to CDC. They
demonstrate the need for an improved, specific laboratory approach to
identify the agents (many of which are presumed to be viral)
responsible for these outbreaks *RF 1,2 *. Transmission of these
viruses is often associated with fecal contamination of *water* sources

used for drinking, swimming, or producing ice *RF 3 *. Additionally,
the contamination of coastal *water* poses a special problem, since the

consumption of seafood is a risk factor for acquiring Norwalk agent
infection and other enteric viral agents.

     The two best-known enteric viral agents, rotavirus (group A) and
Norwalk agent, were first seen in the stools of diarrhea patients by
means of electron microscopy in the early 1970s. Both agents have
proven to be important causes of *gastroenteritis* in this country,
with rotavirus being the most common agent for diarrhea in young
children *RF 4 * and Norwalk agent being common in adults *RF 5 *.
In recent years, enteric adenoviruses, non-group A rotavirus, and
several 27- to 32-nm enteric *viruses,* including other Norwalk-like
agents, caliciviruses, astroviruses, and other enteric viral
pathogens, reportedly have been associated with *gastroenteritis* *RF
1,6 *. Recent advances in identifying and diagnosing some of these
viruses should make it possible to reduce the number of undiagnosed
outbreaks in future investigations. Methods for serologic and
antigenic tests are available for some agents, but the examination of
stool samples by electron microscopy offers the possibility of
identifying agents for which no specific tests are available. The
probability of detecting viral particles by electron microscopy is
greatest if stool specimens are collected during the early stages of
illness, preferably within 12 hours and no later than 48 hours after
onset. Some viral particles may be more stable if stool samples are
stored at 4 degrees C. The following guidelines are currently

coliforms when the chlorination system was not operating.
Fluorescent dye injected into a 5,000-gallon septic tank situated
uphill from the well confirmed that the well was contaminated with
sewage.
4 OF 13.
    This campground was closed immediately and voluntarily by the
owner. Corrective measures included relocating the well, installing
an alarm system to detect malfunctions in the chlorination system,
reconstructing the chlorination system to ensure that chlorine
remains in contact with *water* in a storage tank for 30 minutes before

the *water* is distributed, maintaining a daily log on chlorine
residuals and sample collection points, and posting the yard hydrant
as a nonpotable source of *water.*
5 OF 13.
    New Mexico. An outbreak of *gastroenteritis* occurred among the
92 guests and staff at a cabin lodge in northern New Mexico over the
Labor Day weekend in 1986. The guests arrived Friday, August 29, and
provided their own food for the weekend. The first persons to become
ill developed diarrhea on Saturday morning, within 24 hours after
arrival. By Wednesday, 36 of the guests and staff members reported
symptoms: 34 had diarrhea; 9, vomiting; 14, fever; 22, abdominal
cramps; and 1, bloody stools. There were no deaths or
hospitalizations.
6 OF 13.
    A questionnaire was administered to all 92 guests and staff to
ascertain risk factors for *gastroenteritis.* Guests consisted of
unrelated groups, and they stayed in 18 seaparate cabins. All 36 of
the patients and 37 of the 56 unaffected attendees had drunk *water* at

their cabin. A dose-response relationship was demonstrated between
the amount of *water* consumed and the attack rate. No illness
occurred among the persons who did not drink *water;* 33% of those
drinking 1-2 cups and 59% of those drinking greater than or equal to
3 cups became ill. Five of the 18 cabins were unaffected; three of
these belonged to families who were residents or frequent visitors at
the lodge.
7 OF 13.
    Assuming guests were exposed upon arrival or when they first
drank *water,* the median incubation period was 41 hours (range = 7-110

hours). Symptoms lasted from 2-17 days, with a median of 5 days.
8 OF 13.
    The cabins were supplied with *water* taken from a stream and
processed through a small chlorinator and a storage tank that was
periodically iodized. A filter had been removed recently from the
pipe because it repeatedly became plugged with debris. A severe
rainstorm occurred the evening the guests arrived, resulting in
increased *water* turbidity.
9 OF 13.
    Water samples taken at the cabins and the surface stream that
supplied the cabins were positive for total coliforms and fecal
coliforms. Stool samples from ill patrons were negative for
pathogenic bacteria and parasites, except for one sample, from which

recommended for specimen collection specifically for diagnosing
outbreaks of viral *gastroenteritis.*
1. Stool specimens should be collected in bulk volume
   as soon after the time of disease onset as possible and no
   later than 48 hours after the onset of symptoms.
2. Stool specimens should be refrigerated, not frozen,
   shipped to the laboratory on the same day that the specimen
   is collected.
3. Paired serum specimens that are collected within 1 weel
   of the disease onset (acute phase) and 3 to 4 weeks after the
   onset of symptoms (convalescent phase) from both ill patients
   and controls are required to establish the causal association
   between agents seen in the stools and the illness.
RF 1 OF 6.

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-END OF DISPLAY REQUEST-

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An Outbreak of an Enterovirus-like Illness at a Community Wading Pool: Implications for Public Health Inspection Programs

Dennis D. Lenaway, MS, Robert Brockmann, MS, Gregory J. Dolan, BS, and Federico Cruz-Uribe, MD, MPH

Abstract: In June 1987, following an outbreak of an illness among children participating in a swim class, investigation revealed that 26 children who had swum in the outdoor wading pool were more likely to be ill than those who had not (OR 12.1, 95% CI = 2.9, 74.2). The pool chlorination system was operating improperly prior to onset of illness and chlorine levels were at or very near zero. This report emphasizes the need for operators and inspectors to give special attention to disinfection of wading pools. (*Am J Public Health* 1989; 79:889–890.)

Introduction

Several previous reports have associated outbreaks of enteroviral infections with community swimming pools.[1-4] Keswick, *et al*, surveyed municipal swimming and wading pools to detect enteroviruses and found viral contamination in 10 of 14 swimming pools and all seven wading pools sampled.[5]

On June 24, 1987, the Boulder County Health Department received several phone calls from concerned parents regarding an illness among children who had participated in a swim class offered at a local municipal pool in Longmont, Colorado, prompting an investigation.

The Longmont municipal pool complex houses an indoor 250,000 gallon Olympic-sized pool and an outdoor 20,000 gallon wading pool. Both pools are equipped with high rate sand filters and automatic feed gas chlorination disinfection systems. Pool personnel manually monitor the pool chemistry and perform manual adjustments to the chlorination system as necessary.

The Boulder County Health Department performs semi-annual inspections of the municipal swimming pools within the county. These inspections include a complete survey of the physical facility as well as on-site analysis of the pool water chemistry. In addition, water samples are drawn quarterly and tested for bacteriological contamination.

This report summarizes the results of the outbreak investigation and discusses the implications to public health pool inspection programs.

Methods

We conducted interviews with all children who had participated in the swim class to determine who had been ill; the onset and symptoms of their illness; the days each child attended the swim class; whether they had swum in the indoor main pool or the outdoor wading pool; their swimming behavior, food consumption, and use of drinking fountains; and whether they had attended other summer activities with the same children. We defined a case as a child with a reported fever greater than 101°F, and at least one of the following: malaise, headache, stomachache, nausea, or diarrhea. A re-interview was conducted with the families of those children meeting our case definition to determine the duration of illness. Telephone contacts were made to area pediatricians to consult on diagnoses and confirm symptoms of those who sought medical attention.

A complete physical and chemical pool inspection was performed, as well as bacteriological examination of the pool water for coliforms and total heterotrophic bacteria by standard methods. We obtained a copy of the daily water chemistry log which requires the recording of pH, temperature, turbidity, total alkalinity, and free chlorine levels.

Stool specimens were collected from several ill children meeting the case definition and forwarded to the Colorado Department of Health (CDH) laboratory for analysis of common enteric bacterial pathogens, and coliform density (standard: <1/100 ml). Viral studies were not available.

Results

The two-week swim class was held June 8–19. Of the 63 children who attended the class, 26 met the case definition. No one was hospitalized, and all symptoms resolved an average of 5.7 days after onset (range three to seven days).

All 63 children (100 per cent) reported swimming in the main indoor pool in conjunction with the scheduled swim class. However, the attack rate for swimmers who used the outdoor wading pool was 62 per cent compared to 12 per cent for those who did not use the wading pool (odds ratio 12.1, 95% confidence intervals (CI) = 2.9, 74.2).[6] When the other risk factors mentioned earlier were considered, no association with illness was found.

Inspection of the community pool complex on June 26 showed the main indoor pool to be operating properly. The outdoor wading pool, however, had to be closed. Violations included water quality deficiencies of extremely high turbidity, temperature of 92°F (maximum allowed 84°F), pH of <6.8 (minimum allowed 7.2), and an inoperative flow meter which prevented the calculation of pool water "turnover."

Review of the daily water chemistry log for the period June 8–19 indicated "0" free chlorine readings for five days, no readings taken for six days, and a low disinfection level of 0.1 mg/l free chlorine on one day. The lack of chlorine disinfectant was due to a faulty gas chlorination system. Pool personnel had manually chlorinated the pool; on the day of inspection the level was >3 mg/l. The pool remained closed until adequate repairs were made.

The earliest reported illnesses appeared on the last day of swim class, June 19 (Figure 1). The remaining illnesses from the primary exposure continued to appear through June 24. We estimated five to seven days as the incubation period based on the interval between first use of the wading pool and onset of illness.

Bacteriological testing of the main and wading pool water was negative for coliforms and total heterotrophic bacteria at the time of the inspection. Stool sample results
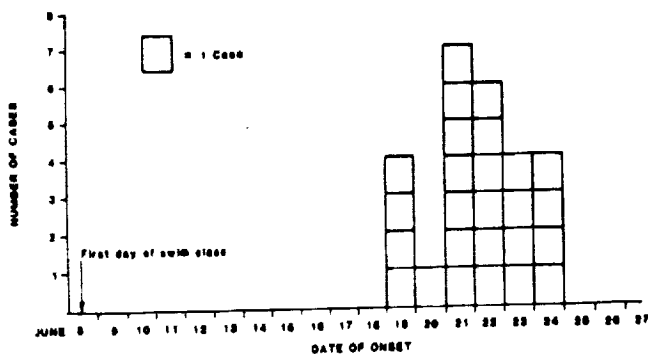
889

FIGURE 1—Enterovirus-like Cases by Date of Onset of Febrile Symptoms, Longmont, Colorado, June 1987

reported by the CDH laboratory were negative for *Salmonella, Shigella, Aeromonas,* and *Campylobacter* species.

Discussion

The lack of chlorination and the strong association between swimming in the wading pool and illness suggest that an infectious agent associated with the wading pool caused the outbreak. The clinical manifestations and course of the illness, estimated incubation time, potential for a fecal-oral route of transmission, and the exclusion of likely bacterial pathogens all suggest a non-polio Enterovirus as a likely etiological agent.

Wading pools are typically small and shallow, with a high surface to volume ratio. The impinging sunlight can quickly deplete chlorine as well as raise the water's temperature. Additionally, some wading pools are plumbed directly into the main pool's operating system, making it difficult to adequately control the wading pool's chemical balance. These physical factors make maintenance of proper disinfection levels and water quality an ongoing task.

Pool operators are often young and inexperienced, and our field staff has found that they tend to neglect wading pools. This is unfortunate, since by virtue of its size and

clientele, the quality of wading pool water is far more likely than that of the main pool to deteriorate in a short period of time. The character of the wading pool water can change dramatically in just a few short hours of heavy use, but the change may go undetected by superficial checks. Even when a critical problem is detected, there is a reluctance by operators to close the pool until the problem is solved.

The wading pool bather load is frequently high and the associated increase in organic load quickly consumes the available free chlorine. Splashing only serves to increase the oxidation and loss of chlorine. Clearly, a large number of young children, still struggling with the complexities of proper personal hygiene, all wading around in, and drinking, waist-deep water is a textbook opportunity for a pathogen that uses the fecal-oral route of transmission.

Pool operators need to monitor and adjust chlorine levels in wading pools more frequently than in the main pool; health agencies need to inspect them more frequently, require better record keeping, establish educational opportunities for operators, and strictly enforce existing regulations. Finally, regulatory agencies must push for legislation which more appropriately addresses the operation and design criteria of wading pools.

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induced, have threshold values below which they do not operate. More importantly, multiple events will not have cumulative effects unless they are essentially simultaneous[3].

Is Manson then one of a virtually simultaneous series of multiple impacts that may have occurred at the K/T boundary? Unfortunately, this is not without its own set of problems. Crater dimensions and other attributes, such as volumes of ejected dust, that might affect the biosphere, do not scale linearly with impact energy. For example, crater dimensions scale approximately as the fourth power of the energy. The impact energy required to form Manson is of the order of 10^{21} joules. A rough mass-balance calculation[4] for the size of projectile required to account for the siderophile anomaly at the K/T boundary suggests that the energy release for the K/T event was of the order to 10^{23} joules. That is enough to form 100 Manson-sized structures or some combination of smaller and larger-sized structures. If Manson is one of several K/T boundary structures, where are the others? The terrestrial cratering record suggests that a Manson-sized structure is formed somewhere on the land surface of the Earth every 5 million years. It is possible that it is simply a coincidence that Manson has an age equivalent to the K/T boundary and that the associated impact is unrelated to the K/T extinction.

The best ages for impact events are obtained using ^{39}Ar–^{40}Ar dating on rocks that have been melted by the impact. These rocks have the best chance of being degassed and having their radiogenic argon clock reset. The samples analysed by Kunk et al. have not been melted, only shocked to much lower pressures. The argon spectra of these samples do not have a good plateau age but rather have a slight U-shape. When dealing with impact melt rocks, the minimum of the U in this type of spectrum is generally taken to represent a maximum age for the event[4]. The fact that two samples give similar ages may mean nothing more than that they happen to have undergone similar shock and post-shock thermal histories, resulting in equivalent ^{40}Ar loss and reset ages. If impact melt rocks are discovered at Manson and also give a similar age then we can be more confident that Manson has an age equivalent to the K/T boundary. □

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AQUATIC VIRUSES

And now, small is plentiful

Evelyn B. Sherr

ON PAGE 467 of this issue[1], Bergh et al. report the use of transmission electron microscopy to make direct counts of femtoplankton — viruses of less than $0.2\ \mu m$ in size[2] in natural waters. They find up to 10^8 viruses per ml, which is three to seven orders of magnitude higher than previous abundance estimates based on plaque-forming units. The earlier estimates had led to the conjecture that instances of bacteriophage infection of suspended bacteria would be rare[3]; now all bets are off.

During the past few decades, aquatic ecologists have been involved in elucidating the trophic roles of ever smaller life forms (somewhat in analogy to the physicists' search for ever smaller sub-atomic particles). In the 1960s and 1970s, nanoplanktonic algae, less than 10–20 μm in size, were a major focus of investigation as the most important component of pelagic primary production[4]. In the 1970s and 1980s, picoplankton, with cells of less than 2 μm in size, were found to be much more abundant and active than previous work had suggested[5,6]. Direct count microscopic methods revealed that the true abundance of heterotrophic bacterioplankton, 10^5–10^7 per ml was much greater than previous estimates of viable bacteria based on plate counts[7,8]. Along with the greater densities of heterotrophic bacteria, the reports of high concentrations of picoalgae, including unicellular bluegreens[9,9] and eukaryotic algae the size of large bacteria[10], were electrifying.

These discoveries have contributed to a revolution in our understanding of the functioning of pelagic food webs. The original concept of a linear food chain in the sea — phytoplankton to copepods to fish — is no longer a viable model for how pelagic ecosystems operate. In fact, we are only now beginning to grapple with the idea that in many aquatic ecosystems, including much of the world ocean, most carbon fixation is carried out by cells too small for copepods to ingest — they pass right through the copepod sieving apparatus. The significance of heterotrophic bacterioplankton has received strong support from the finding that the greatest fraction of particulate organic matter in the water column of the open ocean is in fact metabolically active bacterial cells[11]. It now appears that a complex network of trophic pathways among microbes, including algae, bacteria and protozoa, accounts for the bulk of the carbon and energy flows, with a small fraction of the total microbial production going to support all the other consumers, from copepods to whales[12].

Against this backdrop, the focus in aquatic microbial ecology for the 1990s may well be the trophic and evolutionary implications of the high abundances of viruses in natural waters found by Bergh et al.. As they say in their report, attack by bacteriophages could explain the unaccountably high mortality rates of bacteria measured in some systems. Viral infection can also kill eukaryotic microbes[13], and viruses might account for some of the 'dissolved' DNA found in seawater[14].

The most fundamental implication of high viral abundance is, however, that routine bacteriophage infection of aquatic bacteria is likely to result in significant exchange of genetic material[15]. Natural genetic engineering experiments may have been occurring in bacterial populations for eons. If bacteriophage transduction turns out to be a common mechanism for gene transfer in aquatic ecosystems, then pelagic bacterial assemblages should be able to adapt more rapidly to new situations than has been thought. They might, for example, rapidly develop resistance to antibiotics used in aquaculture, or elaborate enzymes able to act on exotic chemicals introduced into the environment. There is also the disturbing possibility that indigenous bacteria could acquire traits from pathogenic bacteria or artificially engineered bacteria released into lakes and coastal waters[15].

I expect that the discovery reported by Bergh et al. will spur research, only just now beginning, into the genetic composition of bacterioplankton[16], as well as promote further studies of the prevalence and activity of femtoplankton, the smallest 'living' particles in natural waters. □

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cluster of marsupial lineages contrasts with the score of zero to five against it derived from the molecular results[5] (Fig. 1). This statistically significant discrepancy justifies the proposal that the dental and pelvic traits shared uniquely by thylacines and borhyaenids suggest a remarkable amount of convergent or parallel evolution, resulting in the resemblance between these species. The marsupial wolf is thus a striking example of morphological convergence not only to placental wolves but also to South American carnivorous marsupials. This is likely to have been caused by parallel adaptations to similar modes of predation on different continents. The study of the molecular basis for such convergence at the level of the organism is a challenge for biology. □

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High abundance of viruses found in aquatic environments

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THE concentration of bacteriophages in natural unpolluted waters is in general believed to be low[1,2], and they have therefore been considered ecologically unimportant[3]. Using a new method for quantitative enumeration, we have found up to 2.5×10^8 virus particles per millilitre in natural waters. These concentrations indicate that virus infection may be an important factor in the ecological control of planktonic micro-organisms, and that viruses might mediate genetic exchange among bacteria in natural aquatic environments.

The highest total counts of viruses and bacteria were found in samples from the eutrophic lake Plussee (Table 1). We found total counts of viruses of between 5×10^6 and 15×10^6 per ml in marine samples taken during the productive part of the year. Marine samples taken in winter, however, were found to have very low numbers of viruses (Table 1), thus indicating a seasonal variation in the concentration of viruses in natural waters. Our virus counts are 10^3-10^7 times higher than previous reports on virus numbers in natural aquatic environments, which are based

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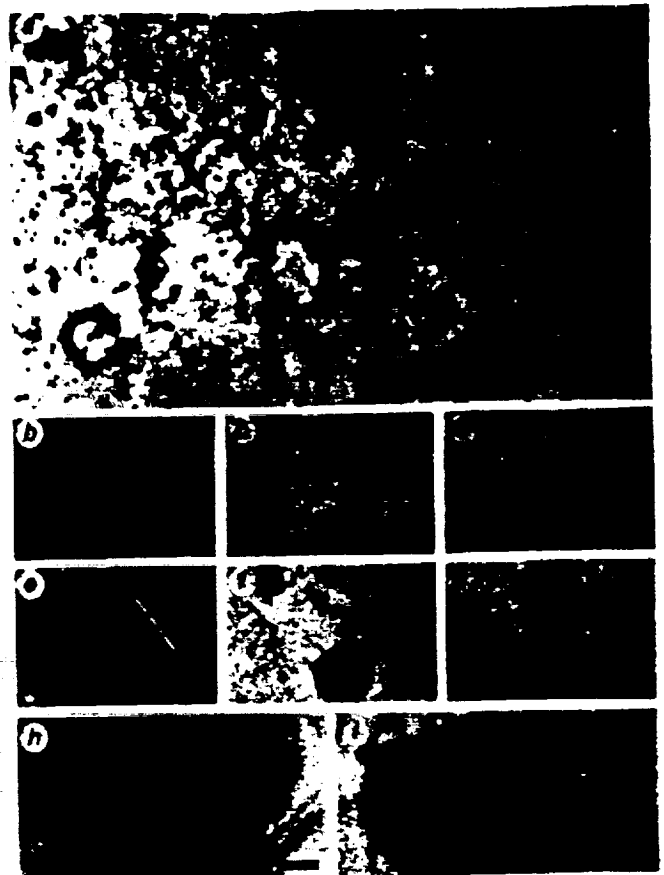
FIG. 1 Phages and bacteria observed in water samples from various natural marine and limnic ecosystems. a, Overview of bacteria and virus-sized particles from Lake Plussee. b-e, Particles classified as bacteriophages according to size and morphology. f-g, Particles of uncertain affiliation, but classified as viral particles in total counts. h, Bacterium with virus-like particles inside. i, Bacterium with phages attached to its surface. The particles, including bacteria, were collected by centrifugation of fixed water samples, 2% glutaraldehyde (Plussee, Chesapeake Bay, North Atlantic), 2% formaldehyde (Barents Sea), or from unfixed samples (Korsfjorden, Raunefjorden). Water samples were filled in centrifuge tubes with plastic-moulded flat bottoms. Grids with carbon-coated formvar film were then dropped onto the flat bottom and the samples were centrifuged (swing-out rotor) at 100,000g for 90 min. After removal of water, the grids were air-dried, stained with uranyl acetate (2%) and total bacteria and virus particles counted at high magnification (×20,000-100,000) in a Jeol 100-CX transmission electron microscope. Scale bars: a, 1 μm; b-d and h, 0.1 μm; e-g 0.05 μm; i, 0.5 μm.

on counts of plaque-forming units using various host bacteria[1,2]. Previously, most marine bacteriophages that have been isolated and described have had a head size larger than 60 nm (ref. 4). We have found, however, that smaller viruses with head size less than 60 nm seem to dominate natural populations (Table 1).

Bacteriophages that can be assigned to the Bradley groups A or B[5] are easily recognized by their tail structures (Fig. 1a (arrowheads), b, c and d). Some examples of virus-like particles, apparently without any tail structure but otherwise of similar size and morphology, are shown in Fig. 1e, f and g. These particles may be bacteriophages of Bradley groups C, D or E; or they may be phages of group A or B that have lost their tail. They may also be viruses relating to eukaryotic hosts such as microalgae.

Most of the virus particles we observed appeared to be free in the water, but some were also associated with bacteria. The bacterium shown in Fig. 1h seems to be in a phase of lytic disruption with numerous virus-like particles inside. Figure 1i

TABLE 1 Total numbers of virus particles and bacteria in samples from various locations

| | | | | Numbers of virus and bacteria (10^6 ml^{-1}) | | | | |
| | | | | | Virus head size | | | Total number |
Location	Sampling depth (m)	Sampling date	Bacteria	30–60 nm	60–80 nm	80–100 nm	>100 nm	of viruses
Plussee, West Germany	0.2	24 May 1988	6.2	166	43.9	36.6	7.3	254
Chesapeake Bay 38°33.5'N, 76°18.5'W	1	27 May 1988	3.2	4.1	2.5	2.5	1.0	10.1
Korsfjorden 60°11.7'N, 5°12.5'E	1	5 October 1988	1.1	2.4	1.6	1.5	0.6	6.1
Raunefjorden 60°16.2'N, 5°12.5'E	1	10 August 1988	0.2	6.1	3.0	0.8	ND	9.9
Raunefjorden 60°16.2'N, 5°12.5'E	1	1 February 1989	0.5	ND	ND	ND	ND	<0.01
Raunefjorden 60°16.2'N, 5°12.5'E	1	13 March 1989	0.5	1.9	1.1	1.2	0.6	4.8
North Atlantic 48°50.1'N, 16°30.8'W	10	8 May 1988	0.3	10.3	4.1	0.2	0.3	14.9
Barents Sea 71°30'N, 31°13'E	30	25 January 1989	0.02	0.02	0.02	0.02	ND	0.06

ND: not detectable (detection limit: 10^4 particles per ml).

shows a cell apparently being infected; several viruses can be seen attached to its surface.

It is often assumed that bacterial production is maintained in balance by protozoan grazing. But recent work[6–9] has suggested that grazing is not always sufficient to explain the mortality of bacteria in aquatic ecosystems. To assess the possible importance of bacteriophages for bacterial mortality in natural waters, we have considered theoretically the rate of phage adsorption. Assuming a population density of bacteria and of phages of 10^6 and 10^7 per ml, respectively, and the formation of 100 different phage-host systems, each containing 1% of the two populations, a first-order equation can be used to describe the adsorption of bacteriophages to host cells[10]. It is possible to calculate that for each of these phage-host systems the rate of phage adsorption is 2.5 min^{-1} ml^{-1}, assuming the adsorption-rate constant determined for T4 phages (0.25×10^{-8} cm^3 min^{-1}) (ref. 10) is valid also for marine phages. The total rate of phage adsorption will then be 250 min^{-1} ml^{-1}, corresponding to 3.6×10^5 day^{-1} ml^{-1}. Thus, as much as one-third of the bacterial population may experience a phage attack each day. If each attack results in infection and phage production, and the burst size is assumed to be 50, the rate of phage production will be 1.8×10^7 phages day^{-1} ml^{-1}. We conclude that the measured concentration of bacteriophages and host cells in natural waters is high enough for phages to be of quantitative importance. If the phages are temperate rather than virulent, phage production will not depend on the infection rate or the concentration of phage-host systems, but on the environment factors inducing phage production and cell lysis.

A high phage-host interaction rate in natural waters implies the possibility of active transfer of genetic information between host populations subjected to infection by the same phage strain. Thus, it is possible that genes may spread from genetically engineered microorganisms introduced into the environment to the indigenous bacterial population.

Our approach for total counting of viruses may prove valuable to establish their role in the ecology of aquatic microorganisms. The interpretation of the total virus counts will, however, have to rely on studies of virus proliferation rates, and on studies of the organisms parasitized by these viruses. The figures we have obtained from coastal, open ocean and freshwater systems suggest that the importance of the viral populations should not be neglected in any aquatic environment. □

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Function of identified interneurons in the leech elucidated using neural networks trained by back-propagation

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MECHANICAL stimulation of the body surface of the leech causes a localized withdrawal from dorsal, ventral and lateral stimuli. The pathways from sensory to motor neurons in the reflex include at least one interneuron[1]. We have identified a subset of interneurons contributing to the reflex by intracellular recording, and our analysis of interneuron input and output connections suggests a network in which most interneurons respond to more than one sensory input, most have effects on all motor neurons and in which each form of the behaviour is produced by appropriate and inappropriate effects of many interneurons. To determine whether interneurons of this type can account for the behaviour, or whether additional types are required, model networks were trained by back-propagation[2] to reproduce the physiologically determined input-output function of the reflex. Quantitative comparisons of model and actual connection strengths show that model interneurons are similar to real ones. Consequently, the identified subset of interneurons could control local bending as part of a distributed processing network in which each form of the behaviour is produced by the appropriate and inappropriate effects of many interneurons.

The main input to the local bending reflex (Fig. 1a) is provided by four pressure-sensitive mechanosensory neurons (P cells)

CHAPTER 12

Water as a Reservoir of Virus
in Nature and Means for Control

JOSEPH L. MELNICK

I. INTRODUCTION

Over 100 different viruses are known to be excreted in human feces. Many of them, particularly Picornaviridae, Adenoviridae, and Reoviridae, are sufficiently thermostable that they remain viable in water for long periods of time. A number also are resistant to conventional procedures, including chlorination, used in wastewater treatment plants and may be found far from the original source of contamination. With the recent recognition of hepatitis A virus and rotaviruses (the causative agents of type B viral gastroenteritis), methods for their detection in water are being developed so that they may be monitored together with the

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conventional enteroviruses in sources of drinking water. Other human viruses that occur in fecally contaminated water, but for which suitable laboratory methods for detection in water have yet to be worked out, include the parvoviruses and the agent of type A viral gastroenteritis.

Removal of viruses from contaminated water includes processes of physical removal and those causing inactivation or destruction of the virus particle. Processes that bring about virus inactivation are preferable to those of simple removal since the latter presents problems in the disposal of infectious virus. The inability of chlorine to act as an effective viral disinfectant in wastewater and the concomitant production of toxic and carcinogenic chlorinated compounds in waters containing organic compounds are factors that are leading to the development of alternate disinfection procedures that can achieve large reductions in pathogenic microorganisms, particularly if wastewater is to be reclaimed and recycled as drinking water. One such method, photodynamic inactivation, in which solar energy can be used as a practical and inexpensive alternative to artificial light for the necessary energy, will be described.

II. OCCURRENCE OF VIRUSES IN WATER

Water contamination by human and animal excreta is the equivalent of saying contamination by bacteria and viruses found in feces and urine. Although viruses of lower animals may be contributing to this pollution, most of our knowledge is based on studies of viruses of humans (see Table 1). Since little can be added to John Fox's (1976) admirable review, I would like to quote relevant parts of it, with some editorial changes for which I take responsibility.

A. Picornaviruses[1]

These relatively hardy agents are capable of surviving for significant periods in the free state in water, such capability varying a bit with particular viruses and/or subgroups (e.g., the vulnerability of rhinovirus to low pH). These latter, although by far the largest subgroup (over 100 serotypes so far recognized), primarily infect the nasal mucosa and are not commonly shed in feces, so that any possible waterborne spread would be virtually restricted to bathing situations. The remaining subgroups, polioviruses, group A and B coxsackieviruses, and echoviruses, are known collectively as enteroviruses which multiply primarily in the alimentary tract and are excreted in substantial amounts in the feces for

[1] Adapted from Fox (1976).

Water as a Reservoir of Virus

TABLE 1
Human Enteric Viruses That May Be Present in Water[a]

Virus group	Number of types	Disease caused
Enteroviruses		
Poliovirus	3	Paralysis, meningitis, fever
Echovirus	34	Meningitis, respiratory disease, rash, diarrhea, fever
Coxsackievirus A	24	Herpangina, respiratory disease, meningitis, fever
Coxsackievirus B	6	Myocarditis, congenital heart anomalies, rash, fever, meningitis, respiratory disease, pleurodynia
New enterovirus types 68–71	4	Meningitis, encephalitis, respiratory disease, acute hemorrhagic conjunctivitis, fever
Hepatitis type A (probably an enterovirus)	1	Infectious hepatitis
Gastroenteritis type A (probably an enterovirus)	2	Epidemic vomiting and diarrhea, fever
Rotavirus (Reovirus family)	?	Epidemic vomiting and diarrhea, chiefly of children
Gastroenteritis type B		
Reovirus	3	Not clearly established
Adenovirus	>30	Respiratory disease, eye infections

[a] Other stable viruses that might contaminate water are SV40-like papovaviruses, which appear in the urine. The JC subtype is associated with progressive multifocal leukoencephalopathy.
Creutzfeld-Jakob disease virus. Like scrapie virus, the CJ virus resists heat and formaldehyde. The virus causes a spongiform encephalopathy, characterized by severe progressive dementia and ataxia.
Parvoviruses. The adeno-associated satellite viruses that are excreted in stools of children survive heating for one hour at 60°C.

varying periods of time, the longest being polioviruses with a mean duration of 50 days and the shortest being echoviruses. The best studied of these are the polioviruses and the least studied are the many serotypes of group A coxsackieviruses that can only be isolated in suckling mice. Numerous studies in developed countries have readily demonstrated the presence of enteroviruses in contaminated streams, in sewage, and in effluents from sewage treatment plants, and enterovirus surveillance of sewage has been used to monitor the impact of live poliovirus vaccination in the community. Although shellfish have not been implicated to date in the transmission of the conventional enteroviruses, clams and oysters

grown in contaminated water do acquire and harbor enteroviruses and viruses can persist in processed and even partially cooked oysters. Few similar studies of viral contamination of water have been published from developing areas but one can assume that, where sanitation is more primitive, gross fecal contamination of water is common and the viruses must be abundantly present.

A few words are now in order concerning the disease potential of enteroviruses. Wild polioviruses are significant entirely because they can cause serious central nervous system (CNS) disease (actually rather infrequently, from 1 in 100 to more than 1 in 1000 infections, depending chiefly on the virulence of the virus and the age of the host). Presently in the U.S. and other developed countries the frequently excreted polioviruses (the group encountered with the greatest frequency in Fox's virus watch studies) are almost entirely vaccine-derived and their pathogenicity is very low, although reversion with respect to neurovirulence during human passage is known to occur. The other enteroviruses are also often causes of CNS disease of usually benign nature (aseptic meningitis), but occasionally resulting in clinically typical paralytic poliomyelitis. While Fox's experience suggests that mild respiratory disease is by far the more typical result of these enterovirus infections and that serious syndromes including CNS involvement must be very infrequent, group B coxsackieviruses in particular do have the potential for causing other significant types of diseases. These include the very uncommon fortable epidemic pleurodynia (Bornholm's disease), pericarditis (chiefly in older persons), serious and often fatal myocarditis in infants, and congenital defects (chiefly cardiac) in infants born of mothers experiencing infection during pregnancy. In summary, enteroviruses can cause very significant disease but, fortunately, do so in only a very small proportion of infections under most circumstances.

This infrequent association with serious disease may well help explain why documentations of waterborne spread of enteroviruses have been so few. A very important principle, best exemplified by the polioviruses, is that the severity of the outcome of infection in a nonimmune host is directly related to host age. In developing countries, where wild enteroviruses are highly prevalent, infections typically are acquired early in life when the risk of serious disease is minimal and most older children and adults are immune. In these areas waterborne spread undoubtedly occurs and may indeed be an important part of the process of natural immunization. As sanitation in some of these areas has improved, reflecting a slow increase in living standards, paralytic poliomyelitis has increased, presumably due to relative interference with virus spread sufficient to delay infections to a somewhat older age but not to prevent them. A similar trend should evolve (perhaps is already evolving) with respect to

disease caused by the other enteroviruses. Meanwhile, the populations most vulnerable to such disease are those of developed countries. Data on the prevalence of immunity to the nonpolio enteroviruses is fragmentary but, in Fox's 1960–1965 study in New York, prevalence of neutralizing antibody among adults to selected viruses ranged from 14% for coxsackievirus B1 to 59% for coxsackievirus A9. In Seattle in 1968 only 10% of adults had antibody to type 30 echovirus. The occurrence in 1968 of a community-wide epidemic of aseptic meningitis due to this virus in Seattle with 44 cases recognized, chiefly in older children and adults, suggests the vulnerability of urban U.S. populations to these agents. Had extensive waterborne spread occurred, the epidemic probably would have been far more dramatic. The moral clearly is that, unless constant vigilance in protection of water supplies is maintained, nonpolio enteroviruses could cause major outbreaks of serious disease.

B. Adenoviruses[1]

Although commonly thought of as respiratory viruses, adenoviruses almost invariably infect the alimentary tract and are abundantly shed in feces. While infections restricted to the alimentary tract cause little or no disease, disease commonly results when other sites, respiratory and conjunctival, are also infected. Fecal shedding of adenoviruses is extremely common among young children and may exceed that of wild enteroviruses in U.S. cities, although enteroviruses almost certainly predominate in developing countries.

Although efforts to detect viruses in sewage-polluted waters have commonly employed methods selected for detection of enteroviruses rather than of adenoviruses, the latter have been detected in a number of such studies. However, the only well-documented waterborne spread of adenoviruses has been in the epidemics of pharyngoconjunctival fever associated with swimming pools. In summary, waterborne spread of adenoviruses is theoretically possible but, for reasons cited, it has not resulted in recognized disease except in relation to swimming pools.

C. Parvoviruses[1]

Most known members of this group of very small DNA viruses, chiefly infect lower animals, e.g., rats, mice, cats (panleucopenia of cats), dogs, swine, and birds. The limited available evidence suggests that as a group these are very stable agents. The first recognized parvoviruses of human origin were the adeno-associated satellite viruses (AAV), which can replicate only with the help of adeno- or (less readily) herpesviruses. Seroepidemiologic studies indicated that antibodies to AAV, especially types 2 and 3, are widely prevalent in young children and that infection is

associated with childhood respiratory disease. AAV, together with adenoviruses, have been recovered from feces and, hence, are almost certainly present in fecally contaminated water. However, data on frequency of excretion are lacking, as is an adequate evaluation of AAV impact on human health.

D. Hepatitis A Virus and Gastroenteritis Type A Virus[1]

The agents of type A viral gastroenteritis and type A viral hepatitis have recently been identified. Work is too limited to classify them with any certainty: their size and morphology indicate that they are either picornaviruses or parvoviruses. The basic fecal–oral transmission of such agents suggests that waterborne spread may be very important wherever sanitation is deficient (or a break occurs in the protection of a water supply).

Hepatitis A virus is excreted in the feces over a relatively extended period and, on the basis of many well-studied epidemics, this agent is often spread via water. Further, the tremendous 1955–1956 epidemic in Delhi, India, caused by gross sewage contamination of the water supply, provided inadvertent evidence for a highly relevant characteristic of the responsible agent, namely, its ability to withstand levels of residual chlorine (greatly raised to combat the emergency), which apparently were adequate to counteract all other enteric pathogens which must also have been present.

As regards gastroenteritis type A, viral particles have been demonstrated by use of immune electron microscopy (IEM) in stool specimens from patients with gastroenteritis. In one outbreak in Norwalk, Ohio, a virus was detected in stool specimens obtained during acute illness. The virus had a diameter of about 27 nm and a buoyant density of 1.38–1.41 g/ml, similar to that of other "pico" viruses.

Particles resembling the Norwalk agent in size and buoyant density were also visualized by IEM in stool filtrates derived from two of six other outbreaks of transmissible nonbacterial gastroenteritis. These two viruses were present in a lower concentration in stool than the Norwalk agent and were detectable by IEM only after stool filtrates were concentrated. The two viruses were distinct antigenically. However, one of the viruses shared antigenic determinants with the Norwalk agent. Thus, there appear to be at least two serotypes among the gastroenteritis type A viruses.

E. Reo- and Rotaviruses

Reoviruses have often been recovered from contaminated surface waters. Although highly infectious, little is known about their ability to

cause disease. A recently discovered member of the Reoviridae, rotavirus, has been established as the major pathogen of nonbacterial infantile diarrhea throughout the world. The virus has been detected in approximately 40% of infants with diarrhea. However, during the winter months, when infection appears to be most common, the frequency of detection has averaged 70%. During the peak of the winter outbreak, the virus has been observed in specimens from 80–90% of infants and young children hospitalized with diarrhea. Large numbers of particles (10^9/g of feces) may be excreted by infected individuals, and so it would not be surprising if contamination of water supplies occurred.

The disease can be induced by the virus in experimental animals. newborn piglets, calves, and rhesus monkeys develop diarrheal illness when given a human stool filtrate containing the virus. The viral shedding patterns in feces of infected gnotobiotic piglets were consistent with those observed for infants with diarrhea.

The human virus has been shown to be related antigenically to Nebraska calf diarrhea virus, the virus of epizootic diarrhea of infant mice. the SA11 virus of monkeys and the O virus (isolated from water flowing from troughs in which the intestines, hence "offal," of both sheep and bovines were washed at the slaughterhouse). Malherbe isolated two other strains of similar agents: one from an algal furrow at the end of a conventional sewage purification plant in Pretoria, South Africa; and the other from a stream receiving effluent from another sewage plant, but at a point in the stream where agricultural land run-off occurred, so that the virus may have come from the land itself and not from the sewage effluent. Three of the viruses of this closely related group cause gastroenteritis in the natural host and some of these agents may find their way into surface water.

F. Papovaviruses[1]

These include the human wart virus plus one or more SV40-like agents associated with progressive multifocal leukoencephalopathy (PML). While this serious disease is extremely rare, seroepidemiological studies indicate that antibody to one of these agents, the JC virus, is widely prevalent (69% of Wisconsin adults) and, hence, infection must occur with some frequency albeit by unknown means and with equally unknown effects other than PML. Still another SV40-like agent, the BK virus, has been recovered from the urine of several renal allograft patients, which means contamination of wastewater. While its relation to human disease remains to be established, serologic studies indicate that primary infection usually occurs in early childhood and that adults commonly possess

antibody. Papovaviruses as a group are unusually stable agents and, in theory, could remain viable in water over long periods.

G. Slow Viruses

The demonstrated existence in lower animals of several so-called slow viruses, especially visna and scrapie (both in sheep), suggests that analogous agents may affect man, a suggestion already supported by the demonstration that Creutzfeldt–Jakob disease and kuru can be experimentally reproduced in lower primates via cell-free filtrates from affected human brains. The kuru agent is highly stable and is thought to infect by ingestion. If these hardy agents should ever appear in water, they would be difficult to remove by any of our current practices.

III. THE PROBLEM

Water problems may vary in different countries, but all will agree that water problems exist now even in the most advanced countries and it takes little foresight to realize that the problems will become even more critical in the not too distant future. Harris and Brecher (1974) aptly point out:

Almost everyone supposes that community water systems are under continuous surveillance by competent state and local health officials, that water samples are scrupulously tested at frequent intervals, that any flaws in a water system will be soon discovered and corrected, and that the water we drink therefore must be safe. Unfortunately, almost everyone supposes wrong.

True, cities in the U.S. no longer suffer from large-scale recurring epidemics of typhoid, cholera, dysentery, and other waterborne bacterial infections. It was those epidemics, with their ghastly death tolls traceable to drinking water, that forced the establishment of community water supplies during the past century. The then-new water systems worked quite well against the hazards they were meant to alleviate. But today, many of the same water systems are overage, dilapidated, substandard in serious respects and barely able to meet peak demands.

Their design is primitive, and they are typically staffed by people trained in an outmoded tradition or not trained at all. As the level of pollution has risen in our sources of raw water, the techniques employed to make that polluted water safe for human consumption have become less and less adequate.

Experience has shown that community water supplies must be tested at frequent intervals if intermittent bacterial contamination is to be spotted. Bacteriological tests are primarily designed to identify coliform bacteria, the types of bacteria found in feces and soil. The standards do not require a total absence of coliforms; a moderate count is permitted. A well-operated water treatment plant can do considerably better than the standards require.

Furthermore, we now know that coliform counts do not correlate with

virus counts. We have had many samples in our laboratory negative for coliforms that were positive for human pathogenic viruses.

The sanitary engineers who built the early community sewage and water systems did not know about viruses, which is understandable, but many modern sanitary engineers still do not know about viruses, which is neither understandable nor excusable. No accepted standards have been set for viruses in recreational or drinking waters, although standards have been proposed, based on present monitoring technology for virus detection. As our detection and monitoring methods improve, we have the obligation to assure members of society that the water they drink is free not only from pathogenic bacteria but from viruses as well.

Viruses occur in water, and waterborne outbreaks of a viral disease such as hepatitis have been well documented. If a virus infection has a short and uniform incubation period, and produces a characteristic, easily recognizable disease, carriage of the virus by water routes can be traced with a fair degree of accuracy.

In contrast to diseases whose spread can be traced in this fashion, the characteristics of many viral diseases are such that their transmission by water is very difficult to recognize. Viruses whose spread is difficult to trace include those viruses that produce clinically observable illness in only a small fraction of the persons who become infected, those that produce diseases with widely variable incubation periods, and those that are easily spread by direct human contact.

These reasons may account for the fact that almost 60% of all documented cases of disease attributable to drinking water in the U.S. were caused by agents of unknown etiology. In addition, at present no field-proven method exists for the detection of the agent of infectious hepatitis in water. These difficulties have led to an emphasis on the detection of enteric viruses in water as an indication of the presence of human pathogenic viruses and the possibility of contracting disease from such water.

Attention is being increasingly paid to problems of viruses in water. In the U.S. the number of waterborne cases of hepatitis type A virus, which behaves as an enterovirus, has been increasing in recent years and is now the most prevalent waterborne disease attributable to a specific etiologic agent. During this same recent period in the U.S. the annual number of waterborne cases of typhoid, a bacterial disease, has decreased at least fivefold.

In many parts of the world increased demands on available water resources due to the concurrent expansion of the world's population and industrial demand make recycling of domestic wastewater inevitable in the future. One of the major problems to be overcome is the development

of adequate methods to ensure the elimination of human pathogenic viruses from reclaimed water. This problem is also compounded by the concern that present water treatment procedures may not regularly be sufficient in preventing viruses from reaching community water supplies.

It has been 30 yr since the first studies on the presence of human enteric viruses in water began in earnest, but their public health significance in water has yet to be ascertained. This has been due in part to the inapparent nature of the infections caused by these viruses and the widespread lack of methodology for detection. Studies have shown that enteric viruses easily survive present sewage treatment methods and many can persist for several months in natural waters.

More than 100 different enteric viruses are known to be excreted in human feces. Table I lists the major groups of enteric viruses that have been found in raw sewage or are known to be present in the feces of infected persons, including healthy carriers. Enteric viruses are excreted in concentrations as high as one million viruses per gram of feces, and can persist for long periods of time in the environment. Reported survival times range up to 168 days in tapwater, seawater, or soil.

The expected average enteric virus density in domestic sewage has been estimated to be about 7000 viruses/liter, but as many as 500,000 viruses/liter have been detected in some parts of the world. The amount of virus present in raw sewage is highly variable depending on such factors as the hygienic level of the population, the incidence of disease in the community, socioeconomic level, and the time of year. In the U.S. peak levels occur in the late summer and early fall. Enteric viruses survive the customary secondary sewage treatment and chlorination as commonly practiced in sufficient numbers to be isolated easily by modern concentration procedures at all times of the year. Thus, it is not surprising that these viruses have been detected in several of the major rivers of the U.S. Since enteric viruses are considerably more resistant to various sewage and water treatment methods than either coliform or enteropathogenic bacteria, the absence of the latter organisms may not guarantee the absence of a viral disease hazard.

Little is known about the occurrence of viruses in drinking water, because until recently methods for the concentration of viruses from large volumes of water have been lacking. A few reports do exist in the literature. In a study during the 1960s in Paris, enteric viruses were detected in 18% of 200 samples, and the average virus concentration was estimated at one infectious unit per 300 liters. More recently, Russian investigators have reported the isolation of enteric viruses on several occasions from drinking water. The water treatment plant from which the water came was found to be functioning normally (the process included

chlorination) during the periods when viruses were recovered. Viruses also have been isolated from drinking water in South Africa. Virological surveys of drinking water supplies are sorely needed to determine if currently practiced water treatment methods are adequate, but unfortunately few laboratories at the present time are equipped or staffed to undertake such studies.

Figure 1 illustrates some of the routes by which viruses from human and animal wastes may find their way to susceptible human hosts (Gerba et al., 1975).

IV. THE SOLUTION

Processes available for virus removal from wastewater include those involving physical removal and those causing inactivation or destruction of the particle. Processes that involve actual removal include sedimentation, adsorption, coagulation and precipitation, and filtration. Conditions that cause inactivation are high pH, chemical oxidation by disinfectants such as halogens, and the recently described process of photooxidation by dyes and light. Processes that bring about virus inactivation are preferable to those of simple removal since the latter present a problem of the disposal of potentially infectious material.

Primary treatment of wastes, involving only settling and retention before discharge, removes little or no viruses. Any virus removal that occurs during this treatment probably results from the sedimentation of viruses associated with sewage solids. Virus removal of up to 90% has been observed after activated sludge treatment, but large variations in removal have been reported. The physical–chemical treatment of sewage can result in large reductions of virus. Alum (aluminum sulfate), lime (calcium hydroxide), and salts of iron compounds as well as polyelectrolytes, are capable of removing as much as 99.99% of virus suspended in water. It has been postulated that coagulation results in the formation of a coagulant–cation–virus complex that settles from solution. The virus is not inactivated by this process, and in fact such coagulation has been used as a method to concentrate viruses from water. The high pH that can be attained during lime treatment can also result in very large reductions of virus. If pH levels above 11 are maintained for sufficiently long periods, 99.9% inactivation of the viruses present can result. Large variations in the reported times required for inactivation exist in the literature, and there is a need for a much closer look at such factors as concentration of organics, time, and temperature.

Under appropriate conditions, viruses are readily adsorbed to a wide

variety of surfaces, including activated carbon, diatomaceous earth, glass, membrane filters, colloidal organic matter, clays, and soil. Adsorption is reversible by alteration of ionic levels or pH or by the addition of competing organic matter. Activated carbon removes virus, but its capacity is soon reached and virus desorption often occurs as organic substances replace the adsorbed virus. Sand filters can remove virus by adsorption onto substances trapped by the sand, but little adsorption onto the sand itself occurs.

Although chlorine treatment has been the mainstay of water disinfection for over 50 yr in the U.S., too little is known about the mechanisms by which it renders the virus nonviable. Chlorine's effectiveness as a viral disinfectant is highly dependent on a number of factors including temperature, pH, the presence of organic matter, and the physical state of the virus (that is, whether it is adsorbed or aggregated). Because of the presence of large amounts of organic matter in the effluents from activated sludge plants, large reductions of virus are not possible because of the combination of the chlorine with ammonia and organics. Application of 8 mg of chlorine per liter of sewage effluent may not decrease the virus concentration. With very high doses (40 mg/liter for 10 min), 99.9% destruction of virus in sewage has been achieved, but then the problems of expense, chlorine toxicity to higher forms of life if such effluents are discharged, and the production of carcinogenic chlorinated hydrocarbons remain. Further complicating the problem is the wide variability in resistance of different enteric viruses to inactivation by chlorine. In a study of the resistance of 25 human enteric viruses, the time required for 99.99% inactivation of the viruses, under the same conditions, varied from 2 min to 2 hr.

Ozone has been widely used in many parts of the world as a disinfectant for water and wastewater, but little information is available to indicate its virucidal efficiency, especially under field conditions, where organic loads are present and act to protect virus from inactivation. One of the main arguments against the widespread use of ozone is that it lacks a residual effect since it has a half-life of only 25 min.

The inability of chlorine to act as an effective disinfectant against viruses in sewage and the possible production of toxic and carcinogenic chlorinated compounds has led to a call for the development of alternate disinfectants (Piecuch, 1975). In addition, disinfection methodology must be developed that is capable of achieving very large reductions of pathogenic microorganisms if reclamation of sewage is to become a reality. In previous work, the inactivation of bacteria, fungi, and viruses by sensitizing these agents with photoreactive dyes and exposing them to white light or monochromatic light to bring about photodynamic oxidation

Fig. 1.   Possible modes of enteric virus transmission by water.

...been described (Wallis and Melnick, 1965; Wallis et al., 1965). Recently, we have been exploring this method as a means of disinfecting sewage.

Photodynamic Inactivation Theory

The photodynamic oxidation reaction has been known since about 1890; however, detailed studies on the inactivation of proteins, nucleic acids, bacteria, and viruses were not performed until the late 1920s and the early 1930s (Spikes and Livingston, 1967). During the late 1950s and early 1960s mechanistic studies were made on the inactivation of free nucleic acids and on intact viruses. The results of these latter studies indicated that the site of the attack was the nucleic acid and involved the purine base guanine, which is a heterodicyclic carbon–nitrogen compound. Also, the studies showed that the rates of inactivation of the guanine moiety of the nucleic acid was much faster in the presence of oxygen and that one mole of oxygen was consumed per mole of guanine photooxidized when sensitized with the dye methylene blue.

B. Laboratory Studies

The photoreactive heterocyclic dye, methylene blue, was chosen for these studies because of its low cost and because the wavelength at which it has maximum absorption (670 nm) was found to penetrate clarified sewage with 15 Jackson turbidity units (JTU) to a depth of 2 in. (5 cm) with a transmission of greater than 80%. The absorption spectrum of methylene blue is shown in Fig. 2. The dye also has a very low toxicity and, in fact, is administered orally or intravenously in treatment of certain diseases of humans and as an antidote (Arena, 1975).

The effectiveness of methylene blue as a photosensitizing dye using white light has been shown to increase with increasing pH. The rate of photooxidation of the nucleotide deoxyguanosine increased 30 times with pH changes from 7 to 10.5.

In our study, the photoirradiation lamps were 4-ft long, 40-W monochromatic lamps (Westinghouse F 44T12/RB/SHO, 1500 M. lamps), and each lamp emitted 2000 μW/cm² of 670-nm light at 1 in. (2.5 cm) from the surface of the lamp. The emission spectrum of the lamp along with the emission spectrum for white light is also shown in Fig. 2. The emission spectrum of the RR lamp, as illustrated, is in the same range in which methylene blue absorbs most strongly.

A number of parameters of the system have been studied (Gerba et al., 1977), using a simple flowthrough irradiation cell shown in Fig. 3. Preincubation of dye–bacteria mixtures resulted in large numbers of coliform

that could be inactivated. Over 100 million bacteria could be inactivated with only a 60-sec exposure to 2000 μW/cm² of 670-nm light containing 1 mg/liter of the dye after sensitization for 4 hr in the dark, while after 8 hr sensitization less than 10 sec was required to inactivate the same number of bacteria.

Poliovirus was found to be readily inactivated. The effect of various concentrations of the dye on the amount of poliovirus that could be inactivated after a 5-min exposure to the light source is shown in Table II. The amount of virus inactivated continued to increase until a concentration of 10 mg/liter was reached. Higher concentrations of dye resulted in a gradual reduction in the amount of virus that could be inactivated. This was attributed to the absorption of the light by the dye in the upper layers of the fluid.

pH and incubation temperature can dramatically affect the photoinactivation of enteroviruses by heterotricyclic dyes. The effect of pH on the photoinactivation of poliovirus using a 5-min exposure to light is shown in Table III. A marked increase in the amount of virus inactivated occurred between pH 9.5 and 10.0. The effect of incubation temperature of the dye–virus mixture on the amount of virus inactivated after exposure to the light source is shown in Table IV.
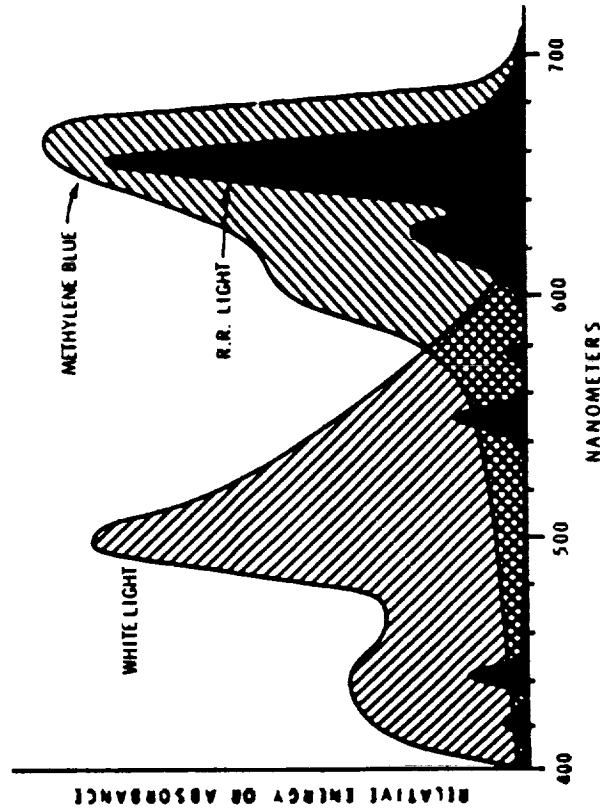

Fig. 2. Nomograph of the absorbance spectrum of methylene blue and the emission spectra of Westinghouse 40-W RR Special SHO tubes and to incandescent and fluorescent white lights.

Water as a Reservoir of Virus

TABLE II
Effect of Dye Concentration on the Photoinactivation of Poliovirus[a]

Dye concentration (mg/liter)	$-\text{Log}_{10}\ N/N_0$[b] of virus inactivated after 5-min exposure to 2000 μW/cm² 670-nm light
0.1	0.04
1	0.65
5	1.84
8	1.89
10	2.28
15	2.12
20	1.72
50	0.17

[a] Samples were sensitized for 24 hr.

[b] $-\text{Log}_{10}\ N/N_0 =$ log of the number of organisms inactivated.

determine the effect of the photodynamic inactivation process scheme on poliovirus. Dye removal was accomplished when desired by adding the sterilized sodium form of Amberlite IR120 to the dye–virus solution and vigorously agitating the mixture while adjusting the pH to 8.5. Virus concentration was performed using 4 liters of the decolorized supernatant sample by the procedures already described (Wallis *et al.*, 1976), which readily allows for concentration of the sample to 20–40 ml.

Secondary effluent was obtained from a package sewage treatment plant that processes municipal sewage. The effluent was pumped into the

TABLE III
Effect of pH on Photodynamic Inactivation of Poliovirus[a]

pH	$-\text{Log}_{10}\ N/N_0$ of virus inactivated after a 5-min exposure to 2000 μW/cm² 670-nm light
10.0	2.07
9.5	1.11
9.0	0.98
8.0	0.49
7.0	0.20
5.5	0.00
4.5	0.00

[a] Virus was sensitized for 24 hr at pH 10.0 with 5 mg/liter dye in glycine buffer.

Fig. 3. Flowthrough irradiation cell.

C. Photoinactivation Using Solar Energy (Melnick *et al.*, 1976)

A large part of the energy near the 670-nm wavelength contained in light from the sun passes through the atmosphere and reaches the surface of the earth (Gates, 1962). Photometer readings indicated that during midday the 670-nm energy reaching the Houston area exceeds 10,000 μW/cm²; even on completely overcast days, a range of 1000–2000 μW/cm² has been observed. Exposure of sensitized virus to sunlight indicated that this source of energy was very effective in the photoinactivation of virus in both sewage and tapwater (Table VI).

D. Pilot Scale Operations (Hobbs *et al.*, 1977)

The experimental procedures were designed to provide high quality water from either tapwater or from sewage secondary effluent and to

The coliform results in the large unit corroborated the earlier data that the photoinactivation of the organisms at pH 10 with methylene blue at 5 mg/liter was very effective.

Continuous flow enterovirus inactivation studies were performed in the prototype flowthrough cell to determine the processing parameters for the photoinactivation process on a pilot plant scale basis. Poliovirus was sensitized at 23°C and pH 10.0 to methylene blue at concentrations of 1, 2, and 5 mg/liter for 4–24 hr. The sensitized virus was then photoinactivated in the large cell for various periods of time, which were accomplished by varying the flowrate to the cell. The results of the tests are shown graphically in Fig. 4 and indicate that the photoinactivation of poliovirus on a large-scale continuous flow basis is feasible and corroborate the earlier bench-scale study results.

The effect of methylene blue dye concentration as determined by this limited number of runs suggested that 1–2 mg/liter of dye was as effective as 5 mg/liter of dye for the photoinactivation of poliovirus. The lower

Fig. 4. Photoinactivation of poliovirus in a pilot-scale flowthrough cell using methylene blue at pH 10.0.

TABLE IV
Effect of Incubation Temperature on Photoinactivation of Poliovirus[a]

Temperature (°C)	−Log₁₀ N/N₀ of virus inactivated	
	Before[b]	After
4	0	1.2
24	0	1.74
37	0.3	2.55

[a] Virus was sensitized in the dark at pH 10.0 with 5 mg/liter dye at the indicated temperature for 24 hr before exposure to 2000 $\mu w/cm^2$ 670-nm light for 3 min.
[b] Before exposure to lighting system.
[c] After exposure to lighting system.

1000-gal storage–flocculation tank and was adjusted to pH 10 and flocculated with ferric chloride.

Photodynamic inactivation of poliovirus was performed in irradiation cells containing 12 and 23 monochromatic lamps. The period of irradiation was controlled by the flowrate. In one cell the 12 monochromatic lamps were arranged horizontally in three stacks so that six equal-size chambers surrounded by four lamps were obtained. In the center of each irradiation chamber, a 1-in. (2.5 cm) ID clear plastic tube was placed, and the tubes were interconnected in a series pattern to give a plug-flow reactor. Each lamp was 1 in. from any adjacent irradiation tube.

TABLE V
Use of Solar Energy for the Photoinactivation of Poliovirus[a]

Time (min)	670-nm light[b] ($\mu w/cm^2$)	Temperature (°C)	−Log₁₀ N/N₀ virus inactivated	
			Sewage (20 JTU)	Tap-water (<1.0 JTU)
0	2700	25	0	0
3	2700	25	0.2	0.9
6	2700	25	0.3	2.4
10	2700	26	0.5	>3.1
30	1800	28	2.1	—

[a] Ten liters of sewage or tapwater were placed in a large metal pan with a depth of approximately 10 cm. The pH was then adjusted to 10.0 and stock poliovirus was added to a final concentration of 9.6×10^3 PFU/ml. Dye to a final concentration of 5 mg/liter was then added as the solutions were continuously mixed by a magnetic stirring bar. Amount of virus inactivated is the loss of titer difference between controls held in the dark.
[b] As measured horizontal to the surface of the liquid. The experiment was performed on a cloudy, overcast day in Houston, Texas.

usage of dye would have a cost benefit not only from less reagent usage but also for a cost reduction on the process requirements for dye removal, if this is deemed necessary.

E. Process and Economic Evaluation

The proposed photodynamic inactivation process for the disinfection of sewage secondary effluent is shown schematically in Fig. 5 and consists of the following steps: (1) adjustment of secondary effluent to pH 10 using caustic soda or lime and ferric chloride addition to aid in flocculation of solids and removal of phosphorus; (2) clarifier for settling of solids; (3) additional step for nitrogen removal if desirable by air stripping; (4) sand or dual media filtration; (5) dye addition to tank for sensitization of the bacteria and viruses with the dye; (6) photodynamic irradiation cell; (7) dye removal by activated carbon, which will also result in effluent beneficiation due to the removal of refractory organic materials; and (8) pH adjustment to pH 9.0–9.5 prior to discharge.

The sequence of process steps proposed for the photodynamic inactivation process, except for the photocell, are the very unit operations for tertiary treatment of secondary effluents for phosphorus and ammonia removal. Also, they are the same sequence of steps used for both the low and high lime processes for raw sewage. Consequently, where physical–chemical operation or tertiary treatment of secondary effluent is being considered, the photodynamic inactivation technique can readily be incorporated into the process without addition of major capital equipment, other than a metering system for the dye, a holding tank for sensitization to occur, and the irradiation cell.

The photodynamic operation has been evaluated on the basis of an enterovirus content in sewage of 1000 plaque-forming units (PFU)/gal, which is to be reduced to 1 PFU/10 gal, or a reduction of 10,000× in virus

content. This level of enterovirus in secondary sewage effluents is now required by ordinance in Montgomery County, Maryland, and has been discussed at the International Conference on Viruses in Water at Mexico City (Berg et al., 1976) and at earlier meetings (Melnick, 1971).

The power requirement was determined for operating such a cell with a volume of 32.6 gal (0.123 m³). The estimated irradiation times at various temperatures, to achieve a 4 log reduction in virus for the flowthrough cell with the lamps in contact with the water were calculated by the FMC Corporation and are recorded in Table VI along with the costs for operating the cell in large and small plants. The chemical costs for the photodynamic oxidation process using 2 mg/liter of methylene blue were also calculated by the FMC Corporation and are given in Table VII.

The effects of photodynamic oxidation on organic molecules have been intensively studied for over a decade and much information is available on how it acts to destroy the integrity of these molecules. Such information is generally lacking for most disinfectants that are commonly used today. It has generally been established that photodynamic oxidation acts directly on the nucleic acid of the virus, resulting in its inactivation. All groups of animal viruses tested have been shown to be sensitive to photodynamic inactivation. Bacteria commonly found in sewage, such as coliforms, are easily photoinactivated as are pathogenic fungi such as Candida albicans.

Organic matter has been shown to greatly reduce the effectiveness of many disinfectants, especially chlorine and ozone. High concentrations of chlorine must be added to obtain small residuals in wastewater. It was shown in this study that sewage organics did not interfere with the action of the dye on microorganisms. Systems utilizing ultraviolet light would be

TABLE VI

Projected Power Costs for Photodynamic Inactivation of Virus[a]

Temperature (°C)	T_4 (hr)	T_1 (min. (10^4 Red))	Rate gallons/min	gallons/day	Cost ($/1000 gal) Package plant (at 2.4¢/kWh)	Large plant (at 1.5¢/kWh)
23	5	45	0.72	1040	1.35	.85
28	5	36	0.90	1304	1.07	.67
38	5	27	1.21	1740	0.80	.50
23	24	22	1.48	2130	0.65	.41
28	24	18	1.80	2590	0.54	.34
35	24	13	2.51	3610	0.39	.24

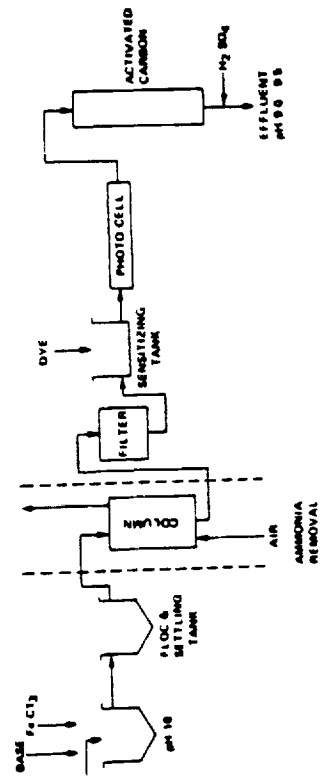[a] As of 1975, using 2 mg/liter methylene blue.

Fig. 5. Proposed process for photodynamic inactivation.

TABLE VII

Chemical Costs for Photodynamic Treatment at pH 10

Reagent	Cost (lb/1000 gal)	(¢/lb)ᵃ	(¢/1000 gal)
Caustic (50%)	3.4	3.5	11.9
Ferric chloride (anhydrous)	0.02	12.5	0.2
Sulfuric acid (93%)	0.5	2.3	1.2
Methylene blue (at 2 mg/liter)	0.017	202.00ᵇ	3.4
Activated carbon	0.04	32.0	1.3
Total chemical			18.0

ᵃ Chemical Market Reporter, June 20, 1975.
ᵇ FMC Corporation projected cost for large scale production.

ineffective in such effluents because of their absorption by the organic matter at the surface of the fluid. Wavelengths in the 670-nm range, though, are not absorbed by the organics and can penetrate to much greater depths. Turbidity can also affect the penetration of visible light into fluids, but under the conditions of this study turbidities of up to 10 JTU did not appear to affect the amount of coliforms or poliovirus that could be inactivated.

Photoinactivation could be most advantageously used in advanced wastewater treatment facilities where a high quality effluent is required. Photoinactivation could easily be incorporated into advanced wastewater treatment plants that use lime flocculation, since alkaline pH levels optimal for sensitizing microorganisms are produced during this process. Facilities that use elevated pH levels for ammonia stripping could also readily incorporate photoinactivation. Such treatment plants also greatly reduce turbidity, allowing for further penetration of light into the effluent. Passage of final effluent from such facilities through granulated activated carbon to remove residual organics is also practiced. This could be taken advantage of for the removal of the dye, since it readily adsorbs to activated carbon. We also conducted studies at a land disposal site in which dye was added to wastewater, and we found that methylene blue quickly becomes undetectable and presumably degraded in the environment. Nevertheless, if desirable, it can be removed to ensure the absence of any potentially, although presently unknown, adverse environmental effects.

The photodynamic inactivation process is more costly as compared to chlorination, breakpoint chlorination, and ozonation using the system

described in this paper. The major cost factors in photodynamic inactivation are the chemical cost for adjusting sewage secondary effluent to pH 10 and the electrical power cost. In physical–chemical and tertiary treatment systems, the chemical cost for pH adjustment is charged against nutrient removal; consequently, the processing costs will be reduced, as well as the reduction in capital costs to only the cost of the dye metering system and the photoinactivation cell. However, the major cost factor is the electrical power for operating the cell, and according to various lamp manufacturers, there does not appear to be any new lighting systems that will increase the efficiency of conversion of the electrical energy to light energy in the 600–700 nm range. The electrical power cost can be reduced by heat treatment during sensitization and by longer sensitization time. Heating the wastewater from 23 to 35°C reduces the irradiation time by half and would materially reduce the electrical power costs as indicated in Table VI. Moreover, solar energy has been shown to be a possible alternative to the use of the high intensity monochromatic lamps for photooxidation using methylene blue. The use of solar energy would eliminate the power costs for the photoinactivation cell, which would reduce the processing costs so that the photodynamic inactivation process would be competitive to the cost for chlorination and ozonation (Melnick et al., 1976).

The amount of 670-nm light in Houston, Texas, was found to be many times that available from the artificial light source that we used in the laboratory. Large reductions of poliovirus in both turbid wastewater and tapwater were easily accomplished, even under heavily overcast skies (Table V). Use of solar energy would greatly reduce operational costs of a system employing photoinactivation. Such a system should be feasible anywhere in the world where some daylight is available year round. It also should be pointed out that sensitization in the dark is not a necessity, since microorganisms can be inactivated by light in the process of becoming sensitized.

The solution to the problem of viruses in water is to prevent their contamination at the source. If sewage is properly treated, our surface waters can be made essentially free of viruses, which in turn reduces the potential of viruses entering our drinking water supplies. To prevent virus contamination of our surface waters, this report describes the feasibility of photodynamic oxidation for disinfection of sewage. Photoinactivation should be of greatest use in systems where large reductions in pathogenic bacteria and viruses are required, such as wastewater recycling systems. Optimal parameters for the photodynamic inactivation of coliforms and poliovirus in sewage by methylene blue have been determined. Temperature, pH, dye concentration, time of sensitization, and time of light

exposure influence the inactivation of coliforms and virus. In contrast to other methods of disinfection, organics in sewage failed to interfere with the photodynamic inactivation of the microorganisms. At first glance, the process and capital costs are greater than for chlorination because of the high cost of electrical power for the lamps and because of the necessity to raise the pH. However, in water treatment plants where the lime-softening process is used for water purification, the added costs of the photodynamic process would be greatly reduced. Furthermore, solar energy was found to be a practical and inexpensive alternative to use of an artificial light source for the photodynamic process. Photodynamic inactivation should be of greatest use where large reductions in pathogenic bacteria and viruses are required, such as water recycling systems. This is particularly true where one wishes to avoid the use of large amounts of chlorine with the subsequent formation of chlorinated carcinogenic compounds, many of which are now appearing in our water supplies.

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CHAPTER 13

Sexually Transmitted Viruses

LAURE AURELIAN AND BRUCE C. STRNAD

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Water-to-Air Transfer of Virus

Abstract. *Bubbles rising through suspensions of the bacteriophages T2 and T4 and of* Escherichia coli *adsorb and eject these particles in droplets that are formed when the bubbles burst. The concentration of the viruses in ejected droplets, determined from electron microscopy, exceeded the suspension concentration by 50 times. Similar results were obtained for* Escherichia coli. *The viability of some of the adsorbed particles was established by biological counts.*

This study concerns the possibility that bubbles adsorb viruses and propel them into the atmosphere when the bubble bursts. Bubbles bursting at a liquid surface eject a tiny jet of fluid into the air. The jet breaks up into a series of tiny drops with the diameter of the uppermost drop one tenth that of the bursting bubble (*1*) and with most of the lower drops having larger diameters. Such jet drops contain much of the material adsorbed to the bubble (*1*) as well as some of the material adsorbed to the surface of the liquid (*2*).

Transport into the atmosphere of red-tide toxins (*3*), organic matter (*4*), and bacteria (*1*) has previously been related to aerosol formation. Gruft *et al.* (*5*) postulated that aerosol of marine origin is a vector of *Mycobacterium intracellulare* (Battey) infection. Whether pathogenic viruses are similarly airborne is clearly of public health concern.

In the present study, the coliphages T2 and T4 were chosen as harmless indicator viruses which, after aerosol ejection, could be assayed biologically and morphologically. If these viruses, with their complex structure and sensitivity to drying and ultraviolet radiation, remained viable and became concentrated during aerosol formation, then it could be assumed that the simpler, less fragile viruses are similarly dispersed.

The jet droplets used in this study were formed by admitting air bubbles (0.03 cm in diameters) under pressure (1 to 2 kg/cm²) (*6*) into the bottom of a liquid column (17 cm in depth) containing *Escherichia coli* and the bacteriophages suspended in buffer. The phage concentration was adjusted to 5×10^8 per milliliter and the bacteria to a concentration of 5×10^8 per milliliter.

Depending on the experiment, different suspending fluids were used in the column. Jet droplets containing phage were seen in the electron microscope when the phages were suspended in tris buffer (0.1*M*, pH 7.6, with 3 percent NaCl). The phage lysate and washed bacteria were added to distilled water. Lysate facilitated adhesion of jet droplets to the carbon film of the electron microscope grid, and upon drying left a distinct circular contour which we used for diameter measurement.

The T2 and T4 bacterial viruses were prepared in high titers from lysates of their host cell B/1.5 (a strain of *E. coli*) grown in synthetic medium (*7*) with heavy aeration. In some experiments the phages were centrifuged away from the lysate and resuspended in buffer before they were added to the column. *Escherichia coli* B/4, a strain that is resistant to T2 and T4, was grown overnight in 1 percent tryptone broth with aeration at 37°C to the stationary phase. The cells were centrifuged and the pellet was suspended in the same buffer as that used in the bubble chamber.

To collect jet droplets for electron microscopy, grids bearing carbon films were held inverted for 30 to 45 seconds about 1 cm above the surface where the bubbles were bursting. In this configuration of emitting bubbles and collecting surface we collected the uppermost two or three droplets of the jet (*8*). The collected droplets were dried in air for 1 minute to minimize subsequent loss of material, stained in saturated uranyl acetate for 2 minutes, rinsed in distilled water, drained, and dried.

The specimens were photographed at two magnifications ($\times 3000$ and $\times 10,000$) by means of a Jeolco 100B electron microscope at 60 kv. Enlarged prints at the lower magnification were used for diameter measurements of the dried droplets and for counting the number of viruses and bacteria in measured areas. The smallest area used in the counts was a quadrant. Only particles showing both a head and an extended tail were counted as virus.

The fields photographed at the higher magnification were randomly selected and provided morphological evidence of intact virus in the droplets.

Viability assays were obtained by holding moist petri dishes containing tryptone agar above the surface of the bubble column for 10 to 40 seconds. Soft agar (0.7 percent) was immediately added to the surface of the dish and spread by vigorous shaking. In the case of the phage assays the soft agar contained the sensitive cell, B/1.5. The dishes were incubated overnight at 37°C.

Electron microscopy showed that both phages and bacteria were transferred from bulk fluid into air by jet droplets. The mean volume calculated from 18 droplets was $2 \times 10^{-8} \pm 1 \times 10^{-8}$ ml ($\pm$ standard deviation) with a range of 4×10^{-8} to 7×10^{-8} ml. Thus the range of diameters of drops ejected from bubbles that were 300 μm in diameter was 24 to 42 μm, the mean being 34 μm. These data corroborate Blanchard's (*1*) rule of thumb that the diameter of the top drop of the jet set is one-tenth the diameter of the bubble. The range of drop sizes results from collecting the uppermost three drops. The second and third drops of the set are smaller and larger, respectively, than the top drop.

The number of phages per droplet was determined from the electron micrograph by counting the number of particles having both a head and a tail per unit area of droplet. Bacteria were easily identified by shape and size. Eighteen separate droplets captured from a col-

Simultaneous Concentration of Four Enteroviruses from Tap, Waste, and Natural Waters

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The efficiency of virus recovery from water was investigated by using a method which enabled the concentration of a mixture of four enteroviruses with determination of their individual recovery efficiencies. The four viruses used (poliovirus 1, coxsackievirus A9, coxsackievirus B1, and echovirus 7) represented each of the four major subgroups of enteroviruses. This method, which was based on selective antibody neutralization, was used to investigate the effects of input water quality on enterovirus concentration by Balston filters (grade C; Balston, Inc., Lexington, Mass.) and organic flocculation. With tap water, the average recovery efficiency of the four viruses was 97%. Concentration from natural waters, including samples from two lakes (Lake Kinneret and the Hula Nature Reserve) and the Mediterranean Sea, resulted in similarly high average recovery efficiencies. Echovirus 7 was recovered with a slightly lower average efficiency from these types of water than were the other viruses. In comparison with other types of water, virus concentration from Jerusalem wastewater generally had a slightly lower efficiency of recovery, ranging from 63 to 75% for each of the viruses, with an overall average of 68%. The ability of each concentration step, membrane filtration or organic flocculation, to recover the viruses from water was assayed. For the filtration step, although there were not large differences in virus recoveries from tap water, echovirus 7 was recovered with the lowest efficiency (72%), and poliovirus 1 was recovered with the highest (87%) efficiency. Overall virus recovery by the filtration step was least efficient for wastewater (73%) and most efficient for seawater (107%). The organic flocculation step was highly efficient, with essentially all of the virus recovered either as individual viruses or as a mixture. Of the types of water tested, viruses were recovered from seawater with the lowest efficiency. In summary, although some differences in virus recovery were observed for various types of water and individual viruses, the range was not large. Thus, the two-step concentration method used was found to be an efficient general method for enterovirus concentration from water.

The pollution of water by low levels of a wide variety of human pathogenic viruses is detectable by techniques designed for concentrating viruses from water. In determining the efficiency of such techniques, a representative virus is chosen and seeded into water, and the water is then processed. The amount of virus in the concentrate is determined, and the recovery is calculated relative to the amount of virus seeded. Generally, the test virus is a laboratory strain of a potentially waterborne virus, and the efficiency of the method is evaluated for one or more such viruses. It is advantageous to use several different virus types in evaluating a concentration method so as to determine its range of applicability.

Current methods of virus concentration have a large degree of variability, in part because of changes in water quality (15) and other as yet undefined factors. Because of this variability, it is difficult to compare the recoveries of different virus types by using the results of separate experiments performed with individual viruses. In this report, we describe the development of a method for determining the individual recoveries of four enteroviruses from a sample seeded with a mixture of the viruses. This method was then used to determine the relative recoveries of the viruses from various types of water by a two-step concentration technique.

MATERIALS AND METHODS

Viruses and virus assays. Poliovirus 1 (Brunhilde strain) grown and titrated by plaque assay in BGM (African

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green monkey kidney) cells as described before (9). Echovirus 7 (Wallace strain), coxsackievirus B1 (Conn-5 strain), and coxsackievirus A9 (Grigg strain) were obtained from the American Type Culture Collection, Rockville, Md., grown, and titrated in BGM cells under the conditions used for poliovirus 1.

Antibodies and virus neutralization. Antibodies to coxsackievirus A9, coxsackievirus B1, and echovirus 7 were obtained from the American Type Culture Collection and were used at dilutions of 1:300. Antibodies to poliovirus type 1 were prepared in rabbits by the inoculation of purified virus as described before (7). Neutralization was performed by mixing of the antibodies with the virus, incubation for 30 min at 37°C, and titration by plaque assay.

Water samples. Water samples were obtained from Jerusalem tap water, Jerusalem wastewater, Lake Kinneret, the Hula Nature Reserve, and the Mediterranean Sea. Samples were stored at 4°C until used. The conductivity of the samples was measured with a conductivity meter (type CDM 3; Radiometer, Copenhagen, The Netherlands), and the chemical oxygen demand was determined by standard methods as described before (1). The conductivity, chemical oxygen demand, and pH values of the samples are shown in Table 1.

Virus concentration. A mixture of four enteroviruses was prepared and used in all concentration experiments. The ranges of input virus titers were 2.4×10^2 to 8.0×10^4 PFU of coxsackievirus A9, 4.0×10^2 to 1.1×10^5 PFU of coxsackievirus B1, 4.7×10 to 2.1×10^5 PFU of echovirus 7, and 1.1×10^2 to 2.0×10^5 PFU of poliovirus 1. Sample volumes were 5 liters each except for wastewater, for which

Viruses and Water Quality

There is a growing concern with the problem of possible viral pollution of water supplies in this country. From an epidemiological point of view, only the infectious hepatitis virus has been considered to be transmitted by water. Gross contamination of drinking water by sewage wastes was responsible for a major epidemic of infectious hepatitis in New Delhi (1955-1956), and resulted in 35,000 cases, 73 deaths, and a case rate of 2,000/100,000 in a single month. In the United States, the annual incidence of infectious hepatitis has remained at a level of 50,000 to 60,000 cases per year during most of the 1952 to 1970 period, while the total annual incidence of typhoid fever dropped continuously from approximately 2,000 cases in 1952 to only 346 cases in 1970. There has been one significant waterborne outbreak of infectious hepatitis during each of the last three years, including the Worcester, Mass, episode that involved 90% of the College of the Holy Cross football team.

The cause of more than 18,000 cases of waterborne gastroenteritis and related diarrheal disease reported from 1946 to 1960 could possibly be ascribed to enteric viruses. Enteric viruses, not necessarily waterborne in nature, have been implicated also in other serious illnesses. Coxsackieviruses have been recognized as the causative agents of idiopathic myocarditis and congenital heart abnormalities, and may trigger an insulin-dependent diabetes. High incidence of mongolism is reportedly associated with hepatitis infection in mothers, while the hemolytic-uremic syndrome has been related with a high degree of enterovirus infection. Although some viruses, which may occur widely in food and water, are not considered to be pathogenic for man, it has been speculated that when they infect an unnatural host, such as man, they may play a role in carcinogenesis.

Many human viruses multiply in the alimentary canal and are excreted in the feces of infected individuals. Their numbers are small when compared with the numbers of excreted bacteria since viruses do not multiply outside of living susceptible cells and decrease in numbers in the receiving waters. At the same time, the smallest amount of virus capable of infecting cells in culture is usually capable of producing at least a symptomless infection in man. Since minimal amounts of virus can produce infection, total removal of viruses from any water for human consumption would appear justified as a sound public health measure.

Human enteric viruses have been recovered from 36% of the surface water samples examined, and there is ample evidence that ground waters can also transmit virus under favorable soil conditions.[1] It is generally agreed that the higher the temperature, the shorter the time the virus survives in water. In general, enteric viruses will survive longer in distilled water than in polluted water, and longer in grossly sewage-polluted water than in moderately polluted water.[2] Poliovirus 1 had a survival time of 19 days at 4C in river water with a "moderate" amount of pollution, but survived 110 days at the same temperature in river water heavily polluted with sewage wastes.[3]

Laboratory methods are currently available to detect and measure the small number of viruses in large volumes of water, but the recovery of one plaque-forming unit of virus from 100 gal of water or more will require even better concentration procedures. Hopefully, a standard method for measuring virus in water may become available in the future and lead to the ultimate development of realistic viral standards for water supplies. The coliform test, a bacterial indicator used for more than 70 years in the sanitary field, is being appraised to determine its dependability as an indicator of viral pollution; positive coliform tests would certainly indicate possible virus contamination, but a negative coliform test might not indicate freedom from viruses.

The most critical tasks for the future in the case of water virology are the removal of pollutants from drinking water in the home and in industry, and from waste water before discharge into the aquatic environment. No single method is currently available that completely removes all viruses from sewage. Conventional waste treatment units provide virus removal efficiencies ranging from 25% to 99%.[4] Terminal disinfection by the use of chlorine has shortcomings as a virucide, since chlorination in the presence of organic material is likely to produce chloramines, a relatively slow viral disinfectant. However, where hypochlorous acid can be maintained in a water, disinfection is readily and rapidly achieved.

Potable water can be treated to be always free of biological pollutants, including viruses, by using current treatment processes and disinfectant measures at all times. Unfortunately, results of the National Community Water Supply Study (1968) show that adequate treatment and disinfection is not being uniformly applied to all systems.[5] Sufficient chlorination of all community water supply systems can be accomplished at a projected cost of only 20 cents per person ($40,000,000) annually in this country, and would eliminate water transmission of enteric viral disease.

Investigation of the potential problems of viruses in water is probably only beginning at this time. Still unanswered questions include what waterborne viruses are important to human health, how to predict the fate of viruses in natural waters, how quantitatively to detect and identify viruses in waters of every quality, and how most effectively to achieve total virus destruction in waste discharges and water supplies. Intensified research will be required to answer these pertinent questions.

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Editorials

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Method for Recovering Viruses from River Water Solids

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Small numbers of virions (poliovirus 1) that had been adsorbed to river water solids were eluted by mixing the solids for 30 min with a 10% solution of beef extract that contained sufficient Na_2HPO_4 to bring the molarity of the salt to 0.05 and sufficient citric acid to bring the pH to 7. The virions were recovered by inoculating the beef extract onto cell cultures. With this method, 39 to 63% of the poliovirions that had been adsorbed onto the river water solids were recovered.

Enteroviruses adsorb readily to many solids in rivers and in other waters. The numbers of virions recovered from the solids in river waters often exceed the numbers of virions recovered from the waters (1, 2, 4, 7, 8). If the viruses in the water environment tend to concentrate in and on the solids in the water, good techniques for recovering viruses from those solids must be developed.

This paper describes a method for recovering viruses from the solids in water.

MATERIALS AND METHODS

Virus. Two preparations of poliovirus 1 (Mahoney) were used in these studies. One had undergone 42 passages in cynomolgus monkey kidney and African green monkey kidney cell cultures. The other preparation had undergone one fewer passage in African green monkey kidney cells and then a final passage in rhesus kidney cells.

Virion assays. Virions were assayed by the plaque technique. BGM cells in passages 222 to 227 were used for the assays. The method for preparing BGM cell cultures and the method for the plaque assay were described earlier (5).

Beef extract. Powdered beef extract (Lab-Lemco) was dissolved in distilled water, and the solution was autoclaved at 121°C for 15 min. In some studies, $Na_2HPO_4 \cdot 7H_2O$ and citric acid, in appropriate quantities, were dissolved with the beef extract powder into the water. The eluting capacity of each new lot of beef extract was compared with that of earlier lots to ascertain that its capacity to elute poliovirus 1 was maximal (3).

River water solids. Water from the Ohio River was collected in 50-gallon (188-liter) drums, and the solids in the water were settled for 4 days. The water above the settled solids was pumped from the drums, and the sediments were centrifuged in 250-ml round-bottom centrifuge bottles at 1,250 × g for 20 min. The water in the centrifuge bottles was decanted and replaced with sediments from the drums. The centrifugation was repeated. This process was continued until the centrifuge bottles were two-thirds full of packed sediments. The sediments were then stored under a thin layer of water at 4°C.

Elution of poliovirus 1 from river water solids. A 15-g sample of the wet river water solids was mixed with 200 ml of distilled water and inoculated with the poliovirions. The virions were mixed with the suspended solids for 30 min on a magnetic stirrer, and the suspension was filtered under suction through a Millipore AP20 fiber glass prefilter pad (diameter, 124 mm) that had been placed in a Büchner funnel. The solids were scraped from the pad. To elute the virions from the solids, the solids were mixed with beef extract or with beef extract with buffer on a magnetic stirrer. Unless otherwise noted, the volume of beef extract or beef extract with buffer used for eluting the virions from the solids was 40 ml, and the time of mixing was 30 min. The mixture was centrifuged at 4°C for 15 min at 1,250 × g. The supernatant was recovered and centrifuged at 4°C for 30 min at 15,000 × g. In one series of tests (Table 2, series 2), this second centrifugation was done at 140,000 × g. To remove contaminating bacteria, the supernatant was filtered through a Swinnex filter apparatus that contained a filter with a diameter of 47 mm and a pore size of 0.45 µm. The virions in the filtrate were assayed by the plaque technique on BGM cells. Unless otherwise noted, the numbers of virions seeded into each test were determined from assays of seed virus preparations diluted into the eluent used for the test (controls).

RESULTS

Elution of poliovirus from river water solids. (i) Elution with beef extract. A 3% solution of beef extract under sonication eluted all of the enterovirions adsorbed to cellulose nitrate membranes. Without sonication, lesser recoveries resulted (3). In repeated tests, a 10% solution of beef extract consistently eluted more of the poliovirions adsorbed to river water solids than a 3% solution of beef extract did (Table 1). Sonication did not improve elution (Berg and Dahling, unpublished data). The eluting effectiveness of the beef extract began to diminish when its concentration was somewhere in the 10 to 20% range (Table 1, series 3, 4, and 5).

Although the volumes of beef extract varied in series 5 because of some losses that occurred when the eluents were prepared, the data in that series suggest strongly that the numbers of viri-

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TABLE 1. *Elution with beef extract of poliovirus 1 from river water solids*

Series no.	Test no.	Beef extract		Virions	
		Concn (%)	Vol (ml)	Seeded (PFU)[a]	Recovered (PFU)
1	1	3	40	102	24
	2	3	40		11
	3	10	40		37
2	1	3	40	82	0
	2	10	40		25
3	1	3	40	78	6
	2	5	40		16
	3	8	40		21
	4	10	40		24
	5	15	40		26
	6	20	40		22
4	1	10	40	216	71
	2	15	40		54
5	1	10	40	121	29
	2	20	25		16
	3	30	35		8
	4	40	30		3
	5	50	30		2

[a] PFU, Plaque-forming units.

ons recovered in elution diminished markedly with beef extract concentrations in excess of 20%. The consistency of beef extract solutions at the 40 and 50% levels was soupy, suggesting that osmotic pressure and possibly other physical factors may have destroyed virions or interfered with their recovery. It is also possible that in 40 and 50% solutions of beef extract, inhibitors reached concentrations that produced an observable effect.

(ii) Elution with beef extract in McIlvaine buffer. A 10% solution of beef extract in McIlvaine buffer of appropriate strength had a greater capacity than a 10% solution of beef extract alone to elute poliovirus 1 from the river water solids to which poliovirions had been adsorbed (Table 2). A 10% solution of beef extract that contained sufficient Na_2HPO_4 to bring the molarity of the salt to only 0.005 M and sufficient citric acid to bring the pH to about 7 did not elute more poliovirions than a 10% solution of beef extract alone did. However, a 10% solution of beef extract that contained sufficient Na_2HPO_4 to bring the molarity of the salt to 0.05 and sufficient citric acid to maintain the pH at about 7 consistently eluted more virions than a 10% solution of beef extract alone did. Since the pH levels of both solutions of beef extract were the same, it would seem that improved elution with the addition of the buffer reflected an effect of one or both of the buffer components.

A 10% solution of beef extract with sufficient Na_2HPO_4 to bring the molarity of the salt to 0.087 and sufficient citric acid to maintain the pH of the solution at 7.1 consistently eluted more virions than a 10% solution of beef extract alone did (Table 3).

Effect of elution interval on yield of poliovirions adsorbed to river water solids. A 10% solution of beef extract (pH 7.1) eluted at least as many poliovirions from river water solids to which the virions had been adsorbed in 30 min as in 60 or 90 min. Similar results were obtained when a 10% solution of beef extract in McIlvaine buffer (pH 7.1) was used as the eluent (Table 3). Studies were not done with shorter elution intervals, and it is possible that maximum elution may be achievable in less than 30 min.

Effect of pH on the elution with beef extract of poliovirions adsorbed to river water solids. In some situations, elution of virions from adsorbents increases at high pH levels. Therefore, studies were undertaken to determine whether elevated pH levels would increase the elution of poliovirions adsorbed to river water solids. Elution with a 10% solution of beef extract at about pH 7 yielded considerably greater numbers of virions than elution at pH levels of about 10 and 11 (Table 4). This was the case whether the 10% solution of beef extract was used alone or in McIlvaine buffer, which had a molarity of 0.13 with respect to Na_2HPO_4. The beef extract in McIlvaine buffer was again a more effective eluent than the beef extract alone.

Effectiveness of 10% beef extract in McIlvaine buffer for eluting poliovirus 1 from river water solids. In 30 min of mixing, 15 g of river water solids in 200 ml of distilled water adsorbed all or almost all of the poliovirus 1 added to such suspensions (unpublished data). Therefore, in determining the effectiveness of a 10% solution of beef extract in McIlvaine buffer for eluting poliovirions, all of the virions added to suspensions of river water solids were assumed to adsorb on the solids. Equal volumes of a suspension of virions were added to a volume of the eluent (control) and to the test system as described in Materials and Methods. The number of virions counted in the control was taken as the number of virions adsorbed to the river water solids in the test system. On this basis, the 10% beef extract in McIlvaine buffer recovered 39 to 63% of the seed poliovirus from the solids (Table 5). The strength of the buffer within the range tested did not affect viral recovery. If less than all of the poliovirions had adsorbed to the solids, then the effectiveness of the eluent would have been proportionately greater than that shown in Table 5.

TABLE 2. *Elution of poliovirus 1 from river water solids with 10% beef extract in McIlvaine buffer*

Series no.	Test no.	pH	Strength of buffer in eluent[a]	Virions	
				Seeded (PFU)[b]	Recovered (PFU)
1	1	7.0	None	178	68
	2	7.0	Na$_2$HPO$_4$ (0.05 M) Citric acid (1.2 g/liter)	204	128
	3	7.1	Na$_2$HPO$_4$ (0.005 M) Citric acid (0.12 g/liter)	204	55
2	1	7.1	None	210	26
	2	7.2	Na$_2$HPO$_4$ (0.05 M) Citric acid (1.2 g/liter)	178	31
	3	7.2	Na$_2$HPO$_4$ (0.005 M) Citric acid (0.12 g/liter)	194	30
3	1	7.1	None	216	71
	2	7.1	Na$_2$HPO$_4$ (0.05 M) Citric acid (1.2 g/liter)	242	107
	3	7.1	Na$_2$HPO$_4$ (0.005 M) Citric acid (0.12 g/liter)	229	57

[a] The volume of eluent in all tests was 40 ml.
[b] PFU, Plaque-forming units.

TABLE 3. *Effect of time on elution of poliovirus 1 from river water solids with 10% beef extract in McIlvaine buffer*

Test no	Elution time (min)	Virions seeded (PFU)	Virions recovered (PFU) with eluent:[a]	
			Beef extract (pH 7.1)	Beef extract in McIlvaine buffer (pH 7.1)
1	30	202	67	
2	30	196		118
3	60	202	67	
4	60	196		119
5	120	202	60	
6	120	196		77

[a] The volume of eluent in all tests was 40 ml. Beef extract in McIlvaine buffer consisted of 10% beef extract and sufficient Na$_2$HPO$_4$ to bring the molarity of the salt to 0.087 and sufficient citric acid (1.25 g/liter) to bring the pH to 7.1. PFU, Plaque-forming units.

TABLE 4. *Effect of pH on elution with 10% beef extract of poliovirus 1 adsorbed to river water solids*[a]

Test no.	pH	pH of eluent adjusted with:	Virions seeded (PFU)[b]	Virions recovered (PFU)
1	7.0	Unadjusted	81	25
2	6.9	McIlvaine buffer[c]		36
3	9.8	Borate buffer[c]		2
4	11.1	NaOH		12

[a] The volume of eluent in all tests was 40 ml.
[b] The numbers of virions seeded were determined from assays of the seed virus preparation diluted into 3% beef extract. PFU, Plaque-forming units.
[c] McIlvaine buffer comprised 0.13 M Na$_2$HPO$_4$ and 3.0 g of citric acid per liter. Borate buffer comprised 0.016 M boric acid, 15 g of KCl per liter, and 8 g of NaOH per liter.

DISCUSSION

Commercially produced beef extracts are by their nature a variable commodity, and this has been reflected in the variable efficiency with which different lots elute viruses (3). Therefore, for seeding experiments, we test each lot of beef extract for its eluting efficiency with the virus under study. For field samples, it has been our practice to determine the eluting effectiveness of beef extract preparations for three different viruses of the family or families targeted for recovery and to use only lots with high recovery efficiencies. It is not clear yet whether the relative eluting ineffectiveness of many lots of beef extract reflects a lack of eluting factors in those lots or whether there is present in those lots materials that are virucidal or inhibitory to the multiplication of viruses. In any event, there is

TABLE 5. *Effectiveness of a 10% solution of beef extract in McIlvaine buffer (pH 7 ± 0.2) for eluting poliovirus 1 from river water solids*

Series no.	Strength of buffer	Virions (PFU)[a]		Elution interval (min)	PFU recovered (%)
		Adsorbed	Eluted		
1	Na$_2$HPO$_4$ (0.05 M) Citric acid (1.2 g/liter)	204	128	30	63
2	Na$_2$HPO$_4$ (0.05 M) Citric acid (1.2 g/liter)	242	107	30	44
3	Na$_2$HPO$_4$ (0.13 M) Citric acid (3 g/liter)	82	36	30	44
4	Na$_2$HPO$_4$ (0.087 M) Citric acid (1.25 g/liter)	196	118	30	60
5	Na$_2$HPO$_4$ (0.087 M) Citric acid (1.25 g/liter)	196	119	60	61
6	Na$_2$HPO$_4$ (0.087 M) Citric acid (1.25 g/liter)	196	77	120	39

[a] PFU, Plaque-forming units.

no guarantee that a lot of beef extract that yields high recoveries of viruses under one set of conditions will do so under others. Certainly, the recoveries of viruses from solids are not as good as they are from membrane filters (3). Moreover a lot of beef extract with high eluting effectiveness for some viruses may not be equally effective for others. And, of course, we should not expect that all viruses within the solids (some of which may be fecal material) in river water are even reached by the eluent.

In experimental laboratory studies, the effectiveness of the recovery technique may also be affected by the length of time that the viruses adsorb to the solids. We have found that the longer adsorption proceeds, the less of the seed virus we are able to recover (unpublished data).

Adsorption of viruses to solids is often best achieved at low pH levels, and elution is often practiced at high pH levels. In our studies, elution was more effective at pH 7 than at pH 10 to 11 (Table 4). It is not clear why. It may be that inhibitors or virucides were produced in the beef extract or potentiated at the higher pH levels.

The presence, in sufficient concentrations, of McIlvaine buffer (Na$_2$HPO$_4$ and citric acid) in a 10% solution of beef extract increased the virus-eluting capability of the extract. The phosphate in the buffer was not the effective agent (unpublished data). Whether the citric acid in the buffer increased the eluting effectiveness of the beef extract solution, and, if so, whether the citric acid alone is a good eluent, is yet to be determined. McIlvaine buffer, in the same strength used in the studies reported here, facilitates the adsorption of enteroviruses to cellulose nitrate membrane filters (3).

The technique described here, at least in laboratory studies, is an effective one. With this technique, we have recovered many different enteroviruses from river water solids. The method is not as practical as it needs to be when small numbers of viruses are to be recovered (especially for laboratories that must purchase cell cultures), because it requires large numbers of cell cultures for assay. At some cost in the recovery efficiency of viruses, the economic problem has been resolved by incorporating as a reconcentration procedure the organic flocculation technique of Katzenelson et al. (6). The results of those studies will be reported elsewhere.

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FATE OF VIRUSES DURING WASTEWATER SLUDGE TREATMENT PROCESSES

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I. OVERVIEW

The pathogens present in sewage fall into four major groups: bacteria, viruses, protozoans, and helminths. A large portion of these tend to become concentrated in sludge fractions during the overall sewage treatment process.[1] This results not only from the pathogens being shed in a solid matrix, but also from subsequent adsorption of pathogens onto the sewage sludge solids and from some of the large pathogens independently settling out with the sludge.[2-5] Knowledge of the fate of the viruses is important since over 130 different types of pathogenic human viruses are known to be excreted in human feces (Table 1). Several additional types of pathogenic human viruses are shed in urine (Table 2) and still others may be introduced to wastewater through the sewer disposal of blood which has not been subjected to proper sterilization treatment (Table 3). Studies have shown that such viruses, at least those of an enteric nature which are shed in feces, can easily survive present methods of sewage treatment.[23] Section II of this article briefly reviews the different types of human viruses which are expected to be present in sewage.

Once viruses are released into the environment, they become susceptible to inactivation by a variety of factors, such as temperature, pH, sunlight, inorganic cations and anions, loss of moisture through evaporation, and antagonism by aerobic microorganisms and microbial products.[24-30] It is important to realize that the persistence of viruses in the environment can be prolonged by certain factors, such as their propensity to adsorb onto particulate surfaces in soil and water.[2] Under appropriate conditions, enteric viruses may persist for several months in wastewater sludges[31] and environmental waters.[26,28,32] It is also important to note that as little as one tissue-culture infectious dose of a virus may be sufficient to cause an infection in humans.[33,34]

Although the human immunodeficiency viruses which cause acquired immune-deficiency syndrome (AIDS) have not yet been found in wastewaters, they could be present as a result of sewer disposal of contaminated blood that has not received adequate sterilization treatment.[20] Although this virus does not normally appear to be spread by the fecal-oral route of transmission, as are enteric viruses, available evidence[20,35] suggests that it may have an environmental stability comparable to that of the enteric viruses. Thus, the sewer disposal of contaminated blood and other similarly contaminated materials without prior sterilization may be a practice that should be viewed with concern.

Viruses can complete a cycle of transmission from wastewater back to man.[32,36] A number of the routes by which this cycling can occur are illustrated in Figure 1. Virus particles associated with sludges can potentially complete all of these transmission routes. Sewage sludge is sometimes intentionally disposed of into surface waters.[37] Raw sewage and sewage sludges are also commonly discharged through sea outfalls.[38-43] Viruses present in these

Table 1
HUMAN VIRUSES THAT ARE SHED IN FECES[4-17]

Virus group	Number of serotypes	Illness caused
Adenovirus	41	Pharyngitis, conjunctivitis, respiratory illness, vomiting, diarrhea
Astrovirus	5	Vomiting, diarrhea
Calicivirus	2	Vomiting, diarrhea
Coronavirus	1	Vomiting, diarrhea
Enterically transmitted Non-A, non-B hepatitis virus	1	Hepatitis
Enterovirus		
Poliovirus	3	Paralysis, meningitis, fever
Coxsackievirus A	24	Herpangina, respiratory illness, meningitis, fever
Coxsackievirus B	6	Myocarditis, congenital heart anomalies, rash, fever, meningitis, respiratory illness, pleurodynia
Echovirus	34	Meningitis, respiratory disease, rash, diarrhea, fever
"Numbered" enteroviruses	4	Meningitis, encephalitis, respiratory illness, acute hemorrhagic conjunctivitis, fever
Hepatitis A virus	1	Hepatitis
Norwalk virus (possibly a calicivirus)	1	Epidemic vomiting and diarrhea
Parvovirus	2	One type possibly associated with enteric infection
Reovirus	3	Not clearly established
Rotavirus	4	Vomiting, diarrhea
"Small Round Viruses" (possibly enteroviruses)	2	Vomiting, diarrhea

Table 2
HUMAN VIRUSES THAT ARE SHED IN URINE[6,18,19]

Virus group	Number of serotypes	Illness caused
BK virus	1	Chronic subclinical infection
Cytomegalovirus	1	Generalized infection, congenital defects
Hepatitis B virus	1	Hepatitis
JC virus	1	Progessive multifocal leukoencephalopathy
Measles (rubeola) virus	1	Rash, fever
Mumps virus	1	Glandular inflammation
Rubella virus	1	Rash, fever, congenital defects

Table 3
HUMAN VIRUSES THAT MAY BE PRESENT IN
DISCARDED BLOOD[4,19,22]

Virus group	Number of serotypes	Illness caused
Hepatitis B virus	1	Hepatitis
Hepatitis C virus (unclassified non-A, non-B)	1	Hepatitis
Hepatitis delta virus	1	Hepatitis
Human immunodeficiency virus	2	Acquired immune deficiency syndrome (AIDS)

Note: Viremia, i.e., the presence of viruses in the blood, occurs transiently in a great many viral illnesses, even though it may not always be readily detectable. The viruses listed in this table are of particular interest because they are transmitted by contaminated blood.
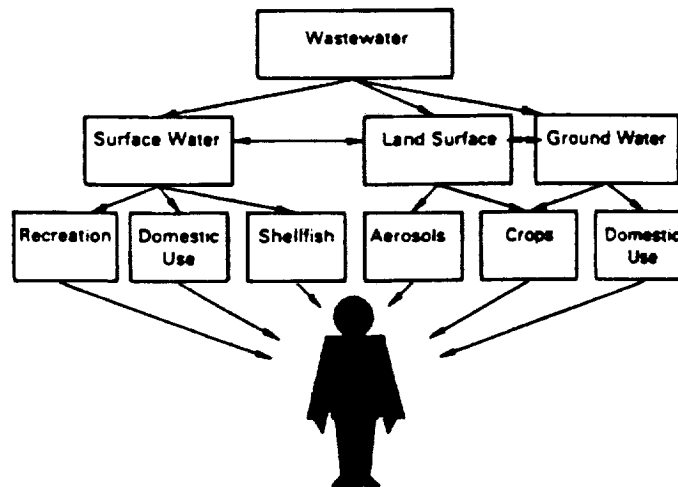
FIGURE 1.   Cycles of virus transmission through the environment.

discarded sludges may be transported over large distances by water flow patterns and thereby contribute to the contamination of drinking water sources, recreational waters, and shell-fishing areas.

Application of sewage treatment-plant sludges onto land surfaces is a common method of disposal and is used to avoid environmental problems which may arise from alternative methods of sludge disposal, such as dispersion into surface waters and incineration. Land application is also viewed as a means of recovering the soil enrichment and fertilizing qualities of sewage sludges.[44-46] The application of human and animal wastes to land for purposes of disposal is, in fact, an ancient practice.[44] The first documented large-scale systems for applying human wastes to soil were built in 1559 in Lower Silesia, Poland, and Breslau, Germany.[47] The practice of collecting wastes for land application spread throughout Europe, and several cities in Great Britain had initiated a practice of "sewage farming" by around 1800.[48] In the U.S., application of sludges to land for the purpose of fertilization has a history dating back to at least 1922.[44] However, the reuse of man's wastes through disposal

to land has been, and still is, accompanied by the perennial transmission of diseases to man.[44] In one study, the incidence of the bacterial diseases shigellosis, salmonellosis, and typhoid fever and the viral disease infectious hepatitis was reported to occur two to four times more frequently in communities practicing wastewater irrigation than in those which did not.[49] However, these results have not been satisfactorily verified.

In addition, land disposal can present a risk of human illness through enteric virus contamination of market fruits and vegetables harvested from sewage-irrigated fields.[50] In this regard, the disposal of sludges may present a more severe problem than that of wastewater effluents, due to the relatively greater concentrations of pathogens which may be present in sludges[51,52] compared with effluents. Another compounding factor is that the adsorption of viruses onto sewage-sludge solids may protect the former against thermal inactivation.[53] Enteroviruses have been known to survive as long as 36 d on lettuce and radishes which had been spray-irrigated with activated sludge[50] and they are capable of surviving on the surface of vegetables for 10 to 15 d at refrigerator temperatures.[54]

Edmonds and Littke[55] have found that microbial aerosols can be generated from the surface of dewatered sewage sludge which has been applied to the land surface. The authors concluded that dry conditions and high wind speeds tended to favor the generation of aerosols from disposed sludge solids. There is also a concern that contaminants present in wastewater sludges which are discarded on land may subsequently contribute to the contamination of surface waters through overland runoff and contaminate groundwaters through leaching processes.[56,57] Hence, despite the obvious benefits to public health and civic pride when nearby lakes and streams appear to be rendered virtually free of pollution through the use of land disposal, the main concern is whether this desirable objective can be achieved without creating alternative health hazards.[23]

Because of their solid nature and high organic content, wastewater sludges cannot be readily disinfected by chlorination. Therefore, a variety of other methods are more commonly used for the treatment of sludges prior to their disposal. These methods include such processes as aerobic and anaerobic digestion, heat drying, irradiation, pasteurization, composting, and liming.[58] The effects of these different processes upon the viral burden of sludges are presented in subsequent sections of this review.

II. A BRIEF INTRODUCTION TO VIRUSES

A. What are Viruses?

Viruses are very small microorganisms which have been divided into families, genera, and species in accordance with classification guidelines prepared by the International Committee on Taxonomy of Viruses.[59] An effort has been made to utilize the Committee's guidelines for viral names throughout this review. Viruses range in size from approximately 18 to 1500 nm or more (a nanometer [nm] is 1 billionth of a meter in length) in their largest dimension. All viruses are characterized as obligate intracellular parasites. As such, they can replicate only within a living host cell. For most virus types, the range of host species in which they can naturally replicate seems limited to only one or, at most, a very few genera. However, host ranges for members of certain other virus groups, such as influenza and some insect-transmitted encephalitises, may, in fact, span phylogenetic order boundaries, encompassing both mammals and birds.

The composition of viruses is relatively simple. They contain only ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) as their genetic material. This material, or genome, is usually surrounded and protected by viral-specified proteins which form a structure known as a capsid. In some virus groups, the capsid takes the form of a rigid or semi-rigid rod. In other groups, capsid proteins are present in the form of a rigid polyhedral shell that generally has the shape of an icosahedron. The polyhedral capsid structures of many virus

groups also bear characteristic, recognizable protein appendages, such as tails and fibers. For some viral groups, the capsid is surrounded by a lipid bilayer membrane, or envelope, that is derived from one of the host cell's membranes during maturation steps which occur late in the viral replicative cycle. Viruses do not contain any metabolic machinery as such, and the limited amount of enzymatic activity associated with individual virus particles, or virions, is generally related to functions which are important for maturation and release of the viral particles from host cells or for steps involved with initiating infection and early phases in the replication of their genome. Virions are not known to contain any intrinsic repair systems as such, which suggests that they would be unable to directly mend damage done to them by environmental factors. Viruses have not been reported to possess any "starvation" or other specific long-term survival modes, as do some bacteria. The organizational structure of viruses and their obvious lack of nutritional requirements when outside a host cell would appear to preclude their possessing any specific "starvation" survival modes.

B. Which Viruses May Be of Concern In Wastewater?

Viruses which merit the greatest concern are those capable of causing human illness. In terms of wastewater sludges, the greatest interest has traditionally rested with viruses that are shed in human fecal material (Table 1). Those which infect animals and are shed in fecal material are often referred to as enteric viruses. Additional virus groups are, of course, contributed to wastewater through their excretion in urine (Table 2). Another, albeit minor, source of human viruses in wastewater are those contained in bodily materials, such as blood (Table 3), for which sewer disposal without prior disinfection may be a common practice and has, in fact, been recommended by the U.S. Centers for Disease Control.[20]

Research studies on the presence of viruses in wastewater sludges have often utilized bacterial viruses, termed bacteriophages, as their subject of interest. Bacteriophages, as well as viruses capable of infecting other types of microorganisms, may be present in wastewater sludges. In fact, bacteriophages are relatively abundant in wastewater sludges.[60] However, because they do not infect animals, bacteriophages cannot be described as enteric in nature. The reasons given for studying bacteriophages have centered primarily around the higher assay costs traditionally associated with human viruses and the requirement for longer assay times when detecting human viruses by older techniques, such as plaque formation. The recent development and standardization of more efficient methods for virus concentration have helped ameliorate the first of these concerns. Use of newer, more rapid, assay methods for detecting human viruses has helped to greatly reduce the second concern.[61]

It must also be realized that bacteriophages generally respond very differently from enteric viruses in terms of their inactivation times when exposed to chemical disinfection agents, such as chlorine. Bacteriophages also have the potential to increase their numbers within wastewater sludges if the proper bacterial host organisms are present. This regrowth cannot occur for human viruses. Because of these and other differences, the use of bacteriophages as indicators of the presence of animal viruses in wastewater sludges requires great caution.

In addition to infecting animals and being intestinally shed, enteric viruses are further characterized by an ability to infect tissues in the throat and gastrointestinal tract. Some enteric viruses are also capable of replicating in other organs of the body as well, where they may cause a variety of recognizable disease syndromes, including hepatitis, meningitis, and paralysis. Table 1 lists known human enteric viruses and the illnesses which they cause. All of the different enteric viruses which are excreted in human feces ultimately find their way into domestic sewage. The average enteric virus density in U.S. domestic sewage has been estimated at about 7000 viruses per liter.[32] It is important to realize that other types of animals also shed a variety of enteric viruses which are naturally associated with their own particular species. Although nonhuman enteric viruses may be present in surface waters

as a result of such processes as direct deposition and runoff from land surfaces, they are not likely to be present in municipal wastewaters in any appreciable numbers. Nevertheless, these viruses are important to persons concerned with the disposal of animal manure wastes. Information on some of these nonhuman enteric viruses are included in this review, and their response to various sludge treatment processes can be expected to approximate that of similarly classified human viruses.

C. Descriptions of the Human Enteric Virus Groups

Adenoviruses are large, nonenveloped viruses which possess icosahedral capsid structures. They have double-stranded DNA genomes and range in diameter from 70 to 80 nm. Adenoviral capsids bear characteristically recognizable fiber-like projections. The name of this group is derived from the initial isolation of the virus from degenerating adenoidal tissue. All of the 41 known serologically distinct types (serotypes) of human adenoviruses can be propagated in laboratory-maintained cell cultures. Most of these adenovirus serotypes cause upper respiratory and conjunctival diseases. Several, however, are associated with gastroenteritis.[7,9,15,61-63] The number of adenoviruses present in raw wastewater sludges was estimated, in one study, to average 10,800 per liter.[60] When subjected to a more sensitive assay technique developed by the same research group,[61] this reported virus level corresponded to an estimated concentration of 54,000 adenoviruses per liter. The number of enteroviruses found in the same sludge samples averaged only 1320 per liter.[60] It has been reported that the number of adenoviruses present in raw primary sludge may exceed that of the enteroviruses by an average factor of 94-fold.[61] It has also been estimated that approximately 80% of the adenoviruses present in wastewater sludges are of serotypes 40 and 41,[61] which cause gastroenteritis.

Astroviruses are small, nonenveloped viruses which have an icosahedral capsid structure. Their size generally ranges from 26 to 32 nm in diameter and their genomes consist of single-stranded RNA. The name of this group is derived from the virus having a star-like appearance when viewed by transmission electron microscopy after negative staining with solutions of electron-opaque metals. There are five known human astrovirus serotypes, at least four of which can be propagated in laboratory cell cultures. This group of viral agents has been found in the stools of humans ill from gastroenteritis.[7] Their level of concentration in wastewater has not been determined. The Snow Mountain Agent, which also causes gastroenteritis in humans, now appears to belong to astrovirus serotype 5.[12]

Caliciviruses are nonenveloped, possess single-stranded RNA genomes, and have an icosahedral capsid structure. Their capsids bear cup-like projections from which the name of this virus group is derived. The caliciviruses range in diameter from 35 to 40 nm.[7] Their level of concentration in wastewater is generally unknown. Several of the human caliciviruses can be propagated in cell cultures.

Coronaviruses are pleomorphic, membrane-enveloped viruses with a diameter of approximately 120 nm. Their geonomes consist of single-stranded RNA and they have rod-shaped capsid structures. This virus group derives its name from the petal-shaped projections, termed "peplomers", which protrude from their envelopes and cause the viruses to have a crown-like appearance when viewed by electron microscopy. They have been associated with gastroenteritis in humans.[10,11,15] Their level of concentration in wastewater remains uncertain and at least some of them can be cultured in the laboratory.

The enterically transmitted non-A, non-B hepatitis virus is nonenveloped and has an icosahedral capsid structure with an approximate diameter of 27 to 34 nm.[17,64] It has been associated with numerous outbreaks of waterborne, enterically transmitted hepatitis, including one that occurred in New Delhi, India, during 1955 to 1956 and involved about 29,000 reported cases.[65] This virus can be propagated using laboratory animals as hosts.

Enteroviruses are nonenveloped and have icosahedral capsids which are approximately

28 nm in diameter. The name of this virus group apparently is derived from its enteric nature and route of transmission. Contained within the enterovirus group are the many different serotypes of human echoviruses, coxsackieviruses A and B, and polioviruses. Also included in this group are the more recently recognized "numbered" enteroviruses (types 68 to 71) and the hepatitis A virus which is now designated as human enterovirus 72. The enterovirus group has traditionally been the one most studied with regard to sewage treatment. The reasons for this are twofold: (1) the historical, but now largely discounted, belief that water served as the major route for the transmission of polioviruses within susceptible human populations and (2) the research efforts conducted on poliomyelitis, which resulted in the development of laboratory techniques for successfully detecting the members of this virus group. All of the human enteroviruses can be replicated in laboratory cell-culture lines. Enterovirus concentrations as high as 400,000 plaque-forming units per liter have been found in raw sewage and levels as high as 500,000 have been reported in sewage effluent.[66] The concentration of enteroviruses in raw wastewater sludge was observed to be as great as 140,000 per liter at one treatment plant.[67] However, the range of enteroviruses present in raw sludge has recently been estimated more conservatively at 5000 to 28,000 per liter.[68] Among those which may be found are both oral vaccine and nonvaccine strains of human poliovirus.[69,70]

Norwalk virus presumably has a single-stranded RNA genome and is known to possess an icosahedral capsid structure. The virus is nonenveloped and has a diameter typically ranging from 26 to 32 nm. This virus is a causative agent of epidemic vomiting and diarrhea. Its name results from the virus initially having been identified in conjuction with an outbreak of gastroenteritis that occurred in Norwalk, OH. This virus has been associated with outbreaks of illness attributed to contaminated water[7] and to some extremely large outbreaks associated with the consumption of shellfish which apparently had been harvested in areas polluted by wastewater.[13] Outbreaks of gastroenteritis attributed to the Norwalk virus, as well as other viruses with short incubation periods, are sometimes followed several weeks later by hepatitis attributed to human enterovirus 72. In these cases, infection by both viral agents was probably concurrent, with the time difference in the appearance of the two illnesses resulting from the longer incubation period associated with human enterovirus 72. This virus group has not yet been successfully replicated in cell-culture lines and its level of concentration in wastewater is uncertain. There is now strong serological evidence to support the belief that the Norwalk virus is a type of calicivirus.[16]

Parvoviruses have single-stranded DNA genomes and an icosahedral structure whose general diameter is approximately 20 nm. They are nonenveloped. The name of this virus group derives from its small physical size. Parvoviruses have been reported to be associated with the blood of infected humans. There is also evidence of parvoviruses and parvolike viruses being present in the stools of individuals during outbreaks of gastrointestinal illness.[7] Some of the human parvoviruses can be propagated in cell-culture lines. Their level of concentration in wastewater remains uncertain.

Reoviruses and rotaviruses represent two different groups within the larger family of reoviruses. They have icosahedral capsids, which range in diameter from approximately 70 to 80 nm, and multisegmented, double-stranded RNA genomes. Members of this virus family are nonenveloped. The name reovirus was derived from an abbreviation of their older name, "Respiratory Enteric Orphan" viruses. As the latter name suggests, reoviruses were known to have a respiratory presence in humans before they were identified with any particular illness. The name rotavirus is derived from the fact that members of this group have a wheel-like appearance when viewed by transmission electron microscopy following negative staining. There are three known serotypes of human reoviruses. Although all three are found in human stools, there remains some uncertainty as to the nature of the illness they cause in humans. Their primary site of infection may, indeed, be the respiratory system. The presence

of these viruses in fecal material could result from their being swallowed in material which is cleared from the respiratory passages. Rotaviruses are clearly associated with gastroenteritis in humans.[7,9,14,15] There are four recognized human rotavirus serotypes, plus additional known human rotaviruses which are considered to be atypical. In humans, rotaviruses are primarily associated with infections in young children, although there have been some outbreaks in adult populations. Both human reoviruses and rotaviruses can be propagated in cell-culture lines. Although both groups are present in wastewaters, their respective levels of concentration in sludges have not been studied extensively.

The "Small Round Viruses" constitute a loosely defined group of agents associated with transmissible vomiting and diarrhea in humans.[8,15] The name of this virus group is derived from its small size and nearly featureless appearance when viewed by transmission electron microscopy. The viruses are nonenveloped, appear to have icosahedral capsids, and are approximately 27 nm in diameter. Thus far, they cannot be propagated in laboratory cell lines and their level of concentration in wastewater remains uncertain.

III. VIRUSES IN THE WASTEWATER TREATMENT PROCESS

A. Partitioning of Viruses during Wastewater Treatment Steps

The extent of virus removal that can be achieved by different steps in the wastewater treatment process has been measured, often with widely varying results. In general, it is known that enteric viruses adsorb well onto particulate organic materials and to hydroxide precipitates of metal cations. Hence, removal of viruses during the course of wastewater treatment is largely associated with their partitioning into the various sludge fractions which are produced during collection of different types of suspended solids, onto which these viruses are either adsorbed[71] or possibly imbedded.[4] The degree to which settling of sewage sludges affects the removal of viruses depends not only upon the nature of the solid itself,[2] be it clay, organic, or metal salt floc, but also upon the specific virus group,[72,73] serotype, and strain which is involved[74] (Table 4). Variation in the degree to which different viruses adsorb onto solids may be related to their protein structure[76] and isoelectric point.[77,78]

Primary, secondary, and tertiary settling processes can remove viruses from produced effluents (Table 5). However, the fact that this "removal" results from the partitioning of viruses into the produced sludge fractions[4,79] can pose an additional handling problem since the adsorption of viruses onto sludge solids may increase their environmental stability.[53] Hence, it might be hazardous to dispose of such sludges without first subjecting them to further treatment that would destroy viral infectivity.[71]

Measurable levels of viruses have been found in grit-chamber solids.[72] As mentioned previously, viruses can be readily found in primary sludge solids collecting during the settling of raw sewage. They can also be detected in mixed-liquor and collected activated sludge solids.[72] The levels of enterovirus removal achieved by primary settling and activated sludge treatment (Table 5) range from 0 to 75 and 40 to 95%, respectively.[3,83] A time-course study of virus persistence through a working activated sludge treatment plant is presented in Table 6. In addition to the adsorption of viruses onto sludge flocs, antagonism by sewage microflora is believed to be a major factor associated with virus removal by the activation process.[2,3]

Another type of sludge-generating treatment consists of lagooning wastewaters with stabilization ponds. This process is very erratic with regard to its virus removal, with efficiencies ranging from 0 to 99.9%. Virus removal during this process is largely due to the partitioning of viruses with a sludge layer that forms at the bottom of the lagoons or ponds.[82] The removal of viruses during this type of wastewater treatment may also be greatly influenced by actual sewage retention time, as opposed to calculated average retention time (i.e., short circuiting), and by pond temperature and season, as summarized by Lance and Gerba.[5] A list of various factors known to affect virus survival in wastewater sludges is presented in Table 7.

Table 4
EFFECT OF SEROTYPE AND STRAIN-DEPENDENT DIFFERENCES ON VIRUS ADSORPTION TO ACTIVATED SLUDGE FLOCS

Virus serotype	Strain	Degree of virus adsorption (%)
Human coxsackievirus B1	60[a]	98.7
Human coxsackievirus B3	Nancy[b]	99.8
Human coxsackievirus B4	V216[c]	77.6
	V240[c]	89.1
Human echovirus 1	Farouk[b]	66.7
	V212[c]	91.8
	V239[c]	94.8
	V248[c]	76.9
	V249[c]	64.6
Human echovirus 7	Wallace[b]	99.1
	71[a]	90.0
Human echovirus 12	Undesignated[b]	98.1
Human echovirus 17	63[a]	97.8
Human echovirus 29	JV-10[b]	99.5
Human poliovirus 1	LSc-2ab[b]	98.2
	59[a]	95.9

[a]   Virus isolated from activated sludge flocs.
[b]   Virus isolated from or derived by sequential passage of clinical materials.
[c]   Virus isolated from groundwater.

Adapted from Hurst, C. J., Ph.D. thesis, Baylor College of Medicine, Houston, 1979.

Table 5
REDUCTION IN VIRUS LEVELS ASSOCIATED WITH WASTEWATER SLUDGE GENERATION PROCESSES

Treatment	Virus type	Efficiency of virus removal from effluent[a] (%)	Ref.
Primary settling	Indigenous population	avg. 94.0—97.3	80
		75	81
		24—83	82
Activated sludge with subsequent settling of the produced solids	Indigenous population	98	81
	Coliphage f2	90.1—98.9	83
Combined primary settling plus activation and subsequent secondary settling	Indigenous population	8—93	84

[a]   Virus removal during these processes occurs primarily through adsorption to and subsequent partitioning with the particulate solids. See References 24 and 78.

Table 6
TIME-COURSE STUDY OF REDUCTION IN NUMBERS OF NATURALLY PRESENT VIRUSES DURING TREATMENT AND DISPOSAL OF WASTEWATER SLUDGE

Sample	Treatments	Solids content (mg/ml)	Infectious units/10 g of sludge (dry wt.)
Raw + return sludge		4.9	57.1
	Mixing, activation by aeration (2 h elapsed time)		
Outlet to settling tanks		5.1	59.8
	Settling, return solids reactivated (6 h elapsed time)		
Reactivated, return sludge		7.4	38.4
	Thickening, aerobic digestion, centrifugation (5 d elapsed time)		
Dried sludge, day 0		69	33.5
	Drying in field		
Dried sludge, day 2		140	10.1
	Further drying in field		
Dried sludge, day 7		184	1.0

Adapted from Hurst, C. J., Ph.D. thesis, Baylor College of Medicine, Houston, 1979.

Table 7
LIST OF FACTORS WHICH AFFECT VIRUS SURVIVAL IN WASTEWATER SLUDGES

Factor	Ref.
Temperature	85, 86, and numerous others
Detergents	87
Dewatering through evaporation	24, 88—91
Ammonia	92, 93
Aerobic microbial activity	94
Aerobic microbial metabolites	95
Dissolved, fiberable products of aerobic and anaerobic digestion	96, 97
pH, elevation through addition of CaO	98

Another old, but still commonly used, method of secondary sewage treatment is that of trickling filtration. In this process, sewage effluents are usually sprayed onto the upper surface of filtration beds composed of cobbles and gravel. The contents of the beds are graded as to size, with the coarsest material at the upper surface. Virus removal involves adsorption of the virus particles onto a film of algae and other microorganisms which forms on the surface of the filter material.[75] Virus removal by trickling filtration is also affected by the retention of sewage solids with which individual viral particles are associated. The

effluents collected from beneath trickling filters may then be subjected to further treatment, such as activated sludge, prior to their environmental release. Virus reductions by trickling filtration have been found to range from 0 to 84%. In general, however, virus removal by this process is small and inconsistent.[1,74] In several tests, increases in virus concentrations have occurred during the course of trickling filtration. The increases were presumably caused by the disintegration of fecal solids and resultant liberation of viruses into the liquid effluent.[74] Of these methods commonly used for secondary treatment of sewage, activated sludge, when correctly practiced, seems to be the most efficient for removing viruses, although, even with this process, virus removals are sometimes poor.[4]

The extent of virus removal achieved during the tertiary treatment of sewage by physical-chemical processes depends upon such factors as the concentration of added chemicals and the pH of the sewage. Coagulation of sewage with aluminum sulfate has been shown to remove 85 to 95% of seeded human poliovirus 1 and 95 to 99.9% of seeded human coxsack-ievirus A2. The precipitation of phosphate from wastewater, in association with other types of precipitates, has also been evaluated. Sproul[74] has reported the removal of 70 to 99.86% seeded human poliovirus 1 from secondary effluent during flocculation of phosphate in association with lime and up to 98% of seeded human poliovirus 1 in conjunction with the precipitation of calcium and magnesium. While the degree of virus removal was as great as 99.9% during the precipitation of magnesium in association with calcium carbonate, it was much lower during the precipitation of calcium alone. The use of ferric chloride coagulation has also been used to remove viruses from sewage effluents.[74] All three of these coagulation procedures (employing aluminum sulfate, lime, and ferric chloride) result in the production of infectious, virus-containing sludges. Hence, the procedures have also been used to concentrate indigenous viruses from sewage samples so as to facilitate viral detection.[71] A very important factor involved with flocculation by excess lime is the high pH which results from this treatment and which may, in itself, cause virus reductions through direct inactivation,[4,99] in addition to that removal which is achieved through settling.

Further removal of viruses from the effluents produced by various chemical coagulation treatments can be achieved by passing effluents through filters composed of coarse sand.[100,101] Virus removal through this type of filtration step does not appear to be due to the direct adsorption of viruses onto the filters. Rather, it seems to result from the sand having trapped small suspended flocs which contain adsorbed viruses and whose size was insufficiently large for them to have settled out during the earlier sedimentation processes. A second explanation of virus removal during the sand filtration of coagulation effluents is that the minute flocs trapped by the sand provide adsorption sites for nonsolids-associated viruses that are contained in the applied effluents.[32] This theory is supported by the work of Guy et al.,[101] who found that a great amount of the virus-adsorbing capacity of sand filters is removed during backwashing, a process which removes these minute flocs from the filters. This reduction in virus removal capacity was quickly regained by subsequent passage of fresh coagulation effluent through the filter. Use of a sand filtration step can increase the removal of human poliovirus from secondary sewage effluent by an additional 82 to 99.8% over that obtained by lime flocculation alone.[100]

Carbon adsorption of residual organics is often practiced in advanced wastewater treatment facilities. Although viruses adsorb to activated carbon, the capacity of carbon to remove viruses is limited and the viruses often break through carbon columns after the passage of only a few bed volumes through such columns.[102] Organic material present in sewage effluent interferes with the adsorption of viruses onto activated carbon and viral desorption from carbon often occurs as organic substances replace the adsorbed virions. The efficiency of virus removal from sewage effluents by activated carbon adsorption generally ranges from 0 to 50%.[3,74] Conversely, the removal of human poliovirus from drinking water has been observed to be much greater than this (78.5%). The latter finding probably is due to the

lower levels of organic materials normally found in drinking water, compared with sewage effluent. The backwashing of charcoal filters also reduces their efficiency for virus removal, which is contrary to what might be expected if the disturbance due to backwashing had resulted in the removal of contaminating solids particles, thereby reopening viral adsorption sites on the surface of the carbon particles.[101] Hence, Guy et al.[101] suggested that carbon adsorption was not the sole or principal mechanism of virus removal by carbon columns. It would appear that virus removal by carbon columns (as by sand filters) is, in large part, dependent upon suspended particulates which are trapped by the columns. In summary, the backwashing of both sand and carbon filters may be expected to yield suspensions of particulates containing entrapped or surface-adsorbed virus particles. These backwashed suspensions should be subjected to processes which collect the suspended solids before any resulting effluents are discharged. The solids collected from these backwashings represent yet another contribution to the problem of wastewater sludge treatment and disposal.

B. Factors Affecting the Stability of Viruses in Wastewater Sludges

Sewage sludges generally contain enteroviruses, as well as members of other enteric virus groups.[60,61] Viruses are not only present in raw sludges, but also in digested and lagoon-dried sludges.[31] For this reason, it is important to learn the extent to which various sludge-processing techniques can be used to achieve viral inactivation. Knowledge of the mechanisms by which this inactivation occurs can help maximize the effectiveness of sludge-processing methods.

A brief list of factors known to affect the stability of viruses in wastewater sludges was presented in Table 7. Overall, studies on virus stability suggest that the most important of factors may be the loss of moisture through evaporation,[24,27] temperature,[28,86] and antagonism associated with aerobic microorganisms.[29] Of these three factors, the best-studied and most predictable with regard to its effect would seem to be temperature. An additional factor important to the stability of viruses in wastewater sludges is pH. Moderately low pH levels are not nearly so deleterious to many of the enteric virus groups as are the greatly elevated pH levels which can be achieved from the addition of lime to wastewater sludges.[6,75,103]

The following sections of this review include written discussions and statistical reviews concerning the influence of these and other factors upon the stability of viruses in wastewater sludges. It is important to note that the rate of viral inactivation observed during any particular treatment process may also be dependent upon virus type. Hence, statistical equations presented in the tables included in this section present information on a variety of different virus groups. The reason for addressing more than a single virus group in this review is that members of all groups may, in fact, be simultaneously present in wastewater sludges. It is hoped that by including data for more than one virus group, where such are available, calculations will be more representative of the inactivation processes being examined. Linear regression analysis has proven to be a very helpful tool in understanding viral inactivation processes[28,29,86,104] and has been used extensively in preparing the accompanying tables. The viral inactivation rates used in the regression calculations are included in the tables.

1. Activation Process

Enteroviruses are commonly found in sludge aeration basins[52] and much of the virus content of wastewater becomes adsorbed to, and concentrated with, the sludge flocs during activation.[3,79] Within the laboratory, inactivation of enteroviruses has been observed to occur during the aeration of activated sludge.[105] Inactivation of indigenous enteroviruses, which predominantly consisted of human echovirus 7, was not observed to occur during a study which examined activation in an actual sewage treatment plant[75] (Table 6). This apparent discrepancy could be due to either differences in the aeration conditions for the two studies or variation in the susceptibility of the different enterovirus serotypes which were

Table 9
RATES OF INACTIVATION FOR VIRUSES SUSPENDED IN SLUDGE DURING ANAEROBIC DIGESTION

Incubation temperature (°C)	Virus type	Rate of inactivation (log base 10 units/d)	Number of days required for 90% inactivation	Ref.
32	Human coxsackievirus B3	0.486	2.06	109
34	Human poliovirus 1	0.240	4.17	85
35	Coliphage MS2	1.167	0.86	79
	Human coxsackievirus A9	1.569	0.64	79
	Human coxsackievirus B3	2.167	0.46	109
	Human coxsackievirus B4	1.009	0.99	79
	Human echovirus 11	0.600	1.67	79
	Human poliovirus 1	1.000	1.00	79
37	Human poliovirus 1	0.384	2.60	85
50	Human poliovirus 1	7.699	0.13	85

Note: Calculation of values for rate of inactivation and number of days required for 90% inactivation was based upon information presented in the listed references. Rates of this type provided in the references were utilized without modification.

Table 10
RATES OF INACTIVATION FOR VIRUSES ADSORBED ONTO FILTERS DURING IMMERSION IN ANAEROBICALLY DIGESTING SLUDGE

Incubation temperature (°C)	Virus type	Rate of inactivation (log base 10 units/d)	Number of days required for 90% inactivation	Ref.
34	Coliphage f2	0.900	1.11	97
36	Bovine parvovirus	0.944	1.06	96
	Human coxsackievirus B5	0.475	2.11	96
	Human rotavirus 1	0.314	3.18	96
54	Coliphage f2	28.54	0.035	97
56	Bovine parvovirus	5.11	0.197	96
	Human coxsackievirus B5	1080	0.00009	96
	Human rotavirus 1	204	0.005	96

Note: Calculation of values for rate of inactivation and number of days required for 90% inactivation was based upon information presented in the listed references. Rates of this type provided in the references were utilized without modification.

digestion. Regression analyses of these viral inactivation rates with respect to digestion temperature are presented in Table 11. The analyses demonstrate that, although the rates of inactivation for viruses which were directly suspended in anaerobically digesting sludge evidenced statistically significant correlations with digestion temperature, the inactivation rates representing filter-adsorbed viruses did not. The reduction in statistical significance (p-value) which occurred when the data representing freely suspended viruses were combined with those representing filter-adsorbed viruses (last analysis presented in Table 11) suggests that the data obtained for inactivation of filter-adsorbed viruses may not closely represent the stability of freely suspended viruses.

In addition to temperature itself, other components in anaerobically digested sludge have also been associated with the inactivation of viruses. Inactivation of human poliovirus in

Table 11
REGRESSION EQUATIONS DESCRIBING INACTIVATION OF VIRUSES IN ANAEROBICALLY DIGESTING SLUDGE

| Description | Table in which data presented | Regression equation | | | |
		Slope	Y-intercept	r-value	p-value
Viruses suspended directly in anaerobically digesting sludge	9	0.420	−13.623	0.944	0.00004
Viruses adsorbed to filters immersed in anaerobically digesting sludge	10	4.076	−145.027	0.575	NS*
Both of the above analyzed as a single group	9 & 10	14.617	−516.102	0.520	0.027

Note: Linear regression analyses were performed with rate of inactivation as the dependent variable vs. incubation temperatures as the independent variable.

* Nonsignificant (p-value >0.05).

anaerobically digested sludge is accompanied by fragmentation of the viral RNA and some breakdown of virion proteins.[110] This irreversible inactivation is due to a factor contained in the supernatant following high-speed centrifugation of the digested sludge. The same inactivating factor was not found in raw sludge,[110] nor was it contained in the pelleted digested sludge solids following the centrifugation step. Both raw sludge and collected anaerobic digester solids were shown to provide a measure of protection for enteroviruses against thermal inactivation. Exposure to the virucidal agent by addition of produced digester sludge supernatant to the sludge solids reversed the protective potential of the solids during heat treatment.[53] The active agent present in the digested sludge has since been determined to be ammonia. The inactivating capabilities of ammonia exist only at pH values >8, when the ammonia is in its uncharged state.[92] The rate of enterovirus inactivation in sludge supernatant has been shown to increase with increasing concentrations of ammonia. This relationship is linear and holds true for ammonia concentrations of up to 1500 mg/l.[93] Some other amine compounds also exhibit virucidal effects on poliovirus and the size of the compound has been shown to be important in its inactivating capacity.[111]

3. Sludge Storage and Dewatering by Evaporation

One of the procedures frequently involved in sludge handling is dewatering. Ward and Ashley showed that dewatering of raw-sludge by evaporation effectively inactivated human poliovirus.[88] The viral inactivation which occurred during the dewatering process was due to irreversible virion disruption, resulting in release of the genomic RNA molecules (which were extensively degraded) from their capsids.[88] The authors concluded that the observed viral inactivation may have been partially caused by the evaporation process itself because similar effects on human poliovirus particles were observed to occur in distilled water after only a partial loss of the water by evaporation. Inactivation of human coxsackieviruses and reoviruses was also found to have occurred, suggesting that evaporation may prove to be a feasible method of inactivating all enteric viruses present in sludge.

Another possible method of inactivating viruses in wastewater sludges would be to simply hold them for an adequate period of time at any temperature, with the rate of inactivation

Table 12
RATES OF VIRUS INACTIVATION IN STORED SLUDGE SAMPLES WHEN EVAPORATION IS PRECLUDED

Sludge type	Incubation temperature (°C)	Virus type	Rate of virus inactivation (log base 10 units/d)	Number of days required for 90% inactivation
Raw primary	−70	Indigenous population	0.00015	6667
			0.00140	714
			0.00023	4348
			0.00189	529
	2	Indigenous population	−0.00617	−162
			0.00517	193
			0.00175	571
			0.00145	690
	23	Indigenous population	0.04530	22.07
			0.02933	34.09
			0.04114	24.31
			0.03449	28.00
Mixed liquor activated	−70	Indigenous population	0.00752	133
			0.03071	32.56
			0.00069	1449
			0.00649	154
	2	Indigenous population	0.01794	55.74
			0.00575	174
			−0.00060	−1667
	23	Indigenous population	0.05919	16.89
			0.04093	24.43
			0 01745	57.31

Note: Calculation of values for rate of inactivation and number of days required for 90% inactivation was based upon information presented in Reference 86.

effect increasing with temperature.[86] Information on the rates at which viral inactivation occurs during storage of sludges and regression analyses of these rate values vs. storage temperature are presented in Tables 12 through 17. In recognition of the important role which evaporative moisture loss plays in the inactivation of viruses in wastewater sludges,[24,88] the information in these tables has been separated into studies of sludge storage when evaporation is precluded (Tables 12 and 13), when evaporation is allowed (Tables 14 and 15), and where the evaporation status of the sludge samples could not be clearly established from the information provided in the articles (Tables 16 and 17).

4. Lagooning of Digested Sludges and Land Surface Disposal

Viruses are frequently detected in sludge solids following their digestion by aerobic or anaerobic processes.[24,108,112] It has been concluded that, despite the best efforts at sludge digestion, total elimination of viruses from the sludges is probably not possible and low levels of viruses will probably be introduced into soil systems by the land application of municipal sludges.[52]

In some cases, wastewater sludges may be air-dried in lagoons prior to being discarded.[31] However, in one study, naturally occurring enteroviruses were shown to survive for several months in a sludge lagoon during the spring and summer seasons in Ottawa, Canada.[31] Based upon information presented in this study, calculations of the relative percentages of samples which were positive for viruses during the general time course of that study are presented in Table 18. This information was also subjected to linear regression analysis in

Table 13
REGRESSION EQUATIONS DESCRIBING DATA
FOR VIRUS INACTIVATION IN STORED SLUDGE
WHEN EVAPORATION IS PRECLUDED

| Description | Regression equation | | r-value | p-value |
	Slope	Y-intercept		
Raw primary sludge	0.00029	0.017	0.649	0 022
Raw mixed-liquor activated	0.00023	0.023	0.504	NS*
Both of the above analyzed as a single group	0.00025	0.020	0.562	0.006

Note: Linear regression analyses were performed with rate of inactivation as the dependent variable vs. incubation temperature as the independent variable. Data used in these analyses were presented in Table 12.

*   Nonsignificant (p-value >0.05).

Table 14
RATES OF VIRUS INACTIVATION IN STORED SLUDGE SAMPLES WHEN
EVAPORATION IS ALLOWED

Sludge type	Incubation temperature (°C)	Virus type	Rate of virus inactivation (log base 10 units/d)	Number of days required for 90% inactivation	Ref.
Mixed-liquor activated	4	Human poliovirus 1	0.076	13.16	95
	15	Human poliovirus 1	1.337	0.748	95
	26	Human coxsackievirus A13	2.523	0.396	95
		Human echovirus 12	2.699	0.371	95
		Human poliovirus 1	1.409	0.710	95
		Human reovirus 3	2.698	0.371	95
		Simian rotavirus SA11	1.921	0.521	95
Anaerobically digested	4	Human poliovirus 1	0.286	3.50	110
	20	Human poliovirus 2	0.296	3.38	110
		Human poliovirus 1	0.789	1.27	110
		Human poliovirus 2	0.873	1.14	110
	28	Human poliovirus 1	1.184	0.84	110
		Human poliovirus 2	1.824	0.55	110

Note: Calculation of values for rate of inactivation and number of days required for 90% inactivation was based upon information presented in the listed references. Rates of this type provided in the references were utilized without modification.

an effort to determine a rate equation for the observed viral inactivation. The resulting regression equation is presented in Table 19.

Wellings et al.[113] have shown that enteroviruses can be found in sludge solids 13 d after their disposal. Hence, the survival of viruses in sewage sludges following land disposal is also a subject of importance. Seeded enteroviruses have been shown to survive for 30 d in digested sludge held in the open at ambient temperatures which ranged between 13 and 28°C.[112] Seeded enteroviruses have also been shown to survive 23 weeks during a normal

Table 15
REGRESSION EQUATIONS DESCRIBING DATA
FOR VIRUS INACTIVATION IN STORED SLUDGE
WHEN EVAPORATION IS ALLOWED

| Description | Regression equation | | | |
	Slope	Y-intercept	r-value	p-value
Mixed-liquor acti-vated sludge	0.09638	−0.24262	0.872	0.010
Anaerobically di-gested sludge	0.04814	0.04086	0.906	0.013
Both of the above analyzed as a single group	0.04981	0.52751	0.522	NS*

Note: Linear regression analyses were performed with rate of inactivation
as the dependent variable vs. incubation temperature as the inde-
pendent variable. Data used in these analyses were presented in
Table 14.

*   Nonsignificant (p-value >0.05).

Table 16
RATES OF VIRUS INACTIVATION IN STORED SLUDGE SAMPLES WITH
EVAPORATION STATUS UNKNOWN

Sludge type	Incubation temperature (°C)	Virus type	Rate of virus inactivation (log base 10 units/d)	Number of days required for 90% inactivation
Digested	4	Human poliovirus 1	0.020	50.00
(process	22	Human coxsackievirus A9	0.043	23.26
unspecified)		Human coxsackievirus B2	0.046	21.74
		Human coxsackievirus B4	0.071	14.08
		Human coxsackievirus B5	0.033	30.30
		Human echovirus 6	0.036	27.78
		Human echovirus 9	0.036	27.78
		Human poliovirus 1	0.045	22.22
		Human poliovirus 3	0.033	30.30

Note: Calculation of values for rate of inactivation and number of days required for 90% inactivation was based
upon information presented in Reference 106.

Danish winter, with temperatures ranging between −12 and 25.7°C.[114] Hurst et al.[24] found
that the inactivation of naturally occurring enteroviruses present in sludge solids following
their land surface disposal correlated with the loss of moisture from the sludge solids. Daily
temperatures ranged between 20 and 31°C. A more recent study at a similar time of year
in a different geographic location having a similar climate was conducted by Farrah and co-
workers.[91] Information from these studies, which address reductions observed in the virus
levels of sludges between the dates of land surface spreading and subsequent samplings,
have been recalculated in terms of log base 10 units of virus titer lost since the day of
spreading and are listed in Table 20. Regression of the observed losses in virus titers for
the sludges following their land disposal demonstrated a high degree of statistical significance
when analyzed separately by individual study (Table 21). An even greater level of statistical

Table 17
REGRESSION EQUATION DESCRIBING DATA
FOR VIRUS INACTIVATION IN STORED SLUDGE
WITH EVAPORATION STATUS UNKNOWN

Description	Regression equation		r-value	p-value
	Slope	Y-intercept		
Digested (process unspecified)	0.00127	0.01492	0.546	0.128

Note: Linear regression analysis was performed with rate of inactivation as the dependent variable vs. incubation temperature as the independent variable. Data used in this analysis were presented in Table 16.

Table 18
PERSISTENCE OF INDIGENOUS VIRUSES DURING
LAGOONING OF ANAEROBICALLY DIGESTED
SLUDGE

Date of sampling	Minimum number of months since last addition of sludge[a]	Percentage of samples positive for presence of viruses[b]
April 1975	1	100 (2 of 2)
May	2	33 (2 of 6)
June	3	66 (2 of 3)
July	4	40 (2 of 5)
October	7	50 (2 of 4)
November	8	25 (1 of 4)
December onward	9	0 (0 of 4)

[a] Last addition of sludge to this lagoon was done in March 1975.
[b] Calculation of values for percentage of samples positive for presence of viruses was based upon information provided in Reference 31.

Table 19
REGRESSION EQUATION DESCRIBING DATA FOR
PERSISTENCE OF INDIGENOUS VIRUSES DURING
LAGOONING OF ANAEROBICALLY DIGESTED
SLUDGE

Description	Regression equation		r-value	p-value
	Slope	Y-intercept		
Percentage of samples positive for viruses	−7.66505	82.08736	0.754	0.050

Note: Linear regression analysis was performed with percentage of samples positive for presence of viruses as the dependent variable vs. number of months since last addition of sludge as the independent variable. Data used in this analysis were presented in Table 18.

Table 20

INACTIVATION OF INDIGENOUS VIRUSES FOLLOWING LAND SURFACE SPREADING OF SLUDGE

Number of days since sludge placed on land	Farrah et al.[a]		Hurst et al.[b]			
			Trial 1		Trial 2	
	Virus titer of sludge (plaque-forming units per g dry wt.)	Log base 10 reduction in virus titer since day of land spreading	Virus titer of sludge (plaque-forming units per g dry wt.)	Log base 10 reduction in virus titer since day of land spreading	Virus titer of sludge (plaque-forming units per g dry wt.)	Log base 10 reduction in virus titer since day of land spreading
0	3.00	0	3.35	0	48.90	0
1	0.80	0.574	—	—	—	—
2	0.41	0.864	1.01	0.521	37.69	0.113
5	—	—	—	—	2.00	1.388
7	0.055	1.737	0.10	1.525	0.22	2.347
9	0.010	2.477	—	—	—	—

[a] Calculations based upon data presented in Reference 91.
[b] Calculations based upon data presented in Reference 24.

Table 21
REGRESSION EQUATIONS DESCRIBING DATA FOR
INACTIVATION OF INDIGENOUS VIRUSES
FOLLOWING LAND SURFACE SPREADING OF SLUDGE

Description	Regression equation		r-value	p-value
	Slope	Y-intercept		
Data from study by Farrah et al.[91]	0.24325	0.20603	0.984	0.002
Data from study by Hurst et al.,[24] trials 1 and 2 combined	0.28769	-0.10325	0.950	0.001
All of the above analyzed as a single group	0.26601	0.03114	0.959	<0.00001

Note: Linear regression analysis was performed with log base 10 reduction in titer since day of spreading as the dependent variable vs. number of days since sludge placed on land as the independent variable. Data used in these analyses were presented in Table 20.

significance was achieved when the two sets of data were analyzed together as a single group (last regression in Table 21).

5. Lime Addition

The elevation of sludge pH through addition of lime appears to be a very effective means of achieving viral inactivation at moderate temperatures. Information on the rates of viral inactivation in wastewater sludges following lime treatment is presented in Table 22.

6. Radiation and Pasteurization

One experimental method being used to rid wastewater sludges of pathogens prior to their land application is a deliberate exposure to high doses of ionizing radiation.[115] This type of treatment can be effective in destroying the replicative potential of parasites and bacterial pathogens.[58] Sludge, however, can highly protect viruses against both ionizing radiation and heat when the two treatments are applied separately. The simultaneous application of both heat and radiation seems to act synergistically and largely overcame the protective effects of the sludge. The simultaneous application of heat and radiation, termed thermoirradiation, also dramatically increases the inactivation of bacterial pathogens and parasites.[115,116] Pasteurization, which involves heating to 75°C for 20 to 25 min, is another very effective method of destroying pathogens in sludge.[58] However, neither irradiation nor pasteurization procedures will, by themselves, stabilize sludge solids against putrefaction, as is achieved by digestion.

7. Effects of Detergents

Ionic detergents are the major components of wastewater sludge which reduce the thermal stability of reoviruses.[117] In this function, cationic detergents seem to be more active than anionic detergents. These same detergents are, nevertheless, able to protect a different viral group, the enteroviruses, against heat. Detergents presumably are the protecting material which Ward et al. previously found to be associated with raw and digested sludge solids.[53] The detergents in wastewater, which are themselves solids associated, are concentrated during

Table 22
EFFECTIVENESS OF LIME TREATMENT FOR INACTIVATION OF SEEDED VIRUSES IN WASTEWATER SLUDGES

Sludge type	Virus type	Lime concentration (kg CaO/m³ sludge)	Rate of virus inactivation (log base 10 units/d)	Number of days required for 90% inactivation
Raw	Human poliovirus 1	0	0.080	12.50
		1.5	0.349	2.87
		3.0	>100	<0.01
Anaerobically digested	Bovine parvovirus	0	0.180	5.55
		1.5	0.171	5.85
		3.0	0.193	5.18
		5.0	0.214	4.67
		7.5	12.00 (est.)	0.083
		10.0	16.20	0.062
	Human poliovirus 1	0	0.121	8.26
		1.5	0.146	6.85
		3.0	0.264	3.79
		5.0	2.15	0.465
		7.5	2.26	0.442
		10.0	24.00	0.042

Note: Calculation of values for rate of inactivation and number of days required for 90% inactivation was based upon information presented in Reference 98.

sludge dewatering processes. The composting of sludge greatly reduces these detergent-related protective effects through degradation of the detergents involved.[99] It appears that the concentration of sludge solids through dewatering may also modify the effects of other sludge components upon the rates of heat inactivation of viruses.[75]

8. Composting

Composting of sludges prior to their disposal on land can stabilize them against putrefaction and can be an effective means of destroying pathogens.[118] However, many variables are involved in the efficiency of this treatment technique, such as temperature[118,119] and the method of composting used (i.e., aerated windrow or static pile). One particular hazard associated with composting is the fact that *Aspergillus fumigatus* and other fungi, such as thermophilic actinomycetes, are known to grow in self-heating organic matter. Some of these fungi may produce disease symptoms in sensitive persons working with or otherwise coming into contact with the composting material.[58] No information could be found which specifically addressed the rate at which viruses are inactivated during the process of sludge composting.

IV. SUMMARY

Numerous types of viruses are present in wastewater sludges. The most important of these are, of course, those capable of causing human illness. The contributing sources of these human viruses are fecal material, urine, and sewer-disposed contaminated blood which has not received proper sterilization treatment. Those viruses which are contributed by fecal material are often referred to as "enteric" viruses and probably should receive the greatest attention because of their fecal-oral route of transmission. Viruses associated with wastewater sludges can potentially survive release into the environment and then be transported by natural processes back to human populations, resulting in the cycling of human illnesses.

This type of human health hazard can be minimized by subjecting wastewater sludges to viral inactivating processes prior to their release into the environment.

The most important factors in achieving viral inactivation appear to be thermal exposure, loss of moisture during evaporative drying, microbial antagonism, exposure to high pH, and irradiation. Effective exposure to these factors can be achieved through a number of available treatment processes, including digestion, pasteurization, liming, lagooning, thermoirradiation, and the allowance of appropriate drying periods following land surface spreading.

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